p122 Protein Enhances Intracellular Calcium Increase to Acetylcholine
Its Possible Role in the Pathogenesis of Coronary Spastic Angina

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Objective—Phospholipase C-δ1 activity is enhanced in patients with coronary artery spasm, and a p122 protein was recently cloned to potentiate phospholipase C-δ1 activity. To investigate the role of p122 in enhanced vasomotility, we examined p122 expression in the cultured skin fibroblasts obtained from patients with and without coronary spasm, intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_i]) at baseline and after stimulation with acetylcholine in the cells transfected with p122, and promoter in genomic DNA.

Methods and Results—p122 protein and gene expression levels in patients with coronary spasm (n=11) were enhanced compared with levels in control subjects (n=9) (P<0.01 for both). [Ca\(^{2+}\)_i], at baseline and the peak increase in [Ca\(^{2+}\)_i], in response to acetylcholine were both 2 times higher in cells transfected with p122 than in those without p122. Conversely, knockdown of p122 resulted in diminished [Ca\(^{2+}\)_i] response. In the p122 promoter analysis, the −228G/A and −1466C/T variants revealed the increase in luciferase activity. Although the −1466C/T variant was similar between 144 patients with coronary spasm and 148 controls, the −228G/A variant was more frequent in male patients than in male controls (P<0.05).

Conclusion—The p122 protein is upregulated in patients with coronary spasm, causing increased [Ca\(^{2+}\)_i] to acetylcholine, and thereby seems to be related to enhanced coronary vasomotility. (Arterioscler Thromb Vasc Biol. 2010;30:1968-1975.)

Key Words: calcium ion ▪ coronary spasm ▪ PLC-δ1 ▪ vasoconstriction ▪ vascular biology ▪ p122 protein

Coronary artery spasm plays an important role in the pathogenesis of variant angina\(^1,2\) and other forms of ischemic heart disease.\(^3\) Considering that esophageal motility is also enhanced in patients with angina pectoris due to coronary spasm (ie, coronary spastic angina [CSA]),\(^4\) the presence of a generalized disorder of smooth muscle contraction is strongly suggested. Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and produces inositol 1,4,5-trisphosphate and diacylglycerol. The inositol 1,4,5-trisphosphate mobilizes Ca\(^{2+}\) from the intracellular stores and elicits rapid contraction of smooth muscle cells.\(^5\) PLC-δ1, an isoform of PLC, is more sensitive to Ca\(^{2+}\) than the other isoforms; therefore, the initial increase in Ca\(^{2+}\) induced by G protein–linked PLC induces the prolonged activation of PLC-δ1 in a positive feedback fashion.\(^6,7\) Indeed, it was reported in loss and gain of function studies that PLC-δ1 is attributable to the sustained contractile response to stimuli.\(^8,9\) PLC-δ1 activity in the cultured skin fibroblasts obtained from patients with CSA was 3 times higher than that from control subjects.\(^10\) Also, there was a significant positive correlation between PLC activities in the skin fibroblasts and coronary artery vasomotilities, suggesting the usefulness of the study using the skin fibroblasts as the surrogate of the smooth muscle cells. For the enhanced enzyme activity, we found a single-base variant (864G-A) in the entire coding region of the PLCδ1 gene in human CSA, resulting in the amino acid replacement of arginine 257 by histidine.\(^11\) However, this PLC-δ1 variant was present in only approximately 10% of the patients with CSA. Therefore, other factors may contribute to or play a more important role in the pathogenesis of coronary spasm.

