Catestatin Gly364Ser Variant Alters Systemic Blood Pressure and the Risk for Hypertension in Human Populations via Endothelial Nitric Oxide Pathway

Malapaka Kiranmayi, Venkat R. Chirasani, Prasanna K.R. Allu, Lakshmi Subramanian, Elizabeth E. Martelli, Bhavani S. Sahu, Durairajpandian Vishnuprabu, Rathnakumar Kumaragurubaran, Saurabh Sharma, Dhanasekaran Bodhini, Madhulika Dixit, Arasambattu K. Munirajan, Madhu Khullar, Venkatesan Radha, Viswanathan Mohan, Ajit S. Mullasari, Sathyamangla V. Naga Prasad, Sanjib Senapati, Nitish R. Mahapatra

Abstract—Catestatin (CST), an endogenous antihypertensive/antiadrenergic peptide, is a novel regulator of cardiovascular physiology. Here, we report case–control studies in 2 geographically/ethnically distinct Indian populations (n≈4000) that showed association of the naturally-occurring human CST-Gly364Ser variant with increased risk for hypertension (age-adjusted odds ratios: 1.483; *P*=0.009 and 2.951; *P*=0.005). Consistently, 364Ser allele carriers displayed elevated systolic (up to ≈8 mm Hg; *P*=0.004) and diastolic (up to ≈6 mm Hg; *P*=0.001) blood pressure. The variant allele was also found to be in linkage disequilibrium with other functional single-nucleotide polymorphisms in the *CHGA* promoter and nearby coding region. Functional characterization of the Gly364Ser variant was performed using cellular/molecular biological experiments (viz peptide–receptor binding assays, nitric oxide [NO], phosphorylated extracellular regulated kinase, and phosphorylated endothelial NO synthase estimations) and computational approaches (molecular dynamics simulations for structural analysis of wild-type [CST-WT] and variant [CST-364Ser] peptides and docking of peptide/ligand with β-adrenergic receptors [ADRB1/2]). CST-WT and CST-364Ser peptides differed profoundly in their secondary structures and showed differential interactions with ADRB2; although CST-WT displaced the ligand bound to ADRB2, CST-364Ser failed to do the same. Furthermore, CST-WT significantly inhibited ADRB2-stimulated extracellular regulated kinase activation, suggesting an antagonistic role towards ADRB2 unlike CST-364Ser. Consequently, CST-WT was more potent in NO production in human umbilical vein endothelial cells as compared with CST-364Ser. This NO-producing ability of CST-WT was abrogated by ADRB2 antagonist ICI 118551. In conclusion, CST-364Ser allele enhanced the risk for hypertension in human populations, possibly via diminished endothelial NO production because of altered interactions of CST-364Ser peptide with ADRB2 as compared with CST-WT. **(*Hypertension*. 2016;68:334-347. DOI: 10.1161/HYPERTENSIONAHA.116.06568.)** ● **Online Data Supplement**

Key Words: chromogranin A ■ genetic association study ■ genetic variation ■ hypertension ■ nitric oxide

Chromogranin A (CHGA) is a ≈50-kDa soluble, acidic glycoprotein that plays an essential role in the formation of catecholamine secretory vesicles in neuronal, endocrine, and neuroendocrine tissues.[1] Expression levels of *CHGA* have been found to be elevated in rodent models of both genetic[2] and acquired forms of hypertension.[3] Elevated plasma CHGA levels are associated with clinical severity and serve as independent prognostic indicators in patients with complicated myocardial infarction,[4] acute coronary syndromes,[5] and chronic heart failure.[6]

CHGA also acts as a prohormone and gets cleaved to give rise to several bioactive peptides,[7] including vasostatin (human $CHGA_{1–76}$, a vasodilator and suppressor of inotropy/lusitropy),[8] pancreastatin (human $CHGA_{250–301}$, a dysglycemic hormone),[9]

Received April 21, 2016; first decision May 14, 2016; revision accepted May 17, 2016.

From the Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, Tamil Nadu, India (M.Kiranmayi, V.R.C., P.K.R.A., L.S., B.S.S., R.K., M.D., S.Senapati, N.R.M.); Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, OH (E.E.M., S.V.N.P.); Department of Genetics, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, Tamil Nadu, India (D.V., A.K.M.); Department of Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education and Research, Chandigarh, India (S.Sharma, M.Khullar); Department of Molecular Genetics, Madras Diabetes Research Foundation, Chennai, Tamil Nadu, India (D.B., V.R., V.M.); Institute of Cardiovascular Diseases, Madras Medical Mission, Chennai, Tamil Nadu, India (A.S.M.); Department of Medicine, University of California San Francisco (P.K.R.A.); and Department of Clinical Biochemistry, University of Cambridge, Cambridge, United Kingdom (B.S.S.).

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.116.06568/-/DC1.

Correspondence to Nitish R. Mahapatra, Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai 600036, India. E-mail nmahapatra@iitm.ac.in

catestatin (CST; human $CHGA_{352–372}$, an antihypertensive and cardiosuppressive agent), parathyroid hormone release inhibitor parastatin (human $CHGA_{356–428}$),[10] and serpinin (human $CHGA_{411-436}$, a myocardial β-adrenergic–like agonist).[11] CST was discovered initially as a physiological brake of the adreno–sympathetic–chromaffin system because of its potent catecholamine release–inhibitory function,[12,13] which it manifests by acting specifically on the neuronal nicotinic acetylcholine receptor.[14,15] Plasma CST level is diminished in hypertensive individuals and even in the normotensive offspring of the established hypertensive patients, suggesting its pathogenic role in the development of hypertension.[16] Consistently, severe hypertension in *CHGA* knockout (and thereby, CST-lacking) mice is rescued by the exogenous administration of CST, revalidating its role as an antihypertensive molecule.[17] Many functionally active DNA variants have been discovered in the promoter, coding and 3′-untranslated regions of the human *CHGA* gene.[7,18] Resequencing of the CST-expressing region of *CHGA* in several human populations has revealed the occurrence of 5 single-nucleotide polymorphisms (SNPs; Table S1 in the online-only Data Supplement). A previous report from our laboratory has confirmed the presence of Gly364Ser (rs9658667) variation and, in addition, discovered a novel SNP, Gly367Val (rs200576557), in a Chennai (South India) population.[19] In this report, we analyzed the effect of the Gly364Ser variation on metabolic/cardiovascular disease states in a larger sample size (n=3200 individuals) in the Chennai population. As part of a replication study, we also genotyped the variant in a geographically/ethnically distinct North Indian population from Chandigarh (n=760 individuals). In both the populations, the 364Ser allele showed strong associations with elevated blood pressure (BP) levels and hypertension.

CST peptides have been found to dose dependently reduce the effect of β-adrenergic stimulation.[20] This reduction is mediated by a nitric oxide (NO)–releasing action of CST in endocardial endothelial cells, rather than a direct myocardial action of the peptide. Studies in the ex vivo models of Langendorff-perfused rat heart,[21] amphibian (*Rana esculenta*) heart,[22] and fish (*Anguilla anguilla*) heart[23] have also documented the antiadrenergic and cardiac inotropy/lusitropy modulatory effects of CST. On the basis of these observations, we questioned whether regulation of NO generation by CST peptides is because of their direct interactions/effects on β-adrenergic receptors (ADRB1/2). To understand the mechanistic basis of differential BP manifestations in the individuals because of CST peptides, we performed biochemical studies to assess NO generation, extracellular regulated kinase (ERK) activation, endothelial nitric oxide synthase (eNOS) phosphorylation, and the direct binding of CST peptides to ADRB1/2. In addition, we used a comprehensive set of computational tools including molecular modeling, docking, and molecular dynamics simulations to analyze the potential of CST-WT and CST-364Ser peptides to bind to ADRB1/2. CST-364Ser peptide exerted altered interactions with ADRB2 and led to diminished endothelial NO production (as compared with the CST-WT peptide), which may account for the increased risk for hypertension in 364Ser carriers.

Methods

The detailed methodologies are included in the online-only Data Supplement.

Human Subjects and Study Design

This case–control study recruited 3200 and 760 unrelated human volunteers in Chennai (South India) and Chandigarh (North India), respectively. The detailed demographic and clinical parameters are given in the Tables S2 and S3.

Each subject gave informed, written consent for the use of their blood samples for genetic and biochemical analyses in this study. This study was approved by the Institute Ethics Committee at Indian Institute of Technology Madras in accordance with Declaration of Helsinki (reference number: IITM IEC No 2007008).

The exon-7 region of *CHGA* was resequenced in 1763 subjects to detect the presence of SNPs in CST, pancreastatin, and parastatin domains. Another 2197 subjects were genotyped for the Gly364Ser SNP by Taqman allelic discrimination method. We also resequenced the *CHGA* promoter region in 581 study subjects using specific primers.[19]

Data Representation and Statistical Analysis

The experimental data results and the phenotypic characteristics in the human study are expressed as mean±SE. Allele frequencies were estimated by gene counting. A Pearson χ^2 test was used to compare the distribution of the genotypes. Statistical analysis was performed using the Statistical Package for Social Sciences version 21.0. Haploview 4.2 was used for linkage disequilibrium (LD) analysis.[24] A P value of <0.05 was chosen as statistically significant. The power of the study was calculated using Quanto version 1.2.4.[25] Meta-analysis was performed using the OpenMeta[Analyst] software (www.cebm.brown.edu/open_meta/).

Synthesis of CST Peptides

The CST wild-type (CST-WT, SSMKLSFRARAYGFRGPGPQL) and CST-364Ser variant (CST-364Ser, SSMKLSFRARAY**S**FRGPGPQL) peptides were synthesized by solid-phase method and purified as described previously.[19]

Measurement of NO Levels and eNOS Activity in Cultured Human Umbilical Vein Endothelial Cells

Experimental procedures involving umbilical cords were reviewed and approved by the Indian Institute of Technology Madras Institutional Ethics Committee in accordance with Declaration of Helsinki revised in 2000 (reference number: IITM IEC No 2009024). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords by digestion with collagenase as described previously.[26] NO levels in HUVECs were measured by 4, 5-Diaminofluorescein diacetate method as described previously.[27] Activation of eNOS in HUVECs by CST peptides was assessed by Western blotting and detection of phospho-eNOS-Ser^{1177}.

Isolation of ADRB1/2-Expressing Plasma Membranes, Radioligand Binding Assays, and Competition Binding Assays

Human Embryonic Kidney-293 (HEK-293) cells stably expressing ADRB1/2 were treated with isoproterenol and CST peptides. Activation of ERK as a measure of ADRB1/2-activation in ADRB1/2 HEK-293 cells was assessed by immunoblotting and detection of phospho-ERK as previously described.[28]

Purification of plasma membranes from control HEK-293 and ADRB1/2 HEK-293 cells was performed as previously described.[29,30] To test the level of ADRB1/2 expression, [^{125}I]-cyanopindolol saturation radioligand binding was performed on the isolated plasma membranes. Competition binding was performed by incubating 20 μg of plasma membranes with saturating concentrations of CST-WT and CST-364Ser peptides in the range of 10 pmol/L to 1 mmol/L.

Homology Modeling of ADRB1/2 Receptors and CST Peptides and Analysis of Peptide–Receptor Interactions

The crystal structure of ADRB2 with resolution 2.4 Å was obtained from protein data bank (PDB ID:2RH1).[31] The structure of ADRB1

was modeled by using the structure of ADRB2 as a template. The 3D structures of CST-WT and CST-364Ser were generated following a similar protocol as proposed earlier.[32] The residue Gly364 in the NMR (nuclear magnetic resonance) structure of CST (PDB ID:1LV4) was mutated to 364Ser using Modeller 9v13.[33] Short energy minimizations were performed on both the peptide structures to optimize the side-chain positions. The minimized structures were subsequently subjected to 300 ns explicit water molecular dynamics simulations to generate an ensemble of refined CST-WT and CST-364Ser conformations.

Protein–protein dockings of CST peptides on ADRB1/2 were performed using ZDOCK algorithm.[34] During molecular docking, CST peptides were allowed to search the extracellular region of the ADRB1/2 receptors to identify the best binding location. Out of the 100 binding modes of CST peptides to ADRB1/2, the best docked complex was identified based on the ZDOCK score.

All the structural figures were rendered using Visual Molecular Dynamics.[35] The CST–ADRB2 interactions were identified using PDBsum,[36] and cyanopindolol–ADRB2 interactions were identified using LigPlot+.[37]

Results

Discovery and Occurrence of the CST-Gly364Ser SNP in Indian Populations

Resequencing of the CST region of *CHGA* in 1763 subjects from an urban Chennai (South Indian) population consisting of type 2 diabetes mellitus (DM)/hypertension cases and controls led to discovery of 2 variants: Gly364Ser (rs9658667) and Gly367Val (rs200576557). Because the Gly364Ser variation was common (>5% minor allele frequency [MAF]), we genotyped additional 918 subjects for this SNP by Taqman allele discrimination method in the same population. We also genotyped the SNP in a population of 519 patients with coronary artery disease (CAD) from the same region (Chennai). The Gly364Ser SNP, which is caused by an A to G transition at the 9559 bp position leading to the substitution of codon GGC by codon AGC at the 364th amino acid position of the mature CHGA protein (Figure S1), was found to occur at 6.34% MAF, that is, in ≈13% of the study population (Table S4). We then performed a second replication study in a population from Chandigarh (North Indian) consisting of hypertensive cases and controls. Here, surprisingly, we found the SNP at a much lower MAF (3.48%), that is, only in ≈7% of the population, without the presence of a single homozygous variant in 760 individuals (Table S4).

Genotype frequencies were found to be in Hardy–Weinberg equilibrium (HWE) in the Chennai DM/hypertension (χ^2=1.286; P=0.256) population, Chennai CAD population (χ^2=0.250; P=0.617), Chandigarh hypertension population (χ^2=1.047; P=0.306), and Chandigarh controls population (χ^2=0.142; P=0.706). The Chennai controls population, however, showed a departure from Hardy–Weinberg equilibrium (χ^2=6.799; P=0.009). On closer observation of the genotypes in this population, we found that the presence of only 2 homozygous variants accounted for this departure.

364Ser Allele Is Associated With Hypertension in Independent Populations

The Chennai population was divided into different disease groups (DM, hypertension, and CAD), and logistic regression analysis was performed under both the genotype (GG versus AG and GG versus AA) and dominant (GG versus AG+AA) genetic models. Because of the small number of homozygous variant individuals (n=18), the recessive model was not used. Gly364Ser allele was found to be associated with hypertension under both the models, with unadjusted odds ratios (OR) of 1.440 (95% confidence interval [CI], 1.072–1.933; P=0.015) for GG versus AG and 1.385 (CI, 1.039–1.846; P=0.026) for GG versus AG+AA (Table 1). The associations retained significance even after adjusting individually for age, sex, and body mass index, as well as all 3 factors together (Table 1). An additional adjustment for antihypertensive medications along with age, sex, and body mass index also showed significant association for GG versus AG+AA at OR=1.694 (CI, 1.018–2.819; P=0.042). Interestingly, stronger associations of the 364Ser allele with hypertension were detected in a subgroup (having body mass index <24) of this population under both dominant genetic model (unadjusted OR, 1.856; CI, 1.234–2.792; P=0.003; age-adjusted OR, 1.983; CI, 1.312–2.997; P=0.001; and sex-adjusted OR, 1.856; CI, 1.227–2.809; P=0.003) and GG versus AG genotype model (unadjusted OR, 1.754; CI, 1.155–2.662; P=0.008; age-adjusted OR, 1.854; CI, 1.216–2.826; P=0.004; and sex-adjusted OR, 1.775; CI, 1.164–2.709; P=0.008). The 364Ser allele also showed higher frequency in subjects having any of the 3 disease states (DM/hypertension/CAD). Although the unadjusted ORs were not significant, adjustment with age yielded modestly significant ORs of 1.325 (CI, 1.024–1.714; P=0.032) and 1.299 (CI, 1.011–1.667; P=0.041) under the genotype and dominant models, respectively.

The replication population (Chandigarh) also showed strong association of the 364Ser allele with hypertension.

Table 1. Association of CST-364Ser Allele With Risk for Hypertension

Population	Genotype	OR (95% CI); *P* Value				
		Unadjusted	Age Adjusted	Sex Adjusted	BMI Adjusted	Age, Sex, and BMI Adjusted
Chennai	GG vs AG	1.440 (1.072–1.933); *P*=0.015	1.483 (1.103–1.994); *P*=0.009	1.431 (1.063–1.926); *P*=0.018	1.441 (1.072–1.938); *P*=0.015	1.469 (1.087–1.984); *P*=0.012
Chennai	GG vs AG+AA	1.385 (1.039–1.846); *P*=0.026	1.429 (1.070–1.907); *P*=0.015	1.380 (1.033–1.845); *P*=0.030	1.393 (1.043–1.858); *P*=0.025	1.424 (1.062–1.909); *P*=0.018
Chandigarh	GG vs AG	2.662 (1.420–4.990); *P*=0.002	2.951 (1.390–6.265); *P*=0.005	2.639 (1.389–5.013); *P*=0.003	n.c.	n.c.

Logistic regression analysis was performed in the Chennai and Chandigarh populations. Odds ratio for hypertension was analyzed. The analyses were done both by the genotype (GG vs AG and GG vs AA) and dominant (GG vs AG+AA) genetic models for the Chennai population. BMI indicates body mass index; CI, confidence interval; n.c., not calculable because of unavailability of BMI data in this study population; and OR, odds ratio.

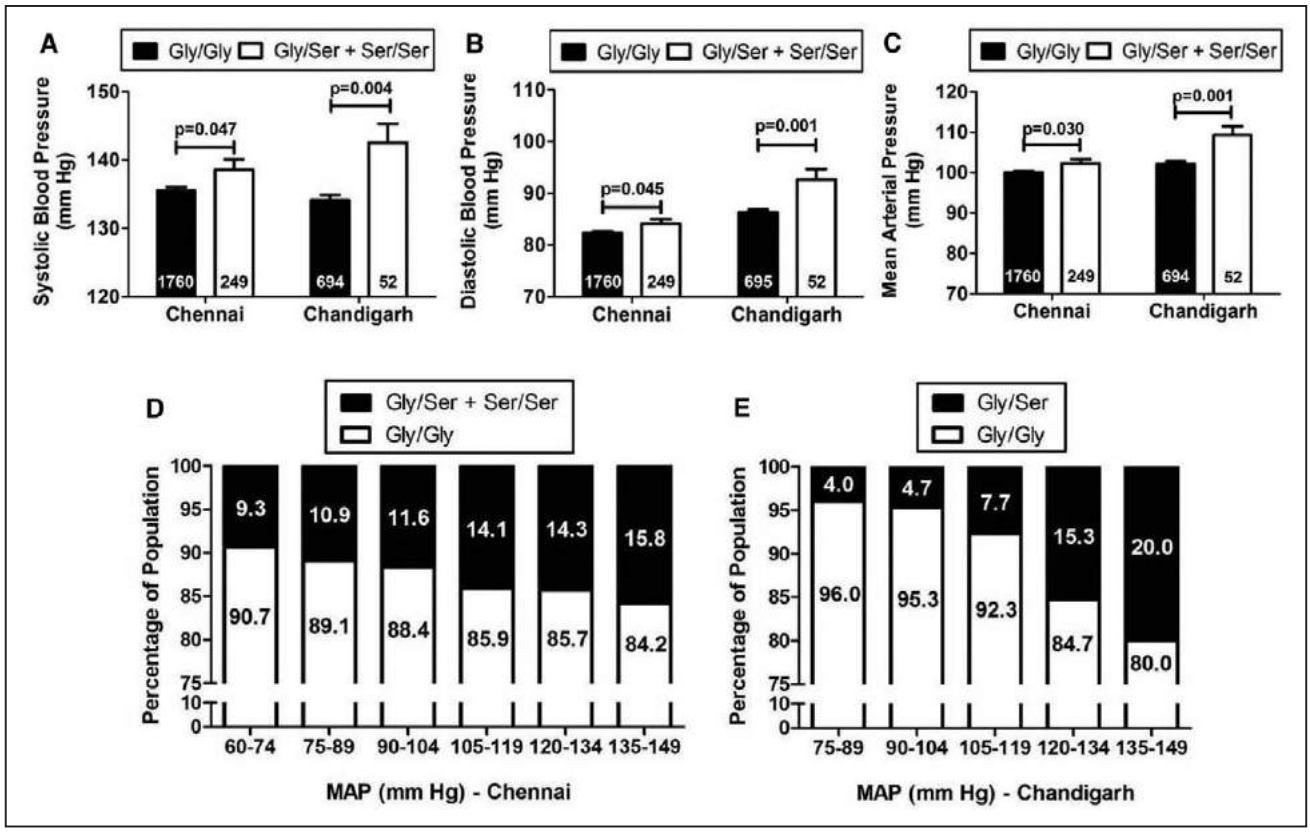

Figure 1. Allele-specific associations of the catestatin (CST) Gly364Ser variation with blood pressure. **A–C**, Data are shown as mean±SE. Systolic blood pressure (**A**), diastolic blood pressure (**B**), and mean arterial pressure (MAP; **C**) levels in the carriers of 364Ser allele were higher (analyzed by independent samples *t* test using SPSS version 21.0) than the wild-type individuals in the overall Chennai and Chandigarh populations. **D** and **E**, Data are shown as percentage. The percentage of individuals harboring the 364Ser allele showed an increase with increase in the range of the MAP levels in both the Chennai (**D**) and Chandigarh (**E**) populations.

