Supplemental Material

Detailed Methods:

Materials

Antibiotic reagents, dispase II, Fura-2 AM, Mag-Fura-2, DAF-FM diacetate, and Lipofectamine 2000 transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA). M199 and FBS were purchased from Mediatech Inc. (Manassas, VA, USA). Anti-SERCA1/2/3, anti-SERCA3, anti-tubulin, anti-actin antibodies, and STIM1 siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-STIM1 and STIM2 antibodies were from Sigma-Aldrich (St Louis, MO, USA). Anti-CD31 antibody and endothelial cell growth supplement (ECGS) were obtained from BD Biosciences (San Jose, CA, USA). Anti-Orai1, 2, 3 antibodies were purchased from ProSci Inc. (Poway, CA, USA). Collagenase II was purchased from Worthington Biochemical (Lakewood, NJ, USA). All other chemicals were from Sigma-Aldrich.

Animal preparation

All investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985). This study was conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee in the University of Illinois at Chicago. Six weeks old male C57BL6 mice were purchased from Harlan Laboratories (Madison, WI, USA) and mice in the diabetic group received a single injection of streptozotocin (133 mg/kg, dissolved in citrate buffer, i.v.). All data was obtained from mice 6 weeks after injection. We chose the time point to perform the experiment in this study on the basis of our experiences in the past studies. In this mouse model, we found that the significant difference of endothelium-dependent vascular relaxation in coronary artery between control and streptozocin (STZ)-treated mice took place approximately 6 weeks after diabetic induction. Plasma glucose levels were 10.1 ± 0.6 mmol/l in control mice and 32.2 ± 0.6 mmol/l in diabetic mice.

Isolation of mouse coronary vascular endothelial cells

MCECs were isolated as described previously[1, 2]. Briefly, dissected heart tissues were enzymatically digested by collagenase II and dispase II and suspended cells were incubated with Dynabeads (Invitrogen) which were prepared as follows; beads coated with sheep anti-rat IgG were incubated with purified rat anti-mouse CD31 monoclonal antibody (1 μg/ml) at 4°C overnight and then washed with PBS containing 0.1% (wt./vol.) BSA and 2 mmol/l EDTA. The cell suspension was incubated with beads for 1 h at 4°C and then beads-attached endothelial cells were captured and isolated by the Dynal magnet (Invitrogen).

Isolation of coronary vascular smooth muscle cells (SMCs)

Mouse coronary SMCs were isolated as described previously[3]. The digested heart material was filtered through sterile 40 μm nylon mesh and washed in 2% FCS–M199. Subsequently, the cells were incubated with Dynabeads which were prepared as follows: M-450 Epoxy dynabeads were incubated with NG2 antibody at 20 μg / 100 μl beads in 100 mM sodium borate buffer at 4°C for two days, washed with PBS containing 0.1% BSA and incubated overnight at 4°C. The cell suspension was incubated with beads for 30 min at 4°C and then the beads attached to SMCs were captured by a Dynal magnet.

Cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) measurement

$[Ca^{2+}]_{cyt}$ in MCECs were measured using a modification of previously described methods[4]. Isolated MCECs were plated on glass slides coated with 5% (wt/vol.) gelatine. After 3 days of recovery from isolation, $[Ca^{2+}]_{cyt}$ was measured by the digital imaging fluorescence microscopy. Cells on cover slips were loaded with the membrane-permeable acetoxymethyl ester form of fura 2 (fura2-AM; 4 μmol/l) for 1 h in the dark at room temperature. The fura2-AM-loaded cells were then superfused with physiological

salt solution [PSS, containing (in mmol/l) 141 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4)] for 30 min at 32˚C to wash away extracellular dye and to permit intracellular cleavage of fura2-AM to active fura2 by esterases. Fura-2 fluorescence (340- and 380-nm excitation; 510-nm emission) from the cells and background fluorescence were imaged using a Nikon TE300 microscope. Data were described as a normalized ratio (F/F_0, $F=I_{340}/I_{380}$, F_0=average of F during first 5 min recording). The peak of $[Ca^{2+}]_{cyt}$ increases ($\Delta F/F_0$) and the Area Under the Curve (AUC) of the peak per individual cell were calculated (see Fig. 1A). The cells were isolated from at least 3 mice to repeat the experiments.