The p122 protein is a dual functional molecule consisting of 1083 amino acid residues.\(^12,13\) One of the functions is the ability to enhance the PIP\(_2\)-hydrolyzing activity of PLC-δ1, and the other is to have GAP activity specific for Rho.\(^14\) It was recently cloned by screening a rat brain expression library with antiserum against purified PLC-δ1 and was shown to enhance the catalytic activity of PLC-δ1 by altering its Michaelis constant or maximum velocity value rather than its Ca\(^{2+}\) dependency.\(^14\)
Table 1. Clinical Profiles of the Study Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients With CSA</th>
<th>Control Subjects</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>58±5</td>
<td>52±6</td>
<td>0.45</td>
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<tr>
<td>Male sex†</td>
<td>8 (73)</td>
<td>7 (78)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Smoking†</td>
<td>7 (64)</td>
<td>4 (44)</td>
<td>0.65</td>
</tr>
<tr>
<td>Hypertension†</td>
<td>4 (36)</td>
<td>5 (56)</td>
<td>0.65</td>
</tr>
<tr>
<td>CCB‡</td>
<td>3</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>ACE-I†</td>
<td>0</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>ARB‡</td>
<td>0</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>Nitrate†</td>
<td>2</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Diabetes mellitus, % HbA1c</td>
<td>5.6±0.3</td>
<td>6.0±0.4</td>
<td>0.30</td>
</tr>
<tr>
<td>Dyslipidemia (LDL-C), mg/dL</td>
<td>139±15</td>
<td>134±11</td>
<td>0.78</td>
</tr>
<tr>
<td>Statin†</td>
<td>2</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

ACE-I indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, Ca²⁺ channel blocker; Hb, hemoglobin; LDL-C, low-density lipoprotein cholesterol; NA, not applicable.

†Data are given as mean±SEM unless otherwise indicated.
‡Data are given as number (percentage).
§Data are given as number affected.

The p122 protein is constitutively localized in caveolin-1–enriched membrane domains by binding caveolin-1 and cholesterol through a GAP domain and an StAR-related lipid transfer domain of the C-terminal region. It was reported that p122 is involved in the reorganization of the actin cytoskeleton and focal adhesions. However, the role of p122 in coronary vasomotility or spasm still remains unclear. In the present study, we examined the possible role of p122 protein in the pathogenesis of coronary spasm and enhanced PLC-δ1 activity.

Methods

This study consisted of determining 3 items: (1) the expression of p122 in the cultured skin fibroblasts obtained from patients with and without coronary spasm, (2) Ca²⁺ signaling after stimulation with acetylcholine (ACh) in the human embryonic kidney (HEK) 293 cells and 2 lines of vascular smooth muscle cells (human coronary artery smooth muscle cells and A7r5 rat aortic smooth muscle cells) transfected with p122 or p122 small interfering RNA (siRNA), and (3) promoter in genomic DNA.

Study Patients

The investigation conformed with the principles outlined in the Declaration of Helsinki. The ethics committee of our institution approved the study protocol. Written informed consent was obtained from all patients before the study.

For the p122 expression study, we enrolled 11 patients with CSA (8 men and 3 women; mean±SEM age, 58±5 years) and 9 control subjects without any history suggestive of angina pectoris who were undergoing cardiac catheterization (7 men and 2 women; mean±SEM age, 52±6 years). The clinical profiles of the patients with CSA and the control subjects are shown in Table 1. Coronary spasm, defined as total or subtotal coronary artery occlusion associated with chest pain and ischemic electrocardiographic change, was induced by intracoronary injection of ACh in all patients with CSA. After intracoronary injection of isosorbide dinitrate, the coronary arteriograms revealed normal or almost normal coronary arteries with a stenosis diameter of less than 50% of the lumen diameter.

The primary skin fibroblasts were obtained from the control subjects and patients with CSA at cardiac catheterization. They were prepared by an explant method, as previously described, to determine the protein and mRNA expression of p122. Fibroblasts from the second to fourth passages were used for the study.

For the p122 promoter assay study, we enrolled 144 patients with CSA (91 men and 53 women; mean±SEM age, 61±11 years) and 148 control subjects without hypertension or any history suggestive of angina pectoris (62 men and 86 women; mean±SEM age, 52±8 years). Coronary spasm was demonstrated during coronary angiographic study in all patients with CSA.

