Orai1-Mediated I_{CRAC} Is Essential for Neointima Formation After Vascular Injury

Wei Zhang, Katharine E. Halligan, Xuexin Zhang, Jonathan M. Bisaillon, José C. Gonzalez-Cobos, Rajender K. Motiani, Guoqing Hu, Peter A. Vincent, Jiliang Zhou, Margarida Barroso, Harold A. Singer, Khalid Matrougui, Mohamed Trebak

Rationale: The molecular correlate of the calcium release-activated calcium current (I_{CRAC}), the channel protein Orai1, is upregulated in proliferative vascular smooth muscle cells (VSMC). However, the role of Orai1 in vascular disease remains largely unknown.

Objective: The goal of this study was to determine the role of Orai1 in neointima formation after balloon injury of rat carotid arteries and its potential upregulation in a mouse model of VSMC remodeling.

Methods and Results: Lentiviral particles encoding short-hairpin RNA (shRNA) targeting either Orai1 (shOrai1) or STIM1 (shSTIM1) caused knockdown of their respective target mRNA and proteins and abrogated store-operated calcium entry and I_{CRAC} in VSMC; control shRNA was targeted to luciferase (shLuciferase). Balloon injury of rat carotid arteries upregulated protein expression of Orai1, STIM1, and calcium–calmodulin kinase IIdelta2 (CamKIIδ2); increased proliferation assessed by Ki67 and PCNA and decreased protein expression of myosin heavy chain in medial and neointimal VSMC. Incubation of the injured vessel with shOrai1 prevented Orai1, STIM1, and CamKIIδ2 upregulation in the media and neointima; inhibited cell proliferation and markedly reduced neointima formation 14 days post injury; similar results were obtained with shSTIM1. VSMC Orai1 and STIM1 knockdown inhibited nuclear factor for activated T-cell (NFAT) nuclear translocation and activity. Furthermore, Orai1 and STIM1 were upregulated in mice carotid arteries subjected to ligation.

Conclusions: Orai1 is upregulated in VSMC during vascular injury and is required for NFAT activity, VSMC proliferation, and neointima formation following balloon injury of rat carotids. Orai1 provides a novel target for control of VSMC remodeling during vascular injury or disease. (Circ Res. 2011;109:534-542.)

Key Words: calcium channels | CRAC channels | vascular smooth muscle proliferation | neointima formation
We and others have demonstrated that Orai1 and STIM1 are upregulated in synthetic proliferative VSMCs.\textsuperscript{9,20} Data from our laboratory showed that either STIM1 or Orai1 knockdown using silencing RNA (siRNA) inhibited SOCE, \( I_{\text{CRAC}} \), and synthetic VSMC proliferation and migration in response to serum and platelet-derived growth factor (PDGF).\textsuperscript{9,21} We also showed that Orai1 and STIM1 were upregulated in medial and neointimal VSMC from rat carotids after balloon injury,\textsuperscript{21} suggesting that these proteins are involved in vascular pathophysiology. Interestingly, 2 recent reports have specifically addressed the role of STIM1 in VSMC remodeling in vivo and independently reported that STIM1 in vivo knockdown inhibited neointima formation after balloon injury in rat carotids.\textsuperscript{22,23} However, the mechanism of action of STIM1 and the exact PM ion channel that mediates STIM1 function during VSMC remodeling in vivo is unclear. In addition to its role in activating Orai1-mediated \( I_{\text{CRAC}} \), STIM1 positively regulates the function of Orai2, Orai3, and Orai1/ Orai3 heteromultimeric channels in their store-operated mode\textsuperscript{24–27} and that of the store-independent Orai1/Oral3 arachidonate-regulated \( C_{a}^{2+} \) (ARC) channel\textsuperscript{28} as well as that of most TRPC channels.\textsuperscript{18,29,30} Furthermore, STIM1 has recently been shown to regulate the function of L-type \( C_{a}^{2+} \) channels in different cell types, including VSMCs.\textsuperscript{31,32}

The significance of Orai1 upregulation during vascular injury, its interdependence on STIM1, and its role in VSMC remodeling and neointima formation in vivo is an important question that has remained unanswered. Therefore, in this study we comparatively tested the role of Orai1 and STIM1 in VSMC proliferation and neointima formation in vivo using the rat carotid artery balloon-injury model. We found that either Orai1 or STIM1 in vivo knockdown is sufficient to inhibit VSMC proliferation and neointima formation to a similar extent. We show that Orai1 is required for nuclear translocation and activation of nuclear factor for activated T cells (NFAT) in VSMCs. Furthermore, we report that Orai1 and STIM1 upregulation is also a feature of another model of vascular remodeling: the carotid ligation in mice, highlighting the importance of Orai1-mediated \( I_{\text{CRAC}} \) in smooth muscle phenotypic switching and unraveling Orai1 as a potential target for vascular occlusive diseases.

**Methods**

All antibodies/reagents concentrations, dilutions, and origins are described in detail in the Online Data Supplement available at http://circres.ahajournals.org. Cell culture, generation of lentiviruses, and surgeries were conducted using standard procedures. An expanded material and methods section is presented in the Online Supplement.

