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

Running title: Shibutani et al.; Vasospasm in transgenic mouse with enhanced PLC-δ1 activity

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Journal Subject Code: [130] Animal models of human disease
Abstract:

*Background* - We reported that phospholipase C (PLC)-δ1 activity was enhanced by 3 times in patients with coronary spastic angina (CSA). 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 (HomoTG) and heterozygous TG (HeteroTG) compared with wild type mice (WT). ST elevation after ergometrine occurred in 17 of 18 HomoTG, 6 of 20 HeteroTG and 3 of 22 WT (p<0.01, HomoTG versus WT; p<0.05, HomoTG versus HeteroTG; p=NS, HeteroTG versus WT). ST elevation was associated with bradyarrhythmias in HomoTG. Focal coronary artery narrowing was documented with microvascular filling technique in 3 of 5 HomoTG after ergometrine but not in any of 7 WT (p<0.05). In the isolated Langendorff hearts, coronary perfusion pressure (CPP) was increased after ergometrine in HomoTG (p<0.01), but not in HeteroTG or WT. CPP increase after prostaglandin F₂α was similar among HomoTG, HeteroTG, and WT. Cultured rat aortic smooth muscle cells transfected with variant PLC-δ1 showed a higher PLC activity than those with wild PLC-δ1 (p<0.05), and further showed greater intracellular Ca²⁺ response to acetylcholine in variant than in wild PLC-δ1 (p<0.05).

*Conclusions* - Increased PLC-δ1 activity enhances coronary vasomotility such as seen in patients with CSA.

**Key words:** vasospasm, genetics, smooth muscle, calcium
Introduction

Coronary artery spasm plays an important role in the pathogenesis of Prinzmetal variant angina\textsuperscript{1,2} and the other acute coronary syndromes.\textsuperscript{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.\textsuperscript{5–7} In addition, the coronary artery constrictor response to diverse constrictor stimuli is enhanced,\textsuperscript{8} and occlusive constriction is readily induced when exposed to such a stimulus. Considering the fact that esophageal motility is enhanced in patients with coronary spastic angina (CSA),\textsuperscript{4} the presence of a generalized disorder of smooth muscle contraction is strongly suggested.

Phospholipase C (PLC), a key enzyme for vasoconstriction, produces inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol by hydrolyzing phosphatidyl inositol 4,5-bisphosphate (PIP\textsubscript{2}). IP\textsubscript{3} mobilizes Ca\textsuperscript{2+} from the intracellular stores and elicits rapid contraction of the vascular smooth muscle cells (VSMC),\textsuperscript{9} whereas diacylglycerol activates protein kinase C (PKC) and initiates sustained contraction by Ca\textsuperscript{2+}-independent mechanism.\textsuperscript{10} We previously demonstrated that PLC activity in cultured skin fibroblasts obtained from patients with CSA was enhanced, and a major PLC isozyme detected in the membrane fraction was the δ\textsubscript{1} isoform.\textsuperscript{11} We further demonstrated PLC-δ\textsubscript{1} 864 G to A mutation resulting in amino acid replacement of arginine 257 by histidine (R257H) in about 10% of the male CSA patients. This
variant PLC-δ1 showed an enhanced enzymatic activity in the physiological range of intracellular free calcium concentration ([Ca^{2+}]_i) compared with the wild type. More recently, we have reported that p122 protein, a positive regulator of PLC-δ1, is upregulated in patients with CSA, thereby causing an increased response of [Ca^{2+}]_i to acetylcholine. Since PLC-δ1 is more sensitive to Ca^{2+} than the other PLC isozymes, initial increase in [Ca^{2+}]_i, 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 direct contribution of the increased PLC-δ1 to the enhanced coronary vasomotility in patients with CSA. In the present study, we generated transgenic (TG) mice with an increased PLC-δ1 activity in VSMCs by overexpressing human R257H variant PLC-δ1 under control of the mouse δ-smooth muscle actin (δ-SMA) promoter. Comparing coronary vasoconstrictor responses among homozygous TG, heterozygous TG, and WT mice showing differential levels of the enzymatic activity (homozygous TG >heterozygous TG >WT mice), we tested the hypothesis that the increased PLC-δ1 activity causes the enhanced coronary vasomotility, leading to coronary spasm, in the mouse model.

