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The Signal Transduction of Abnormal Vascular Smooth Muscle Contraction

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There are two types of vascular smooth muscle contraction. One is normal contraction, which is physiological and Ca^{2+}-dependent. Another is abnormal contraction, which causes vasospasm and Ca^{2+}-independent. Rho-kinase and PKC are known key molecules to mediate the signal transduction of abnormal vascular smooth muscle contraction. Sphingosylphosphorylcholine（SPC）is a member of sphingolipids and induces Ca^{2+}-independent, Rho-kinase-mediated abnormal vascular smooth muscle contraction via the activation of Src family tyrosine kinase（Src-TK）. We found SPC-induced contraction is cholesterol-dependent and suggest the involvement of membrane raft in the signal transduction of abnormal vascular smooth muscle contraction mediated by SPC/Src-TK/Rho-kinase pathway. Eicosapentaenoic acid specifically inhibited abnormal vascular smooth muscle contraction through the inhibition of SPC/Src-TK/Rho-kinase pathway. By functional proteomics, we identified cytoskeleton-related proteins as the candidate of novel molecule to mediate abnormal vascular smooth muscle contraction and investigating their interaction with Rho-kinase. We hope that this interaction could be a new drug target.

Keywords: vascular smooth muscle contraction, functional proteomics, cytoskeleton-related proteins

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TWO TYPES OF VASCULAR SMOOTH MUSCLE CONTRACTION: DIFFERENCES IN FUNCTIONS AND MECHANISMS

Vascular smooth muscle consists of medial layer of blood vessels and has contractile function. There are two types of vascular smooth muscle contraction. One is “normal”（physiological）contraction which regulates blood pressure and blood flow to various organs in the body. Another is “abnormal” contraction（vasospasm）which induces ischemia in critical organs such as brain and heart. Those contraction differs each other not only in its function but also in its mechanism［1］.

Normal vascular smooth muscle contraction is regulated by cytosolic calcium ion（Ca^{2+}）concentration（Fig. 1, blue-shaded area）. When cytosolic Ca^{2+} concentration increases, Ca^{2+} binds to calmodulin（CaM）to activate myosin light chain kinase（MLCK）. MLCK phosphorylates myosin regulatory light chain（MRLC）to activate smooth muscle myosin, enabling it to interact with actin to elicit contraction. When cytosolic Ca^{2+} concentration decreases, MLCK is inactivated and myosin light chain phosphatase（MLCP）dephosphorylated MRLC, resulting in the inactivation of smooth muscle myosin and relaxation. Since normal vascular smooth muscle contraction requires cytosolic Ca^{2+} elevation, it is called Ca^{2+}-dependent contraction［2］.

In contrast, abnormal vascular smooth muscle contraction does not require cytosolic Ca^{2+} elevation（Fig. 1, pink-shaded area）. Rho-kinase and protein kinase C（PKC）are known to be the key molecules to mediate the signal transduction of abnormal vascular smooth muscle contraction. Rho-kinase phosphorylates myosin phosphatase targeting subunit