Protein Expression of p122 by Western Blot Analysis

The fibroblasts were harvested, pelleted, and resuspended in RIPA lysis buffer. Equal amounts of protein, 20 μg per lane, were applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis wells and transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif). After incubating with the primary antibody for p122 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, Calif), the protein bands were detected by the enhanced chemiluminescence plus detection system (Amer sham Pharmacia Biotech, Piscataway NJ).

Gene Expression of p122

Two-step RT-PCR was performed according to the protocol supplied with a commercially available kit (TaqMan Gold RT-PCR kit; Applied Biosystems, Carlsbad, Calif). Oligonucleotide primers and a TaqMan probe for p122 were designed using Primer Express, version 1.5 (Applied Biosystems). The sense and antisense primers were 5′-CCCGGACCTAATCGTCGA TC-3′ and 5′-GCTGCCGAAGAGTGTCGATC AT-3′, respectively. The Taq-Man probe was 5′-TTTGCCTCCTCCAGCAACTTGCCAG G-3′. To assess p122 mRNA stability, the skin fibroblasts were exposed to actinomycin D, 5 μg/mL.

Measurement of Intracellular Ca²⁺ in HEK 293 Cells and Vascular Smooth Muscle Cells

HEK 293 cells and A7r5 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS. Human coronary artery smooth muscle cells (hCASMCs) (Clonetics, Palo Alto, Calif) were cultured in smGM2 medium (human epidermal growth factor, 0.5 ng/mL; insulin, 5 μg/mL; human fibroblast growth factor-B, 2 ng/mL; and 5% FBS), and the cells from the fourth to seventh passages were used for the study.

HEK 293 cells were transfected with muscarine M1 receptor cDNA, the constructed plasmid DNA of the wild-type PLC-δ1, and the constructed plasmid DNA of p122 or the empty vector. hCASMCs were transfected with p122 siRNA (sense, 5′-GAAACGCCUUAA GACACUATT-3′; and antisense, 5′-UAUGGCUUAGUGGCUUUGGCAG UTT-3′ [Takara Biotechnology Co, Ltd, Kyoto, Japan]) or negative control. A7r5 cells were transfected with muscarine M1 receptor CDNA and p122 or the empty vector and with or without PLC-δ1 siRNA (sense, 5′-GAAUCAUGGCUCUUCUAU GACACUATT-3′; and antisense, 5′- AAUGAAGAAGCAGUCUUUGGCUUUGGCAG UTT-3′ [Takara Biotechnology Co, Ltd]).

The expression of the p122 protein and PLC-δ1 was examined by Western blot analysis. After loading with 5-μmol/L fura-2-acetoxyethyl ester, intracellular Ca²⁺ concentration ([Ca²⁺]i), in response to ACh at 10⁻⁶ and/or 10⁻⁷ mol/L, was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm, as previously described. ACh was used because it is widely used for the induction of coronary spasm in Japan.

Measurement of PLC Activity

The PLC assay system included the following components: N-2-hydroxymethylpiperezine-N’-2-ethanesulfonic acid, 50 mmol/L; calcium chloride, 0.1 mmol/L; sodium cholate, 9 mmol/L; tritium-P1P-2, 40,000 counts per minute; and the cell protein, 20 μg. The reaction was discontinued with a combination of chloroform, methanol, and hydrogen chloride, followed by 1N hydrogen chloride containing...
EGTA. After extraction, the aqueous phase was removed for liquid scintillation counting.

**Determination of Initiation of p122 Transcription (5'-Rapid Amplification of cDNA Ends)**
SMART–Rapid Amplification of cDNA Ends (RACE) technology (BD Biosciences, San Jose, Calif) was used to determine the initiation site of p122 transcription. Total RNA isolated from the skin fibroblasts was reverse transcribed, and a complete cDNA with the addition of a BD SMART sequence at the 5' end, was generated according to the manufacturer’s instructions. RACE-ready p122 cDNA was amplified using universal primer mix (BD Biosciences) and gene-specific primer (5'-TGC TGA GGC TGC GGA CGG AAG ACA CC-3'), as recommended by the manufacturers. The PCR products were subcloned into pGEM-T Easy Vector (Promega, Madison, Wis) and then sequenced by a genetic analyzer (ABI PRISM 310).

