Essential Role of Smooth Muscle STIM1 in Hypertension and Cardiovascular Dysfunction

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Objectives—Chronic hypertension is the most critical risk factor for cardiovascular disease, heart failure, and stroke.

Approach and Results—Here we show that wild-type mice infused with angiotensin II develop hypertension, cardiac hypertrophy, perivascular fibrosis, and endothelial dysfunction with enhanced stromal interaction molecule 1 (STIM1) expression in heart and vessels. All these pathologies were significantly blunted in mice lacking STIM1 specifically in smooth muscle (Stim1<sup>SMC</sup>-/). Mechanistically, STIM1 upregulation during angiotensin II–induced hypertension was associated with enhanced endoplasmic reticulum stress, and smooth muscle STIM1 was required for endoplasmic reticulum stress–induced vascular dysfunction through transforming growth factor-β and nicotinamide adenine dinucleotide phosphate oxidase–dependent pathways. Accordingly, knockout mice for the endoplasmic reticulum stress proapoptotic transcriptional factor, CCAAT-enhancer–binding protein homologous protein (CHOP<sup>−/−</sup>), were resistant to hypertension-induced cardiovascular pathologies. Wild-type mice infused with angiotensin II, but not Stim1<sup>SMC</sup>−/− or CHOP<sup>−/−</sup> mice showed elevated vascular nicotinamide adenine dinucleotide phosphate oxidase activity and reduced phosphorylated endothelial nitric oxide synthase, cGMP, and nitrite levels.

Conclusions—Thus, smooth muscle STIM1 plays a crucial role in the development of hypertension and associated cardiovascular pathologies and represents a promising target for cardiovascular therapy. (Arterioscler Thromb Vasc Biol. 2016;36:1900-1909. DOI: 10.1161/ATVBAHA.116.307869.)

Key Words: cardiac hypertrophy ■ endothelial nitric oxide synthase ■ ER stress ■ hypertension ■ nicotinamide adenine dinucleotide phosphate ■ stromal interaction molecule 1 ■ vascular reactivity

Hypertension is a major risk factor for cardiovascular complications, including heart failure and stroke in animal models and patients. Vascular reactivity is of paramount importance in regulating local blood flow and ensuring constant tissue perfusion. We and others reported that hypertension impairs vascular function through reduced endothelial nitric oxide synthase (eNOS) activity and enhanced activation of molecular pathways of stress, including endoplasmic reticulum (ER) stress and oxidative stress. It is well established that increased intracellular calcium (Ca<sup>2+</sup>) concentration is a key second messenger involved in cardiovascular homeostasis in terms of flow-induced dilation and vascular smooth muscle cell (SMC) and cardiomyocyte contractility. Adequate physiological functions of both endothelial cells and SMC require accurate intracellular Ca<sup>2+</sup> regulation. In particular, the ubiquitous store-operated Ca<sup>2+</sup> entry (SOCE) pathway has been shown to regulate many cell functions. In vascular disease states, SOCE is functional in endothelial cells, vascular smooth muscle cells, and cardiomyocytes and plays an essential role in the regulation of proliferation, migration, hypertrophy, and apoptosis. Stromal interaction molecule 1 (STIM1) is an ER Ca<sup>2+</sup> sensor, which plays a critical role in the activation of Orai1 channels at the plasma membrane that mediate SOCE. Physiologically, SOCE is activated on receptor-mediated depletion of inositol-1,4,5-trisphosphate (IP<sub>3</sub>)-sensitive ER Ca<sup>2+</sup> stores. SOCE contributes to intracellular Ca<sup>2+</sup> refilling of the ER and also provides Ca<sup>2+</sup> microdomains crucial for downstream signaling to the nucleus. In smooth muscle, we showed that STIM1 proteins are also required for the activation of another channel contributed by heteromultimeric of Orai1 and Orai3, which is activated by store-independent means involving intracellular actions of the inflammatory lipid second messenger, leukotriene C<sub>4</sub>. Our recent studies determined that the deletion of STIM1 specifically in SMC of mice reduces vascular contractile response to sympathetic stimulation, with no effect on endothelium-dependent relaxation. Others and we showed that increased STIM1 expression is critical for the development of vascular and cardiac remodeling in animal models. The STIM1 expression is also enhanced in vessels of hypertensive rats.
Materials and Methods

One of the most obvious observations from this in vivo study is the reduced body weight of Stim1SMC−/− mice, which is in agreement with previous studies.39 Indeed, regardless of whether mice were infused with saline or with angiotensin II (Ang II), body weight was significantly reduced in Stim1SMC−/− compared to wild-type (WT), Stim1 SMC−/+ and CCAAT-enhancer–binding protein homologous protein (CHOP−/−) mice (Figure 1A). Importantly, in the Ang II–infused mice, hypertension was significantly delayed in Stim1 SMC−/− and CHOP−/− compared with WT and Stim1SMC−/− mice (Figure 1B).

STIM1 and CHOP Deletion Inhibits Hypertension-Mediated Cardiac Hypertrophy and Fibrosis

Cardiac hypertrophy was increased in WT and Stim1SMC−/− mice subjected to Ang II infusion, whereas Stim1SMC−/− and CHOP−/− mice infused with Ang II were protected against cardiac hypertrophy (Figure 1C). Histological examination using collagen-specific Sirius-red staining on heart slices clearly demonstrated that chronic Ang II infusion induces perivascular fibrosis in WT group but not in Stim1SMC−/− and CHOP−/− group (Figure 1D). Similarly, Western blot analysis on heart tissues showed that total and phosphorylated Smad2/3, STIM1, and the ER stress markers BiP (immunoglobulin binding protein), ATF6 (activating transcription factor 6), and CHOP protein expression were increased in the WT group infused with Ang II but not in Stim1SMC−/− and CHOP−/− groups (Figure 1E and 1F). We used reverse transcriptase polymerase chain reaction on heart tissue samples to demonstrate that upregulation of these proteins in the WT group infused with Ang II occur at the mRNA level (Figure 1G–1J). Immunohistochemistry on heart sections showed an increase in STIM1 both in WT and CHOP−/− groups infused with Ang II but not in hearts from Stim1SMC−/− mice infused with Ang II (Figure 1K; Figure IXA in the online-only Data Supplement). However, CHOP protein levels were only increased in WT mice infused with Ang II (Figure 1L; Figure IXB in the online-only Data Supplement). Together, these data suggest that STIM1 is acting upstream of CHOP and ER stress.

