Coronary Vasospasm Induced in Transgenic Mouse With Increased Phospholipase C-δ1 Activity

Shuji Shibutani, MD; Tomohiro Osanai, MD; Toshihiro Ashitate, MD; Shigeki Sagara, MD; Kei Izumiyama, MD; Yoko Yamamoto, MD; Kenji Hanada, MD; Takashi Echizen, MD; Hirofumi Tomita, MD; Takeshi Fujita, MD; Takeshi Miwa, MD; Hiroaki Matsubara, MD; Yoshimi Homma, MD; Ken Okumura, MD

Background—We reported that phospholipase C (PLC)-δ1 activity was enhanced 3-fold in patients with coronary spastic angina. We detected variant PLC-δ1 with replacement of arginine 257 by histidine (R257H) showing increased enzymatic activity. We tested the hypothesis that increased PLC-δ1 activity causes enhanced coronary vasomotility.

Methods and Results—We generated transgenic (TG) mice with human R257H variant PLC-δ1 in vascular smooth muscle cells. PLC enzymatic activity in the coronary artery was increased by 2.57 and 1.89 times, respectively, in homozygous and heterozygous TG compared with wild-type (WT) mice. ST elevation after ergometrine occurred in 17 of 18 homozygous TG, 6 of 20 heterozygous TG, and 3 of 22 WT mice (P<0.01, homozygous TG versus WT; P<0.05, homozygous TG versus heterozygous TG; P=NS, heterozygous TG versus WT). ST elevation was associated with bradyarrhythmias in homozygous TG mice. Focal coronary artery narrowing was documented with the microvascular filling technique in 3 of 5 homozygous TG mice after ergometrine but not in any of 7 WT mice (P<0.05). In the isolated Langendorff hearts, coronary perfusion pressure was increased after ergometrine in homozygous TG mice (P<0.01) but not in heterozygous TG or WT mice. Coronary perfusion pressure increase after prostaglandin F2α was similar among homozygous TG, heterozygous TG, and WT mice. Cultured rat aortic smooth muscle cells transfected with variant PLC-δ1 showed a higher PLC activity than those with WT PLC-δ1 (P<0.05) and furthermore showed greater intracellular Ca2+ response to acetylcholine in variant than in WT PLC-δ1 (P<0.05).

Conclusions—Increased PLC-δ1 activity enhances coronary vasomotility such as that seen in patients with coronary spastic angina. (Circulation. 2012;125:1027-1036.)

Key Words: calcium ▪ genetics ▪ smooth muscle ▪ vasospasm

Coronary artery spasm plays an important role in the pathogenesis of Prinzmetal variant angina1,2 and the other acute coronary syndromes.3,4 We and other investigators have shown that the basal vasomotor tone of the entire coronary artery system of Japanese patients with variant angina is enhanced.5-7 In addition, the coronary artery constrictor response to diverse constrictor stimuli is enhanced,8 and occlusive constriction is readily induced when exposed to such a stimulus. When one considers that esophageal motility is enhanced in patients with coronary spastic angina (CSA),4 the presence of a generalized disorder of smooth muscle contraction is strongly suggested.

Clinical Perspective on p 1036

Phospholipase C (PLC), a key enzyme for vasoconstriction, produces inositol 1,4,5-trisphosphate (IP3) and diacylglycerol by hydrolyzing phosphatidyl inositol 4,5-bisphosphate (PIP2). IP3 mobilizes Ca2+ from the intracellular stores and elicits rapid contraction of the vascular smooth muscle cells,9 whereas diacylglycerol activates protein kinase C and initiates sustained contraction by a Ca2+-independent mechanism.10 We previously demonstrated that PLC activity in cultured skin fibroblasts obtained from patients with CSA was enhanced and that a major PLC
isozyme detected in the membrane fraction was the δ1 isoform. We further demonstrated PLC-δ1 864 G to A mutation resulting in amino acid replacement of arginine 257 by histidine (R257H) in approximately 10% of male CSA patients. This variant PLC-δ1 showed enhanced enzymatic activity in the physiological range of intracellular free calcium concentrations ([Ca²⁺]) compared with the wild type (WT). More recently, we reported that p122 protein, a positive regulator of PLC-δ1, is upregulated in patients with CSA, thereby causing an increased response of [Ca²⁺] to acetylcholine. Because PLC-δ1 is more sensitive to Ca²⁺ than the other PLC isozymes, the initial increase in [Ca²⁺] induced by G protein–linked PLC induces a prolonged activation of PLC-δ1 in a positive feedback fashion. Thus, the increased PLC-δ1 activity caused by either the structural mutation or the increased positive regulator seems to play an important role in the pathogenesis of coronary spasm. However, there has been no proof for the direct contribution of increased PLC-δ1 to the enhanced coronary vasomotility in patients with CSA. In the present study, we generated transgenic (TG) mice with increased PLC-δ1 activity in vascular smooth muscle cells by overexpressing human R257H variant PLC-δ1 under control of the mouse α-smooth muscle actin (α-SMA) promoter. This promoter induces a pattern of transgene expression similar to that of endogenous vascular smooth muscle cell–specific α-actin, the dominant α-actin isoform in the tissue. The resultant recombinant construct was digested with EcoRI and NotI to generate a 7.3-kb DNA fragment consisting of the α-SMA promoter and the variant PLC-δ1 cDNA (Figure 1A). The DNA fragment was then microinjected into the pronuclei of fertilized mouse embryos at the single-cell stage to generate TG mice (C57BL/6 strain), as reported previously. Two lines of homozygous TG mice were produced. Because they had similar phenotypes and responses to stimuli, the data of 1 line of TG mice that manifested greater expression of variant PLC-δ1 were shown in the following sections (see Table I in the online-only Data Supplement for another line). All animals were maintained in the same environment, including constant temperature and humidity and free access to food and water. The experiments were conducted in homozygous TG, heterozygous TG, and WT mice at 20 through 30 weeks of age unless otherwise noted. All procedures were approved by the ethics committee for animal experimentation of Hirosaki University Graduate School of Medicine.

