The molecular mechanism of coronary spasm in iPSC-derived smooth muscle cells: Role of p122 RhoGAP/DLC-1 and phospholipase C-δ1

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Research Article

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Abstract

Background: We previously reported that the activity of phospholipase C-δ1 and the expression of p122RhoGAP/ deleted in liver cancer-1 (p122RhoGAP/DLC-1) were enhanced in skin fibroblasts obtained from patients with coronary spastic angina (CSA). Overexpression of p122RhoGAP/DLC-1 increased intracellular free calcium concentration ([Ca^{2+}]) in response to acetylcholine (ACh). We generated transgenic mice overexpressing vascular smooth muscle-specific p122RhoGAP/DLC-1 (p122-TG) and showed that coronary spasm was induced by ergometrine infusion.

Methods: To examine Ca^{2+} signaling in coronary arteries, we generated induced pluripotent stem cells (iPSCs) from skin fibroblasts of CSA patients (n = 3) and control subjects (n = 3), and differentiated the iPSCs into vascular smooth muscle cells (VSMCs).

Results: We identified the molecular mechanism for enhanced Ca^{2+} signaling involved in the pathogenesis of CSA using iPSC-derived VSMCs. The concentration of [Ca^{2+}]_{i} after ACh stimulation at 10^{-4} M was 53 ± 10 nM in the control iPSC-derived VSMCs and 129 ± 24 nM in the CSA iPSC-derived VSMCs (P < 0.05). Diltiazem hydrochloride (voltage gated Ca^{2+} channel blocker) or xestospongin C [inositol-1,4,5-trisphosphate receptor (IP_{3}R) antagonist] blocked the ACh-induced increase of [Ca^{2+}]_{i} in CSA iPSC-derived VSMCs. Western blot analysis revealed that p122RhoGAP/DLC-1 and IP_{3}R1 protein levels were increased by 2.53 ± 0.88 and 7.53 ± 3.78 times, respectively, in CSA iPSC-derived VSMCs compared with control iPSC-derived VSMCs (all P < 0.05). PLC-δ1 activity in the whole cell was significantly higher (3.12 ± 1.28 times) in CSA iPSC-derived VSMCs compared with control iPSC-derived VSMCs (P < 0.05).

Conclusions: Enhanced PLC-δ1 activity contributes to increased [Ca^{2+}]_{i} in response to ACh in CSA patients concomitantly with enhanced p122RhoGAP/DLC-1 and IP_{3}R1 protein expression.

Background

Coronary artery spasm is defined as abnormal contraction of the epicardial coronary arteries that causes myocardial ischemia. It plays an important role not only in coronary spastic angina (CSA) [1, 2], but also in the pathophysiology of other ischemic heart diseases [3]. Studies have confirmed that CSA enhances the basic vasomotor tone of coronary arteries and the contractile response to various stimuli [4, 5, 6, 7]. These findings suggest that intracellular Ca^{2+} signaling and/or post-receptor mechanisms are involved in the overactivity of vascular smooth muscle cells (VSMC) [8].

We previously demonstrated that in cultured skin fibroblasts from CSA patients, the activity of phospholipase C (PLC)-δ1, an important enzyme in Ca^{2+} signaling, was increased 3-fold and positively correlated with coronary vasomotility [9]. PLC-δ1 is an isoform of PLC, and is more sensitive to Ca^{2+} compared with the other isoforms. Thus, the initial increase in Ca^{2+} induced by G protein-linked PLC-β induces long-term activation of PLC-δ1 in a positive feedback manner [10]. A single base mutation (864G-
A) in the PLC-δ1 gene, in which arginine 257 is replaced with histidine, was observed in 10% of male CSA patients, which indicates enhanced enzyme activity [11]. To further demonstrate a causative role for enhanced PLC-δ1 activity in cutaneous fibroblasts from CSA patients, transgenic mice (PLC-TG) overexpressing mutant PLC-δ1 in a VSMC-specific manner were generated and we confirmed ergometrine-induced coronary spasm in this PLC-TG mouse model [12].

