Common Sodium Channel Promoter Haplotype in Asian Subjects Underlies Variability in Cardiac Conduction

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Background—Reduced cardiac sodium current slows conduction and renders the heart susceptible to ventricular fibrillation. Loss of function mutations in *SCN5A*, encoding the cardiac sodium channel, are one cause of the Brugada syndrome, associated with slow conduction and a high incidence of ventricular fibrillation, especially in Asians. In this study, we tested the hypothesis that an *SCN5A* promoter polymorphism common in Asians modulates variability in cardiac conduction.

Methods and Results—Resequencing 2.8 kb of *SCN5A* promoter identified a haplotype variant consisting of 6 polymorphisms in near-complete linkage disequilibrium that occurred at an allele frequency of 22% in Asian subjects and was absent in whites and blacks. Reporter activity of this variant haplotype, designated HapB, in cardiomyocytes was reduced 62% compared with wild-type haplotype (P=0.006). The relationship between *SCN5A* promoter haplotype and PR and QRS durations, indexes of conduction velocity, was then analyzed in a cohort of 71 Japanese Brugada syndrome subjects without *SCN5A* mutations and in 102 Japanese control subjects. In both groups, PR and QRS durations were significantly longer in HapB individuals ($P \leq 0.002$) with a gene-dose effect. In addition, up to 28% and 48% of variability in PR and QRS durations, respectively, were attributable to this haplotype. The extent of QRS widening during challenge with sodium channel blockers, known to be arrhythmogenic in Brugada syndrome and other settings, was also genotype dependent (P=0.002).

Conclusions—These data demonstrate that genetically determined variable sodium channel transcription occurs in the human heart and is associated with variable conduction velocity, an important contributor to arrhythmia susceptibility. (***Circulation*. 2006;113:338-344.**)

Key Words: arrhythmia ■ conduction ■ death, sudden ■ genetics ■ ion channels

Sudden cardiac death (SCD) accounts for 20% of all mortality in Western countries.[1] One key determinant of normal excitation and conduction of the cardiac impulse is the cardiac sodium channel, responsible for rapid depolarization in most cardiomyocytes. Reduced sodium current predisposes to SCD. For example, although sodium channel blockers have been used for antiarrhythmic therapy, the Cardiac Arrhythmia Suppression Trial (CAST) showed that these agents increase the incidence of SCD.[2] Loss of function mutations in *SCN5A*, the cardiac sodium channel gene, causes ≈20% of cases of the Brugada syndrome, which is associated with a high risk of SCD.[3] Furthermore, there is evidence that such sodium channel mutations also may lead to enhanced fibrosis in myocardial tissue.[4,5]

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The overall hypothesis underlying the work presented here is that variability in regulation of sodium channel expression contributes to interindividual variability in cardiac conduction and consequently can be considered a candidate modulator of arrhythmia susceptibility, especially in the presence of other stressors such as drugs or acute myocardial ischemia.[6] As a first step in testing this hypothesis, we cloned and characterized the proximal promoter region of *SCN5A* and identified multiple cis-acting elements regulating gene expression.[7] We report here identification of an ethnic-specific, common *SCN5A* promoter variant that modulates PR and QRS durations, indexes of cardiac conduction.

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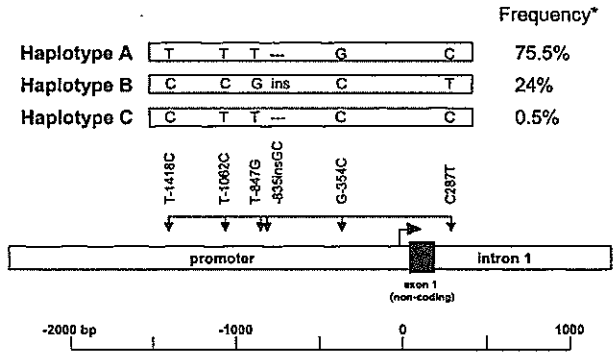

Figure 1. Haplotypes identified in the cardiac sodium channel gene (*SCN5A*) promoter. Nucleotide variations are indicated by their position relative to the major transcription initiation site (+1),[7] with the most frequent nucleotide given below and the least frequent nucleotide given above the position. *Frequency in the Japanese (control) population.

Methods

Identification of Polymorphisms

Resequencing 2.8 kb of the *SCN5A* promoter region in a single individual of Asian origin identified him as a homozygote for 6 DNA polymorphisms in the region: T-1418C, T-1062C, T-847G, −835insGC, G-354C, and C287T (Figure 1). The resequenced region encompassed positions −2190 to 613, relative to the major transcription initiation site[7] of the *SCN5A* promoter, including 2.2 kb upstream of exon 1, exon 1 (which is 173 bp and noncoding), and the proximal 439 bp of intron 1. The fragment was amplified by long and accurate polymerase chain reaction (PCR; TaKaRa kit) with primers F1 and R1 (Data Supplement Table I; see http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.580811/DC1). Further studies described below established that these polymorphisms were common and in near-total linkage disequilibrium, thereby identifying 2 common haplotype blocks, designated HapA and HapB. We also detected a third combination of polymorphisms, designated HapC, in <1% of subjects. In addition to the study populations, 150 white and 100 black individuals were tested for these haplotypes.

Functional Analysis

Generation of Constructs

The 2.8-kb fragment described above was amplified from genomic DNA of HapA- and HapB-homozygous individuals. These fragments were cloned into the pGEM-T Easy vector (Promega), and inserts were subsequently subcloned into the pGL3-basic vector (Promega), which contains the firefly luciferase coding sequence, to generate *SCN5A* promoter–luciferase fusion constructs for reporter assays. These constructs were designated pGL3-Hap A and pGL3-Hap B.

Reporter Activity

Reporter activity was assayed in neonatal mouse cardiomyocytes and in Chinese hamster ovary cells as described in detail previously.[7] In brief, 1 μg pGL3-Hap A or pGL3-Hap B was transfected into neonatal mouse cardiomyocytes or Chinese hamster ovary cells. In each experiment, 0.05 μg pRL-TK plasmid (Promega) encoding Renilla luciferase was cotransfected to normalize for experimental variability caused by differences in cell viability or transfection efficiency. Luminescence was measured 48 hours after transfection with the Dual-Luciferase Reporter Assay System (Promega). The pGL3-basic (promoterless) plasmid was tested in each experiment; its activity level served as the baseline.

Study Participants

Participants in the clinical study were ascertained at the National Cardiovascular Center (Osaka, Japan). All protocols (including molecular screening) were reviewed and approved by the Ethical Review Committee of the National Cardiovascular Center, and informed consent was obtained from all individuals.

The control population consisted of 102 subjects drawn from mutation-negative relatives in congenital long-QT syndrome families in which the causative mutation had been identified. Only 1 person was drawn from each family. There were 67 male and 35 female subjects ranging from 9 to 69 years of age; mean age was 40±14 years (mean±SD).