Reverse Transcription Polymerase Chain Reaction
Total RNA was extracted from homogenized descending aorta with the use of TRIzol reagent according to the protocol of the manufacturer (Qiagen, Valencia, CA), and RNA pellets were resuspended in diethylpyrocarbonate-treated water. cDNA was synthesized with the use of reverse transcription reagents (Applied Biosystems, Foster City, CA). Transgene–specific primers were used to detect the human variant PLC-δ1 transgene product at 710 base pairs (primer forward: 5'-GGATTCACCTAGTATGG ACTCGGGCCGGGACTT-3'; reverse: 5'-AACGTATCCACGGACAGAGT-3').

Western Blot Analysis
The tissue extract was obtained from suspension and homogenization in RIPA lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1% glycerol, 1 mmol/L diithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride). The protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad Laborato-
ties, Hercules, CA). After they were blocked for 1 hour, the membranes were incubated with the primary antibody for PLC-δ1 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The protein bands for antigen-antibody complexes were detected by enhanced chemiluminescence plus detection systems (Amersham Pharmacia Biotech, Piscatway, NJ). Densitometric analysis was performed with Scion image software, and the relative ratio to the protein bands was calculated in each sample.

Immunofluorescence Microscopy
The heart was excised, frozen in liquid nitrogen–cooled isopentane, sectioned from base to apex at a thickness of 8 µm, and then fixed in –20°C methanol for 10 minutes. With the use of the anti-α-SMA antibody and anti-PLC-δ1 antibody, the section was examined to see whether PLC-δ1 was overexpressed in the coronary arteries. Fluorescent images were viewed and captured with the use of the Axioskop, AxioCam, and AxioVision microscope, camera, and software systems (Carl Zeiss Inc, Oberkochen, Germany).

Measurement of PLC Activity
The PLC assay system included the following components: N-2-hydroxyethylpiperazine-Ν'-2-ethanesulfonic acid (50 mmol/L), CaCl₂ (0.1 mmol/L), sodium cholate (9 mmol/L), 3H-PIP2 (40 000 cpn), and the cell protein (20 µg). The reaction was stopped with chloroform/methanol/HCl followed by 1N HCl containing EGTA. After extraction, the aqueous phase was removed for liquid scintillation counting. We measured PLC activities in the coronary artery, aorta, and mesenteric artery.

Blood Pressure Measurement
Blood pressure was determined by the tail-cuff method in conscious mice.

Echocardiography
Transthoracic M-mode images obtained from the short-axis view of the left ventricle (LV) with the use of a Philips HD11 XE and a 15-MHz linear probe were recorded in mice anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). LV end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD), and LV posterior wall thickness were measured, and the data from at least 3 cardiac cycles were averaged. LV fractional shortening (%) was calculated as [(LVEDD-LVESD)/LVEDD]×100.

ECG Recordings and Response to Ergometrine
Two doses (15 and 50 mg/kg) of ergometrine maleate (Sigma, St Louis, MO; 400 mg/mL in dimethyl sulfoxide and diluted with H₂O), which stimulates the serotonergic receptor directly and triggers constriction of vascular smooth muscles, were injected into the jugular vein of the anesthetized mice over 10 minutes. Ergometrine was used because this agent was shown to have the potential to induce coronary spasm in previous mouse models. The doses of ergometrine were also determined according to these previous studies. The ECG lead II was recorded continuously before and after ergometrine injection and was analyzed by the investigators blinded to mouse genotype. We focused on ST-segment changes, particularly ST-segment elevation, compared with the baseline ECG.

