A Gain-of-Function SNP in TRPC4 Cation Channel Protects Against Myocardial Infarction

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Abstract

AIMS. The TRPC4 nonselective cation channel is widely expressed in the endothelium, where it generates Ca\(^{2+}\) signals that participate in the endothelium-mediated vasodilatatory response. This study sought to identify single-nucleotide polymorphisms (SNPs) in the TRPC4 that are associated with myocardial infarction.

METHODS AND RESULTS. Our candidate-gene association studies identified a missense SNP (TRPC4-I957V) associated with reduced risk of myocardial infarction in diabetic patients (OR= 0.61; CI, 0.40-0.95, \(P=0.02\)). TRPC4 was also associated with myocardial infarction in the Wellcome Trust Case-Control Consortium’s genome-wide data: an intronic SNP (rs7319926) within the same linkage disequilibrium block as TRPC4-I957V showed an OR of 0.86 (CI, 0.81-0.94; \(P=10^{-4}\)). Functional studies of the missense SNP were carried out in HEK293 and CHO cells expressing wild type or mutant channels. Patch-clamp studies and measurement of intracellular [Ca\(^{2+}\)] in response to muscarinic agonists and direct G-protein activation showed increased channel activity in TRPC4-I957V transfected cells, compared to TRPC4-WT. Site-directed mutagenesis and molecular modeling of TRPC4-I957V suggested that the gain of function was due to the presence of a less bulky Val-957. This permits a firmer interaction between the TRPC4 and the catalytic site of the tyrosine kinase that phosphorylates TRPC4 at Tyr-959 and facilitates channel insertion into the plasma membrane.

CONCLUSIONS. We provide evidence for the association of a TRPC4 SNP with myocardial infarction in population-based genetic studies. The higher Ca\(^{2+}\) signals generated by TRPC4-I957V may ultimately facilitate the generation of endothelium- and nitric oxide-dependent vasorelaxation, thereby explaining its protective effect at the vasculature.

Key Words: myocardial infarction, TRP ion channels, calcium, genetics of cardiovascular diseases, gene polymorphisms

DISCIPLINE: experimental
OBJECT OF STUDY: vasculature
LEVEL: cellular
EXPERTISE: electrophysiology
INTRODUCTION

The discovery of the TRP family of nonselective ion channels has facilitated our understanding of how animals detect diverse sensory inputs, but also how calcium signaling is generated in a broad range of cellular systems. Different cellular, molecular, and animal model studies have highlighted the relevance of TRP channels in the vasculature where, by shaping Ca\(^{2+}\) signals and modulating cell membrane potential, play important roles in the regulation of vascular tone, remodeling, and integrity. However, from the genetic point of view, only a few reports have studied the relevance of TRP channels at the population level. For example, a single nucleotide polymorphism (SNP) that generates a de novo binding site for nuclear factor-κβ in the promoter region of TRPC6 is associated with pulmonary hypertension; an SNP-inducing loss of activity in TRPV4 has been associated with hyponatremia; and a loss-of-function SNP in TRPV1 has been associated with lower risk of respiratory symptoms in childhood asthma.

Among the TRP channels, TRPC4 channel is widely expressed in the vasculature, where it participates in the generation of intracellular Ca\(^{2+}\) signals that regulate functions such as endothelial permeability, smooth muscle proliferation and, of most importance to this study, endothelium- and nitric oxide (NO)-dependent vasorelaxation. TRPC4 gating is not completely understood. It occurs downstream of both tyrosine kinase and G-protein-coupled receptor activation, with the participation of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) inhibition only relevant to the long TRPC4α splicing isoform. Together, these observations make the TRPC4 channel an interesting target in evaluating contributors to the pathogenesis of human cardiovascular diseases.