**DNA Isolation and Analysis of Genomic DNA for the p122 Promoter**
Genomic DNA was isolated from the whole blood using a kit (QIAamp DNA Blood Mini Kit; Qiagen, Valencia, Calif). The promoter region of p122 was amplified by PCR using 5 sets of primers. Each PCR product was amplified by a kit (BigDye Terminator Cycle Sequencing Kit) and then sequenced by a genetic analyzer (ABI PRISM 310).

**Luciferase Assay for p122 Promoter**
The p122 promoter-luciferase reporter construct, Picagene basic vector (Toyo Ink Mfg Co, Ltd, Tokyo, Japan), was obtained by PCR amplification (LA PCR, TaKaRa) of human p122 promoter between −1599 and 114 relative to the translation start site. The mutated p122 promoter-luciferase reporter constructs, Picagene basic vector −1466C-T and −228G-A, were obtained by direct mutagenesis of single-nucleotide polymorphism sites in Picagene basic vector. The mutations were confirmed by sequencing. The National Institutes of Health 3T3 cells cultured in complete media were transfected with p122 promoter vectors containing or lacking the nucleotide conversions of −1466C-T and −228G-A, using Effectene Transfection Reagent (Qiagen). After incubation for 48 hours, the cell lysates were assayed by the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions.

**Analysis for p122 Promoter Variants at −228 and −1466**
The −228G-A variant of the p122 promoter was analyzed by mutant allele-specific amplification. The promoter region of p122 that spans from nucleotide (nt) −250 to 86 was amplified by PCR using 2 sets of primers (sense, 5'-AAC CGA GGG AAC CGA GGG GGC G-3', to detect the −228A variant, and 5'-AAG CGA GGG AAC CGA GGG GGC A-3', to detect the −228G variant; and antisense, 5'-AGT TGG CCG AGA AGT CCG TGA G-3' to detect both variants). For both variants, a 336-bp amplicon was detected as the PCR product.

The −1466C-T variant was analyzed by restriction fragment length polymorphism with endonuclease Smal I (TaKaRa; Ohtsu, Shiga, Japan). The promoter region of p122 from nt −1599 to −978 was amplified by PCR. Smal I digests the wild-type 621-bp amplicon containing −1466C into 2 fragments (488 and 133 bp), and it does not digest the variant-type 621-bp amplicon containing −1466T.

**Data Analysis**
All data are expressed as mean ± SEM. Categorical variables were compared by χ² analysis. An unpaired t test for comparison of 2 variables and a 1-way ANOVA for multiple comparisons, followed by a Bonferroni test, were used for statistical analysis. Differences were considered significant when P<0.05.

**Results**

**Protein and Gene Expression of p122 in Cultured Fibroblasts**
To assess the protein expression of p122, subconfluent skin fibroblasts, in which PLC-5I 864G/A variant was not detected by single-strand conformation polymorphism analysis, were scraped after 24-hour serum starvation. In these conditions, the protein expression of p122 was constant and reproducible from the second to the fourth passages. As shown in Figure 1A, the ratio of p122 to GAPDH protein was significantly higher by 177±20% in patients with CSA versus control subjects (P<0.001). The level of the ratio in patients with CSA ranged from 1.5 to 3.9; all of them were greater than the value of plus 1 sigma (0.4) of the mean value in the control subjects. In 1 patient with CSA who was