**Materials and Methods**
Construction of vectors and generation of transgenic mice

The R257H variant PLC-δ1 complementary DNA (cDNA) was subcloned into the plasmid pBsKS(-) including a 4.7-kb fragment of the mouse α-SMA promoter. This promoter induces a pattern of transgene expression similar to that of endogenous VSMC-specific α-actin, the dominant α-actin isoform in the tissue. The resultant recombinant construct was digested with EcoRI and NotI to generate ~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.14-16 Two lines of homozygous TG mice were produced. Since they had similar phenotype and responses to stimuli, the data of one line of TG mice that manifested greater expression of variant PLC-δ1 were shown in the following sections (see Supplemental Table 1 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 PCR

Total RNA was extracted from homogenized descending aorta using TRIzol reagent.
according to the protocol of the manufacturer (Qiagen, Valencia, CA), and RNA pellets were resuspended in diethylpyrocarbonate-treated water. cDNA was synthesized using 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’-GGATCCACTAGTATGG ACTCGGGCCGGGACTT-3’, reverse: 5’-AACTGATCCACCGACAGAGT-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 dithiothreitol, and 0.5 mmol/L phenylmethyl-sulfonyl fluoride). The protein was separated by SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). After blocking for one 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 the enhanced chemiluminescence plus detection systems (Amersham Pharmacia Biotech, Piscataway, 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 using 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-N’-2-ethanesulfonic acid (50 mmol/L), CaCl₂ (0.1 mmol/L), sodium cholate (9 mmol/L), ⁴H-PIP₂ (40,000 cpm) and the cell protein (20 μg). The reaction was stopped with chloroform/methanol/hydrogen chloride (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 short axis view of the left ventricle (LV)
using a PHILIPS HD11 XE and a 15MHz 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 wall thickness (PWT) were measured and the data from at least 3 cardiac cycles were averaged. LV fractional shortening (LVFS, %) was calculated as [(LVEDD-LVESD)/LVEDD] x 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 DMSO and diluted with H2O), which stimulates 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 a potential to induce coronary spasm in the 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 the 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 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 analyzed for presence or absence of focal narrowing by the investigators blinded
to the genotype.

**Coronary perfusion pressure in isolated Langendorff-perfused hearts**

Homozygous and heterozygous TG and WT mice were heparinized (0.5U/g body
weight) and anesthetized with 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
(Psysio-tech, Tokyo, Japan) which 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 using a standard
Langendorff setup (Psysio-tech, Tokyo, Japan). Coronary perfusion pressure was continuously
recorded using a pressure-sensing catheter (ADInstruments, 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 into 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 (ACh) was given into the perfusion solution at the final concentration of 1 μmol/L in the presence or absence of PGF₂α. The level of nitric oxide metabolite (NOx) in the perfusion solution was measured by Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI).

**Measurement of [Ca²⁺]ᵢ and IP₃ in A7r5 cells**

A7r5 aortic smooth muscle cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. The cells were subcultured on the coverslips in 12-well plate, and then transfected with both muscarine M1 receptor cDNA (a gift from Dr Tomohiro Kurosaki at Kansai Medical University, Osaka, Japan) and either the human wild type PLC-δ1 or variant PLC-δ1 or empty vector (all, 0.3 μg DNA/well). PLC activities in the three transfected cell groups were measured as described above. After loading with 5 μmol/L fura-2–acetoxymethyl ester, [Ca²⁺]ᵢ in response to ACh at 10⁻⁵ mol/L was measured at excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm as described previously. [Ca²⁺]ᵢ in response to ACh was also measured in the presence of 10 μmol/L BAPTA-acetoxymethyl ester (a high affinity Ca²⁺ chelator).

