STIM1 Regulates Platelet-Derived Growth Factor-Induced Migration and Ca$^{2+}$ Influx in Human Airway Smooth Muscle Cells

Nobukazu Suganuma1*, Satoru Ito1*, Hiromichi Aso1, Masashi Kondo1, Mitsuo Sato1, Masahiro Sokabe2, Yoshinori Hasegawa1

1 Department of Respiratory Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan, 2 Department of Physiology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Abstract

It is suggested that migration of airway smooth muscle (ASM) cells plays an important role in the pathogenesis of airway remodeling in asthma. Increases in intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_{i}$) regulate most ASM cell functions related to asthma, such as contraction and proliferation. Recently, STIM1 was identified as a sarcoplasmic reticulum (SR) Ca$^{2+}$ sensor that activates Orai1, the Ca$^{2+}$ channel responsible for store-operated Ca$^{2+}$ entry (SOCE). We investigated the role of STIM1 in [Ca$^{2+}$]$_{i}$, and cell migration induced by platelet-derived growth factor (PDGF)-BB in human ASM cells. Cell migration was assessed by a chemotaxis chamber assay. Human ASM cells express STIM1, STIM2, and Orai1 mRNA. SOCE activated by thapsigargin, an inhibitor of SR Ca$^{2+}$-ATPase, was significantly blocked by STIM1 siRNA and Orai1 siRNA but not by STIM2 siRNA. PDGF-BB induced a transient increase in [Ca$^{2+}$]$_{i}$, followed by sustained [Ca$^{2+}$]$_{i}$ elevation. Sustained increases in [Ca$^{2+}$]$_{i}$ due to PDGF-BB were significantly inhibited by a Ca$^{2+}$-chelating agent EGTA or by siRNA for STIM1 or Orai1. The numbers of migrating cells were significantly increased by PDGF-BB treatment for 6 h. Knockdown of STIM1 and Orai1 by siRNA transfection inhibited PDGF-induced cell migration. Similarly, EGTA significantly inhibited PDGF-induced cell migration. In contrast, transfection with siRNA for STIM2 did not inhibit the sustained elevation of [Ca$^{2+}$]$_{i}$ or cell migration induced by PDGF-BB. These results demonstrate that STIM1 and Orai1 are essential for PDGF-induced cell migration and Ca$^{2+}$ influx in human ASM cells. STIM1 could be an important molecule responsible for airway remodeling.

Citation: Suganuma N, Ito S, Aso H, Kondo M, Sato M, et al. (2012) STIM1 Regulates Platelet-Derived Growth Factor-Induced Migration and Ca$^{2+}$ Influx in Human Airway Smooth Muscle Cells. PLoS ONE 7(9): e45056. doi:10.1371/journal.pone.0045056

Editor: Ladzlo Csernoch, University of Debrecen, Hungary

Received February 7, 2012; Accepted August 15, 2012; Published September 11, 2012

Copyright: © 2012 Suganuma et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Grants-in-Aid (#22680387) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to S. Ito). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: itori@med.nagoya-u.ac.jp
† These authors contributed equally to this work.

Introduction

Airway remodeling due to repeated airway wall damage and repair plays an important role in the pathophysiology of severe asthma [1]. An increase of airway smooth muscle (ASM) mass due to proliferation and hypertrophy of ASM cells is one of the major pathological features of airway remodeling [1]. In addition, accumulating evidence suggests that ASM cell migration toward the airway epithelium in response to inflammatory mediators such as platelet-derived growth factor (PDGF) contributes to the airway remodeling [2–9]. As a result, the ASM layer in asthmatic patients is in close proximity to airway epithelial cells [6,10], which may lead to increased airway hyperresponsiveness.

Intracellular free Ca$^{2+}$ is a second messenger for ASM cell functions related to asthma, such as contraction, proliferation, and cytokine production [11–14]. Store-operated Ca$^{2+}$ entry (SOCE), originally introduced as capacitative Ca$^{2+}$ entry by Putney [15], is a ubiquitous Ca$^{2+}$ influx pathway in various cell types including ASM cells [11,16–18]. SOCE is activated by a fall in the Ca$^{2+}$ concentration of the sarcoplasmic reticulum (SR) Ca$^{2+}$ stores in muscle cells or endoplasmic reticulum (ER) in non-muscle cells through the binding of inositol-1,4,5-trisphosphate (IP$_{3}$) to the IP$_{3}$ receptor [10]. Importantly, SOCE closely links to the contraction and cell proliferation of ASM cells [11,14,19–21]. Stromal interaction molecule 1 (STIM1) was identified as a key molecule which senses Ca$^{2+}$ concentrations within the SR and reports this information to Orai1, a Ca$^{2+}$-permeable channel responsible for SOCE [22–26]. Peel et al. have demonstrated that SOCE is mediated by STIM1 and Orai1 in human ASM cells [27,28]. However, whether STIM1 is involved in the mechanisms of ASM cell migration is still unknown.

This study was designed to investigate the role of STIM1 in the cell migration and the regulation of intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_{i}$) mediated by a strong chemoattractant, PDGF, in human ASM cells. We demonstrated that both STIM1 and Orai1 are essential for cell migration and elevation of [Ca$^{2+}$]$_{i}$ induced by PDGF in ASM cells.
Materials and Methods

Cell Culture

Primary cultures of normal human bronchial smooth muscle cells from multiple donors were obtained from Lonza (Walkersville, MD). The cells were maintained in culture medium containing 5% FBS, human recombinant epidermal growth factor (1 ng/ml), insulin (10 mg/ml), human recombinant fibroblast growth factor (2 ng/ml), gentamycin (50 mg/ml), and amphotericin B (0.05 mg/ml) (SmGM-2 BulletKit; Lonza) in an atmosphere of 5% CO₂ and 95% air at 37°C [12, 29, 30].

