STIM1 Restores Coronary Endothelial Function in Type1 Diabetic Mice

Irene A. Estrada¹, Reshma Donthamsetty¹, Patryk Debski¹, Meng-Hua Zhou², Shenyuan L. Zhang², Jason X.-J. Yuan³, Wenlong Han⁴ and Ayako Makino¹

¹Section of Endocrinology, Diabetes and Metabolism, ³Section of Pulmonary, Critical Care, Sleep and Allergy, Department of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612, ²Department of Systems Biology and Translational Medicine, Texas A&M Health Science Center, Temple, Texas 76504 and ⁴Tumor Microenvironment Program, Sanford Burnham Medical Research Institute, La Jolla, California 92037.

Running title: STIM1 and Endothelial Function in Diabetes

Subject codes:
[189] Type1 diabetes
[95] Endothelium/vascular type/nitric oxide

Address correspondence to:
Dr. Ayako Makino
Endocrinology, Diabetes, and Metabolism
Department of Medicine
University of Illinois at Chicago
1819 West Polk Street, M/C 640
Chicago, Illinois 60612
Tel: (312) 355-1018
Fax: (312) 413-0437
E-mail: aymakino@uic.edu

In July 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 11.2 days.
ABSTRACT

**Rationale:** The endoplasmic reticulum (ER) is a major intracellular Ca<sup>2+</sup> store in endothelial cells (ECs). The Ca<sup>2+</sup> concentration in the ER greatly contributes to the generation of Ca<sup>2+</sup> signals that regulate endothelial functions. Many proteins, including stromal interaction molecule 1/2 (STIM1/2), Orai1/2/3, and sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase 3 (SERCA3), are involved in the ER Ca<sup>2+</sup> refilling after store depletion in ECs.

**Objective:** This study is designed to examine the role of Ca<sup>2+</sup> in the ER in coronary endothelial dysfunction in diabetes.

**Methods and Results:** Mouse coronary ECs (MCECs) isolated from diabetic mice exhibited i) a significant decrease in the Ca<sup>2+</sup> mobilization from the ER when the cells were treated by SERCA inhibitor, and ii) significant downregulation of STIM1 and SERCA3 protein expression in comparison to the controls. Overexpression of STIM1 restored a) the increase in cytosolic Ca<sup>2+</sup> concentration due to Ca<sup>2+</sup> leak from the ER in diabetic MCECs, b) the Ca<sup>2+</sup> concentration in the ER, and c) endothelium-dependent relaxation that was attenuated in diabetic coronary arteries.

**Conclusions:** Impaired ER Ca<sup>2+</sup> refilling in diabetic MCECs, due to the decrease in STIM1 protein expression, attenuates endothelium-dependent relaxation in diabetic coronary arteries, while STIM1 overexpression has a beneficial and therapeutic effect on coronary endothelial dysfunction in diabetes.

**Keywords:** Diabetic complications, vascular relaxation, cyclopiazonic acid, Ca<sup>2+</sup> homeostasis, endothelial dysfunction

**Non-standard Abbreviations:**
- CA: coronary artery
- EC: endothelial cell
- ER: endoplasmic reticulum
- IP<sub>3</sub>R: inositol triphosphate receptor
- MCEC: mouse coronary endothelial cell
- RyR: ryanodine receptor
- SERCA: sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase
- SOCE: store-operated Ca<sup>2+</sup> entry
- STIM1: stromal interaction molecule
INTRODUCTION

Ischemic heart disease is a major risk for mortality in diabetic patients. Coronary blood flow and coronary vascular resistance are tightly controlled by vascular tone and vascular density. Endothelial cells (ECs) serve as a major player in the regulation of vascular tone and the formation of new vessels. Thus, endothelial dysfunction is considered to be a risk factor for cardiovascular complications in many diseases. In diabetic patients, coronary endothelial dysfunction and vascular rarefaction in the heart cause cardiac ischemia and heart failure due to a shortage of vascular supply versus heart demand. We have recently demonstrated that capillary density in the left ventricle is significantly decreased in the heart of diabetic mice and endothelium-dependent relaxation is significantly attenuated in diabetic coronary arteries (CAs) compared with control CAs.

Endothelial function depends, to various extents, on the changes in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{cyt}}\)]. [Ca\(^{2+}\)\(_{\text{cyt}}\)] is controlled by Ca\(^{2+}\) mobilization from intracellular stores coupled to Ca\(^{2+}\) influx from external medium. In ECs, the endoplasmic reticulum (ER) accounts for approximately 75\% of the total intracellular Ca\(^{2+}\) stores and the [Ca\(^{2+}\)] in the ER significantly determines the generation of important Ca\(^{2+}\) signals that regulate vascular tone. The ER membrane constitutes Ca\(^{2+}\) pumps (sarco/endoplasmic reticulum ATPase (SERCA)) and several classes of intracellular Ca\(^{2+}\)-releasing channels, including the inositol triphosphate receptors (IP\(_3\)Rs) and the ryanodine receptors (RyRs). ECs predominantly express SERCA3 (and also express low level of SERCA2b). Depletion of Ca\(^{2+}\) from the ER activates Ca\(^{2+}\)-permeable channels in the plasma membrane and induces store-operated Ca\(^{2+}\) entry (SOCE) which ensures long term signaling. Stromal interaction molecule (STIM) and Orai were identified recently as essential proteins for SOCE. Recent reports demonstrate that STIM1 serves as a functional sensor for SOCE and therefore contributes to Ca\(^{2+}\) refilling into the ER after store depletion. However, the role of STIM1 in coronary endothelial dysfunction in diabetes is unexplored.

In this study, we demonstrate that the increase in [Ca\(^{2+}\)\(_{\text{cyt}}\)] due to Ca\(^{2+}\) leak from the ER induced by a SERCA blocker (this is an indirect way to measure the Ca\(^{2+}\) concentration in the ER) is significantly inhibited in mouse coronary ECs (MCECs) isolated from diabetic mice compared with ECs from control mice. Protein expression of STIM1 and SERCA3 are significantly lower in MCECs from diabetic mice than in MCECs from control mice. Not only does STIM1 overexpression in diabetic MCECs increase the amount of Ca\(^{2+}\) leakage from the ER and raise [Ca\(^{2+}\)\(_{\text{ER}}\) toward the control level, but it also restores endothelium-dependent relaxation that was attenuated in diabetic CAs. These data suggest that the restoration of increases in [Ca\(^{2+}\)\(_{\text{cyt}}\)] due to Ca\(^{2+}\) leak from the ER by STIM1 overexpression has a beneficial effect on coronary endothelial function, which may subsequently decrease the incident of cardiac ischemia in diabetes.
METHODS

An expanded Methods section is available in the Online Data Supplement.

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. Plasma glucose levels were 10.1 ± 0.6 mmol/l in control mice and 32.2 ± 0.6 mmol/l in diabetic mice.