Endoplasmic reticulum Ca^{2+} concentration ($[Ca^{2+}]_{ER}$) measurement

$[Ca^{2+}]_{ER}$ in MCECs was measured using a modification of previously described methods[5]. Cells on cover slips were loaded with the Mag-fura2-AM (4 μmol/l) for 20 min in the dark at room temperature. The Mag-fura2-AM-loaded cells were then superfused with PSS for 90 min at 32˚C to wash away cytosolic dye. Mag-Fura-2 fluorescence (340- and 380-nm excitation; 510-nm emission) from the cells and background fluorescence were imaged using a Nikon TE300 microscope. Data are described as a ratio ($F=I_{340}/I_{380}$). Mag-fura2 localization in the ER is confirmed by co-staining with ER-Tracker (the marker of the ER, 200 nM, Invitrogen) in Online Figure II. The resting level of $[Ca^{2+}]_{ER}$, the decrease in $[Ca^{2+}]_{ER}$ after CPA treatment, and the AUC per individual cell were calculated (see Fig. 5A).

Western blot analysis

Freshly isolated MCECs were used to measure the protein concentration. Cell lysate was centrifuged at 16,000 g for 10 min at 4°C. Supernatants were used as sample protein. Samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were then incubated with a primary antibody (anti-STIM1 (C-terminus) [1:2000], anti-STIM2 [1:2000], anti-Orai1, 2 or 3 [1:1000], anti-SERCA3 [1:500], anti-tubulin [1:1000], or anti-actin [1:4000]) followed by incubation with an HRP-conjugated secondary antibody. The immunoblots were detected with SuperSignal West Pico reagent (Thermo Fisher Scientific Inc. Rockford, IL, USA). Band intensity was normalized to actin controls and expressed in arbitrary units.

High glucose treatment *ex vivo*

To test the effect of high glucose *ex vivo*, human coronary endothelial cells (HCECs), purchased from Cell Applications (San Diego, CA, USA), were used. For high-glucose treatment (HG), 20 mmol/l glucose was added to the media (the final glucose concentration was 25 mmol/l). This concentration was decided based on the plasma glucose level in diabetic mice used in our experiments. In a control group of cells, equimolar mannitol was added to exclude the potential effect of changes in osmolarity (normal glucose [NG]: glucose concentration, 5 mmol/l). Cells were cultured for 48 hs and used for the protein measurement.

Immunoprecipitation

SERCA3 protein coupled to STIM1 was pulled down using anti-STIM1 (N-terminus) antibody. IP-matrix (ExtraCrus A, Santa Cruz Biotechnology Inc.) was prepared as follows: IP-matrix (40 μl) was incubated with anti-STIM1 antibody (2 μg) at 4°C for 2 hs and then washed with PBS. Cell lysates from HCECs were incubated with IP-matrix at 4°C for overnight. After incubation, IP-matrix was washed 4 times and resuspended with 2x reducing sample buffer. Samples were loaded in the gel as described above for Western blotting.

Construction of Adenoviral Vectors

The positive-mutated-STIM1 driven by CMV promoter in pcDNA3.1 was kindly provided by Dr. J.N. Rao from the University of Maryland[6, 7]. The cDNA of positive-mutated STIM1 was inserted in pENTR/1A vector together with a Tie2 promoter. First, Tie2 promoter was cloned into pENTR/1A using 5’ Sal I and 3’ Kpn I; then STIM1 was cloned under the Tie2 promoter using 5’ Hind III and 3’ Xho I. Adenovirus was generated using ViraPower Adenoviral Expression System (Invitrogen). Replication

deficient adenovirus particles containing the target gene or empty vector (Control-Adv) were generated by *in vivo* recombination in 293 cells, and single plaques were isolated and propagated to achieve high titer. Adenoviral particles were CsCl-purified and quantified by plaque titer assay. The dose of adenovirus to be used was determined based on the overexpression level of the protein, the efficacy of its function as well as the cell viability after infection. The images of STIM1 distribution and the translocation to the plasma membrane after CPA treatment are shown in Online Figure IV.

Adenoviral infection in primary coronary ECs

Cells were infected with an adenovirus carrying an empty vector (Control-Adv) or adenovirus containing STIM1 gene (STIM1-Adv) at the concentration of 500 pfu/cell. Cells were treated with Adv overnight and Adv was washed next day. Two days after infection, cells were used for the experiment.