RT-PCR and Quantitative Real-Time PCR

Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) [17]. RNA was reverse transcribed to cDNA using a Superscript III kit (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) amplification was performed with 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The sequences of the forward and reverse primers, respectively, were STIM1: 5′-CCAGAGGCTCAGCCATAGT-3′ and 5′-CTTCACCA-CAGTCCCTGTC-3′, STIM2: 5′-GCTAAGGGAGGGAGCTGAAT-3′ and 5′-GCTAAGGGAGGGAGCTGAAT-3′, Orai1: 5′-TTCTTACGCTAGGTTGCTGT-3′ and 5′-AATCTCTCTCTCTCAGTGTCTG-3′, GAPDH: 5′-AAGCGATTTGTTGCTATGG-3′ and 5′-TGATGGTCCTGCAAC-GATACCA-3′. Product sizes of the STIM1, STIM2, Orai1, and GAPDH were 481bp, 498bp, 483bp and 498bp, respectively.

Quantitative PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) using the 5-stage program parameters provided by the manufacturer: 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative changes in each mRNA expression compared to an unstimulated control and normalized to GAPDH were quantified by the comparative Ct (2−ΔΔCt) method using Microsoft Excel 2010 [31]. TaqMan Gene Expression Assays for STIM1 (cat # Hs00396337_m1), STIM2 (cat # Hs00956219_m1), Orai1 (cat # Hs00385627_m1), and GAPDH (Hs99999905_m1) genes (Applied Biosystems) in a reaction volume of 20 μL, including 50 ng cDNA were performed.

Cell Transfections of siRNA

Targeting short interfering RNAs (siRNA) and the scrambled siRNA (negative control) were purchased from Invitrogen (Paisley, UK). Cells were transfected with 10 nM predesigned siRNA (Stealth Select RNAi) targeting STIM1, STIM2, and Orai1 or with 10 nM scrambled siRNA (negative control). Lipofectamine RNAiMAX (Invitrogen) was used as a transfection vector. To minimize the possibility of off target effects, three different siRNAs targeting either gene were used. Cells were used for PCR, Western blotting, Ca²⁺ measurement, and migration assays 48 h after siRNA transfection.

Western Blotting

Protein concentrations of cellular lysates were measured by using a protein assay reagent kit (Bio-Rad, Hercules, CA). Equal amounts of lysates, adjusted for protein concentrations, were resolved by SDS-PAGE using a 5–20% linear gradient running gel (Wako, Osaka, Japan). Proteins were transferred to nitrocellulose membranes, and the membranes were blocked in 5% skim milk for 2 h at room temperature, followed by overnight incubation at 4°C with primary antibodies. The membranes were incubated for 1 h at room temperature with a sheep anti-mouse or donkey anti-rabbit secondary antibody. The primary antibodies used were a mouse anti-GOK/Stim1 antibody (BD Biosciences, CA), a rabbit anti-STIM2 antibody (Abcam, Tokyo, Japan), and a mouse anti-Orai1 antibody (Sigma-Aldrich, St. Louis, MO). A polyclonal anti-actin antibody (Sigma-Aldrich) was used for the loading control. Detection was performed with an Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ) [12, 31].

Measurement of Intracellular Ca²⁺ Concentrations

Cells (approximately 50% confluence) grown on glass coverslips (Lab-Tek; Nunc, Rochester, NY) were treated with 3 μM fura-2/AM (Dojin, Kumamoto, Japan) for 25 min at 37°C in normal physiological solution containing (in mM): NaCl 145, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 10, and HEPES 10 (pH 7.40). After the cells were washed with normal physiological solution, the [Ca²⁺], was assessed by the fluorescence of fura-2 using a fluorescence microscope (Fluor20; Nikon, Tokyo, Japan) at room temperature. Data were analyzed using a digital fluorescence imaging system (Aquaicosmos; Hamamatsu Photonics, Hamamatsu, Japan). The excitation wavelengths were set at 340 and 380 nm, and the emission was collected at 510 nm by a photomultiplier. The intensity of the fura-2 fluorescence due to excitation at 340 nm (F340) and 380 nm (F380) was measured after subtraction of the background fluorescence, and the ratio of F340 to F380 (F340/F380 ratio) was used as an indicator of the relative level of [Ca²⁺]. [17, 29]. The fura-2 fluorescence of 7–10 cells per field was analyzed by using individual regions of interest for each experiment and “n” refers to numbers of experiments tested.