The Brugada syndrome population included 80 patients diagnosed with Brugada syndrome, defined as type 1 "coved" ST-segment elevation in V_1 through V_3 (spontaneous in 70 patients, induced by sodium channel blocker in 10 patients).[8] In all patients, physical examination, chest roentgenogram, laboratory values, echocardiography with wall motion analysis, and Doppler screening excluded structural heart disease. Aborted cardiac arrest or ventricular fibrillation (VF) was documented in 30 patients, syncope was identified in 20, and 30 were asymptomatic. All patients had previously been screened for *SCN5A* coding region mutations, and a mutation had been identified in 9 patients. The patient group included 76 male and 4 female subjects ranging from 1 to 76 years of age (mean±SD, 47±16 years).

ECG Phenotypes

ECGs were assessed by an investigator (W.S.) who was blinded to age, gender, and genetic and clinical information. Phenotypes assessed included RR interval, PR interval measured in lead II (PR_{II}), QRS interval measured in leads V_1 (QRS_{V1}) and V_6 (QRS_{V6}), ST amplitude at J point (ST_J), and ST amplitude at 80 ms after the end of the QRS (ST_{80}).

The effects of intravenous administration of sodium channel blockers on these ECG parameters were examined in 49 of 80 Brugada syndrome patients. Pilsicainide (maximum 1 mg/kg at a rate of 0.1 mg · kg^{-1} · min^{-1}) was used in 37 patients, flecainide (maximum 2 mg/kg at a rate of 0.2 mg · kg^{-1} · min^{-1}) was used in 9 patients, and disopyramide (maximum 2 mg/kg at a rate of 0.2 mg · kg^{-1} · min^{-1}) was used in 3 patients.

Genotyping

Genomic DNA was prepared from blood leukocytes. Genotyping for the T-1418C and T-1062C single nucleotide polymorphisms (SNPs) was performed by restriction fragment length polymorphism analysis after PCR amplification with *Ear*I and *Hae*III, respectively. PCR primers used to amplify the 161-bp fragment encompassing the T-1418C SNP were F2 and R2, and those used to amplify the 123-bp fragment encompassing the T-1062C SNP were F3 and R3 (Data Supplement Table II). Genotyping for the other 4 polymorphisms (T-847G, 835insGC, G-354C, and C287T) was done by DNA resequencing of both strands. PCR primers used to amplify the 638-bp fragment encompassing the T-847G, 835insGC, and G-354C polymorphisms were F4 and R4; those used to amplify the 599-bp fragment encompassing the C287T polymorphism were F5 and R5.

Statistical Analysis

Using the individual genotypes for the 6 polymorphisms, we estimated haplotype frequencies using an E-M algorithm.[9] The haplotype frequencies were used to calculate the probabilities of the haplotype pairs compatible with the genotype combinations of the multiple heterozygous patients using Bayes' theorem. Observed haplotype pair frequencies were compared with those expected under Hardy-Weinberg equilibrium in the Brugada syndrome population and control population separately with a χ^2 test. To compare haplotype pair frequencies among Brugada syndrome patients and control subjects, Fisher's exact test was used.

All quantitative phenotypes were normally distributed, and data are expressed as mean±SD. Continuous ECG phenotypes were compared between *SCN5A* mutation–negative Brugada syndrome patients, *SCN5A* mutation–positive Brugada syndrome patients, and control subjects by ANOVA adjusted for age and gender, followed by a post hoc test for pairwise comparisons. Student *t* tests were used

to compare the after-drug-challenge continuous ECG phenotypes between *SCN5A* mutation–negative and –positive Brugada syndrome patients. Correlations between quantitative phenotypes before and after sodium channel blockade are expressed as Pearson correlation coefficients (r). For comparison of the proportion of male subjects, Fisher's exact test was used.

The effect of haplotype pairs on the continuous ECG phenotypes was tested in the Brugada syndrome patients and control subjects separately by ANOVA with adjustment for age and gender. The 9 *SCN5A* mutation–positive Brugada syndrome patients were treated as a separate category (7 HapA/HapA homozygotes, 2 HapA/HapB heterozygotes, pooled). The 2 individuals with the rare HapC variant (1 patient from each group) were excluded from analyses. In all analyses, the proportion of variance attributable to the haplotype pair (R^2) was calculated and corrected for the effects of age and gender.

Differences in reporter gene expression activity between HapA and HapB were examined for statistical significance with Student's t test. Throughout, values of $P<0.05$ were interpreted as being significant. All statistical analyses were done with SAS software (version 9, SAS Institute).

Multiple Testing

When a Bonferroni correction for the 24 statistical models is used to compare the continuous ECG phenotypes, the significance level for the overall probability values is 0.002. Similarly, the Bonferroni-corrected significance levels for the pairwise comparisons between 3 and 4 groups is 0.017 and 0.008, respectively.

Results

Haplotypes

The 6 polymorphisms were in near-complete linkage disequilibrium, with only 2 (similar) discordant haplotypes (of 364; <1%), each occurring in 1 subject from each population. We designated HapA as containing all common alleles and HapB as containing all minor alleles (Figure 1). The discordant haplotype was designated HapC. The estimated frequencies of HapA, HapB, and HapC were 0.755, 0.240, and 0.005 in the control subjects and 0.782, 0.211, and 0.007 in the *SCN5A* mutation–negative Brugada syndrome patients, respectively. Haplotype distributions were in Hardy-Weinberg equilibrium ($P>0.05$) in both populations. No significant difference in haplotype frequencies was observed between the Brugada syndrome group and the control subjects. The haplotypes were absent in white and black samples.

Functional Analysis

In cardiomyocytes, reporter activity of HapB was markedly reduced, by 62%, compared with HapA: 5.5±0.4 (mean±SE) versus 14.5±2.8 (normalized activity units; n=9 each; $P=0.006$; Figure 2). A similar trend was seen in the noncardiac cells: 2.7±0.3 versus 3.6±0.3 (n=13 each; $P=0.04$; Figure 2).

Phenotypic Characteristics of the Control and Brugada Syndrome Patient Populations

The decreased reporter activity for HapB suggested that individuals carrying this promoter haplotype would display ECG-detectable conduction slowing. Accordingly, the relationships between genotype and ECG intervals were evaluated in the control and Brugada syndrome populations.

ECG data are shown in Table 1. As expected, Brugada syndrome patients had significantly longer conduction intervals (PR_{II}, QRS_{V1}, QRS_{V6}) and greater ST-segment elevation

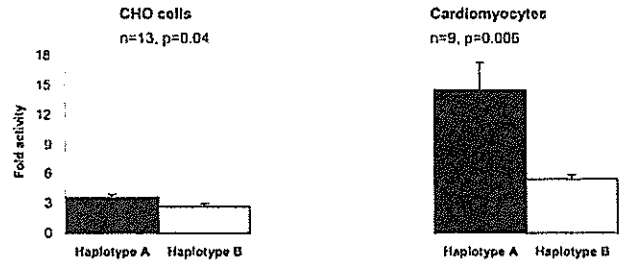

Figure 2. Reporter activity of *SCN5A* promoter haplotypes A and B. Firefly luciferase expression levels, which report the activities of the inserted *SCN5A* sequence, were divided by coexpressed Renilla luciferase activities and expressed as relative luciferase units.[7] Data are presented as mean±SE (vs empty vector). CHO indicates Chinese hamster ovary.