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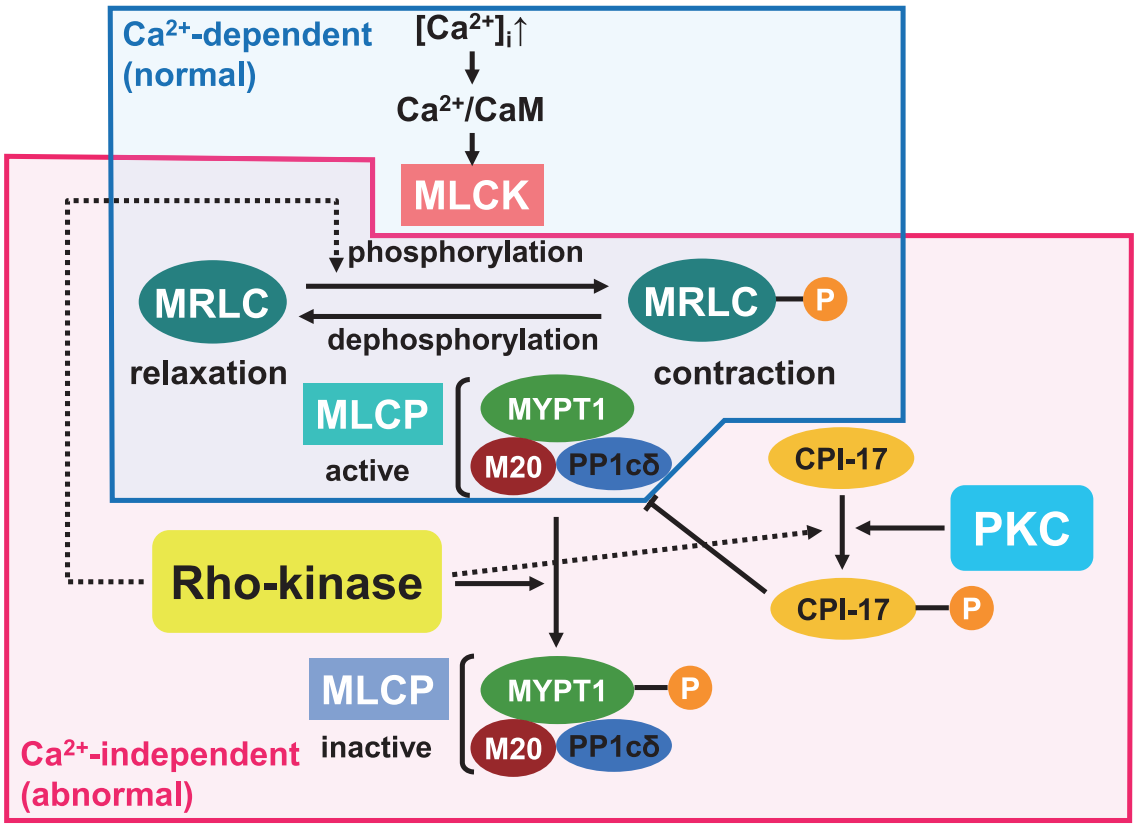

Fig. 1. Molecular mechanisms of Ca^{2+}-dependent and Ca^{2+}-independent vascular smooth muscle contraction. While the molecular mechanism of Ca^{2+}-dependent vascular smooth muscle contraction is shown in the blue-shaded area, that of Ca^{2+}-independent vascular smooth muscle contraction is in the pink-shaded area.

1 (MYPT1), a regulatory subunit of MLCP, resulting in MLCP inactivation [3]. PKC phosphorylates CPI-17, an endogenous inhibitory protein of MLCP [4]. Also, Rho-kinase is reported to phosphorylate CPI-17 to inhibit MLCP [5], or directly phosphorylate MRLC [6]. Those pathway results in MLCP inhibition and increased MRLC phosphorylation, leading to smooth muscle contraction without cytosolic Ca^{2+} elevation. This mechanism is called Ca^{2+}-sensitization of vascular smooth muscle contraction or Ca^{2+}-independent contraction.

SPC/Src-TKs/Rho-kinase PATHWAY TO MEDIATES ABNORMAL VASCULAR SMOOTH MUSCLE CONTRACTION

Sphingosylphosphorylcholine (SPC) is a member of sphingolipid and generated by the *N*-deacylation of sphingomyelin which is abundant in cell membrane [7]. Simultaneous measurement of cytosolic Ca^{2+} concentration using Fura-2 and vascular smooth muscle contraction revealed that SPC induced Ca^{2+}-independent contraction in porcine coronary [8,9] and bovine middle cerebral arteries (Fig. 2) [10]. Furthermore, in α-toxin permeabilized vascular smooth muscle strips, which you can make small pores on plasma membrane of vascular smooth muscle to control cytosolic Ca^{2+} concentration and introduce molecules less than 1 kDa, SPC induced vascular smooth muscle contraction at a constant cytosolic Ca^{2+} level of pCa6.3, which was inhibited by Rho-kinase inhibitor Y-27632 [8,10]. Also, dominant-negative Rho-kinase inhibited the SPC-induced contraction whereas PKC pseudosubstrate peptide PKCα19-31 did not inhibit the SPC-induced contraction in β-escin-permeabilized vascular smooth muscle strips, which you can make small pores on plasma membrane of vascular smooth muscle to control cytosolic Ca^{2+} concentration and introduce molecules up to 150 kDa (Fig. 3) [10]. These findings suggest that SPC induced abnormal vascular smooth muscle contraction which is mediated by Rho-kinase rather than PKC. Interestingly, SPC-induced contraction did not require GTP either in