Cell Migration Assay

Cell migration was measured using a modified Boyden chamber (Chemotaxicell; Kurabo, Osaka, Japan). Chambers with 8-μm pores were coated with type-1 collagen (Nitta Gelatin Inc., Osaka, Japan). Confluent ASM cells were brought to a quiescent state overnight by incubation in DMEM/F-12 cell culture medium (Invitrogen) containing 0.1% FBS before being used in a migration assay. Cells (2×10⁴) suspended in 400 μL of DMEM/F-12 containing 0.1% FBS were placed in the upper chamber. PDGF-BB (Sigma-Aldrich) dissolved in DMEM/F-12 containing 0.1% FBS was inserted in the wells of the lower chamber. The cells were transferred to the upper wells, and after incubation for 6 h at 37°C in a 5% CO₂ incubator, the non-migrated cells on the upper membranes, and the membranes were blocked in 5% skim milk for 2 h at room temperature, followed by overnight incubation at 4°C with primary antibodies. The membranes were incubated for 1 h at room temperature with a sheep anti-mouse or donkey anti-rabbit secondary antibody. The primary antibodies used were a mouse anti-GOK/Stim1 antibody (BD Biosciences, CA), a rabbit anti-STIM2 antibody (Abcam, Tokyo, Japan), and a mouse anti-Orai1 antibody (Sigma-Aldrich, St. Louis, MO). A polyclonal anti-actin antibody (Sigma-Aldrich) was used for the loading control. Detection was performed with an Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ) [12, 31].

Statistical Analysis

All data are expressed as means ± SD. Analysis of variance followed by the Bonferroni test for post hoc analysis or t-test was used to evaluate the statistical significance. SigmaPlot11.0; Systat Software Inc., San Jose, CA). P<0.05 was considered statistically significant.

Results

Expression of STIM1, STIM2, and Orai1 in Human Airway Smooth Muscle Cells

We initially measured the expression of STIM1, STIM2, and Orai1 in human ASM cells. Expression of STIM1, STIM2, and
Orai1 mRNAs assessed by RT-PCR is shown in Figure 1A. Next, the cells were transfected with siRNA sequences for STIM1 (siSTIM1), STIM2 (siSTIM2), or Orai1 (siOrai1). Real-time quantitative PCR data showed that transfection of siSTIM1, siSTIM2, and siOrai1 induced a large decrease in mRNA levels of target genes without altering mRNA levels of non-target genes (Figure 1B). Average mRNA levels for STIM1, STIM2, and Orai1 normalized to GAPDH in the cells transfected with siRNAs were 3.0%, 4.2%, and 0.6%, respectively (n = 4, P < 0.001 vs. scrambled siRNA) (Figure 1B). Three different siRNAs targeting the same gene were tested and gave similar mRNA expression results (data not shown). These findings demonstrate that the siRNAs used in the present study had no off-target effects. The effects of siRNA transfection on protein levels of STIM1, STIM2, and Orai1 were assessed by Western blotting. Transfection with siRNAs for STIM1, STIM2, and Orai1 inhibited protein expression of STIM1, STIM2, and Orai1, respectively (Figure 1C, 1D, 1E). STIM1 protein expression as assessed by the STIM1/actin ratio was significantly lower in the cells transfected with siSTIM1 than the control cells transfected with scrambled siRNA (n = 3, P < 0.001) (Figure 1C). Similarly, the STIM2/actin ratio and Orai1/actin ratio were significantly lower in the cells transfected with siSTIM2 (n = 3, P < 0.001) (Figure 1D) and siOrai1 (n = 3, P < 0.001) (Figure 1E) than the control cells transfected with scrambled siRNA.

**Store-Operated Ca2+ Entry Induced by Thapsigargin**

It is established that thapsigargin, an inhibitor of SR Ca2+-ATPase (SERCA), induces SOCE by depleting SR Ca2+-stores in ASM cells [11]. When 5 μM thapsigargin (Calbiochem, La Jolla, CA) was applied to the cells in the nominally Ca2+-free solution, a transient increase in the F340/F380 ratio, a measure of [Ca2+]i, due to Ca2+-release from the SR Ca2+-stores was observed (Figure 2A and 2B). When 2 mM Ca2+ was added to the extracellular solution, the F340/F380 ratio was quickly increased to approximately 1.0 and then dropped to 0.6–0.7 and sustained (Figure 2A). This sustained increase (plateau phases) in the F340/F380 ratio was abolished by replacing the extracellular solution with Ca2+-free solution or application of 2 mM EGTA (n = 6, P < 0.001) (Figure 2A and 2B), demonstrating that the second phase was due to sustained activation of SOCE.

**Role of STIM1 and Orai1 in Store-Operated Ca2+- Entry**

Next, we assessed the role of STIM1, STIM2, and Orai1 in SOCE in human ASM cells. The effects of a knockdown of STIM1, STIM2, and Orai1 genes with siRNA on SOCE were examined. Representative records of the effects of 5 μM thapsigargin on the F340/F380 ratio in cells transfected with siSTIM1 and siOrai1 are shown in Figure 3A and 3B, respectively. The transient increase in F340/F380 ratio in the nominally Ca2+-free solution due to Ca2+-release was still observed in the cells transfected with siSTIM1 (Figure 3A) or siOrai1 (Figure 3B). In contrast, the second increase in F340/F380 ratio due to SOCE was strongly inhibited by siSTIM1 transfection (Figure 3A). The rapid increase in the F340/F380 ratio was not observed (Figure 3A) and the F340/F380 ratio of the plateau phase was significantly lower in siSTIM1-transfected cells (n = 6, P < 0.001) (Figure 3C). Similarly, SOCE activated by 5 μM thapsigargin was significantly inhibited in the cells transfected with siOrai1 (n = 6, P < 0.001) (Figure 3B and 3C). Moreover, simultaneous transfection with siSTIM1 and siOrai1 also significantly inhibited SOCE by 5 μM thapsigargin (n = 6, P < 0.001) (Figure 3C). The increase in the F340/F380 due to SOCE (plateau phase) was not affected by siSTIM2 or scrambled siRNA (n = 6) (Figure 3C).

