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STIM1 Restores Coronary Endothelial Function in Type 1 Diabetic Mice

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Abstract

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

Objective—This study is designed to examine the role of Ca^{2+} in the ER in coronary endothelial dysfunction in diabetes.

Methods and Results—Mouse coronary ECs (MCECs) isolated from diabetic mice exhibited (1) a significant decrease in the Ca^{2+} mobilization from the ER when the cells were treated by SERCA inhibitor, and (2) significant downregulation of STIM1 and SERCA3 protein expression in comparison to the controls. Overexpression of STIM1 restored (1) the increase in cytosolic Ca^{2+} concentration due to Ca^{2+} leak from the ER in diabetic MCECs, (2) the Ca^{2+} concentration in the ER, and (3) endothelium-dependent relaxation that was attenuated in diabetic coronary arteries.

Conclusions—Impaired ER Ca^{2+} 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^{2+} homeostasis; endothelial dysfunction

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.

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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.[1–4] 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.[5–10] We have recently demonstrated that capillary density in the left ventricle is significantly decreased in the heart of diabetic mice and that endothelium-dependent relaxation is significantly attenuated in diabetic coronary arteries (CAs) compared with control CAs.[11]

Endothelial function depends, to various extents, on the changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). $[Ca^{2+}]_{cyt}$ 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 regulates vascular tone.[12] 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_3Rs) and the ryanodine receptors (RyRs). ECs predominantly express SERCA3 (and also express low level of SERCA2b).[13] 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.[14,15] 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.[16–18] However, the role of STIM1 in coronary endothelial dysfunction in diabetes is unexplored.

In this study, we demonstrate that the increase in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} leak from the ER induced by a SERCA blocker (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 is 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 $[Ca^{2+}]_{ER}$ toward the control level, but it also restores endothelium-dependent relaxation that was attenuated in diabetic CAs. These data suggest that the restoration of an increase in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} leak from the ER by STIM1 overexpression has a beneficial effect on coronary endothelial function, which may subsequently decrease the incidence 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-week-old male C57BL6 mice were purchased from Harlan Laboratories (Madison, WI); mice in the diabetic group received a single injection of streptozotocin (133 mg/kg, dissolved in citrate buffer, IV). All data were 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±SEM. Bonferroni tests for multiple statistical comparisons and Student *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 $[Ca^{2+}]_{cyt}$ 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 nitric oxide 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 μmol/L) in the absence of extracellular Ca^{2+}. The rise in $[Ca^{2+}]_{cyt}$ during CPA treatment in ECs superfused with Ca^{2+}-free solution is often referred to as an indirect estimation of $[Ca^{2+}]_{ER}$. As shown in Figure 1B and 1C (1st $\Delta F/F_0$ and 1st AUC), the rise in $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]_{cyt}$ (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 $[Ca^{2+}]_{cyt}$ through SOCE by adding extracellular Ca^{2+} in the presence of CPA. As shown in Figure 1C (described as 2nd $\Delta F/F_0$ and 2nd 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) (Figure 2). These data suggest that hyperglycemia leads to a decrease in $[Ca^{2+}]_{ER}$ in ECs.

Hyperglycemia Decreases the Protein Expression of STIM1 and SERCA3

$[Ca^{2+}]_{ER}$ is regulated by the activity of Ca^{2+} pump (SERCA) and several classes of Ca^{2+} release channels (IP_3Rs and RyRs) and by SOCE (eg, 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 (Figure 3A). HG-treatment in ECs ex vivo not only downregulated STIM1 and SERCA3 protein expression (Figure 3B), but also inhibited the coupling of STIM1-SERCA3 in comparison to ECs treated with NG (Figure 3C). The attenuated rise of $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]_{cyt}$ Due to Ca^{2+} Leak From the ER During CPA Stimulation and Increases the $[Ca^{2+}]_{ER}$ in Diabetic MCECs

An increase in $[Ca^{2+}]_{cyt}$ in ECs leads to vascular relaxation, thus restoring the level of Ca^{2+} in the ER of diabetic MCECs helps improve endothelium-dependent relaxation. Enhanced SOCE and a subsequent increase in $[Ca^{2+}]_{cyt}$ 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 Ca^{2+} leak from the ER during CPA stimulation in diabetic MCECs toward the control level. In addition, direct measurement of $[Ca^{2+}]_{ER}$ demonstrates that STIM1 overexpression in diabetic MCECs increases the resting

level of $[Ca^{2+}]_{ER}$ and the amount of Ca^{2+} released from the ER after CPA treatment toward the level seen in control MCECs (Figure 5). These data suggest that STIM1 regulates Ca^{2+} refilling into the ER in diabetic ECs.