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.

RESULTS

Hyperglycemia attenuates the rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to Ca\(^{2+}\) leakage from the ER.

Ca\(^{2+}\) is an essential signaling element for endothelial functions, including endothelium-dependent vascular relaxation by activating the endothelial NO synthase (a Ca\(^{2+}\)-dependent enzyme)\(^{19, 20}\) and Ca\(^{2+}\)-activated K\(^{+}\) channels in ECs (which leads to hyperpolarization)\(^{21-23}\). Ca\(^{2+}\) in the ER contributes greatly to Ca\(^{2+}\)-dependent endothelial function\(^{24-28}\). We first tested whether MCECs isolated from diabetic mice altered Ca\(^{2+}\) leak from the ER after stimulation by cyclopiazonic acid (CPA, a SERCA inhibitor, 10 \(\mu\)mol/l) in the absence of extracellular Ca\(^{2+}\). The rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) during CPA treatment in ECs superfused with Ca\(^{2+}\)-free solution is often referred to as an indirect estimation of [Ca\(^{2+}\)]\(_{\text{ER}}\). As shown in Figure 1B and C (1\(^{st}\) \(\Delta\)F/F\(_0\) and 1\(^{st}\) AUC), the rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to CPA-mediated Ca\(^{2+}\) leak was significantly attenuated in MCECs isolated from diabetic mice (red tracings) compared with ECs from control mice (black tracings). The resting level of [Ca\(^{2+}\)]\(_{\text{cyt}}\) (referred as F\(_0\) in the graph) was not significantly different between control ECs and diabetic ECs (control: 1.87 ± 0.06, diabetic: 1.79 ± 0.05, P=0.33). We then tested the increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) through SOCE by adding extracellular Ca\(^{2+}\) in the presence of CPA. As shown in Fig. 1C (described as 2\(^{nd}\) \(\Delta\)F/F\(_0\) and 2\(^{nd}\) AUC), there was no significant difference in SOCE between control and diabetic MCECs. The exposure of ECs to high glucose (HG) significantly attenuated the Ca\(^{2+}\) leak from the ER compared with ECs treated with normal glucose (NG) (Fig. 2). These data suggest that hyperglycemia leads to a decrease in [Ca\(^{2+}\)]\(_{\text{ER}}\) in ECs.

Hyperglycemia decreases the protein expression of STIM1 and SERCA3.

[Ca\(^{2+}\)]\(_{\text{ER}}\) is regulated by the activity of Ca\(^{2+}\) pump (SERCA) and several classes of Ca\(^{2+}\) release channels (IP\(_{3}\)Rs and RyRs), and by SOCE (e.g., STIM1/2, Orai1-3). We used freshly isolated MCECs to determine STIM1 and SERCA3 protein expression. MCECs from diabetic mice exhibited significantly lower protein expression of STIM1 and SERCA3 than control MCECs (Fig. 3A). HG-treatment in ECs \(\text{ex vivo}\) not only downregulated STIM1 and SERCA3 protein expression (Fig. 3B), but also inhibited the coupling of STIM1-SERCA3 in comparison to ECs treated with NG (Fig. 3C). The attenuated rise of [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to Ca\(^{2+}\) leak from the ER during CPA stimulation shown in Figure 1, might be due to the
attenuated ER refilling resulting from the decrease in SERCA3 and STIM1 protein expression in diabetic MCECs.

Overexpression of STIM1 significantly enhances the rise of \([\text{Ca}^{2+}]_{\text{cyt}}\) due to \(\text{Ca}^{2+}\) leak from the ER during CPA stimulation and increases the \([\text{Ca}^{2+}]_{\text{ER}}\) in diabetic MCECs.

An increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in ECs leads to vascular relaxation, thus restoring the level of \(\text{Ca}^{2+}\) in the ER of diabetic MCECs helps improve endothelium-dependent relaxation. Enhanced SOCE and a subsequent increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in smooth muscle cells by upregulated STIM1 and/or STIM2 are, however, one of the important causes for systemic\(^{29}\) and pulmonary\(^{30}\) hypertension. Thus, we designed an adenovirus that encoded STIM1 positive mutant with a Tie2 promoter, which can be selectively expressed in ECs\(^{31,32}\). As shown in Figure 4, overexpression of the STIM1 restored the level of \(\text{Ca}^{2+}\) leak from the ER during CPA stimulation in diabetic MCECs toward the control level. In addition, direct measurement of \([\text{Ca}^{2+}]_{\text{ER}}\) demonstrates that STIM1 overexpression in diabetic MCECs increases the resting level of \([\text{Ca}^{2+}]_{\text{ER}}\) and the amount of \(\text{Ca}^{2+}\) released from the ER after CPA treatment toward the level seen in control MCECs (Fig. 5). These data suggest that STIM1 regulates \(\text{Ca}^{2+}\) refilling into the ER in diabetic ECs.

It has to be emphasized that, in these \textit{ex vivo} experiments, we did not overexpress STIM1 in diabetic MCECs above the level seen in control cells. Since STIM1 is significantly downregulated in coronary endothelial cells in diabetic mice, we actually restored the expression level of STIM1 in diabetic MCECs to the level similar to the STIM1 expression level in control MCECs. Furthermore, our experiments demonstrated that overexpression of STIM1 in HEK293 cells had no inhibitory effect on Orai1 expression and function; STIM1 overexpression actually enhances the increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to \(\text{Ca}^{2+}\) influx through SOC/Orai1 channels and augments \(\text{Ca}^{2+}\) currents through SOC/Orai1 channels (Online Figure VII).

Overexpression of STIM1 restores endothelium-dependent vascular relaxation that was attenuated in diabetic CAs.

Diabetic CAs exhibit attenuated endothelium-dependent relaxation (assessed by ACh-induced relaxation), but not smooth muscle cell-dependent relaxation (assessed by SNP)\(^{11}\). STIM1-Adv or control-Adv was infected in CAs dissected from control or diabetic mice and isometric tension experiments were performed 24 hours after infection. Figure 6A demonstrates that 10 \(\mu\text{M}\) CPA-induced relaxation was significantly attenuated in diabetic CAs infected with control-Adv compared with control CAs infected with control-Adv. However, STIM1 overexpression in diabetic CAs significantly augmented CPA-induced vascular relaxation close to the level shown in control CAs (Fig. 6A). ACh-induced relaxation was significantly attenuated in diabetic CAs compared with control CAs (Fig. 6B), whereas \(10^{-4}\text{ M SNP}-\text{induced vascular relaxation was not significantly different between control and diabetic CAs (control CAs; }99.7 \pm 3.5, \text{ diabetic CAs; }103.3 \pm 8.2, \text{ } P=0.70\). STIM1 overexpression significantly increased ACh-induced vascular relaxation in diabetic CAs, while there was no difference in SNP-induced relaxation between diabetic CAs infected with control Adv and diabetic CAs infected with STIM1-Adv (Fig. 6C). In addition, we measured and compared NO production in control and diabetic MCECs. As shown in Figure 6D-E, NO production at the resting condition and during CPA treatment in the absence of extracellular \(\text{Ca}^{2+}\) is decreased in diabetic MCECs compared to control MCECs, which is partially, but significantly, restored toward the control level by STIM1 overexpression in diabetic MCECs. These data suggest that downregulated STIM1 protein expression in coronary ECs leads to a decrease in \(\text{Ca}^{2+}\) release from the ER, and subsequently attenuates endothelium-dependent relaxation in diabetic CAs.
Inhibition of STIM1 attenuates the rise in \([Ca^{2+}]_{cyt}\) due to \(Ca^{2+}\) release/leakage from the ER during CPA treatment and decreases \([Ca^{2+}]_{ER}\) in coronary ECs.