**Effects of PDGF on Intracellular Ca2+ Concentrations**

Effects of PDGF-BB on [Ca2+]i, were investigated. Application of PDGF-BB (10 ng/mL) to the normal physiological solution containing 2 mM Ca2+ induced a transient increase in the F340/F380 ratio, followed by a sustained increase in the F340/F380 ratio (Figure 4A). Application of EGTA (2 mM) abolished the sustained increase in the F340/F380 ratio by PDGF-BB (Figure 4A). PDGF-BB transiently increased the F340/F380 ratio in nominally Ca2+-free solution (Figure 4B). However, the increase in the F340/F380 ratio by application of PDGF-BB returned to the baseline level in Ca2+-free solution (Figure 4B). There was no significant difference between peak F340/F380 ratios elicited by PDGF-BB in the cells in the normal (control) and nominally Ca2+-free solutions (n = 6) (Figure 4C). The sustained increases in the F340/F380 ratios by application of PDGF-BB were significantly lower in the nominally Ca2+-free solution or the normal solution with 2 mM EGTA than those in the normal solution (control) (n = 6, P < 0.001) (Figure 4D). These results indicate that the initial transient increase and the subsequent sustained phase (plateau phase) are due to Ca2+-release from the SR and Ca2+ influx from the extracellular side, respectively.

**Role of STIM1 in PDGF-Induced Intracellular Ca2+ Elevation**

We next evaluated whether STIM1 and Orai1 mediate increases in [Ca2+]i by PDGF-BB. The transient increase in the F340/F380 ratio induced by PDGF-BB was still observed (Figure 5A) but the F340/F380 ratio gradually decreased close to the baseline level within 5 min after PDGF-BB application in the cells transfected with siSTIM1 (Figure 5A). Similarly, PDGF-BB induced the transient increase in the F340/F380 ratio in the cells transfected with siOrai1 (Figure 5B). In contrast, the PDGF-BB-induced elevation of the F340/F380 ratio was not reduced by siSTIM2 transfection (Figure 5C). The peak F340/F380 ratio was not affected by transfection with siSTIM1, siOrai1, or siSTIM2 (n = 6) (Figure 5D). The F340/F380 ratio in the plateau phase was significantly lower in the siSTIM1- or siOrai1-transfected cells than in the cells transfected with scrambled siRNA (negative control) (n = 6, P < 0.001) (Figure 5E). Simultaneous transfection with siSTIM1 and siOrai1 also significantly inhibited the PDGF-BB-induced increase in the F340/F380 ratio (n = 6, P < 0.05) (Figure 5E) without affecting the peak F340/F380 ratio (Figure 5D), similar to transfection with siSTIM1 or siOrai1 alone. In contrast, the PDGF-BB-induced elevation of the F340/F380 ratio was not inhibited by siSTIM2 (n = 6) (Figure 5E).

**Roles of STIM1 and Orai1 in Cell Migration Induced by PDGF**

The roles of STIM1 and Orai1 in PDGF-induced cell migration were investigated using a chemotaxis assay. Migrating cell numbers were significantly increased by treatment with PDGF-BB (10 ng/mL, 6 h) compared with time-matched control cells (n = 6, P < 0.001) (Figure 6A). Moreover, cell migration induced by PDGF-BB (10 ng/mL, 6 h) was also significantly inhibited by 1 mM EGTA (n = 6, P < 0.001) (Figure 6A). There was no significant difference in baseline cell migration (in cell culture media with 0.1% FBS, 6 h) between the control and EGTA-treated cells (Figure 6A).

Next, cells were transfected with siRNAs targeting STIM1, STIM2, Orai1, or the negative control (scrambled siRNA). Transfection with siSTIM1, siOrai1, or both siSTIM1 and siOrai1 significantly inhibited PDGF-BB-induced cell migration (n = 6, P < 0.001 vs. scrambled siRNA) (Figure 6B). In contrast,
Figure 1. Expression of STIM1, STIM2, and Orai1. A: Expression of STIM1, STIM2, Orai1, and GAPDH mRNAs detected by RT-PCR in human airway smooth muscle (ASM) cells is shown. Negative indicates a negative control. The product sizes for STIM1, STIM2, Orai1, and GAPDH were 481bp, 498bp, 483bp, and 498bp, respectively. B: Effects of siRNA-targeted knockdown of STIM1, STIM2, and Orai1 mRNAs on the change in mRNA expression over control normalized to the reference gene GAPDH are shown (n = 4). Changes in mRNA expression were assessed by quantitative real-time PCR. Effects of siRNA transfection targeting STIM1 (siSTIM1) (C), STIM2 (siSTIM2) (D), and Orai1 (siOrai1) (E) on changes in protein levels were assessed by Western blot. STIM1, STIM2, and Orai1 protein levels expressed as the target protein/actin ratio in the cells transfected with siSTIM1 (C), siSTIM2 (D), or siOrai1 (E) and scrambled siRNA (negative control) are compared (n = 3). The control value without siRNA transfection is defined as 100%. *Significantly different from the values of the scrambled siRNA condition (P<0.05). Bars represent means ± SD.

doi:10.1371/journal.pone.0045056.g001
siSTIM2 or scrambled siRNA did not affect the PDGF-induced cell migration (n = 6) (Figure 6B).