It must be emphasized that in these ex vivo experiments, we did not overexpress STIM1 in diabetic MCECs above the level seen in control cells. Because 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 $[Ca^{2+}]_{cyt}$ due to Ca^{2+} influx through SOC/Orai1 channels and augments 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 acetylcholine [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 μmol/L 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 (Figure 6A). ACh-induced relaxation was significantly attenuated in diabetic CAs compared with control CAs (Figure 6B), whereas 10^{-4} mol/L SNP-induced vascular relaxation was not significantly different between control and diabetic CAs (control CAs, 99.7±3.5; diabetic CAs, 103.3±8.2; P=0.70). STIM1 overexpression significantly increased ACh-induced vascular relaxation in diabetic CAs, whereas there was no difference in SNP-induced relaxation between diabetic CAs infected with control Adv and diabetic CAs infected with STIM1-Adv (Figure 6C). In addition, we measured and compared nitric oxide production in control and diabetic MCECs. As shown in Figure 6D and 6E, nitric oxide production at the resting condition and during CPA treatment in the absence of extracellular Ca^{2+} is decreased in diabetic MCECs compared with 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 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 (Figure 7).

Treatment of Free Fatty Acid Significantly Decreases STIM1 mRNA and Protein Levels but HG Decreases STIM1 Protein Expression Without Changing the mRNA Level in Mouse Coronary ECs

In diabetes, plasma glucose and free fatty acid (FFA) levels are both significantly increased, and these changes trigger and progress vascular complications. We tested whether FFA or

HG affects STIM1 mRNA levels and found that only FFA decreases STIM1 mRNA, however both HG and FFA significantly decrease STIM1 protein expression in ex vivo (Figure 8).

Discussion

The major findings of the current study are that (1) 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 (2) 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 (eg, for 10–15 minutes) 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 (eg, by a decreased level of $[Ca^{2+}]$ 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 (Figure 1). HG treatment in normal ECs significantly lowers CPA-mediated Ca^{2+} leak from the ER compared with NG-treated ECs (Figure 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.

STIM, a functional sensor of stored $[Ca^{2+}]$ in the ER, and Orai, a pore-forming subunit of SOC, were identified as essential proteins for SOCE.[14,15] In ECs, STIM1 and Orai1 are both highly expressed.[16] 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 (Figure 3A). Ex vivo experiments using HG reveal that HG treatment not only lowers STIM1 and SERCA3 protein expression (Figure 3B) but also decreases the coupling of STIM1-SERCA3 (Figure 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 must be examined.

One would expect to see that the decreased STIM1 protein expression in diabetic MCECs should attenuate SOCE-mediated $[Ca^{2+}]_{cyt}$ increase. However, contrary to our expectations, our data showed that there was no significant difference in the increase of $[Ca^{2+}]_{cyt}$ due to SOCE between control MCECs and diabetic MCECs (Figure 1C, lower panels). It has been reported that STIM1 regulates Ca^{2+} refilling into the ER not only via the 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 Ca^{2+} due

to SOCE via SERCA on the ER membrane. The direct refilling mechanism is that the 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 may be more efficient than the indirect mechanism in refilling 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 $[Ca^{2+}]_{ER}$, but insufficient to regulate SOCE-mediated global increase in $[Ca^{2+}]_{cyt}$. In addition, it is possible that other STIM1-independent mechanisms for SOCE (eg, TRPC1) may be compensatorily affected in diabetic MCECs, which makes the amplitude of SOCE comparable between control and diabetic MCECs.