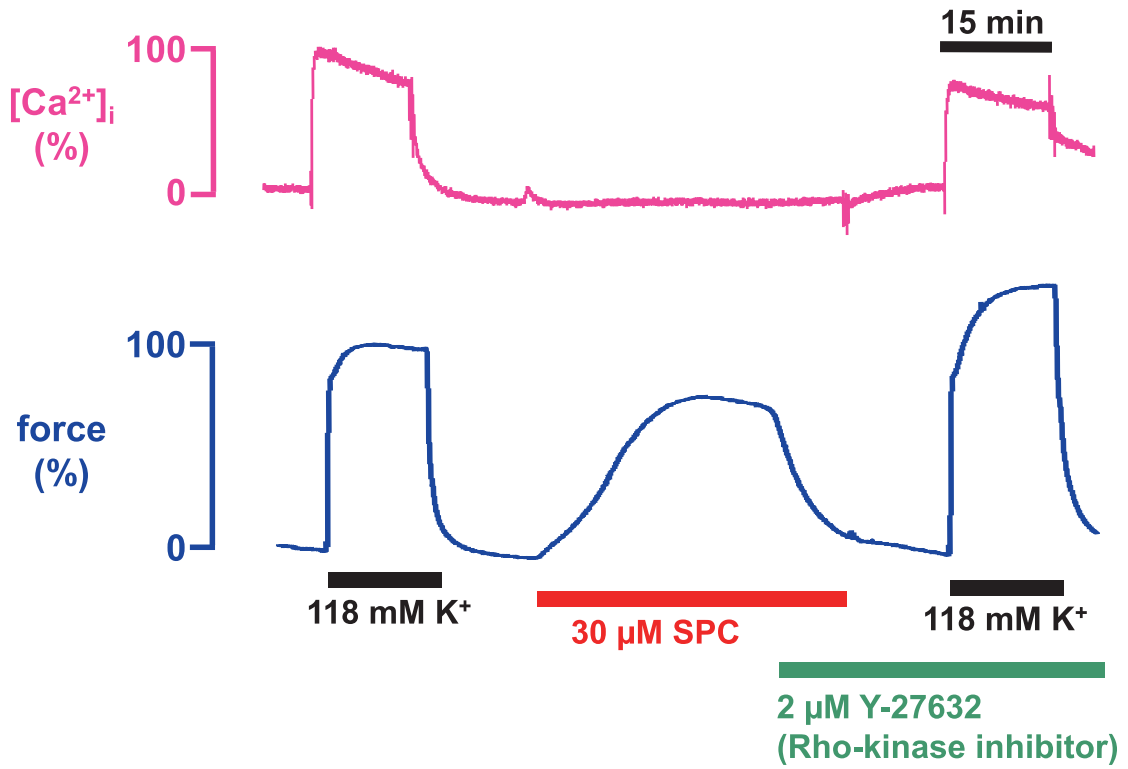

Fig. 2. SPC induced Ca^{2+}-independent vascular smooth muscle contraction which is mediated by Rho-kinase. Simultaneous measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) using Fura-2 and vascular smooth muscle contraction (force) revealed that SPC induced Ca^{2+}-independent contraction, which was inhibited a Rho-kinase inhibitor Y-27632 in bovine middle cerebral artery. (Figure adapted from Fig. 1. in Ref. 10.)