Discussion

This study highlights the novel role of STIM1 in migration of ASM cells. The main findings are that (1) SOCE activation by thapsigargin was inhibited by siRNAs targeting STIM1 and Orai1, (2) a sustained increase in [Ca\(^{2+}\)]\(\text{i}\) induced by PDGF-BB was also inhibited by siSTIM1 and siOrai1, (3) STIM1 and Orai1 were essential for PDGF-induced ASM cell migration, and (4) STIM2 was not involved in these mechanisms. To our knowledge, this is the first report which demonstrates an essential role of STIM1 and Orai1 in migration and increases in [Ca\(^{2+}\)]\(\text{i}\), induced by PDGF in human ASM cells.

STIM1 was identified as the key molecule for SOCE [23,25]. STIM1 predominantly exists in the SR or ER and has its N-terminal sensing Ca\(^{2+}\) domain in the SR/ER lumen and its C-terminal Orai1 coupling site in the cytosol [32]. When the amount of Ca\(^{2+}\) contents within the SR or ER is decreased, STIM1 rapidly forms oligomers and activates Orai1, a highly Ca\(^{2+}\)-selective plasma-membrane cation channel [24,26]. It has been reported that STIM1 and Orai1 regulate SOCE in human and rat ASM cells [21,27,28]. In our results, SOCE activated by thapsigargin was inhibited by siSTIM1 and siOrai1. In contrast, STIM2 is not involved in the regulation of SOCE in human ASM cells despite its expression (Figures 1 and 3). These are consistent with the findings reported by Peel et al. [27]. In human myoblasts, STIM2 regulates SOCE similarly to STIM1 [33]. Thus, the discrepancy in the role of STIM2 in the regulation of SOCE arises from the difference in cell types.

Figure 2. Store-Operated Ca\(^{2+}\) Entry Induced by Thapsigargin.

Store-operated Ca\(^{2+}\) entry (SOCE) activated by thapsigargin. A: Representative traces of the F\(_{340}/F_{380}\) ratio, a measure of intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(\text{i}\)), by 5 \(\mu\)M thapsigargin (TPG). After the cells were treated with 5 \(\mu\)M thapsigargin in the nominally Ca\(^{2+}\)-free solution, 2 mM Ca\(^{2+}\) was added to the solution. At the end, 2 mM EGTA was added. B: The F\(_{340}/F_{380}\) ratios in nominally Ca\(^{2+}\)-free solution (Ca\(^{2+}\)-free), in response to 5 \(\mu\)M thapsigargin in the Ca\(^{2+}\)-free solution due to Ca\(^{2+}\) release, in the normal solution containing 2 mM Ca\(^{2+}\) with thapsigargin due to SOCE (the plateau phase), and in the normal solution with thapsigargin and 2 mM EGTA (SOCE+EGTA). Bars represent the means \(\pm\) SD (n = 6). Significantly different from values in the Ca\(^{2+}\)-free solution (*) and of SOCE (#) (P<0.05).

doi:10.1371/journal.pone.0045056.g002

Figure 3. Role of STIM1 and Orai1 in Store-Operated Ca\(^{2+}\) Entry.

Roles of STIM1 and Orai1 in SOCE. Representative changes in the F\(_{340}/F_{380}\) ratio due to 5 \(\mu\)M thapsigargin (TPG) in cells transfected with siRNA targeting STIM1 (A) and Orai1 (B). After the cells were treated with thapsigargin in the nominally Ca\(^{2+}\)-free solution, 2 mM Ca\(^{2+}\) was added to the solution. C: The F\(_{340}/F_{380}\) ratios in response to 5 \(\mu\)M thapsigargin in the normal solution due to SOCE with or without (control) siRNA treatment. The cells transfected with scrambled siRNA, siSTIM1, siOrai1, both siSTIM1 and Orai1, or siSTIM1. Bars represent the means \(\pm\) SD (n = 6). *Significantly different from the values of the cells transfected with scrambled siRNA (P<0.05).

doi:10.1371/journal.pone.0045056.g003
In the present study, increases in \([Ca^{2+}]_i\) due to PDGF-BB were significantly inhibited by knockdown of STIM1 and Orai1 with siRNA in human ASM cells (Figure 5). PDGF binds to PDGF receptors, members of receptor tyrosine kinases, which involve \(\alpha\) and \(\beta\) subtypes [34]. PDGF-BB activates \(\alpha\) and \(\beta\) receptors both of which are expressed in human ASM cells [34,35]. It is known that PDGF receptors cause phospholipase C\(\gamma\) activation and intracellular \(Ca^{2+}\) mobilization [34,36]. Indeed, stimulation of human ASM cells by PDGF-BB transiently elicited elevation of \([Ca^{2+}]_i\) even in the \(Ca^{2+}\)-free solution (Figure 4), demonstrating that PDGF-BB induces \(Ca^{2+}\) release from the SR. This transient increase of \([Ca^{2+}]_i\) was still observed in the cells transfected with siSTIM1 or siOrai1 (Figure 5). Therefore, STIM1 and Orai1 are not involved in the mechanisms of \(Ca^{2+}\) release from the SR via IP\(3\) receptors. In contrast, the sustained increase of \([Ca^{2+}]_i\) due to PDGF-BB was abolished in the \(Ca^{2+}\)-free solution and largely inhibited by siSTIM1 and siOrai1 (Figures 4 and 5). These findings demonstrate that STIM1 and Orai1 regulate the \(Ca^{2+}\) influx pathway for the sustained \([Ca^{2+}]_i\) elevation by PDGF-BB in human ASM cells. It has been reported that SOCE mediated by STIM1/Orai1 is crucial in the PDGF-induced increase of \([Ca^{2+}]_i\) in vascular smooth muscle cells [37–39], consistent with our findings in ASM cells.

