Isoprostane, an “Intermediate Phenotype” for Oxidative Stress
Heritability, Risk Trait Associations, and the Influence of Chromogranin B Polymorphism

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Objectives
The purpose of this study is to understand whether isoprostane, a biomarker of oxidative stress, is subject to heritable control; whether it shares heritability with other cardiometabolic risk traits; and finally whether genetic variation at a specific candidate locus contributes to isoprostane variability.

Background
Isoprostane marks oxidative stress, and elevated isoprostane excretion might be involved in cardiovascular target organ damage. Here we used the classical twin pair method to probe the role of heredity in generating the isoprostane trait.

Methods
Trait heritability ($h^2$) and shared genetic determination among traits (pleiotropy, genetic covariance, $r_g$) were estimated by variance components in twin pairs. Because the isoprostane and Chromogranin B (CHGB) traits shared $r_g$, we examined the CHGB locus for effects on the traits.

Results
Urinary isoprostane excretion was substantially heritable ($h^2 = 65.8 \pm 4.3\%$), and the isoprostane trait aggregated with multiple traits (CHGB, catecholamines, autonomic/baroreceptor, and renal function), including several features of the metabolic syndrome (body mass index, insulin resistance, dyslipidemia). Isoprostane excretion also aggregated with systemic hypertension. Twin studies demonstrated genetic covariance (pleiotropy) for the isoprostane and CHGB traits ($r_g = 0.27$), and therefore we investigated the CHGB locus for trait effects. A common variant in the 3'-UTR of CHGB (C+84A) associated with plasma CHGB as well as isoprostane excretion. The C+84A disrupted an A/U-rich messenger ribonucleic acid stability element, and in transfected luciferase/3'-UTR plasmids, the C+84 and +84A alleles differed markedly in reporter expression in chromaffin and neuroblastoma cells, whereas site-directed mutagenesis confirmed the importance of this variant within the context of the A/U-rich motif.

Conclusions
Isoprostane excretion is substantially heritable and shares joint genetic determination with CHGB as well as multiple features of the metabolic syndrome. A common polymorphism in the 3'-UTR (C+84A) of CHGB, which disrupts an A/U-rich messenger ribonucleic acid stability element, associates with not only CHGB secretion but also excretion of isoprostane. We propose a chain of events whereby CHGB genetic variation results in oxidative stress, with isoprostane formation. The results suggest novel links among the catecholaminergic system, oxidative pathways, and systemic hypertension. (J Am Coll Cardiol 2010;56:1338–50) © 2010 by the American College of Cardiology Foundation

Oxidative stress is characterized by an imbalance between increased exposure to free radicals and antioxidant defenses. Oxidative damage of any of the biomolecules can theoretically contribute to disease development. Indeed, an increasing amount of evidence suggests that oxidative stress is linked to either the primary or secondary pathophysiologic mechanisms of multiple acute and chronic human diseases (1,2). Oxidative stress might also play an important role in...
the pathophysiology of essential hypertension (3, 4) and cardiovascular diseases (5–7), although the origin and significance of such oxidative damage is still incompletely understood. Inflammation might contribute to or result from tissue injury after oxidative stresses, inflammatory mediators generated by pathogens, genetic factors, or depletion of endogenous anti-inflammatory mediators. The inflammatory response might be accompanied by release of oxygen radicals (8, 9) that deplete endogenous nitrovasodilators, causing vasoconstriction (10, 11), thereby raising BP.

Isoprostanes are chemically stable end-products of lipid peroxidation derived from arachidonic acid (12). A series of isoprostanes might be generated, but most interest has focused on F2-isoprostanes, particularly on 8-iso-PGF2α (also known as 8-epi-PGF2α or 15-isoprostane F2t) because of its biological activity (13). As products of lipid peroxidation, F2-isoprostanes are considered to be reliable markers of enhanced systemic oxidative stress in vivo (14, 15). In the human circulation, isoprostanes are present mainly in their ester forms, whereas only hydrolyzed isoprostanes are excreted in the urine. The presence of detectable concentrations of isoprostanes in biological fluids implies continuing lipid peroxidation, despite the presence of a complex network of antioxidant defenses. As well as being the best available marker of oxidative stress, some isoprostanes have biological activities that might be relevant to the pathophysiology of disease (16). The 8-iso-PGF2α is a potent vasoconstrictor in the rat, and its action has been shown to be mediated at least in part via interaction with a TXA2/PGH2 receptor (13). Isoprostanes have also been shown to enhance vasoconstriction in hypercholesterolemia both in vitro (13, 17) and in vivo (18). Thus, 8-iso-PGF2α is not only a marker of but also a participant in the pathophysiology of disease.