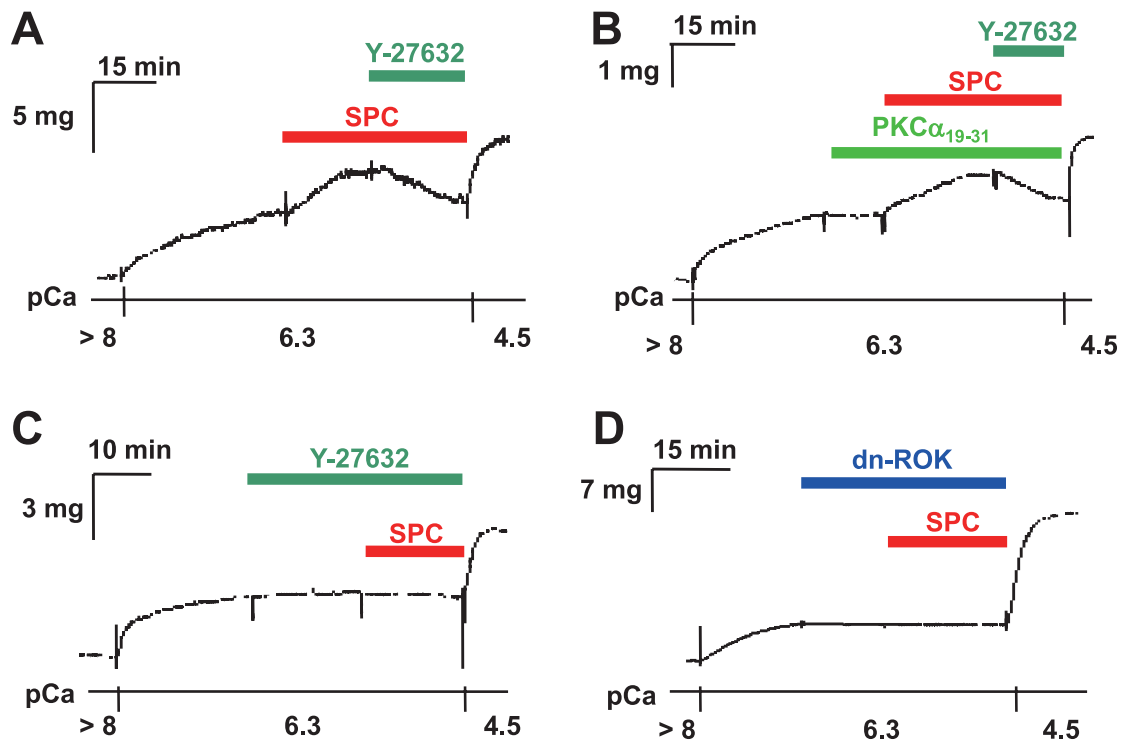

Fig. 3. SPC induced abnormal vascular smooth muscle contraction which is mediated by Rho-kinase rather than PKC. In β-escin-permeabilized vascular smooth muscle strips, A: SPC induced contraction at a constant Ca^{2+} level of pCa6.3, which was inhibited by a Rho-kinase inhibitor Y-27632. B: PKCα19-31, a pseudosubstrate PKC peptide, did not inhibit the SPC-induced contraction. C: Y-27632 pretreatment inhibited the SPC-induced contraction. D: Dominant-negative Rho-kinase (dn-ROK) inhibited the SPC-induced contraction. (Figure adapted from Fig. 3. in Ref. 10.)