Because there were no homozygous variant individuals identified in this population, the logistic regression analysis based on both genotype and dominant models yielded the same results. The unadjusted OR was highly significant at 2.662 (CI, 1.420–4.990; *P*=0.002; Table 1). The association persisted even after adjusting for age at OR=2.951 (CI, 1.390–6.265; *P*=0.005), sex at OR=2.639 (CI, 1.389–5.013; *P*=0.003), and age and sex together at OR=2.862 (CI, 1.359–6.028; *P*=0.006) (Table 1). Adjusting for smoking and dyslipidemia as well (data for which was not available for a large section of our study subjects) would have made this study stronger.

Association of 364Ser Variant With Elevated BP Levels in Independent Populations

In the primary Chennai population, there was a significant trend of increased BP levels in the carriers of the 364Ser allele as compared with the WT individuals. Initially, we compared the BP levels among the different genotype groups in 2069 individuals from the overall DM/hypertension population. The variant individuals had ≈2.5 mm Hg higher systolic BP (SBP; *P*=0.045), ≈1.5 mm Hg higher diastolic BP (DBP; *P*=0.074), and ≈2 mm Hg higher mean arterial pressure (MAP; *P*=0.043) levels than the WT individuals. Next, to adjust for the effect of the antihypertensive medications, 60 individuals without information for antihypertensive medication were removed, and the analysis was repeated after adjusting the BP values in the remaining 2009 individuals.[38] After drug adjustment, 364Ser carriers displayed ≈3 mm Hg higher SBP (*P*=0.047), ≈2 mm Hg higher DBP (*P*=0.045), and ≈2.5 mm Hg higher MAP (*P*=0.030) levels as compared with Gly364 carriers (Figure 1A–1C). Adjusting for age via ANCOVA further strengthened the association for SBP (*P*=0.031), DBP (*P*=0.044), and MAP (*P*=0.025; Table 2).

In the Chandigarh population as well, we found 364Ser to be associated with elevated BP levels. The variant allele–carrying individuals showed ≈8 mm Hg higher SBP (*P*=0.004), ≈6 mm Hg higher DBP (*P*=0.001), and ≈7 mm Hg higher MAP (*P*=0.001) levels than the WT individuals (Figure 1A–1C). For the hypertensive cases, the pretreatment BP levels were considered for association.

We further divided our Chennai and Chandigarh populations into different BP ranges and calculated the frequencies of the 364Ser allele in each range. With an increase in the severity of the disease, there was an increase in the percentage of people harboring the variant allele (for Chennai: linear-by-linear association χ^2=3.99 and *P*=0.046 and for Chandigarh: linear-by-linear association χ^2=12.89 and *P*=0.0003; Figure 1D and 1E).

The unadjusted power of the study for the hypertensive Chennai population was 65.6% and on adjusting for age, sex, and body mass index, the power of the study stood at 73.1%. For the Chandigarh population, the unadjusted power was 98.5%, whereas power adjusted for age and sex was 98.3%.

Table 2. Meta-Analysis of the 364Ser Allele Effects on Blood Pressure in Asian Populations

Population	Parameter	Gly/Gly			Gly/Ser+Ser/Ser			Effect Size	95% Confidence Interval		Unadjusted *P* Value	Adjusted *P* Value (ANCOVA)
		n	Mean	SEM	n	Mean	SEM		Lower Boundary	Upper Boundary		
Chennai/ South Indian	SBP	1760	135.50	0.54	249	138.57	1.52	3.07	–0.09	6.23	0.047	0.031
	DBP	1760	82.33	0.31	249	84.12	0.84	1.79	0.03	3.55	0.045	0.044
	MAP	1760	100.04	0.37	249	102.3	1.00	2.26	0.17	4.35	0.030	0.025
Chandigarh/ North Indian	SBP	694	134.12	0.76	52	142.54	2.70	8.42	2.92	13.92	0.004	0.009
	DBP	695	86.33	0.49	52	92.65	2.00	6.32	2.28	10.36	0.001	0.004
	MAP	694	102.24	0.55	52	109.29	2.07	7.05	2.85	11.25	0.001	0.003
Ibaraki, Saitama, Shizuoka/ Japanese	SBP	301	132.00	1.14	42	138.20	2.72	6.20	0.42	11.98	0.055	0.048
	DBP	301	80.30	0.60	42	82.00	1.37	1.70	–1.23	4.63	0.314	n.s.
	MAP	301	100.7	0.86	42	104.50	1.97	3.80	–0.41	8.01	0.117	n.s.
	PP	301	51.70	0.72	42	56.10	1.92	4.40	0.382	8.418	0.030	0.025
Overall population	SBP	2755	…	…	343	…	…	5.21	1.92	8.50	<0.01	…
	DBP	2756	…	…	343	…	…	2.76	0.40	5.11	0.02	…
	MAP	2755	…	...	343	…	…	3.93	1.12	6.73	<0.01	…

Meta-analysis was performed using the data for the effect size of the Gly364Ser SNP in the 3 Asian populations of Chennai, Chandigarh, and Japan. The data for the Japanese population were derived from the study by Choi et al.[59] Age-adjusted ANCOVA was performed in the Chennai and Chandigarh populations. For the Japanese population, the ANCOVA was performed considering gender, age, BMI, antihypertensive medication, diabetes, dyslipidemia, and smoking as covariates. The three independent populations displayed directionally concordant effect on blood pressure. Meta-analysis results show an overall significant effect of elevated blood pressure in 364Ser allele carriers. BMI indicates body mass index; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure calculated from SBP and DBP; n.s., not significant; and SBP, systolic blood pressure.

364Ser Allele Is in LD With *CHGA* Promoter SNPs and Neighboring Exon-7 Coding Variants

Previous studies have discovered the occurrence of common SNPs in the *CHGA* promoter as well as coding regions.[18,19,39] Many of these SNPs have been found to be functionally active, either in altering the transcriptional activity of the promoter[18] or the potencies of the peptides they are found in.[18,32,39,40] We had the complete genotyped data for 581 individuals for the Gly364Ser SNP, 8 *CHGA* promoter SNPs: -1106G→A (rs9658628), -1018A→T (rs9658629), -1014T→C (rs9658630), -988T→G (rs9658631), -462G→A (rs9658634), -415T→C (rs9658635), -89C→A (rs7159323), and -57C→T (rs9658638); and 3 other neighboring SNPs in the *CHGA* exon-7: Gly297Ser (rs9658664), Arg381Trp (rs729940), and Glu403Glu (rs729939). To test whether the Gly364Ser variant is likely to be segregated with any of these other common SNPs, and whether association with the variant is also being contributed by them, we performed an LD analysis using the genotyped data for the 581 subjects. The Gly364Ser variant was found to be in LD with 7 out of the 11 polymorphisms: 4 in promoter (-1106G→A, -1018A→T, -415T→C, and -57C→T) and 3 in *CHGA* exon-7 (Gly297Ser, Arg381Trp, and Glu403Glu; Figure S2).

CST Peptides Differ in NO Production Ability in HUVECs

To investigate the increase in BP levels in the presence of the 364Ser allele, we tested whether the WT and variant peptides differ in their efficacies in inducing NO production in vascular endothelial cells. Initially, we treated HUVECs with 2 doses of CST-WT (0.1 and 1 nmol/L), both of which significantly increased ($P<0.001$) the NO levels as compared with the basal condition (Figure 2A). The NO indices followed the order: 1 nmol/L CST-WT (2.50) > 0.1 nmol/L CST-WT (2.35) > basal (1.90) (Figure 2B). Because at both the doses the peptide showed significant effect, we then chose to continue with a dose of 1 nmol/L to compare the activities of the variant and the WT peptide. Treatment of HUVECs with CST-WT or CST-364Ser showed that NO indices were increased with both the peptides compared with basal ($P<0.001$), but however, the increase with CST-WT was significantly higher than CST-364Ser variant ($P<0.001$; Figure 2C). The NO indices in HUVECs were in the following order: CST-WT (22.85) > CST-364Ser (19.26) > basal (16.85) (Figure 2D). Interestingly, when both the peptides were added in an equimolar ratio as a representative of the heterozygous condition, the NO index (19.89) was in between that of the WT and variant peptide (Figure 2C and 2D).

In view of the direct links between stimulation of cardiovascular β-adrenergic receptors and NO generation,[41] we sought to test whether the NO effects of the CST peptides are routed through their interactions with the β-adrenergic receptors ADRB1 and ADRB2. Accordingly, we treated HUVECs with ADRB1/2 antagonists (viz CGP 20712 for ADRB1 and ICI 118551 for ADRB2), followed by treatment with the CST peptides. The ADRB2 antagonist was found to significantly blunt the NO-increasing effect of the CST-WT peptide (NO index for CST-WT: 9.09 versus NO index for CST-WT+ICI 118551: 8.01), whereas it did not do the same in the case of CST-364Ser peptide (NO index for CST-364Ser: 8.80 versus NO index for CST-364Ser+ICI 118551: 8.36; Figure 2E and

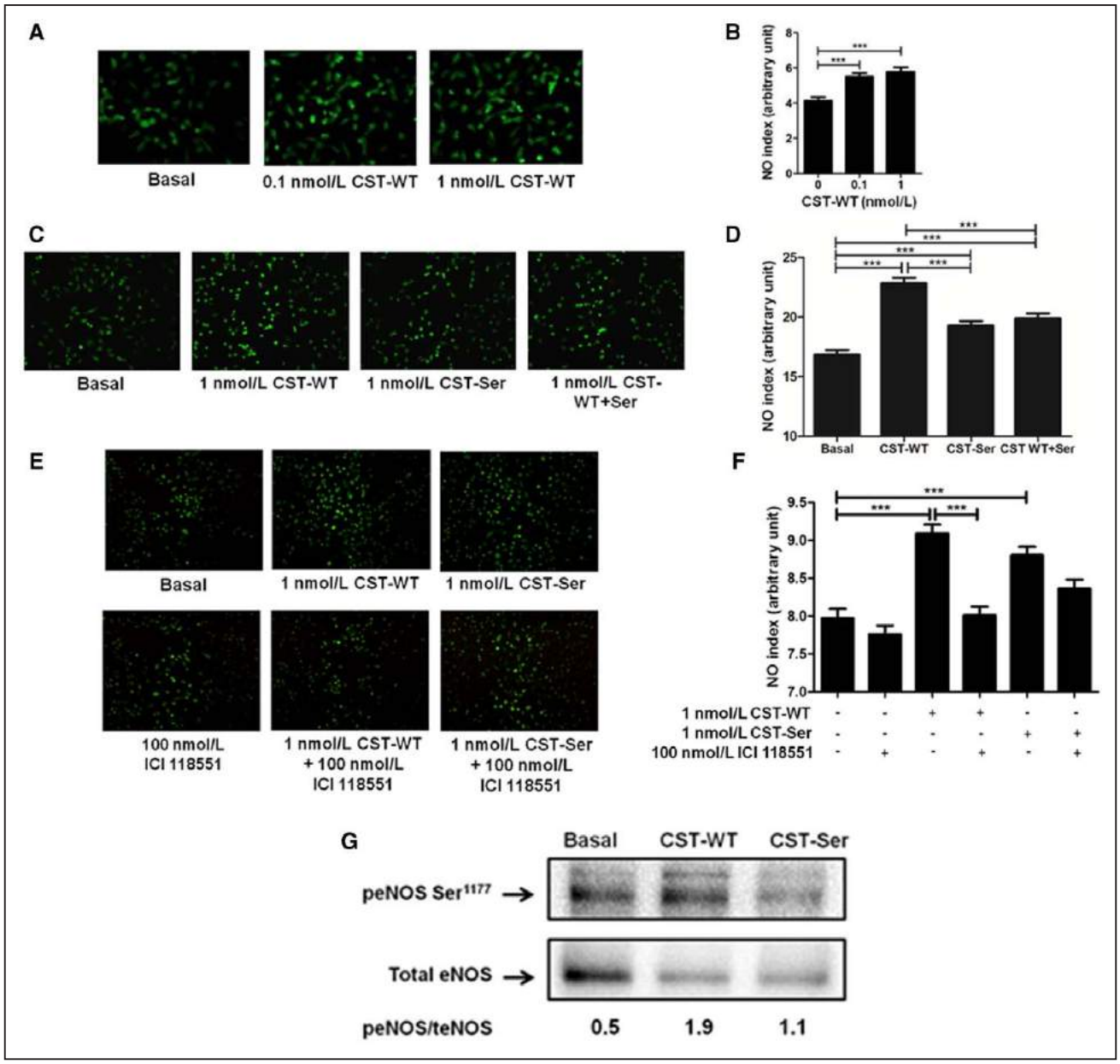

Figure 2. Effect of catestatin (CST) peptides on NO production in human umbilical vein endothelial cells (HUVECs). The fluorescence intensities (NO indices) were calculated by Image J analysis and plotted as mean±SE. The experimental groups were compared by 1-way ANOVA followed by Tukey multiple comparison post-test. **A** and **B**, Representative images for the treatment of HUVECs with different doses of CST wild-type peptide (CST-WT; 0.1 nmol/L and 1 nmol/L). ***P<0.001; 1-way ANOVA F=15.71 and P<0.0001; n=50 cells per condition. **C** and **D**, Representative images for the treatment of HUVECs with CST peptides. ***P<0.001; 1-way ANOVA F=37.15 and P<0.0001; n=450 cells per condition. The order for efficacy of peptides in NO production: CST-WT>CST-WT+Ser>CST-364Ser>basal. **E** and **F**, Representative images for the treatment of HUVECs with CST peptides and ADRB2 antagonist ICI 118551. ***P<0.001; 1-way ANOVA F=19.65 and P<0.0001; n=450 cells per condition. **G**, Representative Western blot of 3 independent experiments showing phosphorylated Ser^{1177}-eNOS (peNOS) and total eNOS (teNOS) levels on treatment of HUVECs with CST peptides. The peNOS/teNOS values have been indicated below each lane.

2F). ADRB1 antagonist failed to show any inhibition of the NO levels produced by both peptides (Figure S3).

Next, to assess the effect of CST peptides on eNOS activity, we checked the phosphorylation levels of eNOS at Ser^{1177} residue, after treatment with the CST peptides. CST-WT increased the phosphorylation at Ser^{1177} of eNOS as compared with CST-364Ser (Figure 2G). Because phosphorylation at Ser^{1177} leads to activation of eNOS, these results suggest an overall higher eNOS activity in cells treated with CST-WT.

Differential Interactions of CST Peptides With ADRB1/2: Experimental Evidence

We then tested the interactions of the CST peptides with ADRB1/2 to see whether their altered interactions with either of the receptors can explain their differential NO effects. Competition binding assays were performed with [^{125}I]-cyanopindolol using HEK-293 cells stably expressing human ADRB1/2. The levels of ADRB1/2 expression were assessed by performing radioligand binding using saturating concentrations of [^{125}I]-cyanopindolol

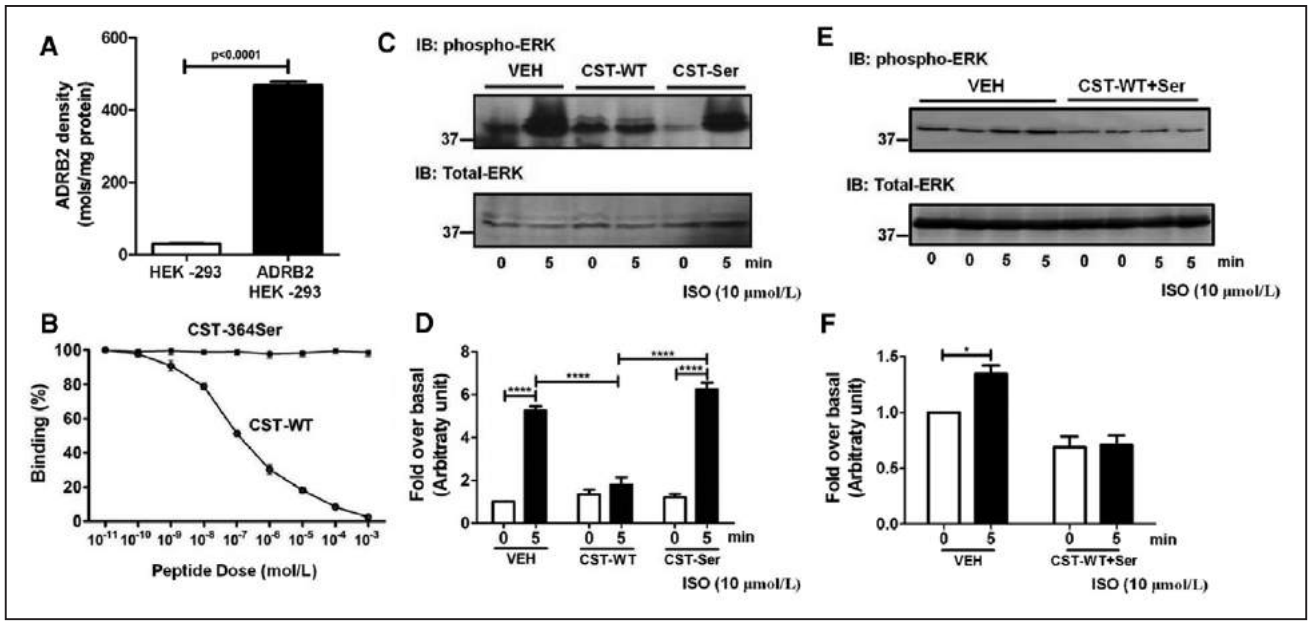

Figure 3. Binding of catestatin (CST) peptides to ADRB2 receptor and downstream effects. **A**, ADRB2 HEK-293 cells showed ≈16-fold higher expression of ADRB2 (P<0.0001 by 2-tailed unpaired t test) as compared with control HEK-293 cells. Data are shown as ADRB2 levels normalized with total protein. **B**, Data are shown as percentage binding of the radioligand cyanopindolol. With increasing doses of CST wild-type peptide (CST-WT; 10 pmol/L to 1 mmol/L), the ligand got completely displaced (P<0.0001; F=2300; R^2=0.998), whereas with increasing doses of CST-364Ser, there was no effect. The experimental groups were compared by 1-way ANOVA followed by Tukey multiple comparison post-test. **C** and **D**, Representative western blot (**C**) and quantitative representation of the densitometric analysis from 4 to 6 independent experiments (**D**) showing phosphorylated ERK (pERK) and total ERK levels on treatment with CST peptides and isoproterenol (ISO). ISO (10 μmol/L) showed an increase in pERK levels at 5 minutes in the vehicle (VEH) condition, reflecting the activation of ADRB2. However, this increase was inhibited on pretreatment with CST-WT (10 μmol/L). ****P<0.0001. On the contrary, pretreatment with CST-364Ser (10 μmol/L) showed levels of activation similar to the vehicle. The experimental groups were compared by 2-tailed t test. **E** and **F,** Representative Western blot (**E**) and quantitative representation of the densitometric analysis from 4 independent experiments (**F**) showing pERK and total ERK levels on treatment with equimolar ratios of CST-WT and CST-364Ser peptides and ISO. ISO (10 μmol/L) showed an increase in pERK levels at 5 min in the vehicle (VEH) condition. However, this increase was inhibited upon pretreatment with CST-WT+Ser (10 μmol/L). *P<0.05. The experimental groups were compared by 2-tailed t test. IB indicates immunoblotting.

on plasma membranes isolated from ADRB1/2-expressing HEK-293 cells. ADRB1 HEK-293 cells showed ≈109-fold (P<0.0001) higher levels of ADRB1 expression (Figure S4A) because HEK-293 cells have very sparse endogenous expression of ADRB1. ADRB2 HEK-293 cells showed ≈16-fold (P<0.0001) higher level of ADRB2 expression compared with control HEK-293 cells (Figure 3A) because HEK-293 cells do have some level of endogenous ADRB2s. Plasma membranes from ADRB1/2 HEK-293 cells were then subjected to an indirect competition ligand-binding assay wherein we tested for the ability of increasing doses (10 pmol/L to 1 mmol/L) of CST-WT or CST-364Ser peptides to displace saturating concentrations of labeled cyanopindolol. In ADRB2 HEK-293 cells, CST-WT peptide competitively displaced the [^{125}I]-cyanopindolol with increasing concentrations (P<0.0001; F=2300; R^2=0.998) in contrast to CST-364Ser peptide which did not displace or compete with cyanopindolol (Figure 3B). However, in ADRB1 HEK-293 cells, both the peptides failed to displace [^{125}I]-cyanopindolol with increasing concentrations (Figure S4B).