(ST_J, ST_{80}) compared with control subjects. Heart rate was not significantly different between the 2 populations. In addition, we found differences between *SCN5A* mutation–positive and *SCN5A* mutation–negative Brugada syndrome patients similar to those previously reported[10]: Mutation-positive subjects had significantly longer baseline PR and QRS intervals and longer RR intervals. Data on the subset of Brugada syndrome patients who underwent drug challenge are presented in Table 2. For all ECG parameters investigated, highly significant ($P<0.0001$) correlations were present between measures before and after drug challenge (Table 2). As previously reported, *SCN5A* mutation–positive patients displayed longer PR and QRS intervals after challenge with sodium channel blockers compared with *SCN5A* mutation–negative patients.[10]

Haplotype Pair Effects

PR and QRS durations were significantly longer in HapB individuals in both study populations (Brugada syndrome and control subjects: $P\leq0.002$ for PR_{II}; $P<0.0001$ for QRS_{V1} and QRS_{V6}; Figure 3). In the control population, PR_{II}, QRS_{V1}, and QRS_{V6} intervals showed a gene-dose effect, being longest in HapB homozygotes, intermediate in HapA/HapB heterozygotes, and shortest in HapA homozygotes. A similar pattern was observed in the *SCN5A* mutation–negative Brugada syndrome patient group. As discussed earlier, these analyses excluded data in the 2 individuals with HapC. PR_{II}, QRS_{V1}, and QRS_{V6} means (±SD) per haplotype group for the 2 populations are listed in the Data Supplement Table II. Both the overall and pairwise probability values were highly statistically significant even after correction for multiple testing.

The amount of variance (R^2) in PR and QRS intervals explained by the haplotype pair after correction for age and gender is shown in Table 3. As can be seen, a significant proportion of variance in PR and QRS intervals, both at baseline (both groups) and after drug challenge (Brugada syndrome group), was attributable to the haplotype. No significant association was found between haplotype and RR, ST_J, and ST_{80} in either population (data not shown).

Drug Challenge and Haplotype

The haplotype pairs were also highly associated with conduction intervals (PR_{II}, QRS_{V1}, QRS_{V6}) after sodium channel

TABLE 1. Baseline ECG Characteristics of the Control and Brugada Syndrome Patient Populations

		Brugada Syndrome Patients			Pairwise Comparison *P*	
	Control Subjects	*SCN5A*$^{-ve}$	*SCN5A*$^{+ve}$	Overall *P*	*SCN5A*$^{-ve}$ vs *SCN5A*$^{+ve}$	*SCN5A*$^{-ve}$ vs Control Subjects
n	102	71	9			
Male, n (%)	67 (66)	67 (94)	9 (100)	<0.0001	1.000	<0.0001
Age, y	40.0±14.2	46.5±16.3	51.1±8.4	0.005	0.376	0.005
RR, ms	925.3±130.0	913.7±134.3	1055.6±154.2	0.012	0.003*	0.572
PR_{II}, ms	162.3±21.8	180.4±20.4	238.9±26.7	<0.0001*	<0.0001*	<0.0001*
QRS_{V1}, ms	93.8±11.8	104.9±19.3	142.2±19.1	<0.0001*	<0.0001*	<0.0001*
QRS_{V6}, ms	87.4±12.4	100.2±19.1	139.4±21.6	<0.0001*	<0.0001*	<0.0001*
ST_J, mV	0.10±0.05	0.30±0.14	0.34±0.18	<0.0001*	0.249	<0.0001*
ST_{80}, mV	0.18±0.10	0.25±0.12	0.24±0.13	0.001*	0.778	0.001*

Values are given as mean±SD.
*Below the Bonferroni-corrected overall or pairwise significance levels (see Multiple Testing).

blockade in 44 *SCN5A* mutation–negative Brugada syndrome patients who underwent drug challenge (for PR_{II}, QRS_{V1}, QRS_{V6}, $P<0.0001$; Figure 3). PR_{II}, QRS_{V1}, and QRS_{V6} means (±SD) per haplotype group are listed in the Data Supplement Table II. Here also, overall and pairwise probability values were highly statistically significant even after correction for multiple testing.

In addition, the extent of QRS widening (ΔQRS) after drug challenge was genotype dependent, and a gene-dose effect was also observed (ΔQRS_{V6}: HapB/HapB=30 ms [mean± SD]; HapA/HapB=24.2±7.9; HapA/HapA=17.8±7.2; $P=0.002$; Figure 4). A similar trend was seen for extent of PR widening (ΔPR) after drug challenge (ΔPR_{II}: HapB/HapB=40 ms; HapA/HapB=33.8±13.2; HapA/HapA=28.6±8.3; $P=0.05$).

Discussion

We demonstrate that a set of 6 *SCN5A* promoter polymorphisms found in Asian subjects are in near-complete linkage disequilibrium, have a significant impact on sodium channel expression in vitro, account for a large proportion of variance in ECG conduction parameters in 2 independent Japanese populations, and represent pharmacogenetic markers predicting variable drug response.

Twin studies have identified strong genetic effects for ECG parameters, including PR and QRS durations.[11–14] Indeed, associations have been reported between ECG parameters and single coding region nonsynonymous (amino acid–changing) SNPs in ion channel genes.[15,16] However, common functional variants in regulatory regions that strongly modulate basal ECG intervals have not previously been identified; 1 preliminary report has suggested an association between a potassium channel promoter polymorphism and QRS axis in women only.[17] Only recently has the concept of tightly linked polymorphisms (constituting a haplotype block) been applied to understanding variability in cardiac electrophysiology. In 1 study, a small degree of variance (<1%) in QT interval in a central European population could be attributed to single SNPs and haplotype blocks in 4 potassium channel genes.[18]

TABLE 2. Clinical Characteristics of the Brugada Syndrome Patients After Sodium Channel Blocker Challenge

	SCN5A^{-ve}	*SCN5A*$^{+ve}$	*P*	*r*, Before and After Sodium Channel Blockade
n	44	5		
Male, n (%)	42 (95)	5 (100)	1.000	
Age, y	46.3±14.8	52.0±5.4	0.397	
aRR, ms	892.3±113.1	956.0±99.4	0.234	0.94
aPR_{II}, ms	209.6±25.1	278.0±35.6	<0.0001*	0.95
$aQRS_{V1}$, ms	124.1±16.1	166.0±17.8	<0.0001*	0.92
$aQRS_{V6}$, ms	119.2±17.1	166.0±17.8	<0.0001*	0.92
aST_J, mV	0.51±0.21	0.78±0.25	0.013	0.84
aST_{80}, mV	0.41±0.17	0.70±0.31	0.109	0.63

Values are given as mean±SD. Pearson correlation coefficients (*r*) observed between measures before and after sodium channel blocker challenge ($P<0.0001$). Mean baseline ECG parameters for the 44 *SCN5A*$^{-ve}$ and 5 *SCN5A*$^{+ve}$ patients (not shown) were very similar to those for the total patient group given in Table 1.
*Below the Bonferroni-corrected overall significance levels (see Multiple Testing).