Microvascular Filling Study
The microvascular filling technique was applied to the coronary artery as described previously. Briefly, homozygous TG and WT mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg), and then either ergometrine (50 mg/kg) or vehicle was administered intravenously from the jugular vein, followed by the infusion of Microfil, a liquid latex medium. The coronary arteries filled with the Microfil were sectioned from base to apex at a thickness of 8 µm, and then fixed in –20°C methanol for 10 minutes. With the use of the anti-α-SMA antibody and anti-PLC-δ1 antibody, the section was examined to see whether PLC-δ1 was overexpressed in the coronary arteries. Fluorescent images were viewed and captured with the use of the Axioskop, AxioCam, and AxioVision microscope, camera, and software systems (Carl Zeiss Inc, Oberkochen, Germany).

Coronary Perfusion Pressure in Isolated Langendorff-Perfused Hearts
Homozygous and heterozygous TG and WT mice were heparinized (0.5 U/g body wt) and anesthetized with an intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). The hearts were then rapidly excised and transfused via a 20-gauge cannula (Physio-tech, Tokyo, Japan) that was placed immediately distal of the intact aortic valve, as described previously. The hearts were perfused at a constant flow (2 mL/min) with Krebs-Henseleit solution (in mmol/L:120 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 10 glucose, 25 NaHCO₃, 1.25 CaCl₂) equilibrated with 95% O₂ and 5% CO₂ at 37°C with the use of a standard Langendorff setup (Physio-tech). Coronary perfusion pressure was recorded continuously with the use of a pressure-sensing catheter (AD Instruments, Bella Vista, Australia) connected to the perfusion cannula. The hearts were equilibrated for at least 20 minutes before experiments. Each of ergometrine and prostaglandin F₂α (PGF₂α) was given in the perfusion solution at the final concentration of 1 and 10 µmol/L, respectively, in Langendorff-perfused hearts for 20 minutes. During the experiment, the hearts were maintained at 38°C via a water-jacketed tissue-organ bath.

To assess endothelial function in TG and WT mice, acetylcholine was given in the perfusion solution at the final concentration of 1 µmol/L, in the presence or absence of PGF₂α. The level of nitric oxide metabolite in the perfusion solution was measured by a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI).

Measurement of [Ca²⁺] and IP₃ in A7r5 Cells
A7r5 aortic smooth muscle cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were subcultured on the coverslips in 12-well plates and then transfected with both muscarinic M1 receptor cDNA (a gift from Dr Tomohiro Kurosaki at Kansai Medical University, Osaka, Japan) and either the human WT PLC-δ1, or the PLC-δ1 transgene were identified by polymerase chain reaction of genomic DNA. Neonatal mortality was not significantly different between TG mice and WT mice. Both homozygous and heterozygous
TG mice showed no early mortality. Trend of body weight after birth was not statistically different between TG mice and their control littermates. Systolic blood pressure was modestly but significantly elevated in homozygous TG mice (n=30) compared with WT mice (n=27) (111±6 versus 105±6 mm Hg; P<0.001). Echocardiographic study demonstrated that LV fractional shortening, LVESD, LVEDD, or LV posterior wall thickness was not statistically different between TG mice and WT mice (Table 1).

Expression of Variant PLC-δ1 in TG Mice

The bands of reverse transcription polymerase chain reaction on total mRNA extracted from the aorta of homozygous TG and WT mice are shown in Figure 1B. The mRNA of the human R257H variant PLC-δ1 transgene was detected only in TG mice at 710 base pairs. The distribution of human variant PLC-δ1 mRNA expression levels is shown in Figure 1C. Variant PLC-δ1 was expressed in overall tissues, especially in the aorta, heart, and skin. As shown in Figure 1D, the endogenous mouse PLC-δ1 protein was seen at 70 kDa and slightly at 85 kDa in the aorta from WT mice (left lane), whereas the human variant PLC-δ1 protein was superimposed at 85 kDa in TG mice (right lane). The protein expression of PLC-δ1 was increased 2-fold in the aorta obtained from homozygous TG mice compared with that from WT (Figure 1E).