We also showed that p122RhoGTPase-activating protein/deleted in liver cancer-1 (p122RhoGAP/DLC-1) protein, a positive regulator of PLC-δ1, is upregulated in skin fibroblasts of CSA patients [13]. We generated transgenic mice (p122-TG) that overexpressed p122RhoGAP/DLC-1 specifically for VSMCs. Coronary spasm was induced in these p122-TG mice following ergometrine injection [14]. Recently, we discovered that p122RhoGAP/DLC-1 forms a complex with IQ motif-containing GTPase-activating protein 1 (IQGAP1) and PLC-δ1, which move along microtubules and enhance PLC activity [15]. Recently, p122RhoGAP/DLC-1 was cloned by screening a rat brain expression library with antiserum derived from purified PLC-δ1 and it was identified as a bifunctional molecule consisting of 1083 amino acids [16, 17]. One function is its interaction with PLC-δ1 and the enhancement of its ability to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2). The other is a GAP activity specific for Rho [10]. Overall, we demonstrated that enhanced PLC-δ1 and p122RhoGAP/DLC-1, both of which were identified in human skin fibroblasts from CSA patients, may be attributable to human coronary spasm. However, the underlying molecular mechanism of coronary spasm in humans remains unclear, because experimental data have been derived from the human skin fibroblasts rather than VSMCs. We hypothesize that PLC-δ1, p122RhoGAP/DLC-1, and IQGAP1 enhance coronary artery spasm and are involved in Ca2+ signaling similar to the results of previous studies using skin fibroblasts. We generated induced pluripotent stem cells (iPSCs) from the skin fibroblasts of CSA patients and control subjects and differentiated these iPSCs into VSMCs. Using these VSMCs, we examined the molecular mechanism for enhanced Ca2+ signaling in the pathogenesis of CSA, focusing on the role of PLC-δ1, p122RhoGAP/DLC-1, IQGAP1, and their downstream molecules, such as voltage-dependent Ca2+ channels, associated with Ca2+ influx.

**Methods**

**Study patients**

The ethics committee of our institution approved the study protocol (2013-086). Written informed consent was obtained from all patients prior to the study. The study population included three Japanese patients with CSA who suffered from severe chest pain at rest (one man and two women with a mean age of 63 ± 6 years) and three control subjects without hypertension or any history suggestive of angina pectoris (two men and one woman with a mean age of 68 ± 12 years). A coronary arteriographic study was performed in all patients by cardiologists at our hospital. Coronary spasm, defined as the total or subtotal occlusion or severe vasoconstriction of the coronary artery (99% stenosis, subtotal occlusion; >90% stenosis, severe vasoconstriction) associated with chest pain and ischemic ECG change, was induced by intracoronary injection of acetylcholine (ACh) in all CSA patients. After intracoronary injection of isosorbide dinitrate,
coronary arteriograms revealed normal or almost normal coronary arteries with a stenosis diameter of ≤ 50% of the lumen diameter of all patients.

**iPSC generation**

Human skin fibroblasts were prepared by an explant method as described previously [9]. They were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in an environment of 5% CO₂ and 95% air. Episomal vectors (pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL) were obtained from Addgene (Cambridge, MA, USA) and were introduced into the fibroblasts. These episomal vectors (1 µg of each) were electroporated into 6 × 10⁵ fibroblasts with the Neon Transfection System (Thermo Fisher Scientific) using a 100 µl kit according to the manufacturer's instructions (conditions for electroporation: pulse voltage: 1650 V, pulse width: 10 ms, pulse number: 3) [18]. Following electroporation, the cells were maintained in fibroblast medium for 6 days, which was replaced with embryonic stem cell (ESC) medium consisting of DMEM/HAM F-12 (Thermo Fisher Scientific) supplemented with 20% KnockOut Serum Replacement, 1% non-essential amino acids (Invitrogen), 1 mM L-Glutamine (Thermo Fisher Scientific), 0.1 mM β-mercaptoethanol (Thermo Fisher Scientific), and 4 ng/ml human recombinant basic fibroblast growth factor (bFGF) (R&D Systems, MN, USA) for reprogramming. The medium was changed daily until iPSC colonies were formed.