In addition to the store depletion-mediated STIM1 interaction with Orai1,[16] direct interaction between SERCA3 and TRPC channels (eg, human TRPC1 and TRPC6) can be stimulated by extracellular ligand and store depletion to form the STIM/SERCA3/TRPC complex.[35–37] In human platelets, SERCA3 forms the macromolecular protein complex with TRPC1/6 and IP_3R 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 Ca^{2+} refilling into the ER (eg, via a direct refilling mechanism without a global increase in $[Ca^{2+}]_{cyt}$) 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 $[Ca^{2+}]_{ER}$ 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 Ca^{2+} refilling into the ER in diabetic ECs.

The increase in $[Ca^{2+}]_{cyt}$ 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.[29,30,40–42] 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 must be selective in ECs, we designed the adenovirus that carries the Tie2 promoter so that STIM1 is only expressed in ECs[31,32] (Figure 4A through C and Online Figures IV and VI). After infection of STIM1-Adv in diabetic MCECs, the CPA-induced rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} leak from the ER (Figure 4D) and the $[Ca^{2+}]_{ER}$ (Figure 5) were significantly increased toward the level similar to control MCECs. These data suggest that STIM1 regulates $[Ca^{2+}]_{ER}$ in normal ECs and that overexpression of STIM1 (or restoration of the expression level of STIM1) in diabetic MCECs helps to increase the Ca^{2+} release from the ER and thus endothelium-dependent coronary vasodilatation.

We also examined the effect of STIM1 on 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 $[Ca^{2+}]_{cyt}$ 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 Ca^{2+} uptake or refilling.

Endothelial dysfunction is considered to be a major risk factor for cardiovascular complications in diabetes.[44–46] 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,[11,47–50] endothelium-dependent relaxation is

significantly attenuated in the coronary artery,[6,9,11,51,52] and endothelial cell permeability is increased.[53] 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 mobility 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 nitric oxide production (Figure 6). These data suggest that a decrease in Ca^{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 (Figure 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^{2+} leak from the ER, SOCE, and $[Ca^{2+}]_{ER}$ in normal ECs. Downregulation of STIM1 with siRNA significantly inhibited the CPA-induced increase in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} leakage from the ER and the CPA-mediated increases in $[Ca^{2+}]_{cyt}$ due to SOCE and significantly decreased $[Ca^{2+}]_{ER}$ in ECs. Furthermore, we demonstrate that STIM1 overexpression fully restores not only $[Ca^{2+}]_{ER}$ in diabetic MCECs but also endothelium-dependent relaxation in diabetic coronary artery in Figures 4 to 6. These data suggest that STIM1 may play a 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 endothelial cells. In diabetes, the plasma levels of glucose and 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, whereas only FFA decreases the mRNA expression of STIM1 (Figure 8). These observations suggest that increased protein degradation (eg, ubiquitinylation) and microRNA-mediated posttranscriptional modulation are potential mechanisms involved in the HG-mediated decrease in STIM1 protein level.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

ACh	acetylcholine
CA	coronary artery
EC	endothelial cell
ER	endoplasmic reticulum
IP_3R	inositol triphosphate receptor
MCEC	mouse coronary endothelial cell
RyR	ryanodine receptor
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SOCE	store-operated Ca^{2+} entry
STIM1	stromal interaction molecule

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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 (eg, 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 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 a 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 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.