Two days after STIM1 siRNA transfection in coronary ECs by electroporation, \(Ca^{2+}\) release/leakage from the ER during CPA treatment, SOCE, and \([Ca^{2+}]_{ER}\) were examined. Our data demonstrate that STIM1 downregulation not only decreases the SOCE but also attenuates the increase in \([Ca^{2+}]_{cyt}\) due to \(Ca^{2+}\) leak from the ER and significantly decreases \([Ca^{2+}]_{ER}\) in ECs (Fig. 7).

Treatment of free fatty acid (FFA) significantly decreases STIM1 mRNA and protein levels, but HG decreases STIM1 protein expression without changing the mRNA level in mouse coronary ECs.

In diabetic, plasma glucose and FFA levels are both significantly increased and these changes trigger and progress vascular complications. We tested whether FFA or HG affects STIM1 mRNA level and found that only FFA decreases STIM1 mRNA although both HG and FFA significantly decrease STIM1 protein expression \textit{ex vivo} (Fig. 8).

**DISCUSSION**

The major findings of the current study are that \(a)\) downregulation of STIM1 protein expression in diabetic MCECs leads to attenuated coronary vascular relaxation due to the decrease in stored \(Ca^{2+}\) in the ER and \(b)\) STIM1 expression in diabetic MCECs restores the endothelial function, which may subsequently decrease the incidence of cardiac ischemia in diabetes.

Inhibition of SERCA (by CPA or thapsigargin) causes a transient increase in \([Ca^{2+}]_{cyt}\) of ECs due to \(Ca^{2+}\) leakage from the ER and induces endothelium-dependent relaxation (EDR). Prolonged treatment of ECs with CPA or thapsigargin (e.g., for 10-15 min) depletes \(Ca^{2+}\) from the ER and, thus inhibits ACh-induced vasodilation.\(^{24-27}\). These observations suggest that a rise in \([Ca^{2+}]_{cyt}\) in ECs due to \(Ca^{2+}\) release from the ER is crucial for EDR. In addition to inducing EDR, the sufficient \([Ca^{2+}]_{ER}\) and the rise in \([Ca^{2+}]_{cyt}\), due to \(Ca^{2+}\) release from the ER, also contribute to stimulating EC migration and proliferation.\(^{33,34}\) Taken together, \(Ca^{2+}\) released from the ER plays an important role in endothelial function. In other words, the attenuated \(Ca^{2+}\) release from the ER (e.g., by a decreased level of \([Ca^{2+}]_{ER}\) in the ER) exerts a maladaptive effect on physiological functions of endothelium. Coronary ECs isolated from diabetic mice exhibit a significant attenuation of the rise in \([Ca^{2+}]_{cyt}\) during CPA treatment compared with coronary ECs isolated from control mice, whereas \(Ca^{2+}\) influx via SOC is not significantly different between control and diabetic MCECs (Fig. 1). HG treatment in normal ECs significantly lowers CPA-mediated \(Ca^{2+}\) leak from the ER compared with NG-treated ECs (Fig. 2). These data imply that decreased \(Ca^{2+}\) release from the ER in coronary ECs by hyperglycemia is one of the causes for attenuated coronary vascular relaxation in diabetes.

STIM1, a functional sensor of stored \([Ca^{2+}]_{ER}\) in the ER, and Orai, a pore-forming subunit of SOC, were identified as essential proteins for SOCE.\(^{14,15}\) In ECs, STIM1 and Orai are both highly expressed. When the ER \(Ca^{2+}\) store is depleted, STIM1 undergoes a conformational change, which allows it to multimerize, translocate to the ER-plasma membrane junction (or puncta), bind with Orai1 tetramers on the plasma membrane, activate SOC, and induce SOCE. The STIM1-mediated SOCE not only contributes to a sustained rise in \([Ca^{2+}]_{cyt}\) but also contributes to \(Ca^{2+}\) refilling into the ER by interacting with SERCA (Online Figure V).\(^{17,18}\) Our data demonstrate that protein expressions of STIM1 and SERCA3 are significantly decreased in MCECs isolated from diabetic mice compared with control MCECs (Fig. 3A). \textit{Ex vivo} experiments using HG reveal that HG treatment not only lowers STIM1 and SERCA3 protein expression (Fig. 3B), but also decreases the coupling of STIM1-SERCA3 (Fig. 3C). Further experiments are required in order to define the detailed mechanisms that cause the decrease in STIM1-SERCA3.
coupling. Although STIM2 is not predominant among STIM subtypes, MCECs express STIM2 and its function in diabetic MCECs also needs to be examined.

One would expect to see that the decreased STIM1 protein expression in diabetic MCECs should attenuate SOCE-mediated \([\text{Ca}^{2+}]_{\text{cyt}}\) increase. However, contrary to our expectation, our data showed that there was no significant difference of the increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to SOCE between control MCECs and diabetic MCECs (Fig. 1C, lower panels). It has been reported that STIM1 regulates \(\text{Ca}^{2+}\) refilling into the ER not only via SOC-mediated indirect pathway, but also via direct interaction with SERCA in other cell types\(^{17,18}\). The indirect refilling mechanism is mainly caused by the uptake of increased cytosolic \(\text{Ca}^{2+}\) due to SOCE via SERCA on the ER membrane. The direct refilling mechanism is that the \(\text{Ca}^{2+}\) ions entered cell via SOC can be “directly” sequestered by the SERCA without slowly diffusing into the cytosol due to the STIM1-SERCA interaction and this mechanism might be more efficient than the indirect mechanism in refilling \(\text{Ca}^{2+}\) into the ER. We hypothesize that the degree of decrease in STIM1 protein expression (as shown in diabetic MCECs) is sufficient to affect the level of \([\text{Ca}^{2+}]_{\text{ER}}\), but insufficient to regulate SOCE-mediated global increase in \([\text{Ca}^{2+}]_{\text{cyt}}\). In addition, it is possible that other STIM1-independent mechanisms for SOCE (e.g., TRPC1) may be compensatorily affected in diabetic MCECs, which makes the amplitude of SOCE comparable between normal and diabetic MCECs.