**Characterization of human iPSC colonies and iPSC-derived VSMCs**

Alkaline phosphatase staining (APS) was carried out using the Leukocyte Alkaline Phosphatase kit (86-R; Sigma-Aldrich, MO, USA). For immunocytochemistry, human iPSCs or iPSC-derived VSMCs were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with phosphate buffered saline (PBS) (Thermo Fisher Scientific), the cells were treated with 0.1% Triton X-100 for 10 min at room temperature. The cells were then treated with PBS containing 5% normal horse serum (26050-070; Thermo Fisher Scientific). The following primary antibodies were used: stage-specific embryonic antigen-4 (SSEA-4, 21704, Santa Cruz Biotechnology, TX, USA), TRA-1-60 (21750, Santa Cruz Biotechnology), alpha smooth muscle actin (α-SMA, ab5694; Abcam, Cambridge, UK), and calponin (ab46794; Abcam). The following secondary antibodies were used: FITC-goat anti-rabbit IgG (Thermo Fisher Scientific) and Texas Red-X goat anti-rabbit IgG (Thermo Fisher Scientific).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized from 2.5 µg of RNA with SuperScript VILO Master Mix (11755050; Thermo Fisher Scientific) in a 20 µl reaction volume. The cDNA samples were subjected to PCR amplification with primers specific to human VSMC genes (Table 1).
Table 1.
Primers used for RT-PCR

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<th>Gene</th>
<th>Primer sequence</th>
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<td></td>
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Induction of differentiation of human iPSCs into VSMCs

iPSCs were differentiated into VSMCs using previously published protocols [19, 20, 21]. Briefly, human iPSCs were manually lifted, cut, and transferred to an ultra-low attachment dish (Corning, Corning, NY, USA) to generate floating embryoid bodies (EBs). The EBs were cultured in human ESC culture medium without bFGF supplement. Six-day-old EBs were transplanted into 60-mm dishes pre-coated with 0.1% gelatin and cultured for an additional 6 days in DMEM containing 10% FBS. The cells from the EBs were then dissociated by digestion with TrypLE Express (Thermo Fisher Scientific). In addition, they were cultured on iMatrix-551 (rLN511E) (Nippi, Tokyo, Japan)-coated dishes in complete smooth muscle growth medium 2 (SmGM2, Lonza, Walkersville, MD, USA) until the cells reached 80–90% confluence. To differentiate the cells into more mature VSMCs, they were cultured on a gelatin-coated dish under differentiation conditions, which consisted of SmGM2 basal medium plus 5% FBS for 7 days.

Flow cytometry

Human iPSCs were dissociated into single cells by digestion with 0.05% trypsin. After re-suspending in PBS, they were fixed in 4% paraformaldehyde solution (pH 7.5) at room temperature for 20 min and washed twice with PBS. They were permeabilized with PBST (0.1% Triton X-100 in PBS) for 10 min.
blocking with Blocking Solution (5% horse serum in PBS) for 30 min, the cells were double-stained for calponin (C2687; Sigma–Aldrich) and α-SMA (ab124964; Abcam). After washing with PBS, the cells were incubated with PBS containing a secondary antibody coupled to Cy5 (A10523; Thermo Fisher Scientific) and FITC (616511; Thermo Fisher Scientific), in which both were diluted to 1:1000 at room temperature for 1 hour. The cells were then washed twice with PBS and analyzed by flow cytometry (BD FACSaria II).