Immunofluorescence

Two days after Control- or STIM1-Adv infection in MCECs, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% (vol/vol) Triton X-100, and blocked 5% (wt./vol.) BSA in PBS. Cells were incubated overnight with the primary antibodies (anti-STIM1 C-terminus-antibody), followed by secondary anti-rabbit antibody, conjugated with Alexa488 (Invitrogen). The fluorescence intensity of the cells (indicative of expression level of STIM1) is assessed by the difference between the cell intensity (Ic) and background intensity (Ib) (Ic-Ib). To show the distribution of STIM1 (Online Figure IV), STIM1 was stained with anti-STIM1 N-terminus-antibody and nuclei were stained with Hoechst33342 (Invitrogen). Images were captured with an Eclipse Ti-E; Nikon deconvolution microscope system (Nikon, Tokyo, Japan). Using a 60× (numerical aperture 1.4) lens, images of ~5 serial optical sections, spaced by 0.25 μm, were acquired. The data sets were deconvolved using ImagePro-PLUS 7.0 software (Media Cybernetics Inc.). SERCA was stained with anti SERCA, followed by secondary anti-mouse antibody, conjugated with Alexa594 (Invitrogen).

Adenoviral infection in mouse CA *ex vivo*

The heart together with the aorta was removed and the coronary artery was dissected. Control-Adv or STIM1-Adv was delivered through the aorta (4.5×10^{11} pfu/ml; 0.5 mL in Hank's balance salt solution). The CAs were then left at 37°C for 30 min and incubated for additional 24 hs at 4°C before being used for the isometric tension measurement experiment.

Isometric tension measurement in coronary arterial ring

Isometric tension measurement in CAs was performed as described previously[8]. Briefly, third-order small CAs were dissected from the hearts and then cut into 1-mm segments. The CA rings were mounted on a myograph (DMT-USA, Inc. Ann Arbor, MI, USA) using thin stainless wires (20 μm in diameter) and the resting tension was set at 0.1 g. CAs were allowed to equilibrate for 60 min with intermittent washes every 20 min. After equilibration, each CA ring was contracted by treatment with $PGF_{2\alpha}$ to generate similar contraction level in all groups ($PGF_{2\alpha}$ concentration: control, $1.1 \times 10^{-6} \pm 3.3E\text{-}07$ M; diabetic, $6.3 \times 10^{-6} \pm 1.3E\text{-}06$ M, P=0.01. Absolute contraction value: control, 0.14 ± 0.03 g; diabetic, 0.07 ± 0.01 g, P=0.08). Acetylcholine was administrated in a dose dependent manner (1 nmol/l to 100 μmol/l).

Cytosolic NO measurement

Cytosolic NO in MCECs was measured using a modification of previously described methods[9]. Cells on cover slips were loaded with the DAF-FM diacetate (5 μmol/l) for 30 min in the dark at room temperature. The DAF-FM-DA-loaded cells were then superfused with PSS for 30 min at 32˚C to remove excess probe and complete de-esterification of the intracellular diacetates. DAF-FM (495-nm excitation; 515-nm emission) from the cells and background fluorescence were imaged using an Eclipse Ti-E; Nikon microscope. DAF intensity (F) was calculated as follows; background intensity (I_b) was subtracted from cell intensity (I_c) [$F=I_c-I_b$]. Resting level of NO was measured, normalized by the value in control ECs infected with control Adv and described as arbitrary unit. NO does not disassociate from DAF-FM after

binding, it is thus a cumulative amount of NO within the cells will be shown as a fluorescence signal. Time-dependent changes in cellular DAF-FM intensity were expressed as percentage change from baseline (F/F_0). Linear regression was used to quantify the rate of DAF fluorescence rise during CPA treatment (between time 15 and 24) (see Fig.6E). The slope was described as $d(F/F_0)/dt$.

STIM1 siRNA transfection in HCECs

Downregulation of STIM1 in HCECs was achieved using the STIM1 siRNA (0.2 nmol/$5x10^4$ cells) and the Nucleofector technology (Lonza Walkersville Inc., Walkersville, MD, USA)[2]. Two days after transfection, cells were used for the experiments. Specific protein knockdown was verified with western blotting.

Adenoviral infection in mouse CA *in vivo*

To demonstrate the selective gene transduction in ECs by Tie2 promoter-adenovirus vector, we used Adv-Tie2-EGFP to visualize the GFP expression *in vivo*. Adv-Tie2-EGFP (10^{12} pfu/ml, 0.01 ml/g bwt) was injected through the tail vein. One week after the injection, the heart was dissected and, embedded in OCT compound (Sakura Finetek U.S.A., Inc. Torrance, CA), frozen in 2-methylbutane precooled with liquid nitrogen, then kept at -80°C until sectioned. Sections (6 μm) were fixed in 4% formaldehyde and CAs were photographed in sequence by a CCD camera connected to a fluorescence microscope with a 20x objective lens (Online Figure VI).