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**Figure 1.** A, Protein expression in the skin fibroblasts of p122 normalized by human GAPDH in patients with CSA (n=11) and control subjects (n=9). B, p122 mRNA level normalized by human GAPDH in patients with CSA (n=11) and control subjects (n=9). C, Representative amplification curves for p122 and GAPDH in RT-PCR (40 cycles). D, Changes in p122 mRNA levels after the addition of actinomycin D, 5 μg/mL, in patients with CSA (n=4) and control subjects (n=4).
diagnosed as having an acute myocardial infarction due to a coronary spasm, the level of the ratio of p122 to GAPDH was 3.9, which was the highest value among the patients with CSA. In the same culture condition, p122 gene expression was increased in patients with CSA compared with control subjects by 37±7% (P<0.01) (Figure 1B), suggesting that upregulation of p122 protein expression is partially caused by enhanced gene expression. Figure 1C illustrates the representative amplification curves for p122 and GAPDH mRNA after the blockade of mRNA synthesis by actinomycin D. p122 mRNA was decreased at 5 and 10 hours in a time-dependent manner, and the ratio of p122 to GAPDH mRNA in patients with CSA was similar to that in control subjects (Figure 1D), suggesting that the p122 increase in patients with CSA is independent of mRNA stability but dependent on the transcription and/or translation level.

**Effects of p122, p122 siRNA, or PLC-δ1 siRNA Transfection on the Response of [Ca\(^{2+}\)]\(_i\) to ACh**

In the HEK 293 cells transfected with plasmid DNA of p122, a single immunoreactive compound was confirmed at 122 kDa (Figure 2A), and the ratio of p122 to GAPDH protein was increased by approximately 2 times compared with the cells transfected with empty vector instead of p122, being comparable to the degree of its increased expression in patients with CSA. HEK 293 cells per se showed no [Ca\(^{2+}\)]\(_i\) response to ACh, whereas those expressing muscarine M1 receptor and PLC-δ1 showed a constant [Ca\(^{2+}\)]\(_i\) response to ACh. Figure 2B illustrates the representative waveforms of [Ca\(^{2+}\)]\(_i\), in response to ACh. ACh at both 10\(^{-6}\) and 10\(^{-5}\) mol/L caused a rapid transient increase in [Ca\(^{2+}\)]\(_i\), followed by a lower but sustained phase of the increase. In HEK 293 cells transfected with p122, the transient increase in [Ca\(^{2+}\)]\(_i\) was augmented and the sustained phase was prolonged. As shown in Figure 2C, the [Ca\(^{2+}\)]\(_i\) level at baseline was 23±1 nmol/L in the cells without p122 and 39±2 nmol/L in cells with p122 (P<0.01). The peak increase in [Ca\(^{2+}\)]\(_i\) from baseline after ACh at 10\(^{-5}\) mol/L was higher than that after ACh at 10\(^{-6}\) mol/L in both types of cells (P<0.05 for both) (Figure 2D). The peak increase in [Ca\(^{2+}\)]\(_i\) from baseline after ACh was significantly greater in the cells transfected with p122 than in those without p122 (68±6 versus 33±4 nmol/L at 10\(^{-6}\) mol/L ACh and 128±11 versus 67±8 nmol/L at 10\(^{-5}\) mol/L ACh, respectively; P<0.01 for both).

As shown in Figure 3A, [Ca\(^{2+}\)]\(_i\) at baseline was decreased from 68±6 to 44±3 nmol/L in the knockdown of p122 with siRNA in hCASMCs (n=6, P<0.05). The peak increase in [Ca\(^{2+}\)]\(_i\) from baseline after ACh at 10\(^{-5}\) mol/L was decreased (n=6, P<0.05). By the knockdown of p122, PLC-δ1 protein expression was unchanged, but PLC activity was decreased.

As shown in Figure 3B, [Ca\(^{2+}\)]\(_i\) at baseline was increased from 76±12 to 123±35 nmol/L by the overexpression of p122 in A7r5 cells (n=6, P<0.05). The peak in [Ca\(^{2+}\)]\(_i\), from baseline after ACh at 10\(^{-5}\) mol/L was decreased (n=6, P<0.05). PL e-δ1 protein expression was also enhanced by p122 overexpression (n=6, P<0.05). Furthermore, in this condition, knockdown of PLC-δ1 with siRNA cancelled the elevated baseline [Ca\(^{2+}\)]\(_i\) level and abolished the increase in [Ca\(^{2+}\)]\(_i\) in response to ACh and enhancement of PLC activity.