In addition to the store depletion-mediated STIM1 interaction with Orai\(^{16}\), direct interaction between SERCA3 and TRPC channels (e.g., human TRPC1 and TRPC6) can be stimulated by extracellular ligand and store depletion to form STIM/SERCA3/TRPC complex\(^{35-37}\). In human platelets, SERCA3 forms the macromolecular protein complex with TRPC1/6 and IP\(_3\)R that is activated by store depletion or thrombin to mediate SOCE. In pulmonary vascular endothelial cells, Orai1 interacts with TRPC and regulates the ion selectivity and activation kinetics of store-operated channels\(^{38,39}\). Physical interaction among TRPC/Orai, SERCA and STIM in vascular endothelial cells would significantly enhance the efficiency for \(\text{Ca}^{2+}\) refilling into the ER (e.g., via a direct refilling mechanism without a global increase in \([\text{Ca}^{2+}]_{\text{cyt}}\)) and maintaining the normal endothelial function. The data from our study indicate that downregulated STIM1 (and SERCA3) in diabetic MCECs leads to a decrease in \([\text{Ca}^{2+}]_{\text{ER}}\) and an inhibition of endothelium-dependent coronary vasodilation. It is possible that dysfunctional (or inhibited) interaction among STIM1, SERCA3 and TRPC is also involved in the attenuated \(\text{Ca}^{2+}\) refilling into the ER in diabetic ECs.

The increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in smooth muscle cells (SMCs) causes smooth muscle contraction and SMC proliferation, which lead to the increase in vascular tension and resistance. Indeed, upregulated STIM1/2 in SMCs has been implicated in several cardiovascular diseases\(^{39,40,42}\). In contrast, recent reports have highlighted the importance of STIM1 in endothelial physiological functions\(^{16,39,43}\). This current study is the first report to demonstrate the pathophysiological role of STIM1 in diabetic ECs. Since STIM1 overexpression needs to be selective in ECs, we designed the adenovirus that carries the Tie2 promoter so STIM1 is only expressed in ECs\(^{31,32}\) (Fig. 4A-C, Online Figure IV and VI). After infection of STIM1-Adv in diabetic MCECs, the CPA-induced rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to \(\text{Ca}^{2+}\) leak from the ER (Fig. 4D) and the \([\text{Ca}^{2+}]_{\text{ER}}\) (Fig. 5) were significantly increased toward the level similar to control MCECs. These data suggest that STIM1 regulates \([\text{Ca}^{2+}]_{\text{ER}}\) in normal ECs and that overexpression of STIM1 (or restoration of the expression level of STIM1) in diabetic MCECs helps to increase the \(\text{Ca}^{2+}\) release from the ER and thus endothelium-dependent coronary vasodilatation.

We also examined the effect of STIM1 on \(\text{Ca}^{2+}\) leak from the ER in control MCECs (Online Figure I); overexpression of STIM1-Adv in control MCECs did not increase the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) after CPA treatment. It might be because that 1) the endogenous level of STIM1 protein is very high in control MCECs, therefore overexpression of exogenous STIM1 is unable to further enhance its function, and 2) the amount of SERCA in control MCECs is low and insufficient to bind to extra STIM1 to enhance \(\text{Ca}^{2+}\) uptake or refilling.
Endothelial dysfunction is considered to be a major risk factor for cardiovascular complications in diabetes. The main physiological roles of ECs are the regulation of vascular tone, new vessel formation, and vascular wall permeability. It has been reported that in Type1 diabetic animal models as well as in human diabetic patients, capillary density in the heart is progressively decreased, endothelium-dependent relaxation is significantly attenuated in the coronary artery, and endothelial cell permeability is increased. Augmented tension and increased resistance in the coronary artery lead to an insufficient delivery of oxygen to the cardiac myocyte and causes cardiac ischemia, a leading cause of mortality and morbidity in diabetes. Therefore, a decrease in vascular contractility in coronary arteries should have beneficial effects on coronary vascular complications in diabetes. ACh-induced relaxation was significantly decreased in CAs in diabetic mice compared with control CAs. STIM1 overexpression not only restored CPA-induced vascular relaxation, but also augments ACh-induced relaxation to the level shown in control CAs through increasing NO production. These data suggest that a decrease in Ca\textsuperscript{2+} release from the ER due to downregulated STIM1 protein expression in ECs is one of the causes that lead to attenuated endothelium-dependent relaxation in CAs in diabetes.

In coronary arterial SMCs, the protein expression level of STIM1 is slightly (but statistically significant) upregulated in diabetic mice in comparison to control mice. However, the 40K+-mediated coronary vasoconstriction was actually decreased in coronary arteries isolated from diabetic mice (Online Figure III). We will further examine the function of STIM1 in diabetic SMCs in the future.

We have shown the lower expression level of SERCA3 protein in diabetic MCECs than in control MCECs (Fig. 3). These data imply that overexpression of SERCA3 may also have beneficial effects on coronary endothelial dysfunction in diabetes. In Figure 7, we examined the effect of STIM1 knockdown on the Ca\textsuperscript{2+} leak from the ER, SOCE and [Ca\textsuperscript{2+}]\textsubscript{ER} in normal ECs. Downregulation of STIM1 with siRNA significantly inhibited the CPA-induced increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to Ca\textsuperscript{2+} leakage from the ER and the CPA-mediated increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to SOCE and significantly decreased [Ca\textsuperscript{2+}]\textsubscript{ER} in ECs. Furthermore, we demonstrate that STIM1 overexpression fully restores not only [Ca\textsuperscript{2+}]\textsubscript{ER} in diabetic MCECs but also endothelium-dependent relaxation in diabetic coronary artery in Figure 4-6. These data suggest that STIM1 might play the prominent role in coronary endothelial dysfunction in diabetes. Further studies are required to determine the role of SERCA3 and its interaction with STIM1 and TRPC channels in diabetes.

We are not sure why and how STIM1 protein expression level is altered in diabetic coronary endothelia cells. In diabetes, the plasma levels of glucose and free fatty acid (FFA) are both elevated. In our ex vivo experiments, we showed that exposure of EC to high glucose and FFA significantly decreases the protein expression of STIM1, while only FFA decreases the mRNA expression of STIM1 (Fig. 8). These observations suggest that increased protein degradation (e.g., ubiquitinylation) and microRNA-mediated posttranscriptional modulation are potentially mechanisms involved in HG-mediated decrease in STIM1 protein level.

Taken together, our data suggest that the restoration of effective and sufficient Ca\textsuperscript{2+} release from the ER by STIM1 overexpression improves endothelium-dependent relaxation in diabetic CAs, and may be a novel strategy to develop therapeutic approach for cardiac ischemia in diabetes.