Urinary and/or plasma isoprostanes are increased in and associated with several features of the metabolic syndrome, including hypercholesterolemia (19, 20), hypertension (21), and diabetes mellitus (22). In addition to their association with cardiovascular risk factors, isoprostanes are also increased in patients with established vascular disease (23). Familial correlations indicate that hypertension is, at least in part, a genetically determined condition (24) and likely further enhanced by environmental factors. Here we hypothesized that 8-iso-PGF2α is also subject to genetic determination and tested this hypothesis by evaluating the heritability (h²) of the trait, where h² is the fraction of trait variance accounted for by genetic variance (h² = Vg/Vp).

We estimated h² with the classical twin method (25), with both monozygotic and dizygotic twin pair correlations. We evaluated physiological traits and biochemical traits for shared genetic determination, with the genetic covariance (26) in twin pairs. Because blood pressure (BP) is adrenergically mediated, we coupled estimates of twin h² with allelic variation at chromogranin B (CHGB), which was first described in 1985 (27, 28) as a major catecholamine storage vesicle core protein and seems to play a necessary role in the biogenesis of catecholamine secretory vesicles (29, 30).

Methods

Twin and sibling pairs. Twin recruitment proceeded by access to a population birth record-based twin registry (31) as well as by newspaper advertisement, as described by us (32). We recruited 519 individuals from 194 twin pairs and 131 additional siblings of twins and other sibling pairs. From the 194 complete twin pairs, there were 130 monozygotic pairs (25 M/M and 105 F/F pairs) and 64 DZ pairs (13 M/M, 39 F/F, and 12 M/F pairs). Twin zygosity assignment was based on self-identification, with further confirmation by the presence or absence of heterozygosity at the (TCAT)n microsatellite (32) (unshared alleles necessarily indicating dizygotic status). The 519 subjects were all white (European ancestry); ethnicity was established by self-identification as well as by identification for both parents and all 4 grandparents. Twin ages were 14 to 81 years. Four hundred sixty-three individuals were normotensive, and the rest were hypertensive (41 treated with 1 to 2 antihypertensive medications; 15 untreated); BP status (high vs. normal) was defined by history (medical record or self-report), presence or absence of antihypertensive medications, and measurement of seated BP by arm cuff (hypertension: >140 mm Hg systolic BP and/or >90 mm Hg diastolic BP). Self-reported family history of hypertension (in a first-degree relative before the age of 60 years) was determined as described (33). Although self-reported family history of hypertension is generally accurate (34), in family studies with ascertainment of both probands and relatives (35), the specificity of a positive family history of hypertension is high (>90%) (i.e., few false positives); however, the sensitivity is variable (i.e., false negatives might occur). None of the subjects had a history of renal failure, and serum creatinine concentrations were ≤1.5 mg/dl. Definitions of subject characteristics are according to previous reports from our laboratory. Subjects were volunteers from southern California, and each subject gave informed, written consent; the protocol was approved by the University of California at San Diego (UCSD) Human Research Protection Program.

Physiological phenotyping in vivo. Subjects were studied before genotyping. Wild-type and variant subjects were studied during the same time interval. Brachial arterial cuff BP (in mm Hg) and heart rate (in beats/min) (n = 519) were obtained in seated subjects with a DynaPulse device (PulseMetric, Vista, California), as previously described and validated. Triplicate determinations of BP and heart rate were made, until each value was within ±10% of the mean
value. To probe autonomic control of the circulation in twin pairs, BP and heart rate were recorded continuously and noninvasively for 5 min in seated, resting subjects with a radial artery applanation device as well as thoracic electrocardiography electrodes and dedicated sensor hardware (Colin Pilot, Colin Instruments, San Antonio, Texas) and software (ATLAS, WR Medical Electronics, Stillwater, Minnesota; and Autonomic Nervous System, Tonometric Data Analysis [ANS-TDA], Colin Instruments). Baroreceptor coupling and baroreceptor slope were quantified as previously described (36).