Effect of STIM1 and CHOP Deletion on Vascular Reactivity in Hypertension

The constriction of mesenteric resistance artery (MRA) in response to phenylephrine was significantly reduced in Stim1SMC−/− compared to WT, Stim1SMC−/+ and CHOP−/− mice (Figure 2A). However, this reduction in constriction in Stim1SMC−/− was not altered in Ang II–infused hypertensive mice (Figure 2D). The constriction to a thromboxaneA2 agonist (U-46619) was similar in all groups (Figure 2B). However, in hypertension, thromboxaneA2 receptor–induced constriction was enhanced but to a lesser extent in STIM1SMC−/− and CHOP−/− groups infused with Ang II (Figure 2E). The endothelium-dependent relaxation of MRA was identical in all control saline-infused groups (Figure 2C). However, in mice infused with Ang II, the endothelium-dependent relaxation in response to acetylcholine was impaired in WT and Stim1SMC−/+ groups (Figure 2F). Interestingly, the endothelium-dependent relaxation was only partially inhibited (~50%) in Stim1SMC−/− mice infused with Ang II and was comparable to control levels in CHOP−/− mice infused with Ang II (Figure 2F).

Western blots demonstrated a more pronounced reduction in eNOS phosphorylation in WT mice infused with Ang II compared with Stim1SMC−/− mice infused with Ang II, with the CHOP−/− mice infused with Ang II showing normal levels of eNOS phosphorylation (Figure 2G). Similarly, the cGMP and nitrite/nitrate levels (markers for NO signaling) were less reduced in Stim1SMC−/− mice infused with Ang II by comparison to WT mice infused with Ang II (Figure 2H and 2I). However, CHOP−/− mice infused with Ang II had essentially normal levels of cGMP and nitrite/nitrate (Figure 2H and 2I). Consistent with eNOS, cGMP, and nitrite/nitrate data mentioned above, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (Figure 2K), p47phox expression (Figure VA in the online-only Data Supplement), mRNA levels of ROS NADPH oxidase (NOX) isoforms (Nox2 and 4; Figure VI in the online-only Data Supplement), and 8-OHD (8-hydroxy-2′-deoxyguanosine; Figure VIIA in the online-only Data Supplement) were significantly elevated in WT mice infused with Ang II compared with Stim1SMC−/− and CHOP−/− mice infused with Ang II, with NADPH oxidase activity in CHOP−/− mice infused with Ang II, showing levels comparable to those of control saline-infused mice (Figure 2K).

The results obtained earlier with the MRA were confirmed in conductance arteries (thoracic aortas). Indeed, contractility in response to phenylephrine (Figure 1A and IB in the online-only Data Supplement).
online-only Data Supplement) and thromboxaneA2 analogue (Figure IC and ID in the online-only Data Supplement), the relaxation to acetylcholine (Figure IE and IF in the online-only Data Supplement), eNOS levels (Figure IG in the online-only Data Supplement), cGMP and nitrite/nitrate (Figure IH and II in the online-only Data Supplement), and NADPH oxidase activity (Figure III in the online-only Data Supplement). We also measured p47phox expression (Figure VB in the online-only Data Supplement) and 8-OHd (Figure VII in the online-only Data Supplement) in thoracic aorta were similar to the results observed in MRA.

To study the involvement of eNOS coupling in vascular reactivity, we measured the expression of eNOS T495 phosphorylation and the phosphorylated and total PKC (protein kinase Cα/β) in MRA and thoracic aorta (Figure V in the online-only Data Supplement). Our data indicate that there was no difference in the expression of eNOS T495 (Figure VB in the online-only Data Supplement) among groups; however, the ratio P-PKCα/β/T-PKCα/β was significantly increased in WT mice infused with Ang II compared with STIM1SMC−/− and CHOP−/− mice infused with Ang II (Figure V A and VB in the online-only Data Supplement).
Supplement). Our data showed that the increased NADPH oxidase in the WT group infused with Ang II was significantly reduced after treatment with L-NAME and apocynin (Figure VIII in the online-only Data Supplement).

**STIM1 and CHOP Deletion Inhibits Vascular ER Stress in Hypertension**

Because STIM1 is an ER protein of major importance in the maintenance of ER Ca^{2+} homeostasis, and because specific lack of STIM1 in SMC protects against disruption of vascular and cardiac function during hypertension, we reasoned that STIM1 upregulation that occurs in hypertension might be mediating negative cardiovascular effects through exacerbation of ER stress. Indeed, we found that the expression of ER stress marker proteins BiP and ATF6 were significantly enhanced in MRA from WT mice infused with Ang II compared with all other groups (Figure 2J). STIM1 protein expression in MRA was greatly augmented in WT mice infused with Ang II compared with control saline-infused mice (Figure 2L). STIM1 protein expression was also significantly increased in Stim1SMC^{−/−} mice and CHOP^{−/−} mice infused with Ang II compared with control saline-infused mice, but this increase was less pronounced in these 2 groups of mice compared with WT mice infused with Ang II (Figure 2L). The increase in STIM1
expression observed in MRA from Stim1SMC−/− mice most likely reflects contributions from endothelial cells and possibly adventitial fibroblasts.23

We also demonstrated that changes in protein expression observed earlier occur at the mRNA levels. Indeed, all ER stress markers BiP, ATF6, and CHOP mRNA levels, as well as STIM1 mRNA levels, were increased after Ang II infusion, and these increases were significantly decreased in Stim1SMC−/− and blunted in CHOP−/− mice infused with Ang II (Figure 2M–2P).