**SOURCES OF FUNDING**
This work was supported in part by the grants of DK083506 and HL115578 (A. Makino) and HL066012 and HL098053 (J. Yuan) from the National Institutes of Health.

**DISCLOSURES**
None.
REFERENCES


18. Jousset H, Frieden M, Demaurex N. STIM1 knockdown reveals that store-operated Ca\textsuperscript{2+} channels located close to sarco/endoplasmic Ca\textsuperscript{2+} ATPases (SERCA) pumps silently refill the endoplasmic reticulum. J Biol Chem. 2007;282:11456-11464.

DOI: 10.1161/CIRCRESAHA.112.275743


35. Redondo PC, Jardin I, Lopez JJ, Salido GM, Rosado JA. Intracellular Ca\(^{2+}\) store depletion induces the formation of macromolecular complexes involving hTRPC1, hTRPC6, the type II IP3 receptor and SERCA3 in human platelets. *Biochim Biophys Acta.* 2008;1783:1163-1176.


49. Warley A, Powell JM, Skepper JN. Capillary surface area is reduced and tissue thickness from capillaries to myocytes is increased in the left ventricle of streptozotocin-diabetic rats. Diabetologia. 1995;38:413-421.


FIGURE LEGENDS

Figure 1. Hyperglycemia significantly inhibits the rise in \([Ca^{2+}]_{cyt}\) due to Ca\(^{2+}\) release/leakage from the ER during cyclopiazonic acid (CPA) treatment in coronary ECs. A. Typical record of the change in \([Ca^{2+}]_{cyt}\) in coronary ECs and the parameters used for the statistical analysis. 1\(^{st}\) peak describes the rise in \([Ca^{2+}]_{cyt}\) by CPA treatment in the absence of extracellular Ca\(^{2+}\) (indirect indicator of \([Ca^{2+}]_{ER}\)) and 2\(^{nd}\) peak indicates store-operated Ca\(^{2+}\) entry (SOCE). B. Averaged record of the change in \([Ca^{2+}]_{cyt}\) in coronary ECs isolated from control (black) and diabetic mice (red). Three days after EC isolation, ECs were used for \([Ca^{2+}]_{cyt}\) measurement with Fura-2-AM. Thirty minutes after preincubation of cells with physiological salt solution with Ca\(^{2+}\) (Ca\(^{2+}\)-PSS), extracellular solution was switched to Ca\(^{2+}\) free-PSS. 10 \(\mu\)M CPA was added in Ca\(^{2+}\) free-PSS to determine the \([Ca^{2+}]_{ER}\) indirectly. Data are described as a normalized ratio (F/F\(_0\), F=I\(_{340}\)/I\(_{380}\), F\(_0\)=average of F during first 5 min recording in Ca\(^{2+}\)-PSS). C. Summarized data of \(\Delta F/F_0\) and Area Under the Curve (AUC). Control (Cont, open bars); n=18 cells, diabetic (Dia, solid bars); n=34 cells. Data are mean ± SE. \(P*<0.05\) vs. Cont.

Figure 2. High-glucose treatment over 48 hours significantly inhibits the rise in \([Ca^{2+}]_{cyt}\) due to Ca\(^{2+}\) release/leakage from the ER during CPA treatment in coronary ECs. 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). Normal glucose (NG, open bars); n=22 cells, high glucose (HG, solid bars); n=17 cells. Data are mean ± SE. \(P*<0.05\) vs. NG.

Figure 3. Hyperglycemia downregulates protein expression of STIM1 and SERCA3 in coronary ECs. A. Freshly isolated coronary ECs from control mice (Cont, open bars) or diabetic mice (Dia, solid bars) were used to determine the protein concentration of STIM1 and SERCA3 by Western blot. Data are normalized by the signal of actin. STIM1; n=4 in each group, SERCA3; n=6 in each group. Data are mean ± SE. \(P<0.05\) vs. Cont. B. Two days after the exposure of ECs to NG (open bars) or HG (solid bars), cells were lysed and protein concentration of STIM1 and SERCA3 was measured. STIM1; n=7 in each group, SERCA3; n=3 in each group. Data are mean ± SE. \(P<0.05\) vs. NG. C. Immunoprecipitation (IP) of SERCA3 with STIM1. After NG or HG treatment, cells were lysed and IP was performed using anti-STIM1 N-terminus antibody. Immunoblotting (IB) was determined using anti-SERCA3 antibody. The same membrane was used for IB with anti-STIM1 C-terminus to normalize the data. For each group, n=3. Data are mean ± SE. \(P<0.05\) vs. NG.

Figure 4. STIM1 overexpression restores the attenuated increase in \([Ca^{2+}]_{cyt}\) due to Ca\(^{2+}\) release/leakage from the ER during CPA treatment in MCECs isolated from diabetic mice. A. Construction of STIM1 positive mutant with the Tie2 promoter in an adenoviral vector. B. STIM1 adenovirus (Adv.) infection in HCECs upregulates STIM1 protein concentration determined by WB. C. STIM1 protein expression level in MCECs after Adv. infection. Representative images showing STIM1 protein expression level determined by immunofluorescence in control MCECs infected with control-Adv. (Cont EC-Cont Adv), diabetic MCECs infected with control-Adv (Dia EC-Cont Adv), and diabetic MCECs infected with STIM1 Adv (Dia EC-STIM1 Adv). Dark dots in the cells are beads used for cell isolation. Bar = 20 \(\mu\)m. The lower panel showing the summarized data of STIM1 expression level (intensity). Cont EC-Cont Adv; n= 104 cells, Dia EC-Cont Adv; n=143 cells, Dia EC-STIM1 Adv; n=151 cells. Data are mean ± SE. \(P<0.05\) vs. Cont EC-Cont Adv. D. STIM1 overexpression in MCECs isolated from diabetic mice significantly increased the rise in \([Ca^{2+}]_{cyt}\) due to Ca\(^{2+}\) leakage, but not by SOCE, toward the level of control MCECs. 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 ECs infected with control Adv (Cont EC-Cont Adv, open bars), n=18, diabetic ECs infected with control Adv (Dia EC-Cont Adv, open bars), n=22, and diabetic ECs infected with STIM1 Adv (Dia EC-STIM1 Adv, hatched bars), n=25. Data are mean ± SE. \(P<0.05\) vs. Cont EC-Cont Adv.
STIM1 overexpression increases the $[\text{Ca}^{2+}]_{\text{ER}}$ in MCECs isolated from diabetic mice. A. Typical record of the change in $[\text{Ca}^{2+}]_{\text{ER}}$ in control MCECs and the parameters used for the statistical analysis. B. Averaged record of the change in $[\text{Ca}^{2+}]_{\text{ER}}$ in control MCECs infected with control-Adv. (Cont EC-Cont Adv, black tracing), diabetic MCECs infected with control-Adv (Dia EC-Cont Adv, red tracing), and diabetic MCECs infected with STIM1 Adv (Dia EC-STIM1 Adv, blue tracing). C. Summarized data of resting level of $[\text{Ca}^{2+}]_{\text{ER}}$, $\Delta$Peak, and the AUC (the change in $[\text{Ca}^{2+}]_{\text{ER}}$ after CPA treatment). Cont EC-Cont Adv (open bars); $n=25$, Dia EC-Cont Adv (solid bars); $n=21$, and Dia EC-STIM1 Adv (hatched bars); $n=30$. Data are mean ± SE. *$P<0.05$ vs. Cont EC-Cont Adv. # $P<0.05$ vs. Dia EC-Cont Adv. ANOVA was performed to test the statistical difference between the groups.