Coronary artery disease (CAD) is the leading cause of death in the developed world. The pathophysiology of myocardial infarction (MI), one of the clinical manifestations of CAD, involves a reduced blood supply to the myocardium. This blood supply is inversely related to the resistance in the myocardial vascular bed, which in turn depends on a calcium
signal generated in the vascular smooth muscle (VSM).\textsuperscript{17} Through modulation of the calcium signal, several VSM ion channels participate in setting the level of coronary arterial tone\textsuperscript{18-20} and a few of them have shown a genetic association with CAD, its treatment, or cardiovascular diseases that are risk factors for CAD.\textsuperscript{21-26} Endothelial cells modulate VSM tone following the release of vasomotor regulators or electrical coupling between endothelial cells and VSM, mechanisms in which endothelial ion channels are also involved,\textsuperscript{27,28} including TRPC4.\textsuperscript{13}

Myocardial infarction is a complex disease whose heritability component\textsuperscript{29} has been extensively reevaluated in the last few years by genome wide association studies (GWAS).\textsuperscript{30} Novel loci have appeared, although in most cases the functional connection between loci and phenotype remains to be elucidated. One exception seems to be an SNP in 1p13 that alters lipid metabolism by generating a new transcription factor binding site.\textsuperscript{31} Therefore, identification of functional genetic variants associated with MI is still necessary to improve our knowledge of the disease pathophysiology. The present study sought to identify SNPs in the TRPC4 that are associated with MI.
METHODS

Gene polymorphism identification.

The 11 exons of human TRPC4 were amplified from genomic DNA using PCR and analyzed by direct sequencing in 50 healthy subjects. A novel SNP was found in exon 11 (A3104G, later included in SNP databases as rs73184536, NM_016179), corresponding to an isoleucine to valine mutation at position 957 of the protein (I957V). The TRPC4-I957V variant was identified by dideoxynucleotide sequencing (ABI PRISM BigDye Terminator 3.0; Applied Biosystems) and confirmed by sequencing of the second strand, using forward and reverse primers.

Following the identification of the TRPC4-I957V polymorphism in the last exon of the TRPC4 gene by direct sequencing, this variant was analyzed in every study participant. The DNA samples were analyzed by TaqMan assay (ABI PRISM 7900HT; Applied Biosystems), using 5’-GGTGGAGGACACGGTTCCTA-3’ and 5’-GTGACTGTGTCTGGGAGGTTAGTTAG-3’ primers and FAM-ACTCTAGTGTAGACTATGAT and VIC-AGGACTCTAGTATAGACTATGAT probes for the V (G base) and I (A base) alleles, respectively. All participants gave written informed consent. The study conformed with the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of the IMIM Institut de Recerca Hospital del Mar and Universitat Pompeu Fabra.

Myocardial infarction case–control study.

We used a case–control design. Between January 1996 and December 2002, we recruited 1025 patients (188 women, 18.3%) with a first MI consecutively admitted to the only reference coronary unit in the catchment area in Girona, Spain. The 3899 participants from 2 cross-sectional studies in the same area served as controls and were judged free of angina or MI by medical records, physical examination, and electrocardiography. Standardized smoking and diabetes mellitus questionnaires and details of recruitment and measured variables are already described.32
Cell culture and transfection

Chinese Hamster Ovary (CHO) fibroblast cells (from the European Collection of Cell Cultures) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Culture media contained 1% streptomycin with penicillin. Cells were incubated at 37°C in an atmosphere of 95% air-5% CO₂. The CHO cells were transiently transfected with 0.3 µg pEGFP and 3 µg of either pcDNA3-hTRPC4β WT, pcDNA3-hTRPC4β I957V, or hTRPC4β Y959F using 8 equivalents of polyethylenimine (Fermentas MBI). Cells were used 24-48 hours after transfection. Human TRPC4-I957V mutant was generated by site-directed mutagenesis with primers 5’-GAAGAGGACTCTAGTGACTATGATCTAAACCTCC-3’ and 5’-GGAGGTCTTAGATCATAGTCTACTAGACTGTCCTCTCC-3’. With the Quick Change® Site Directed Mutagenesis kit (Stratagene), TRPC4-Y959F double mutants were generated using primers 5’- GAA GAG GAC TCT AGT ATA GAC TTT GAT CTA AAC CTC CCA GAC -3’ and 5’- GTC GAG GAG GTT TAG ATC AAA GTC TAT ACT AGA GTC CTC TTC -3’ for TRPC4-Ile957 variant and primers 5’- GAA GAG GAC TCT AGT GTA GAC TTT GAT CTA AAC CTC CCA GAC -3’ and 5’- GTC GAG GAG GTT TAG ATC AAA GTC TAT ACT AGA GTC CTC TTC -3’ for TRPC4-Val957 variant (changes underlined). Mutants were verified by sequencing with ABiPrism 3.1 BigDye kit.