Intracellular IP₃ concentrations were measured by IP₃ enzyme-linked immunosorbent assay.
(ELISA) kit (Cusabio Biotech Co., Ltd., Wuhan, China). The expression of muscarine M1 receptor was confirmed by Western blot analysis using the primary antibody for muscarine M1 receptor and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). ACh was used since we found it could induce [Ca\(^{2+}\)]\(_i\) response in A7r5 cells\(^{13}\) and it is widely used for the induction of coronary spasm in patients in Japan.\(^{11}\)

**Statistical analysis**

All continuous data are shown as mean±SD. A student’s t-test was used to compare mean values where appropriate. Differences in proportions were analyzed by chi-square test or Fisher’s exact probability test. For the analysis of coronary perfusion pressure, the paired t-test was used. For the analyses of ECG study and [Ca\(^{2+}\)]\(_i\) responses to ACh in cultured cells, one-way ANOVA followed by Bonferroni multiple comparison tests was used. p<0.05 was considered to indicate a statistical significance.

**Results**

**Phenotype of TG mice at baseline**

Two founders containing the variant PLC-δ1 transgene were identified by PCR 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 vs 105±6 mmHg, p<0.001). Echocardiographic study demonstrated that either of LVFS, LVESD, LVEDD, or PWT was not statistically different between TG mice and WT mice (Table 1).

**Expression of variant PLC-δ1 in TG mice**

The bands of RT-PCR 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 kD and slightly at 85 kD in the aorta from WT mice (left lane), whereas the human variant PLC-δ1 protein was superimposed at 85 kD in TG mice (right lane).

The protein expression of PLC-δ1 was increased by 2 times in the aorta obtained from homozygous TG mice compared with that from WT (Figure 1E).

Figure 2 illustrates immunofluorescence microscopy of the 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. Co-staining 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 respectively (both p<0.05).

**ECG changes after ergometrine**

Either of 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) being 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%), 6 of 20 heterozygous TG (30%) and 17 of 18 homozygous TG mice (94%) by 50 mg/kg (Figure 3B). As shown in Figure 3C, ST segment elevation after ergometrine was sometimes followed by complete or advanced atrioventricular (AV) block (0/22 in WT, 0/20 in heterozygous TG, and 3/18 in homozygous TG, p<0.05), leading to cardiac arrest.

**Coronary artery narrowing demonstrated by microvascular filling study**

Figure 4 shows the representative photographs of the coronary arteries of WT and homozygous TG mice treated with vehicle (upper panels) and ergometrine at 50 mg/kg (lower panels). In the group treated with vehicle, no focal spasm was observed in any portions of the artery in either WT or TG mice. In the group treated with ergometrine, focal narrowing of the coronary artery was documented in 3 of 5 TG mice, but none in 7 WT mice (p<0.05).

**Coronary perfusion pressure in isolated Langendorff-perfused hearts**

Effect of ergometrine: Figure 5A shows the representative tracings of coronary perfusion pressures before and after treatment with ergometrine at 1 μmol/L. After ergometrine, coronary perfusion pressure was unchanged in WT mice, while 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 was not in either heterozygous TG or WT mice.

Effect of PGF$_{2\alpha}$: Figure 5C shows the representative tracings of coronary perfusion pressures before and after treatment with PGF$_{2\alpha}$ at 10 µmol/L. 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 ACh: Coronary perfusion pressure was unchanged after ACh in both WT and homozygous TG mice. In the presence of PGF$_{2\alpha}$, however, it was decreased after ACh by 20±12 and 16±9 mmHg 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 ACh in the presence of PGF$_{2\alpha}$.