Figure 2 illustrates immunofluorescence microscopy of heart sections from homozygous TG and WT mice. Each of PLC-δ1 (left panel) and α-SMA immunoreactivities (middle panel) was seen consistently in both large and small vessels in the heart. Both vessels were more strongly stained by anti-PLC-δ1 antibody in TG mice than in WT mice, indicating that variant PLC-δ1 was expressed only in TG mice, whereas there was no difference in the immunoreactivity to α-SMA between TG and WT mice. Costaining with anti-PLC-δ1 and anti-α-SMA antibodies (right panel) confirmed that variant PLC-δ1 was expressed in the coronary artery.

PLC Activity

PLC enzymatic activities in the coronary artery, aorta, and mesenteric artery were compared among WT, heterozygous TG, and homozygous TG mice (Table 2). The activities in the coronary artery and the aorta were increased by 1.89±0.27 and 1.91±0.49 times, respectively, in heterozygous TG and by 2.57±0.13 and 2.41±0.48 times, respectively, in homozygous TG compared with WT mice (all P<0.05). PLC activities were higher in homozygous TG mice than in heterozygous TG mice (both P<0.05). In the mesenteric artery, the activity was slightly increased in homozygous TG compared with WT and heterozygous TG mice (both P<0.05).

ECG Changes After Ergometrine

Baseline heart rate, QRS duration, or PR interval was not statistically different among WT, heterozygous TG, and homozygous TG mice. As shown in Figure 3A, in an anesthetized homozygous TG mouse, intravenous injection of ergometrine at 50 mg/kg promptly induced ST-segment elevation (indicated by arrows) associated with PR-interval prolongation and without apparent change in the heart rate. ST elevation was induced in none of 22 WT (0%), 1 of 20 heterozygous TG (5%), and 6 of 18 homozygous TG mice (33%) by ergometrine at 15 mg/kg and in 3 of 22 WT (14%)

Table 1. Echocardiographic Analyses of Heart Dimension and Function in Phospholipase C-δ1–Overexpressing Transgenic and Wild-Type Mice

<table>
<thead>
<tr>
<th>Heart Dimension</th>
<th>Wild-Type Mice (n=10)</th>
<th>Homozygous Transgenic Mice (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interventricular septum, mm</td>
<td>0.72±0.09</td>
<td>0.68±0.06</td>
</tr>
<tr>
<td>LV posterior wall, mm</td>
<td>0.72±0.12</td>
<td>0.70±0.06</td>
</tr>
<tr>
<td>LV end-diastolic dimension, mm</td>
<td>3.78±0.15</td>
<td>3.68±0.18</td>
</tr>
<tr>
<td>LV fractional shortening, %</td>
<td>38.8±4.5</td>
<td>40.7±1.8</td>
</tr>
</tbody>
</table>

LV indicates left ventricular.

Table 2. Phospholipase C Activity in the Coronary Artery, Aorta, and Mesenteric Artery Obtained From Wild-Type Mice and Heterozygous and Homozygous Transgenic Mice

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Wild-Type Mouse</th>
<th>Heterozygous Transgenic Mouse</th>
<th>Homozygous Transgenic Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery (n=4)</td>
<td>0.32±0.08</td>
<td>0.61±0.09†</td>
<td>0.82±0.04†</td>
</tr>
<tr>
<td>Aorta (n=4)</td>
<td>0.41±0.12</td>
<td>0.68±0.21*</td>
<td>0.99±0.20†</td>
</tr>
<tr>
<td>Mesenteric artery (n=4)</td>
<td>0.58±0.04</td>
<td>0.57±0.06</td>
<td>0.99±0.20†</td>
</tr>
</tbody>
</table>

P<0.05 by 1-way ANOVA.

*P<0.05 vs wild-type mice.

†P<0.05 vs wild-type mice and heterozygous transgenic mice.
6 of 20 heterozygous TG (30%), and 17 of 18 homozygous TG mice (94%) by ergometrine at 50 mg/kg (Figure 3B). As shown in Figure 3C, ST-segment elevation after ergometrine was sometimes followed by complete or advanced atrioventricular block (0/22 in WT, 0/20 in heterozygous TG, and 3/18 in homozygous TG mice; \( P < 0.05 \)), leading to cardiac arrest.

Coronary Perfusion Pressure in Isolated Langendorff-Perfused Hearts

Effect of Ergometrine

Figure 5A shows representative tracings of coronary perfusion pressures before and after treatment with ergometrine at 1 \( \mu \text{mol/L} \). After ergometrine, coronary perfusion pressure was unchanged in WT mice, whereas it was abruptly elevated in homozygous TG mice. As shown in Figure 5B, coronary perfusion pressure at baseline was similar among WT (\( n = 8 \)), heterozygous TG (\( n = 7 \)), and homozygous TG mice (\( n = 11 \)). After ergometrine, coronary perfusion pressure was significantly increased in homozygous TG but not in either heterozygous TG or WT mice.