**Results**

**VSMC Orai1 and STIM1 Knockdown Using Lentiviral Particles-Encoding shRNA Abrogates SOCE and \( I_{\text{CRAC}} \)**

ShRNA sequences targeting either Orai1 or STIM1 were cloned under the control of the H1 promoter in the lentiviral vector pFUGW-GFP, which separately encodes green fluorescent protein (GFP) under the control of the Ub-c promoter. Lentiviral particles were produced and used to infect synthetic cultured rat aortic VSMCs. These viral particles were efficient at infecting 90% to 95% of VSMCs, as visualized by GFP fluorescence (Figure 1A). Infection of VSMC with either shOrai1 or shSTIM1 viruses caused marked decrease in cell number (Figure 1A), consistent with previous studies demonstrating a role for Orai1/STIM1 in VSMC proliferation.\textsuperscript{9,21} Real-time PCR showed that shOrai1 viruses reduced their respective mRNA levels with no significant effect on STIM1 mRNA levels; similar results were obtained with shSTIM1 viruses (Figure 1B). Orai1 and STIM1 protein knockdown after shRNA treatment was documented by Western blotting (Figure 1C and 1D). ShOrai1 viruses reduced Orai1 mRNA and protein levels by \( 88.7\% \pm 0.76\% \) and \( 67.4\% \pm 9.34\% \), respectively, and shSTIM1 viruses reduced STIM1 mRNA and protein levels by \( 88.8\% \pm 0.53\% \) and \( 98.1\% \pm 0.66\% \), respectively; \( n = 3 \) (Figure 1B and 1D). Furthermore, shOrai1 had no significant effect on expression of Orai2 and Orai3 homologs as measured using real-time PCR (Supplemental Figure 1A), ruling out potential cross-effects of shOrai1 on Orai2 and Orai3. \( C_{a}^{2+} \) imaging was performed on selected VSMCs displaying strong GFP fluorescence, thus selecting only cells with almost complete knockdown. ShOrai1 and shSTIM1 viruses inhibited the extent of SOCE activated by thapsigargin in VSMC by \( 87.7\% \pm 2.38\% \) and \( 74.8\% \pm 2.98\% \), \( n = 21 \) and 19, respectively, in comparison with cells infected with shLuciferase lentivirus (Figure 1E and 1F); the remaining SOCE in cells infected with shSTIM1 viruses despite almost complete STIM1 protein downregulation is likely due to native STIM2, consistent with previous studies.\textsuperscript{10} The inhibition of SOCE on Orai1 knockdown could be effectively rescued by expressing siRNA-resistant cyan fluorescent protein (CFP)-Orai1 clone in VSMC (Supplemental Figure 1B and 1C). Previous studies from our laboratory and others have shown that Orai2 and Orai3 do not contribute to SOCE in rat and human VSMCs.\textsuperscript{9,21,33} Importantly, shOrai1 and shSTIM1 viruses dramatically inhibited \( I_{\text{CRAC}} \) (by comparison with shLuciferase) measured by whole cell patch clamp after passive store depletion with dialysis of 20 mmol/L of the chelator BAPTA through the patch pipette (Figure 1G through 1K). \( I_{\text{CRAC}} \) was measured in a bath
solution containing 20 mmol/L Ca^{2+} (Ca^{2+} I_{CRAC}) and was amplified using short pulses (30 seconds) of standard divalent-free (DVF; Na^{+} I_{CRAC}) solutions as described previously.\textsuperscript{,8,9} I_{CRAC} showed typical current depotentiation in DVF solutions (Figure 1G). ShOrai1 inhibited Ca^{2+} I_{CRAC} and Na^{+} I_{CRAC} by 90% and 95%, respectively [Na^{+} I_{CRAC} values are reported as Ca^{2+} I_{CRAC} since Ca^{2+} I_{CRAC} values are too small to be reliable: control, 0.39±0.02 pA/pF, n=3; shOrai1, 0.02±0.01 pA/pF, n=4 at −100 mV]. ShSTIM1 inhibited Ca^{2+} I_{CRAC} and Na^{+} I_{CRAC} by 92% and 96%, respectively [shSTIM1, 0.01±0.004 pA/pF (Na^{+} I_{CRAC}) at −100 mV; n=5 (Figure 1K)]. Current-voltage (I/V) relationships for Na^{+} I_{CRAC} and Na^{+} I_{CRAC} currents taken at −100 mV from several independent VSMCs infected with shLuciferase, shOrai1, or shSTIM1 lentiviruses were taken where indicated by the color-coded asterisks.

Rat Carotid Balloon Injury Causes Increased Orai1/STIM1 Protein Expression and VSMC Proliferation

We performed balloon injury on rat left carotids as described in Methods; right carotids were used as internal controls. Carotid injury causes significant neointima formation at 7 days.
days postinjury with peak neointima observed at 14 days postinjury (Figure 2A), consistent with previous studies.⁵³ Left injured and right control carotid arteries were isolated, sectioned, and subjected to immunofluorescence (IF) staining using Orai1, STIM1, Ki67, and myosin heavy chain (MHC) specific antibodies, as described in the Methods. Figure 2B shows IF staining on carotid arteries 7 days postinjury in which Orai1 and STIM1 proteins are clearly upregulated in neointimal VSMCs from left injured carotid arteries in comparison with right control carotids. IF staining also shows increased expression of the proliferative marker Ki67 and decreased expression of vascular smooth muscle cell (VSMC) contractile marker myosin heavy chain (MHC) in neointimal layers of balloon-injured left carotid artery sections in comparison with right noninjured control carotids. C, Western blotting on medial and neointimal VSMC from balloon-injured left carotid arteries in comparison with the medial layer from sham-operated arteries showing increased protein expression of Orai1, STIM1, and the proliferative marker proliferating cell nuclear antigen (PCNA) in balloon-injured left carotid arteries 14 days postinjury. A = adventitia; M = media; NI = neointima; L = lumen.