Effect of STIM1 and CHOP Deletion on Vascular Transforming Growth Factor-β and Reactive Oxygen Species in Hypertension

ER stress is an important determinant in the initiation of oxidative stress and transforming growth factor-β (TGF-β) signaling. Our previous studies showed that induction of ER stress in mice causes endothelial dysfunction and inhibits vascular reactivity through TGF-β and reactive oxygen species–dependent mechanisms.3 Therefore, we sought to determine the contribution of oxidative stress and TGF-β signaling in mediating vascular dysfunction downstream of STIM1 and ER stress using WT, Stim1SMC−/−, Stim1SMC−/+ and CHOP−/− mice. We incubated MRA ex vivos with the TGF-β inhibitor, SB431542, or the NADPH oxidase inhibitor, gp-91-stat. In the control groups of WT, Stim1SMC−/+ and Stim1SMC−/− and CHOP−/− saline-infused mice, the inhibition of TGF-β and NADPH oxidase had no effect on the endothelium-dependent relaxation of MRA (Figure 3A, 3C, 3E, and 3G). Similar results were obtained when thoracic aortas were used instead of MRA (Figure IIA, IIC, IIE, and IIG in the online-only Data Supplement). Interestingly, when mice were infused with Ang II, the NADPH oxidase inhibitor gp-91-stat improved endothelium-dependent relaxation of MRA from WT and Stim1SMC−/+ mice (Figure 3B and 3D) but had no effect in thoracic aorta (Figure IIB and IID in the online-only Data Supplement). Reciprocally, the inhibition of TGF-β improved endothelium-dependent relaxation in thoracic aorta of WT and Stim1SMC−/+ mice infused with Ang II (Figure IIB and IID in the online-only Data Supplement) but had no effect in MRA.

Figure 3. Effect of inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and transforming growth factor-β (TGF-β) signaling on vascular reactivity in stromal interaction molecule 1 (STIM1) and CCAAT-enhancer–binding protein homologous protein (CHOP) knockout mice. Wire myograph vascular reactivity showing endothelial-dependent relaxation in response to acetylcholine (ACh) before and after incubation with TGF-β inhibitors (SB431542) and NADPH oxidase inhibitor (gp91 ds-tat) in mesenteric resistance arteries (MRA) from wild-type (WT) mice infused with saline or angiotensin II (Ang II; A and B, n=5), heterozygous Stim1 knockout specifically in SMC (Stim1SMC−/+; C and D, n=5), homozygous Stim1 knockout specifically in SMC (Stim1SMC−/−; E and F, n=5), homozygous CHOP knockout specifically in SMC (CHOP−/−; G and H, n=5). Two-way repeated measured ANOVA followed by Tukey’s post hoc test were applied for A–H. *P<0.05 between WT+Ang II+gp91 ds-tat vs WT+Ang II. **P<0.05 between WT+Ang II+SB vs WT+Ang II+gp91 ds-tat. †P<0.05 between WT vs Stim1SMC−/+ + Ang II+gp91 ds-tat vs Stim1SMC−/− + Ang II+gp91 ds-tat vs Stim1SMC−/− + Ang II+SB. §P<0.05 between WT vs Stim1SMC−/+ + Ang II+gp91 ds-tat vs Stim1SMC−/− + Ang II+gp91 ds-tat vs Stim1SMC−/− + Ang II+SB.
Consistent with a role for SMC STIM1 in promoting vascular dysfunction through ER stress–dependent mechanisms, endothelium-dependent relaxation of MRA and thoracic aorta was only partially inhibited in homozygous Stim1SMC−/− mice and was preserved in CHOP−/− mice infused with Ang II. Furthermore, in both Stim1SMC−/− and CHOP−/− mice, the TGF-β inhibitor and the NADPH oxidase inhibitor were without effect (Figure 3F and 3H; Figure IIF and IIH in the online-only Data Supplement).

**Relationship Between ER Stress and STIM1**

To directly establish the relationship between ER stress and STIM1, we injected WT, Stim1SMC−/+, and Stim1SMC−/− mice with the ER stress inducer tunicamycin followed by measurements of vascular reactivity after 2 weeks. Tunicamycin significantly reduced the body weight in WT and heterozygous Stim1SMC−/+ mice but had a noticeably smaller effect on the body weight of homozygous Stim1SMC−/− mice (Figure 4A); please note that as shown earlier, untreated Stim1SMC−/− mice have reduced body weight compared with WT and Stim1SMC−/+ mice. Tunicamycin treatment had marginal effects on blood pressure that was essentially normal in all groups of mice (Figure 4B). Interestingly, endothelium-dependent relaxation in both MRA (Figure 4C) and thoracic aorta (Figure IIIA in the online-only Data Supplement) was significantly impaired in WT and heterozygous Stim1SMC−/+ mice injected with tunicamycin, whereas it was protected by ≈50% in homozygous Stim1SMC−/− mice infused with tunicamycin. Consistently, infusion with tunicamycin caused a more pronounced reduction in total levels of eNOS in MRA (Figure 4D) and thoracic aorta (Figure IIB in the online-only Data Supplement) from WT and heterozygous Stim1SMC−/+ mice compared with homozygous Stim1SMC−/− mice.

Ex vivo experiments using the NADPH oxidase and TGF-β inhibitors as described earlier were performed to determine the contributions of oxidative stress and TGF-β signaling during ER stress induction in WT, Stim1SMC−/+, and Stim1SMC−/− mice. Consistent with the results from Figure 3 and Figure II in the online-only Data Supplement, the NADPH oxidase inhibitor gp-91-stat greatly improved endothelium-dependent relaxation of MRA from WT and Stim1SMC−/+ mice treated with tunicamycin (Figure 5A and 5B), compared with the effect observed in thoracic aorta (Figure IVA and IVB in the online-only Data Supplement). Reciprocally, the inhibition of TGF-β signaling greatly improved endothelium-dependent relaxation in thoracic aorta of WT and Stim1SMC−/+ mice treated with tunicamycin compared with the effect in MRA (Figure 5A and 5B; Figure IVA and IVB in the online-only Data Supplement). Consistent with results obtained with Ang II infusion, endothelium-dependent relaxation in MRA and aortas from homozygous Stim1SMC−/− mice was only partially inhibited by tunicamycin treatment, and additional treatment with the NADPH oxidase inhibitor or the TGF-β inhibitor was with no effect (Figure 5C; Figure IVC in the online-only Data Supplement). The main finding in this study is summarized in Figure X in the online-only Data Supplement.