**Measurement of intracellular Ca$^{2+}$ in iPSCs-derived VSMCs**

For intracellular Ca$^{2+}$ measurements, primary cultured human iPSC-VSMCs were used. After loading with 5 µmol/L fura-2–AM, intracellular free calcium concentration ([Ca$^{2+}$]$_i$) in response to ACh at $10^{-5}$ through $10^{-3}$ mol/L was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm as described previously [22]. Calibration was done using ionomycin followed by ethylene glycol tetraacetic acid (EGTA)-Tris. An increase in [Ca$^{2+}$]$_i$ released from endoplasmic reticulum-Ca$^{2+}$ stores in response to ACh was measured in Ca$^{2+}$-free solution. ACh was used because it is widely used in the clinic to induce coronary spasm in Japan [7, 9].

**Chemicals**

The drugs used were diltiazem hydrochloride (voltage gated Ca$^{2+}$ channel blocker; Tokyo Chemical industry, Tokyo, Japan) and xestospongin C (inositol-1,4,5-trisphosphate receptor (IP$_3$R) 1 antagonist; FUJIFILM Wako Chemicals, Osaka, Japan).

**Determination of protein expression**

Protein samples were treated with a solution containing 3% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol and incubated at 50°C for 30 min. They were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking for 1 hour, the membranes were incubated with primary antibody for p122RhoGAP/DLC-1 (612020; BD Biosciences, NJ, USA), PLCδ1 (ab134936; Abcam), IP$_3$R1 (D53A5, Cell Signaling Technology, MA, USA), Ca$_v$1.2 (ACC-003; Alomone Labs, Jerusalem, Israel), IQGAP1 (610611; BD Biosciences), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-25778S, Santa Cruz Biotechnology) at 4°C overnight. As a secondary antibody, horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) was used. After SDS-PAGE, western blot analysis was performed using an ECL detection system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions. Densitometry analysis was done using scion image software and the relative ratio to GAPDH was calculated for each sample.

**Measurement of PLC activity**

The PLC assay included the following components: N-2-hydroxyethylpiperazin-N’-2-ethanesulfonic acid, 50 mmol/L; calcium chloride, 0.1 mmol/L; sodium cholate, 9 mmol/L; $^3$H-PIP$_2$ (40,000 counts per minute), and 20 µg of cell protein. The reaction was stopped with a combination of chloroform, methanol, and
hydrogen chloride and extracted with 1N hydrogen chloride containing EGTA. After extraction, the aqueous phase was removed for liquid scintillation counting.

**Statistical analysis**

Data analysis was performed using JMP statistical software (version 11.0, Cary, NC, USA) and were expressed as the mean ± standard deviation. Comparisons of two variables were tested by paired or unpaired t-tests, as appropriate by F-test, and multiple comparisons were tested using Tukey–Kramer’s test. P-values of < 0.05 were considered statistically significant in a one-tail test.

**Results**

**Establishment of human iPSCs from skin fibroblasts**

Figure 1a and b show the morphological and biological characteristics of human skin fibroblasts and undifferentiated human iPSCs obtained from control and CSA subjects. The colonies of human iPS cells were picked, expanded for several passages, and then checked for APS activity. The established iPSC strain colonies exhibited positive staining for APS activity (Fig. 1a). Expression of pluripotency markers was also evaluated using immunofluorescent staining. Representative iPSC colonies strongly expressed the pluripotency markers, SSEA-4 and TRA1-60, on the cell surface (Fig. 1a). As shown in Fig. 1b, the expression of NANOG, OCT3/4, and SOX2 mRNA in human iPSC lines were validated by RT-PCR using the primers shown in Table 1. This suggests that all of the iPSCs expressed undifferentiated ES cell marker genes.

Differentiation of human iPSCs into vascular VSMCs and analyses of vascular VSMC markers by flow cytometry and RT-PCR

During the final stage of differentiation, the cells were growing into a spindle shape. Immunofluorescent staining revealed that the VSMC marker proteins, such as α-SMA and calponin, were positively expressed (Fig. 1c). In all six cell lines, flow cytometry revealed that more than 95.5% of the cells were co-stained with α-SMA and calponin. The expression of the VSMC markers, α-SMA, calponin, caldesmon, and SM22α, in the six cell lines were also validated by RT-PCR (Fig. 1d).