NOx levels in the perfusion solution were increased after ACh at 1 µmol/L from 2.9±1.4 µmol/L to 22.1±1.8 µmol/L in WT mice (p<0.05) and from 3.7±1.0 µmol/L 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 Ach, and PLC activity in rat smooth muscle cells transfected with wild type and variant PLC-δ 1

Figure 6A illustrates the expression of muscarine M1 receptor, and the representative
waveforms of \([\text{Ca}^{2+}]_i\), after ACh administration in the cells transfected with human variant or wild type PLC-\(\delta\) 1 in the presence or absence of extracellular \(\text{Ca}^{2+}\). Muscarine M1 receptor was expressed in the cells transfected with its receptor cDNA, but was barely detectable in those without transfection. ACh induced a biphasic increase in \([\text{Ca}^{2+}]_i\), consisting of a rapid transient increase followed by a lower but sustained phase (waveforms in black). A biphasic increase in \([\text{Ca}^{2+}]_i\) was greater in the cells transfected with variant PLC-\(\delta\) 1 than that in the cells transfected with wild type PLC-\(\delta\) 1 or without transfection. When ACh was administered in \(\text{Ca}^{2+}\)-free buffer by the addition of EGTA, the transient increase in \([\text{Ca}^{2+}]_i\) was still greater in the cells transfected with variant PLC-\(\delta\) 1 than in those with wild type PLC-\(\delta\) 1, but the sustained phase was abolished in both cells (waveforms in red). In the cells loaded with both fura-2 and BAPTA, ACh failed to elevate \([\text{Ca}^{2+}]_i\).

\([\text{Ca}^{2+}]_i\) at baseline was elevated in the cells transfected with variant PLC-\(\delta\) 1 (126±9 nmol/L, n=4) and wild type PLC-\(\delta\) 1 (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 \([\text{Ca}^{2+}]_i\) at baseline between variant and wild type PLC-\(\delta\) 1. The peak increase in \([\text{Ca}^{2+}]_i\) from the baseline value after ACh at \(10^{-5}\) mol/L was significantly greater in the cells transfected with variant PLC-\(\delta\) 1 (165±31 nmol/L, n=4) than in those transfected with wild type PLC-\(\delta\) 1 (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 wild PLC-δ₁ compared with those without transfection (Figure 6D).

IP₃ level (pg/mg protein) was increased at 15 seconds after ACh 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 ACh and the degree of change after ACh from the 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 co-localization of α-SMA immunostaining, indicating the increased PLC-δ₁ expression in the coronary artery VSMCs 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, being associated with
AV block in some mice, while it did 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. Further, cultured rat aortic smooth muscle cells transfected with variant PLC-δ 1 showed enhanced [Ca^{2+}]_i response to ACh compared with those transfected with wild type PLC-δ 1 and those only with endogenous PLC-δ 1 in parallel with the levels of PLC activity. These findings strongly suggest the crucial role of the enhanced PLC-δ 1 activity in the pathogenesis of enhanced coronary vasomotility.

**Generation of TG mouse with increased PLC-δ 1 enzymatic activity**

PLC-δ isoforms are more sensitive to Ca^{2+} than the other ones, and an increase in [Ca^{2+}], within the physiological range is sufficient to stimulate PLC-δ 1. The initial transient increase in [Ca^{2+}]_i induced by IP3 in turn contributes to the prolonged activation of PLC-δ 1 in a positive-feedback fashion. We previously detected R257H mutation of PLC-δ 1 in patients with CSA, and showed that its enzymatic activity was significantly higher at the physiological [Ca^{2+}]_i than that of the wild type. We further showed that [Ca^{2+}]_i at baseline and the peak increase from the baseline after ACh were found to be both greater in the cells transfected with the variant PLC-δ 1 than those in the wild type. In the present study, ACh induced a biphasic
increase in [Ca^{2+}], consisting of a rapid transient increase followed by a sustained phase, and in the cells transfected with variant PLC-δ1, both transient increase in [Ca^{2+}], and sustained phase were enhanced. When ACh was added in Ca^{2+}-free buffer, the transient phase of [Ca^{2+}], was still greater in the cells transfected with the variant PLC-δ1 than those with the wild type PLC-δ1, but the sustained phase was abolished in both cells. These suggest that PLC-δ1 enhanced not only IP$_3$-mediated mobilization of intracellular Ca$^{2+}$ store but the following sustained phase which is dependent on influx of extracellular Ca$^{2+}$. Since A7r5 cells express voltage-gated calcium channels constitutively, the increase in the sustained phase of [Ca$^{2+}$] by PLC-δ1 seems to be due to activation of the Ca$^{2+}$ channels following a spike increase in [Ca$^{2+}$]. Thus, PLC-δ1 induces the extreme increase in [Ca$^{2+}$] by IP$_3$ followed by influx of extracellular Ca$^{2+}$ as expected. Ca$^{2+}$-independent mechanisms via diacylglycerol-PKC pathway may also be involved in the action of PLC-δ1.