**Sequencing of Genomic DNA for the p122 Promoter**

As shown in Figure 4A, we analyzed the promoter sequence (between nt −1599 and 114) of the p122 genomic DNA...
obtained from the whole blood of 6 patients with CSA and 6 control subjects. Then, we compared the sequence of the p122 genomic DNA obtained from the patients with CSA with that of the p122 genomic DNA previously reported (GenBank accession No. AF514295). Eight different nucleotides were found at position $\text{H11002} 1466$ (C to T), $\text{H11002} 1319$ (T to C), $\text{H11002} 833$ (T to A), $\text{H11002} 572$ (T to G), $\text{H11002} 276$ (G to A), $\text{H11002} 228$ (G to A), $\text{H11002} 144$ (G to C), and $\text{H11002} 14$ (T to C).

p122 Promoter Assay

To investigate the contribution of genomic DNA variants to gene expression, a luciferase assay was performed using mutated p122 promoter constructs. We conventionally named the p122 promoter obtained from a control subject containing neither $-1466T$ nor $-228A$ variant as wild type. As shown in Figure 4B, luciferase activities in the cells transfected with $-1466T$ and $-228A$ variant promoter constructs were both significantly increased compared with those with wild-type promoter construct by $1.34\pm0.27$ times ($P<0.03$) and $1.57\pm0.34$ times ($P<0.001$), respectively.

Incidence of $-228G-A$ Variant in Genomic DNA

To examine the variance at nt $-228$, we amplified the corresponding promoter region by PCR using genomic DNA of 144 patients with CSA and 148 control subjects and examined the presence of this variant by the mutant allele-specific amplification method. Figure 5A (left and center panels) demonstrates 2 representative patterns of the PCR

Figure 3. A, The effects of knockdown of p122 with siRNA in human coronary artery smooth muscle cells. Representative waveforms of $[\text{Ca}^{2+}]_i$ are shown in the middle panel. $[\text{Ca}^{2+}]_i$ at baseline, peak increase in $[\text{Ca}^{2+}]_i$ from baseline after $10^{-5}$ mol/L ACh, and PLC activity in control and knockdown cells are shown in the lower panel. B, The effects of p122 overexpression with and without PLC $\delta 1$ knockdown in A7r5 (rat aortic smooth muscle) cells. Representative waveforms of $[\text{Ca}^{2+}]_i$ are shown in the upper panel, and representative bands of protein expression of each parameter are shown in the middle panel, $[\text{Ca}^{2+}]_i$ at baseline, peak increase in $[\text{Ca}^{2+}]_i$, from baseline after $10^{-5}$ mol/L ACh, and PLC activity in control cells and p122 overexpression with and without PLC $\delta 1$ knockdown are shown in the lower panel. *$P<0.05$ vs each control (C).

Figure 4. A, Sequence analysis of the p122 promoter region on genomic DNA. Eight different nucleotide sites were found by using 5 sets of primers. B, Effect of genomic DNA variants on p122 promoter activity. Luciferase activity of the wild-type reporter construct was arbitrarily set as 1, and the mean $\pm$ SEM values of relative luciferase activity from 3 independent experiments are shown, each performed in duplicate, *$P=0.03$ and **$P<0.001$ (n=6). Ex1 indicates exon 1; Luc, luciferase gene.
products, corresponding to the −228G/G normal homozygote and the −228A/A homozygote. As shown in Figure 5B, the incidence of the −228A/A homozygote or −228G/A heterozygote variant was greater in male patients with CSA than in male control subjects (9 of 91 patients with CSA versus 1 of 62 control subjects; \( P < 0.05 \)). However, by the analysis of the T-allele influence to the risk of CSA, no significant effects were found in the additive (G versus A allele), dominant (GG versus AA and GA alleles), and recessive (GG and AG versus AA alleles) models (Table 2).