Overexpression of STIM1 restores CPA- and ACh-induced relaxation in diabetic CAs and increases NO production in diabetic MCECs. A. After precontraction of the CAs, CPA- (10 $\mu$M) induced vascular relaxation was observed. Relaxation was calculated versus the magnitude of the contraction induced by PGF$_{2\alpha}$ and described as %. Control CAs infected with control Adv (Cont CA-Cont Adv, open bar), $n=4$; diabetic CAs infected with control Adv (Dia CA-Cont Adv, solid bar), $n=5$; diabetic CAs infected with STIM1 Adv (Dia CA-STIM1 Adv, hatched bar), $n=3$. Data are mean ± SE. *$P<0.05$ vs. Cont CA-Cont Adv. $^*P<0.05$ vs. Dia CA-Cont Adv. B. Endothelium-dependent relaxation was determined by ACh-induced relaxation. After preconstruction of CAs, ACh was administrated with a dose dependent manner. Cont CA-Cont Adv (open circles), $n=4$; Dia CA-Cont Adv (solid circles), $n=5$; Dia CA-STIM1 Adv (solid triangles), $n=4$. Data are mean ± SE. *$P<0.05$ vs. Cont CA-Cont Adv. $^*P<0.05$ vs. Dia CA-Cont Adv. C. Endothelium-independent relaxation was determined by SNP-induced relaxation. After preconstruction of CAs, SNP was administrated with a dose dependent manner. Dia CA-Cont Adv (solid circles), $n=3$; Dia CA-STIM1 Adv (solid triangles), $n=3$. Data are mean ± SE. D. Resting level of DAF intensity was obtained from the average intensity of first 2-4 min during Ca$^{2+}$ PSS perfusion. Cont EC-Cont Adv (open bar), $n=44$; Dia EC-Cont Adv (solid bar), $n=49$; Dia EC-STIM1 Adv (hatched bar), $n=44$. Data are mean ± SE. *$P<0.05$ vs. Cont EC-Cont Adv. $^*P<0.05$ vs. Dia EC-Cont Adv. E. NO production due to Ca$^{2+}$ release/leakage from the ER during CPA treatment in coronary ECs. Left graph showing a typical record of DAF-FM intensity change indicated as F/F$_0$. The slope between time 15 ($t_{15}$) and time 24 ($t_{24}$) (total 9 min) was calculated (gray line) and used as an indication of NO production in response to CPA $[\text{d}(\text{F/F}_{0})/\text{d}t]$. Right panel showing the summarized data of $[\text{d}(\text{F/F}_{0})/\text{d}t]$ during CPA treatment ($t_{15}$-$t_{24}$). Cont EC-Cont Adv (open bar), $n=44$; Dia EC-Cont Adv (solid bar), $n=49$; Dia EC-STIM1 Adv (hatched bar), $n=44$. Data are mean ± SE. *$P<0.05$ vs. Cont EC-Cont Adv. $^*P<0.05$ vs. Dia EC-Cont Adv. ANOVA was performed to test the statistical difference between the groups.

STIM1 downregulation attenuates the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca$^{2+}$ release/leakage from the ER during CPA treatment and decreases $[\text{Ca}^{2+}]_{\text{ER}}$ in coronary ECs. A. STIM1 siRNA transfection in HCECs downregulates STIM1 protein expression determined by Western blot. Values are mean ± SE (n=2). *$P<0.05$ vs. Control siRNA. B. Summarized data of the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 siRNA (open bars); n=25 cells, STIM1 siRNA (solid bars); n=27 cells. Data are mean ± SE. P*<0.05 vs. Control siRNA. C. Summarized data of resting level of $[\text{Ca}^{2+}]_{\text{ER}}$, $\Delta$Peak, and the AUC (the change in $[\text{Ca}^{2+}]_{\text{ER}}$ after CPA treatment). Control siRNA (open bars); n=24, STIM1 siRNA (solid bars); n=30. Data are mean ± SE. *$P<0.05$ vs. Control siRNA.

High glucose (HG) and free fatty acid (HF) treatment downregulate protein expression of STIM1, whereas only HF decreases mRNA level of STIM1, in mouse coronary ECs. A and B. STIM1 protein (A, n=5 in each group) and mRNA (B, n=6 in each group) levels were measured in ECs treated with NG (open bars) or HG (solid bars) for 48 hs. Data are mean ± SE. *$P<0.05$ vs. NG. C and D.
STIM1 protein (A, n=3 in each group) and mRNA (B, n=6 in each group) levels were measured in ECs treated with vehicle (NF, open bars) or HF (solid bars) for 24 hs. Data are mean ± SE. *P<0.05 vs. NF.

Novelty and Significance

What Is Known?

- Coronary vascular endothelial dysfunction is implicated in the development and progression of cardiac ischemia and heart failure due to a decrease in coronary blood flow.
- The Ca^{2+} concentration in the endoplasmic reticulum (ER) is important for generating critical Ca^{2+} signals to mediate endothelium-dependent vasodilation.
- Stromal interaction molecule (STIM) protein (e.g., STIM1) is an important regulator that activates Ca^{2+}-permeable channels in the plasma membrane after depletion of Ca^{2+} from the ER and refill Ca^{2+} into the ER.

What New Information Does This Article Contribute?

- Protein expression of STIM1 is significantly downregulated and the Ca^{2+} concentration in the endoplasmic reticulum (ER) is markedly decreased in coronary endothelial cells in diabetes in comparison to coronary endothelial cells isolated from controls.
- Downregulated STIM1 in diabetic coronary endothelial cells contributes to the decreased Ca^{2+} concentration in the ER and results in significant inhibition of endothelium-dependent coronary vasodilation.
- Restoration of STIM1 protein expression in coronary endothelial cells has beneficial effect on coronary endothelial dysfunction in diabetes.