Figure 2. Orai1/STIM1 proteins and balloon carotid injury in rats. A, H&E staining of balloon-injured left carotid artery sections showing increased neointima as early as 7 days after injury in comparison with sham-operated arteries (intact). Neointima formation peaks at 14 days postinjury and is still prominent 21 days postinjury. B, IF staining with specific antibodies shows increased protein expression of Orai1 and STIM1 in neointima of left injured carotid arteries 7 days postinjury in comparison with right noninjured control carotids. C, Western blotting on medial and neointimal VSMC from balloon-injured left carotid arteries in comparison with the medial layer from sham-operated arteries showing increased protein expression of Orai1, STIM1, and the proliferative marker proliferating cell nuclear antigen (PCNA) in balloon-injured left carotid arteries 14 days postinjury.

Effects of in Vivo Infection With shOrai1 and shSTIM1 Lentiviruses on VSMC Proliferation and Protein Expression

A series of vascular injury experiments were conducted on left carotids of rats to evaluate the effect of in vivo knockdown of Orai1 and STIM1 on VSMC proliferation and protein expression. Left carotids were injured and treated
with either shLuciferase, shOrai1, or shSTIM1 lentiviruses for 30 minutes as outlined in the Methods; right carotids were neither injured nor treated with lentiviruses (NT) and were used throughout as internal controls. As shown in Figure 3A, left carotid arteries were successfully infected by lentiviruses as evidenced by positive expression of GFP; right noninfected carotids show no GFP expression. ShOrai1 inhibited upregulation of Orai1 protein as well as that of STIM1 and CamKII\textsubscript{δ2}, 14 days postinjury. Similarly, shSTIM1 inhibited upregulation of STIM1 protein as well as that of Orai1 and CamKII\textsubscript{δ2}. ShOrai1 and shSTIM1 inhibited VSMC proliferation as evidenced by reduced protein expression of proliferating cell nuclear antigen (PCNA) in medial and neointimal protein extracts from balloon-injured left carotids treated with shOrai1 and shSTIM1 lentiviruses in comparison with uninjured left carotids treated with shLuciferase lentiviruses. B, Statistical analyses on Orai1, STIM1, CamKII\textsubscript{δ2}, and PCNA protein expression data on extracts of medial and neointimal VSMC from balloon-injured left carotids and treated with shLuciferase, shOrai1, or shSTIM1 lentiviruses. Data represent densitometry on protein bands with average ±SEM from 6 rats per condition, determined using Image J and normalized to β-actin expression. C through D, IF using specific anti-Orai1 (C) and anti-STIM1 (D) antibodies on left carotid sections (14 days after injury) injured and treated with shLuciferase, shOrai1, or shSTIM1 lentiviruses (bottom) and corresponding right noninjured nontreated control carotids (top).
IF experiments using antibodies against Orai1, STIM1, and CamKIIβ2 were performed on right (control) and left carotids (injured and treated with shRNA), 14 days postinjury (Figure 3C and 3D). Orai1 (Figure 3C), STIM1 (Figure 3D), and CamKIIβ2 (Supplemental Figure III) protein expression are clearly increased in medial and neointimal VSMC from injured and shLuciferase-treated left carotids. Treatment with shOrai1 caused decrease protein expression of Orai1 (Figure 3C; see also Supplemental Figure IV), STIM1 (Figure 3D) and CamKIIβ2 (Supplemental Figure III) in medial and neointimal VSMC in comparison with shLuciferase-treated carotids; similar results were obtained when injured left carotids were treated with shSTIM1 viruses (Figure 3C and 3D). Secondary antibodies controls are shown in Supplemental Figure IV.

Orai1 and STIM1 in Vivo Knockdown Prevents Neointima Formation

Left carotid injury followed by in vivo lentiviral infection with shLuciferase, shOrai1, and shSTIM1 were performed as described above. Figure 4A shows H&E staining of carotid artery cross-sections from sham-operated control uninjured (intact) and balloon-injured and treated with lentiviral particles encoding either shLuciferase (shLuc; n = 7), shOrai1 (n = 5), or shSTIM1 (n = 5) 14 days after injury. Treatment with shOrai1 or shSTIM1 dramatically attenuated neointima formation in comparison with shLuciferase (Figure 4A). Figure 4B shows statistical analysis of neointima and media layer areas (in mm²) from left injured carotids and intima and media layer areas from right noninjured carotids obtained from 5 to 7 independent rats per condition and determined using the Image J software. The size of neointima is dramatically increased 14 days after injury, and this increase is largely attenuated by shOrai1 and shSTIM1. However, the medial layer area from injured carotids treated with shLuciferase is not significantly different from that of carotids injured and treated with shOrai1 or shSTIM1 or that of noninjured right carotid controls (Figure 4B). Figure 4C represents statistical analysis of area ratios of neointima/media (N/M) and clearly shows increased N/M ratios in injured and shLuciferase-treated left carotids; these N/M ratios were substantially reduced in left carotid arteries that were injured and treated with either shOrai1 or shSTIM1 lentiviruses (Figure 4C). Supplemental Table I depicts data on vessel and lumen size measurements from conditions.

We next sought to evaluate whether STIM1 and Orai1 contribution to VSMC proliferation is mediated by constitutive STIM1/Orai1-mediated SOCE due to precoupling of STIM1/Orai1 in synthetic proliferative VSMCs. We failed to detect any basal (in absence of store depletion) SOCE activity in synthetic VSMCs using either Ca²⁺ imaging or patch clamp electrophysiology (Supplemental Figure V). Confocal microscopy on ectopically expressed eYFP-STIM1 in VSMCs showed no STIM1 puncta under resting conditions; STIM1 puncta was observed only after thapsigargin treatment (Supplemental Figure VIA). Similarly, coexpression of eYFP-STIM1 and CFP-Orai1 showed colocalization only after store depletion (Supplemental Figure VIB), suggesting that the contribution of STIM1/Orai1 to VSMC remodeling is likely regulated by growth/vasoactive factors.