Effect of PGF\(_{2\alpha}\)

Figure 5C shows representative tracings of coronary perfusion pressures before and after treatment with PGF\(_{2\alpha}\) at
Coronary perfusion pressure was increased after PGF$_{2\alpha}$ to a similar degree in WT, heterozygous TG, and homozygous TG mice without any statistical difference (Figure 5D).

**Effect of Acetylcholine**

Coronary perfusion pressure was unchanged after acetylcholine in both WT and homozygous TG mice. In the presence of PGF$_{2\alpha}$, however, it was decreased after acetylcholine by 20±12 and 16±9 mm Hg in WT and homozygous TG mice (n=4 in each), respectively (P=NS, WT versus homozygous TG). Heart rate in WT and homozygous TG mice was unchanged after acetylcholine in the presence of PGF$_{2\alpha}$.

Nitric oxide metabolite levels in the perfusion solution were increased after acetylcholine at 1 μmol/L from 2.9±1.4 to 22.1±1.8 μmol/L in WT mice (P<0.05) and from 3.7±1.0 to 23.9±1.4 μmol/L in homozygous TG mice (P<0.05) (P=NS, WT versus homozygous TG).

**Responses of [Ca$^{2+}$]$_i$ and IP$_3$ to Acetylcholine and PLC Activity in Rat Smooth Muscle Cells Transfected With WT and Variant PLC-δ1**

Figure 6A illustrates the expression of muscarine M1 receptor and representative waveforms of [Ca$^{2+}$]$_i$ after acetylcholine administration in the cells transfected with human variant or WT PLC-δ1 in the presence or absence of extracellular Ca$^{2+}$. Muscarine M1 receptor was expressed in the cells transfected with its receptor cDNA but was barely detectable in those without transfection. Acetylcholine induced a biphasic increase in [Ca$^{2+}$]$_i$, consisting of a rapid transient increase followed by a lower but sustained phase (waveforms in black). A biphasic increase in [Ca$^{2+}$]$_i$ was greater in the cells transfected with variant PLC-δ1 than in those transfected with WT PLC-δ1 or without transfection. When acetylcholine was administered in Ca$^{2+}$-free buffer by the addition of EGTA, the transient increase in [Ca$^{2+}$]$_i$ was still greater in the cells transfected with variant PLC-δ1 than in those with WT PLC-δ1, but the sustained phase was abolished in both cells (waveforms in red). In the cells loaded with both fura-2 and BAPTA, acetylcholine failed to elevate [Ca$^{2+}$]$_i$.

[Ca$^{2+}$]$_i$ at baseline was elevated in the cells transfected with variant PLC-δ1 (126±9 nmol/L; n=4) and WT PLC-δ1 (109±7 nmol/L; n=4) compared with those without transfection (73±12 nmol/L; n=4) (both P<0.05) (Figure 6B). There was no difference in [Ca$^{2+}$]$_i$ at baseline between variant and WT PLC-δ1. The peak increase in [Ca$^{2+}$]$_i$ from the baseline value after acetylcholine at $10^{-5}$ mol/L was significantly greater in the cells transfected with variant PLC-δ1 (165±31 nmol/L; n=4) than in those transfected with WT PLC-δ1 (93±11 nmol/L;
n = 4) and those without transfection (59 ± 9 nmol/L; n = 4) (both P < 0.05) (Figure 6C).

PLC activity was increased by 2.99 ± 0.23 times in the cells transfected with variant PLC-δ1 and by 2.04 ± 0.15 times in those with WT PLC-δ1 compared with those without transfection (Figure 6D).

IP3 level (pg/mg protein) was increased at 15 seconds after acetylcholine at 10⁻⁵ mol/L from 70.4 ± 11.8 to 386.0 ± 75.3 in the cells with transfection of variant PLC-δ1 and from 50.2 ± 3.0 to 149.0 ± 18.4 in the cells without transfection (both P < 0.05). IP3 level after acetylcholine and the degree of change after acetylcholine from baseline (5.51 times versus 2.98 times) were both greater in the cells transfected with variant PLC-δ1 than in those without transfection (both P < 0.05).