Single nucleotide polymorphisms at CHGB. Deoxyribonucleic acid (DNA) was prepared from leukocytes in ethylenediaminetetraacetic acid–anticoagulated blood, with PureGene extraction kits (Gentra Biosystems, Minneapolis, Minnesota).

Single nucleotide polymorphism assays. Genotypes at CHGB (30) in twin samples were scored on amplified DNA by extension-based methods: mass spectrometry (Sequenom, La Jolla, California), or pyrosequencing (Biotech, Uppsala, Sweden). The CHGB 3′-UTR variant C +84A (rs28281) was in Hardy Weinberg Equilibrium (chi-square = 4.09, p = 0.152), with allele frequencies of C = 60.5%/A = 39.5% in subjects of European ancestry. The position of CHGB 3′-UTR C/A variant rs28281 can be described either in the 3′-UTR (C +84A, numbered in base pairs [bp] downstream of the translation termination codon) or in the gene (C13612A, numbered in bp downstream from the cap [transcription initiation] site).

Characterization of CHGB genetic variation in cell. Sympathochromaffin cell culture. Rat pheochromocytoma (chromaffin) PC12 cells were grown in Dulbecco's modified Eagle/high-glucose medium supplemented with 5% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum (Gemini Bio-Products, Woodland, California), 100 U/ml penicillin G and 100 mg/ml streptomycin (Invitrogen, Carlsbad, California) at 37°C, 6% carbon dioxide, in either 10-cm plates or 12-well plates. Human neuroblastoma SH-SY-5Y cells (ATCC) were grown at 37°C with 5% carbon dioxide, in 10-cm plates in 50% Advanced MEM (Invitrogen) and Ham's F12 (Invitrogen) supplemented with 10% of heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin.

CHGB 3′-UTR/luciferase reporter activity assays. The entire 308-bp CHGB 3′-UTR fragment was amplified from human genomic DNA with the following primers: sense, 5′-GGTCTAGACTGTCATTGGAGCGGTGG-3′; antisense, 5′-GGTCTAGAGGTCTCAGGTGAATTCTTTT-3′. The XbaI (TCTAGA) restriction (ligation) site is given in italics. The polymerase chain reaction product and pGL3-Promoter vector (with SV40 as promoter; Promega, Madison, Wisconsin) were each digested by restriction enzyme XbaI separately, and the insert was then ligated into the vector with T4 DNA ligase. Correct insert orientation was confirmed by asymmetric double digestion with restriction enzyme MfeI, which cuts once within the insert, and nearby in the vector at position 2103, within the SV40 pA cassette. The primers for site-directed mutagenesis at C +84A (on this template: A → C) are as follows (mutation in bold type): sense, 5′-CTGAAAGACACATTATATTACCAGGGGAGATTGTA-3′; antisense, 5′-TACCTTTCTGCCCCCTGGGTAGATAATGGTTGCTTTCCAG-3′. Point mutations were confirmed by dideoxy sequencing. The PC12 pheochromocytoma cells were transfected (at 50% to 60% confluence, 1 day after 1:4 splitting) with 1 µg of firefly luciferase/CHGB 3′-UTR reporter plasmid and 10 ng of the Renilla luciferase expression plasmid pRL-CMV (Promega) (as an internal transfection efficiency control) per well, by the liposome method (Superfect, Qiagen, Valencia, California). Firefly and Renilla luciferase activities in the cell lysates were measured 8 to 36 h after transfection, with the Dual Luciferase reporter assay system (Promega), and the results were expressed as the ratio of firefly/Renilla luciferase activity, as described previously (37). Each experiment was repeated a minimum of 3 times. Luciferase activity of C/A heterozygosity was estimated by a 1:1 mix of the C or A allele plasmids.