Orai1/STIM1 Knockdown Inhibits NFAT Nuclear Translocation and Activity

To delineate downstream pathways involved in Orai1- and I.CRAC-mediated increase in VSMC proliferation, we sought to evaluate the contribution of VSMC Orai1-mediated I.CRAC to the activation of the transcription factor, nuclear factor for activated T cells (NFAT), a known downstream target of I.CRAC in lymphocytes. Synthetic VSMC were transfected with a plasmid encoding NFAT-GFP fusion protein and siOrai1, siSTIM1, or nontargeting control siRNA, and NFAT nuclear translocation was monitored by following GFP fluorescence before and after activation of SOCE by 2 μmol/L thapsigargin for 15 minutes. Knockdown of either Orai1 (n = 15) or STIM1 (n = 11) drastically inhibited NFAT nuclear translocation in
comparison with siRNA control (n=28; Figure 5A and 5B). The average percentage of inhibition of NFAT nuclear translocation was 85.4% for siOrai1 and 92.3% for siSTIM1.

We also used a luciferase reporter assay to directly measure NFAT activity on VSMC transfection with siOrai1, siSTIM1, or control siRNA as described in Methods. NFAT activity was determined using the NFAT-driven luciferase construct (pIL2-Luc) cotransfected with the Renilla-luciferase vector (as an internal control) in VSMC previously transfected with different siRNA. SiOrai1- (n=6) and siSTIM1-transfected VSMCs (n=4) displayed essentially abrogated luciferase activities in comparison with control siRNA-transfected cells (n=6; Figure 5C).

Orai1/STIM1 Are Upregulated in Carotids From Mice Subjected to Ligation Injury

To determine a potential broader role of Orai1/STIM1, we investigated the upregulation of Orai1 and STIM1 in carotid artery sections from mice subjected to disturbed flow or increased shear stress due to carotid ligation. IHC staining with specific antibodies against Orai1 and STIM1 in ligation-injured carotids (at day 21 postligation) from mice shows marked upregulation of Orai1 and STIM1 proteins in media and neointima from ligation-injured carotids in comparison with control (Figure 6); Orai1 and STIM1 staining was correlated with that of the VSMC marker smooth muscle α-actin (SMα-actin) on contiguous sections, indicating that cells with upregulated Orai1 and STIM1 are VSMCs (Figure 6). STIM1 staining in intact vessels was almost undetectable at the dilution of primary antibody used for injured sections (1:2000) and became visible at a dilution of primary antibody of 1:1200 (see Figure 6, inset). These data suggest that Orai1 and STIM1 upregulation is a more general feature of VSMC remodeling shared by other models of vascular occlusive diseases.

Discussion

Orai1 and STIM1 have recently emerged as central molecular players for SOCE and ICRAC as well as store-independent ARC channels.5,36 Both Orai1 and STIM1 are upregulated in synthetic proliferative VSMC in comparison with quiescent freshly isolated VSMC.9,20 STIM1 is clearly regulating an increasing number of ion channels, and future research is likely to extend further the list of channels, transporters, and

Figure 5. Orai1 and STIM1 knockdown prevents nuclear factor for activated T cells (NFAT) nuclear translocation and activity. A, Vascular smooth muscle cell (VSMCs) were transfected with a plasmid encoding NFAT- green fluorescent protein (GFP) after transfection with either siRNA against Orai1 (siO1), STIM1 (siS1), or nontargeting siRNA control (siNT). NFAT-GFP nuclear translocation in response to thapsigargin (2 μmol/L for 15 minutes) was monitored under fluorescence microscope. B, Data were quantified using Image J, and efficiency of NFAT-GFP translocation was represented as a ratio of GFP fluorescence in nucleus/cytosol. C, Luciferase reporter assays with and without 2 μmol/L thapsigargin treatment of VSMCs transfected with siOrai1 (siO1), siSTIM1 (siS1), or nontargeting siRNA (siNT).

Figure 6. Orai1/STIM1 proteins are upregulated in carotid arteries from mice subjected to ligation. IHC staining with specific antibodies against Orai1 and STIM1 in ligation-injured carotids from mice shows a marked upregulation of Orai1 and STIM1 proteins in media and neointima from ligation-injured carotids 21 days postligation in comparison with their respective control vessels. Contiguous sections from the same vessels were also stained with anti-SMα-actin antibody as a vascular smooth muscle cell (VSMC) marker. Dilutions used for primary antibodies are anti-Orai1, 1:400; anti-STIM1, 1:2000 (inset section dilution was 1:1200 for control vessel); anti-SMα-actin, 1:800.
pumps regulated by STIM1. Indeed, in addition to Orai1-mediated IC^RAC\,
STIM1 was shown to regulate Orai2 and Orai3 channels in their store-operated mode,\textsuperscript{24,26} as well as the store-independent ARC channels mediated by Orai1/Orai3 heteromers.\textsuperscript{28,37} STIM1 also regulates the function of all TRPC channels, with the exception of TRPC7\textsuperscript{18} and has recently been shown by 2 independent groups to control the function of L-type Ca\textsuperscript{2+} channels.\textsuperscript{31,32}

Therefore, this study set out to specifically test the involvement of Orai1 in neointima formation by comparison with STIM1 and to determine the significance of Orai1 upregulation in synthetic proliferative VSMC from injured arteries. Inhibition of Orai1 upregulation during carotid injury using lentiviral particles encoding shRNA prevented Orai1 protein increase 14 days postinjury. Interestingly, Orai1 in vivo knockdown also prevented upregulation of STIM1 and CamKII\textsuperscript{52}. Importantly, Orai1 in vivo knockdown inhibited VSMC proliferation assessed by PCNA and dramatically reduced neointima formation.