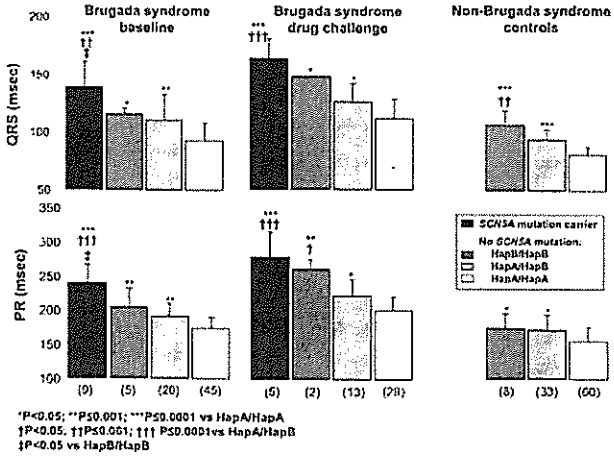

Figure 3. *SCN5A* promoter haplotype effects on durations of QRS_{V6} and PR_{II} in Brugada syndrome patients at baseline and after challenge with sodium channel blocking agents and in non-Brugada syndrome control subjects. Patient numbers are indicated in parentheses. Genotype effects on QRS_{V1} were similar to those on QRS_{V6} because of a high correlation between these 2 parameters (Pearson's coefficient, $r=0.96$). Data are presented as mean±SD. For Bonferroni-corrected significance levels for pairwise comparisons, refer to the Multiple Testing section in Patients and Methods.

In contrast, the *SCN5A* promoter haplotype we report here explained a remarkable proportion of variance in conduction parameters in the Japanese subjects studied (Table 3). Such associations could arise because the haplotypes studied are, in turn, in linkage disequilibrium with other functionally important variants in regulatory or other regions of the gene. However, in this case, the in vitro functional studies indicate that the effect is attributable to a variant within the haplotype block; at this point, the specific variant mediating this effect has not been identified.

A principal determinant of cardiac conduction in atrial and ventricular muscle is the sodium current; sodium channel blockers prolong PR and QRS durations, an effect also seen with loss of function mutations in *SCN5A*.[3] Critical degrees of conduction slowing represent a final common pathway to VF,[19] so dissection of the genetic determinants of cardiac conduction in the general population is a key step to understanding variable susceptibility to common arrhythmias resulting from conduction slowing, as in myocardial ischemia

TABLE 3. Variance Explained by the Haplotype Pair

	R^2, %		
	Control Subjects	Brugada Syndrome Baseline	Brugada Syndrome Drug Challenge
PR_{II}	12.2	28.4	33.0
QRS_{V1}	47.6	26.4	33.0
QRS_{V6}	48.5	24.9	36.2

or heart failure.[19] Thus, the data we present here implicate the *SCN5A* promoter variant HapB, which slowed conduction in normal subjects and exacerbated conduction slowing in those with Brugada syndrome, as a candidate modulator of variability in risk of SCD. Importantly, imposition of further depression of sodium channel function by administration of sodium channel blocking drugs further exacerbated conduction slowing in a gene-dose-dependent fashion. Studies in large numbers of subjects at risk for SCD are required to further establish the role of this and other regulatory region polymorphisms in modulating that risk.

Differences in disease penetrance and expression have been widely reported in the cardiac sodium and other channelopathies.[20–23] Relatives carrying an *SCN5A* mutation identical to that of the proband may be clinically unaffected,[20] and family members may display different phenotypes, eg, Brugada syndrome or conduction disease.[23] Genetic variants like the one presented here are obvious candidate modulators of this variability in phenotypic expression. Interindividual variability also has been noted in response to pharmacological challenge with sodium channel blockers in Brugada syndrome patients.[20,24] In some patients, even some carrying an *SCN5A* mutation, drug challenge fails to unmask a Brugada syndrome ECG. The significantly greater increases in PR and QRS durations with sodium channel blockade in HapB carriers thus identify variability in expression of the drug target, the sodium channel, as a key mediator of this variable drug effect. It is thus possible that other sodium channel blocker response phenotypes such as the increased mortality with sodium channel blockers in the CAST[2] was determined by variable sodium channel expression. DNA samples from that important clinical trial were not archived, so this question will remain unanswered. More generally, the data

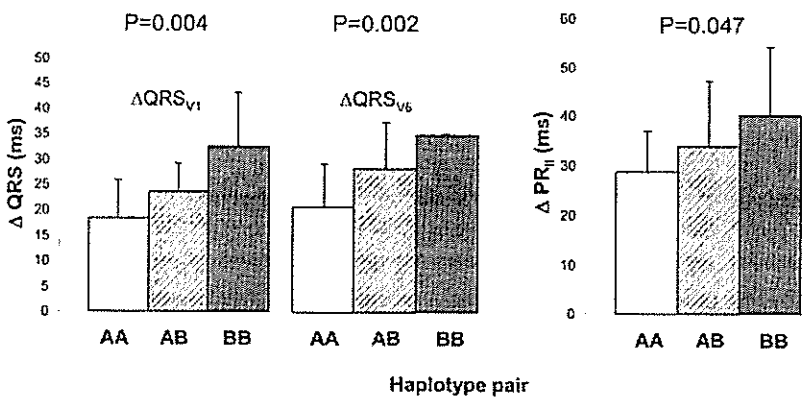

Figure 4. *SCN5A* promoter haplotype effects on extent of QRS (ΔQRS_{V1} and ΔQRS_{V6}) and PR (ΔPR_{II}) widening after sodium channel blockade. AA, n=29; AB, n=13; BB, n=2. Data are presented as mean±SD. The Bonferroni-corrected significance level is 0.002.

indicate that sodium channel function is additively suppressed by drug challenge, Brugada syndrome mutations, and the HapB regulatory variant. Although a strong reduction in reporter gene activity was observed for HapB compared with HapA in vitro, the extent to which this reduction translates proportionately into reduced sodium channel density in vivo is unknown.

Brugada syndrome is endemic in Asia, where the disorder is also known as sudden unexplained nocturnal death syndrome[25]; in fact, the incidence is higher in Asia than in the United States and Europe.[26] Because HapB is common in Asians and absent in whites and has a large negative impact on cardiac conduction, a long-recognized feature of Brugada syndrome,[27] it may logically contribute to differences in Brugada syndrome incidence as a function of ethnicity. In this study, PR and QRS durations in individuals matched for haplotype were consistently longer in the Brugada syndrome group compared with control subjects; thus, the greatest conduction slowing was in those subjects with Brugada syndrome and the HapB/HapB genotype. Indeed, control HapB/HapB subjects had longer QRS durations than did those with manifest Brugada syndrome and the commoner HapA/HapA genotype. Thus, although the minor allele is quite common, it alone may give rise to one part of the spectrum of loss of sodium channel function that constitutes the Brugada syndrome. However, data at this stage do not indicate that HapB per se leads to Brugada syndrome.