To further check whether binding of these peptides has consequences in β-adrenergic signaling, receptor activation was assessed by determining the phosphorylation status of ERK. ADRB1/2 HEK-293 cells were pretreated with either CST-WT or CST-364Ser peptide followed by ADRB agonist isoproterenol stimulation. Although in ADRB2 HEK-293 cells, pretreatment with CST-WT inhibited ADRB2-mediated ERK activation (P<0.0001), CST-364Ser had no appreciable effects in altering ERK (Figure 3C and 3D). Interestingly, treatment with equimolar ratios of CST-WT and CST-364Ser elicited a similar response to that of CST-WT alone (Figure 3E and 3F). In ADRB1 HEK-293 cells, on the contrary, both the peptides failed to show any detectable effects in altering ERK levels (Figure S4C and S4D). These studies suggest that CST-WT may be acting as an inhibitor/antagonist to ADRB2 function in contrast to CST-364Ser which does not alter the ADRB2 function. Moreover, this differential effect seems to be limited to the ADRB2 receptor only and not the ADRB1 isoform.

Structures of the CST Peptides and Their Differential Interactions With ADRB1/2: Computational Analysis

To explore whether the differential effects of the peptides in vitro can be attributed to any structural differences between them, we generated in silico models of CST peptides and CST-ADRB1/2 complexes and performed structural analysis on them using protein–protein modeling and molecular dynamics simulations (Figure 4). The CST-WT structure was found to comprise a metastable antiparallel β-sheet and a random coil (Figure 4A). Its N-terminal β-strand was stabilized by interactions between Lys355, Leu356, and Ser357, whereas the C-terminal β-strand was stabilized by Gly369, Pro370, and Gln371. Interestingly, the mutation of Gly364 to 364Ser drastically changed the secondary

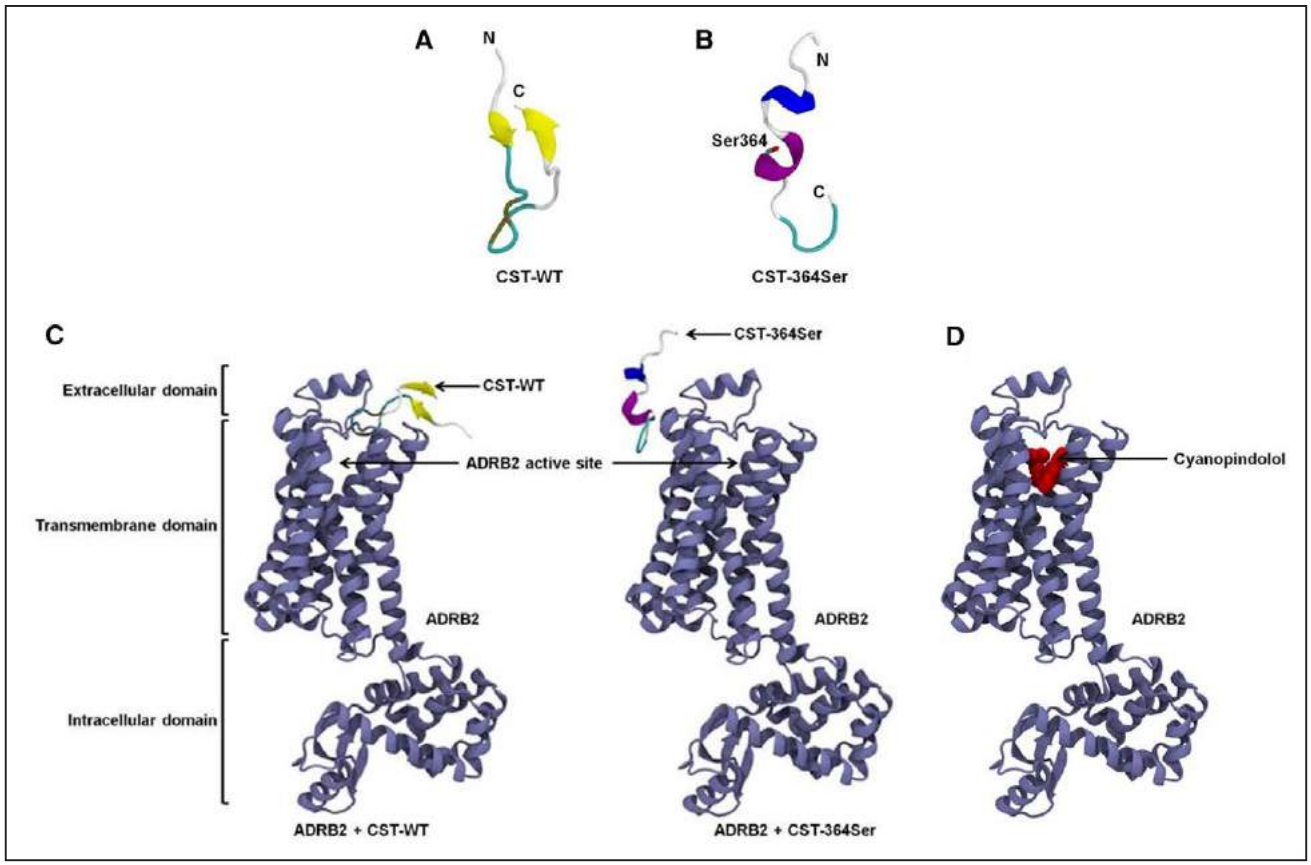

Figure 4. Structures of catestatin (CST) peptides and complexes of CST peptides/cyanopindolol with ADRB2 receptor. The time-averaged structures of CST-WT (**A**) and CST-364Ser (**B**) are shown in cartoon representation. The 364Ser mutation in the CST-364Ser peptide is shown in a stick representation. **C**, Snapshots of CST-WT (**left**) and CST-364Ser (**right**) docked to ADRB2. ADRB2: violet, β-sheet in CST-WT: yellow, α-helix in CST-364Ser: purple, and 3_{10} helix in CST-364Ser: blue. **D**, Snapshot of the docked complex of cyanopindolol (red, van der Waals representation) and ADRB2 (violet, cartoon representation).

structural content of CST. CST-364Ser displayed a metastable 3_{10} helix and a stable α-helix in the central region (Figure 4B). The 3_{10} helix was stabilized by residues Leu356, Ser357, and Phe358, and the stable α-helix comprised Arg361, Ala362, Tyr363, and Ser364. All residues of the time-averaged structures of both peptides from simulations are in the allowed regions of the Ramachandran plot (Figure S5), thus validating the proposed models of the peptides.

The binding of CST peptides to ADRB1/2 was explored via protein–protein docking in which both CST-WT and CST-364Ser were allowed to sample the extracellular region of the modeled ADRB1/2 (Figure S6) independently. In case of ADRB1, both the CST peptides did not show any significant binding to the ADRB1 active site (Figure S7). On the contrary, in case of ADRB2, they were found to bind to different locations of the receptor (Figure 4C). Although the thumb-like structure of CST-WT could fit into the ligand entry site of ADRB2 because of its shape complementarity, CST-364Ser failed to dock into the ligand entry site because of its linear stretched structure and difference in secondary structural content compared with CST-WT. It instead bound to the outer surface of the receptor, away from the CST-WT binding site. A brief 50 ns molecular dynamics simulation was performed on each of these CST-ADRB2 complexes in lipid bilayer for further refinement, but no significant change in binding mode was observed. The ZDOCK score (calculated based on surface complementarity, electrostatistics, and statistics potential) was 1197 for CST-WT and ADRB2 and 1067 for CST-364Ser and ADRB2, implying better binding of CST-WT to ADRB2.

To reconfirm the binding of CST-WT to ADRB2, the high-affinity ligand cyanopindolol was docked to the peptide–receptor complexes. In >100 attempts for protein-ligand docking through AutoDock,[42] cyanopindolol could never bind to the CST-WT–ADRB2 complex, whereas it bound effectively to the CST-364Ser–ADRB2 complex in all the attempts. This further proves the complete occlusion of the receptor's ligand-binding pocket by CST-WT and out-of-pocket binding of CST-364Ser to ADRB2 (Figure 4C).

To check the competitive binding of CST-WT and cyanopindolol to ADRB2, we produced the cyanopindolol–ADRB2 complex by protein-ligand docking (Figure 4D). Similar to the available crystal structure of cyanopindolol–ADRB1 complex (PDB ID: 4BVN),[43] cyanopindolol was found to bind to the hollow region of ADRB2 formed by the 7 transmembrane helices. A closer look at the interactions involved in the CST-WT–ADRB2 and cyanopindolol–ADRB2 complex formation revealed that even though cyanopindolol binds deep into the pocket, there were 2 common ADRB2 residues (viz Phe165 and Thr167) which interacted with both CST-WT (Figure 5A) and cyanopindolol (Figure 5C). Of note,

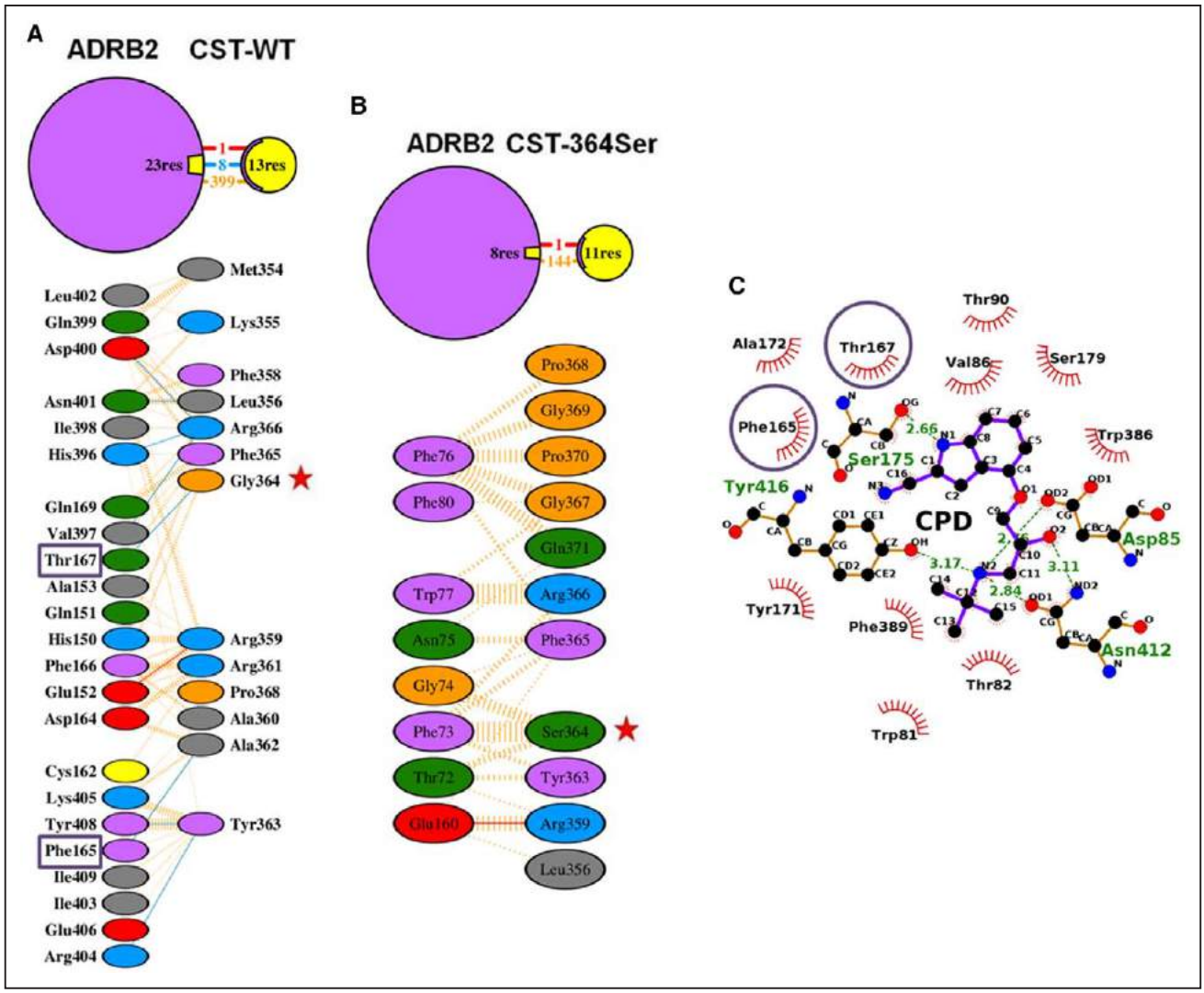

Figure 5. Molecular interactions in the complexes of catestatin (CST) peptides or cyanopindolol with ADRB2. **A** and **B**, Binding interactions in CST-WT–ADRB2 (**A**) and CST-364Ser–ADRB2 (**B**) complexes. Hydrogen bonds: blue lines, hydrophobic contacts: orange lines, and salt bridges: red lines. Each residue is color coded based on its nature with aliphatic residues in gray; aromatic residues in pink; negatively charged residues in red; positively charged residues in cyan; neutrally charged residues in green; Pro and Gly in orange; and Cys in yellow. Gly364Ser polymorphism: red stars. **C**, Binding interactions of cyanopindolol with ADRB2. Hydrophobic interactions: red spiked semicircles and hydrogen-bonding interactions: green dotted lines with distance values indicated. Common interacting residues of ADRB2 with CST-WT and cyanopindolol are highlighted using purple boxes in (**A**) and purple circles in (**C**), respectively.

ADRB2:Thr167 interacts with CST-WT:Gly364 strongly through hydrogen bonds during the CST-WT–ADRB2 complex formation, thus making CST-WT:Gly364 a crucial residue for complex formation. Therefore, it is not surprising that a mutation at this particular residue of CST makes its binding to ADRB2 active site weaker or there is no binding. The interactions in the CST-364Ser–ADRB2 complex formation involve 0 hydrogen bonds (in contrast to 8 in CST-WT–ADRB2 complex) and 1 salt bridge. The remaining interactions are hydrophobic in nature, including the ones involving 364Ser, making this binding a weak one (Figure 5B). An analysis of the Gly364Ser mutation using the polyphen-2 tool (which estimates the possible impact of an amino acid substitution on the structure and function of a human protein with the help of sequence, phylogenetic, and structural information characterizing the substitution) predicted this particular SNP to be possibly damaging with a score of 0.528 (sensitivity: 0.8; specificity: 0.9).[44]

Discussion

CST: A *CHGA*-Derived Antihypertensive Peptide

Recent studies have provided ample evidence to testify CST as a multifunctional peptide with diverse roles in the regulation of cardiovascular/metabolic functions.[45,46] Given that its precursor *CHGA* is a candidate gene for essential hypertension,[47] CST's role as an antihypertensive agent has been an interesting topic of research. The primary evidence for this was found when the administration of exogenous CST resulted in the rescue of the hypertensive and hyperadrenergic phenotypes exhibited by *CHGA* knockout mice.[17,48] The role of CST as a potent vasodilator in vivo has also been well documented both in rats[49] and in humans.[50] CST also seems to be capable of modulating the components of the brain-stem circuitry (rostral and caudal ventrolateral medulla) that regulate BP.[51,52]

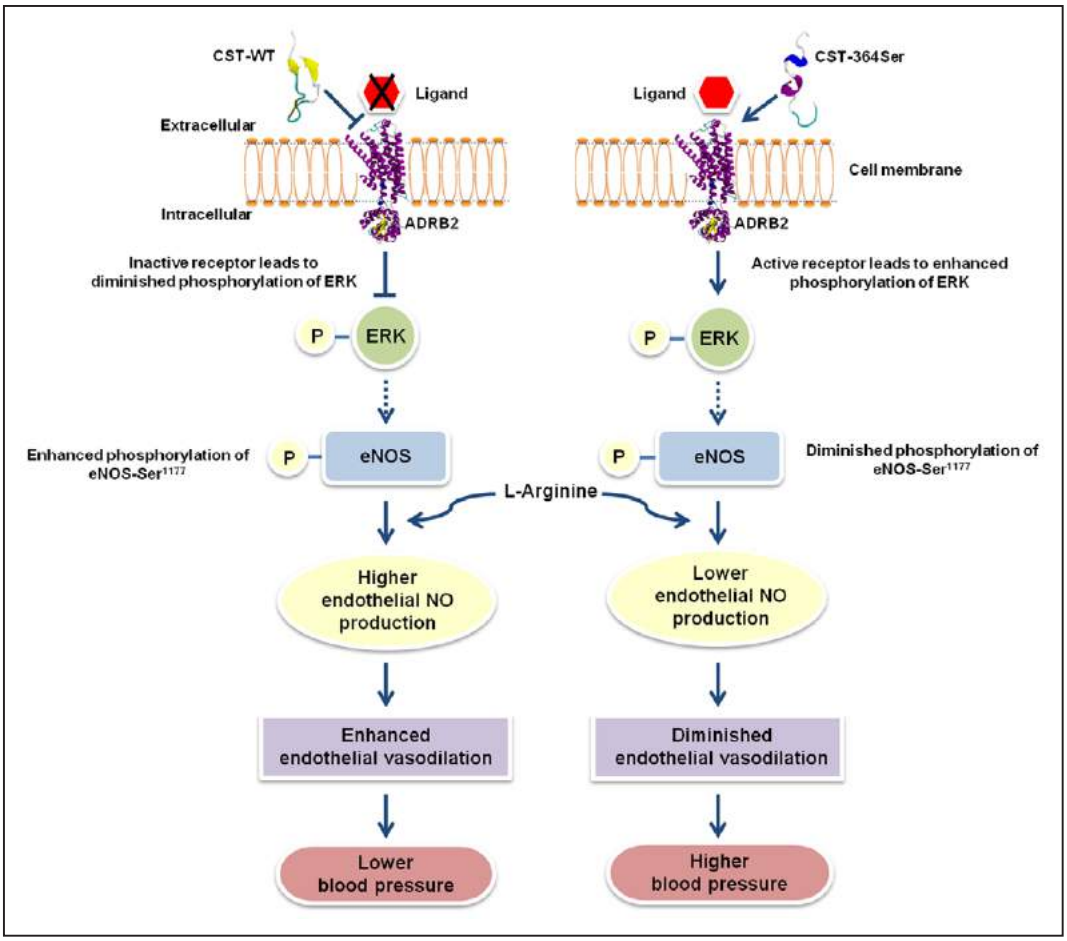

Figure 6. A schematic representation of the plausible mechanistic basis for the effects of catestatin (CST) peptides on blood pressure via modulation of NO pathway. The CST-364Ser peptide does not interact at the ligand-binding site of ADRB2 unlike CST-WT because of differences in their secondary structures. Their differential interactions with ADRB2 result in diminished antagonization of ADRB2 and enhanced activation/phosphorylation of extracellular regulated kinase (ERK) by CST-364Ser. The altered ERK activation between the CST peptides may result in diminished phosphorylation of eNOS-Ser^{1177} and consequently lower endothelial nitric oxide synthase (eNOS) activity in the case of CST-364Ser. These cellular/molecular processes lower the NO levels in vascular endothelial cells in the carriers of CST-364Ser allele leading to endothelial dysfunction and thereby increasing their risk for hypertension.

The Occurrence of the Gly364Ser SNP in Diverse Ethnic World Populations

The discovery of a functionally active variant of CST (Gly364Ser) in a Southern Californian population held promise of providing small, yet important, clues in elucidating the mechanism for the development of hypertension.[18] However, because of the vast difference in the genetic makeup of the different ethnic populations across the world, it would be irrational to generalize the magnitude and direction of allelic effect sizes across populations.[53,54] A study in the Population Architecture using Genomics and Epidemiology (PAGE) consortium of multi-ancestry populations has ably demonstrated that differential LD between common polymorphisms (tag-SNPs) and functional variants within diverse populations significantly contribute to diluting the effect sizes among these populations.[55] The immense variation in the distribution of genotypes for the Gly364Ser polymorphism in different world populations foreshadows a similar distortion in the effect sizes in these populations (Table S5). Overall, the SNP seems to be occurring in 3 strata of ethnic groups. The first stratum, with a high allelic frequency (6% to 8%), includes the Asian and Hispanic groups. The European ethnicity forms the second stratum, which has a moderate frequency (2% to 5%). The third stratum, with the lowest frequency (0% to 1%), consists of the African populations. In our previous study,[19] we reported the discovery of the Gly364Ser polymorphism in an urban Chennai population (n=1010 individuals) at an MAF of ≈8.0%. In this study, we have expanded the sample size to 3200 individuals consisting of DM/hypertension/CAD/controls from the same population. 364Ser allele displayed an MAF of 6.34% in the Chennai DM/hypertension/CAD population, which seems to be consistent with the frequencies observed in other Asian populations. Interestingly, the ethnically distinct Chandigarh population displayed a much lesser MAF of 3.48%, which is closer to that observed in the European stratum than the Asian one. The distribution of the genotype frequencies differed significantly between Chennai DM/hypertension/CAD population and Chandigarh population (χ^2=18.01 and P=0.0001). Thus, CST region of *CHGA* seems to be displaying significant genetic variations among different world ethnic populations. In the evolutionary context,

the 364Ser variant has not been detected in other mammals (Figure S8). Some mammals (eg, Giant panda), however, have Asp at the 364th position.