Both Orai1 and STIM1 knockdown inhibited NFAT nuclear translocation and transcriptional activity, providing a transcriptional pathway downstream of Orai1 and IC^RAC-mediated Ca\textsuperscript{2+} entry for the control of VSMC proliferation. The upregulation of Orai1 and STIM1 appears to be a feature of proliferative VSMC in other models of vascular diseases such as carotid ligation model in mice, highlighting the universality of IC^RAC Ca\textsuperscript{2+} entry pathway in VSMC remodeling regardless of whether the trigger for this remodeling is acute mechanical injury with removal of endothelium or chronic disturbance of flow or shear stress-induced injury.

The fact that Orai1 knockdown is as efficient as STIM1 in preventing neointima formation in vivo suggests that Orai1 can be a potential target for treatment of VSMC remodeling during vascular diseases. Conceptually, Orai1 would offer a far better target than STIM1 for the following reasons: (1) by its virtue of being an ion channel expressed at the PM, Orai1 is more accessible than STIM1 that is mainly expressed in the ER and (2) targeting STIM1 could be associated with side effects because STIM1 is involved in controlling a plethora of ion channels in addition to Orai1-mediated IC^RAC. However, Orai1 and IC^RAC are prominently functional in the immune system; the main feature of Orai1-deficient patients and mice is severe immunodeficiency.\textsuperscript{38–40} Thus, although it is logical to presume that any potential drug against Orai1 and IC^RAC in the vasculature might have subtle differences that would endow them with different sensitivities to drugs in comparison with other tissues.

In summary, this study provides evidence that Orai1 could be used as a target for vascular occlusive diseases. Future studies aimed at better understanding Orai1 molecular organization and its mechanisms of regulation in VSMC are likely to realize the use of Orai1 as an efficient target for treatment of vascular occlusive diseases.

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Disclosures

None.

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Orai1 and STIM1 are two critical components of the CRAC channel (l_{CRAC}) that regulate Ca^{2+} entry in nonexcitable cells. The role of Orai1 in vascular function and disease is only beginning to emerge. Our work shows that Ca^{2+} entry through Orai1 and l_{CRAC} is essential for driving VSMC remodeling in vivo during vascular disease such as restenosis. In vivo knockdown of Orai1 by shRNA in a rat balloon injury model prevents upregulation of Orai1, STIM1, and CamKII{delta} in VSMC and inhibits VSMC proliferation and neointima formation. Orai1 knockdown in VSMC inhibits NFAT nuclear translocation and activation, providing a potential mechanism for Orai1-dependent regulation of VSMC remodeling. These findings reveal a role for Orai1 and l_{CRAC} in driving VSMC proliferation during vascular disease. Orai1 targeting with specific drugs might have the potential for treatment of vascular occlusive diseases.
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Detailed Methods

Cell culture
Primary rat VSMCs were isolated from aortas of male Sprague-Dawley rats (150g) by enzymatic dispersion as described before¹. VSMCs were cultured in media containing DMEM (45%), Ham’s F12 (45%), and 10% fetal bovine serum (FBS) with L-glutamine and antibiotics.

Antibodies and Reagents
Polyclonal antibody specific for Orai1 was purchased from Alomone (#ACC-062). STIM1 monoclonal antibody was from BD Biosciences (#610954). STIM1 polyclonal antibody was a gift from Drs. Jonathan Soboloff and Donald Gill (Temple University). Polyclonal antibody specific for CaMKIIδ2 was described earlier². Polyclonal antibodies specific for proliferating cell nuclear antigen (PCNA) protein and green fluorescent protein (GFP) were purchased from Abcam. Monoclonal antibody specific for β-actin was purchased from Sigma. Monoclonal antibody specific for rat Ki67 was purchased from Dako. Rat-specific siRNA sequences were (siOrai1: CCUGUGGCCUGGUGUUUAU; siSTIM1: UAAGGGAAGACCUCAAUUA; siRNA non-targeting control #1 obtained from Dharmacon). Cell culture media and supplies and most of the other reagents were purchased from Fisher Scientific.

Lentiviral Particles
Rat specific Orai1, STIM1 and Luciferase shRNA were cloned in the lentiviral vector pFUGW-GFP³ using standard protocols and their sequences are as follows:
Orai1: GACCGACAGTTCCAGGAGCTCAACGAGCT
STIM1: GATGATGCCAATGGTGATGTGGATGTGGA
Luciferase: CGTACGCAGAATACTTCGA

Viral particles were generated using standard protocols. PolyJet was used as a transfection reagent (SignaGen) to transfect HEK293FT cells (Invitrogen). Briefly, the lentiviral constructs pCMV-VSVG, pCMV-dR8.2 and pFUGW-GFP-shSTIM1/shOrai1/shLuciferase were co-transfected into a flask of 95% confluent HEK293FT cells. Cell culture media with viral particles were collected at 48h and 72h after transfection and was concentrated using Amicon Ultra-15 filter by centrifugation.

Balloon Injury and Lentiviral Treatment

All Animals protocols, including surgeries were approved by the Institutional Animal Care and Use Committee at the Albany Medical College Animal Resource Facility, which is licensed by the USDA and N.Y.S. Department of Public Health, Division of Laboratories and Research. Male Sprague-Dawley rats (400~450g, Taconic Farms, Germantown, NY) were anesthetized with ketamine (80mg/kg) and xylazine (5mg/kg). Following a midline cervical incision and muscular tissues separation, the left common carotid artery was exposed and blunt dissection was performed alongside the artery by dull forceps to expose the carotid artery bifurcation into the internal/external branches. Blood flow cessation was performed by arterial clamps, with the following steps. A 2F Fogarty balloon catheter (Edwards) was introduced via a small arteriotomy in the external carotid artery and advanced to the common carotid artery. The balloon was inflated by 1.6atm pressure and was inserted and withdrawn three times. Sham-operated rats were treated in the same manner except that no balloon was inserted in the vessel. Concentrated lentiviral solutions encoding shOrai1, shSTIM1 or shLuciferase (50µl) were infused into the injured segment of the common carotid artery and incubated for 30 minutes. After the 30min viral treatment, leftover viral solutions in lumen were aspirated and disposed off to avoid dissemination of viral solutions into the systemic circulation. A permanent ligation was placed in the external carotid artery, and then blood flow in the common carotid artery and its internal branch was restored. Following the suturing of incision, rats were treated by the analgesic Buprenex (0.20mg/kg) delivered through intramuscular injection and allowed to recover.