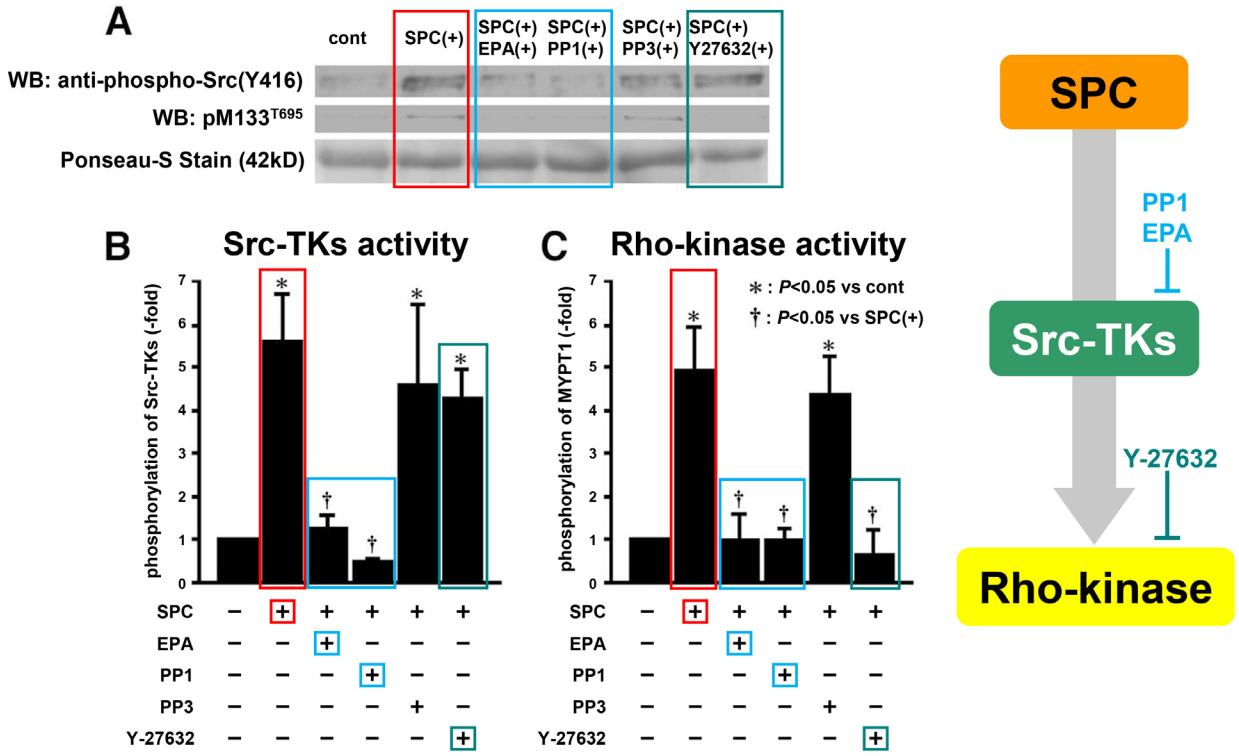

Fig. 4. SPC induced Rho-kinase activation through the activation of Src-TKs in vascular smooth muscle cells.
A: Vascular smooth muscle strips were incubated with vehicle (cont) or SPC in the absence (SPC(+)) or presence of Src-TK inhibitors of EPA (SPC(+) EPA(+)) and PP1 (SPC(+) PP1(+)), a PP1's inactive analog PP3 (SPC(+) PP3(+)), and a Rho-kinase inhibitor Y-27632 (SPC(+) Y-27632(+)). Src-TK activity was analyzed by western blot using anti-phospho-Src (Y416) antibody to detect the autophosphorylation of Src-TKs. Rho-kinase activity was analyzed by western blot using anti-pM133^{T695} antibody to detect the phosphorylation of MYPT1. B: Summary of Src-TK activity in each sample. C: Summary of Rho-kinase activity in each sample. While Src-TK inhibitors EPA and PP1 inhibited both SPC-induced activations of Src-TKs and Rho-kinase, a Rho-kinase inhibitor Y-27642 inhibited only SPC-induced activations of Rho-kinase, but not of Src-TKs. (Figure adapted from Fig. 8. in Ref. 9.)

α-toxin- or β-escin-permeabilized vascular smooth muscle strips [8,10], suggesting that SPC activates Rho-kinase with no requirement of small G protein RhoA.

In addition, intracisternally injected SPC induced vasospasm *in vivo* in canine [11]. Also, SPC concentration in cerebrospinal fluid was increased in human patients of subarachnoid hemorrhage (SAH) compared to non-SAH patients such as brain tumor and normal pressure hydrocephalus [12]. These *in vivo* and clinical findings suggest that SPC is a novel messenger to induce vasospasm through the activation of Rho-kinase.