Discussion

Major Findings

The coronary arteries in the present TG mice were stained by anti-PLC-δ1 antibody more strongly than those in WT mice with colocalization of α-SMA immunostaining, indicating increased PLC-δ1 expression in the coronary artery vascular smooth muscle cells of TG mice. PLC activity in the coronary artery was also found to be increased by 2.57 and 1.89 times in homozygous and heterozygous TG mice, respectively, compared with WT mice. The enhanced coronary vasomotility in TG mice was demonstrated by the following experiments: Intravenous ergometrine injection elicited ST-segment elevation in almost all TG mice, which was associated with atrioventricular block in some mice, whereas it did so only in a small number of heterozygous TG and WT mice; focal narrowing of the coronary artery was observed after ergometrine only in TG mice; and coronary perfusion pressure in the Langendorff-perfused hearts was abruptly increased after ergometrine only in homozygous TG mice and not in heterozygous TG or WT mice. Furthermore, cultured rat aortic smooth muscle cells transfected with variant PLC-δ1 showed enhanced [Ca²⁺]i response to acetylcholine compared with those transfected with WT PLC-δ1 and those with only endogenous PLC-δ1 in parallel with the levels of PLC activity. These findings strongly suggest the crucial role of enhanced PLC-δ1 activity in the pathogenesis of enhanced coronary vasomotility.

Generation of TG Mouse With Increased PLC-δ1 Enzymatic Activity

PLC-δ isozymes are more sensitive to Ca²⁺ than the other isoforms of PLC, and an increase in [Ca²⁺]i within the physiological range is sufficient to stimulate PLC-δ1. The initial transient increase in [Ca²⁺]i, induced by IP3, in turn contributes to the prolonged activation of PLC-δ1 in a positive-feedback fashion. We previously detected R257H mutation of PLC-δ1 in patients with CSA and showed that its enzymatic activity was significantly higher at the physiological [Ca²⁺]i than that of WT. We further showed that [Ca²⁺]i at baseline and at the peak increase from baseline...
after acetylcholine were both found to be greater in cells transfected with the variant PLC-δ1 than those in the WT.12 In the present study, acetylcholine induced a biphasic increase in \([\text{Ca}^{2+}]_i\), consisting of a rapid transient increase followed by a sustained phase, and in cells transfected with variant PLC-δ1, both transient increase in \([\text{Ca}^{2+}]_i\), and sustained phase were enhanced. When acetylcholine was added in \(\text{Ca}^{2+}\)-free buffer, the transient phase of \([\text{Ca}^{2+}]_i\), was still greater in the cells transfected with the variant PLC-δ1 than in those with the WT PLC-δ1, but the sustained phase was abolished in both cells. These findings suggest that PLC-δ1 enhanced not only IP3-mediated mobilization of intracellular \(\text{Ca}^{2+}\) store, but the following sustained phase, which is dependent on influx of extracellular \(\text{Ca}^{2+}\). Because A7r5 cells express voltage-gated calcium channels constitutively, the increase in the sustained phase of \([\text{Ca}^{2+}]_i\) by PLC-δ1 seems to be due to activation of the \(\text{Ca}^{2+}\) channels after a spike increase in \([\text{Ca}^{2+}]_i\). Thus, PLC-δ1 induces the extreme increase in \([\text{Ca}^{2+}]_i\), by IP3 followed by influx of extracellular \(\text{Ca}^{2+}\), as expected. \(\text{Ca}^{2+}\)-independent mechanisms via the diacylglycerol/protein kinase C pathway may also be involved in the action of PLC-δ1.

To generate PLC-δ1–overexpressing TG mice with increased PLC enzymatic activity, we used human R257H variant PLC-δ1 transgene rather than human WT PLC-δ1 transgene. This was because the activity of variant PLC-δ1 was higher than that of WT.12 In fact, PLC activity in the coronary artery of the homozygous and heterozygous TG mice was increased by 2.57 and 1.89 times, respectively, compared with WT mice. We reported previously that the activities of PLC in the cultured skin fibroblasts obtained from patients with CSA and from those without CSA but with coronary artery disease were enhanced by 2.68 and 1.38 times, respectively, compared with WT mice. We reported previously that the activities of PLC in the cultured skin fibroblasts obtained from patients with CSA and from those without CSA but with coronary artery disease were enhanced by 2.68 and 1.38 times, respectively, compared with WT mice. The present study demonstrated that PLC activity was increased not only IP3-mediated mobilization of intracellular \(\text{Ca}^{2+}\) store, but the following sustained phase, which is dependent on influx of extracellular \(\text{Ca}^{2+}\). Because A7r5 cells express voltage-gated calcium channels constitutively, the increase in the sustained phase of \([\text{Ca}^{2+}]_i\) by PLC-δ1 seems to be due to activation of the \(\text{Ca}^{2+}\) channels after a spike increase in \([\text{Ca}^{2+}]_i\). Thus, PLC-δ1 induces the extreme increase in \([\text{Ca}^{2+}]_i\), by IP3 followed by influx of extracellular \(\text{Ca}^{2+}\), as expected. \(\text{Ca}^{2+}\)-independent mechanisms via the diacylglycerol/protein kinase C pathway may also be involved in the action of PLC-δ1.