Biochemical phenotyping. Isoprostane in urine. As an index of systemic oxidative stress, we used the urinary content of 8-iso-PGF2α (also known as 8-epi-PGF2α or 15-isoprostane F2t), a stable product of arachidonic acid formed on nonenzymatic oxidation. Untimed (“spot”) urine samples were collected in the morning after at least a 3-h fast, temporarily stored at 0°C for up to 1 h, and then maintained at −80°C until analysis; urinary content of 8-iso-PGF2α was indexed to creatinine concentration (measured by autoanalyzer) (Beckman-Coulter, Brea, California) and expressed as pg/mmol creatinine. The content of 8-iso-PGF2α was determined with a commercially available enzyme-linked immunosorbent assay (AMS Biotechnology [Europe], Ltd., Abingdon, United Kingdom). In brief, after sample dilution, 15-isoprostane F2t competes with the same antigen conjugated to horseradish peroxidase for binding to a polyclonal antibody-coated microplate. Tetramethylbenzidine substrate addition results in blue color inversely proportional to the amount of 15-isoprostane F2t. Addition of an acid stop solution causes a color change to yellow, for which absorbance is read at 450 nm. The assay sensitivity was 50 pg, with a dynamic range of 0.1 to 10 ng/ml, and the average intra-assay coefficient of variation for 24 replicate samples was 1.72%. Cross-reactivities were undetectable (<0.01%) for prostaglandins F2α, E2, D2, or arachidonic acid. The correlation between this method and GC/MS was $r^2 > 0.8$ ($t = 0.89$), as has been noted for previous isoprostane immunosassays (38,39). Urinary content of 8-iso-PGF2α was indexed to creatinine concentration (measured by autoanalyzer) (Beckman-Coulter) and expressed as pg/mmol creatinine.
CATECHOLAMINES. Blood samples for measurement of plasma catecholamines were drawn into ethylenediaminetetraacetic acid anticoagulant tubes and promptly chilled to 0°C before separation of plasma and storage at −70°C. Urine samples were similarly stored. Batched, previously unthawed samples were subjected to a sensitive radioenzymatic assay based on catechol-O-methylation (40). Intraassay coefficients of variation were: norepinephrine 4%, and epinephrine 13%. Inter-assay coefficients of variation were: norepinephrine 10%, and epinephrine 16%. Urine catecholamine values were normalized to creatinine excretion in the same sample.

CHROMOGRANIN B. The CHGB region-specific radioimmunoassays (CHGB[439–451], CHGB[566–577]) were based on synthetic peptides, as previously described (41,42). The [125I]-radiolabeling of each peptide was enabled by an endogenous or adventitious (terminal) Tyr residue. Polyclonal rabbit antisera were developed for the synthetic CHGB regions as described (43).

OTHER ASSAYS. Plasma lipids and apolipoproteins (224 individuals) were measured as previously described (44). Plasma insulin, leptin, and C-reactive protein were measured by immunoassay. Plasma glucose and plasma and urine electrolytes were measured by autoanalyzer (Beckman-Coulter).

Calculations. Endogenous insulin sensitivity (or resistance) was estimated from fasting plasma glucose and insulin values by HOMostatic Assessment model (HOMA), an index of insulin resistance (45).

Statistical analyses. Descriptive statistics (mean ± SEM) were computed across all of the twins, with generalized estimating equations (PROC GENMOD in SAS [SAS, Cary, North Carolina]) to take into account intra–twin-pair correlations (46). For isoprostane excretion values (pg/mmol), initial skewness = 5.3 ± 0.1, and kurtosis = 50.0 ± 0.2; therefore, values were Log10-transformed for parametric statistical analyses, including h² by Sequential Oligogenic Linkage Analysis Routines (SOLAR) and generalized estimating equations (post-Log10-transformation, skewness = −1.3 ± 0.3, and kurtosis = 6.2 ± 0.2). Trait-on-trait nonparametric Spearman correlations were performed with 1 individual per twin pair, to avoid false positive conclusions from nonindependent observations.

Estimates of h² (h² = V_G/V_P, where V_G is additive genetic variance and V_P is total phenotypic variance) were obtained with the variance-component methodology implemented in the SOLAR package (47,48). This method maximizes the likelihood of assuming a multivariate normal distribution of phenotypes in twin pairs (monozygotic vs. dizygotic) with a mean dependent on a particular set of explanatory covariates. The null hypothesis (H₀) of no h² is tested by comparing the full model, which assumes genetic variation (V_G), and a reduced model, which assumes no genetic variation, with a likelihood ratio test. All h² estimates were adjusted for age and sex, because of the effects of these covariates on several traits (Table 1).