Recently, several reports have shown that STIM1 and Orai1 are also involved in mechanisms of \(Ca^{2+}\) influx independent of \(Ca^{2+}\) stores. Xiao et al. demonstrated that heating to above 40°C induces clustering of STIM1 without depleting \(Ca^{2+}\) stores in Jurkat T cells [40]. Following cooling the cell off to 25°C, Orai1 was activated [40]. However, in our experimental conditions, it is unlikely that such temperature change-dependent activation of STIM1 and Orai1 is involved in the PDGF-induced \(Ca^{2+}\) influx. In a study by Liu et al. [41], \(Ca^{2+}\) influx via reverse mode Na\(^+\)/\(Ca^{2+}\) exchange in
Ca\(^{2+}\) exchange (NCX) was regulated by SR Ca\(^{2+}\) store depletion and STIM1 in human ASM cells. In their report, the histamine-induced increase of [Ca\(^{2+}\)]\(_i\) was partially inhibited by the reverse mode NCX inhibitor KB-R7943 \[41\]. In our preliminary results, PDGF-induced migration of human ASM cells was not significantly inhibited by KB-R7943 (data not shown). These observations suggest that contribution of the reverse mode NCX to PDGF-induced Ca\(^{2+}\) influx is much less than that of Orai1 in ASM cells. In another report by Feng et al. \[42\], Orai1 was activated by Secretory Pathway Ca\(^{2+}\)-ATPase 2 (SPCA2) independently of ER Ca\(^{2+}\) stores or STIM1 in breast cancer cells. By contrast to their results, we demonstrated that siOrai1 blocked the thapsigargin-induced SOCE and PDGF-induced [Ca\(^{2+}\)]\(_i\) elevation (Figures 3 and 5). Moreover, simultaneous transfection with siSTIM1 and siOrai1 did not have additive effects on the PDGF-induced [Ca\(^{2+}\)]\(_i\) elevation (Figure 5). Therefore, SPCA2-dependent, STIM1-independent Orai1 activation is not likely involved in the PDGF-induced influx by PDGF-BB. Taken together, the PDGF-induced increase of [Ca\(^{2+}\)]\(_i\) is mostly via SOCE in human ASM cells. Nevertheless, possible involvement of store-independent mechanisms in PDGF-induced [Ca\(^{2+}\)]\(_i\) elevation cannot be ruled out.

Activation of PDGF receptors strongly promotes migration of ASM cells \[2,7,34\]. We found that transfection with siSTIM1, siOrai1, or both reduced the PDGF-induced migration of human ASM cells as assessed by the chemotaxis assay (Figure 6). It was determined that STIM1 and Orai1 regulate PDGF-evoked migration in a wound-healing assay using vascular smooth muscle.

---

**Figure 5. Role of STIM1 in PDGF-Induced Intracellular Ca\(^{2+}\) Elevation.** Roles of STIM1 and Orai1 in [Ca\(^{2+}\)]\(_i\) elevation induced by PDGF-BB. Representative changes in the F\(_{340}/F_{380}\) ratios with 10 ng/mL PDGF-BB in the cells transfected with siSTIM1 (A) and siOrai1 (B), and siSTIM2 (C) are shown. Transient (peak) (D) and sustained increases (plateau phase) (E) in the F\(_{340}/F_{380}\) ratio in response to PDGF-BB with or without (control) siRNA treatment are compared. Bars represent the means ± SD (n = 6). *Significantly different from the values of the control cells treated with 10 ng/mL PDGF-BB plus scrambled siRNA (P < 0.05).

doi:10.1371/journal.pone.0045056.g005
Figure 6. Roles of STIM1 and Orai1 in Cell Migration Induced by PDGF. Roles of STIM1 and Orai1 in cell migration induced by PDGF-BB (10 ng/mL, 6 h) are shown. Cell migration was assessed by a chemotaxis assay. A: Effects of PDGF-BB on migrated cell numbers with or without (control) 1 mM EGTA treatment (n = 6). Baseline (black column) denotes the time-matched number of cells that migrated without PDGF-BB treatment. Significantly different from the values of the baseline (*) and by PDGF-BB alone (#) (P < 0.05). B: Effects of siRNA treatment targeting STIM1, STIM2, Orai1, and both STIM1 and Orai1 on migrating cell numbers induced by PDGF-BB (n = 6). *Significantly different from the values of the time-matched control cells treated with PDGF-BB plus scrambled siRNA (negative control, NC) (P < 0.05). Bars represent means ± SD.

doi:10.1371/journal.pone.0045056.g006

cells [37,39]. Furthermore, both STIM1 and Orai1 were implicated in cell migration in breast cancer cells and cervical cancer cells [43,44]. Zou et al. demonstrated the involvement of STIM1 and Orai1 in the cell proliferation evoked by PDGF-BB in rat ASM cells [21]. These previous findings using different cell types and species also support our results. Taken together, STIM1 and Orai1 regulate PDGF-induced migration of human ASM cells. In contrast, STIM2 does not contribute to the migration or [Ca²⁺]i elevation evoked by PDGF-BB in human ASM cells.