More generally, the data fit nicely the concept that individuals vary in their ability to maintain sodium channel function to protect against the arrhythmia-prone substrate and identify HapB as a variant that contributes to such variable "antifibrillatory reserve."[10,28]

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Disclosures

Drs Shimizu and Miyamato are applying for a Japanese domestic patent based on this work. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

The sodium current determines conduction velocity in the heart, and reducing sodium current predisposes to VF. Sodium channel blockers increased sudden death after MI in CAST, and at least some cases of the Brugada syndrome, in which structurally normal hearts are prone to VF, are due to loss of function mutations in the cardiac sodium channel gene *SCN5A*. Thus, variability in the synthesis of sodium channels could contribute to variable conduction velocity in heart and to VF susceptibility. This study represents an important first step to testing that hypothesis. A set of 6 DNA variants were identified in the *SCN5A* promoter, the region of the gene directing transcriptional activity. The variants are common but only in Asian subjects and are in tight linkage disequilibrium; ie, subjects have either wild-type sequences or all 6 variants, defining a haplotype block called HapB here. HapB sequences not only reduced transcriptional activity in vitro but also predicted slower conduction velocity, assessed by PR and QRS durations, in both Japanese control and Brugada syndrome subjects. The longest QRS durations were in Brugada syndrome patients homozygous for HapB (≈7%) challenged with sodium channel blockers. Indeed, normal subjects homozygous for HapB had longer QRS durations than Brugada syndrome patients homozygous for wild-type sequences. These data support the idea that common *SCN5A* promoter variants modulate conduction velocity and thus susceptibility to VF in response to challenges such as other arrhythmogenic mutations, sodium channel blocking drugs, or acute ischemia. In addition, HapB may contribute to the higher prevalence of Brugada syndrome in Asians.

A −786T>C polymorphism in the endothelial nitric oxide synthase gene reduces serum nitrite/nitrate levels from the heart due to an intracoronary injection of acetylcholine

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We identified a −786T>C polymorphism in the eNOS gene, and this polymorphism was strongly associated with coronary spasm. The present study aimed to elucidate whether the −786T>C polymorphism or acetylcholine (ACh)-induced coronary spasm affects serum nitrite/nitrate (NOx) levels. The study population comprised three groups: (i) 26 patients without coronary spasm in the left anterior descending coronary artery (LAD) with the T/T genotype (group A); (ii) 20 patients with coronary spasm in the LAD with the T/T genotype (group B); and (iii) 16 patients with coronary spasm in the LAD with the C/T genotype (group C). Paired blood samples were obtained from the coronary sinus (CS) and the aortic tract (Ao) before and after an intracoronary injection of ACh. Serum NOx and plasma lactate levels were measured. The delta NOx level was calculated as the serum concentration of NOx in the CS minus that in the Ao. We compared lactate extraction ratios (LERs) and delta NOx levels between the three groups. The LERs after the provocation test in groups A, B and C were 18.9±2.4%, −0.5±3.9% and −13.5±4.2%, respectively. The LER in group C was significantly lower than in group B. The delta NOx levels after the provocation test in groups A, B and C were 11.5±1.7 μmol/l, 10.4±3.5 μmol/l and −2.1±4.8 μmol/l, respectively. The delta NOx levels in group C were significantly lower ($P<0.05$). Although the NOx level was significantly increased after the provocation test in group A ($P<0.05$), the NOx level was significantly decreased after the provocation test in group C ($P=0.001$). In group B, the provocation test did not significantly change the delta NOx level. In conclusion, the −786T>C polymorphism reduces the NOx level from the heart due to an intracoronary injection of ACh, and thereby predisposes the patients to severe coronary spasm. *Pharmacogenetics and Genomics* 16:339–345 © 2006 Lippincott Williams & Wilkins.

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Introduction

Coronary spasm plays an important role in the pathogenesis of not only variant angina, but also of ischemic heart diseases in general, including other forms of angina pectoris, acute myocardial infarction, and sudden death [1]; however, the precise mechanism of the pathogenesis remains to be elucidated.

We have shown that an intracoronary injection of acetylcholine (ACh) results in severe vasoconstriction in coronary spasm patients, whereas ACh causes coronary vasodilation in subjects with healthy coronary arteries [1–3]. ACh-induced vasodilation is mediated by NO released from the endothelium [4,5]. These results suggest that the endothelium in the coronary arteries of coronary spasm patients is dysfunctional and that, as a consequence, NO release in response to ACh is lessened. Indeed, we have previously shown that basal, ACh-stimulated and flow-dependent NO activities are decreased in both the coronary and brachial arteries of coronary spasm patients [6,7].

In the endothelium of both animals and humans, the synthesis of nitric oxide (NO) from the amino acid

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L-arginine is catalysed by the endothelial nitric oxide synthase (eNOS) enzyme [4], and the resulting continuously generated NO serves to maintain basal vascular tone [4,5]. We previously reported that the –786T > C polymorphism in the 5′-flanking region of the eNOS gene was an independent risk factor for coronary spasm, as was smoking, although to a lesser degree [8,9]. As assessed by reporter gene assays, the –786C allele resulted in a significant reduction in eNOS gene promoter activity [8]. We also reported that the replication protein A1 represses the eNOS gene with the –786C allele, acting as a repressor of the eNOS gene transcription [10].

The present study aimed to elucidate whether an intracoronary injection of ACh increases NO production from the heart in coronary spasm patients and in non-coronary spasm patients; moreover, whether and how the –786T > C polymorphism of the eNOS gene affects NO levels from the heart.

Materials and methods

Study population

We analysed 64 subjects who were admitted consecutively at Kumamoto University Hospital. All subjects underwent coronary angiography with an intracoronary injection of ACh for evaluation of chest pain. Coronary spasm is defined as total or subtotal occlusion of the coronary artery associated with chest pain and/or ischemic electrocardiographical changes. After an intracoronary injection of isosorbide dinitrate (ISDN), the patients' coronary arteries appeared to be normal and exhibited no significant organic stenosis (< 50% luminal diameter).

Subsequently, we divided these subjects into three groups: (i) 26 T/T genotype patients who had no coronary spasm in the left coronary arteries (group A); (ii) 20 T/T genotype patients who had coronary spasm in the left coronary arteries after an intracoronary injection of ACh (group B); and (iii) 16 C/T genotype patients who had coronary spasm in the left coronary arteries after an intracoronary injection of ACh (group C). There were two C/T genotype patients without coronary spasm in the left coronary arteries. There were no patients with the C/C genotype.

When considering the clinical characteristics of the study patients, hypertension was operationally defined as a blood pressure > 145/90 mmHg, whereas diabetes mellitus was defined as fasting venous blood glucose levels ≥ 126 mg/dl or > 200 mg/dl on an oral glucose tolerance test. Cigarette smoking included current smokers and ex-smokers.

Written informed consent was obtained from all patients. The study was also in agreement with the guidelines of and approved by the ethics committee of Kumamoto University Graduate School of Medical Sciences.

Cardiac catheterization

All medications being taken by the study participants were discontinued at least 48 h prior to cardiac catheterization. Coronary arteriography was performed in the morning when the subjects were in a fasting state. After baseline arteriography of the left and right coronary arteries, an intracoronary injection of ACh was administrated, as previously described [3]. Two consecutive doses (50 and 100 μg) of ACh were injected, 4 min apart, into the left coronary artery; angiography was performed, and completed within 30 s of each injection. Then, 50 μg of ACh was injected into the right coronary artery and angiography was again performed 4 min apart. Finally, both left and right coronary arteriograms were taken after an intracoronary injection of 1 mg of ISDN. We evaluated the degree of organic stenosis after the injection of ISDN.