Pleiotropy (genetic covariance for 2 correlated, heritable traits; the cross-product of the trait heritabilities) (26) was estimated as the parameter ρ_G in SOLAR (47). Sequential Oligogenic Linkage Analysis Routines was also used to estimate the environmental covariance, as parameter ρ_E.

Haplotypes were inferred from common single nucleotide polymorphisms (minor allele frequency >10%) of CHGB by the HAP algorithm (49), which can also generate a likely phylogeny for each variant. Pair-wise linkage disequilibrium (LD) between common SNPs was quantified as r²=100, by Haploview (50).

Data were stored in Microsoft Access, and analyses were conducted in SPSS (SPSS, Chicago, Illinois), SAS, or SOLAR.

Results

Heritability of isoprostane excretion. Trait h² (the proportion of trait variation accounted for by genetic variation; h² = V_G/V_P) was estimated from twin pair correlations and is shown in Table 1, expressed as percentage (i.e., h² scaled from 0% to 100%). Urinary isoprostane excretion h² is also displayed graphically, within the context of other features of the metabolic syndrome, in Figure 1. Heritability was significant (p = 5.48E-22) for isoprostane excretion, at 65.8 ± 4.3% of trait variance. Table 1 lists h² for a variety of traits, including multiple features of the metabolic syndrome as well as a number of autonomic traits, both biochemical (e.g., catecholamines) and physiological (e.g., heart rate variability).

Isoprostane trait correlations. Nonparametric (Spearman) correlations of urinary isoprostane excretion with other traits are also shown in Table 1. Several phenotypes correlated with urinary isoprostane excretion, both physical/physiological and biochemical. Prominent correlations were found for urinary catecholamine excretion, plasma CHGB_439 – 451, glomerular filtration rate, and several components of the metabolic syndrome: C-reactive protein, leptin, insulin, HOMA, and vascular compliance.

Urinary isoprostane excretion extreme groups: associated traits. The Online Table 1 and Online Figure 1 dichotomize the twins/siblings above and below the urinary isoprostane median value of approximately 22.9 pg/mmol (normalized to creatinine). Individuals with higher urinary isoprostane excretion displayed a number of significant trait differences, both biochemical (chromogranin, catecholamine, inflammatory) and physiological (body mass index, insulin resistance, cardiac, autonomic, and renal). Once again, several of the isoprostane-associated traits are components of the metabolic syndrome.

Pleiotropy (shared h²): genetic covariance with isoprostane excretion. We tested several heritable traits that correlated with urinary isoprostane excretion for shared genetic determination (pleiotropy) with urinary isoprostane excretion (Table 1, Fig. 2). The results indicate that urinary isoprostane excretion shares significant genetic determina-
tion (pleiotropy, $\rho_C$) with body mass index, HOMA, catecholamines, CHGB, leptin, baroreceptor coupling, and oxidized phospholipids. For CHGB, $\rho_C$ with isoprostane was 0.27 ± 0.10 ($p = 0.007$). By contrast, several correlated traits also shared significant environmental determination (environmental covariance, $\rho_E$) with isoprostane: HOMA, oxidized phospholipids, and arterial distensibility. Two traits displayed both $\rho_C$ and $\rho_E$ with isoprostane: HOMA and oxidized phospholipids.

Figure 2 illustrates the substantial shared hereditary determination ($\rho_C$), without significant environmental co-determination ($\rho_E$), for isoprostane excretion with
plasma leptin, norepinephrine, oxidized phospholipids, and CHGB.

Hypertension and isoprostane excretion. Online Figure 1 stratifies 519 subjects by isoprostane quantile and BP status: hypertensive (n = 56) versus normotensive (n = 463). We observed that subjects with elevated isoprostane excretion were over-represented in the hypertension group (p = 0.0126).