Free fatty acid treatment *ex vivo*

To test the effect of free fatty acid (FFA) *ex vivo*, mouse coronary endothelial cell line, purchased from Cedarlane Laboratories Ltd. (Burlington, Ontario, Canada), were used. Palmitic acid are conjugated with BSA and then applied to the cells at 150 μmol/l (HF: high FFA). In a control group of cells, equimolar BSA was added (NF: normal FFA). Cells were cultured for 24 hs and used for the measurement of protein and mRNA level of STIM1.

Assay of STIM1 mRNA

STIM1 mRNA level was measured by real time quantitative PCR. RNA from ECs was isolated using the RNeasy Plus Micro kit (QIAGEN, Chatsworth, CA). Briefly, mouse coronary ECs were lysed in buffer RLT Plus by passing 10 times through a 21g needle and the manufacturer's instructions were followed to purify RNA. cDNA was made by reverse transcription of DNAse-free RNA templates using SuperScript III First-Strand Synthesis SuperMix (Life Technologies, Carlsbad, CA). Primers for mSTIM1 are as follows: Fw-TGAAGAGTCTACCGAAGCAGA, Rv-AGGTGCTATGTTTCACTGTTGG. The primers of endogenous reference gene, 18S ribosomal RNA, are; Fw- GTAACCCGTTGAACCCCATT, Rv-CCATCCAATCGGTAGTAGCG. Measurements were made in triplicate with a Bio-Rad CFX Real Time PCR System. The efficiency correlated ΔCt method was used to determine the level, in arbitrary units, of STIM1 RNA relative to 18S.

Overexpression of STIM1 and Orai1 in HEK293 cells

The positive-mutated-STIM1 driven by CMV promoter in pcDNA3.1 was transfected into HEK293 cells (10^5 cells) retiometrically using Lipofectamine2000. CMV-Orai1 plasmid was obtained from Addgene (Cambridge, MA). 48 hs after transfection, cells were used for the experiments.

Whole-Cell Recording

Whole-cell recordings were done on transfected HEK cells as described previously[6, 10]. Transfected cells were selected by fluorescence from GFP-tagged Orai1. Currents were recorded using an EPC-10 Mac-driven patch clamp amplifier (HEKA Elektronik). Command voltage protocol generation and data acquisition were done using Patchmaster (HEKA Electronik). The membrane potential was held at 0 mV, and 230-ms voltage ramps from -110 to 90 mV were delivered every 2 s. The standard Cs^+-containing pipette solution consisted of (mM) the following: 150 cesium glutamate, 0.5 $CaCl_2$, 1 EGTA, and 10

HEPES; pH = 7.3. This solution was supplemented with 10 mM $MgCl_2$ to inhibit the endogenous Mg^{2+}-inhibited cation (MIC/TRPM7) channels. The standard extracellular solution contained (mM) the following: 150 NaCl, 2 $CaCl_2$, 4.5 KCl, 10 Glucose, 10 HEPES; pH = 7.3. The liquid junction potential was corrected during data acquisition. Cell and pipette capacitances were compensated during recording by software; series resistance was not compensated. The current at break-in was subtracted as leak. 2 mM thapsigargin was then added to the bath to evoke CRAC current. All recordings were performed at room temperature. Data were analyzed with OriginPro 8 software (OriginLab). The normalized peak current (pA/pF) at -110 mV of individual cells were collected for comparison. Analyzed data are presented as mean ± SE (Online Figure VII).

Statistical Analysis

Values are expressed as mean ± SE. Bonferroni tests for multiple statistical comparisons and Student's *t*-test for unpaired samples were carried out to identify significant differences. Differences were considered to be statistically significant when $P<0.05$.