Mice carotid artery ligation model

C57BL/6 male mice (3-4-month old) were anesthetized by intraperitoneal injection of a mixed solution of xylazine (5mg/kg body weight) and ketamine (80mg/kg body weight). The left common carotid artery was dissected and completely ligated near the carotid bifurcation.

Sections, Hematoxylin/Eosin staining, Immunofluorescence and Immunohisto-chemistry

Rats were euthanized at different time points by asphyxiation in a CO₂ chamber and fragments of carotid arteries were placed in a cryoprotective embedding medium OCT then snap frozen in liquid nitrogen. The specimen was then stored at -80°C or sectioned in a Leica CM3050 cryostat. Sections were treated by pre-cooled acetone for 10 minutes at 4°C and air dried. Mice with ligated carotids were sacrificed 21days after injury in CO₂ chamber and both the left (ligated) and right (intact, used as a control) carotid arteries were collected and embedded in paraffin. The specimen was then stored at -80°C or sectioned in a Leica CM3050 cryostat. Sections were treated by pre-cooled acetone for 10 minutes at 4°C and air dried. Hematoxylin/Eosin (H&E) staining was performed following standard protocols, except that the incubation time with Hematoxylin was increased to 3 minutes and the time with Eosin-Y was decreased to 15 seconds. For Immunofluorescence staining, the sections were fixed with the pre-cooled acetone for 10 minutes at 4°C and rinsed with 1 x PBS. The sections were then incubated in a PBS washing buffer containing 0.1% Triton X-100 for 10 minutes. The sections were incubated for 30 minutes in blocking buffer (1 x PBS/5% goat serum/0.5% fish
gelatin/0.1% Triton X-100). After blocking, the sections were treated overnight with primary antibody diluted in blocking buffer at 4°C. (Rabbit-anti-STIM1 1:50, Rabbit anti-Orai1 1:75, Rabbit-anti-CaMKIIδ2 1:50, Mouse-anti-smooth muscle myosin heavy chain, SM-MHC 1:50; and Mouse anti-Ki67 1:50). The sections were rinsed with washing buffer and then incubated with secondary antibody diluted in blocking buffer containing DAPI for 2 hours at room temperature. 488nm anti-rabbit secondary antibody (Molecular Probes) or 647nm anti-mouse secondary (Molecular Probes) diluted 1:200. Finally, the sections were mounted with anti-fade mounting media (Sigma). The sections were imaged using confocal microscopy at 63x.

For Immunohistochemistry staining, frozen OCT sections were rinsed with 1x PBS 3 times and were fixed using either acetone at -20°C or 4% paraformaldehyde at room temperature for 10 minutes. Then sections were rinsed with 1x PBS and endogenous peroxidase was quenched by incubating sections in 0.3% H₂O₂ for 10 minutes followed by rinsing with 1x PBS. The sections were then incubated in blocking buffer (1x PBS/0.1% Tween-20/2% BSA, 5-10% serum) for 30-60 minutes, rinsed with 1x PBS, and incubated with primary antibody (diluted in 1x PBS/0.1% Tween-20/2%BSA) and incubated overnight at 4°C. Primary antibodies dilutions were as follows: anti-Orai, 1:400; anti-STIM1, 1:2000 (Inset in Figure 6, 1:1200); anti-SMα-actin, 1:800.

Secondary antibodies were incubated with sections for 60 minutes at room temperature and sections were labeled by fresh ABC (Avidin/Biotinylated Enzyme Complex) for 30 minutes at room temperature. Substrate DAB (1 drop DAB chromagen in 1mL DAB buffer) was added to sections for 2-5 minutes. Hematoxylin staining was performed for 45 seconds. Between every step, sections were rinsed with 1x PBS. Paraffin sections were deparaffinized by Xylene for 10 minutes 2 times, rehydrated by ethanol (100%, 95%, 70%), rinsed with dH₂O, and treated by Citra Plus 1X for 20-60 minutes. Then sections were rinsed by 1x PBS and incubated in blocking buffer (1x PBS/0.1% Tween-20/2% BSA, optional 1-2% serum) for 30 minutes. The remaining steps were similar to those used for OCT sections.

**Protein Extraction and Western Blotting**

The media (and neointima) of the carotid arteries were harvested by cutting open longitudinally and peeling off the adventitia. Tissues were used for protein extraction which was performed on ice. In brief, tissues were cut into small pieces and grinded with a homogenizer in the presence of cell lysis buffer: 10% Phosphatase Inhibitor, 10% Proteinase Inhibitor, and 1% PMSF in RIPA buffer (50mM Tris HCl, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2mM EDTA). After sonication and centrifugation of the cell lysate, proteins were quantified by BCA assay and then loaded in a 10-12% polyacrylamide gel at 10µg per lane. Diluted primary antibodies: Anti-Orai1 1:2000, Anti-STIM1 1:250 (BD biosciences), Anti-PCNA 1:100; Anti-GFP 1:1,000; Anti-CaMKIIδ2 1:1,000 and Anti-β-actin 1:40,000 were incubated overnight at 4°C, and diluted secondary antibodies (anti-rabbit 1:20,000 or anti-mouse 1:10,000) were incubated for 1 hour at room temperature. Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Photos were taken by Fuji LAS4000 Imaging Station and band densities were quantified using the Image J software (NIH).