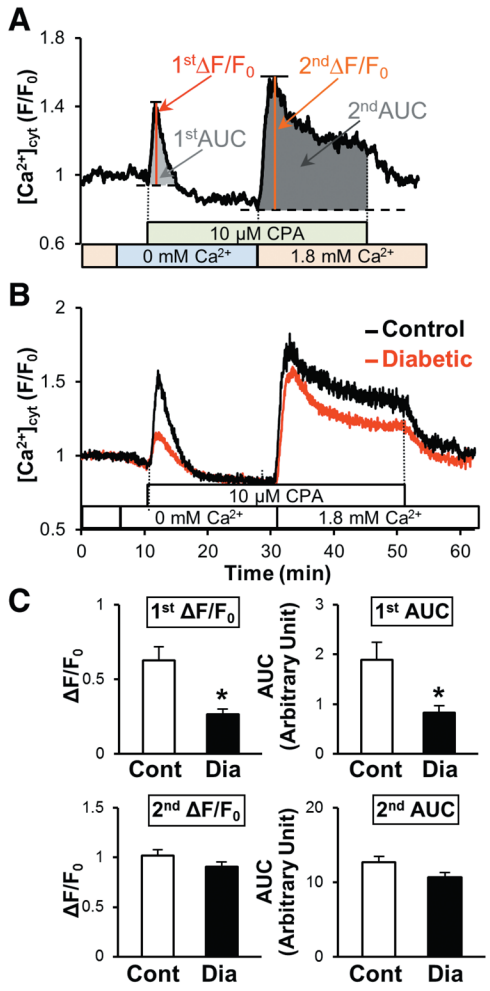

Figure 1. Hyperglycemia significantly inhibits the rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release/leakage from the ER during cyclopiazonic acid (CPA) treatment in coronary ECs

A, Typical record of the change in $[Ca^{2+}]_{cyt}$ in coronary ECs and the parameters used for the statistical analysis. First 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 2nd 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 μmol/L 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 minutes 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±SEM. *P**<0.05 versus Cont.

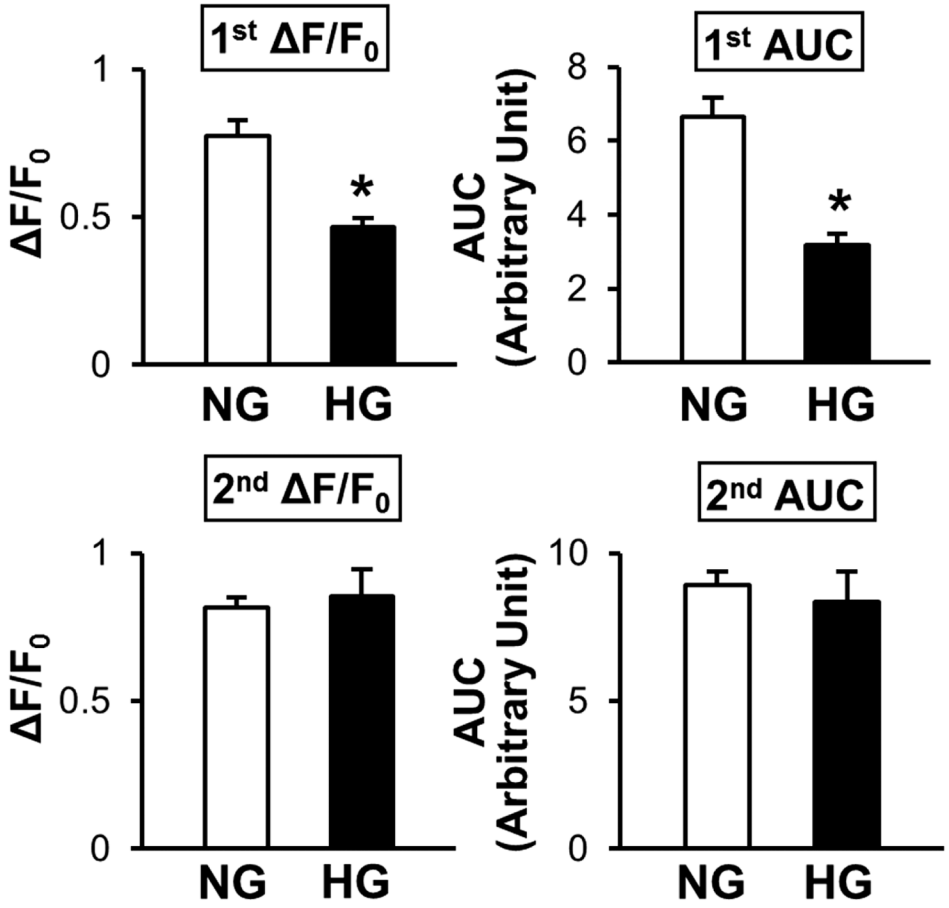

Figure 2. High-glucose treatment over 48 hours significantly inhibits the rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release/leakage from the ER during CPA treatment in coronary ECs

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 1st area under the curve [AUC]) and SOCE (2^{nd} $\Delta F/F0$ and 2^{nd} AUC). Normal glucose (NG, open bars); n=22 cells, high glucose (HG, solid bars); n=17 cells. Data are mean±SEM. *P<0.05 versus NG.