Incidence of −1466C-T Variant in Genomic DNA
As shown in Figure 5A (right panel), 2 nucleotide patterns of the −1466C/C normal homozygote and the −1466C/T heterozygote were demonstrated. The −1466C/C normal homozygote and the −1466C/T heterozygote were present in 120 (83.3%) and 24 (16.7%), respectively, of the 144 patients with CSA. The −1466C/C normal homozygote and the −1466C/T heterozygote were present in 125 (88.0%) and 17 (12.0%), respectively, of the 142 control subjects. The incidence of the −1466C/T heterozygote tended to be more frequent in patients with CSA than in control subjects (\( P = 0.08 \)), but it did not reach statistical significance.

Discussion
The major findings of this study were as follows. Protein expression of p122 was enhanced by approximately 3 times in patients with CSA, being associated with the enhanced gene expression. Baseline \([\text{Ca}^{2+}]_i\) and the peak \([\text{Ca}^{2+}]_i\) in response to ACh were both higher in HEK 293 cells and vascular smooth muscle cells transfected with p122 than in those without p122. The 8 variants of the nucleotide were detected in the p122 promoter, and the −228G/A and −1466C/T variants revealed the increase in promoter activity. Thus, p122 protein is upregulated in patients with CSA, and its enhancement may be involved in the increased coronary vasomotility via the increased \([\text{Ca}^{2+}]_i\) response.

Role of p122 in Enhanced Vasomotility
The novel regulatory protein p122 plays 2 important roles in signaling pathways. One of the dual functions is the ability to enhance the \(\text{PIP}_2\)-hydrolyzing activity of PLC-δ1 and the other is a GAP activity specific for Rho. Regarding the GAP activity, it was recently reported that p122 is involved in the
reorganization of the actin cytoskeleton and focal adhesions. However, the role of p122 as a positive regulator for PLC-δ1 in coronary spasm still remains unclear. In the present study, we showed that the protein expression of p122 in the cultured skin fibroblasts obtained from patients with CSA was upregulated by 3 times compared with control. All protein levels of p122 in patients with CSA exceeded the highest level in control subjects and were greater than the +1 σ of the mean. Therefore, it is likely that patients with CSA could be distinguished from control subjects by the protein level of p122. The gene expression of p122 was increased by 37% in patients with CSA compared with the control, and p122 mRNA stability was similar between patients with CSA and control subjects, suggesting that upregulation of p122 protein expression is not fully, but is significantly, dependent on enhanced gene expression. Homma and Emori showed that recombinant PLC-δ1 catalyzes the hydrolysis of PIP_2 in a Ca^{2+}-dependent manner; in the presence of p122, its activity is 5- to 10-fold increased in the range of physiological Ca^{2+} concentration. Thus, it is conceivable that upregulation of p122 protein observed in patients with CSA is responsible for the high activity of PLC-δ1.

By using 3 kinds of cultured cells (HEK 293 cells transfected with muscarine M1 receptor, PLC-δ1, and p122; hCASMCs with p122 siRNA; and A7r5 cells with muscarine M1 receptor and p122 or PLC-δ1 siRNA), we tested the hypothesis that p122 upregulation, such as that observed in patients with CSA, is associated with increased [Ca^{2+}], response to ACh. In these cells, p122 was upregulated approximately 2 times compared with the cells without transfection with p122, and it was comparable to the degree of upregulated p122 protein in patients with CSA. We used ACh because it is widely used as a provocation test for coronary spasm in the clinical setting in Japan. It binds the muscarine M1 receptor linked to the Gq–PLC-β pathway. The results showed that the peak increase in [Ca^{2+}], in response to ACh was augmented and the sustained phase was prolonged in the cells transfected with p122. In addition, [Ca^{2+}] at baseline was elevated in the cells transfected with p122. These results are consistent with the previous findings of the 864G-A variant of PLC-δ1, in which the conformational change is associated with upregulation of PLC-δ1 activity. These characteristics seem to explain the pathogenesis of CSA in which the basal vascular tone and the vasoconstrictor response to the diverse stimuli were both enhanced. Previously, PLC activity was positively correlated not only with basal coronary artery tone but with the maximal and averaged constrictor responses of the coronary artery to ACh. This suggests a critical role of p122 protein in the genesis of coronary spasm.