**Figure 4.** Effect of tunicamycin-induced endoplasmic reticulum (ER) stress on vascular reactivity in stromal interaction molecule 1 (STIM1) knockout mice. Body weight (BW; A, n=5) and systolic blood pressure (SBP) measured by tail cuff machine (B, n=5), wire myograph vascular reactivity showing endothelial-dependent relaxation in response to acetylcholine (ACh; C, n=5) and endothelial nitric oxide synthase (eNOS) levels determined by an ELISA kit (D, n=3) in mesenteric resistance arteries (MRA) from wild-type (WT), heterozygous (Stim1SMC−/+), and homozygous Stim1 knockout specifically in SMC (Stim1SMC−/−) mice treated with saline or tunicamycin. Two-way repeated measured ANOVA followed by Tukey’s post hoc test were applied for A–C. One-way ANOVA followed by Bonferroni post hoc test were applied for D. *P<0.05 between STIM1SMC−+/+Tunic vs WT, STIM1SMC−/−, Stim1SMC−/+Nucina, WT+Tunica vs WT, STIM1SMC−/−, Stim1SMC−/+Tunica. **P<0.05 between STIM1SMC−+/+Tunica, WT+Tunica vs WT, STIM1SMC−/−, Stim1SMC−/+Tunica. *P<0.05 between STIM1SMC−+/+Tunica vs STIM1SMC−/−Tunica. †P<0.05 between STIM1SMC−/−, STIM1SMC−/+Tunica vs STIM1SMC−/−+Tunica, WT+Tunica. ‡P<0.05 between STIM1SMC−/−Tunica vs STIM1SMC−/−+Tunica and WT+Tunica.
Discussion

The present study illustrates a novel mechanism connecting STIM1 to ER stress in mediating cardiac hypertrophy and vascular dysfunction in hypertension. The role of STIM1 in controlling several physiological processes, such as endothelial and smooth muscle functions has been well established. Others and we have previously shown that STIM1 is a master regulator of cardiovascular function; STIM1 protein expression is upregulated in vascular smooth muscle during vascular remodeling that is associated with phenotypic switching of smooth muscle from contractile to proliferative phenotypes. The prevention of STIM1 upregulation using in vivo delivery of siRNA into vessels of living rats inhibits vascular remodeling and neointimal hyperplasia. Although STIM1 expression is increased in vessels from hypertensive rats, the role of STIM1 in hypertension and associated cardiac and vascular dysfunction remained unknown. Our data demonstrate a significant upregulation of STIM1 expression in heart and arteries during hypertension-induced cardiac damage and vascular dysfunction. We used mice with targeted gene deletion of STIM1 specifically in smooth muscle to demonstrate that lack of STIM1 in smooth muscle prevents hypertension and hypertension-induced cardiac hypertrophy and vascular dysfunction through abrogation of ER stress.

It is well established that hypertension in animals and patients triggers cardiac hypertrophy and cardiac fibrosis. We show that cardiac hypertrophy and fibrosis induced by Ang II were associated with enhanced STIM1 expression. Interestingly, STIM1 deletion in SMC reduced Ang II–induced cardiac hypertrophy and fibrosis, suggesting that STIM1 in SMC is an important contributor to the development of cardiac damage in hypertension. Our data are supported by studies from our group and others showing increased STIM1 expression in neointimal hyperplasia and cardiac hypertrophy. Additionally, our data indicate that ER stress markers were increased in the heart from WT mice infused with Ang II, which is in agreement with previous publications. STIM1 is a key regulator of Ca²⁺ homeostasis by supporting communications between the ER and plasma membrane. Therefore, STIM1 upregulation could lead to ER stress and either directly or indirectly dictates the development of cardiovascular complications under hypertensive conditions. Thus, we determined that ER stress markers were reduced in the heart from Stim1SMC−/− and CHOP−/− mice infused with Ang II. Our data showed that STIM1 expression was increased in the heart from CHOP−/− mice infused with Ang II, suggesting that STIM1 is upstream of the ER stress marker (CHOP), but likely they are part of a positive loop.

It is well established that hypertension is associated with impaired vascular reactivity in animal models and patients. but the mechanisms involved in vascular dysfunction during hypertension are not fully understood. Here we report that vasoconstriction in response to phenylephrine is significantly reduced in Stim1SMC−/− compared to WT mice, consistent with previous studies. This effect was independent of hypertension because vasoconstriction in response to phenylephrine in Stim1SMC−/− mice infused with Ang II is also reduced. The vasoconstriction in response to phenylephrine in CHOP−/− mice with or without Ang II was similar to that in WT mice.

Figure 5. Effect of inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and transforming growth factor-β (TGF-β) signaling on vascular reactivity in tunicamycin-treated stromal interaction molecule 1 (STIM1) knockout mice. Wire myograph vascular reactivity showing endothelial-dependent relaxation in response to acetylcholine (ACh) before and after incubation with TGF-β inhibitor (SB431542) and NADPH oxidase inhibitor (gp91 ds-tat) in mesenteric resistance arteries from wild-type (WT) mice treated with saline or tunicamycin (A, n=5), heterozygous Stim1 knockout specifically in SMC (Stim1SMC−/+ mice treated with saline or tunicamycin (B, n=5), and homozygous Stim1 knockout specifically in SMC (Stim1SMC−/−) mice treated with saline or tunicamycin (C, n=5). Two-way repeated measured ANOVA followed by Tukey’s post hoc test were applied for A–C. *P<0.05 between WT, WT+Tunica+gp91 ds-tat vs Sham+Tunica, WT+Tunica+SB. $P<0.05 between STIMSMC−/+ vs STIMSMC−/++Tunica+gp91 ds-tat. @P<0.05 between STIMSMC−/−+Tunica, STIMSMC−/−+Tunica+SB, STIMSMC−/−+Ang II, STIMSMC−/−+Tunica+SB. MRA indicates mesenteric resistance artery.
Under control conditions (ie, in the absence of Ang II infusion), the vasoconstriction in response to the thromboxane A2 analog U46619 was identical in all groups. However, in mice infused with Ang II, the U46619-induced contraction was augmented in WT and heterozygous Stim1SMC−/− mice and to a lesser extent in Stim1SMC−/− and CHOP−/− mice. The difference in the vasoconstriction response between phenylephrine and U46619 could be explained by the fact that thromboxane A2 receptor signaling is not coupled to STIM1, whereas α1-adrenoceptor signaling is.