Attenuated endothelium-dependent coronary vasodilation is an important cause for cardiac ischemia and cardiovascular complications in diabetes. This study provides compelling evidence that the decreased Ca^{2+} concentration in the endoplasmic reticulum and the downregulated expression of STIM proteins in coronary endothelial cells play an important pathogenic role in diabetic endothelial dysfunction. Overexpression (or restoration of) of STIM1 protein in diabetic coronary endothelial cells increases (or restores) Ca^{2+} concentration in the ER, enhances nitric oxide (NO) production, and restores endothelium-dependent relaxation in diabetic coronary arteries. These findings yield critical information on the new targets that can be used to develop novel therapeutic approaches for cardiovascular complications and cardiac ischemia in diabetes.
Figure 1. Hyperglycemia significantly inhibits the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca$^{2+}$ release leakage from the ER during cyclopiazonic acid (CPA) treatment in coronary ECs. A. Typical record of the change in $[\text{Ca}^{2+}]_{\text{cyt}}$ in coronary ECs and the parameters used for the statistical analysis. 1st peak describes the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ by CPA treatment in the absence of extracellular Ca$^{2+}$ (indirect indicator of $[\text{Ca}^{2+}]_{\text{ER}}$) and 2nd peak indicates store-operated Ca$^{2+}$ entry (SOCE). B. Averaged record of the change in $[\text{Ca}^{2+}]_{\text{cyt}}$ in coronary ECs isolated from control (black) and diabetic mice (red). Three days after EC isolation, ECs were used for $[\text{Ca}^{2+}]_{\text{cyt}}$ measurement with Fura-2-AM. Thirty minutes after preincubation of cells with physiological salt solution with Ca$^{2+}$ (Ca$^{2+}$-PSS), extracellular solution was switched to Ca$^{2+}$ free-PSS. 10 μM CPA was added in Ca$^{2+}$ free-PSS to determine the $[\text{Ca}^{2+}]_{\text{ER}}$ indirectly. Data are described as a normalized ratio (F/F$_{0}$, F=I$_{340}$/I$_{380}$, F$_{0}$=average of F during first 5 min recording in Ca$^{2+}$-PSS). C. Summarized data of ΔF/F$_{0}$ and Area Under the Curve (AUC). Control (Cont, open bars); n=18 cells, diabetic (Dia, solid bars); n=34 cells. Data are mean ± SE. $P^{*}<0.05$ vs. Cont.
Figure 2. High-glucose treatment over 48 hours significantly inhibits the rise in [Ca^{2+}]_{cyt} due to Ca^{2+} release/leakage from the ER during CPA treatment in coronary ECs. Summarized data of the rise in [Ca^{2+}]_{cyt} due to Ca^{2+} release/leakage from the ER (1st ΔF/F_0 and 1st AUC) and SOCE (2nd ΔF/F_0 and 2nd AUC). Normal glucose (NG, open bars); n=22 cells, high glucose (HG, solid bars); n=17 cells. Data are mean ± SE. P*<0.05 vs. NG.
Figure 3. Hyperglycemia downregulates protein expression of STIM1 and SERCA3 in coronary ECs. A. Freshly isolated coronary ECs from control mice (Cont, open bars) or diabetic mice (Dia, solid bars) were used to determine the protein concentration of STIM1 and SERCA3 by Western blot. Data are normalized by the signal of actin. STIM1; n=4 in each group, SERCA3; n=6 in each group. Data are mean ± SE. *P<0.05 vs. Cont. B. Two days after the exposure of ECs to NG (open bars) or HG (solid bars), cells were lysed and protein concentration of STIM1 and SERCA3 was measured. STIM1; n=7 in each group, SERCA3; n=3 in each group. Data are mean ± SE. *P<0.05 vs. NG. C. Immunoprecipitation (IP) of SERCA3 with STIM1. After NG or HG treatment, cells were lysed and IP was performed using anti-STIM1 N-terminus antibody. Immunoblotting (IB) was determined using anti-SERCA3 antibody. The same membrane was used for IB with anti-STIM1 C-terminus to normalize the data. For each group, n=3. Data are mean ± SE. *P<0.05 vs. NG.
Figure 4. STIM1 overexpression restores the attenuated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca$^{2+}$ release/leakage from the ER during CPA treatment in MCECs isolated from diabetic mice. 

A. Construction of STIM1 positive mutant with the Tie2 promoter in an adenoviral vector. 

B. STIM1 adenovirus (Adv.) infection in HCECs upregulates STIM1 protein concentration determined by WB. 

C. STIM1 protein expression level in MCECs after Adv. infection. Representative images showing STIM1 protein expression level determined by immunofluorescence in control MCECs infected with control-Adv. (Cont EC-Cont Adv), diabetic MCECs infected with control-Adv (Dia EC-Cont Adv), and diabetic MCECs infected with STIM1 Adv (Dia EC-STIM1 Adv). Dark dots in the cells are beads used for cell isolation. Bar = 20 μm. The lower panel showing the summarized data of STIM1 expression level (intensity). Cont EC-Cont Adv; n= 104 cells, Dia EC-Cont Adv; n=143 cells, Dia EC-STIM1 Adv; n=151 cells. Data are mean ± SE. *P<0.05 vs. Cont EC-Cont Adv. #P<0.05 vs. Dia EC-Cont Adv. 