The p122 protein has another function, a GAP activity for Rho. It was reported that the Rho-kinase inhibitor fasudil attenuated the constrictor response of the coronary artery to ACh and prevented the occurrence of chest pain in patients with CSA. By its GAP activity, p122 may antagonize the development of coronary spasm like the Rho-kinase inhibitor. On the other hand, Ca^{2+} mobilization induced by PLC-δ1 activation may upregulate Rho and Rho kinase in the vascular smooth muscle. Thus, the role of p122 in the regulation of Rho is complicated, and the relation of p122 to the genesis of coronary spasm via Rho activity remains to be determined.

**Possible Mechanisms for Enhanced Protein Expression of p122**

Some variants that upregulate mRNA expression may be involved in upregulation of p122 protein. Therefore, we first clarified the translation start site of the p122 by 5' RACE procedure and then sequenced the p122 genomic DNA coding the promoter from −1599 through 92 by the genetic analyzer. According to the reported sequence, we found 2 sites of single allele mutation at −228G-A and −1466C-T, both of which resulted in increased p122 promoter activity. We analyzed the incidence of these variants in the relatively small, but strictly defined, population in whom coronary spasm was confirmed by total or subtotal coronary artery occlusion after intracoronary injection of ACh. The result showed that the incidence of −228G-A was more frequent in male patients with CSA than in male control subjects, suggesting that this variant is a possible candidate responsible for upregulation of p122 protein in CSA. The mechanism of enhancement of p122 promoter activity by the −228G-A variant may be related to transcription factor SP because this variance causes loss of binding to SP1 in its region. Upregulation of p122 protein was observed in the skin fibroblasts, but the variant of p122 promoter should be present in the overall tissues, including the coronary arteries. Therefore, upregulation of p122 might play an important role in the pathogenesis of CSA in humans. It is obvious that the p122 protein in patients with CSA is upregulated. However, other factors or a combination of factors may contribute to or play a more important role in the mechanism for the mRNA or protein upregulation of p122. Further studies are required.

**Study Limitations**

Upregulation of p122 was shown in the skin fibroblasts obtained from patients with CSA. Further studies are required to confirm whether this abnormality is present in the coronary artery, although it is impossible to obtain the coronary artery smooth muscle cells from the patients with CSA. In addition, we showed a possible role of p122 in the pathogenesis of CSA in the Japanese. It remains unclear whether this is observed in the overall population.

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**Disclosures**

None.

**References**


p122 Protein Enhances Intracellular Calcium Increase to Acetylcholine: Its Possible Role in the Pathogenesis of Coronary Spastic Angina

Reiichi Murakami, Tomohiro Osanai, Hirofumi Tomita, Satoko Sasaki, Atsushi Maruyama, Ken Itoh, Yoshimi Homma and Ken Okumura

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An erratum has been published regarding this article. Please see the attached page for:
/content/30/11/e178.full.pdf

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In the article, “p122 Protein Enhances Intracellular Calcium Increase to Acetylcholine: Its Possible Role in the Pathogenesis of Coronary Spastic Angina” by Murukami et al, which appeared in the October 2010 issue of the journal (*Arterioscler Thromb Vasc Biol*. 2010;30:1968–1975; DOI: 10.1161/ATVBAHA.110.203083), the publisher omitted a few corrections from the final, printed version of the article:

1. Page 1968, abstract, Objective, line 4 “([Ca^{2+}])” should have appeared as “([Ca^{2+}]_i)”  
2. Page 1968, abstract, Methods and Results, line 1, the first sentence “Phospholipase C-δ1 activity…” should be deleted  
3. Page 1968, abstract, Key Words, “calcium” and “coronary vasospasm” should be removed from the list  
4. Page 1974, 1st column, 3rd paragraph, line 2, “ρ” should have appeared as “Rho”

The online version has been corrected.

The publisher sincerely regrets the error.

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