Furthermore, Src family tyrosine kinases (Src-TK) activity is required for SPC/Rho-kinase pathway to mediate abnormal vascular smooth muscle contraction. PP1, a selective inhibitor of Src-TK, inhibited the SPC-induced contraction in porcine coronary artery [9]. PP1 also inhibited the SPC-induced activation of Src-TK and Rho-kinase while Rho-kinase inhibitor Y-27632 inhibited only the SPC-induced activation of Rho-kinase but not of Src-TK [9], suggesting that Src-TK acts as an intermediate between SPC and Rho-kinase (Fig. 4). Src-TK belongs to nonreceptor tyrosine kinase and currently 9 members are reported in human including c-Src, Blk, Fyn, Yes, Lyn, Lck, Hck, Fgr, and Yrk [13]. Since the expression of c-Src and Fyn were confirmed in vascular smooth muscle cells and tissues [9], subcellular localization of those proteins was analyzed using confocal microscopy. As a result, SPC induced the translocation of Fyn, but not c-Src, from cytosol to plasma membrane [9]. Those results suggested that Fyn, rather than c-Src, is likely to be involved in the signal transduction of abnormal vascular smooth muscle contraction mediated by SPC/Rho-kinase pathway.

THE INVOLVEMENT OF CHOLESTEROL AND MEMBRANE RAFTS IN THE SIGNAL TRANSDUCTION OF ABNORMAL VASCULAR SMOOTH MUSCLE CONTRACTION

We found the SPC-induced contraction is cholesterol-dependent. In the vascular smooth muscle strips obtained from normocholesterolemic human patients, SPC did not induce contraction, whereas SPC induced remarkable contraction in the vascular smooth muscle strips obtained from hypercholesterolemic patients (Fig. 5) [14]. To confirm cholesterol dependency, we treated vascular smooth muscle strips obtained from hypercholesterolemic rabbit with β-cyclodextrin to deprive cholesterol and found that SPC-induced contraction was markedly inhibited in those cholesterol-depleted vascular smooth muscle strips.

To clarify the molecular mechanisms of the dependency of abnormal vascular smooth muscle contraction on cholesterol, we focused on membrane raft, a membrane microdomain enriched with cholesterol and sphingolipids. When we analyzed the subcellular localization of Rho-kinase and a membrane raft marker protein caveolin-1 in vascular smooth muscle cells, SPC induced the translocation of Rho-kinase from cytosol to plasma membrane, which was inhibited by cholesterol depletion by β-cyclodextrin [14]. Interestingly, in those β-cyclodextrin-treated vascular smooth muscle cells, caveolin-1 was deprived from plasma membrane, suggesting the destruction of membrane rafts [14]. Those findings suggest that SPC induces the translocation of Rho-kinase from cytosol to membrane rafts to conduct the signal transduction of abnormal smooth muscle contraction.

EPA SPECIFICALLY INHIBITED ABNORMAL VASCULAR SMOOTH MUSCLE CONTRACTION THROUGH THE INHIBITION OF SPC/Src-TK/Rho-kinase PATHWAY

Since SPC induced the translocation of Fyn from the cytosol to plasma membrane in vascular smooth muscle cells [9], a substance which can prevent Fyn from its translocating to plasma membrane is likely to be a good candidate for the drug to treat abnormal vascular smooth muscle contraction. After extensive screening, eicosapentaenoic acid (EPA) was found to inhibit the SPC-induced translocation