Association of the CST-364Ser Allele With Hypertension

The logistic regression analysis revealed a significant association of the 364Ser allele with hypertension in both the primary (Chennai) and the replication (Chandigarh) populations (Table 1). This was supported by the observation of elevated BP levels in the 364Ser carriers in both the study populations. Despite the difference in the frequency of the Gly364Ser polymorphism in these 2 ethnically distinct populations, its effect on BP levels seems to replicate well. The higher occurrence of the variant allele in the population with an increase in BP ranges further provides the evidence of association of the 364Ser allele with hypertension (Figure 1D and 1E). Overall, the 364Ser allele seems to act as a risk allele for hypertension development in Indian populations.

Why has Gly364Ser not been detected in the genome-wide association studies performed on cardiovascular diseases till date? First, most of these genome-wide association studies were performed in either European or American populations wherein the MAFs for this SNP are much lower than those in Indian populations (Table S5). Because of the low MAFs, large sample sizes would be required to identify any significant association with this SNP in these populations. Second, almost all the genechip arrays (for example, Illumina Human550K Bead chip and Illumina Human610K Bead chip) used in these reported studies as well as other studies performed among Asians[56,57] did not harbor the Gly364Ser SNP. Therefore, the fact that these genome-wide association studies did not identify Gly364Ser as a risk variant for hypertension is not surprising.

We found the 364Ser allele to be in LD with the *CHGA* promoter SNPs at -1106, -1018, -415, and -57 bp positions (Figure S2). The 8 SNPs in the *CHGA* promoter form haplotypes which differ from each other in terms of their transcriptional activity.[18] The minor alleles at -1018, -415, and -57 bp positions of the *CHGA* promoter give rise to one of the 5 common *CHGA* promoter haplotypes (GTTTGCCT) which shows higher promoter activity as compared with the WT consensus haplotype (GATTGTCC).[18] This would mean that the 364Ser carriers may have a more active promoter leading to greater levels of the parent CHGA molecule being produced. Elevated levels of CHGA are associated with elevated BP levels.[58] Therefore, the hypertensive effect of the 364Ser allele might be getting manifested through increased *CHGA* promoter activity as well. 364Ser allele is also in modest LD with Gly297Ser, a functionally active SNP in pancreastatin that seems to alter the risk for type 2 diabetes mellitus in an Indian population.[32] Thus, the association of the 364Ser allele with elevated plasma glucose levels (Figure S9) may be an effect of it being a bystander.

Of note, in a Southern Californian population, the 364Ser allele displayed association with diminished DBP levels, especially in men; however, the effect was not consistently observed for SBP or in women.[39] Conversely, the 364Ser allele was associated with elevated SBP and MAP levels in a Japanese population (Table 2).[59] Thus, 364Ser allele seems to exert directionally concordant effects on BP in several Asian populations (South Indian, North Indian, and Japanese) although not in Caucasians. This is similar to a previous study reporting that the 12Ala allele in the peroxisome proliferator–activated receptor-γ2 did not offer the same protective role in Indians as it did in Caucasians.[60] Such contradictory associations of an allele provide evidence for heterogeneity in different populations and underscore the need for carrying out association studies in diverse ethnic populations to draw more accurate conclusions in each population.

Mechanistic Basis for Elevated BP Level in the Carriers of 364Ser: Influence of the Endothelial NO Pathway

It is well established that the endothelium plays an important role in regulating arterial BP. The manifestation of hypertension through impaired endothelium-dependent vasodilation as well as reduced NO production has been well documented in both animal[61,62] and human[63,64] studies. It is, therefore, not surprising that the hypertensive and hyperadrenergic phenotype in the *CHGA*-KO mice was accompanied by lowered NO levels.[65] The attenuation of such a phenotype on the exogenous administration of CST would therefore have to route through restoration of NO levels. In a study performed in BAE-1 (bovine aortic endothelium) cells,[20] CST-WT was shown to induce a wortmannin-sensitive, Ca^{2+}-independent increase in NO production and eNOS Ser^{1179} phosphorylation, whereas CST-364Ser was found to be ineffective. CST-WT has also been shown to dose dependently induce a NO-cGMP dependent cardiosuppression in the in vitro frog heart.[66] In light of this, we asked whether CST-WT and CST-364Ser differ in their ability to generate NO in HUVECs. Indeed, CST-364Ser displayed lower efficacy to produce NO in HUVECs (Figure 2D), thus corroborating the elevated BP levels in the 364Ser allele–carrying individuals. Consistently, carriers of 364Ser allele showed diminished (by ≈1090 mm/s) brachial artery pulse-wave velocity (indicating increased endothelial dysfunction) in a Japanese population.[59] eNOS is known to be activated via phosphorylation at its Ser^{1177} residue[67,68] HUVECs treated with CST-WT showed increased levels of phosphorylation at Ser^{1177} sites of eNOS as compared with HUVECs treated with CST-364Ser (Figure 2G). In case of CST-WT, there was a ≈3.8-fold increase in Ser^{1177} phosphorylation levels over basal; CST-364Ser, on the other hand, showed only a ≈2.2-fold increase in Ser^{1177} phosphorylation levels over basal. This is indicative of increased eNOS activity in case of CST-WT as compared with CST-364Ser which goes in corroboration with our observations of increased NO levels on treatment with CST-WT as compared with CST-364Ser in HUVECs.

Direct links between stimulation of cardiovascular β-adrenergic receptors and NO generation in endothelial cells are well established.[41] In isolated human umbilical vein, the vasorelaxation response to either the nonselective β-adrenergic agonist isoproterenol or to the cAMP analogue dibutyryl cAMP is attenuated by the NOS inhibitor NG-monomethyl-L-arginine. Thus, the β-adrenergic receptor–mediated vasorelaxation response in this system seems to be largely NO dependent and mediated through the elevation of cAMP

levels.[69] Likewise, in HUVECs as well, it has been shown that either β-adrenergic receptor–stimulated or β-adrenergic receptor–independent elevation of intracellular cAMP levels results in increased NOS activity.[69] In HUVECS, the inhibition of eNOS activity in the presence of the ADRB2 antagonist ICI 118551 and not ADRB1 antagonist CGP 20712 shows that this effect is mediated exclusively through ADRB2.[70] Consistently, we found that the elevation in NO levels mediated by CST-WT peptide was abrogated in the presence of ICI 118551 but not CGP 20712 (Figure 2F; Figure S3).

On the basis of the above findings, we postulated that our in vitro observation of differential effects of CST peptides on NO production via regulation of eNOS activity in HUVECs might be because of the differential interaction of CST peptides with ADRB2. Indeed, our computational analysis showed that by virtue of differences in secondary structures, CST-WT blocks the binding of the ligand to ADRB2 (by competing with it for the active site), whereas CST-364Ser binds at a site that keeps the agonist-binding pocket within ADRB2 intact (Figure 4C). Consistent with the computational prediction, competitive binding assays showed that although CST-WT was able to significantly displace the high-affinity β-adrenergic receptor ligand cyanopindolol, CST-364Ser failed to do the same even at high concentration (Figure 3B). Furthermore, the inhibition of agonist isoproterenol-stimulated increase in phospho-ERK levels by CST-WT (but not by CST-364Ser) in ADRB2 HEK-293 cells points toward an antiadrenergic role of the WT peptide but not the variant peptide (Figure 3C and 3D). This is consistent with a previous report that CST-WT lowers the phospho-ERK levels in Langendorff-perfused rat hearts.[21]

In contrast to the effective ability of CST-WT to bind to ADRB2, our computational studies show that CST peptides bind to the outer surface of the ADRB1 receptor and are thus incapable of blocking the agonist-binding pocket in the receptor. These observations are further supported by our competitive binding assays with ADRB1 (Figure S4B). Thus, the antiadrenergic role of CST-WT seems to be mediated primarily through the ADRB2 receptor and may underlie the differential BPs observed with the variants being expressed in patients.

Conclusions

We discovered a naturally occurring, common genetic variation, Gly364Ser, within the antihypertensive peptide CST, a proteolytic fragment of the prohormone chromogranin A that is expressed in secretory vesicles of endocrine, neuroendocrine, and neuronal cell types. The 364Ser allele showed association with elevated levels of SBP, DBP, and MAP in human subjects across 2 independent and ethnically/geographically distinct Indian populations. Corroborating these findings, the carriers of the 364Ser allele displayed enhanced risk for hypertension. This is on same lines of a recent Japanese study which found similar associations of the 364Ser allele with hypertension in their population. Genetic association studies of this chromogranin A locus with hypertension and other metabolic diseases need to be performed in additional ethnic populations to evaluate whether the results are of general importance across the overall world population as well. Our in cella and in silico analyses provided molecular/mechanistic underpinnings for the diminished effects of the CST-364Ser peptide (as compared with the CST-WT peptide) in the modulation of the endothelial NO pathway (via differential binding to ADRB2 and differential activation of ERK and eNOS phosphorylation) that might lead to an increased disease risk in carriers of the 364Ser allele. A schematic of our hypothesis/findings are presented in Figure 6. These results have implications for inter-individual variations in BP homeostasis and ultimately for pathogenesis of hypertension.

Perspectives

The neuroendocrine secretory granule protein chromogranin A is emerging as an important regulator of cardiovascular pathophysiology; it acts as precursor for several bioactive peptides including the antihypertensive and cardioprotective peptide CST. We discovered a nonsynonymous genetic variation (Gly364Ser that occurs in a large section of the worldwide human population) within the CST domain. The 364Ser allele was associated with profound elevated BP (up to ≈8 mm Hg SBP and ≈ 6 mm Hg DBP) and enhanced risk (by ≈48%) for hypertension in its carriers in several Asian populations. These findings contribute toward potential clinical use of functional genetic variations to predict the risk for hypertension and preventive intervention in asymptomatic patients for better management of cardiovascular disease burden.

Acknowledgments

We acknowledge all the individuals who voluntarily participated in this study. We thank the high-performance computational facility at Indian Institute of Technology Madras (IITM). We also thank Suvro Chatterjee, Anna University, Chennai, for his help. M. Kiranmayi and R. Kumaragurubaran thank Council of Scientific and Industrial Research for fellowship. P.K.R. Allu and D. Vishnuprabu received research fellowships from University Grants Commission. V.R. Chirasani, L. Subramanian, and S. Sharma would like to thank IITM, Department of Science and Technology, and Indian Council of Medical Research for fellowships, respectively.

Sources of Funding

This work was supported by grants BT/PR9546/MED/12/349/2007 and BT/PR12820/BRB/10/726/2009 from the Department of Biotechnology (DBT), Government of India. This work was also supported in part by grants SR/SO/HS-084/2013A from the Science and Engineering Research Board, BT/PR4820/MED/12/622/2013 from DBT, Government of India, and NIH RO1 HL HL089473.

Disclosures

None.

References

1. Kim T, Zhang CF, Sun Z, Wu H, Loh YP. Chromogranin A deficiency in transgenic mice leads to aberrant chromaffin granule biogenesis. *J Neurosci*. 2005;25:6958–6961. doi: 10.1523/JNEUROSCI.1058-05.2005.
2. O'Connor DT, Takiyyuddin MA, Printz MP, Dinh TQ, Barbosa JA, Rozansky DJ, Mahata SK, Wu H, Kennedy BP, Ziegler MG, Wright FA, Schlager G, Parmer RJ. Catecholamine storage vesicle protein expression in genetic hypertension. *Blood Press*. 1999;8:285–295.
3. Takiyyuddin MA, De Nicola L, Gabbai FB, Dinh TQ, Kennedy B, Ziegler MG, Sabban EL, Parmer RJ, O'Connor DT. Catecholamine secretory vesicles. Augmented chromogranins and amines in secondary hypertension. *Hypertension*. 1993;21:674–679.
4. Estensen ME, Hognestad A, Syversen U, Squire I, Ng L, Kjekshus J, Dickstein K, Omland T. Prognostic value of plasma chromogranin A levels in patients with complicated myocardial infarction. *Am Heart J*. 2006;152:927.e1–927.e6. doi: 10.1016/j.ahj.2006.05.008.

5. Jansson AM, Røsjø H, Omland T, Karlsson T, Hartford M, Flyvbjerg A, Caidahl K. Prognostic value of circulating chromogranin A levels in acute coronary syndromes. *Eur Heart J*. 2009;30:25–32. doi: 10.1093/eurheartj/ehn513.
6. Ceconi C, Ferrari R, Bachetti T, Opasich C, Volterrani M, Colombo B, Parrinello G, Corti A. Chromogranin A in heart failure; a novel neurohumoral factor and a predictor for mortality. *Eur Heart J*. 2002;23:967–974. doi: 10.1053/euhj.2001.2977.
7. Barbosa JA, Gill BM, Takiyyuddin MA, O'Connor DT. Chromogranin A: posttranslational modifications in secretory granules. *Endocrinology*. 1991;128:174–190. doi: 10.1210/endo-128-1-174.
8. Aardal S, Helle KB, Elsayed S, Reed RK, Serck-Hanssen G. Vasostatins, comprising the N-terminal domain of chromogranin A, suppress tension in isolated human blood vessel segments. *J Neuroendocrinol*. 1993;5:405–412.
9. Tatemoto K, Efendić S, Mutt V, Makk G, Feistner GJ, Barchas JD. Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. *Nature*. 1986;324:476–478. doi: 10.1038/324476a0.
10. Fasciotto BH, Trauss CA, Greeley GH, Cohn DV. Parastatin (porcine chromogranin A347-419), a novel chromogranin A-derived peptide, inhibits parathyroid cell secretion. *Endocrinology*. 1993;133:461–466. doi: 10.1210/endo.133.2.8344192.
11. Loh YP, Koshimizu H, Cawley NX, Tota B. Serpinins: role in granule biogenesis, inhibition of cell death and cardiac function. *Curr Med Chem*. 2012;19:4086–4092.
12. Simon JP, Bader MF, Aunis D. Secretion from chromaffin cells is controlled by chromogranin A-derived peptides. *Proc Natl Acad Sci USA*. 1988;85:1712–1716.
13. Mahata SK, O'Connor DT, Mahata M, Yoo SH, Taupenot L, Wu H, Gill BM, Parmer RJ. Novel autocrine feedback control of catecholamine release. A discrete chromogranin a fragment is a noncompetitive nicotinic cholinergic antagonist. *J Clin Invest*. 1997;100:1623–1633. doi: 10.1172/JCI119686.
14. Mahata SK, Mahata M, Wen G, Wong WB, Mahapatra NR, Hamilton BA, O'Connor DT. The catecholamine release-inhibitory "catestatin" fragment of chromogranin a: naturally occurring human variants with different potencies for multiple chromaffin cell nicotinic cholinergic responses. *Mol Pharmacol*. 2004;66:1180–1191. doi: 10.1124/mol.104.002139.
15. Sahu BS, Mohan J, Sahu G, Singh PK, Sonawane PJ, Sasi BK, Allu PK, Maji SK, Bera AK, Senapati S, Mahapatra NR. Molecular interactions of the physiological anti-hypertensive peptide catestatin with the neuronal nicotinic acetylcholine receptor. *J Cell Sci*. 2012;125(pt 9):2323–2337. doi: 10.1242/jcs.103176.
16. O'Connor DT, Kailasam MT, Kennedy BP, Ziegler MG, Yanaihara N, Parmer RJ. Early decline in the catecholamine release-inhibitory peptide catestatin in humans at genetic risk of hypertension. *J Hypertens*. 2002;20:1335–1345.
17. Mahapatra NR, O'Connor DT, Vaingankar SM, Hikim AP, Mahata M, Ray S, Staite E, Wu H, Gu Y, Dalton N, Kennedy BP, Ziegler MG, Ross J, Mahata SK. Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog. *J Clin Invest*. 2005;115:1942–1952. doi: 10.1172/JCI24354.
18. Wen G, Mahata SK, Cadman P, Mahata M, Ghosh S, Mahapatra NR, Rao F, Stridsberg M, Smith DW, Mahboubi P, Schork NJ, O'Connor DT, Hamilton BA. Both rare and common polymorphisms contribute functional variation at CHGA, a regulator of catecholamine physiology. *Am J Hum Genet*. 2004;74:197–207. doi: 10.1086/381399.
19. Sahu BS, Obbineni JM, Sahu G, Allu PK, Subramanian L, Sonawane PJ, Singh PK, Sasi BK, Senapati S, Maji SK, Bera AK, Gomathi BS, Mullasari AS, Mahapatra NR. Functional genetic variants of the catecholamine-release-inhibitory peptide catestatin in an Indian population: allele-specific effects on metabolic traits. *J Biol Chem*. 2012;287:43840–43852. doi: 10.1074/jbc.M112.407916.
20. Bassino E, Fornero S, Gallo MP, Ramella R, Mahata SK, Tota B, Levi R, Alloatti G. A novel catestatin-induced antiadrenergic mechanism triggered by the endothelial PI3K-eNOS pathway in the myocardium. *Cardiovasc Res*. 2011;91:617–624. doi: 10.1093/cvr/cvr129.
21. Angelone T, Quintieri AM, Brar BK, Limchaiyawat PT, Tota B, Mahata SK, Cerra MC. The antihypertensive chromogranin a peptide catestatin acts as a novel endocrine/paracrine modulator of cardiac inotropism and lusitropism. *Endocrinology*. 2008;149:4780–4793. doi: 10.1210/en.2008-0318.
22. Mazza R, Gattuso A, Mannarino C, Brar BK, Barbieri SF, Tota B, Mahata SK. Catestatin (chromogranin A344-364) is a novel cardiosuppressive agent: inhibition of isoproterenol and endothelin signaling in the frog heart. *Am J Physiol Heart Circ Physiol*. 2008;295:H113–H122. doi: 10.1152/ajpheart.00172.2008.
23. Imbrogno S, Garofalo F, Cerra MC, Mahata SK, Tota B. The catecholamine release-inhibitory peptide catestatin (chromogranin A344-363) modulates myocardial function in fish. *J Exp Biol*. 2010;213(pt 21):3636–3643. doi: 10.1242/jeb.045567.
24. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 2005;21:263–265. doi: 10.1093/bioinformatics/bth457.
25. Gauderman WJ. Sample size requirements for matched case-control studies of gene-environment interaction. *Stat Med*. 2002;21:35–50.
26. Giri H, Muthuramu I, Dhar M, Rathnakumar K, Ram U, Dixit M. Protein tyrosine phosphatase SHP2 mediates chronic insulin-induced endothelial inflammation. *Arterioscler Thromb Vasc Biol*. 2012;32:1943–1950. doi: 10.1161/ATVBAHA.111.239251.
27. Kesavan R, Potunuru UR, Nastasijević B, T A, Joksić G, Dixit M. Inhibition of vascular smooth muscle cell proliferation by Gentiana lutea root extracts. *PLoS One*. 2013;8:e61393. doi: 10.1371/journal.pone.0061393.
28. Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, Le Corvoisier P, Violin JD, Wei H, Lefkowitz RJ, Rockman HA. Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J Clin Invest*. 2007;117:2445–2458. doi: 10.1172/JCI31901.
29. Naga Prasad SV, Barak LS, Rapacciuolo A, Caron MG, Rockman HA. Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by beta-adrenergic receptor kinase 1. A role in receptor sequestration. *J Biol Chem*. 2001;276:18953–18959. doi: 10.1074/jbc.M102376200.
30. Vasudevan NT, Mohan ML, Gupta MK, Hussain AK, Naga Prasad SV. Inhibition of protein phosphatase 2A activity by PI3Kγ regulates β-adrenergic receptor function. *Mol Cell*. 2011;41:636–648. doi: 10.1016/j.molcel.2011.02.025.
31. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science*. 2007;318:1258–1265. doi: 10.1126/science.1150577.
32. Allu PK, Chirasani VR, Ghosh D, Mani A, Bera AK, Maji SK, Senapati S, Mullasari AS, Mahapatra NR. Naturally occurring variants of the dysglycemic peptide pancreastatin: differential potencies for multiple cellular functions and structure-function correlation. *J Biol Chem*. 2014;289:4455–4469. doi: 10.1074/jbc.M113.520916.
33. Webb B, Sali A. Comparative protein structure modeling using MODELLER. *Curr Protoc Bioinformatics*. 2014;47:5.6.1–5.632. doi: 10.1002/0471250953.bi0506s47.
34. Pierce BG, Wiehe K, Hwang H, Kim BH, Vreven T, Weng Z. ZDOCK server: interactive docking prediction of protein-protein complexes and symmetric multimers. *Bioinformatics*. 2014;30:1771–1773. doi: 10.1093/bioinformatics/btu097.
35. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996;14:33–38, 27.
36. de Beer TA, Berka K, Thornton JM, Laskowski RA. PDBsum additions. *Nucleic Acids Res*. 2014;42(Database issue):D292–D296. doi: 10.1093/nar/gkt940.
37. Laskowski RA, Swindells MB. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J Chem Inf Model*. 2011;51:2778–2786. doi: 10.1021/ci200227u.
38. Cui JS, Hopper JL, Harrap SB. Antihypertensive treatments obscure familial contributions to blood pressure variation. *Hypertension*. 2003;41:207–210.
39. Rao F, Wen G, Gayen JR, et al. Catecholamine release-inhibitory peptide catestatin (chromogranin A(352-372)): naturally occurring amino acid variant Gly364Ser causes profound changes in human autonomic activity and alters risk for hypertension. *Circulation*. 2007;115:2271–2281. doi: 10.1161/CIRCULATIONAHA.106.628859.
40. Taupenot L, Harper KL, O'Connor DT. The chromogranin-secretogranin family. *N Engl J Med*. 2003;348:1134–1149. doi: 10.1056/NEJMra021405.
41. Queen LR, Ferro A. Beta-adrenergic receptors and nitric oxide generation in the cardiovascular system. *Cell Mol Life Sci*. 2006;63:1070–1083. doi: 10.1007/s00018-005-5451-2.
42. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. *J Comput Chem*. 2009;30:2785–2791. doi: 10.1002/jcc.21256.