**Comparison of ACh-induced intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in human iPSC-derived VSMCs**

Figure 2a and b illustrate representative waveforms of [Ca\(^{2+}\)]\(_i\) after the addition of an incremental dose of ACh in human iPSC-derived VSMCs obtained from control and CSA subjects (2a; Ca\(^{2+}\)-containing buffer, 2b; Ca\(^{2+}\)-free buffer). In Ca\(^{2+}\)-containing buffer, the ACh-induced increase in Ca\(^{2+}\) consisted of a transient phase (mobilization from intracellular Ca\(^{2+}\) stores and influx of extracellular Ca\(^{2+}\)) and a subsequent sustained phase (influx of extracellular Ca\(^{2+}\)). In Ca\(^{2+}\)-free buffer, the ACh-induced increase in Ca\(^{2+}\) exhibited a transient phase (mobilization from intracellular Ca\(^{2+}\) store). In Ca\(^{2+}\)-containing buffer, the level
of \([\text{Ca}^{2+}]_i\) at baseline was 79 ± 27 nM in control iPSC-derived VSMCs and 95 ± 33 nM in CSA iPSC-derived VSMCs (P < 0.05, n = 30) (Fig. 2c). Figure 2d shows dose-related changes in \([\text{Ca}^{2+}]_i\) after ACh in human iPSC-derived VSMCs obtained from control and CSA subjects in \(\text{Ca}^{2+}\)-containing buffer. The peak increase in \([\text{Ca}^{2+}]_i\) from baseline was significantly greater in the CSA iPSC-derived VSMCs compared with the control iPSC-derived VSMCs (129 ± 24 vs. 53 ± 10 nM at 10\(^{-4}\) M ACh and 217 ± 32 vs. 93 ± 29 nM at 10\(^{-3}\) M ACh, respectively; both P < 0.001, n = 3). Figure 2e shows the dose-related changes in \([\text{Ca}^{2+}]_i\) after ACh in human iPSC-derived VSMCs obtained from control and CSA subjects using a \(\text{Ca}^{2+}\)-free buffer. The peak increase in \([\text{Ca}^{2+}]_i\) from baseline after ACh was significantly greater in the CSA iPSC-derived VSMCs compared with the control iPSC-derived VSMCs (72 ± 10 vs. 30 ± 5 nM at 10\(^{-4}\) M ACh and 124 ± 14 vs. 51 ± 7 nM at 10\(^{-3}\) M ACh, respectively; both P < 0.001, n = 3). For subsequent experiments, 10\(^{-4}\) M ACh was used for intracellular \([\text{Ca}^{2+}]_i\) measurements.

**Effects of diltiazem on ACh-induced \(\text{Ca}^{2+}\) influx in human iPSC-derived VSMCs**

Figure 3a illustrates representative waveforms of \([\text{Ca}^{2+}]_i\) after the addition of ACh (10\(^{-4}\) M) at incremental doses of diltiazem in human iPSC-derived VSMCs obtained from control and CSA subjects using a \(\text{Ca}^{2+}\)-containing buffer. Pretreatment with diltiazem 10\(^{-5}\) M did not affect ACh-induced \([\text{Ca}^{2+}]_i\) levels in control or CSA iPSC-derived VSMCs, whereas diltiazem 10\(^{-4}\) M attenuated the ACh-induced \([\text{Ca}^{2+}]_i\) increase from 134 ± 9 nM to 72 ± 10 nM in the CSA iPSC-derived VSMCs (P < 0.001, n = 3) (Fig. 3a and 3b). Diltiazem 10\(^{-4}\) M also slightly, but significantly, suppressed the ACh-induced increase in \([\text{Ca}^{2+}]_i\) in control iPSC-derived VSMCs, (56 ± 4nM without diltiazem vs. 42 ± 1nM with diltiazem, P < 0.05, n = 3).