Endothelium-dependent relaxation of arteries depends on the activation of eNOS and cGMP signaling. The deletion of STIM1 and CHOP did not alter the endothelium-dependent relaxation when compared with WT. However, after infusion of Ang II, the endothelium-dependent relaxation and eNOS phosphorylation were impaired in WT and Stim1SMC−/− mice but protected in Stim1SMC−/− and CHOP−/− mice infused with Ang II. These data indicate that in hypertension, enhanced smooth muscle STIM1 and ER stress impair vascular endothelium-dependent relaxation. Several experimental and clinical evidence have linked the enhanced production of reactive oxygen species to hypertension. We showed an increase in NADPH oxidase activity in mice infused with Ang II, and this increase was blunted in Stim1SMC−/− and CHOP−/− mice infused with Ang II. We previously reported that TGF-β, an important cytokine produced by smooth muscle, plays a major role in the impairment of endothelium-dependent relaxation in conductance arteries, whereas NADPH oxidase signaling is involved in resistance arteries. Consistent with our previous studies, we found that in WT mice infused with Ang II, the inhibition of NADPH oxidase improves endothelium-dependent relaxation in resistance arteries, whereas TGF-β inhibition improves endothelium-dependent relaxation in the thoracic aorta. Interestingly, in Stim1SMC−/− mice infused with Ang II, the inhibition of NADPH oxidase and TGF-β pathway did not improve further the endothelium-dependent relaxation, suggesting that STIM1 and NADPH oxidase/TGF-β pathways are not additive and likely mediating their effects through the same pathway. To determine the relationship between STIM1 and ER stress independently of hypertension, we treated mice with the ER stress inducer, tunicamycin. In agreement with our previous studies, tunicamycin did not enhance arterial blood pressure while impairing vascular endothelium-dependent relaxation in WT and heterozygous Stim1SMC−/− mice. However, in homozygous Stim1SMC−/− mice, endothelium-dependent relaxation was protected after injection of tunicamycin. These results suggest a circular effect between STIM1 and ER stress and the interplay: how STIM1 regulates ER stress and how ER stress regulates STIM1.

The mechanism by which SMC-specific deletion of STIM1 protects the endothelium-dependent relaxation in hypertension is still unknown. It is likely that SMC STIM1 regulates factors released by SMC that interact with endothelial cells and, therefore, eNOS activity. Future studies are needed to determine the link between SMC STIM1 and the endothelium-dependent relaxation.

The important implication from these data is that direct induction of ER stress independently of hypertension impairs vascular endothelium-dependent relaxation, and specific deletion of STIM1 in smooth muscle overcomes ER stress-induced vascular dysfunction. Our study provides new insights into the in vivo contribution and molecular mechanisms of smooth muscle STIM1 in hypertension-induced cardiac damage and vascular dysfunction. Therefore, specific targeting of smooth muscle STIM1 has the potential to overcome hypertension-induced cardiovascular complications.

Limitations
Previous studies by Giachini et al showed that normotensive and spontaneously hypertensive female rats have reduced store-operated Ca2⁺ entry compared with normotensive and hypertensive males, respectively, and that was because of reduced expression of Orai1 and STIM1 in females. The same authors showed that when spontaneously hypertensive females were ovariectomized, aortas from these female rats showed increased contraction and enhanced Orai1 expression, with no changes in STIM1 expression, suggesting that female sex hormones may downregulate Orai1-mediated Ca2⁺ entry, thus, contributing to vascular protection in females. In fact, studies by Flourakis et al showed that the expression of Orai1 in prostate cancer is controlled by androgen, whereas our previous studies showed that estrogen regulates the expression of the Orai3 isoform in breast cancer. Taken together, these data suggest that potentially a smaller ratio of Orai1 to Orai3 in females compared with males might contribute to vascular protection observed in females. Because STIM1 regulates the function of all 3 Orai isoforms, it is likely that our current findings have major relevance to both females and males. Clearly, additional studies comparing males and females side by side, similar to those of Giachini et al, would be required to conclusively address this issue.

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Disclosures
None.

References


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**Highlights**

- We have found that protein expression of the Ca\(^{2+}\) sensor stromal interaction molecule 1 is enhanced in heart and vessels of hypertensive mice.
- We show that abrogating stromal interaction molecule 1 expression specifically in smooth muscle protects against hypertension and associated cardiovascular dysfunction.
- Smooth muscle cell stromal interaction molecule 1 deletion protects the cardiovascular system likely through the modulation of the endoplasmic reticulum stress.
Essential Role of Smooth Muscle STIM1 in Hypertension and Cardiovascular Dysfunction

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Figure Supp I.- STIM1 and CHOP mediate hypertension-induced cardiac damage and fibrosis. Wire Myograph vascular reactivity showing Contraction response to phenylephrine (A, B, n=5-6) and thromboxaneA2 analogue U-46619 (C, D, n=5), and endothelial-dependent relaxation in response to acetylcholine (E, F, n=5) in thoracic aorta from in mesenteric resistance arteries from WT, heterozygous (Stim1SMC-/-) and homozygous Stim1 knockout specifically in smooth muscle (Stim1SMC-/-), and CHOP knockout (CHOP-/-) mice infused with saline or Ang II. cGMP levels were determined using a sandwich enzyme-linked immunosorbent assay (G, n=3) Elisa showing eNOS levels (I, n=3) and nitrites/nitrate levels were determined using the Griess reaction (H, n=3), and NADPH oxidase using lucigenin chemiluminescence assays (J, n=5), in thoracic aortas from WT, Stim1SMC-/- and CHOP-/- mice infused with saline or Ang II.