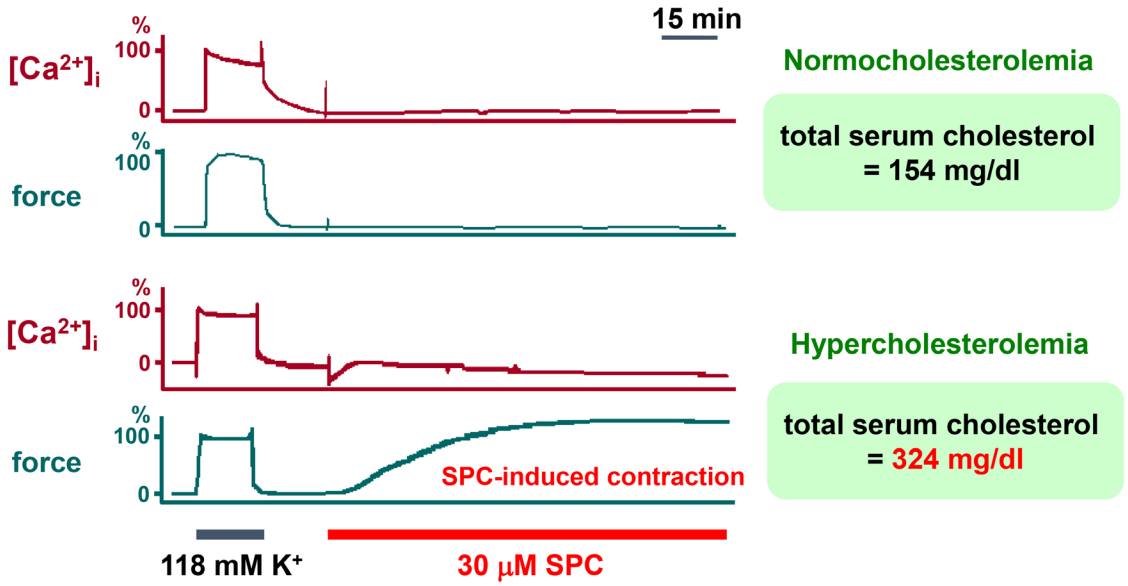

Fig. 5. SPC-induced contractions of human vascular smooth muscle depend on serum cholesterol levels. While SPC did not induced contraction in vascular smooth muscle strip obtained from a human patient with normal cholesterol level (upper panel), SPC induced remarkable Ca^{2+}-independent contraction in in vascular smooth muscle strip obtained from a human patient with high cholesterol level (lower panel). (Figure adapted from Fig. 1. in Ref. 14.)

of Fyn from cytosol to plasma membrane [9]. EPA is a n-3 polyunsaturated fatty acid enriched in fish oil and approved as a drug (EPADEL in Japan and VASCEPA in USA). EPA has various health benefits such as prevention of platelet aggregation [15], lowering blood triglyceride level [16,17], and anti-inflammatory effect [18]. In porcine coronary arterial smooth muscle strips, EPA inhibited the SPC-induced Ca^{2+}-independent contraction without affecting depolarization-induced cytosolic Ca^{2+} elevation and Ca^{2+}-induced contraction [9]. EPA also inhibited the SPC-induced activations of Src-TK and Rho-kinase [9]. Those findings suggest that EPA blocks the signal transduction of abnormal vascular smooth muscle contraction by blocking the activations of Src-TK and Rho-kinase.

Furthermore, intracisternaly injected EPA inhibited the SPC-induced vasospasm as well as cerebral vasospasm in SAH model in canine basilar artery[11]. In addition, a prospective, multicenter, randomized study revealed the efficacy of EPA for cerebral vasospasm in human SAH patients [19]. Those findings support that EPA is a magic bullet for the prevention and treatment of abnormal vascular smooth muscle contraction.

FUNCTIONAL PROTEOMICS: STRATEGIES TO FIND NOVEL SIGNALING MOLECULES TO MEDIATE ABNORMAL VASCULAR SMOOTH MUSCLE CONTRACTION

Although EPA has a potent and selective inhibitory effect on abnormal vascular smooth muscle contraction, its route is limited to oral administration and therefore it is difficult to administrate EPA to a patient who is unconscious and can't ingest orally. To explore another drug targets for the development of injectable drugs, we applied functional proteomics to screen novel signaling molecules downstream of Fyn tyrosine kinase. In the functional proteomics, we enriched tyrosine-phosphorylated proteins and identified them using tandem mass spectroscopy. We identified cytoskeleton-related proteins as the candidate of novel signaling protein to mediate abnormal vascular smooth muscle contraction and investigating their interaction with known signaling molecules such as Rho-kinase. Hopefully, these interactions could be the new drug target for the treatment and prevention of abnormal vascular smooth muscle contraction.

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Conflict of Interests

Author has received patent royalties of P5186679 and P5504405 from OrientalBio Co., Ltd.

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