43. Miller-Gallacher JL, Nehmé R, Warne T, Edwards PC, Schertler GF, Leslie AG, Tate CG. The 2.1 Å resolution structure of cyanopindolol-bound β1-adrenoceptor identifies an intramembrane Na+ ion that stabilises the ligand-free receptor. *PLoS One*. 2014;9:e92727. doi: 10.1371/journal.pone.0092727.
44. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. *Nat Methods*. 2010;7:248–249. doi: 10.1038/nmeth0410-248.
45. Mahapatra NR. Catestatin is a novel endogenous peptide that regulates cardiac function and blood pressure. *Cardiovasc Res*. 2008;80:330–338. doi: 10.1093/cvr/cvn155.
46. Mazza R, Tota B, Gattuso A. Cardio-vascular activity of catestatin: interlocking the puzzle pieces. *Curr Med Chem*. 2015;22:292–304.
47. Sahu BS, Sonawane PJ, Mahapatra NR. Chromogranin A: a novel susceptibility gene for essential hypertension. *Cell Mol Life Sci*. 2010;67:861–874. doi: 10.1007/s00018-009-0208-y.
48. Gayen JR, Gu Y, O'Connor DT, Mahata SK. Global disturbances in autonomic function yield cardiovascular instability and hypertension in the chromogranin a null mouse. *Endocrinology*. 2009;150:5027–5035. doi: 10.1210/en.2009-0429.
49. Kennedy BP, Mahata SK, O'Connor DT, Ziegler MG. Mechanism of cardiovascular actions of the chromogranin A fragment catestatin in vivo. *Peptides*. 1998;19:1241–1248.
50. Fung MM, Salem RM, Mehtani P, Thomas B, Lu CF, Perez B, Rao F, Stridsberg M, Ziegler MG, Mahata SK, O'Connor DT. Direct vasoactive effects of the chromogranin A (CHGA) peptide catestatin in humans in vivo. *Clin Exp Hypertens*. 2010;32:278–287. doi: 10.3109/10641960903265246.
51. Gaede AH, Pilowsky PM. Catestatin in rat RVLM is sympathoexcitatory, increases barosensitivity, and attenuates chemosensitivity and the somatosympathetic reflex. *Am J Physiol Regul Integr Comp Physiol*. 2010;299:R1538–R1545. doi: 10.1152/ajpregu.00335.2010.
52. Gaede AH, Pilowsky PM. Catestatin, a chromogranin A-derived peptide, is sympathoinhibitory and attenuates sympathetic barosensitivity and the chemoreflex in rat CVLM. *Am J Physiol Regul Integr Comp Physiol*. 2012;302:R365–R372. doi: 10.1152/ajpregu.00409.2011.
53. Dorajoo R, Blakemore AI, Sim X, Ong RT, Ng DP, Seielstad M, Wong TY, Saw SM, Froguel P, Liu J, Tai ES. Replication of 13 obesity loci among Singaporean Chinese, Malay and Asian-Indian populations. *Int J Obes (Lond)*. 2012;36:159–163. doi: 10.1038/ijo.2011.86.
54. Ntzani EE, Liberopoulos G, Manolio TA, Ioannidis JP. Consistency of genome-wide associations across major ancestral groups. *Hum Genet*. 2012;131:1057–1071. doi: 10.1007/s00439-011-1124-4.
55. Carlson CS, Matise TC, North KE, et al; PAGE Consortium. Generalization and dilution of association results from European GWAS in populations of non-European ancestry: the PAGE study. *PLoS Biol*. 2013;11:e1001661. doi: 10.1371/journal.pbio.1001661.
56. Hiura Y, Tabara Y, Kokubo Y, Okamura T, Miki T, Tomoike H, Iwai N. A genome-wide association study of hypertension-related phenotypes in a Japanese population. *Circ J*. 2010;74:2353–2359.
57. Guo Y, Tomlinson B, Chu T, Fang YJ, Gui H, Tang CS, Yip BH, Cherny SS, Hur YM, Sham PC, Lam TH, Thomas NG. A genome-wide linkage and association scan reveals novel loci for hypertension and blood pressure traits. *PLoS One*. 2012;7:e31489. doi: 10.1371/journal.pone.0031489.
58. Takiyyuddin MA, Parmer RJ, Kailasam MT, Cervenka JH, Kennedy B, Ziegler MG, Lin MC, Li J, Grim CE, Wright FA. Chromogranin A in human hypertension. Influence of heredity. *Hypertension*. 1995;26:213–220.
59. Choi Y, Miura M, Nakata Y, et al. A common genetic variant of the chromogranin A-derived peptide catestatin is associated with atherogenesis and hypertension in a Japanese population. *Endocr J*. 2015;62:797–804. doi: 10.1507/endocrj.EJ14-0471.
60. Radha V, Vimaleswaran KS, Babu HN, Abate N, Chandalia M, Satija P, Grundy SM, Ghosh S, Majumder PP, Deepa R, Rao SM, Mohan V. Role of genetic polymorphism peroxisome proliferator-activated receptor-gamma2 Pro12Ala on ethnic susceptibility to diabetes in South-Asian and Caucasian subjects: evidence for heterogeneity. *Diabetes Care*. 2006;29:1046–1051. doi: 10.2337/diacare.2951046.
61. Lüscher TF, Diederich D, Weber E, Vanhoutte PM, Bühler FR. Endothelium-dependent responses in carotid and renal arteries of normotensive and hypertensive rats. *Hypertension*. 1988;11(6 pt 2):573–578.
62. Fu-Xiang D, Jameson M, Skopec J, Diederich A, Diederich D. Endothelial dysfunction of resistance arteries of spontaneously hypertensive rats. *J Cardiovasc Pharmacol*. 1992;20(suppl 12):S190–S192.
63. Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation*. 1993;87:1468–1474.
64. Panza JA, García CE, Kilcoyne CM, Quyyumi AA, Cannon RO, 3rd. Impaired endothelium-dependent vasodilation in patients with essential hypertension. Evidence that nitric oxide abnormality is not localized to a single signal transduction pathway. *Circulation*. 1995;91:1732–1738.
65. Gayen JR, Zhang K, RamachandraRao SP, Mahata M, Chen Y, Kim HS, Naviaux RK, Sharma K, Mahata SK, O'Connor DT. Role of reactive oxygen species in hyperadrenergic hypertension: biochemical, physiological, and pharmacological evidence from targeted ablation of the chromogranin a (Chga) gene. *Circ Cardiovasc Genet*. 2010;3:414–425. doi: 10.1161/CIRCGENETICS.109.924050.
66. Mazza R, Pasqua T, Gattuso A. Cardiac heterometric response: the interplay between Catestatin and nitric oxide deciphered by the frog heart. *Nitric Oxide*. 2012;27:40–49. doi: 10.1016/j.niox.2012.04.003.
67. Michell BJ, Chen Zp, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, Kemp BE. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J Biol Chem*. 2001;276:17625–17628. doi: 10.1074/jbc.C100122200.
68. Butt E, Bernhardt M, Smolenski A, Kotsonis P, Fröhlich LG, Sickmann A, Meyer HE, Lohmann SM, Schmidt HH. Endothelial nitric-oxide synthase (type III) is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases. *J Biol Chem*. 2000;275:5179–5187.
69. Ferro A, Queen LR, Priest RM, Xu B, Ritter JM, Poston L, Ward JP. Activation of nitric oxide synthase by beta 2-adrenoceptors in human umbilical vein endothelium *in vitro*. *Br J Pharmacol*. 1999;126:1872–1880. doi: 10.1038/sj.bjp.0702512.
70. Yao K, Xu B, Liu YP, Ferro A. Effects of beta-adrenoceptor stimulation on endothelial nitric-oxide synthase phosphorylation of human umbilical vein endothelial cells. *Acta Pharmacol Sin*. 2003;24:219–224.

Novelty and Significance

What Is New?

- This is the first study that analyzes the association of the Gly364Ser variant in the antihypertensive peptide catestatin with the risk for hypertension in independent Asian populations.
- This study provides evidence for the direct interaction of catestatin peptides with β-2 adrenergic receptor for the first time to our knowledge.

What Is Relevant?

- This study identifies a novel blood pressure–regulating locus that seems to play an important role to alter the risk for hypertension in several Asian populations.

Summary

Directionally concordant replication of the association of catestatin 364Ser variant allele with elevated blood pressure in independent human populations suggests a causal role for this genetic variant. Consistently, the 364Ser allele enhanced the risk for hypertension in these study populations. Moreover, our receptor–peptide interaction studies provided evidence for differential interactions of the wild-type and variant catestatin peptides with β-2 adrenergic receptor that might be responsible for the altered risk for hypertension in their carriers.

A Common Tag Nucleotide Variant in *MMP7* Promoter Increases Risk for Hypertension via Enhanced Interactions With CREB (Cyclic AMP Response Element-Binding Protein) Transcription Factor

Lakshmi Subramanian, Sakthisree Maghajothi, Mrityunjay Singh, Kousik Kesh, Ananthamohan Kalyani, Saurabh Sharma, Madhu Khullar, Suma M. Victor, Snehasikta Swarnakar, Shailendra Asthana, Ajit S. Mullasari, Nitish R. Mahapatra

Abstract—MMP (matrix metalloproteinase)-7—a potent extracellular matrix degrading enzyme—is emerging as a new regulator of cardiovascular diseases. However, potential contributions of *MMP7* genetic variations to hypertension remain unknown. In this study, we probed for the association of a tag single-nucleotide polymorphism in the *MMP7* promoter (−181A/G; rs11568818) with hypertension in an urban South Indian population (n=1501). The heterozygous AG genotype significantly increased risk for hypertension as compared with the wild-type AA genotype (odds ratio, 1.60 [95% CI, 1.25–2.06]; $P=2.4\times10^{-4}$); AG genotype carriers also displayed significantly higher diastolic blood pressure and mean arterial pressure than wild-type AA individuals. The study was replicated in a North Indian population (n=949) (odds ratio, 1.52 [95% CI, 1.11–2.09]; $P=0.01$). Transient transfection experiments using *MMP7* promoter-luciferase reporter constructs revealed that the variant −181G allele conferred greater promoter activity than the −181A allele. Computational prediction and structure-based conformational and molecular dynamics simulation studies suggested higher binding affinity for the CREB (cyclic AMP response element-binding protein) to the −181G promoter. In corroboration, overexpression/downregulation of CREB and chromatin immunoprecipitation experiments provided convincing evidence for stronger binding of CREB with the −181G promoter. The −181G promoter also displayed enhanced responses to hypoxia and epinephrine treatment. The higher promoter activity of −181G allele translated to increased MMP7 protein level, and *MMP7* −181AG heterozygous individuals displayed elevated plasma MMP7 levels, which positively correlated with blood pressure. In conclusion, the *MMP7* A-181G promoter polymorphism increased MMP7 expression under pathophysiological conditions (hypoxic stress and catecholamine excess) via increased interactions with CREB and enhanced the risk for hypertension in its carriers. **(*Hypertension*. 2019;74:1448-1459. DOI: 10.1161/HYPERTENSIONAHA.119.12960.)** ● **Online Data Supplement**

Key Words: catecholamine ■ hypertension ■ hypoxia ■ MMP7 ■ single nucleotide polymorphism ■ transcriptional regulation

MMPs (matrix metalloproteinases) degrade the components of extracellular matrix (ECM) and thereby contribute to tissue remodeling and developmental processes. MMPs cleave bioactive molecules, chemokines, cytokines, growth factors involved in physiological processes including inflammation, angiogenesis, and wound healing.[1] To date, 24 different MMPs have been identified and classified into collagenases, stromelysins, gelatinases, matrilysins, and membrane-type MMPs based on their domain and substrate specificities. Expressions of MMP genes are primarily regulated at the transcriptional level, and activities of MMPs are inhibited by tissue inhibitors of MMPs.[2]

MMP7—the smallest MMP (molecular weight, 28 kDa)—is a secreted matrilysin with specificity for a broad range of substrates (*viz.* fibronectin, elastin, type IV collagen, and proteoglycans). MMP7 also cleaves non-matrix substrates (eg, proheparin-binding epidermal growth factor, Fas ligand, E-cadherin, β2 adrenergic receptor, and TNF-α [tumor necrosis factor-α]) and activates other MMPs (*viz.* pro-MMP2 and pro-MMP9).[3] Serum/plasma MMP7 levels are elevated in several types of cancers, atherosclerosis, hypertension, chronic kidney disease, and diabetes mellitus.[4–7] Since uncontrolled proteolytic processes leading to vascular remodeling act as an important determinant of

Received March 6, 2019; first decision March 23, 2019; revision accepted September 26, 2019.

From the Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India (L.S., S.M., A.K., N.R.M.); Drug Discovery Research Center, Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad, Haryana, India (M.S., S.A.); Drug Development Diagnostic and Biotechnology Division, Indian Institute of Chemical Biology, Kolkata, India (K.K., S.SW.); Department of Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education and Research, Chandigarh, India (S. Sharma, M.K.); and Institute of Cardiovascular Diseases, Madras Medical Mission, Chennai, India (S.M.V., A.S.M.).

The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.119.12960.

Correspondence to Nitish R. Mahapatra, Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai 600036, India. Email nmahapatra@iitm.ac.in

cardiovascular complications, MMPs are expected to serve as potential mediators in cardiovascular diseases (CVDs).[8] Indeed, knockdown of *MMP7* in the SHR (spontaneous hypertensive rat) model attenuates hypertension.[9] MMP7 transcriptionally activates MMP2, and knockdown of *MMP7* prevents the progression of Ang II (angiotensin II)–induced hypertension and cardiac hypertrophy.[10] Genetic polymorphisms in *MMP7* have been associated with coronary artery disease, acute myocardial infarction, multiple sclerosis, rheumatoid arthritis, and several cancers.[7,11] Among the most widely studied single nucleotide polymorphisms (SNPs) of *MMP7*, the promoter SNPs A-181G (rs11568818) and C-153T (rs11568819) displayed allele-specific effects and modulated gene expression via differential interaction with nuclear-binding proteins.[11,12] However, potential association of regulatory variants in *MMP7* gene with hypertension has not been reported to date. Here, we aimed to study the association of the common tag SNP A-181G in the *MMP7* gene promoter with hypertension in 2 geographically distinct Indian populations and to elucidate the mechanism governing the allele-specific effects of the polymorphism using systematic computational and experimental analyses.

Materials and Methods

The detailed Materials and Methods are provided in the online-only Data Supplement. The authors declare that all supporting data are available within the article and the online-only Data Supplement.

Human Subjects

The study population comprised of 1354 hypertensive and 1096 normotensive individuals attending the outpatient departments of The Madras Medical Mission, Chennai, and the Postgraduate Institute of Medical Education and Research, Chandigarh, during 2012 to 2018. The study was approved by the Institutional Ethics Committee at Indian Institute of Technology Madras. Demographic, physiological, and biochemical parameters of both the study populations are listed in Tables S1 and S2 in the online-only Data Supplement.

Genotyping of *MMP7* −181A/G Polymorphism

Human genomic DNA samples were polymerase chain reaction amplified using specific primers for the *MMP7* promoter region (−302 to −153 bp), purified, and genotyped (Figure S1).

Cloning and Mutagenesis

MMP7 −181A/G promoter-reporter constructs (harboring −230bp to +22 bp region of *MMP7* gene) were generated using pGL3-Basic vector. *MMP7* −181A/G promoter-cDNA constructs were generated by replacing the firefly luciferase cDNA in *MMP7* promoter-reporter constructs with *MMP7* cDNA.

Cell Lines, Transfection, and Reporter Assays

MMP7 promoter-reporter constructs and β-gal (β-galactosidase) expression plasmid were transfected into IMR-32, SH-SY5Y, H9c2, and N2a cell lines. Cotransfection experiments with CREB (cyclic AMP response element-binding protein) and KCREB (dominant-negative CREB) expression plasmids,[13] CREB siRNA oligos, and treatments (epinephrine and hypoxia) were performed in IMR-32 and H9c2 cells. Luciferase, β-gal, and total protein assays were performed,[14] and promoter activities were expressed as luciferase/β-gal or luciferase/μg of protein.

Experiments were also performed to mimic the homozygous and heterozygous conditions *in cella* with *MMP7* promoter-reporter constructs and by transfecting *MMP7* promoter-cDNA constructs to estimate MMP7 levels *in vitro*.

Western Blotting

Immunoblotting experiments were performed to detect overexpression or downregulation of CREB, phospho-CREB, and HIF-1α (hypoxia-inducible factor 1α) after transfection experiments/epinephrine treatment/hypoxia and to measure the influence of *MMP7* −181G and −181A promoter variants on the MMP7 levels *in vitro* using specific antibodies.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were performed in N2a and H9c2 cells transfected with *MMP7* −181G and *MMP7* −181A promoter-reporter constructs with/without treatment with epinephrine (5 μM) or hypoxia.[5] Immunoprecipitated DNA in case of −181G and −181A alleles due to CREB/phospho-CREB binding were quantified by quantitative polymerase chain reaction using fold enrichment method relative to IgG signal.

Computational Analysis

The homology model of CREB1 was built using MODELLER v11.9[15] based on the template 1DH3 (*Mus musculus* mCREB1). The *MMP7* −181G and *MMP7* −181A promoter DNA models were built using 3DDART[16] and SCFBio (http://www.scfbio-iitd.res.in/research/drugdna.html). Protein-DNA docking was performed with HDOCK[17] and HADDOCK[18] tools. Molecular dynamics simulation study for all CREB1-*MMP7* (protein-DNA) complexes/models was performed using Desmond[19,20] tool in Maestro.

Data Presentation and Statistical Analysis

Genotype-phenotype associations were tested by 1-way ANOVA with post hoc tests using Statistical Package for Social Sciences. Promoter-reporter transfection results were expressed as mean±SEM from representative experiments. Statistical significance was calculated by Student *t* test, 1-way ANOVA, or 2-way ANOVA with post hoc tests, as applicable, using Prism 5 program.

Results

Identification and Linkage Disequilibrium Analysis of SNPs in the *MMP7* Promoter

SNPs occurring at a frequency ≥1% in the 5-kb region of *MMP7* promoter in the South Asian population of the 1000 Genomes project were identified from dbSNP database (Figure S2; Table S3). Of the 10 SNPs identified, 7 were common polymorphisms (occurring at a frequency of ≥5%). Pairwise linkage disequilibrium analysis was performed to predict nonrandom association of the alleles of these 10 SNPs and to shortlist tag SNPs. Eight of the 10 SNPs constituted a haplotype block suggesting that these alleles could be inherited together (Figure S2B). Additionally, linkage disequilibrium tag SNP selection tool from SNPinfo[21] web server predicted the rs11568818 (*MMP7* A-181G) polymorphism as a tag SNP; it occurred at a minor allele frequency (MAF) of 0.36 in the overall 1000 Genomes population and 0.43 in the South Asian superpopulation. This tag SNP was in linkage disequilibrium with SNPs at −1378 bp (rs17098318) and −1773 bp (rs17881620) (Figure S2B).

Occurrence of *MMP7* −181 SNP in Indian Populations

The *MMP7* A-181G SNP occurred at an MAF of 0.47 (≈72.4% of the subjects) in the Chennai population (n=1501). MAF of this SNP in a geographically distinct Chandigarh population (n=949) was 0.41 (≈64.3% of the subjects). The genotypic

frequencies in both populations were in Hardy-Weinberg equilibrium (Chennai population: X^2 P=0.94 and Chandigarh population: X^2 P=0.44) (Table S4).