Two-way repeated measured ANOVA followed by Tukey's Post-Hoc test was applied for figures (A, B, C, D, E, F). One-way ANOVA followed by Bonferroni Post-Hoc test was applied for figures (G, H, I, J).

*p<0.05 between STIM1SMC-/- vs WT, STIM1SMC-/-, and CHOP-/-, $p<0.05 between STIM1SMC-/- + AngII vs WT + Ang II, STIM1SMC-/- + Ang II, CHOP-/- + Ang II, ?p<0.05 between STIM1SMC-/- + AngII, CHOP-/- + Ang II VS WT + Ang II, STIM1SMC-/- + Ang II, #p<0.05 between WT + Ang II, STIM1SMC-/- + Ang II vs STIM1SMC-/- + AngII, ^p<0.05 between WT + Ang II, STIM1SMC-/- + Ang II
vs STIM^{SMC/-} + AngII vs CHOP^{+/−} + Ang II. @p<0.05 between WT + Ang II vs WT, STIM^{SMC/-}, STIM^{SMC/-} + AngII, CHOP^{−/−}, CHOP^{−/−} + Ang II. $p<0.05$ between STIM^{SMC/-} vs STIM^{SMC/-} + AngII. %p<0.05 between CHOP^{+/−} vs CHOP^{−/−} + Ang II.
Figure Supp. II.- STIM1 and CHOP regulate hypertension-induced vascular damage.
Wire Myograph vascular reactivity showing endothelial-dependent relaxation in response to acetylcholine before and after incubation with TGF-β inhibitor (SB431542) and NADPH oxidase inhibitor (gp91 ds-tat) in thoracic aorta from:

- WT mice infused with saline or AngII (A, B, n=5)
- Heterozygous Stim1 knockout specifically in SMC (Stim1SMC-/+ mice) infused with saline or AngII (C, D, n=5)
- Homozygous Stim1 knockout specifically in SMC (Stim1SMC-/- mice) infused with saline or AngII (E, F)
- CHOP knockout (CHOP-/- mice) infused with saline or AngII (G, H, n=5)

Two-way repeated measured ANOVA followed by Tukey’s Post-Hoc test was applied for figures (A, B, C, D, E, F, G, H).

* p<0.05 between WT + Ang II + gp91 ds-tat, WT + Ang II vs WT + Ang II + SB. ^p<0.05 between STIMSMC-/+ + Ang II + gp91 ds-tat, STIMSMC-/+ + Ang II vs STIMSMC-/- + Ang II + SB.
Figure Supp. III.- Effect of Tunicamycin-induced ER stress on vascular reactivity in STIM1 knockout mice. Wire Myograph vascular reactivity showing endothelial-dependent relaxation in response to acetylcholine (A, n=5) and eNOS levels (B, n=3) in thoracic aorta from WT, heterozygous (Stim\textsuperscript{SMC+/+}) and homozygous Stim1 knocko ut specifically in SMC (Stim\textsuperscript{SMC-/-}) mice treated with saline or Tunicamycin.

Two-way repeated measured ANOVA followed by Tukey's Post-Hoc test was applied for figure (A). One-way ANOVA followed by Bonferroni Post-Hoc test was applied for figure (B).

$^{s}p<0.05$ between STIM$^{SMC+/+}$ + Tunica vs WT, STIM$^{SMC-/+}$, STIM$^{SMC-/-}$. *$p<0.05$ between STIM$^{SMC+/+}$ + Tunica, WT + Tunica vs STIM$^{SMC-/+}$ + Tunica, WT, STIM$^{SMC-/+}$, STIM$^{SMC-/-}$. **$p<0.05$ between STIM$^{SMC-/+}$ + Tunica vs STIM$^{SMC-/-}$ + Tunica and WT + Tunica.
**Figure Supp. IV.**- Effect of inhibition of NADPH oxidase and TGF-β signaling on Vascular reactivity in tunicamycin-treated STIM1 knockout mice. Wire Myograph vascular reactivity showing endothelial-dependent relaxation in response to acetylcholine before and after incubation with TGF-β inhibitor (SB431542) and NADPH oxidase inhibitor (gp91 ds-tat) in thoracic aorta from:

- WT mice treated with saline or Tunicamycin (A, n=5)
- Heterozygous Stim1 knockout specifically in SMC (Stim1SMC+/+) mice treated with saline or Tunicamycin (B, n=5)
- Homozygous Stim1 knockout specifically in SMC (Stim1SMC-/-) mice treated with saline or Tunicamycin (C, n=5)

Two-way repeated measured ANOVA followed by Tukey's Post-Hoc test was applied for figures (A, B, C)

* p<0.05 between WT + Tunica, WT + Tunica + gp91 ds-tat vs WT + Tunica + SB.
$ p<0.05$ between STIMSMC+/+ vs STIMSMC++ + Tunica + SB. @p<0.05 between STIMSMC++ + Tunica + SB vs STIMSMC+/+ + Tunica, STIMSMC++ + Tunica + gp91 ds-tat. p<0.05 between STIMSMC++ vs STIMSMC++ + Tunica, STIMSMC++ + Tunica + gp91 ds-tat, STIMSMC++ + STIMSMC++ + Tunica + SB.
Figure Supp. V.- Effect of STIM1 and CHOP deletion on hypertension-induced uncoupling eNOS and NADPH oxidase. Western blot analysis and quantification for uncoupled eNOS markers (eNOS T495, P and T-PKCα/β) and NADPH oxidase subunit p47 phox in mesenteric arteries (MRA, A, n=4) and thoracic aorta (B, n=4) from:
- WT mice infused with saline or Angiotensin II
- CHOP knockout mice infused with saline or Angiotensin II.
- Homozygous Stim1 knockout specifically in SMC (Stim1<sup>SMC-/-</sup>) mice infused with saline or Angiotensin II.
One-way ANOVA followed by Bonferroni Post-Hoc test was applied for figures (A, B).