**Effects of xestospongin C on ACh-induced \(\text{Ca}^{2+}\) mobilization from intracellular store in human iPSC-derived VSMCs**

To evaluate the amount of \(\text{Ca}^{2+}\) release from endoplasmic reticulum \(\text{Ca}^{2+}\) stores, the increase in \([\text{Ca}^{2+}]_i\) by ACh and the effect of xestospongin C were examined in a \(\text{Ca}^{2+}\)-free buffer. Figure 3c illustrates representative waveforms of \([\text{Ca}^{2+}]_i\) after the addition of ACh (10\(^{-4}\) M) at incremental doses of xestospongin C in control and CSA iPSC-derived VSMCs. As shown in Fig. 3d, pretreatment of human iPSC-derived VSMCs with 10 µM xestospongin C significantly suppressed ACh-induced \(\text{Ca}^{2+}\) release from endoplasmic reticulum \(\text{Ca}^{2+}\) stores in the control and CSA subjects (42 ± 8 nM to 16 ± 3 nM in the control iPSC-derived VSMCs, P < 0.01, and 79 ± 9 nM to 35 ± 6 nM in the CSA iPSC-derived VSMCs, P < 0.001, n = 3).

**Protein expression in human iPSC-derived VSMCs**
To assess the expression of p122RhoGAP/DLC-1, PLCδ1, IP₃R1 Caᵥ1.2, and IQGAP1 protein, the cells were scraped after 16 hours of serum starvation. Representative bands obtained by western blotting are shown in Fig. 4a. p122RhoGAP/DLC-1 and IP₃R1 protein levels were significantly increased by 2.53 ± 0.88 and 7.53 ± 3.78-fold, respectively, in CSA iPSC-derived VSMCs compared with that of the control iPSC-derived VSMCs (Fig. 4b and c, P < 0.05, n = 3). PLC-δ1, Caᵥ1.2, and IQGAP1 protein expression was similar between the control and CSA iPSC-derived VSMCs.

**PLC activity**

As shown in Fig. 4d, PLC activity in the whole cell was significantly higher (3.12 ± 1.28-fold) in CSA iPSC-derived VSMCs compared with the control iPSC-derived VSMCs (P < 0.05, n = 3).

**Discussion**

In this study, we identified the molecular mechanism responsible for the enhancement of Ca²⁺ signaling in the pathogenesis of CSA. We succeeded in generating VSMCs derived from iPS cells from control and CSA subjects.

First, we confirmed that CSA iPSC-derived VSMCs enhanced the [Ca²⁺]ᵢ response to ACh, which involved Ca²⁺ mobilization from endoplasmic reticulum Ca²⁺ stores and Ca²⁺ influx. ACh was used because it is widely used in the clinic as a provocation test for coronary spasm. The peak increase in [Ca²⁺]ᵢ from baseline after ACh stimulation was significantly greater in CSA iPSC-derived VSMCs compared with control iPSC-derived VSMCs. The amount of [Ca²⁺]ᵢ release from the endoplasmic reticulum Ca²⁺ stores was also significantly greater in CSA iPSC-derived VSMCs compared with control iPSC-derived VSMCs. The PLC-δ1 isozyme is more sensitive to Ca²⁺ compared with the other isozymes. When [Ca²⁺]ᵢ is in a physiological range (10 to 1000 nmol/L), it is sufficient to activate PLC-δ1 [11]. Therefore, the initial transient increase in [Ca²⁺]ᵢ induced by IP₃, in turn, contributes to the long-term activation of PLC-δ1 in a positive feedback manner [12]. Similarly, [Ca²⁺]ᵢ at baseline is regulated by PLC-δ1, because it is maintained at concentrations that activate PLC-δ1. Therefore, [Ca²⁺]ᵢ at baseline was elevated in the CSA iPSC-derived VSMCs that exhibited enhanced PLC-δ1 activity. These results are consistent with the pathogenesis of CSA, in which both the basal vascular tone and the vasoconstrictor response to stimuli are enhanced.