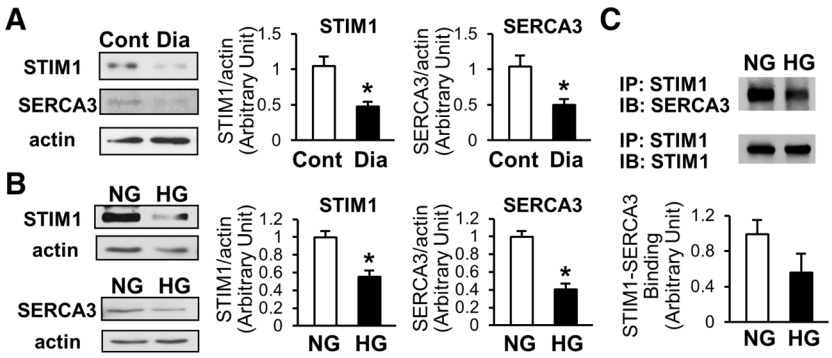

Figure 3. Hyperglycemia downregulates protein expression of STIM1 and SERCA3 in coronary ECs

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±SEM. **P*<0.05 versus 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±SEM. **P*<0.05 versus 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±SEM. **P*<0.05 versus NG.

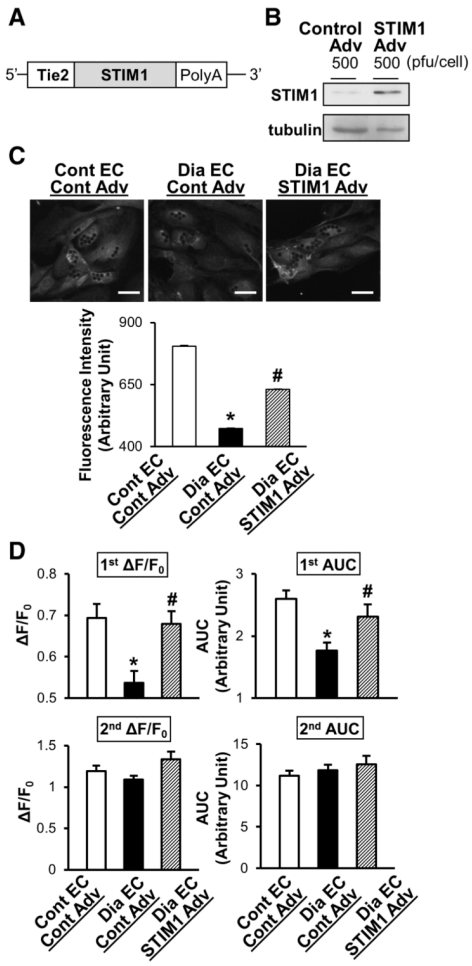

Figure 4. STIM1 overexpression restores the attenuated increase in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release/leakage from the ER during CPA treatment in MCECs isolated from diabetic mice

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 Western blot. **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. Lower panel shows 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±SEM. *P<0.05 versus Cont EC–Cont Adv. #P<0.05 versus Dia 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} area under the curve [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, solid bars), n=22; and diabetic ECs infected with STIM1 Adv (Dia EC–STIM1 Adv, hatched bars), n=25. Data are mean±SEM. *P<0.05 versus Cont EC–Cont Adv. #P<0.05 versus Dia EC–Cont Adv. ANOVA was performed to test the statistical difference between the groups.