*p<0.05 between WT + Ang II vs WT, CHOP<sup>−/−</sup>, CHOP<sup>−/−</sup> + Ang II, STIM<sup>SMC−/−</sup> and STIM<sup>SMC−/−</sup> + Ang II.
Figure Supp. VI.- Effect of STIM1 and CHOP deletion on NADPH oxidase subunits during hypertension. Real Time PCR showing the mRNA expression of Nox1 (A, n=3-4), Nox2 (B, n=3-4) and Nox4 (C, n=3-4) in mesenteric arteries (MRA) from:
- WT mice infused with saline or Angiotensin II
- CHOP knockout mice infused with Angiotensin II.
- Homozygous Stim1 knockout specifically in SMC (Stim1^{SMC/-}) mice infused with Angiotensin II.
One-way ANOVA followed by Bonferroni Post-Hoc test was applied for figures (A, B).

*p<0.05 between WT + Ang II vs WT, CHOP^{/-} + Ang II and STIM^{SMC/-} + Ang II. #p<0.05 between STIM^{SMC/-} + Ang II vs WT and CHOP^{/-} + Ang II.
Figure Supp. VII.- Effect of STIM1 and CHOP deletion on oxidative stress.
Immunostaining showing the expression of 8-hydroxy-2-deoxyguanosine (8-OHD) in mesenteric arteries (MRA, A, n=3) and thoracic aorta (B, n=3) from:
- WT mice infused with saline or Angiotensin II
- CHOP knockout mice infused with saline or Angiotensin II.
- Homozygous Stim1 knockout specifically in SMC (Stim1^{SMC/-}) mice infused with saline or Angiotensin II.
**Figure Supp. VIII.- Effect of STIM1 and CHOP deletion on NADPH oxidase activity during hypertension.** Luminometer showing the NADPH oxidase activity in the presence and absence of L-NAME and Apocynin in mesenteric arteries (MRA, A, n=4), heart (B, n=4) and thoracic aorta (C, n=4) from:
- WT mice infused with saline or Angiotensin II
- CHOP knockout mice infused with Angiotensin II.
- Homozygous Stim1 knockout specifically in SMC (Stim1^SMC^/-) mice infused with Angiotensin II.

One-way ANOVA followed by *Bonferroni Post-Hoc test* was applied for figures (A, B, C)

*p*<0.05 between WT + Ang II vs WT ± L-NAME or Apocynin, WT + Ang II ± L-NAME or Apocynin, STIM1^SMC^/- + Ang II ± L-NAME or Apocynin and CHOP^+/+ + Ang II ± L-NAME or Apocynin.
**Figure Supp. IX.** - Effect of Angiotensin II on STIM1 and CHOP in heart. Quantification of STIM1 (A) and CHOP (B) in heart (n=4) from:
- WT mice infused with saline or Angiotensin II
- CHOP knockout mice infused with Angiotensin II.
- Homozygous Stim1 knockout specifically in SMC (Stim1SMC-/-) mice infused with Angiotensin II.

One-way ANOVA followed by Bonferroni Post-Hoc test was applied for figures (A, B).

*\( p<0.05 \) between WT+ Ang II vs WT, STIMSMC-/-, STIMSMC-/- + Ang II, CHOP-/- and CHOP-/- + Ang II.

\( #p<0.05 \) between WT vs STIMSMC-/-, STIMSMC-/- + Ang II, CHOP-/-.

\( ^\wedge p<0.05 \) between CHOP-/- + Ang II vs WT, STIMSMC-/-, STIMSMC-/- + Ang II, CHOP-/-.

\( %p<0.05 \) between STIMSMC-/- + Ang II vs WT, STIMSMC-/-, CHOP-/- and CHOP-/- + Ang II.
Figure Supp. 9.- schematic summarizing the main findings.
Essential Role of Smooth Muscle STIM1 in Hypertension and Cardiovascular Dysfunction

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Short title: STIM1 and cardiovascular dysfunction

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MATERIALS AND METHODS

Mice
All experiments were performed according to the American Guidelines for the Ethical Care of Animals and were approved by institutional Animal Care and Use Committees at Tulane University and EVMS. Wild type littermates (8 weeks-old males) and CHOP knockout mice (8 weeks-old males) were purchased from Jackson Laboratories (Bar Harbor, ME). Male STIM1 smooth muscle-specific knockout mice (Stim1SMC−/−, 8 to 10 week-old) as well as heterozygote mice (Stim1SMC+/−, 8 to 10 week-old) were generated in our laboratory using stim1 floxed mice provided by Stefan Feske (NYU)1 and SM22α-Cre obtained from Jackson Laboratories.1,2 All mice were housed in groups of five, maintained at a temperature of 23 °C with 12 h light/dark cycles and fed a solid standard diet (Na+ content 0.4%) and water. Mice were divided into 8 groups: 1) wild type mice infused with saline, (WT, n=10); 2) wild type mice infused with Ang II (400 ng/kg/min) for 4 weeks (WT + Ang II, n=10); 3) Stim1SMC+/− mice infused with saline (Stim1SMC+/−, n=10); 4) Stim1SMC+/− mice infused Ang II (Stim1SMC+/− + Ang II, n=10); 5) Stim1SMC−/− mice infused with saline (Stim1SMC−/−, n=10); 6) Stim1SMC−/− mice infused Ang II (Stim1SMC−/− + Ang II, n=10); 7) CHOP−/− mice infused with saline (CHOP−/−, n=10); 8) CHOP−/− mice infused with Ang II (CHOP−/− + Ang II, n=10). The infusion was performed using subcutaneous miniosmotic pumps and the body weight and systolic blood pressure were recorded weekly. Systolic blood pressure (SBP) was measured using the CODA tail-cuff blood pressure system (Kent Scientific Torrington, USA). Arterial blood pressure measurements were performed at the same time of the day (between 9 am and 11 am) in order to avoid the influence of the circadian cycle, and the value of SBP was obtained by estimating the average of 10 measurements. At the end of the treatment period, mice were sacrificed and tissues (Thoracic aorta, mesenteric resistance artery and heart) were harvested immediately, placed in PSS solution (composition in mM: NaCl 118; KCl 4.7; CaCl2 2.5; KH2PO4 1.2; MgSO4x7H2O 1.2; NaHCO3 25 and glucose 11, pH=7.4) and processed appropriately for further studies. Blood samples were centrifuged at 2500 rpm for 10 min at 4 °C to obtain plasma, which was immediately stored at -80 °C. Heart was used to determine hypertrophy and fibrosis. Vascular reactivity was evaluated in aorta and mesenteric resistance arteries.