The expression of IP₃R1 protein was significantly increased by 7.5-fold in CSA iPSC-derived VSMCs compared with control iPSC-derived VSMCs. The inositol 1,4,5-trisphosphate/calcium (IP₃/Ca²⁺) signaling pathway directly controls various processes. For example, it influences metabolism, secretion, fertilization, proliferation, and smooth muscle contraction [23]. Increased activity of the IP₃/Ca²⁺ signaling pathway contributes to increased myogenic tone, which results in hypertension and cardiac disease [24]. In human pulmonary arterial VSMCs, up-regulation of the Ca²⁺-sensing receptor increases
the activity of the IP$_3$/Ca$^{2+}$ signaling pathway [25]. In hypertensive rats, IP$_3$R up-regulation in VSMCs is associated with enhanced IP$_3$-dependent Ca$^{2+}$ release and sensitization, which increases VSMC contraction in response to agonist stimulation [26]. Thus, enhanced expression of IP$_3$R1 may contribute to enhanced coronary VSMC contraction. Further studies are needed to identify the relationship between IP$_3$R expression and CSA pathogenesis.

We previously demonstrated a 3-fold increase in the expression of p122RhoGAP/DLC-1 protein in cultured skin fibroblasts from CSA patients compared with the controls. In the present study, we also showed enhanced expression of p122RhoGAP/DLC-1 protein in CSA iPSC-derived VSMCs. In contrast, IQGAP1 protein expression was upregulated 1.4-fold in CSA dermal fibroblasts compared with the control in the previous study [15]; however, we found no difference in IQGAP1 protein expression in iPSC-derived VSMCs in the present study. Importantly, PLC activity in CSA skin fibroblasts was increased 3-fold compared with the control [13] and similar results were obtained using CSA iPSC-derived VSMCs in the present study, although no difference was observed in protein expression. These findings indicate that enhanced expression of p122RhoGAP/DLC-1 in CSA iPSC-derived VSMCs may contribute to increased PLC activity without affecting PLC-δ1 protein expression. Furthermore, we demonstrated that Cav1.2 protein expression did not differ between CSA and control iPSC-derived VSMCs. Consistently, we recently showed that there was no difference in Cav1.2 protein expression in the aorta between PLC-TG and wild-type mice [27]. Furthermore, the inhibitory effect of diltiazem on intracellular calcium concentration was also observed in the present study using CSA iPSC-derived VSMCs and in our recent animal study [27]. These similarities suggest that the elucidation of the mechanism and development of therapeutic drugs for coronary spasm that were only tested in animal models can now be performed on living human cells using iPSC-derived VSMCs, thus providing useful tools to accelerate further research in this field.

**Conclusions**

p122RhoGAP/DLC-1 and IP$_3$R1 proteins are overexpressed in human iPSC-derived VSMCs obtained from CSA patients, and thereby enhance the intracellular Ca$^{2+}$ response to ACh. This indicates that overexpressed p122RhoGAP/DLC-1 and IP$_3$R1 proteins play a causal role in the pathogenesis of coronary spasm that is associated with human CSA.

**Study limitations**

There are some confounding factors in this study that may affect the results including the sample size, selection bias, and the age of the patients.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APS</td>
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<td>CSA</td>
<td>Coronary spastic angina</td>
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DMEM  Dulbecco's Modified Eagle's medium
EB  Embryoid bodies
EGTA  Ethylene glycol tetraacetic acid
ESC  Embryonic stem cell
FBS  Fetal bovine serum
PBS  Phosphate buffered saline
SDS  Sodium dodecyl sulfate
VSMC  Vascular smooth muscle cells

Declarations

Ethics approval and consent to participate

The ethics committee at our institution approved the study protocol (2013-086). Written informed consent was obtained from all patients before the study.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

MT performed the experiments, analyzed the results, and wrote the manuscript. KO, and TY helped design the experiments and interpreted the data. TO helped write the manuscript. HT designed and supervised the study, analyzed the results, and wrote the manuscript. All authors read and approved the final manuscript.