To generate PLC-δ1-overexpressing TG mice with the increased PLC enzymatic activity, we used human R257H variant PLC-δ1 transgene rather than human wild type PLC-δ1 transgene. This was because the activity of variant PLC-δ1 was higher than that of wild type.$^{12}$ 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 previously reported that the activity of PLC in the cultured skin fibroblasts obtained from patients with CSA
was enhanced by 2.68 times compared with that from control subjects, and that from patients without CSA but with coronary artery disease by 1.38 times. Thus, the increased PLC activity found in homozygous TG mice is relevant to the activity in patients with CSA. The modest increase in PLC activity in heterozygous TG mice may be relevant to that in patients without CSA but with coronary artery disease. Further, the heterozygous and homozygous TG mice enable us to perform the experiments with three different levels of PLC activity (homozygous TG > heterozygous TG > WT mice). Therefore, our TG mice are a novel animal model to help understanding the role of PLC-δ1 in the enhanced coronary vasomotility seen in the CSA patients.

Enhanced responses to ergometrine in homozygous TG mice

Ergometrine is an ergot alkaloid that stimulates serotonergic receptors and triggers contraction of the VSMC. This contraction is dependent on Ca²⁺ mobilization and not on Ca²⁺ sensitivity of the contractile elements. We used three methods to examine the coronary vasomotility to ergometrine. First, we observed the ECG changes after intravenous ergometrine administration. In the previous SUR2 and Kir6.1-null mouse models, ST segment elevation was demonstrated spontaneously or after intravenous ergometrine, and in some animals, AV block was observed. In the present study, intravenous ergometrine induced ST elevation in almost all homozygous TG mice, being associated with AV 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 using this technique.\textsuperscript{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 the isolated Langendorff-perfused hearts. A previous study on Kir6.1-null mice revealed an abrupt increase in the perfusion pressure after administration of methylergometrine.\textsuperscript{19} The present study similarly demonstrated the abrupt increase in the perfusion pressure after ergometrine only in homozygous TG mice and not in heterozygous TG and WT mice. Further, we comparatively examined the effect of another vasoconstrictor PGF\textsubscript{2a}, and showed a similar increase in the pressure among homozygous and heterozygous TG and WT mice. It is known that PGF\textsubscript{2} elicits vasoconstriction by an actin-associated mechanism for RhoA kinase activation, which is called as Ca\textsuperscript{2+} sensitization.\textsuperscript{27}

Thus, the enhanced response to ergometrine and not to PGF\textsubscript{2a} in homozygous TG mice seems to be closely related to the increased PLC-\(\delta\) 1 activity which contributes to the prolonged enhanced Ca\textsuperscript{2+} response in a positive-feedback fashion, but not to Ca\textsuperscript{2+} sensitization. Thus, the present study clearly showed the enhanced responses to ergometrine in TG mice with the increased PLC-\(\delta\) 1 enzymatic activity.