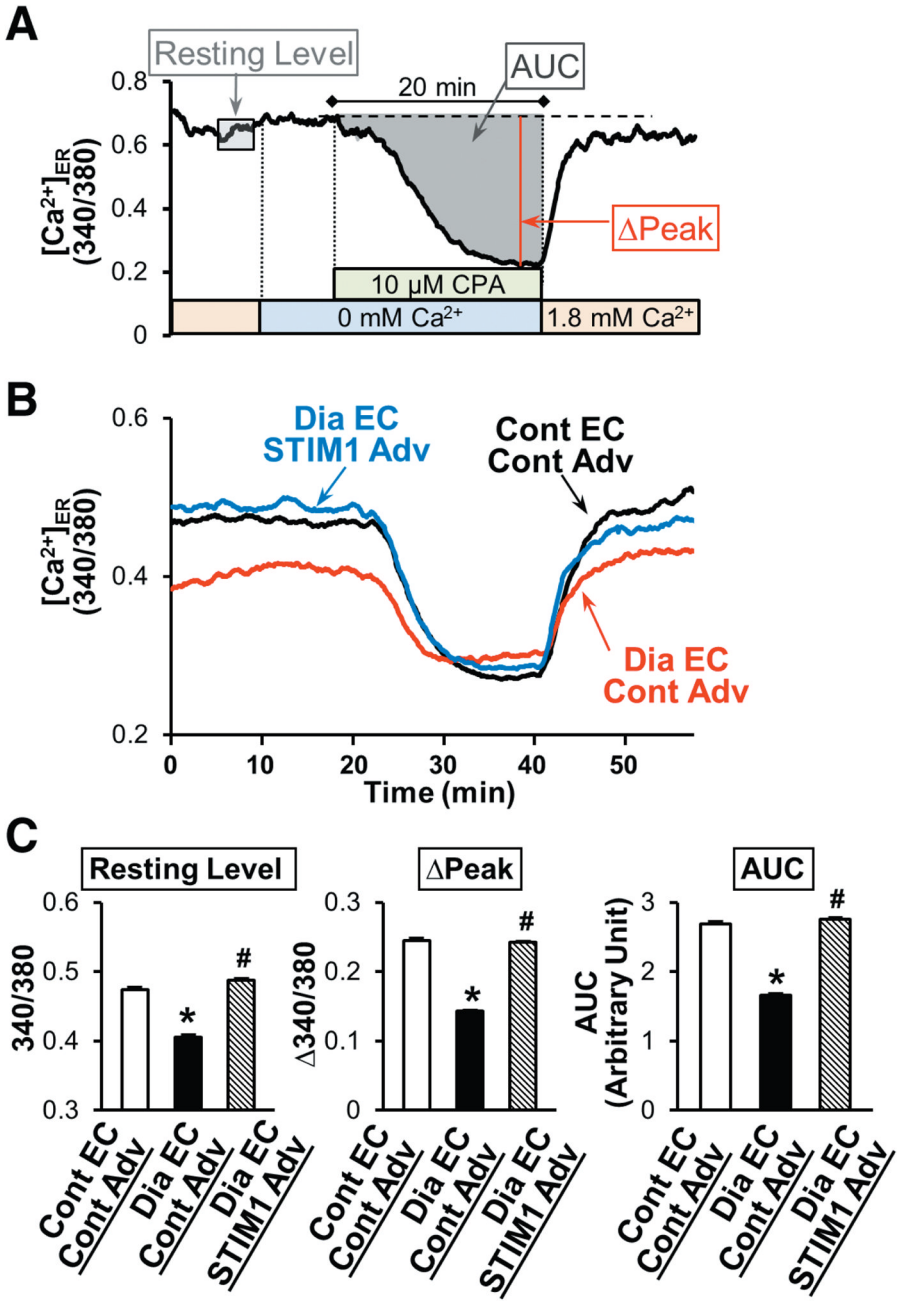

Figure 5. STIM1 overexpression increases the $[Ca^{2+}]_{ER}$ in MCECs isolated from diabetic mice

A, Typical record of the change in $[Ca^{2+}]_{ER}$ in coronary ECs and the parameters used for the statistical analysis. **B**, Averaged record of the change in $[Ca^{2+}]_{ER}$ 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 $[Ca^{2+}]_{ER}$, ΔPeak, and the area under the curve (AUC; the change in $[Ca^{2+}]_{ER}$ 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±SEM. *P<0.05 versus Cont EC–

Cont Adv. #$P<0.05$ versus Dia EC–Cont Adv. ANOVA was performed to test the statistical difference between the groups.