Blood sampling and assays

Paired blood samples were obtained from the aorta and the coronary sinus immediately following the injection of 100 μg of ACh into the left coronary artery and these samples were then immediately placed in an ice bath. After centrifuging of the blood sample for 10 min, the serum and plasma were packed in a freezer at –80°C for subsequent determination of nitrite/nitrate and lactate concentrations.

Screening for the –786T > C polymorphism of the eNOS gene by the allele-specific oligonucleotide method

The allele-specific oligonucleotide method was used to determine the presence of the –786T > C polymorphism. This method has been described previously [9]. In brief, polymerase chain fragments 236-bp in length, including the –786T/C site, were blotted in duplicate onto nylon membranes. Hybridization was accomplished with ^{32}P-radiolabelled oligonucleotides corresponding to either the –786T sequence (5′-GGG TCA GCC AGC CAG GGA A-3′: probe for the –786T sequence) or the –786C sequence (5′-GGG TCA GCC GGC CAG GGA A-3′: probe for the –786C sequence).

Measurement of plasma lactate concentration and serum nitrite/nitrate concentration

Plasma lactate concentrations were measured using an enzyme assay [12]. We determined the lactate extraction ratio (LER) as: 100 × [plasma lactate concentration at aorta (Ao)–plasma lactate concentration at coronary sinus (CS)]/lactate concentration at Ao. Serum nitrite/nitrate concentrations were measured using a flow injection autoanalyser (TCI-NOX1000, Tokyo Kasei Kogyo, Tokyo, Japan) which is based on the Griess Reaction methodology [11]. The samples were passed through a column containing copper-coated cadmium, which reduced all nitrate to nitrite; the nitrite was then detected by

reacting it with a Griess reagent; and, finally, nitrite/nitrate concentrations were then measured spectrophotometrically at 540 nm.

We analysed delta serum nitrite/nitrate (NOx) levels as: NOx concentration at the CS–NOx concentration at the Ao.

Statistical analysis

Continuous variables were compared using two-tailed unpaired *t*-tests. Categorical variables were compared by chi-square analysis with Fisher's exact probability test. LER and delta NOx levels were compared using two-tailed unpaired *t*-tests between the study groups. Comparison of delta NOx levels before and after an intracoronary injection of ACh was performed using the paired *t*-test. Linear regression analysis was used to correlate delta NOx level and LER. $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of the study population

The clinical characteristics of this study population are shown in Table 1. There were no significant differences between the three groups regarding age, gender, cigarette smoking, hypertension, diabetes mellitus, total cholesterol level, or body mass index.

Lactate extraction ratios

Before an intracoronary injection of ACh, LERs in groups A, B and C were $39.0 \pm 2.3\%$, $36.1 \pm 2.7\%$ and $36.3 \pm 3.1\%$, respectively (Fig. 1). There were no significant differences between the three groups. After an intracoronary injection of ACh, LERs in groups A, B and C were $19.0 \pm 2\%$, $-0.5 \pm 4\%$ and $-13.5 \pm 4\%$, respectively (Fig. 1). In the patients with the –786T/T genotype, LER was significantly lower in the patients with coronary spasm (group B) than in the patients without coronary spasm (group A) ($P < 0.0001$). In the coronary spasm patients, LER was significantly lower in the patients with the –786C/T genotype (group C) than in the patients with the –786T/T genotype (group B) ($P < 0.03$). The LERs in two patients with the –786C/T genotype, without coronary spasm, were 63.4% and 6.3% after an intracoronary injection of ACh.

Serum nitrite/nitrate levels

Serum NOx levels in the aorta and the coronary sinus before and after the provocation test are shown in Table 2 There were no significant differences in the serum NOx levels between the three groups regarding each part or blood sampling time. Subsequently, we analysed the delta NOx level as: NOx at the CS–NOx concentration at the Ao.

Before the provocation test, the delta NOx levels in groups A, B and C were 7.6 ± 2.2 μmol/l, 12.9 ± 3.8 μmol/l and 14.8 ± 3.9 μmol/l, respectively (Fig. 1). There were no significant differences between the three groups. After the provocation test, the delta NOx levels in groups A, B and C were 11.5 ± 1.7 μmol/l, 10.4 ± 3.5 μmol/l and -2.1 ± 4.8 μmol/l, respectively (Fig. 1). The delta NOx levels in group C were significantly lower ($P < 0.05$). We compared the delta NOx levels before and after the provocation test in each group as shown in Fig. 2. Although the delta NOx level was significantly increased after the provocation test in group A ($P < 0.05$), the delta NOx level was significantly decreased after the provocation test in group C ($P < 0.001$). In group B, the provocation test did not significantly change the delta NOx level. In one of the two patients with the –786C/T genotype without coronary spasm, the delta NOx levels before and after the provocation test were 46.5 μmol/l and 38.1 μmol/l, respectively; the delta NOx levels of the other patient before and after the provocation test were 20.3 μmol/l and 18.4 μmol/l, respectively. The delta NOx levels in the two patients with the –786C/T genotype without coronary spasm basically decreased after the provocation test.

Correlation between delta NOx level and LER

Before the provocation test, the delta NOx level did not significantly correlate with LER ($r = -0.042$, $P =$ NS) (Fig. 3). After the provocation test, the delta NOx level had a significant positive correlation with LER ($r = 0.346$, $P < 0.005$) (Fig. 3).

Discussion

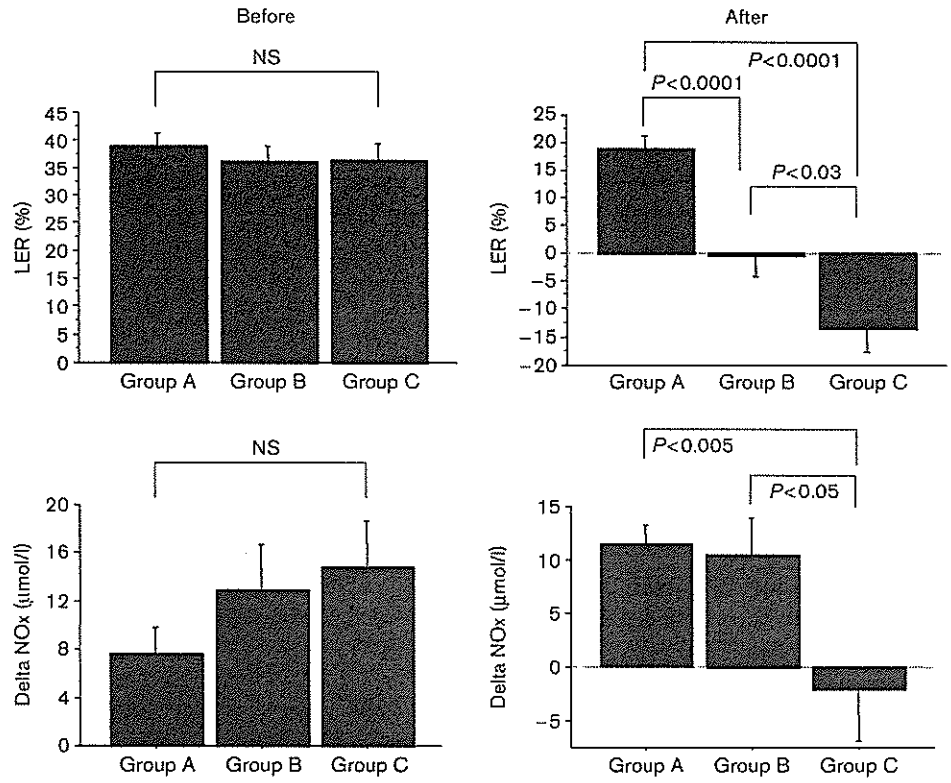
The vascular endothelium plays an important role in the regulation of regional blood flows by releasing an endothelium derived relaxing factor, a major component