References

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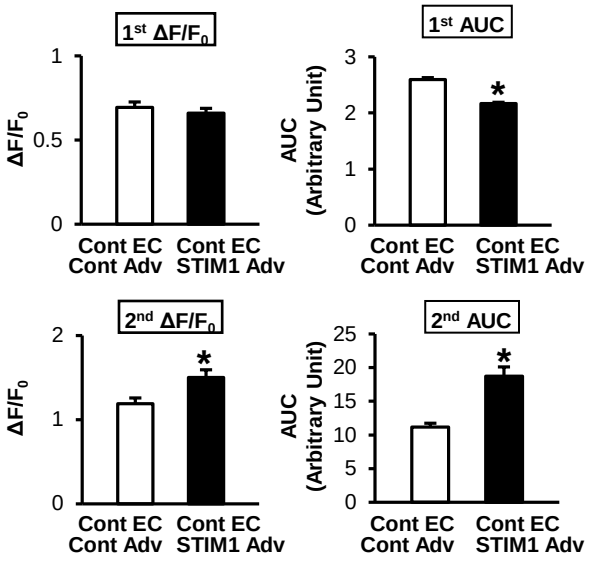
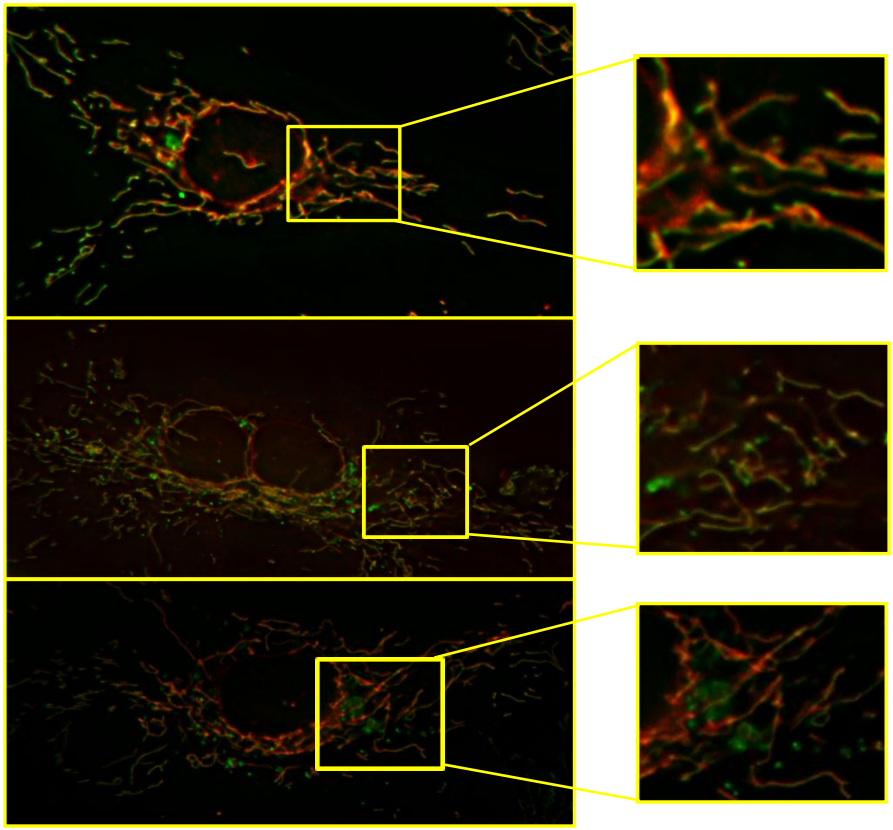
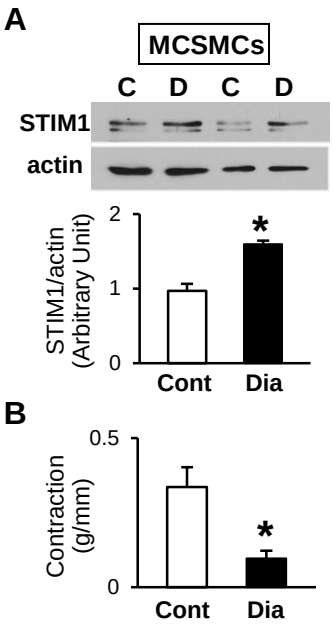