MMP7 −181AG Genotype Is Associated With Hypertension in Indian Populations

Logistic regression analysis was performed to estimate the relative risk contributed by the variant G allele toward hypertension by both genotypic (AA versus AG and AA versus GG) and dominant (AA versus AG+GG) models. The −181AG heterozygous genotype displayed association with hypertension risk as compared with the −181AA genotype with odds ratios (ORs) of 1.60 ([95% CI, 1.25–2.04] P=1.6×10^{-4}) in Chennai population and 1.49 ([95% CI, 1.12–1.97]; P=0.006) in Chandigarh population (Table). These associations remained significant even after adjusting for age/sex/body mass index (Chennai population: OR, 1.60 [95% CI, 1.25–2.06]; P=2.4×10^{-4} and Chandigarh population: OR, 1.52 [95% CI, 1.11–2.09]; P=0.01). Although the homozygous variant GG genotype did not show a statistically significant OR, the dominant model (AG+GG) exhibited a significantly strong association with hypertension in both Chennai (adjusted OR, 1.37 [95% CI, 1.09–1.73]; P=0.006) and Chandigarh (adjusted OR, 1.46 [95% CI, 1.09–1.98]; P=0.013) populations (Table).

Differential Activities of *MMP7* −181A/G Promoter-Reporter Constructs in Cultured Cardiomyoblast and Neuroblastoma Cells

To test the functional role of −181A and −181G alleles, *MMP7* promoter-reporter constructs harboring A/G allele were transfected into cardiomyoblast (H9c2) and neuroblastoma (IMR-32, SH-SY5Y, and N2a) cell lines (Figure 1). *MMP7* −181G construct consistently displayed higher promoter activity than the −181A construct in all these cell lines (H9c2: ≈1.7-fold, P<0.01; IMR-32: ≈1.4-fold, P<0.001; SH-SY5Y: ≈1.3-fold, P<0.05; N2a: ≈1.5-fold, P<0.05) (Figure 1).

Computational Analyses Reveal Differential Interactions of CREB With *MMP7* −181A/G Promoters

To probe for potential differential interactions of transcription factors at the −181 bp position that could contribute to the higher *MMP7* promoter activity in case of the −181G allele, computational analysis was performed using ConSite/MatInspector/P-Match programs. The transcription factor CREB was predicted to bind to the −181G allele with higher affinity by both ConSite and P-Match. The Transfac position-weight matrix for CREB with binding scores for −181A and −181G alleles is shown in the Figure S3. Structure-based conformational and molecular dynamics simulation studies were next performed using models of CREB and *MMP7* promoters.

The CREB1 homology model was generated for the DNA binding region corresponding to residues 280-341 of CREB1 based on DP-Bind and PredictProtein tool predictions. Models of *MMP7* −181A and *MMP7* −181G promoters were also generated, and docking of the CREB1 structure to the promoter DNA structures followed by molecular dynamics simulations was performed (Figure 2A; Figure S4; Table S5).

The average structure was extracted from the first 40 ns simulation to generate the comparable stable interaction map between *MMP7*-promoter DNA and CREB1. In case of CREB1-chain A:*MMP7* −181A complex, N293 and R301 were the 2 main amino acids that participated in the interactions with the promoter DNA segment. The residue N293 formed total 4 hydrogen bonds (*viz.* one with A24 nucleotide at DNA-chain C, 2 with A25 nucleotide at DNA-chain C, and 1 with T23 nucleotide at DNA-chain D) (Figure 2B and 2D); the residue R301 formed 1 hydrogen bond with T22 nucleotide at DNA-chain D. On the contrary, the CREB1-chain A:*MMP7* −181G complex, apart from having interactions similar to that of *MMP7* −181A complex, also established 3 additional hydrogen bonds with residue R289 (2 hydrogen bonds with G23 nucleotide at DNA-chain C [i.e., the −181G variant nucleotide] and 1 hydrogen bond with T25 nucleotide at DNA-chain D) (Figure 2C and 2E).

The 2-dimensional interaction map of the *MMP7* −181A and *MMP7* −181G systems reflects the same interaction pattern (Figure S5). The interaction map displays 2 regions of interactions for CREB1-chain A, between 22-26 nucleotide on *MMP7* DNA:chain C (i.e., −180 to −184 bp of *MMP7* promoter) and 21-22 nucleotide on *MMP7* DNA:chain D (i.e., −185 to −186 bp of *MMP7* promoter). Similarly, CREB1-chain B interacts with 29-31 nucleotide on *MMP7* DNA:chain C (i.e., −187 to −189 bp of *MMP7* promoter) and 15-18 nucleotide on *MMP7* DNA:chain D (i.e., −189 to −192 bp of *MMP7* promoter). Despite differences in the van der Waals contacts made by the amino acid residues of CREB1-chain A and CREB1-chain B with the *MMP7* wild-type and mutated promoters, the direct hydrogen bonds between the *MMP7* −181G nucleotide and a hot-spot residue R289 could facilitate the favorable binding of CREB to the *MMP7* −181G promoter with higher affinity over *MMP7* −181A promoter.

Table. Association of *MMP7* −181A/G Polymorphism With Hypertension Risk in Indian Populations

MMP7 Promoter Genotype	Chennai Population				Chandigarh Population			
	Logistic Regression (Unadjusted), n=1501		Logistic Regression (Age, Sex, and BMI Adjusted), n=1453		Logistic Regression (Unadjusted), n=949		Logistic Regression (Age and Sex Adjusted), n=949	
	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
AA	1 (ref)	…	1 (ref)	…	1 (ref)	…	1 (ref)	…
AG	1.60 (1.25–2.04)	1.6×10^{-4}	1.60 (1.25–2.06)	2.4×10^{-4}	1.49 (1.12–1.97)	0.006	1.52 (1.11–2.09)	0.010
GG	0.99 (0.75–1.33)	0.98	1.01 (0.75–1.36)	0.94	1.14 (0.78–1.66)	0.49	1.31 (0.86–2.01)	0.210
AG+GG	1.37 (1.09–1.73)	0.006	1.39 (1.09–1.75)	0.007	1.38 (1.06–1.80)	0.017	1.46 (1.09–1.98)	0.013

BMI indicates body mass index; CI, confidence interval; MMP7, matrix metalloproteinase-7; and OR, odds ratio.

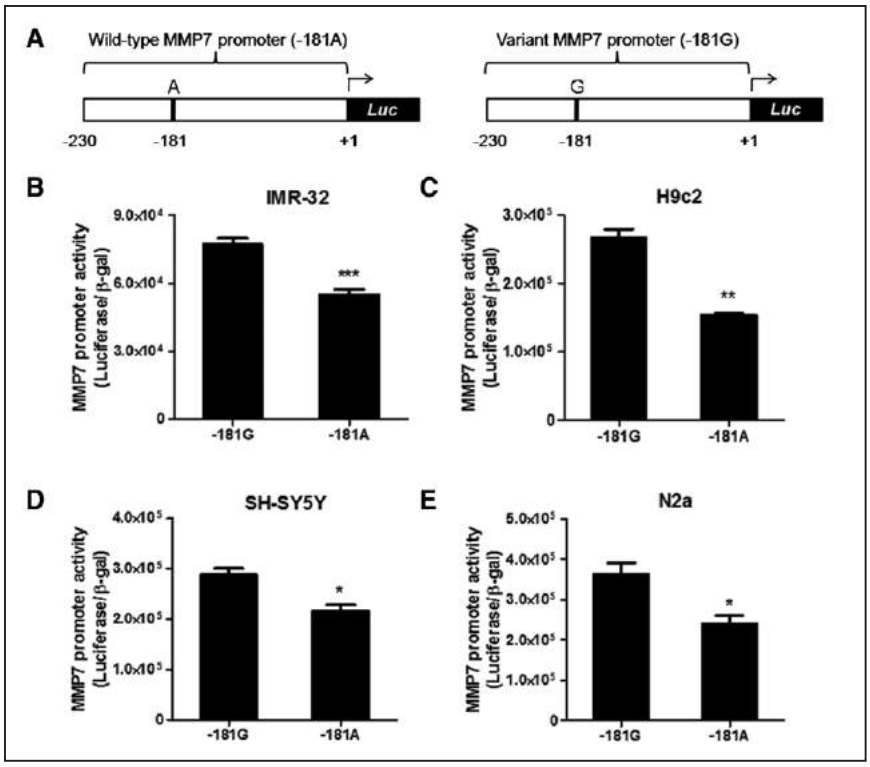

Figure 1. Differential promoter activities of *MMP7* promoter-reporter constructs. **A**, Schematic representation of the *MMP7* promoter-reporter constructs. **B**–**E**, *MMP7* −181G and −181A promoter-reporter constructs were transfected into rat cardiomyoblast H9c2 (**B**) neuroblastoma cell lines IMR-32 (**C**), SH-SY5Y (**D**), and N2a (**E**) along with β-gal (β-galactosidase) expression plasmid. Results are expressed as mean±SEM of triplicate values of the ratio of luciferase/β-gal activity. **P*<0.05, ***P*<0.01, and ****P*<0.001 with respect to −181G construct.

Experimental Evidence for Interactions of CREB With *MMP7* −181A/G Promoters

In view of the prediction of enhanced interactions of CREB with the *MMP7* −181G allele (Figure 2; Figure S5), experimental validation of differential activation of *MMP7* −181A/G promoters by CREB was performed. Overexpression of CREB increased activities of *MMP7* promoter-reporter constructs in a concentration-dependent manner; the −181G construct

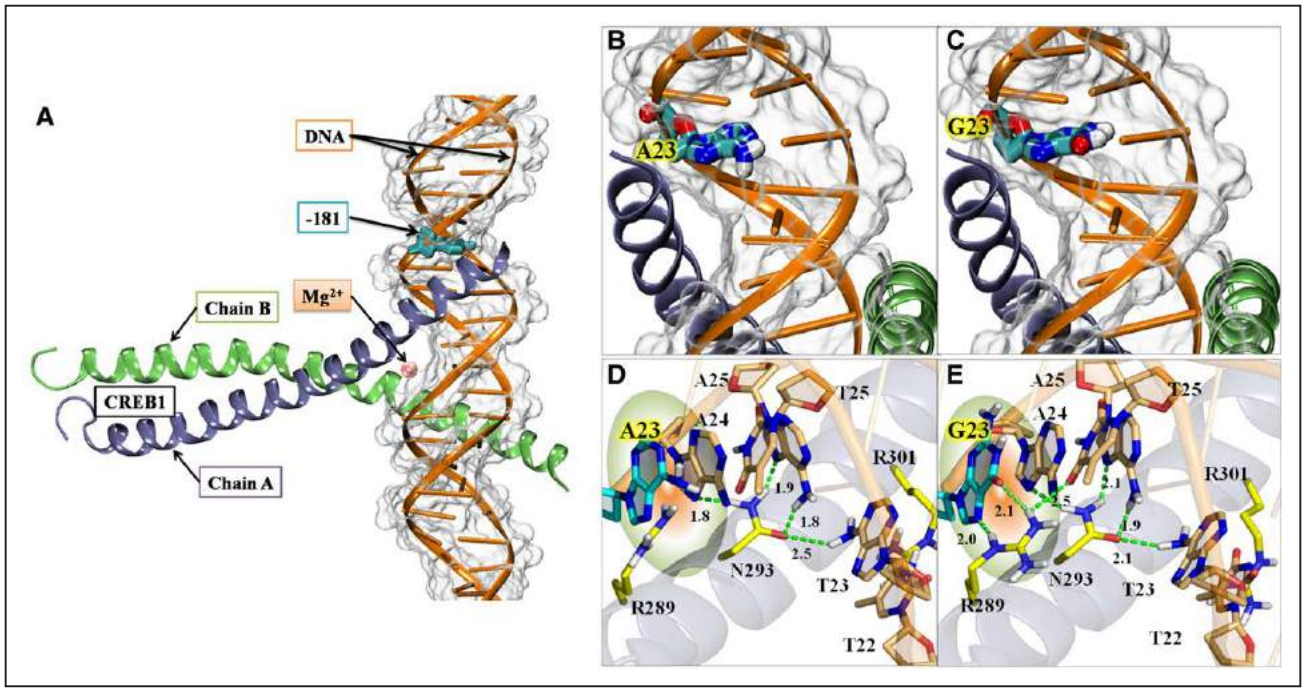

Figure 2. Schematic diagram of interactions of *MMP7*-promoter DNA with CREB (cyclic AMP response element-binding protein)-1 transcription factor. **A**, Representative energy minimized model of CREB1:wild-type *MMP7* complex rendered in new cartoon representation. DNA is shown in orange color; CREB1-chain A and CREB1-chain B are shown in ice-blue and green colors, respectively. DNA is shown in transparent white surface view as well. Position of the −181 bp nucleotide and Mg^{2+} has been indicated. **B**, Positioning of the nucleotide −181A (labeled as A23) in wild-type promoter. **C**, Positioning of the nucleotide −181G (labeled as G23) in the mutant promoter. Comparison of wild-type (**D**) and mutant (**E**) *MMP7*-promoter DNA interactions with CREB1 in an enlarged view. Amino acids are indicated by single-letter codes and based on their positions in the CREB1-chain A. Amino acids and nucleotides are rendered in licorice and colored atom-wise; C, light orange; N, blue; O, red; H, white. Hydrogen bonds are shown in green dotted lines.

exhibited significantly higher promoter activity than the −181A construct in IMR-32 (1-way ANOVA F=162.9, $P<0.0001$) and H9c2 cells (1-way ANOVA F=43.3, $P<0.0001$) (Figure S6A and S6B). Overexpression of CREB was confirmed by Western blotting. In corroboration, cotransfection of KCREB plasmid lead to a highly significant concentration-dependent decrease (at least 60%) in the promoter activity of *MMP7* −181G construct in both cell types (IMR-32: 1-way ANOVA F=13.72, $P<0.0001$; H9c2: 1-way ANOVA F=11.45, $P<0.001$) (Figure S6C and S6D). The −181A construct did not show any change in promoter activity in response to KCREB cotransfection. Downregulation of CREB using siRNA also reduced the promoter activity of −181G construct as compared with the negative control oligo in IMR-32 (by ≈45%; 1-way ANOVA F=7.13, $P<0.05$) and H9c2 (by ≈60%; 1-way ANOVA F=79.02, $P<0.0001$) cells; −181A construct did not show consistent reduction in promoter activity under similar conditions (Figure 3A and 3B). Downregulation of CREB was confirmed by Western blotting.

Further, chromatin immunoprecipitation assays were performed to study the interaction and preferential binding of CREB with the *MMP7* −181G promoter in the context of chromatin. Quantitative polymerase chain reaction of purified, CREB-immunoprecipitated chromatin from N2a and H9c2 cells transfected with *MMP7* −181G and −181A promoter constructs revealed that the enrichment of the *MMP7* −181G allele was ≈1.7- and ≈1.8-fold higher, respectively, as compared with the −181A allele ($P<0.05$; Figure 3C and 3D).

Enhanced Response of *MMP7* −181G Promoter to Epinephrine: Crucial Role for CREB

Since elevated levels of catecholamines are associated with hypertension, we evaluated the effect of epinephrine and the concomitant role of CREB, if any, on *MMP7* −181G and −181A promoter activities. Indeed, epinephrine treatment augmented the promoter activity of *MMP7* −181G construct in a concentration-dependent manner in IMR-32 (≤2.5-fold; 1-way ANOVA F=22.03, $P<0.0001$) and in H9c2 (≈2.2-fold; 1-way ANOVA F=17.10, $P<0.0001$) cells; on the contrary, *MMP7* −181A construct did not show significant increase in promoter activity (Figure S7A and S7B). Consistently, epinephrine treatment enhanced phospho-CREB levels in a concentration-dependent manner in these cell lines (Figure S7C and S7D). Further, to test whether CREB mediated the activation of *MMP7* −181G promoter in response to epinephrine, IMR-32 and H9c2 cells were cotransfected with KCREB and *MMP7* −181G or −181A promoter-reporter constructs followed by epinephrine treatment. Epinephrine, which augmented the promoter activity of *MMP7* −181G construct significantly, failed to evoke a similar response in the KCREB cotransfected condition in IMR-32 (2-way ANOVA genotype effect: F=56.05, $P<0.0001$; treatment effect: F=15.30, $P<0.0001$) and H9c2 (2-way ANOVA genotype effect: F=16.50, $P<0.001$; treatment effect: F=17.58, $P<0.0001$) cells. The −181A construct did not show any significant difference in promoter activity upon epinephrine treatment either in the presence or absence of KCREB (Figure S7E and S7F).

In chromatin immunoprecipitation assays using *MMP7* −181G/A promoter-transfected and epinephrine-treated cells, the −181G allele displayed significantly higher fold enrichment with CREB (N2a: ≈5.0-fold; H9c2: ≈1.8-fold) and phospho-CREB (N2a: ≈4.5-fold; H9c2: ≈3.5-fold) as compared with the −181A allele (Figure S7G and S7H). Thus, the epinephrine induced activation of *MMP7* −181G promoter activity appears to be strongly mediated by CREB.

Hypoxic Stress Activates *MMP7* −181G Promoter via CREB

Since hypoxia is known to increase blood pressure (BP) and is a major contributor to cardiac pathophysiology, the effect of hypoxia on *MMP7* −181G and −181A promoter-reporter constructs was studied. IMR-32 and H9c2 cells were subjected to hypoxia after transfection with −181G/A promoter constructs for 12 hours with/without CREB and KCREB cotransfection. The *MMP7* −181G construct displayed significantly enhanced promoter activity under hypoxia in both IMR-32 (≈1.7-fold) and H9c2 (≈2.3-fold) cells; however, no significant increase was observed with the −181A construct (Figure 4A and 4B). Upon CREB overexpression, hypoxia treatment further increased the *MMP7* −181G promoter activity (≈5.7-fold in IMR-32 and ≈7.5-fold in H9c2) as compared with *MMP7* −181A construct in IMR-32 (2-way ANOVA genotype effect: F=39.7, $P<0.0001$; treatment effect: F=267.8, $P<0.0001$) and H9c2 (2-way ANOVA genotype effect: F=30.97, $P<0.0001$; treatment effect: F=73.01, $P<0.0001$) cells. On the contrary, hypoxia resulted in only a modest increase in promoter activity in the KCREB cotransfected cells. Under hypoxia, concomitant with HIF-1α (IMR-32: ≈6.6-fold; H9c2: ≈7.9-fold), CREB (IMR-32: ≈1.4-fold; H9c2: ≈1.8-fold), and phospho-CREB (IMR-32: ≈1.5-fold; H9c2: ≈1.3-fold) levels were also found to be elevated (Figure 4C and 4D).

Further, interactions of CREB/phospho-CREB with −181G/A alleles under hypoxia were probed in N2a and H9c2 cells transfected with *MMP7* promoter-reporter constructs. The fold enrichment for CREB (N2a: ≈1.5-fold; H9c2: ≈1.4-fold) and phospho-CREB (N2a: ≈2.3-fold; H9c2: ≈1.9-fold) in case of the −181G allele under hypoxia was significantly higher (Figure 4E and 4F) than the −181A allele (N2a: 1-way ANOVA F=63.7, $P<0.0001$; H9c2: 1-way ANOVA F=28.0, $P<0.0001$).

MMP7 −181G Promoter Results in Higher MMP7 Levels *In Vitro* and *In Vivo*

By generating recombinant plasmids wherein hMMP7 cDNA was placed under the control of *MMP7* −181G and *MMP7* −181A promoters, we probed whether the differential activities of G/A alleles result in altered MMP7 levels *in vitro* (Figure S8A). Expression of these plasmids in N2a (Figure S8B and S8C) and H9c2 (Figure S8D and S8E) cells revealed significantly elevated levels of MMP7 in case of −181G promoter-driven *MMP7* cDNA (N2a: ≈1.6-fold, $P<0.0001$; H9c2: ≈1.4-fold, $P<0.05$) as compared with −181A promoter-driven *MMP7* cDNA suggesting higher activity of −181G promoter in the genomic context.