**Pathogenesis of coronary vasospasm**
The main feature of coronary spasm is the enhanced contractility of VSMC of the coronary artery, as shown in this model. Endothelial dysfunction due to reduced NO bioavailability or abnormal NO synthase has been also suggested as a genesis of coronary spasm. Since 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 concerned with coronary artery spasm. The mutation of endothelial NO 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, and -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 ACh-induced NOx release to the coronary perfusion solution between homozygous TG and WT mice. Further, the decrease in coronary perfusion pressure after ACh in the presence of PGF\(_{2\alpha}\) was not difference between homozygous TG and WT mice. These support that the enhanced responses 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 present TG mice did not show early mortality or sudden cardiac death unlike Kir6.1-null\textsuperscript{19} and SUR2-null mice,\textsuperscript{18,21} although the enhanced coronary vasomotility, the most specific phenotype, was similar among the models. Also, basal coronary perfusion pressure was not increased in the present mice unlike the other mouse models.\textsuperscript{18,19} In the mice overexpressing dominant negative Kir6.x pore-forming subunits in cardiac myocytes, an increased mortality was noted after the age of 4-5 months despite no phenotype of coronary spasm.\textsuperscript{31} In the mice overexpressing dominant negative Kir6.x pore-forming subunits in the endothelium, coronary spasm was not observed, but basal coronary perfusion pressure was elevated.\textsuperscript{20} Thus, there are diverse phenotypes in the specific molecule mutant-based spasm models, and there may be no close relationship between the 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 between TG mice with variant PLC- δ 1 and TG overexpressing wild type human PLC- δ 1. The quantitative comparison between the two homozygous TG mice overexpressing variant and wild type human PLC- δ 1 is very difficult because of the difficulty in controlling the expression levels of the inserted transgenes.\textsuperscript{32} We therefore compared the responses of $[Ca^{2+}]_b$ to ACh among cultured rat aortic smooth muscle cells only with the
endogenous rat PLC-δ 1 and those transfected with human variant or wild type PLC-δ 1. The results demonstrated the increased PLC activity in the cells transfected with variant PLC-δ 1 followed by those with wild type PLC-δ 1 compared with those with no transfection but only with the endogenous rat PLC-δ 1. In the cells with variant PLC-δ 1, the increased [Ca^{2+}]_{i} response to ACh was also demonstrated compared with those with wild type PLC-δ 1, being consistent with our previous findings in human embryonic kidney 293 cells transfected with variant and wild type PLC-δ 1.\textsuperscript{12}

**Conclusions**

Increased PLC-δ 1 activity found in the present homozygous TG mice causes the enhanced coronary vasomotility such as seen in patients with CSA.

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

**Funding Sources:** This study was partly supported by Grant-in-Aid for Scientific Research 18590758 from the Ministry of Education, Culture, Sports, Science and Technology of Japan to Dr. Okumura, and 18890012 from the Japan Society for the Promotion of Science to Dr. Tomita.

**Conflict of Interest Disclosures:** None
References:


Table 1. Echocardiographic analyses of heart dimension and function in phospholipase C (PLC)-d1 overexpressing transgenic (TG) and wild type (WT) mice

<table>
<thead>
<tr>
<th></th>
<th>WT mice (n=10)</th>
<th>Homozygous TG mice (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS (mm)</td>
<td>0.72 ± 0.09</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.72 ± 0.12</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.78 ± 0.15</td>
<td>3.68 ± 0.18</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>38.8 ± 4.5</td>
<td>40.7 ± 1.8</td>
</tr>
</tbody>
</table>

IVS, interventricular septum; LVPW, left ventricular posterior wall; LVEDD, left ventricular end-diastolic dimension; LVFS, left ventricular fractional shortening

Table 2. Phospholipase C (PLC) activity in the coronary artery, the aorta, and the mesenteric artery obtained from wild type (WT) mice and heterozygous and homozygous transgenic (TG) mice

<table>
<thead>
<tr>
<th>Tissues</th>
<th>PLC activity (pmol/mg protein/min)</th>
<th>WT mice</th>
<th>Heterozygous TG mice</th>
<th>Homozygous TG mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary A. (n=4)</td>
<td>0.32 ± 0.08</td>
<td>0.61 ± 0.09*</td>
<td>0.82 ± 0.04**</td>
<td></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>
<td></td>
</tr>
<tr>
<td>Mesenteric A. (n=4)</td>
<td>0.58 ± 0.04</td>
<td>0.57 ± 0.06</td>
<td>0.99 ± 0.20**</td>
<td></td>
</tr>
</tbody>
</table>

A: Artery P<0.05 by one way ANOVA
* P<0.05 vs WT mice
** P<0.05 vs WT mice and Heterozygous TG mice