Migration of ASM cells is not only essential for development of hollow airways and the respiratory system but also important for airway remodeling in asthma [3,4]. Nevertheless, the mechanisms of ASM cell migration are not fully elucidated yet. It has been proposed that the dynamics of the cytoskeleton and multiple signal transduction pathways, including intracellular Ca²⁺ signaling, are involved in processes of cell motility and migration [3,36,45,46]. It is well established that activation of myosin light-chain kinase (MLCK) and subsequent myosin light chain phosphorylation is the main downstream pathway of [Ca²⁺]i elevation in smooth muscle contraction [47]. In contrast, Carlin et al. demonstrated that PDGF-induced cell migration was not inhibited by MLCK inhibitors in human ASM cells [3]. Essentially the same results were observed in our preliminary experiments (data not shown). Thus, it is unlikely that MLCK is involved in the mechanism of ASM cell migration enhanced by PDGF. Further studies are necessary to identify the downstream pathways of SOCE in PDGF-induced ASM cell migration.

Because SOCE is a major influx pathway both in muscle and non-muscle cells, STIM1 and Orai1 proteins play critical roles in homeostasis of the immune system and normal development. Mutations in STIM1 and Orai1 genes are clinically characterized by severe immunodeficiency and congenital myopathy in human patients [48]. Mice lacking STIM1 or Orai1 gene die perinatally of unknown causes [48]. On the other hand, involvement of STIM1 and Orai1 in the pathogenesis of several diseases has also been reported [48]. Baba et al. reported that STIM1 is essential for mast cell activation and immunoglobulin E-mediated anaphylactic responses in mice [49]. Another possibility is that SOCE mediated by STIM1 and Orai1 is involved in the pathophysiology of cardiovascular diseases. In rat carotid artery, vascular smooth muscle cell proliferation in vitro and neointima formation after balloon injury in vivo were significantly inhibited by a knockdown of the STIM1 gene [50]. Bisallon et al. demonstrated that mRNA levels of STIM1 and Orai1 were upregulated in a balloon-injured carotid artery compared with the control in rat models [37]. Therefore, SOCE is likely to contribute to vascular remodeling. ASM cells are the main effector cells of airway narrowing in asthma [51]. Recently, Sathish et al. found that both STIM1 and Orai1 are upregulated by TNF-α in human ASM cells [52]. Because the present and previous results have demonstrated that SOCE tightly regulates the contraction, proliferation, and migration of ASM cells [11,14,19,21], STIM1 and Orai1 may be involved in mechanisms of the pathophysiology of airway diseases such as asthma and COPD.

In summary, STIM1 and Orai1, key molecules for SOCE, regulate PDGF-induced migration and Ca²⁺ influx of human ASM cells. Our findings suggest that STIM1 and Orai1 may be important molecules responsible for airway remodeling in asthma.

Acknowledgments

We thank Ms. Katherine Ono for providing language help.

Author Contributions

Conceived and designed the experiments: SI YH. Performed the experiments: NS SI HA MK M. Sato. Analyzed the data: NS SI. Contributed reagents/materials/analysis tools: NS SI MK M. Sato. Wrote the paper: NS SI M. Sokabe.

References

1. Lazaar AL, Panettieri RA Jr (2003) Airway smooth muscle: a modulator of airway remodeling in asthma. J Allergy Clin Immunol 116: 488–495.
2. Carlin SM, Roth M, Black JL (2003) Urokinase potentiates PDGF-induced chemotaxis of human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 284: L1020–L1026.
3. Gerthoffer WT (2008) Migration of airway smooth muscle cells. Proc Am Thorac Soc 5: 97–105.
4. Goncharova EA, Goncharov DA, Krymskaya VP (2006) Assays for in vitro monitoring of human airway smooth muscle (ASM) and human pulmonary arterial vascular smooth muscle (VSM) cell migration. Nat Protoc 1: 2933–2939.
5. Hedges JC, Dechert MA, Yamboliev IA, Marin JL, Hickey E, et al. (1999) A role for p38MAPK/HSN27 pathway in smooth muscle cell migration. J Biol Chem 274: 24211–24219.

6. Joubrer F, Hamid Q (2003) Role of airway smooth muscle in airway remodeling. J Allergy Clin Immunol 116: 713–716.

7. Krymkaya VP, Goncharova EA, Ammit AJ, Lim PN, Goncharov DA, et al. (2005) Src is necessary and sufficient for human airway smooth muscle cell proliferation and migration. FASEB J 19: 420–430.

8. Madison JM (2003) Migration of airway smooth muscle cells. Am J Respir Cell Mol Biol 29: 8–11.

9. Parameswaran K, Cox G, Radford K, Janssen LJ, Schani R, et al. (2002) Cysteinyl leukotrienes promote human airway smooth muscle migration. Am J Respir Crit Care Med 166: 730–742.

10. James AL, Maxwell PS, Pearce-Pinto G, Elliot JG, Carroll NG (2002) The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. Am J Respir Crit Care Med 166: 1590–1595.

11. Ito S, Kume H, Yamaki K, Katoh H, Nishio H, et al. (2002) Regulation of capacitative and noncapacitative receptor-operated Ca\(^{2+}\) entry by Rho-kinase in tracheal smooth muscle. Am J Respir Cell Mol Biol 26: 491–498.

12. Iwata S, Ito S, Iwaki M, Kondo M, Sashio T, et al. (2009) Regulation of endothelin-1-induced interleukin-6 production by Ca\(^{2+}\) influx in human airway smooth muscle cells. Eur J Pharmacol 605: 15–22.