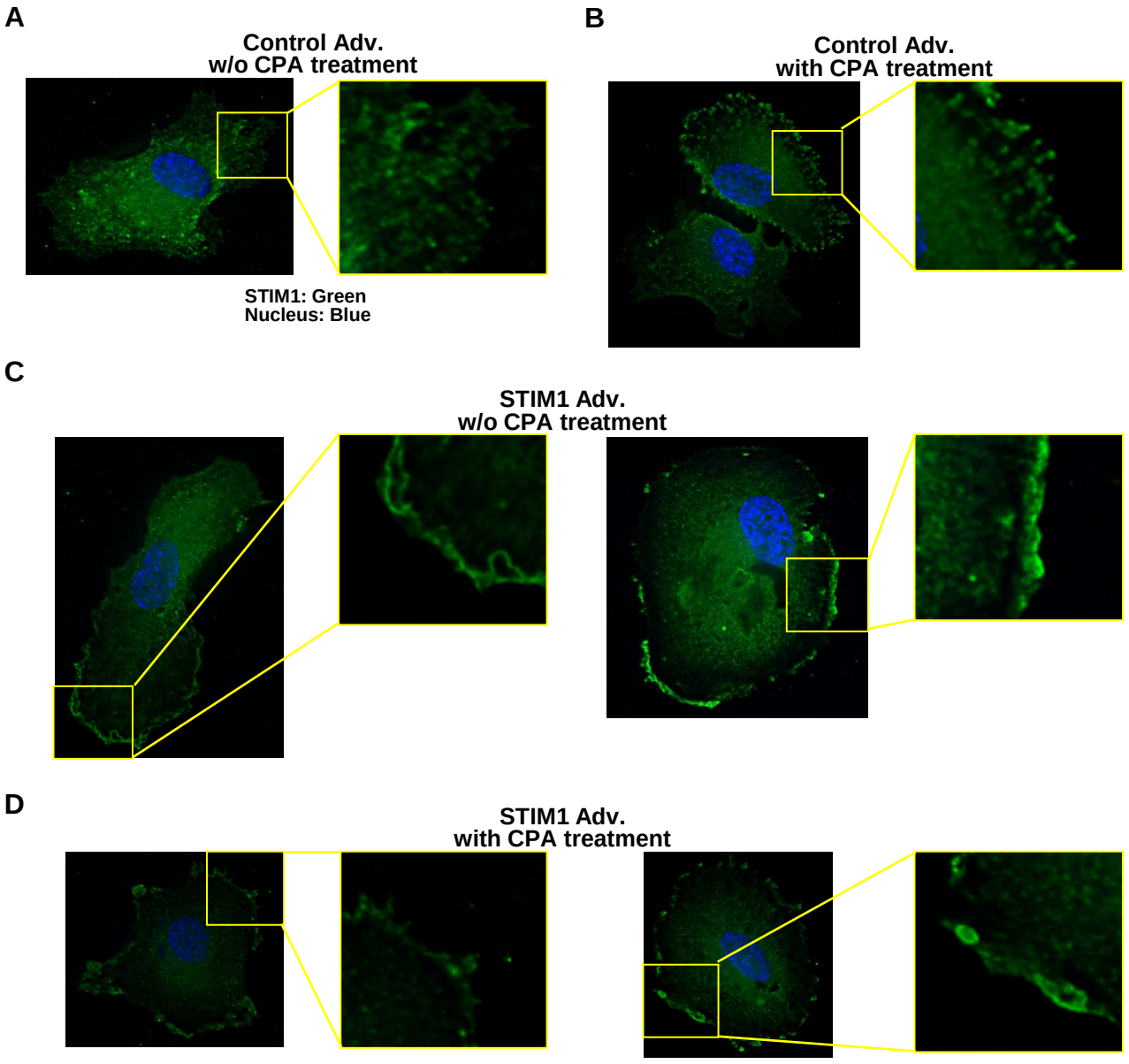
Online Figure I.
Overexpression of STIM1 in control MCECs does not increase the rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release/leakage from the ER during CPA treatment. Summarized data of the rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release/leakage from the ER (1^{st} $\Delta F/F_0$ and 1^{st} AUC) and SOCE (2^{nd} $\Delta F/F_0$ and 2^{nd} AUC). Control MCECs infected with control-Adv (Cont EC Cont Adv, while column); n=18 cells, control MCECs infected with STIM1-Adv (Cont EC STIM1 Adv, black column); n=18 cells. Data are mean ± SE. P*<0.05 vs. Cont EC Cont Adv.