Table 1 Clinical characteristics of the study subjects

	Group A: non-coronary spasm with –786T/T ($n=26$)	Group B: coronary spasm with –786T/T ($n=20$)	Group C: non-coronary spasm with –786C/T ($n=16$)	*P*
Age (years)	61 ± 13	64 ± 9	60 ± 15	NS
Men : women (ratio)	12 : 14	10 : 10	9 : 7	NS
Cigarette smoking, *n* (%)	10/26 (38)	10/20 (50)	10/16 (63)	NS
Hypertension, *n* (%)	8/26 (31)	8/20 (40)	4/16 (25)	NS
Diabetes mellitus, *n* (%)	1/26 (4)	4/20 (20)	4/26 (25)	NS
Total cholesterol (mg/dl)	184 ± 29	187 ± 24	184 ± 35	NS
Body mass index (kg/m^2)	24.0 ± 3.2	23.0 ± 1.5	23.5 ± 3.7	NS

Data are mean ± SD except where indicated.

Fig. 1



Lactate extraction ratio (LER) before (left) and after (right) an intracoronary injection of acetylcholine. Delta nitrite/nitrate (NOx) levels before (left) and after (right) an intracoronary injection of acetylcholine. The delta NOx indicates the difference in serum NOx level between the coronary sinus (CS) and the aorta (Ao) [delta NOx (CS–Ao)]. Values are expressed as the means ± SEM.

Table 2 Serum nitrite/nitrate levels (μmol/l) in aorta and coronary sinus before and after provocation test

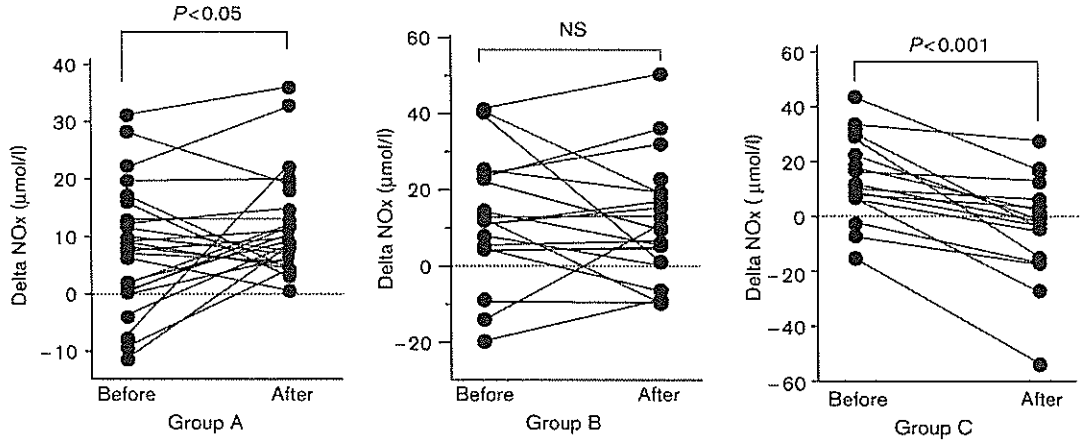
	Before		After	
	Aorta	Coronary sinus	Aorta	Coronary sinus
Group A: non-coronary spasm with −786T/T (n=26)	54.9±31.2	62.4±34.1	41.1±27.0	52.7±30.5
Group B: coronary spasm with −786T/T (n=20)	45.1±22.2	58.0±24.8	38.1±18.9	48.4±20.8
Group C: non-coronary spasm with −786C/T (n=16)	49.9±21.8	64.7±22.9	52.1±29.5	50.0±15.8

Data are mean±SD.

of which is endothelial NO [4,5]. An intracoronary injection of ACh results in severe vasoconstriction in coronary spasm patients, whereas ACh causes coronary vasodilation in subjects with healthy coronary arteries [1–3]. ACh-induced vasodilation is mediated by NO released from the endothelium [4,5]. Because NO is a labile substance with a short half-life and decomposes rapidly into nitrite and nitrate (NOx), its direct measurement has proved to be difficult [13]. It has been reported that increases in the serum NOx levels in rats treated with endotoxin were inhibited by the coadministration of NO synthase inhibitor nitro-L-argine methyl ester, suggesting that the NOx level reflects endogenous NO production [14].

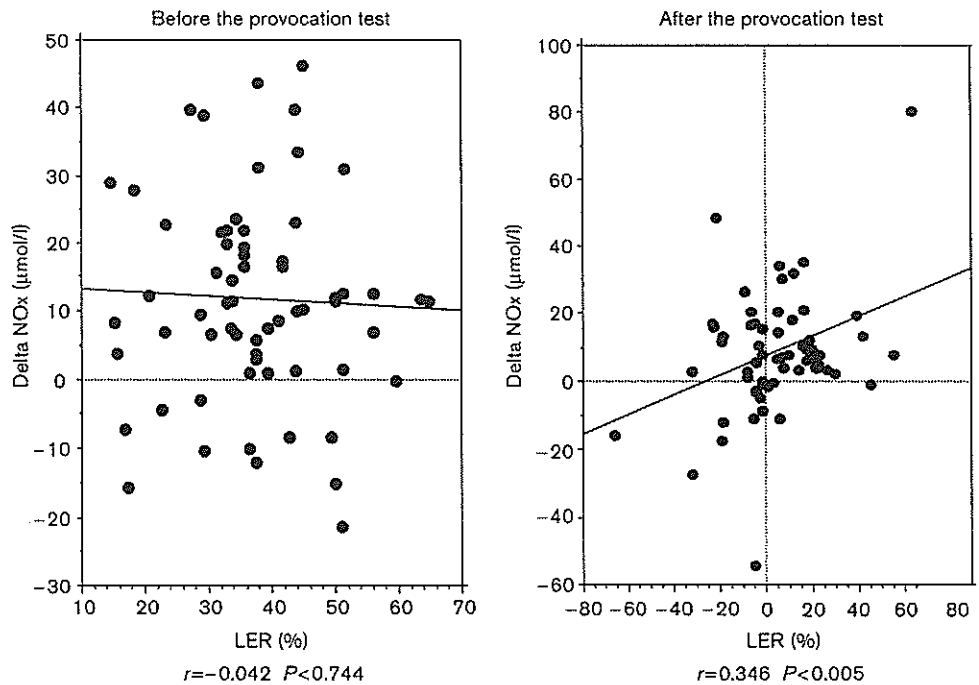
In the present study in coronary spasm patients, LER was significantly lower in the patients with the –786C/T genotype of the eNOS gene than in the patients with the –786T/T genotype after an intracoronary injection of ACh. The –786T > C polymorphism possibly causes coronary spasm and contributes to the severity. Naber *et al.* [15] reported that myocardial lactate uptake was reversed into net lactate production after an intracoronary injection of acetylcholine in subjects with the –786C allele. Our results on LER is in agreement with their report. As for possible actions to increase the severity of coronary spasm, the –786C > T polymorphism significantly reduced delta NOx levels in coronary spasm patients after the provocation test.