Patch-clamp recordings

Cells were plated in 35-mm plastic dishes and mounted on the stage of an Inverted Olympus IX70 microscope and the whole-cell currents were recorded with an Axon200A amplifier as previously described. The bath solution contained either (in mM) 140 NaCl, 2.5 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5 Glucose, 10 HEPES or 120 CsCl, 10 Heps, or 12 Glucose; pH was adjusted to 7.4 with Tris, 305 mosmol/l. The pipette solution contained (in mM) 80 CsCl, 1 MgATP, 1 EGTA, 10 HEPES, 5 Glucose, 500 µM CaCl₂ (calculated free Ca²⁺ concentration was 67.5 nM) and 88 µM GTPγS, a G-protein activating analogue of GTP; pH was adjusted to
7.4 with CsOH and 300 mOsm. Patch electrodes of 3-5 MΩ were used. Membrane potential was held at 0 mV and ramps from -110 mV to +100 mV (400 ms) were applied at frequency of 0.2 Hz. Data was acquired at 10 kHz and low-pass filtered at 1 kHz. Single-channel recordings were carried out using a bath solution containing (in mM): 140 NaCl, 2.5 KCl, 0.5 EGTA, 0.5 MgCl₂, 5 Glucose, and 5 Hepes (pH=7.4 with Tris). The pipette solution contained (in mM) 120 CsCl, 10 HEPES, 12 Glucose and 0.5 MgCl₂ (pH=7.4 with Tris). Single-channel data were filtered at 2.5 kHz. Experiments were performed at room temperature. PP2, an inhibitor of the Src family of tyrosine kinases, and the negative control PP3 (Calbiochem, Merck4Biosciences) were used at 5 µM.

**Measurement of intracellular [Ca²⁺]**

Cells were plated onto glass coverslips, loaded with 5 µM of Fura-2AM for 30 minutes at room temperature, washed out thoroughly, and bathed in an isotonic solution containing (in mM): 140 NaCl, 2.5 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10 HEPES (305 mosmol/l, pH 7.4 with Tris). Ca²⁺-free solutions were obtained by replacing CaCl₂ with an equal amount of MgCl₂ plus 0.5 mM EGTA. Carbamylcholine chloride (Cch, Sigma), a muscarinic receptor agonist, was added to the bath solution as indicated in the figure legend. All experiments were carried out at room temperature as previously described.³³ AquaCosmos software (Hamamatsu Photonics) was used to capture the fluorescence ratio at 505 nm, obtained following excitation at 340 and 380 nm. Images were computed every 5 s.

**Molecular modeling of TRPC4-Fyn kinase interaction**

We used molecular modeling techniques to investigate how a single point mutation at position -2 (aa 957) from Tyr-959 in the human TRPC4 channel could affect the interaction between a tyrosine kinase and the short sequence fragment containing the target tyrosine (see Supplemental methods for further details). We used the structural data available for the Fyn kinase that phosphorylates and modulates TRPC4. An overview of the model obtained for the
FYN kinase in complex with I$^{957}$DY$^{959}$DLN is shown in Supplementary Figure S2 and subsequent figures. Additional details of methods are included in the Supplement.

**Statistical analysis**

Deviation from Hardy-Weinberg equilibrium was assessed using a Chi-square test with 1 degree of freedom. Adjusted odds ratios (ORs) of MI risk and their 95% confidence intervals were estimated for Val-carriers (ValVal +IleVal genotypes) versus IleIle genotype by unconditional logistic regression analysis. Interaction between the genetic variant and age, sex, or diabetes was also assessed for impact on MI risk.

Functional data were expressed as means±S.E.M of n (number of cells analyzed). Statistical analysis was performed with Student’s unpaired t test or one-way analysis of variance (ANOVA) with Tukey’s test for post hoc comparison of means, using SigmaPlot or OriginPro software. The criterion for a significant difference was a final value of $P <0.05$.