Figure Legends:

Figure 1. Characteristics of the human variant phospholipase C (PLC)-δ1-overexpressing transgenic (TG) mice. A, Schematic map of the α-smooth muscle actin-human R257H variant PLC-δ1 transgenic construct. B, RT-PCR performed on total mRNA in the aorta isolated from the
R257H PLC-δ1 TG mice and wild type (WT) mice. C in the panel B indicates a positive control of transgene positive genomic DNA. PCR was conducted using primers specific to the human R257H variant PLC-δ1 transgene to yield the expected product of 710 base pairs. C, The gene expression of human variant PLC- δ1 mRNA in TG mice. D, Protein expression of human variant PLCδ1 in the aorta. E, Comparison of PLC- δ1 protein expression between WT and TG mice.

**Figure 2.** Immunofluorescence microscopy of the heart sections containing the large (upper panels) and small (lower panels) coronary arteries. The antibodies used are indicated at the top of the columns. Left panels show staining with an anti- phospholipase C (PLC)-δ1 antibody demonstrating the increase in PLC-δ1 immunoreactivity in TG mice (red). Middle panels show anti-smooth muscle actin reactivity to mark vascular smooth muscle cells (green). Right panels show merged images.

**Figure 3.** Representative ECG tracings and responses to ergometrine in wild type (WT) and transgenic (TG) mice. A, Representative tracings of ECG before (left) and after (right) intravenous administration of ergometrine in anesthetized TG mouse. Intravenous injection of ergometrine elicited ST segment elevation. B, Incidence of ST segment elevation after ergometrine in WT and heterozygous and homozygous TG mice. C, ECG showing atrioventricular (AV) block and bradycardia seen in TG mice. The arrows indicate P waves.

**Figure 4.** Microvascular filling with Microfil of the coronary arteries of wild type (WT) and homozygous transgenic (TG) mice. Hearts were perfused with microfil latex compound, fixed,
dehydrated, and cleared with methylsalicylate. Focal narrowing (arrows) in the coronary artery after injection of ergometrine was observed in TG but not in WT mice.

**Figure 5.** Coronary perfusion pressures measured in isolated Langendorff hearts of wild type (WT) and homozygous and heterozygous transgenic (TG) mice. **A**, Representative traces of coronary perfusion pressures before and after injection of ergometrine at 1 μmol/L in WT (left) and homozygous TG (right) mice. **B**, Change in coronary perfusion pressure before (open bar) and after (closed bar) injection of ergometrine at 1 μmol/L in WT and heterozygous and homozygous TG mice. **C**, Representative traces of coronary perfusion pressures before and after injection of prostaglandin F2α (PGF2α) at 10 μmol/L in WT (left) and homozygous TG (right) mice. **D**, Change in coronary perfusion pressures before (open bar) and after (closed bar) injection of PGF2α at 10 μmol/L in WT and heterozygous and homozygous TG mice.

**Figure 6.** [Ca^{2+}]_i responses to acetylcholine (ACh) in cultured rat aortic smooth muscle cells (A7r5 cells) only with endogenous phospholipase C (PLC)-δ1 and transfected with human variant or wild type PLC-δ1. **A**, The representative waveforms of [Ca^{2+}]_i in response to ACh and the expression of muscarine M1 receptor. **B** and **C**, Baseline [Ca^{2+}]_i and peak increase in [Ca^{2+}]_i from the baseline after 10^{-5}M ACh, respectively, in the 3 cell groups. **D**, PLC activities in the 3 cell groups.
A

Baseline

After ergometrine

Lead II

PQ prolongation

ST elevation

B

Incidence of ST elevation (%)

WT Heterozygous Homozygous

p<0.05

Heterozygous TG

Homzygous TG

p<0.01

Ergometrine 15mg/kg

Ergometrine 50mg/kg

C

Complete AV block

Lead II
Coronary Vasospasm Induced in Transgenic Mouse with the 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.* published online January 20, 2012;
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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

* P<0.05 vs WT mice and Heterozygous TG mice