Fig. 2



Delta nitrite/nitrate (NOx) levels before and after an intracoronary injection of acetylcholine in groups A, B and C. The delta NOx indicates the difference in serum NOx level between the coronary sinus (CS) and the aorta (Ao) [delta NOx (CS–Ao)].

Fig. 3



Correlation between delta nitrite/nitrate (NOx) levels [coronary sinus (CS)–aorta (Ao)] and lactate extraction ratio (LER) before (left) and after (right) an intracoronary injection of acetylcholine.

We have reported that the –786T > C polymorphism enhanced the vasoconstriction response due to an intracoronary injection of ACh [9,16]. We suggested that reducing the ACh-induced NO production from the coronary endothelial cells in the patients with the –786T > C polymorphism causes significant

vasoconstriction. Although the ACh-induced NO is mainly generated by the endothelial cells, both endothelial cells and cardiomyocytes are thought to be potential sources of NO generation when a state of hypoxia exists in the heart. Node *et al.* [17] reported that NO production from the heart is increased in ischemic hearts, and after exertion, in patients with effort angina. These results suggest that hypoxia possibly accounts for an increase in NO production from the heart, including from coronary arterial endothelial cells and/or from cardiomyocytes. Han *et al.* [18] reported that hypoxic red blood cells (RBCs) generate HbFe(II)NO, and that the NO consumption rate therefore increases. The NO level is possibly reduced under the hypoxic condition because of an increase in the NO consumption rate of RBCs. In the present study, for non-coronary spasm patients with the –786T/T genotype (group A), NO was possibly generated from endothelial cells due to the intracoronary injection of ACh; furthermore, their coronary arteries did not produce coronary spasm. In coronary spasm patients with the –786T/T genotype (group B), an intracoronary injection of ACh caused coronary spasm. Although the NO consumption rate possibly increases in hypoxic RBCs, the total NO level in the serum was maintained at an overall high level in group B. The increase in NO production from the heart, including from the endothelial cells and/or from the cardiomyocytes, under an ischemic condition, immediately relaxed the coronary arteries. After an intracoronary injection of ACh, there was no significant difference in the delta NOx levels between groups A and B. Although the coronary spasm patients with the –786T/T genotype have high delta NOx levels before and after the provocation test, some of them possibly have coronary spasm for reasons other than the reduced NO production from the heart. In coronary spasm patients with the –786C allele (group C), reduced NO production from the endothelial cells due to the intracoronary injection of ACh caused coronary spasm, and an insufficient supply of NO production from the heart under this ischemic condition prolonged coronary spasm. An increase in the NO consumption rate in hypoxic RBCs possibly leads to a still more critical spasm state. Previously, we reported that the –786T > C polymorphism is strongly associated with coronary spasm and also with myocardial infarction without organic stenosis [19]; furthermore, we suggested that this polymorphism is possibly associated with the severity of coronary spasm. The –786T > C polymorphism reduced NO production from the heart, even in an ischemic condition, and predisposed the patients to a prolonged coronary spasm, leading to myocardial infarction without organic stenosis. Also, endothelial dysfunction and oxidative stress are known to be crucially involved in the pathogenesis of coronary spasm [20–24]. A decrease in NO production possibly increases oxidative stress and predisposes the patients with the –786C allele to coronary spasm.

There are some reports regarding systemic circulating NOx levels and the –786T > C polymorphism [10,25,26]. Although there is a low tendency for the systemic circulating NOx level in subjects with the –786C allele, there are few reports stating that it is clearly low. It is possible that there is not enough of a significant difference in the systemic circulating NOx level to classify this as being due to the genotype of the –786T > C polymorphism because of the influences of either meal and/or individual levels of oxidative stress. In the present study, an intracoronary injection of ACh significantly increased delta NOx levels in subjects without coronary spasm without the –786C allele, although it did not significantly change the delta NOx levels in subjects with coronary spasm without the –786C allele, and it significantly decreased the delta NOx level in subjects with coronary spasm with the –786C allele. There was a difference of sufficient magnitude in delta NOx levels before and after the provocation test to classify the genotype of the –786T > C polymorphism, even in coronary spasm patients. It is well known that NO plays a key role in the regulation of vascular tone [4,5,27,28] and has vasoprotective effects by scavenging superoxide radicals and suppressing platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation [29–31]. A decrease in the delta NOx level possibly affects the cardiovascular system and leads to severe vasoconstriction. Furthermore, Tanus-Santos *et al.* [32] reported that the –786C allele decreases platelet-derived NO. The –786C allele may accelerate platelet aggregation and serve as a risk factor for cardiovascular disease. Indeed, it was reported that the –786C allele is associated with coronary spasm [8], myocardial infarction [19] and coronary organic stenosis [33].

In conclusion, the –786T > C polymorphism reduces NO production from the heart due to an intracoronary injection of ACh, and thus predisposes patients to a prolonged and more severe coronary spasm.

Study limitation

In the present study population, there were two non-coronary spasm patients with the –786C/T genotype and there were no patients with the –786C/C genotype, this is possibly because the study population was relatively small in size. However, we have previously reported that the frequencies of these patients are relatively low in the Japanese population [8,9,19]. In both patients with the –786C/T genotype without coronary spasm, delta NOx levels basically decreased after the provocation test. Even in the case of non-coronary spasm patients, the –786C allele possibly suppresses NO production from the heart, which is due to an intracoronary injection of ACh. Further studies in a larger population group, including many non-coronary spasm patients with the –786C allele and many patients with the –786C/C genotype, will be beneficial to further elucidate this topic.

NO is generated by NO synthase (NOS), which exists as a family of related but distinct isoforms, including neuronal (nNOS) [34,35], inducible (iNOS) [36,37], and endothelial (eNOS) [4] isoforms. It has been reported that eNOS is detected in the endothelial cells overlying normal human aortas, fatty streaks and advanced atherosclerotic lesions, whereas iNOS and nNOS are not detectable in normal vessels, although widespread production of these two isoforms has been found in early and advanced lesions associated with macrophages, endothelial cells and mesenchymal-appearing intimal cells [38]. In the present study, we did not distinguish which isoform of NOS produces NO from the heart before or after the provocation test.

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