**Real-time PCR**

Experiments were conducted as described previously. In brief, total RNA was extracted from cells using a Qiagen RNeasy Mini Kit following the manufacturer’s protocol. cDNA was made from 0.5 µg of RNA reverse transcribed using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA) and SuperScript III reverse transcriptase (Invitrogen). PCR reactions were completed using Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The sense and antisense primers targeting rat STIM1, and Orai1 were described earlier. Real-time PCR analysis was performed using a Bio-Rad iCycler and iCycler iQ Optical System Software (Bio-Rad Laboratories). PCR reactions were performed using Bio-Rad iQ SYBR Green.
Supermix. The PCR protocol started with 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 54.3°C, and 45 s at 72°C. Quantification was measured as sample fluorescence crossed a predetermined threshold value that was just above the background. Expressions of STIM1, or Orai1 were compared to those of the housekeeping gene rpL32 (Forward: 5'-GCCCAAGATCGTCAAAAAGA; Reverse: 5'-CGATGGCTTTTCGGTTCTTA) and were measured using comparative threshold cycle values as described previously.

Ca²⁺ measurements

Ca²⁺ measurements were performed as described previously¹, ⁴, ⁵. Briefly, coverslips with attached cells were mounted in a Teflon chamber and incubated at 37°C for 45 min in culture media containing 4 μM Fura-2/acetoxyethyl ester (Molecular Probes, Eugene, OR, USA). Cells were then washed and bathed in HEPES-buffered saline solution (140 mM NaCl, 1.13 mM MgCl₂, 4.7 mM KCl, 2 mM CaCl₂, 10 mM D-glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH) for at least 10 min before Ca²⁺ measurements were made. For Ca²⁺ measurements, fluorescence images of several cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2; Intracellular Imaging Inc., Cincinnati, OH, USA). Fura-2 fluorescence at an emission wavelength of 510 nm was induced by exciting Fura-2 alternately at 340 and 380 nm. The 340/380 ratio images were obtained on a pixel-by-pixel basis. All experiments were conducted at room temperature.

Whole-cell patch-clamp electrophysiology

VSMCs were used for whole-cell patch-clamp recordings as described previously¹ and have a capacitance of 41.75 ± 11.71 pF. Patch pipettes of 2.5- to 4-MΩ were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL, USA) with a P-97 flaming/brown micropipette puller (Sutter Instrument Company, Novato, CA, USA). Axopatch 200B and Digidata 1440A (Molecular Devices Corp., Sunnyvale, CA, USA) with pCLAMP 10 software were used for data acquisition and analysis. VSMCs were infected with shRNA-encoding lentiviruses for 5 days before recordings. VSMCs were seeded on round coverslips 36 h before experiments. VSMCs were washed with bath solution containing 135 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM MgSO₄, 10 mM HEPES, 20 mM CaCl₂, and 10 mM glucose (pH was adjusted to 7.4 with NaOH). Pipette solution contained 145 mM Cs-methanesulfonate, 20 mM Cs-1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), 8 mM MgCl₂, and 10 mM HEPES (pH adjusted to 7.2 with CsOH). Divalent-free (DFV) bath solution contained 155 mM Na-methanesulfonate, 10 mM N-hydroxyethylendiaminetriacetic acid, 1 mM EDTA, and 10 mM HEPES (pH 7.4, adjusted with HCl). Only cells with tight seals (>10 GΩ) were selected to break in. On obtaining a GΩ seal and break-in, recordings were made from cells with <10 MΩ series resistance. Cells were maintained at a 0 mV holding potential during experiments and subjected to voltage ramps from +100 to −150 mV lasting 250 ms every 2 s. “Reverse” ramps were designed to inhibit Na⁺ channels, 10 μM verapamil was included in bath solution to inhibit L-type calcium channels, and 3 μM nimodipine was added to the bath solution to generally stabilize membrane patches and reach better seals.

NFAT-GFP translocation and NFAT luciferase activity assays

Due to the interference of shLuciferase lentivirus with the luciferase assay and that of GFP fluorescence encoded by the lentiviral particles with that of NFAT-GFP, we used siRNA electroporation instead of shRNA lentiviral infection of VSMC. Rat VSMCs were transfected by siRNA against STIM1, Orai1 or non-targeting control using electroporation (sequences provided above). After 3 days, these cells were transfected with pEGFP plasmid encoding nuclear factor for activated T cells (NFAT)-GFP fusion protein (addgene) or co-transfected with pIL-2-Luc (a generous gift from Dr. Fernando Macian) and Renilla-luciferase plasmid (minTK pRL). Twenty-four hours after transfection, cells were treated with 2µM Thapsigargin for 15 minutes. NFAT
nuclear translocation was monitored by the GFP tag under the fluorescence microscope. NFAT activity was measured 24 hours after treatment with thapsigargin for 15 minutes in cell lysates by (firefly-)Luciferase assay using the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI), and normalized to Renilla-luciferase activity.

**Statistical analysis**
Data are expressed as means ± SE, and statistical analysis using One-way ANOVA was done with Origin software (OriginLab, Northampton, MA). *, ** and *** indicates p values < 0.05, 0.01 and 0.001 respectively. Differences were considered significant when \( P < 0.05 \).