Enhanced Responses to Ergometrine in Homozygous TG Mice

Ergometrine is an ergot alkaloid that stimulates serotonergic receptors and triggers contraction of the vascular smooth muscle cells.17 This contraction is dependent on \(\text{Ca}^{2+}\) mobilization and not on \(\text{Ca}^{2+}\) sensitivity of the contractile elements.26 We used 3 methods to examine coronary vasomotility in response to ergometrine. First, we observed ECG changes after intravenous ergometrine administration. In the previous SUR2,18,21 and Kir6.1-null mouse models,19 ST-segment elevation was demonstrated spontaneously or after intravenous ergometrine, and atrioventricular block was observed in some animals. In the present study, intravenous ergometrine induced ST elevation in almost all homozygous TG mice, associated with atrioventricular block, but in only a few of heterozygous TG and WT mice. Second, the microvascular filling technique was used to visualize the coronary artery. Spontaneous focal coronary artery narrowing was demonstrated in SUR2-null mouse with the use of this technique.18,21 We also observed focal coronary artery narrowing after ergometrine only in homozygous TG and not in WT mice. Third, coronary artery perfusion pressure change after ergometrine was examined in isolated Langendorff-perfused hearts. A previous study in Kir6.1-null mouse revealed an abrupt increase in the perfusion pressure after administration of methylergometrine.19 The present study similarly demonstrated the abrupt increase in perfusion pressure after ergometrine only in homozygous TG mice and not in heterozygous TG and WT mice. Furthermore, we comparatively examined the effect of another vasoconstrictor, PGF2α, and showed a similar increase in pressure among homozygous and heterozygous TG and WT mice. It is known that PGF2α elicits vasoconstriction by an actin-associated mechanism for RhoA kinase activation, which is called \(\text{Ca}^{2+}\) sensitization.27 Thus, the enhanced response to ergometrine and not to PGF2α, in homozygous TG mice seems to be closely related to the increased PLC-δ1 activity, which contributes to the prolonged enhanced \(\text{Ca}^{2+}\) response in a positive-feedback fashion but not to \(\text{Ca}^{2+}\) sensitization. Thus, the present study clearly showed the enhanced responses to ergometrine in TG mice with increased PLC-δ1 enzymatic activity.

Pathogenesis of Coronary Vasospasm

The main feature of coronary spasm is enhanced contractility of vascular smooth muscle cells of the coronary artery, as shown in this model. Endothelial dysfunction due to reduced nitric oxide bioavailability or abnormal nitric oxide synthase has also been suggested as a genesis of coronary spasm. Because endothelial thickening is associated with supersensitivity to serotonin,26,28 vascular endothelial damage induced by environmental factors such as smoking, dyslipidemia, and genetic factors may be involved in coronary artery spasm. The mutation of endothelial nitric oxide synthase was reported to be related to coronary artery spasm.29 NADH/NADPH oxidase p22 phox gene 242C→T is a susceptibility locus for coronary artery spasm in men, as well as −1171/5A→6A in the stromelysin-1 gene and −634C→G in the interleukin-6 gene in women.30 With the use of the isolated Langendorff-perfused heart, the present study demonstrated no difference in acetylcholine-induced nitric oxide metabolite release to the coronary perfusion solution between homozygous TG and WT mice. Furthermore, the decrease in coronary perfusion pressure after acetylcholine in the presence of PGF2α was not different between homozygous TG and WT mice. These findings support the hypothesis that the enhanced response to ergometrine is independent of endothelial dysfunction. With regard to systemic peripheral arterioles, the elevation of arterial blood pressure in this model was modest, in contrast to the markedly enhanced response in the coronary artery. This may be related to the fact that the increase in PLC
activity in the mesenteric arteries of homozygous TG mice was modest.

The TG mice in the present study did not show early mortality or sudden cardiac death, unlike Kir6.1-null and SUR2-null mice, although enhanced coronary vasomotility, the most specific phenotype, was similar among the models. In addition, basal coronary perfusion pressure was not increased in mice in the present study, unlike the other mouse models. In the mice overexpressing dominant negative Kir6.1 pore-forming subunits in cardiac myocytes, increased mortality was noted after the age of 4 to 5 months despite no phenotype of coronary spasm. In the mice overexpressing dominant negative Kir6.1 pore-forming subunits in the endothelium, coronary spasm was not observed, but basal coronary perfusion pressure was elevated. Thus, there are diverse phenotypes in the specific molecule mutant-based spasm models, and there may be no close relationship between enhanced coronary vasomotility and sudden cardiac death or elevated basal coronary perfusion pressure.