**RESULTS**

**Genetic association studies**

We identified an SNP in *TRPC4* (A3104G, later renamed as rs73184536, NM_016179) corresponding to an isoleucine to valine mutation at position 957 of the protein (I957V). The frequency of the Val allele was 0.07 and the genotype frequencies in the representative random population sample of 3899 participants were 86.2%, 13.4% and 0.4% for the IleIle, IleVal and ValVal genotypes, respectively. The observed genotype frequencies fitted the Hardy-Weinberg equilibrium. The ValVal homozygote and IleVal heterozygote subjects were analyzed together because of the low prevalence of the former.

We evaluated the association of *TRPC4-I957V* SNP with MI risk in a case-control study. The main characteristics of the sample are shown in table 1. We observed a statistically significant interaction ($P = 0.049$) between the genetic variant and diabetes on MI risk.
Multivariate analysis showed a protective effect of the 957V allele against MI risk only in diabetic subjects (adjusted OR = 0.61; 95% CI, 0.40-0.95, \( P = 0.02 \)) (Table 2).

The association of TRPC4 with MI was also tested in the Wellcome Trust Case-Control Consortium (WTCCC), which included 1988 cases with coronary artery disease (CAD) and 5380 healthy controls. The nonsynonomous rs73184536 SNP (TRPC4-I957V) was not included in the chip used in this study. Therefore, we analyzed SNPs within or near the TRPC4 chromosomal region (13q13:37.108-37.341). One SNP within the TRPC4 gene (rs7319926), belonging to the same linkage disequilibrium block as TRPC4-I957V, was associated with lower prevalence of CAD (OR = 0.87; CI 0.81-0.94; \( P = 0.0002 \)).

**Functional analysis of TRPC4-I585V variant**

To evaluate the mechanism by which TRPC4-I957V might be protective against cardiovascular disease, we expressed and functionally tested wild-type (TRPC4-WT) and mutant channels (TRPC4-I957V) in CHO cells. Similar levels of expression were observed between TRPC4-WT and TRPC4-I957V channels in CHO cells (Supplementary Figure S1).

We measured cytosolic \([\text{Ca}^{2+}]\) using the fura-2 sensor and cationic currents using the whole-cell and single-channel modes of the patch-clamp technique. TRPC4 is a nonselective cation channel that responds to the muscarinic agonist carbachol (Cch), epidermal growth factor, and other stimuli that use G-protein-mediated signaling\(^{14,15}\).

Figure 1A shows representative \([\text{Ca}^{2+}]\) traces obtained from CHO cells transfected with control GFP, TRPC4-WT, or TRPC4-I957V and stimulated with 100 \( \mu \text{M} \) Cch using an add-back \([\text{Ca}^{2+}]\) protocol. The first peak reflects \([\text{Ca}^{2+}]\) exit from the endoplasmic reticulum (ER) following the production of inositol trisphosphate (IP\(_3\)) and the activation of its receptor at the ER. The second peak reflects the \([\text{Ca}^{2+}]\) entry component following muscarinic receptor activation by carbachol and was clearly modified by the presence of TRPC4, which was significantly larger with TRPC4-I957V. A quantitative analysis of the \([\text{Ca}^{2+}]\) signal, calculating the mean area under the second peak as an indicator of the magnitude of the \([\text{Ca}^{2+}]\) entry, is
shown in Figure 1B. Following ER Ca\(^{2+}\) depletion by thapsigargin and subsequent activation of store-operated calcium entry mechanisms, no difference in Ca\(^{2+}\) entry was observed between cells transfected with GFP, TRPC4-WT and TRPC4-I957V (Fig. 1C-D), consistent with the current view that TRPC4 is not a key player in store-operated calcium entry in the endothelium.\(^{34}\)

Mutant channel was also evaluated using electrophysiological techniques. Gating of TRPC4 currents with 88 µM GTP\(_{\gamma}\)S in transfected cells occurred within a few minutes following the whole-cell configuration and showed the characteristic TRPC4 double rectification current/voltage (I/V) curves (Figure 2A-B). In agreement with the [Ca\(^{2+}\)] data, TRPC4-I957V channels showed higher response than TRPC4-WT channels. Mean channel response (at -100 mV) is shown in Figure 4C. Interestingly, TRPC4-I957V protein appears to exert a dominant positive effect on the WT protein, as co-expression of both TRPC4-WT and TRPC4-I957V proteins in the same cell produces an increase in current density similar to that of TRPC4-I957V protein alone (Figure 4C).