Furthermore, we analyzed plasma MMP7 levels in a section of normotensive/untreated subjects from the study population after stratification into *MMP7* promoter genotypes; *MMP7* −181AG individuals displayed higher plasma MMP7 levels (Figure 5A) than −181AA and −181GG individuals (1-way ANOVA F=3.06, $P<0.05$). Although plasma MMP7

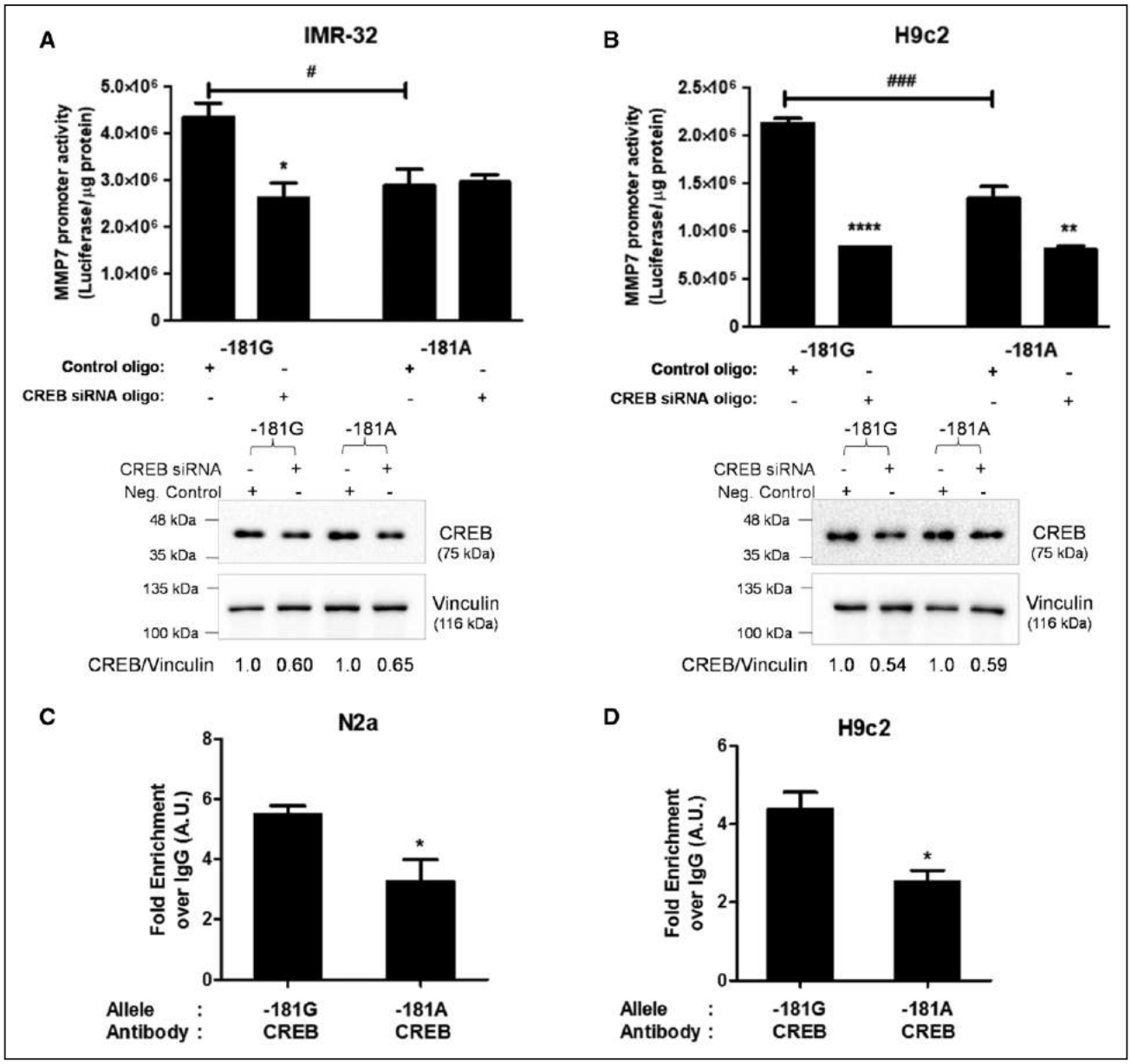

Figure 3. Enhanced interactions of CREB (cyclic AMP response element-binding protein) with *MMP7* −181G allele. **A** and **B**, siRNA-mediated knockdown of CREB decreases the *MMP7* −181G promoter activity. IMR-32 (**A**) and H9c2 (**B**) cells were transfected with *MMP7* −181G or −181A constructs along with control siRNA oligo or CREB siRNA oligo. Assays were performed after 48 h of transfection. Results are expressed as mean±SEM of triplicate values of the ratio of luciferase activity/μg protein. **P*<0.05, ***P*<0.01, and *****P*<0.0001 as compared with the basal activity of the corresponding construct. #*P*<0.05 and ###*P*<0.001 as compared with the basal activity of *MMP7* −181G construct. Downregulation of CREB was confirmed by Western blotting. **C** and **D**, Binding of CREB to −181G and −181A alleles of *MMP7* promoter. Chromatin immunoprecipitation of N2a (**C**) and H9c2 (**D**) cells transfected with −181G or −181A construct was performed using antibody against CREB/control IgG. Immunoprecipitated chromatin was subjected to quantitative polymerase chain reaction. Fold enrichment in case of CREB antibody over IgG control is shown. **P*<0.05.

levels were modestly higher in individuals of −181GG genotype than those with −181AA genotype, the difference was not statistically significant. Thus, the plasma levels of MMP7 follow the same trend of association displayed by the −181AG individuals in the logistic regression analysis toward hypertension risk (Table).

Activities of *MMP7* Promoter Constructs in Diploid Combinations *in Cella*

In view of the association of *MMP7* −181AG genotype with hypertension risk, as well as higher MMP7 levels *in vivo*, we sought to test whether this observation could also be demonstrated in transfected cultured cells. We transfected IMR-32 and H9c2 cells with −181G or −181A promoter-reporter plasmids in 3 diploid combinations: −181G/G (ie, only −181G construct), −181G/A (ie, equimolar amounts of −181G and −181A constructs), and −181A/A (ie, only −181A construct) to mimic the homozygous variant, heterozygous and wild-type conditions, respectively. Interestingly, both −181G and −181G/A transfected conditions showed similar extent of elevated promoter activity (≈1.8-fold and ≈1.75-fold, respectively, in IMR-32 cells; ≈1.4-fold and ≈1.3-fold, respectively, in H9c2 cells) as compared with the −181A construct alone (IMR-32: 1-way ANOVA F=63.74, *P*<0.0001;

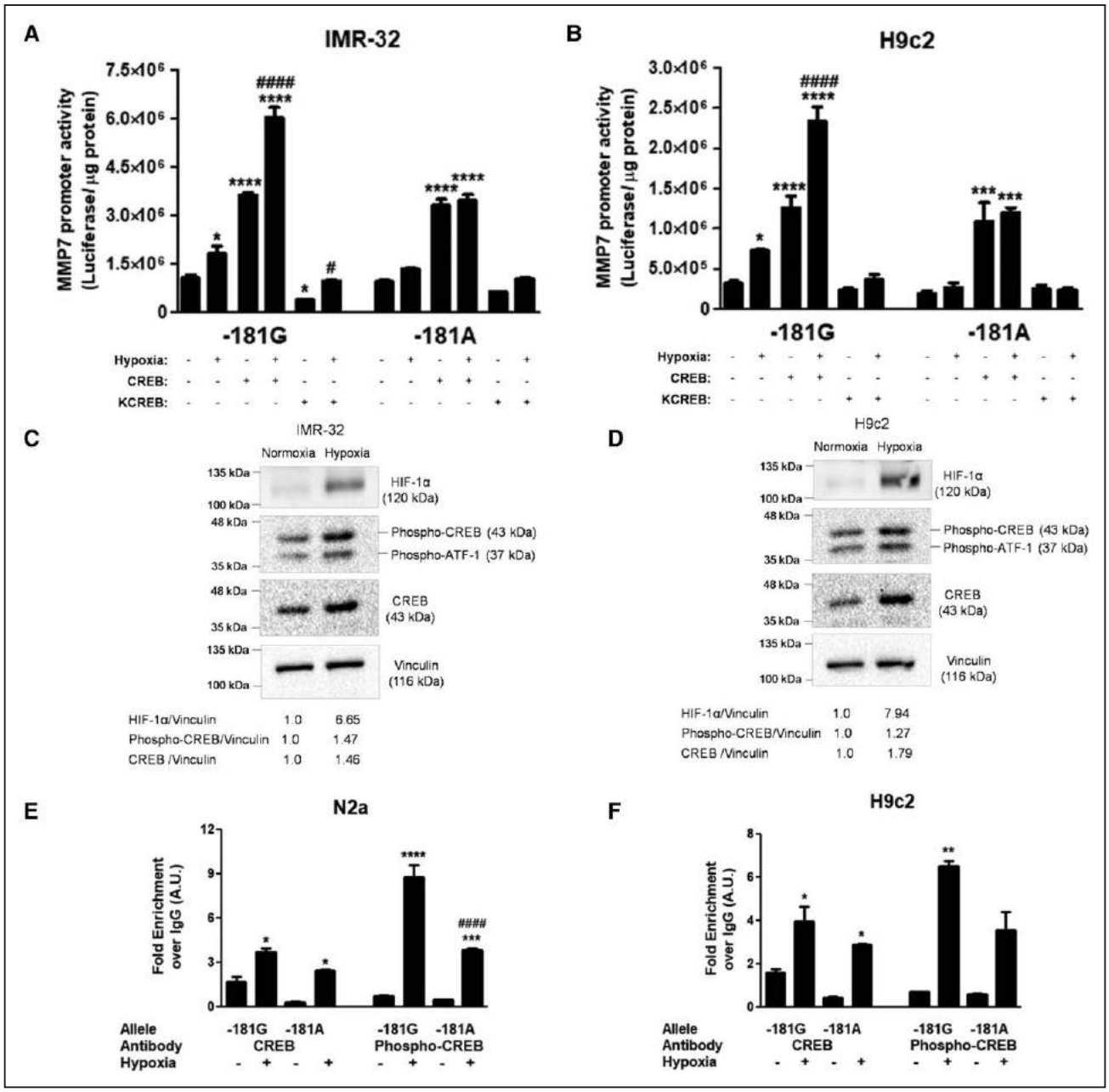

Figure 4. Effect of hypoxia on *MMP7* promoter: crucial role for CREB (cyclic AMP response element-binding protein). **A** and **B**, *MMP7* −181G and −181A promoter-reporter constructs were transfected into IMR-32 (**A**) and H9c2 (**B**) cells with/without CREB and KCREB (dominant-negative CREB) expression plasmid and subjected to 12 h of hypoxia. Results are expressed as mean±SEM of triplicate values of luciferase activity/μg protein. **P*<0.05, ****P*<0.001, and *****P*<0.0001 when compared with basal activity of the respective construct and #*P*<0.05 and ####*P*<0.0001 as compared with the corresponding normoxic condition. IMR-32 (**C**) and H9c2 (**D**) cells were subjected to hypoxia for 12 h, and Western blotting of the total proteins was performed probing for HIF-1α (hypoxia-inducible factor 1α), CREB, phospho-CREB, and vinculin. **E** and **F**, Chromatin immunoprecipitation of N2a and H9c2 cells transfected with *MMP7* −181G or −181A promoter construct, with/without exposure to hypoxia was performed using antibody against CREB, phospho-CREB, and preimmune IgG. Fold enrichment in case of CREB/phospho-CREB antibody over preimmune IgG is shown. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001 when compared with the respective untreated condition and ####*P*<0.0001 as compared with the corresponding treatment in case of *MMP7* −181G promoter construct.

and H9c2: 1-way ANOVA F=51.37, *P*<0.001) (Figure 5B). These results suggest that the *MMP7* −181G allele may act in a dominant manner while impacting the promoter activity.

Association of *MMP7* −181AG Genotype With BP

To evaluate potential associations of the *MMP7* promoter SNP with cardiovascular traits, inferential statistics were performed using demographic/physiological/biochemical parameters of the study subjects after stratifying them based on their genotypes. The BP data were adjusted for antihypertensive drugs according to Cui et al.[22] We observed higher mean arterial pressure (MAP) (≈2–3 mm Hg) and diastolic BP (DBP) (≈2–3 mm Hg) in individuals of *MMP7* −181AG genotype than −181AA or −181GG individuals in both Chennai (MAP: 1-way ANOVA F=3.15, *P*<0.05; DBP: 1-way ANOVA F=3.69, *P*<0.05) and Chandigarh (MAP: 1-way ANOVA F=3.02, *P*<0.05; DBP: 1-way ANOVA F=4.21, *P*<0.05) populations (Figure 5C and 5D). The MAP and DBP levels in

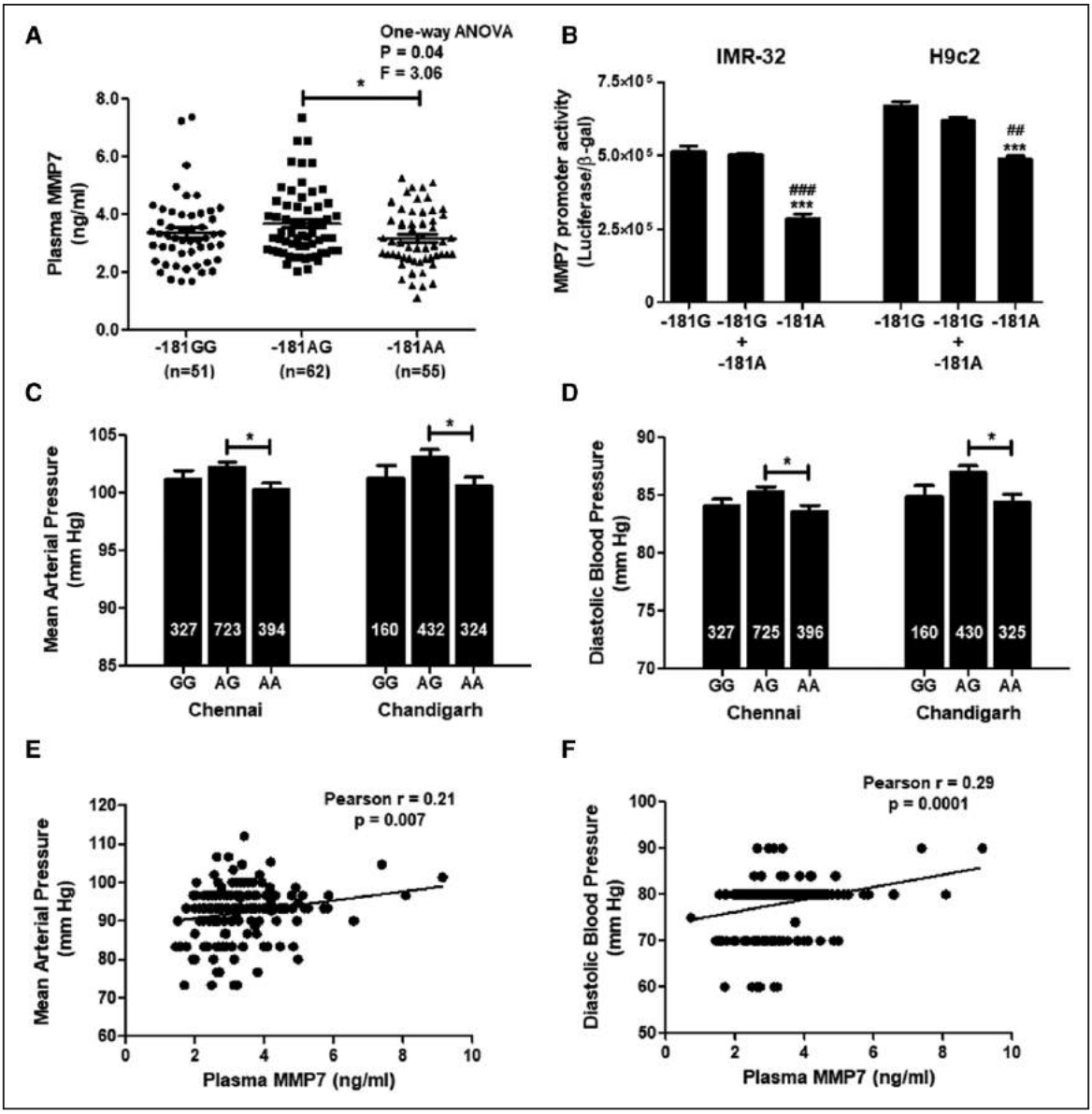

Figure 5. Allele-specific association of *MMP* (matrix metalloproteinase)-*7*-181 promoter genotypes with plasma MMP7 levels and blood pressure in Indian populations. **A**, Plasma MMP7 levels in −181GG, −181AG, and −181AA individuals as measured by ELISA. **B**, IMR-32 and H9c2 cells were transfected with 1 μg each of *MMP7* −181G or −181A or 500 ng each of −181G and −181A constructs. Results are mean±SEM of triplicate values of luciferase/β-gal activity. ***P<0.001 as compared with the −181G and ##P<0.01, ###P<0.001 as compared with the −181G+−181A conditions. **C** and **D**, *MMP7* −181AG individuals display higher mean arterial pressure (**C**) and diastolic blood pressure (**D**) in Chennai and Chandigarh populations. *P<0.05 **E** and **F**, Correlation of diastolic blood pressure (**E**) and mean arterial pressure (**F**) with MMP7 levels. MMP7 levels showed significant positive correlation with diastolic blood pressure and mean arterial pressure. Pearson r and P for the correlations are indicated.

these subjects positively correlated with plasma MMP7 levels (MAP: Pearson r=0.21, P<0.01; DBP: Pearson r=0.29, P<0.001) (Figure 5E and 5F). *MMP7* −181AG individuals also displayed a trend toward higher systolic BP (≈3 mm Hg), although the difference was not statistically significant (Figure S9). However, a modest positive correlation was observed with plasma MMP7 levels in these individuals (Pearson r=0.17, P<0.05) (Figure S9).

Discussion

Overview

Hypertension—a major risk factor for CVD—is characterized by several complex pathophysiological mechanisms including cardiac remodeling.[23] The remodeling that occurs initially as an adaptive response results in an altered ECM content ultimately leading to cardiovascular dysfunction.[24] MMPs play a crucial role in cardiac remodeling by degrading ECM components, which provide structural and mechanical support to the vasculature.[25] MMP7 cleaves a wide range of substrates including ECM components, vasoactive ligands, growth factor receptors, proinflammatory molecules, and other MMPs.[26] Knockdown of *MMP7* in SHR model attenuated hypertension and stopped the development of cardiac hypertrophy.[9] MMP7 is involved in early stages of agonist-induced hypertension (serving as a transcriptional regulator of MMP2) wherein knockdown of MMP7 and TACE (TNF-α

converting enzyme) prevented hypertension, as well as the development of cardiac hypertrophy.[10] Since expression of MMP7 is known to be tightly regulated at transcriptional level, polymorphisms in the promoter region may affect the gene expression by altering the binding affinities of transcription factors. Such functional polymorphisms could potentially alter susceptibility to pathophysiological phenotypes.[11] Two polymorphisms in the *MMP7* promoter (A-181G and C-153T) have been associated with the risk of coronary artery disease[11] but association of these variants with hypertension risk has not been investigated.

Occurrence and Association of *MMP7*A−181G Polymorphism With Hypertension in Human Populations

Pairwise linkage disequilibrium analysis of common SNPs in the *MMP7* upstream promoter region (Figure S2) identified *MMP7* A-181G as a tag SNP, which was further analyzed. Occurrence of this polymorphism in Indian populations (MAFs, 0.47 and 0.41; Table S4) was similar to that in European (MAF, 0.44), South Asian (MAF, 0.43), and African (MAF, 0.45) superpopulations of the 1000 Genomes project. However, frequency of this polymorphism was lesser in American superpopulation (MAF, 0.34) and the least in East Asian superpopulation (MAF, 0.085) suggesting its ethnicity-dependent occurrence in different World populations.

Next, we probed for association of the *MMP7* A-181G polymorphism with hypertension risk. The −181AG genotype conferred at least ≈1.5-fold higher risk of hypertension in both North Indian (Chandigarh) and South Indian (Chennai) populations; the dominant model also displayed a highly significant association with hypertension risk (Table). One limitation of the present study is that the −181GG genotype did not show a statistically significant association with hypertension. However, to the best of our knowledge, this is the first report on association of *MMP7* A-181G SNP with hypertension although many studies demonstrated its association with several types of cancers.[5,7,27–30] Of note, a previous study (using high-order gene-gene interaction analysis) in a North Indian population predicted that individuals carrying the combination of *MMP7* −181AG+GG, MMP9 668RQ+QQ, *AT1* (*Ang II type 1*) 1166 AC+CC and *NFKB1* -94 ATTG Ins/Ins genotypes had a significantly higher risk for left ventricular dysfunction (adjusted OR, 8.14; P=0.003).[31] It would be interesting to perform large-scale studies on the potential association of *MMP7* A-181G SNP with CVD states in different human populations.