Limitations
We compared the responses to ergometrine among homozygous and heterozygous TG mice and WT mice and did not compare responses between TG mice with variant PLC-δ1 and TG overexpressing WT human PLC-δ1. The quantitative comparison between the 2 homozygous TG mice overexpressing variant and WT human PLC-δ1 is difficult because of difficulty in controlling the expression levels of the inserted transgenes.22 We therefore compared the responses of [Ca2++]i to acetylcholine among cultured rat aortic smooth muscle cells only with endogenous rat PLC-δ1 and those transfected with human variant or WT PLC-δ1. The results demonstrated increased PLC activity in the cells transfected with variant PLC-δ1, followed by those with WT PLC-δ1, compared with those with no transfection but only with endogenous rat PLC-δ1. In the cells with variant PLC-δ1, the increased [Ca2+]i response to acetylcholine was also demonstrated compared with those with WT PLC-δ1, which is consistent with our previous findings in human embryonic kidney 293 cells transfected with variant and WT PLC-δ1.12

Conclusions
Increased PLC-δ1 activity found in homozygous TG mice in the present study causes enhanced coronary vasomotility such as that seen in patients with CSA.

Acknowledgments
We gratefully thank Tomoko Yoshida for her excellent technical support in the isolated Langendorff-perfused heart study.

Sources of Funding
This study was supported in part by grant-in-aid for scientific research 18590758 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to Dr Okumura and grant 18890012 from the Japan Society for the Promotion of Science to Dr Tomita.

Disclosures
None.

References
Coronary spasm is involved in the pathogenesis of not only variant angina, but the other acute coronary syndromes; however, its precise mechanism still remains unclear. We reported that phospholipase C-1 (PLC-1) activity in cultured skin fibroblasts obtained from patients with coronary spastic angina (CSA) was increased 3-fold compared with that from control subjects. We also detected R257H variant PLC-1 enzymatic activity. We further found that p122 protein, a positive regulator of PLC-1 activity caused by either structural mutation or increased positive regulator is likely to be related to the pathogenesis of coronary spasm. However, there has been no proof for a direct contribution of increased PLC-1 to enhanced coronary vasoconstrictor response to ergometrine in vascular smooth muscle cells by overexpressing human R257H variant PLC-1 in cultured skin fibroblasts obtained from patients with coronary spastic angina (CSA). In this study, we generated transgenic mice with increased PLC-1 activity in vascular smooth muscle cells by overexpressing human R257H variant PLC-1. Our transgenic mouse showed increased PLC enzymatic activity in the coronary artery and enhanced coronary vasoconstrictor response to ergometrine compared with that from wild-type mouse. In particular, intravenous ergometrine administration induced ST elevation on ECG and focal coronary artery narrowing such as that seen in CSA patients in the transgenic but not in the wild-type mouse. Thus, this transgenic mouse is a novel animal model of CSA and would provide a novel tool for revealing the mechanism for coronary spasm and investigating the treatment of CSA.
Coronary Vasospasm Induced in Transgenic Mouse With Increased Phospholipase C-δ1 Activity

Shuji Shibutani, Tomohiro Osanai, Toshihiro Ashitate, Shigeki Sagara, Kei Izumiyama, Yuko Yamamoto, Kenji Hanada, Takashi Echizen, Hirofumi Tomita, Takeshi Fujita, Takeshi Miwa, Hiroaki Matsubara, Yoshimi Homma and Ken Okumura

_Circulation._ 2012;125:1027-1036; originally published online January 20, 2012; doi: 10.1161/CIRCULATIONAHA.111.064303

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2012 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circ.ahajournals.org/content/125/8/1027

Data Supplement (unedited) at:

http://circ.ahajournals.org/content/suppl/2012/01/20/CIRCULATIONAHA.111.064303.DC1
Supplemental Table 1. Incidence of ST segment elevation after ergometrine in wild type (WT) and heterozygous and homozygous transgenic (TG) mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Ergometrine 15mg/kg</th>
<th>Ergometrine 50mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ST elevation (-) (+)</td>
<td>ST elevation (-) (+)</td>
</tr>
<tr>
<td>WT (n=17)</td>
<td>16 1</td>
<td>16 1</td>
</tr>
<tr>
<td>Heterozygous TG (n=13)</td>
<td>12 1</td>
<td>10 3</td>
</tr>
<tr>
<td>Homozygous TG (n=16)</td>
<td>12 4 *</td>
<td>0 16 *</td>
</tr>
</tbody>
</table>

* P<0.05 vs WT mice and Heterozygous TG mice