TRPC4 activity is regulated by tyrosine kinase phosphorylation,\(^{15}\) a process that may be engaged via cross-activation secondary to muscarinic receptor activation.\(^{35}\) Ile-957 resides close to a tyrosine (Tyr-959) that, when phosphorylated, regulates TRPC4 activity by inserting more channels into the plasma membrane.\(^{15}\) In fact, single-channel analysis using the cell-attached mode of the patch-clamp technique showed a 3-fold increase in the number of active TRPC4-I957V channels compared to TRPC4-WT channels following carbachol stimulation (Figure 3). The mean single-channel currents were similar to that reported previously for mouse TRPC4 (20) and no differences were observed between WT and I957V channels (2.7±0.2 pA and 2.8±0.2 pA for WT and mutant channel, respectively; n=4). This observation is consistent with a higher insertion of mutant channels into the membrane, although we cannot fully discard that mutant channels also presented an increased open probability, thereby facilitating the occurrence of multiple channels within a membrane patch.
The presence of different amino acid residues within ±5 positions from the target tyrosine modifies phosphorylation rates.\textsuperscript{36} It might occur that I957V substitution affects channel phosphorylation and insertion into the membrane, which would offer an explanation for the higher activity of TRPC4-I957V. Prediction of the phosphorylation rate of the peptide I(V)-D-Y\textsuperscript{959}-D-L-N (corresponding to the TRPC4 sequence around Y959) using the NetPhos 2.0 server suggests an increase of the phosphorylation rate from 0.7 to 0.8 with the I957V change. Besides, molecular dynamics simulations over 1 ns, using the structural data available for the Fyn kinase that phosphorylates and modulates TRPC4\textsuperscript{15} and I(V)-D-Y\textsuperscript{959}-D-L-N peptide as the substrate, showed that the presence of a less bulky Val permits the substrate to approach Lys-431 of the Fyn kinase more closely and to establish a firmer interaction (Supplementary Figures S2-S6).

To further investigate at the molecular level the link between gain of channel function and tyrosine phosphorylation, we replaced Tyr-959 by the nonphosphorylated phenylalanine (Phe-959) in both TRPC4-WT and TRPC4-I957V. Y959F substitution decreased activation of TRPC4-I957V to levels similar to those obtained with TRPC4-WT (Figure 4A-B). Mean responses are shown in Figure 4C. We also evaluated the sensitivity of TRPC4-WT and TRPC4-I957V channels to the tyrosine kinase inhibitor PP2, finding that PP2 reduced channel activity by 50% in TRPC4-WT, without a significant effect on TRPC4-I957V (Figure 4D). The nonactive analog of PP2 (PP3) did not affect either WT or I957V channels (n=4, results not shown). Finally, the direct effect of I957V substitution on TRPC4 phosphorylation was evaluated (Supplementary Figure S7). HEK293 cells transfected with FLAG-TRPC4-WT or FLAG-TRPC4-I957V were stimulated with epidermal growth factor (EGF) and TRPC4 immunoprecipitated with an anti-FLAG antibody. Immunoprecipitated TRPC4 was assessed for tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine antibody (PY20). Tyrosine phosphorylation of TRPC4-I957V showed a 2-fold increase compared to TRPC4-WT.
DISCUSSION