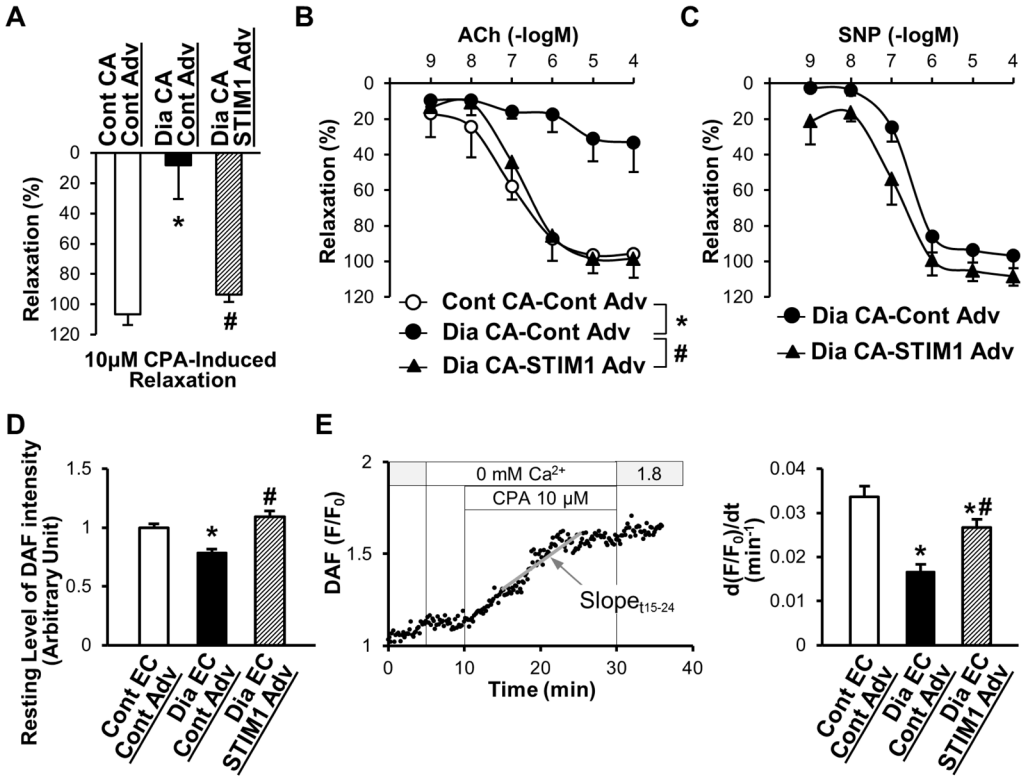

Figure 6. Overexpression of STIM1 restores CPA- and ACh-induced relaxation in diabetic CAs and increases nitric oxide (NO) production in diabetic MCECs

A, After precontraction of the CAs, CPA-induced (10 μmol/L) vascular relaxation was observed. Relaxation was calculated versus the magnitude of the contraction induced by $PGF_{2\alpha}$ and described as percentage. 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±SEM. **P*<0.05 versus Cont CA– Cont Adv. #*P*<0.05 versus 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±SEM. **P*<0.05 versus Cont CA–Cont Adv. #*P*<0.05 versus 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±SEM. **D**, Resting level of DAF intensity was obtained from the average intensity of first 2 to 4 minutes 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±SEM. **P*<0.05 versus Cont EC–Cont Adv. #*P*<0.05 versus Dia EC–Cont Adv. **E**, NO production due to Ca^{2+} release/leakage from the ER during CPA treatment in coronary ECs. Left graph shows 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 minutes) was calculated (gray line) and used as an indication of NO production in response to CPA [$d(F/F_0)/dt$]. Right panel shows the summarized data of $d(F/F_0)/dt$ 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±SEM. **P*<0.05 versus Cont EC–Cont Adv. #*P*<0.05

versus Dia EC–Cont Adv. ANOVA was performed to test the statistical difference between the groups.