Allele-Specific Effect of CREB in Activation of *MMP7* −181G Promoter in Basal and Pathophysiological Conditions

Transient transfections of *MMP7* promoter-reporter constructs displayed consistently higher promoter activity of the −181G promoter than the −181A promoter across different cell lines (Figure 1). Detailed computational analyses, CREB overexpression/downregulation studies, and chromatin immunoprecipitation assays affirmed stronger interaction of CREB transcription factor with the −181G promoter in neuronal/cardiomyoblast cells (Figures 2 and 3; Figures S5 and S6). This is consistent with our previous finding that CREB interacts with the −181G allele with higher affinity in adenocarcinoma cells.[5] The higher promoter activity of *MMP7* −181G allele has also been attributed to the generation of a putative binding site (NGAAN) for a heat-shock transcription factor in U937 macrophage cells.[11]

CREB is a ubiquitously expressed leucine-zipper transcription factor with well-known roles in cell proliferation, differentiation, and survival. The role of CREB in the development of CVDs has been recently recognized.[32] Several kinases (including protein kinase A/B/C) activate CREB by phosphorylating the Ser-133 residue which in turn recruits the CREB-binding protein to activate gene expression.[33] Transgenic mice overexpressing cardiomyocyte-specific dominant-negative CREB developed dilated cardiomyopathy and displayed reduced cardiac contractility upon isoproterenol treatment.[34] Phospho-CREB levels were also elevated in cerebral arteries of hypertensive rats.[35] Involvement of CREB in Ang II–induced IL (interleukin)-6 expression in vascular smooth muscle cells was attributed to a crucial cyclic AMP response element site. Ang II activated several kinases via AT1 receptor to result in CREB phosphorylation suggesting that CREB may have a role to play in the vascular remodeling associated with cardiac hypertrophy, heart failure, and atherosclerosis.[36] In view of these reports, our findings on the transcriptional regulation of MMP7 by CREB provide new insights into molecular mechanisms of CVDs.

Since stress elevates catecholamine levels through the hypothalamic-pituitary-adrenal axis[37] and hypertension is characterized by elevated levels of vasoconstrictive agonists such as catecholamines, we checked whether epinephrine (a catecholamine and known activator of CREB) exhibited any allele-specific effect with respect to the *MMP7* −181A/G promoters. Indeed, the *MMP7* −181G construct displayed a concentration-dependent increase in promoter activity in response to increasing doses of epinephrine with a concomitant increase in phospho-CREB levels (Figure S7). Epinephrine treatment also enhanced promoter occupancy of CREB/phospho-CREB for the −181G allele in the context of chromatin (Figure S7). Thus, −181G allele-carrying hypertensive individuals may have higher expression of *MMP7* due to catecholamine excess. Interestingly, isoproterenol activated MMP7 in gastric cancer cells and increased expression of MMP7 in gastric cancer tissue was observed at the sites where β2-adrenergic receptor was overexpressed.[38] Thus, epinephrine may play a key role in activation of *MMP7* −181G promoter in hypertension and other stress-induced cardiac complications.

Similarly, hypoxia is a common pathophysiological condition in CVDs including atherosclerosis and heart failure. Normotensive Sprague-Dawley rats developed sustained arterial hypertension when subjected to hypobaric hypoxia.[39] The hypoxic environment in the brain stem of SHR animals resulted in elevated sympathetic activity and a corresponding increase in arterial BP.[40] Moreover, HIF-1α signaling plays an important role in macrophage activation, which in turn contributes to the tissue remodeling processes and influences the severity of CVDs.[41] In addition to HIF-1α, hypoxic response

may also be mediated by NF-κB (nuclear factor-κB) and CREB. Interestingly, CREB/ATF-1 may also bind to the HIF-1 DNA recognition site.[42] In adrenal medullary PC12 cells, hypoxia resulted in Ser-133 phosphorylation of CREB, which persisted up to 24 hours.[43] In line with these reports, hypoxia significantly enhanced the promoter activity of *MMP7* −181G construct under basal as well as CREB cotransfected conditions via increased promoter occupancy of CREB (Figure 4). In corroboration, primary human monocyte-derived macrophages displayed elevated *MMP7* mRNA levels under hypoxia.[44] Thus, the A-181G polymorphism governs *MMP7* gene expression under basal, as well as pathophysiological, conditions.

Genotype-Phenotype Correlations of *MMP7* A-181G Polymorphism

Consistent with the higher transcription/translation of *MMP7* in the presence of the −181G allele *in vitro* (Figure S8), plasma MMP7 level was significantly higher in heterozygous AG carriers as compared with wild-type AA individuals (Figure 5A). Interestingly, the −181AG heterozygous combination also yielded significantly higher promoter activity than the −181A wild-type upon expression of diploid combinations of −181A/−181G constructs in cultured cells (mimicking the homozygous and heterozygous conditions), indicating possible enhancement of *MMP7* gene expression in the heterozygous individuals (Figure 5B). Indeed, previous reports suggested that altered expression of MMPs in different individuals could result due to the polymorphisms in the regulatory regions (eg, promoter) of MMP genes.[2]

Of note, in a preliminary study, hypercholesterolemic patients with coronary artery disease possessing the −181G allele presented with smaller reference luminal diameters before percutaneous transluminal coronary angioplasty than patients with the wild-type allele suggesting a functional role for the *MMP7* A-181G polymorphism in the matrix remodeling associated with coronary artery disease.[11] We also probed for association of the *MMP7* A-181G polymorphism with biochemical parameters (*viz.* total cholesterol, triglycerides, LDL [low-density lipoprotein], and HDL [high-density lipoprotein] cholesterol and blood glucose levels) that may act as comorbidities to CVD; however, no significant correlations were observed. Nonetheless, the significantly higher DBP and MAP observed in individuals of AG genotype (Figure 5C and 5D) corroborated with their increased hypertension risk (Table). The higher BP in *MMP7* −181AG individuals might result from enhanced proteolytic cleavage of the β2-adrenergic receptor by MMP7 as MMP7 level is elevated in these individuals because of higher promoter activity associated with the −181G allele. The cleavage of β2-adrenergic receptor may suppress the normal vasodilatory stimulus provided by this receptor upon agonist binding resulting in a lack of vasodilatory input to the arterial/arteriolar tone; this signaling cascade may lead to elevation of arterial BP.[45] Further studies are required to establish the mechanisms modulating BP homeostasis in these individuals.

In summary, we genotyped a common naturally occurring polymorphism (A-181G) in the upstream regulatory region of *MMP7* gene in 2 geographically distinct Indian populations. The *MMP7* A-181G polymorphism showed strong association with increased hypertension risk in our study populations. The −181G allele-containing promoter displayed higher promoter activity than the −181A promoter in basal/pathophysiological conditions (hypoxia, catecholamine excess) due to preferential binding of CREB, translating to increased MMP7 levels *in vitro*. The risk genotype (−181AG) also had a correlative association with increased plasma MMP7 levels and BP suggesting that this functional regulatory polymorphism may contribute to cardiovascular risk.

Perspectives

Dysregulated proteolytic processes modulated by MMPs result in cardiac remodeling and play a major role in CVDs. MMP7—a potent metalloproteinase with a wide range of ECM/non-ECM substrates—is implicated in several cancers and atherosclerosis. This study sheds light on the association of a highly frequent tag SNP in the promoter region of *MMP7* (A-181G; rs11568818) with increased BP and higher risk for hypertension in Indian population. This work also provides a plausible transcription regulatory mechanism behind the observed elevated levels of MMP7 in the carriers of the variation. Thus, this study paves way toward better understanding of the role of interindividual variations and functional regulatory polymorphisms in conferring disease risk, which may help to develop preventive strategies for individuals predisposed to hypertension and the related cardiovascular complications.

Acknowledgments

We acknowledge all the volunteers who participated in this study. We thank David Ginty (Harvard Medical School) for the CREB (cyclic AMP response element-binding protein) expression plasmid (VP16–CREB+bZIP) and Richard H. Goodman (Vollum Institute, Oregon Health Sciences University) for the CREB dominant-negative plasmid (KCREB). L. Subramanian acknowledges the clinical research team at Madras Medical Mission, Chennai, for helping with the sample collection and Abrar Ali Khan, Vikas Arige, and Amrita Anand for their support during the study.

Sources of Funding

This study was supported, in part, by grants from the Department of Biotechnology, Department of Science and Technology, and Ministry of Human Resource Development, Government of India.

Disclosures

None.

References

1. Löffek S, Schilling O, Franzke CW. Series "matrix metalloproteinases in lung health and disease": biological role of matrix metalloproteinases: a critical balance. *Eur Respir J*. 2011;38:191–208. doi: 10.1183/09031936.00146510
2. Ye S. Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. *Matrix Biol*. 2000;19:623–629.
3. Chiao YA, Zamilpa R, Lopez EF, Dai Q, Escobar GP, Hakala K, Weintraub ST, Lindsey ML. *In vivo* matrix metalloproteinase-7 substrates identified in the left ventricle post-myocardial infarction using proteomics. *J Proteome Res*. 2010;9:2649–2657. doi: 10.1021/pr100147r

4. Ban CR, Twigg SM, Franjic B, Brooks BA, Celermajer D, Yue DK, McLennan SV. Serum MMP-7 is increased in diabetic renal disease and diabetic diastolic dysfunction. *Diabetes Res Clin Pract*. 2010;87:335–341. doi: 10.1016/j.diabres.2010.01.004
5. Kesh K, Subramanian L, Ghosh N, Gupta V, Gupta A, Bhattacharya S, Mahapatra NR, Swarnakar S. Association of MMP7 -181A→G promoter polymorphism with gastric cancer risk: Influence of nicotine in differential allele-specific transcription via increased phosphorylation of cAMP-response element-binding protein (CREB). *J Biol Chem*. 2015;290:14391–14406. doi: 10.1074/jbc.M114.630129
6. Nilsson L, Jonasson L, Nijm J, Hamsten A, Eriksson P. Increased plasma concentration of matrix metalloproteinase-7 in patients with coronary artery disease. *Clin Chem*. 2006;52:1522–1527. doi: 10.1373/clinchem.2006.067439
7. Zhang J, Jin X, Fang S, Wang R, Li Y, Wang N, Guo W, Wang Y, Wen D, Wei L, Dong Z, Kuang G. The functional polymorphism in the matrix metalloproteinase-7 promoter increases susceptibility to esophageal squamous cell carcinoma, gastric cardiac adenocarcinoma and non-small cell lung carcinoma. *Carcinogenesis*. 2005;26:1748–1753. doi: 10.1093/carcin/bgi144
8. Schmid-Schönbein GW. An emerging role of degrading proteinases in hypertension and the metabolic syndrome: autodigestion and receptor cleavage. *Curr Hypertens Rep*. 2012;14:88–96. doi: 10.1007/s11906-011-0240-9
9. Wang X, Chow FL, Oka T, Hao L, Lopez-Campistrous A, Kelly S, Cooper S, Odenbach J, Finegan BA, Schulz R, Kassiri Z, Lopaschuk GD, Fernandez-Patron C. Matrix metalloproteinase-7 and ADAM-12 (a disintegrin and metalloproteinase-12) define a signaling axis in agonist-induced hypertension and cardiac hypertrophy. *Circulation*. 2009;119:2480–2489. doi: 10.1161/CIRCULATIONAHA.108.835488
10. Odenbach J, Wang X, Cooper S, Chow FL, Oka T, Lopaschuk G, Kassiri Z, Fernandez-Patron C. MMP-2 mediates angiotensin II-induced hypertension under the transcriptional control of MMP-7 and TACE. *Hypertension*. 2011;57:123–130. doi: 10.1161/HYPERTENSIONAHA.110.159525
11. Jormsjö S, Whatling C, Walter DH, Zeiher AM, Hamsten A, Eriksson P. Allele-specific regulation of matrix metalloproteinase-7 promoter activity is associated with coronary artery luminal dimensions among hypercholesterolemic patients. *Arterioscler Thromb Vasc Biol*. 2001;21:1834–1839. doi: 10.1161/hq1101.098229
12. Richards TJ, Park C, Chen Y, Gibson KF, Peter Di Y, Pardo A, Watkins SC, Choi AM, Selman M, Pilewski J, Kaminski N, Zhang Y. Allele-specific transactivation of matrix metalloproteinase 7 by FOXA2 and correlation with plasma levels in idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2012;302:L746–L754. doi: 10.1152/ajplung.00319.2011
13. Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science*. 1999;286:2358–2361. doi: 10.1126/science.286.5448.2358
14. Sonawane PJ, Sahu BS, Sasi BK, Geedi P, Lenka G, Mahapatra NR. Functional promoter polymorphisms govern differential expression of HMG-CoA reductase gene in mouse models of essential hypertension. *PLoS One*. 2011;6:e16661. doi: 10.1371/journal.pone.0016661
15. Webb B, Sali A. Protein structure modeling with MODELLER. *Methods Mol Biol*. 2014;1137:1–15. doi: 10.1007/978-1-4939-0366-5_1
16. van Dijk M, Bonvin AM. 3D-DART: a DNA structure modelling server. *Nucleic Acids Res*. 2009;37(Web Server issue):W235–W239. doi: 10.1093/nar/gkp287
17. Yan Y, Zhang D, Zhou P, Li B, Huang SY. HDOCK: a web server for protein-protein and protein-DNA/RNA docking based on a hybrid strategy. *Nucleic Acids Res*. 2017;45(W1):W365–W373. doi: 10.1093/nar/gkx407
18. van Zundert GCP, Rodrigues JPGLM, Trellet M, Schmitz C, Kastritis PL, Karaca E, Melquiond ASJ, van Dijk M, de Vries SJ, Bonvin AMJJ. The HADDOCK2.2 web server: user-friendly integrative modeling of biomolecular complexes. *J Mol Biol*. 2016;428:720–725. doi: 10.1016/j.jmb.2015.09.014
19. *Schrödinger Release 2017-2: Desmond Molecular Dynamics System*. New York, NY: D. E. Shaw research; 2017. Maestro-desmond interoperability tools, schrödinger, New York, NY 2017.
20. Mattapally S, Singh M, Murthy KS, Asthana S, Banerjee SK. Computational modeling suggests impaired interactions between NKX2.5 and GATA4 in individuals carrying a novel pathogenic D16N NKX2.5 mutation. *Oncotarget*. 2018;9:13713–13732. doi: 10.18632/oncotarget.24459
21. Xu Z, Taylor JA. SNPinfo: integrating GWAS and candidate gene information into functional SNP selection for genetic association studies. *Nucleic Acids Res*. 2009;37(Web Server issue):W600–W605. doi: 10.1093/nar/gkp290
22. Cui JS, Hopper JL, Harrap SB. Antihypertensive treatments obscure familial contributions to blood pressure variation. *Hypertension*. 2003;41:207–210. doi: 10.1161/01.hyp.0000044938.94050.e3
23. Bashey RI, Cox R, McCann J, Jimenez SA. Changes in collagen biosynthesis, types, and mechanics of aorta in hypertensive rats. *J Lab Clin Med*. 1989;113:604–611.
24. Intengan HD, Schiffrin EL. Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis. *Hypertension*. 2001;38(3 Pt 2):581–587. doi: 10.1161/hy09t1.096249
25. Birkedal-Hansen H. Proteolytic remodeling of extracellular matrix. *Curr Opin Cell Biol*. 1995;7:728–735. doi: 10.1016/0955-0674(95)80116-2
26. Pardo A, Selman M. Role of matrix metalloproteases in pulmonary fibrosis. In: Lagente V, Boichot E, eds. *Matrix Metalloproteinases in Tissue Remodelling and Inflammation*. Basel: Birkhäuser Basel; 2008:39–55.
27. Yang X, Liu Y, Yang Y, Li B. Update meta-analysis on MMP-7 -181A>G polymorphism and cancer risk: evidence from 25 studies. *Gene*. 2013;521:252–258. doi: 10.1016/j.gene.2013.03.079
28. Lu ZQ, Wang YM, Cao YY, Zhang QJ, Zhang XH, Li YH, Wang HS, Xie HL, Jiao BH, Zhang JH. [Correlations of polymorphisms in matrix metalloproteinase-3 and -7 promoters to susceptibility to brain astrocytoma]. *Ai Zheng*. 2007;26:463–468.
29. Li Y, Jin X, Kang S, Wang Y, Du H, Zhang J, Guo W, Wang N, Fang S. Polymorphisms in the promoter regions of the matrix metalloproteinases-1, -3, -7, and -9 and the risk of epithelial ovarian cancer in China. *Gynecol Oncol*. 2006;101:92–96. doi: 10.1016/j.ygyno.2005.09.058
30. Singh H, Jain M, Mittal B. MMP-7 (-181A>G) promoter polymorphisms and risk for cervical cancer. *Gynecol Oncol*. 2008;110:71–75. doi: 10.1016/j.ygyno.2008.03.007
31. Mishra A, Srivastava A, Mittal T, Garg N, Mittal B. Genetic predisposition to left ventricular dysfunction: a multigenic and multianalytical approach. *Gene*. 2014;546:309–317. doi: 10.1016/j.gene.2014.05.060
32. Ichiki T. Role of cAMP response element binding protein in cardiovascular remodeling: good, bad, or both? *Arterioscler Thromb Vasc Biol*. 2006;26:449–455. doi: 10.1161/01.ATV.0000196747.79349.d1
33. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol*. 2001;2:599–609. doi: 10.1038/35085068
34. Fentzke RC, Korcarz CE, Lang RM, Lin H, Leiden JM. Dilated cardiomyopathy in transgenic mice expressing a dominant-negative CREB transcription factor in the heart. *J Clin Invest*. 1998;101:2415–2426. doi: 10.1172/JCI2950
35. Wellman GC, Cartin L, Eckman DM, Stevenson AS, Saundry CM, Lederer WJ, Nelson MT. Membrane depolarization, elevated Ca(2+) entry, and gene expression in cerebral arteries of hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2001;281:H2559–H2567. doi: 10.1152/ajpheart.2001.281.6.H2559
36. Funakoshi Y, Ichiki T, Takeda K, Tokuno T, Iino N, Takeshita A. Critical role of cAMP-response element-binding protein for angiotensin II-induced hypertrophy of vascular smooth muscle cells. *J Biol Chem*. 2002;277:18710–18717. doi: 10.1074/jbc.M110430200
37. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. *Annu Rev Physiol*. 2005;67:259–284. doi: 10.1146/annurev.physiol.67.040403.120816
38. Shi M, Liu D, Duan H, Han C, Wei B, Qian L, Chen C, Guo L, Hu M, Yu M, Song L, Shen B, Guo N. Catecholamine up-regulates MMP-7 expression by activating AP-1 and STAT3 in gastric cancer. *Mol Cancer*. 2010;9:269. doi: 10.1186/1476-4598-9-269
39. Vaziri ND, Wang ZQ. Sustained systemic arterial hypertension induced by extended hypobaric hypoxia. *Kidney Int*. 1996;49:1457–1463. doi: 10.1038/ki.1996.205
40. Marina N, Ang R, Machhada A, Kasymov V, Karagiannis A, Hosford PS, Mosienko V, Teschemacher AG, Vihko P, Paton JF, Kasparov S, Gourine AV. Brainstem hypoxia contributes to the development of hypertension in the spontaneously hypertensive rat. *Hypertension*. 2015;65:775–783. doi: 10.1161/HYPERTENSIONAHA.114.04683
41. Abe H, Semba H, Takeda N. The roles of hypoxia signaling in the pathogenesis of cardiovascular diseases. *J Atheroscler Thromb*. 2017;24:884–894. doi: 10.5551/jat.RV17009
42. Kvietikova I, Wenger RH, Marti HH, Gassmann M. The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. *Nucleic Acids Res*. 1995;23:4542–4550. doi: 10.1093/nar/23.22.4542

43. Beitner-Johnson D, Millhorn DE. Hypoxia induces phosphorylation of the cyclic AMP response element-binding protein by a novel signaling mechanism. *J Biol Chem*. 1998;273:19834–19839. doi: 10.1074/jbc.273.31.19834
44. Burke B, Giannoudis A, Corke KP, Gill D, Wells M, Ziegler-Heitbrock L, Lewis CE. Hypoxia-induced gene expression in human macrophages: implications for ischemic tissues and hypoxia-regulated gene therapy. *Am J Pathol*. 2003;163:1233–1243. doi: 10.1016/S0002-9440(10)63483-9
45. Rodrigues SF, Tran ED, Fortes ZB, Schmid-Schönbein GW. Matrix metalloproteinases cleave the beta2-adrenergic receptor in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2010;299:H25–H35. doi: 10.1152/ajpheart.00620.2009

Novelty and Significance

What Is New?

- This is the first study, to the best of our knowledge, demonstrating the association of *MMP* (*matrix metalloproteinase*)*7* A-181G (rs11568818) polymorphism with hypertension risk.
- This study provides evidence for the role of CREB (cyclic AMP response element-binding protein) in allele-specific transcriptional activation of *MMP7* –181G promoter resulting in higher MMP7 protein levels.

What Is Relevant?

- The study identified a highly prevalent tag SNP that conferred hypertension risk in Indian populations. This finding may be useful for developing preventive strategies for individuals predisposed to hypertension and its complications.

Summary

We identified a common genetic variation (A-181G) in the promoter region of *MMP7* gene that shows strong association with increased hypertension risk in 2 geographically distinct populations consisting a total of ≈2450 hypertensive/normotensive subjects. The –181G allele-containing promoter displayed higher activity than the –181A promoter under basal/pathophysiological conditions due to preferential binding with transcription factor CREB. Carriers of the risk genotype AG also had increased plasma MMP7 levels and blood pressure suggesting that this functional regulatory polymorphism may act as a predictor for cardiovascular risk.