**Legends to supplementary figures**
**Supplementary Figure I:** A. Real-time PCR experiment showing that Orai1 knockdown with shRNA has no significant effect on Orai2 and Orai3 mRNA while significantly decreasing Orai1 mRNA levels. B. Fura2 Ca\(^{2+}\) imaging experiment showing inhibition of SOCE upon Orai1 knockdown and rescue of SOCE with expression of CFP-Orai1 into Orai-silenced VSMCs. Traces are averages of several cells as indicated. C. Statistical analysis on 3-4 independent Fura2 experiments similar to B.

**Supplementary Figure II:** IHC using specific anti-GFP antibody as an indication of shOrai1 virus infection of rat left carotid sections (14 day post injury) injured and treated with shOrai1 lentivirus. Sections stained with secondary antibody alone (control) are also shown. Bottom panels are magnified from regions of vessel sections where indicated.

**Supplementary Figure III:** IF using specific anti-CamKII\(\delta\)2 antibody on rat left carotid sections (14 day post injury) injured and treated with shLuciferase, shOrai1 or shSTIM1 lentiviruses (bottom) and corresponding right non-injured non-treated control carotids (top).

**Supplementary Figure IV:** IF on rat left carotid vessel sections (14 day after injury) balloon-injured and treated with shLuciferase lentivirus (A,C,E) or shOrai1 virus (B, D, F) and stained with either anti-Orai1 antibody (C, D) or secondary antibodies alone (control; A,B,E,F)

**Supplementary Figure V:** A. Fura2 Ca\(^{2+}\) imaging experiments in proliferative synthetic rat VSMCs probing for constitutive Ca\(^{2+}\) entry activity using the “Ca\(^{2+}\) off/ Ca\(^{2+}\)” on protocol. Cells are incubated with nominally free Ca\(^{2+}\) solution (-Ca\(^{2+}\)) and Ca\(^{2+}\)-containing solution (2mM; +Ca\(^{2+}\)) where indicated. 10 \( \mu \)M ionomycin is added where shown as a control for the Fura2 signal. B. Representative whole cell patch clamp experiment in a VSMC cell dialyzed with a pipette solution where Ca\(^{2+}\) concentration if buffered to 150nM using BAPTA. No basal current is detected up to 10 min after break-in.

**Supplementary Figure VI:** A. eYFP-STIM1 expression in proliferative VSMCs (1.5 \( \mu \)g plasmid DNA) shows typical STIM1 fibrilar distribution under basal conditions under confocal microscope. Addition of thapsigargin (2 \( \mu \)M) caused redistribution of STIM1 into sustained relatively small puncta (Size of puncta is dependent on STIM1 expression level; not shown). B. Co-expression of eYFP-STIM1 (green channel) and CFP-Orai1 (red channel) shows no significant basal co-localization of STIM1/Orai1. Co-localization occurred only after store depletion with thapsigargin. Right hand graphs show co-localization of STIM1 and Orai1 signals in a cross-section of the cell.
Supplemental Table I: Carotid vessel and lumen size measurements on left injured (L) and right control (R) from rats treated with either shLuc (n=7), shOrai1 (n=5) and shSTIM1 (n=5). * P<0.05 compared to right carotid (R) from shLuc. Lack of significant decrease in lumen size after injury, despite neointima formation, is likely due to compensatory vessel remodeling in response to mechanical injury, as discussed previously.

<table>
<thead>
<tr>
<th>samples</th>
<th>Vessel size (mm²)</th>
<th>Lumen size (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R(intact)</td>
<td>L(injured)</td>
</tr>
<tr>
<td>shLuc</td>
<td>0.32±0.018</td>
<td>0.40±0.024*</td>
</tr>
<tr>
<td>shOrai1</td>
<td>0.29±0.010</td>
<td>0.35±0.023</td>
</tr>
<tr>
<td>shSTIM1</td>
<td>0.36±0.029</td>
<td>0.35±0.032</td>
</tr>
</tbody>
</table>

References

Supplemental Figure I

A

mRNA (Normalized to Control)

shLuc  Orai1  Orai2  Orai3

0.0  0.4  0.8  1.2  1.6

B

siControl  siOrai1  siOrai1+CFP-Orai1

Ca^{2+}  2mM

Thapsigargin  2μM

Ratio 340/380

Time(s)

n=25  n=14  n=23

C

ΔRatio 340/380

siControl  siOrai1  CFP-Orai1 Rescue

n=3, 79  n=3, 95  n=4, 69

**
14 days after balloon injury

Supplemental Figure II
Supplemental Figure III

14 days after balloon injury

shLuciferase
CaMKIIδ2 (green)
TOPRO-3 (blue)

shOrai1

shSTIM1

M

R-Control

M

L-Injured

N.I.

M

L-Injured

N.I.

M

L-Injured
14 days after balloon injury

Supplemental Figure IV

Alexa 488- 2⁰ (green) alone

A

N.I.

shLuciferase

M

shOrai1

B

M

E

N.I.

C

M

shLuciferase

shOrai1

D

M

Alexa 647- 2⁰ (red) alone

C

M

shLuciferase

shOrai1

D

M

F

M
**Supplemental Figure V**

**A**

- Plot showing the ratio of 340/380 over time.
- Two conditions: +Ca²⁺ (2 mM) and -Ca²⁺.
- The time range is from 0 to 1200 seconds.
- The x-axis represents time in seconds.
- The y-axis represents the ratio 340/380.

**B**

- Graph showing membrane potential with time.
- Conditions include VSMC-Control, 150nM Ca²⁺ in pip, DVF, and 0.2pA/pF.
- Voltage range is from -100 to 100 mV.
- Current range is from -1.0 to 0.4 pA/pF.
- Time scale is 2 minutes.

**Legend**

- Iono (10μM)
- 150nM Ca²⁺ in pip