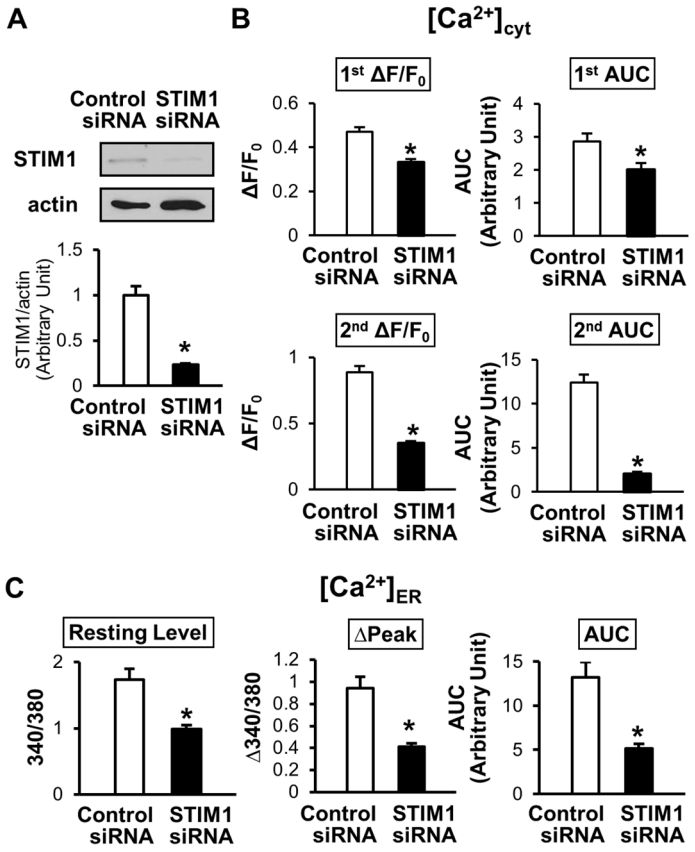

Figure 7. STIM1 downregulation attenuates the rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release/leakage from the ER during CPA treatment and decreases $[Ca^{2+}]_{ER}$ in coronary ECs

A, STIM1 siRNA transfection in HCECs downregulates STIM1 protein expression determined by Western blot. Values are mean±SEM (n=2). **P*<0.05 versus control siRNA. **B**, Summarized data of the rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release/leakage from the ER (1st $\Delta F/F_0$ and 1st area under the curve [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 ±SEM. **P*<0.05 versus control siRNA. **C**, Summarized data of resting level of $[Ca^{2+}]_{ER}$, ΔPeak, and the AUC (the change in $[Ca^{2+}]_{ER}$ after CPA treatment). Control siRNA (open bars), n=24; STIM1 siRNA (solid bars), n=30. Data are mean±SEM. **P*<0.05 versus control siRNA.

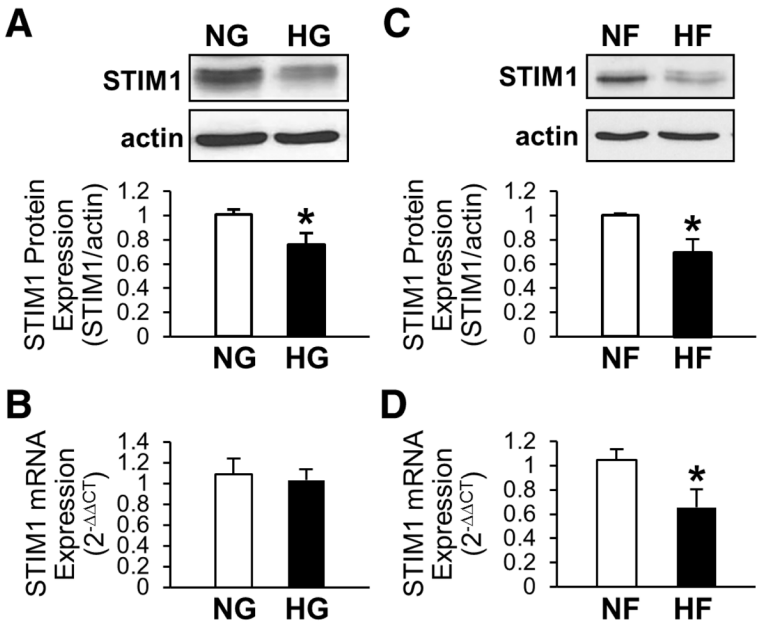

Figure 8. High glucose (HG) and free fatty acid (HF) treatment downregulate protein expression of STIM1, whereas only HF decreases mRNA level of STIM1, in mouse coronary ECs

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 hours. Data are mean±SEM. **P*<0.05 versus 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 hours. Data are mean±SEM. **P*<0.05 versus NF.