We have identified a genetic variation in TRPC4 (I957V) that links increased channel activity to a protective effect against MI. This gain of function determines higher Ca\textsuperscript{2+} signals that may facilitate the generation of endothelium- and NO-dependent vasorelaxation mediated by TRPC4.\textsuperscript{13} The enhanced channel activity of the genetic variant appears to be related to a firmer interaction of the channel with a regulatory tyrosine kinase that phosphorylates and regulates channel insertion into the membrane and its activity. This observation may also offer a clue to the interpretation of the protective effect of TRPC4-I957V against MI in diabetic patients, who present endothelial dysfunction typically associated with a reduced NO-dependent vasorelaxation.\textsuperscript{37,38} Therefore, it is conceivable that the association of the TRPC4-I957V polymorphism with protection against MI in diabetic patients might be due, at least in part, to the phosphorylation-mediated gain-of-function of the mutated channel in the endothelium. The higher Ca\textsuperscript{2+} signals generated by TRPC4-I957V may ultimately facilitate the generation of endothelium- and NO-dependent vasorelaxation, which would compensate the loss of tyrosine phosphorylation and subsequent reduction of NO-mediated vasodilatation described in diabetic vessels.\textsuperscript{39}

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CONFLICT OF INTEREST

Conflict of interest: none declared
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FIGURE LEGENDS

Figure 1.
Calcium signals generated by TRPC4. (A) Charbachol-stimulated Ca\(^{2+}\) entry in CHO cells transected with control GFP, human TRPC4-WT, and TRPC4-I957V. Ca\(^{2+}\) signals were generated by 100 µM charbachol in Ca\(^{2+}\)-free solutions (white box, reflecting intracellular Ca\(^{2+}\) release), followed by addition of 1.2 mM Ca\(^{2+}\) to the bathing solution (black box) to detect Ca\(^{2+}\) influx. (B) Mean Ca\(^{2+}\) entry calculated from the area under the curve in the presence of Ca\(^{2+}\) is also shown. (C), Thapsigargin-stimulated Ca\(^{2+}\) entry in CHO cells transected with control GFP, TRPC4-WT and TRPC4-I957V. Ca\(^{2+}\) signals were generated by 1 µM TG in Ca\(^{2+}\)-free solutions, followed by addition of 1.2 mM Ca\(^{2+}\) to the bathing solution to detect Ca\(^{2+}\) influx. (D), Mean Ca\(^{2+}\) entry calculated from the area under the curve in the presence of Ca\(^{2+}\). No statistically significant differences were observed under any condition. Number of recorded cells given in parenthesis.

Figure 2
Whole-cell TRPC4 currents. Time course of whole-cell cationic currents at –100 mV in CHO cells transfected with TRPC4-WT (A) or TRPC4-I957V (B) and dialyzed with a pipette solution containing 88 µM GTP-γS. Insets show current-voltage relationship of peak whole-cell cationic currents recorded at the time indicated by the colored symbols.

Figure 3
Single-channel recordings of TRPC4-WT and TRPC4-I957V channels. (A), Unitary currents of human TRPC4-WT channels recorded in cell-attached patches of transfected CHO cells before (top) and after the addition of 100 µM charbacol (bottom) using a pipette potential of +60 mV. (B), Unitary currents of human TRPC4-I957V channels recorded under identical conditions as in A. Traces showed a maximum of 2 active channels for cells expressing TRPC4-WT and 5 active channels in the case of
cells expressing TRPC4-I957V. Dash line indicates channel close state; dotted lines indicate the unitary current levels of different channels (up to five). (C), Mean increase in the number of active channels in response to 100 µM carbachol obtained in cells expressing TRPC4-WT and TRPC4-I957V.

**Figure 4.**

Current-voltage relations of peak whole-cell cationic currents obtained from CHO cells transfected with TRPC4-WT-Y959F (A) or the double mutant TRPC4-I957V-Y959F (B), recorded at basal and maximal activation conditions. (C), Mean current densities at -100 mV recorded from CHO cells expressing different constructs: GFP, TRPC4-WT, TRPC4-I957V and TRPC4-WT/TRPC4-I957V. (D), PP2 (5 µM) inhibition of TRPC4 currents expressed as \( \frac{I_{PP2}}{I_{max}} \). Number of recorded cells given in parenthesis.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
### Table 1. Main characteristics of the sample. (*) Mean (standard deviation). (**) Current smoker or former <1year. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure

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### Table 2. Adjusted Odds Ratio of myocardial infarction risk for 957V-carriers in the whole sample and stratified by diabetes mellitus status. Model 1: adjusted by age, sex, hypertension, antihypertensive therapy, and diabetes mellitus. Model 2: adjusted by age, sex, hypertension, and antihypertensive therapy.