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References


Figure 1

Establishment of human induced pluripotent stem cell (iPSC) from skin fibroblasts.

a) Human skin fibroblasts and morphology of undifferentiated human iPSCs from control and CSA subjects. Alkaline phosphatase staining (APS) and immunocytochemistry of SSEA-4 and TRA1-60 were performed.

b) RT-PCR analysis of NANOG, OCT3/4, and SOX2 in iPSC lines from control and CSA subjects.

c) Immunofluorescence staining of vascular smooth muscle cells (VSMC) markers in iPSC-derived VSMCs from control and CSA subjects. Red staining shows α-smooth muscle actin (αSMA) and green staining shows calponin. Blue staining shows nuclei. Flow cytometry analysis for α-SMA and calponin in human iPSC-derived VSMCs.

d) RT-PCR analysis of VSMC markers, αSMA, calponin, caldesmon, and SM22α in iPSC-derived VSMCs from control and CSA subjects.

Figure 2

Comparison of acetylcholine (ACh)-induced intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) in human iPSC-derived VSMCs.

a and b) Representative waveforms of [Ca\(^{2+}\)]\(_i\) after the addition of incremental doses of ACh in human iPSC-derived VSMCs from control and CSA subjects in a Ca\(^{2+}\)-containing buffer (upper row) and Ca\(^{2+}\)-free buffer (lower row).

c) [Ca\(^{2+}\)]\(_i\) at baseline in human iPSC-derived VSMCs from control and CSA subjects (n = 30).

d) Dose-related changes in [Ca\(^{2+}\)]\(_i\) after ACh stimulation in human iPSC-derived VSMCs from control and CSA subjects using a Ca\(^{2+}\)-containing buffer (n = 3).

e) Dose-related changes in [Ca\(^{2+}\)]\(_i\) after ACh stimulation in human iPSC-derived VSMCs from control and CSA subjects in a Ca\(^{2+}\)-free buffer (n = 3).

Figure 3

Effects of diltiazem and xestospongin C on ACh-induced Ca\(^{2+}\) influx in human iPSC-derived VSMCs.
a) Representative waveforms of $[Ca^{2+}]_i$ after addition of ACh ($10^{-4}$ M) and the effect of incremental doses of diltiazem in human iPSC-derived VSMCs from control and CSA subjects in a $Ca^{2+}$-containing buffer.

b) Dose-dependent changes in $[Ca^{2+}]_i$ after diltiazem treatment in human iPSC-derived VSMCs from control and CSA subjects in a $Ca^{2+}$-containing buffer ($n = 3$).

c) Representative waveforms of $[Ca^{2+}]_i$ after addition of ACh ($10^{-4}$ M) and the effect of incremental doses of xestospongin C in human iPSC-derived VSMCs from control and CSA subjects in a $Ca^{2+}$-free buffer.

d) Dose-dependent changes in $[Ca^{2+}]_i$ after xestospongin C treatment in human iPSC-derived VSMCs from control and CSA subjects in a $Ca^{2+}$-free buffer ($n = 3$).

Figure 4

Protein expression and phospholipase C (PLC) activity of human iPSC-derived VSMCs.

a) Western blot analysis of p122RhoGAP/DLC-1, PLCδ1, IP$_3$R1, Ca$_v$1.2, IQGAP1, and GAPDH protein expression in human iPSC-derived VSMCs from control and CSA subjects.

b and c) Protein expression of p122RhoGAP/DLC-1 and IP$_3$R1 normalized to GAPDH in human iPSC-derived VSMCs from control and CSA subjects ($n = 3$).

d) PLC activity in human iPSC-derived VSMCs from control and CSA subjects ($n = 3$).