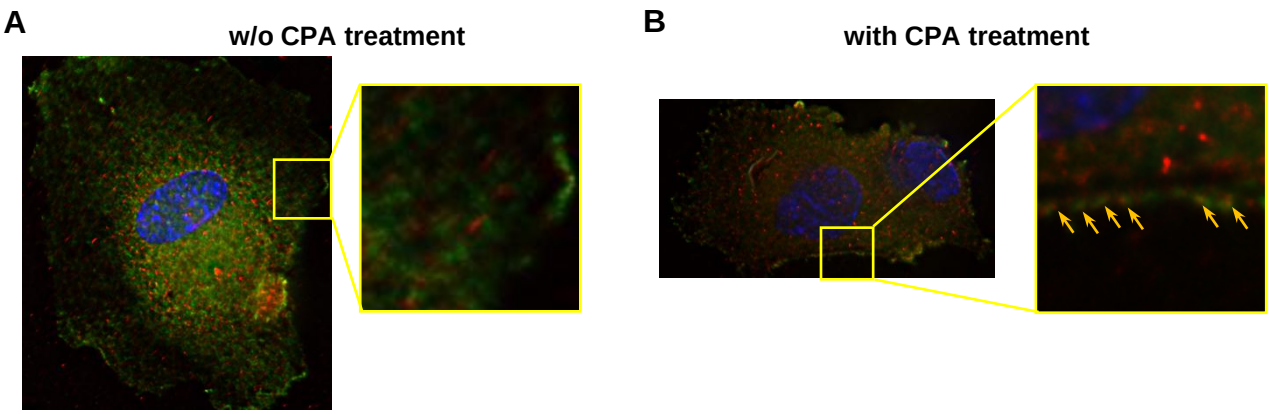
Online Figure II.
Co-localization of Mag-Fura2 signal with ER-Tracker signal in ECs. ECs were stained with Mag-Fura2 (a detector of $[Ca^{2+}]_{ER}$, 4 μM, green) and ER-tracker (a marker of ER, 200 nM, red). For Mag-Fura2 image, the signal obtained by 380nm/ex, 510nm/em is shown in green. The right microphotograph is showing the magnified image selected in the yellow square in the left image. Mag-fura2 signal (green) is co-localized with red signal (shown in yellow) except the dots with strong green signal (it might be the signal from Golgi).



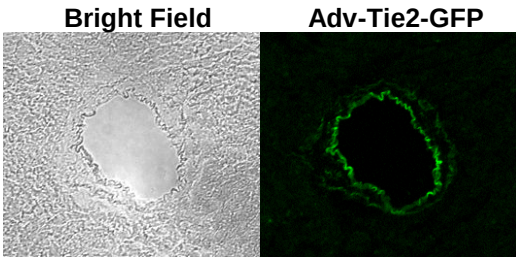
Online Figure III.
Mouse coronary smooth muscle cells (SMCs) from diabetic mice exhibit higher STIM1 protein expression than the control, but 40K$^+$-mediated contraction was significantly attenuated in diabetic CAs compared with control CAs. A. Western blotting. Control SMCs (Cont); white column, diabetic SMCs (Dia); black column. n=2 respectively. Data are mean ± SE. P*<0.05 vs. Cont. **B.** Vascular contraction induced by 40K$^+$ in CAs. Control CAs (Cont); white column, diabetic CAs (Dia); black column. n=4 respectively. Data are mean ± SE. P*<0.05 vs. Cont.