13. Perez-Zoghbi JF, Karrier C, Ito S, Shepherd M, Alraishdan Y, et al. (2009) Ion channel regulation of intracellular calcium and airway smooth muscle function. J Pharmacol Ther 32: 398–407.

14. Sweeney M, McDaniel SS, Platoshyn O, Zhang S, Yu Y, et al. (2002) Role of the N-type calcium channel in airway smooth muscle. Respir Res 7: 119.

15. Putney JW Jr (1986) A model for receptor-regulated calcium entry. Cell Calcium 9: 223–235.

16. Ay B, Prakash YS, Pabelick CM, Sieck GC (2004) Store-operated Ca\(^{2+}\) influx in human pulmonary microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol L265–L273, 2012.

17. Ito S, Suki B, Kume H, Numaguchi Y, Ishii M, et al. (2010) Actin cytoskeleton regulation in epithelial cells. Am J Physiol Lung Cell Mol Physiol 43: 26–34.

18. Ito S, Suki B, Kume H, Numaguchi Y, Ishii M, et al. (2010) Actin cytoskeleton regulates stretch-activated Ca\(^{2+}\) influx in human pulmonary microvascular endothelial cells. Am J Respir Cell Mol Biol 43: 26–34.

19. Zou JJ, Gao YD, Geng S, Yang J (2011) Role of STIM1/Orai1-mediated store-operated Ca\(^{2+}\) influx in airway smooth muscle. Eur J Pharmacol 650: 15–22.

20. Peel SE, Liu B, Hall IP (2010) Reverse mode Na\(^{+}\)/Ca\(^{2+}\) exchange mediated by STIM1 contributes to Ca\(^{2+}\) influx in airway smooth muscle following stimulation. Respir Res 11: 168.

21. Deng X, Wang Y, Zhou Y, Soboloff J, Gill DL (2009) STIM and Orai: dynamic intermembrane coupling to control cellular calcium signals. J Biol Chem 284: 22437–22447.

22. Heldin CH, Westmark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79: 1213–1316.

23. Bonner JC, Badgett A, Landroos PM, Coin PG (1996) Basic fibroblast growth factor induces expression of the PDGF receptor-alpha on human bronchial smooth muscle cells. Am J Physiol 271: L480–L486.

24. Wei C, Wang X, Chen M, Ouyang K, Song LS, et al. (2009) Calcium flickers steer cell migration. Nature 457: 901–905.

25. Bisalou JM, Motiani RR, Gonzalez-Cobos JC, Potier M, Halligan KE, et al. (2010) Essential role for STIM1/Orai-mediated calcium influx in PDGF-induced smooth muscle migration. Am J Physiol Cell Physiol 298: C993–C1005.

26. Li J, McKown L, Ojebi O, Stacey M, Foster R, et al. (2011) Nanomolar potency and selectivity of a Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel inhibitor against store-operated Ca\(^{2+}\) entry and migration of vascular smooth muscle cells. Br J Pharmacol 164: 382–393.

27. Potier M, Gonzalez JC, Motiani RK, Abdullah I, Bisalou JM, et al. (2009) Evidence for STIM1- and Orai-dependent store-operated calcium influx through ICRAC in vascular smooth muscle cells: role in proliferation and migration. FASEB J 23: 2425–2437.

28. Xiao B, Coste B, Madhur J, Patapoutian A (2011) Temperature-dependent STIM1 activation induces Ca\(^{2+}\) influx and modulates gene expression. Nat Chem Biol 7: 351–358.

29. Liu B, Peel SE, Fox J, Hall IP (2010) Reverse mode Na\(^{+}\)/Ca\(^{2+}\) exchange regulated by STIM1 contributes to Ca\(^{2+}\) influx in airway smooth muscle following stimulation. Respir Res 11: 168.

30. Feng M, Grice DM, Faddy HM, Nguyen N, Leitch S, et al. (2010) Store-independence of Orai by SCPC2A in mammary tumors. Cell 143: 84–98.

31. Chen YF, Chia WT, Chen YT, Lin PY, Huang HJ, et al. (2011) Calcium store sensor stromal-interaction molecule 1-dependent signaling plays an important role in cervical cancer growth, migration, and angiogenesis. Proc Natl Acad Sci U S A 108: 15225–15230.

32. Yang S, Zhang JH, Huang XY (2009) Orai1 and STIM1 are critical for breast tumor cell migration and metastasis. Cancer Cell 15: 124–134.

33. Brundage RA, Fogarty KE, Tuft RA, Fay FS (1991) Calcium gradients following agonist stimulation. Respir Res 11: 168.

34. Feske S (2010) CRAC channelopathies. Phaeths Arbor 467: 411–435.

35. Baba Y, Nishida K, Fujii Y, Hirano T, Hikida M, et al. (2008) Essential function of p38(MAPK)/HSP27 pathway in smooth muscle cell migration. J Biol Chem 283: 22501–22505.

36. Zuyderduyn S, Sukkar MB, Fust A, Dhaliwal S, Burgess JK (2008) Treating asthma using anti-SCPC2A. Pulm Pharmacol Ther 21: 343–348.

37. Weber SE, Boulanger CM, Dikalov SI, Constantine NG, Weihrauch J, et al. (2009) Caveolin-1 regulation of store-operated Ca\(^{2+}\) influx in human airway smooth muscle. Eur Respir J 39: 1471–1479.