The CHGB gene: pleiotropic genetic associations with CHGB secretion and isoprostane excretion. Because isoprostane excretion associated (Online Table 1) and aggregated (Online Fig. 1) with CHGB expression and shared genetic determination with CHGB (Fig. 2), we tested whether genetic variation at the CHGB locus might also influence the isoprostane trait. To visualize patterns of SNP associations, we constructed pair-wise LD maps from 3 common SNPs (minor allele frequencies >5%) in the regulatory regions (promoter or 3’-UTR) of CHGB. Two blocks of LD were noted by Haploview (49)(Fig. 3). The most common haplotype spanning the locus (haplotype-1: CTC) associated with isoprostane in twins: carriers of haplotype-1 (1 or 2 copies; n = 196), as compared with subjects without haplotype-1 (0 copies; n = 137), displayed reduced isoprostane excretion (28.7 ± 1.79 pg/mmol vs. 25.0 ± 1.17 pg/mmol, chi-square = 5.24, p = 0.022).

Because 3’-UTR SNP C+84A seemed to reside in a block of relatively low LD with the promoter region (r² = 0.19 to 0.20) (Fig. 3), we tested the effect of the 3’-UTR variant separately (Table 2); A allele homozygosity (A/A) elevated plasma CHGB (p = 0.0123) as well as isoprostane excretion (p = 0.0111). For the 2 promoter SNPs (A-296C, A-261T), no associations with isoprostane excretion were found (Table 2).

CHGB 3’-UTR variant function in cells. To test whether the CHGB 3’-UTR C+84A variant is itself functional, we inserted the 308-bp 3’-UTR into a reporter plasmid just downstream from the luciferase open reading frame and derived the minor allele version (+84A) by site-directed mutagenesis (Fig. 4A). Luciferase activities of C+84, +84A, and the “empty” vector were measured sequentially after transfection into chromaffin/PC12 cells (Fig. 4B). The C+84 allele displayed gain-of-function as compared with the empty vector, whereas the +84A variant had loss-of-function. By 36 h, the A allele destabilized expression by approximately 47% compared with the C allele (p = 0.001). Results of transfection of CHGB 3’-UTRs into human neuroblastoma (SH-SY5Y) cells were similar to those in rat pheochromocytoma (PC12) cells.
To pursue the mechanism of action of C/H1100184A, we created a series of mutants in the luciferase/3'-UTR plasmid (Fig. 4A), followed by transfection into PC12 cells (Fig. 4C). Compared with the wild-type (C+84) allele, luciferase reporter activity fell by approximately 21% (p <
when the entire A/U-rich element was deleted. Manipulation of the wild-type C+84 allele, through either deletion of 1 base or replacement by the +84A variant, resulted in more substantial (approximately 31% to 41%) falls in gene expression. The results suggest that the A/U-rich region regulates CHGB expression and that C+84A serves as a crucial switch within this element.

**CHGB 3′-UTR C+84A within an A/U-rich messenger ribonucleic acid element: bioinformatics.** We aligned C+84A and its flanking sequences in humans and other mammalian species by Clustal-W. The C+84A lies in a substantially conserved region of the messenger ribonucleic acid (mRNA) (Fig. 5). The C+84 (human major) allele is found in nonhuman primates (chimp, Rhesus), whereas nonprimate mammals have a T allele at this position. Only the human has the +84A variant, extending an A/U (A/T)-rich region (TTTATATA; position +84 in bold type) suggesting a motif that could affect the mRNA stability and hence overall gene expression (51); a decline in expression with extension of this element is consistent with its role in mRNA instability. Such A/U-rich elements (or “ARE”s) might be targets of trans-acting factors that mediate mRNA instability, such as the Hu family of proteins (52,53).

**Discussion**

**Overview.** The current study demonstrates that isoprostane excretion is substantially heritable (h² = 65.8 ± 4.3%, p = 5.48E-22) and both correlated (Online Table 1) and aggregated (Online Fig. 1) with several components of the metabolic syndrome; indeed, isoprostane displays substantial genetic co-determination with several such traits (Fig. 2), and the isoprostane trait is associated with systemic hypertension (Fig. 6).