D. STIM1 overexpression in MCECs isolated from diabetic mice significantly increased the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca$^{2+}$ leakage, but not by SOCE, toward the level of control MCECs. Summarized data of the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca$^{2+}$ release/leakage from the ER (1$^{\text{st}}$ ΔF/F$_0$ and 1$^{\text{st}}$ AUC) and SOCE (2$^{\text{nd}}$ ΔF/F$_0$ and 2$^{\text{nd}}$ AUC). Control ECs infected with control Adv (Cont EC-Cont Adv, open bars), n=18, diabetic ECs infected with control Adv (Dia EC-Cont Adv, solid bars), n=22, and diabetic ECs infected with STIM1 Adv (Dia EC-STIM1 Adv, hatched bars), n=25. Data are mean ± SE. *P<0.05 vs. Cont EC-Cont Adv. #P<0.05 vs. Dia EC-Cont Adv. ANOVA was performed to test the statistical difference between the groups.
Figure 5. STIM1 overexpression increases the \([\text{Ca}^{2+}]_{\text{ER}}\) in MCECs isolated from diabetic mice. A. Typical record of the change in \([\text{Ca}^{2+}]_{\text{ER}}\) in coronary ECs and the parameters used for the statistical analysis. B. Averaged record of the change in \([\text{Ca}^{2+}]_{\text{ER}}\) in control MCECs infected with control-Adv. (Cont EC-Cont Adv, black tracing), diabetic MCECs infected with control-Adv (Dia EC-Cont Adv, red tracing), and diabetic MCECs infected with STIM1 Adv (Dia EC-STIM1 Adv, blue tracing). C. Summarized data of resting level of \([\text{Ca}^{2+}]_{\text{ER}}\), ΔPeak, and the AUC (the change in \([\text{Ca}^{2+}]_{\text{ER}}\) after CPA treatment). Cont EC-Cont Adv (open bars); n=25, Dia EC-Cont Adv (solid bars); n=21, and Dia EC-STIM1 Adv (hatched bars); n=30. Data are mean ± SE. *P<0.05 vs. Cont EC-Cont Adv. #P<0.05 vs. Dia EC-Cont Adv. ANOVA was performed to test the statistical difference between the groups.
Figure 6. Overexpression of STIM1 restores CPA- and ACh-induced relaxation in diabetic CAs and increases NO production in diabetic MCECs. A. After precontraction of the CAs, CPA- (10 μM) induced vascular relaxation was observed. Relaxation was calculated versus the magnitude of the contraction induced by PGF2α and described as %. Control CAs infected with control Adv (Cont CA-Cont Adv, open bar), n=4; diabetic CAs infected with control Adv (Dia CA-Cont Adv, solid bar), n=5; diabetic CAs infected with STIM1 Adv (Dia CA-STIM1 Adv, hatched bar), n=3. Data are mean ± SE. *P<0.05 vs. Cont CA-Cont Adv. #P<0.05 vs. Dia CA-Cont Adv. B. Endothelium-dependent relaxation was determined by ACh-induced relaxation. After precontraction of CAs, ACh was administrated with a dose dependent manner. Cont CA-Cont Adv (open circles), n=4; Dia CA-Cont Adv (solid circles), n=5; Dia CA-STIM1 Adv (solid triangles), n=4. Data are mean ± SE. *P<0.05 vs. Cont CA-Cont Adv. #P<0.05 vs. Dia CA-Cont Adv. C. Endothelium-independent relaxation was determined by SNP-induced relaxation. After precontraction of CAs, SNP was administrated with a dose dependent manner. Dia CA-Cont Adv (solid circles), n=3; Dia CA-STIM1 Adv (solid triangles), n=3. Data are mean ± SE. D. Resting level of DAF intensity was obtained from the average intensity of first 2-4 min during Ca2+ PSS perfusion. Cont EC-Cont Adv (open bar), n=44; Dia EC-Cont Adv (solid bar), n=49; Dia EC-STIM1 Adv (hatched bar), n=44. Data are mean ± SE. *P<0.05 vs. Cont EC-Cont Adv. #P<0.05 vs. Dia EC-Cont Adv. E. NO production due to Ca2+ release/leakage from the ER during CPA treatment in coronary ECs. Left graph showing a typical record of DAF-FM intensity change indicated as F/Fo. The slope between time 15 (t15) and time 24 (t24) (total 9 min) was calculated (gray line) and used as an indication of NO production in response to CPA [d(F/Fo)/dt]. Right panel showing the summarized data of d(F/Fo)/dt during CPA treatment (t15-t24). Cont EC-Cont Adv (open bar), n=44; Dia EC-Cont Adv (solid bar), n=49; Dia EC-STIM1 Adv (hatched bar), n=44. Data are mean ± SE. *P<0.05 vs. Cont EC-Cont Adv. #P<0.05 vs. Dia EC-Cont Adv. ANOVA was performed to test the statistical difference between the groups.
Figure 7. STIM1 downregulation attenuates the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to $\text{Ca}^{2+}$ release/leakage from the ER during CPA treatment and decreases $[\text{Ca}^{2+}]_{\text{ER}}$ in coronary ECs. A. STIM1 siRNA transfection in HCECs downregulates STIM1 protein expression determined by Western blot. Values are mean ± SE (n=2). *P<0.05 vs. Control siRNA. B. Summarized data of the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to $\text{Ca}^{2+}$ release/leakage from the ER (1st $\Delta F/F_0$ and 1st AUC) and SOCE (2nd $\Delta F/F_0$ and 2nd AUC). Control siRNA (open bars); n=25 cells, STIM1 siRNA (solid bars); n=27 cells. Data are mean ± SE. *P<0.05 vs. Control siRNA. C. Summarized data of resting level of $[\text{Ca}^{2+}]_{\text{ER}}$, ΔPeak, and the AUC (the change in $[\text{Ca}^{2+}]_{\text{ER}}$ after CPA treatment). Control siRNA (open bars); n=24, STIM1 siRNA (solid bars); n=30. Data are mean ± SE. *P<0.05 vs. Control siRNA.
Figure 8. High glucose (HG) and free fatty acid (HF) treatment downregulate protein expression of STIM1, whereas only HF decreases mRNA level of STIM1, in mouse coronary ECs. 

A and B. STIM1 protein (A, n=5 in each group) and mRNA (B, n=6 in each group) levels were measured in ECs treated with NG (open bars) or HG (solid bars) for 48 hs. Data are mean ± SE. *P<0.05 vs. NG. C and D. STIM1 protein (A, n=3 in each group) and mRNA (B, n=6 in each group) levels were measured in ECs treated with vehicle (NF, open bars) or HF (solid bars) for 24 hs. Data are mean ± SE. *P<0.05 vs. NF.
STIM1 Restores Coronary Endothelial Function in Type1 Diabetic Mice
Irene A. Estrada, Reshma Donthamsetty, Patryk Debski, Meng-Hua Zhou, Shenyuan L. Zhang, Jason X.-J. Yuan, Wenlong Han and Ayako Makino

Circ Res. published online August 14, 2012;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2012/08/14/CIRCRESAHA.112.275743

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/08/14/CIRCRESAHA.112.275743.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
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. 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. 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 Ca²⁺ ([Ca²⁺]_{cyt}) measurement
[Ca²⁺]_{cyt} in MCECs were measured using a modification of previously described methods. Isolated MCECs were plated on glass slides coated with 5% (wt/vol.) gelatine. After 3 days of recovery from isolation, [Ca²⁺]_{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 (ExtraCru 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 x 10¹¹ 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 previously⁸. 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 PGF₂α to generate similar contraction level in all groups (PGF₂α concentration: control, 1.1 x 10⁻⁶ ± 3.3E-07 M; diabetic, 6.3 x 10⁻⁶ ± 1.3E-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 methods⁹. 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 (I₀) was subtracted from cell intensity (I₀) [F=I₀-I₀]. 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/F₀). 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/F₀)/dt.

**STIM1 siRNA transfection in HCECs**
Downregulation of STIM1 in HCECs was achieved using the STIM1 siRNA (0.2 nmol/5x10⁴ 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 (10¹² 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 (10⁵ 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. 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 CaCl₂, 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**


Online Figure I.

Overexpression of STIM1 in control MCECs does not increase the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to \(\text{Ca}^{2+}\) release/leakage from the ER during CPA treatment. Summarized data of the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to \(\text{Ca}^{2+}\) release/leakage from the ER (1st \(\Delta F/F_0\) and 1st AUC) and SOCE (2nd \(\Delta F/F_0\) and 2nd 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 $\left[Ca^{2+}\right]_{\text{ER}}$, 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. \( \text{Ca}^{2+} \) 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 ΔF/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).