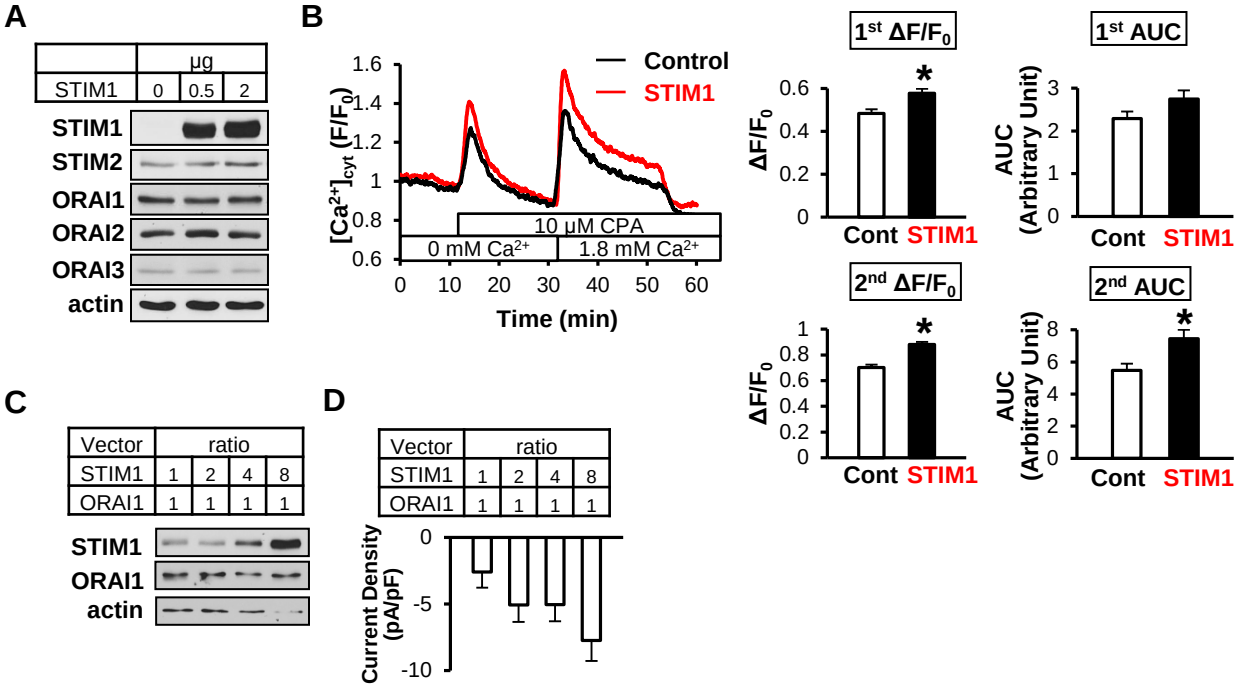
Online Figure IV.
STIM1 Adv infection induces STIM1 protein translocation in the plasma membrane without CPA stimulation. ECs were stained with rabbit anti-STIM1 followed by anti rabbit Alexa488 (shown in green color). Blue signal shows nucleus. The right microphotograph is showing the magnified image selected in the yellow square in the left image. **A**. EC infected with control Adv. without CPA treatment. **B**. ECs infected with control Adv. with CPA treatment. **C**. ECs infected with STIM1 Adv. without CPA treatment. **D**. ECs infected with STIM1 Adv. with CPA treatment. ECs infected with constitutively active STIM1 Adv. exhibit the STIM1 distribution in the plasma membrane even without CPA treatment.



Online Figure V.
Co-localization of SERCA and STIM1 after CPA treatment in ECs. ECs were stained with rabbit anti-STIM1 and mouse anti-SERCA followed by anti-rabbit Alexa488 (STIM1, green) and anti-mouse Alexa594 (SERCA, red). Blue signal shows nucleus (Hoechst staining). The right microphotograph is showing the magnified image selected in the yellow square in the left image. **A**. EC without CPA treatment. **B**. ECs with CPA treatment. Ca^{2+} depletion in the ER by CPA induces co-localization of STIM1 and SERCA (shown in yellow arrow).



Online Figure VI.
Selective overexpression of GFP in coronary ECs by Tie2-GFP Adv. The heart was dissected for imaging one week after the Adv. injection (10^{12} pfu/ml, 0.01 ml/g bwt) through the tail vein. This data suggests that Tie2-promoter transduces the gene selectively in ECs.



Online Figure VII.

Overexpression of STIM1 has no inhibitory effects on the expression and function of Orai1 channels. A. Effect of STIM1 overexpression on ORAI1 (and ORAI2/3) protein expression levels in HEK293 cells. The plasmid vector which encodes CMV-driven STIM1 was transfected in HEK293 cells (10^5 cells) and cells were lysed after 48 hours incubation. **B.** Effect of STIM1 overexpression on the increase in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} leakage from the ER and the increase in $[Ca^{2+}]_{cyt}$ due to SOCE in HEK293 cells. 0.5 μg of STIM1-encoded vector was transfected in the cells and $[Ca^{2+}]_{cyt}$ was measured using Fura-2. Data are described as a normalized ratio (F/F_0, $F=I_{340}/I_{380}$, F_0=average of F during the first 5-min recording in Ca^{2+}-containing PSS). Right panels show the summarized data of $\Delta F/F_0$ and Area Under the Curve (AUC). Control (Cont, white bars); n=53 cells, STIM1-transfected cells (STIM1, red bars); n=51 cells. Data are mean ± SE. *P<0.05 vs. Cont. **C.** Effect of STIM1 on ORAI protein expression in HEK293 cells co-transfected with STIM1 and ORAI1 in different ratio. All cells are transfected with the same concentration of ORAI1 and only STIM1 concentration was changed ratiometrically. **D.** Effect of STIM1 on Ca^{2+} current in cells co-transfected with STIM1 and ORAI1 in different ratio. Whole-cell Ca^{2+} currents were measured after store depletion by the SERCA inhibitor thapsigargin (2 μM) in cells co-transfected with STIM1 and ORAI1 at the ratio (STIM1:ORAI1) of 1:1, 2:1, 4:1 and 8:1 (n=7-9 in each group).