Because CHGB and isoprostane shared h² (ρC) (Fig. 2), we tested the CHGB locus for the effects of allelic variation on CHGB and isoprostane traits and found that common functional variation in the CHGB 3′-UTR associated with both traits (Table 2). The results suggest novel functional links among adrenergic pathways, oxidative stress, and hypertension. An unusual feature of this study was the use of the twin method, to dissect out complex features of the mode of inheritance of isoprostane, including h² and pleiotropy (Figs. 1 and 2).

**Role of catecholamines and isoprostanes in hypertension.** Catecholamines are known to trigger oxidative stress through adrenergic receptors in cardiovascular and renal target cells, thereby activating NADPH oxidase isoforms (54–56). Genetic variation at the CHGB locus alters catecholamine secretion in both humans and experimental animals (30,32). We have previously demonstrated evidence of increased oxidative stress in human hypertension, as indicated by elevated hydrogen peroxidase generation (8,9).

The 8-iso-PGF2α not only is a marker of oxidative stress but also participates in the pathophysiology of disease through its direct vasoconstrictive effects, as evidenced in the pulmonary artery (57), coronary arteries (58), cerebral arterioles (59), and portal vein (60). Elevated 8-iso-PGF2α levels have previously been associated with longitudinal risk of developing hypertension (21).

Urinary excretion of isoprostane correlated positively with glomerular filtration rate (ρ = 0.125, p = 0.008) (Table 1). Although we did not measure the plasma concentration of isoprostane, urine and plasma values generally correlate well (61); however, there are occasional discrepancies (62).

**Complex inheritance of isoprostane: pleiotropy.** The twin design allowed us to explore pleiotropy (shared genetic determination of 2 or more traits), documented as the genetic covariance (ρG) (Table 1 and Fig. 2) between correlated traits. Of the many traits correlated or associated with isoprostane excretion, pleiotropy was noted for catecholamine secretion, CHGB, leptin, baroreceptor coupling, apolipoprotein A, and oxidized phospholipids. What specific genes might jointly contribute to such traits? We explored the role of genetic variation at the CHGB locus, because CHGB and isoprostane shared ρC (Fig. 2), yet polymorphisms at other genes not directly scored in this report could jointly contribute to multiple facets of the metabolic syndrome.

**Functional nature of polymorphism at CHGB.** The CHGB is abundant within catecholamine secretory vesicles and might be necessary for inducing secretory granule formation (29). The twin data indicate that the concentrations of CHGB circulating fragments are under substantial genetic control (Table 1), suggesting that

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**Table 2: Associations Among CHGB Genetic Variants, Isoprostane Excretion, and Plasma CHGB**

<table>
<thead>
<tr>
<th>CHGB Regulatory SNP</th>
<th>Genotype</th>
<th>n</th>
<th>Isoprostane Excretion, pg/mmol (log[10])</th>
<th>Plasma CHGB 439–451, nmol/l</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SEM</td>
<td>Chi-Square</td>
</tr>
<tr>
<td>Promoter A-296C (rs236140)</td>
<td>A/A</td>
<td>112</td>
<td>1.39 ± 0.02</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>A/C or C/C</td>
<td>187</td>
<td>1.45 ± 0.03</td>
<td>2.31</td>
</tr>
<tr>
<td>Promoter A-261T (rs236141)</td>
<td>A/A</td>
<td>112</td>
<td>1.39 ± 0.02</td>
<td>2.31</td>
</tr>
<tr>
<td></td>
<td>A/T or T/T</td>
<td>187</td>
<td>1.34 ± 0.02</td>
<td>2.31</td>
</tr>
<tr>
<td>3′-UTR C+84A (C13612A in gene, rs2821)</td>
<td>C/C or C/A</td>
<td>273</td>
<td>1.34 ± 0.02</td>
<td>6.45</td>
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<tr>
<td></td>
<td>A/A</td>
<td>61</td>
<td>1.44 ± 0.02</td>
<td>6.45</td>
</tr>
</tbody>
</table>

By generalized estimating equations; age-, sex- and glomerular filtration rate (GFR)-adjusted. *Significant effects (p < 0.05).

CHGB = chromogranin B; SNP = single nucleotide polymorphism.
Figure 4 Functional Evaluation of Common Allelic Variation in the CHGB 3'UTR: C+84A (C13612A)

(A) Human CHGB 3'UTR reporter plasmid construction. The 308-bp CHGB 3'UTR fragment was amplified from human genomic deoxyribonucleic acid and (pGL3-Promoter, with the SV40 early promoter) (Promega, Madison, Wisconsin), just 3' to the luciferase open reading frame (ORF) but upstream from the polyadenylation (pA) signal. Correct insert orientation was confirmed by asymmetric double digestion with restriction enzyme MfeI, which cuts once within the insert, and nearby in the vector at position 2103, within the SV40 pA cassette. Variants were created by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, California), and mutations were verified by direct sequencing before use. Point mutations were confirmed by dideoxy sequencing. (B) CHGB common 3'UTR C+84A polymorphism affects transfected gene expression. When transfected into chromaffin (rat pheochromocytoma, PC12) cells, 3'UTR variant C+84A influenced luciferase reporter gene expression in vitro, in a direction (C>A) coordinate with the in vivo effects. Compared with the empty pGL3-Promoter vector alone, the C+84A exhibits a gain of function, whereas +84A has a loss of function. At 36 h, the A allele destabilized expression by approximately 47% (compared with the C allele). (C) CHGB 3'UTR: Function of A/U (A/T)-rich element containing C+84A on luciferase reporter activity in transfected PC12 chromaffin cells. Several mutations were created in the A/U-rich region. Removal of the entire A/U-rich region resulted in an approximately 21% fall in gene expression. Manipulation of the wild-type C+84 allele, through either deletion of 1 base or replacement by the +84A variant, resulted in more substantial (approximately 31% to 41%) declines in gene expression. ANOVA = analysis of variance; CHGB = chromogranin B.
circulating CHGB might be a useful “intermediate phenotype” (63) in the pathogenesis of hypertension. The CHGB has both extracellular and intracellular roles in the neuroendocrine system (64), and its deficiency is associated with dysregulated catecholamine secretion and hypertension (32).

Several findings in this study suggested that polymorphism in the 3′-UTR of CHGB might be functionally important for inter-individual variation in autonomic as well as oxidative traits. First of all, the 3′-UTR region associated with plasma CHGB concentration as well as isoprostane excretion (Table 2). Second, 3′-UTR common polymorphism C/H1100184A influenced gene expression in cella (Fig. 4B), perhaps by altering an A/U-rich stability motif in a conserved sequence within the mRNA (Fig. 4C). Third, the changes conferred by allelic variation at C/H1100184A seemed to be directionally consistent: the C/H1100184 (major) allele elevated gene expression both in cella (Fig. 4B) and in vivo (Fig. 7). Finally, because the 3′-UTR region of CHGB displays only minimal LD with the remainder of the CHGB locus (Fig. 3), it is perhaps not surprising that the 3′-UTR might exert a relatively independent contribution to the regulation of CHGB expression.

Conclusions and Perspectives
Isoprostane excretion is a highly heritable trait, displaying joint genetic determination (pleiotropy) with such traits as CHGB, catecholamines, leptin, baroreceptor coupling, lipids, and oxidized phospholipids. On the
basis of pleiotropic genetic control of isoprostane and CHGB (Fig. 2), we tested the role of CHGB genetic variation and found that a common polymorphism in the CHGB 3′-UTR that alters an A/U-rich mRNA stability element (Fig. 4C) contributes to trait variation in CHGB and isoprostane (Table 2). Therefore we propose the following model to unify our results in a parsimonious fashion: CHGB 3′-UTR C+84A variation (C→A at position +84) destabilizes CHGB mRNA, leading to decreased CHGB expression, prompting unregulated catecholamine release (32), thereby triggering oxidative stress (54,55). The twin method used here initially focused on healthy individuals rather than subjects with overt cardiovascular disease; only longitudinal/follow-up studies can establish the ultimate risk conferred by CHGB genotype or elevated isoprostane in these subjects. However, our results suggest novel pathophysiological links between the CHGB gene and oxidative stress, inflammation, and hypertension and suggest new strategies for probing the role and actions of oxidative stress within this setting.

REFERENCES


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Key Words: catecholamine • CHGB • hypertension • isoprostane excretion • metabolic syndrome • oxidative stress • twin study.

APPENDIX

For accompanying tables and figures, please see the online version of this article.