In another set of experiments, we used eight-weeks-old male C57/BL6 wild-type mice, STIM1 smooth muscle-specific knockout mice (Stim1SMC−/−, 8 to 10 week-old) and their homologous heterozygous (Stim1SMC+/−, 8 to 10 week-old). Mice were divided into 6 groups: 1) wild-type received vehicle, (WT, n=10); 2) wild type group received intra-peritoneal injection of Tunicamycin (Tunica, 1 mg/kg, 2 injections/week for two weeks, WT + Tunica, n=10); 3) Stim1SMC+/− group received vehicle (Stim1SMC+/−, n=10); 4) Stim1SMC+/− group received Tunicamycin (Stim1SMC+/− + Tunica, n=10); 5) Stim1SMC−/− group received vehicle (Stim1SMC−/−, n=10); 6) Stim1SMC−/− group received Tunicamycin (Stim1SMC−/− + Tunica, n=10). Body Weight and Systolic Blood Pressure were measured weekly. At the end of treatment, mice were sacrificed, and aorta and mesenteric resistance arteries (MRA) were immediately harvested and placed in PSS solution for vascular reactivity studies and biochemical assays.

Cardiac Fibrosis and hypertrophy
The atrium was removed from the heart; all epicardial fat was removed, and the right and the left ventricles were separated. Transverse sections of the left ventricle were fixed in 4% of formalin, embedded in paraffin and cut into 4µm thick sections. Slices were stained with the collagen-specific Sirius-red (Sigma-Aldrich, USA). At least eight areas of the left ventricle from each heart were captured using a high-resolution digital camera (Olympus DP50, Japan). Cardiac hypertrophy was determined by evaluating heart weight/tibia length ratios as previously described3.
Vascular Reactivity
MRA and thoracic aorta from WT, CHOP^{−/−}, Stim1^{SMC^{−/−}} and Stim1^{SMC^{−/+}} mice, infused with either saline or Ang II or injected with Tunicamycin, were carefully cleaned of fat and connective tissue and then cut into rings (2 mm in length). MRA and thoracic aorta were mounted in a small vessel dual chamber myograph for measurement of isometric tension. After a 30 min equilibration period in PSS solution bubbled with carbogen at 37°C and pH=7.4, arteries were stretched to their optimal lumen diameter for active tension development. After one-hour incubation, cumulative concentration responses to phenylephrine (PE, 3.10^{−8}−10^{−4} M) and thromboxane analog (U46619, 10^{−10}−10^{−5} M) were obtained. In another series of experiments, rings were pre-constricted with U46619 (3.10^{−7} mol/L) and when a steady maximal contraction was reached, cumulative concentration-response curves were obtained for acetylcholine (ACh, 10^{−8}−3.10^{−5}) and sodium nitroprusside (SNP, 10^{−8}−10^{−5}).

To determine the role of NADPH oxidase and TGFβ1 in impaired endothelium-dependent relaxation in hypertensive mice, aorta and MRA from all groups were incubated with NADPH oxidase and TGFβ1 inhibitors: gp91 ds-tat (100 µM) for 30 minutes and SB431542 (10 µM) for 1h respectively, then endothelium-dependent relaxation was determined after pre-contraction with U46619. The same protocol was used for mice treated with Tunicamycin (1 mg/Kg).

Western blot analysis
Western blot analysis was determined in lysates from mesenteric arteries and hearts using specific antibodies against phosphorylated (Serine 1177) and total-eNOS, eNOS P^{T495}(#9574S), phosphorylated and total PKCa/β (# 9375S, # 2056S), CHOP (# Ab108994), ATF6 (# Ab 37149) and Bip (# 3177S) (1:1000 dilution, Cell Signaling Technology, Inc, USA), p47phox (sc-17845), P-Smad2,3 (# 8822Sß) and T-Smad1,2,3 (# Sc7960) (1:500 dilution, Santa Cruz Biotechnology, Inc) as previously described.

Immunohistochemistry
Hearts, mesenteric resistance arteries and thoracic aorta were fixed in 4% of paraformaldehyde followed by zinc-saturated formalin and paraffin-embedding for either immunostaining or immunoperoxidase staining using the Vectastain ABC Kit. The hearts sections were incubated overnight with the anti-STIM1 antibody (1:200, Cell Signaling Technology, Inc) and anti-CHOP antibody (1:200, Cell Signaling Technology). Mesenteric resistance and thoracic aorta sections were incubated with anti 8-hydroxy-2-deoxyguanosine (8-OHD), marker of oxidative stress (1:200, Abnova; #MAB1998). At least eight sections from each sample were captured using a high-resolution digital camera (Olympus DP50, Japan).

Nitrites and Nitrates levels
A number of nitrites, the end product of NO metabolism was measured in aorta and MRA tissue samples by the Griess reaction. Optical density at 550 nm wavelengths was measured using a Spectramax 250 microplate reader (Molecular Devices, CA). Nitrite concentrations were calculated by establishing a standard curve with known sodium nitrite concentrations.

Colorimetric Determination of cGMP
The cGMP levels were measured in MRA lysates in all groups of mice. Measurements were performed using a sandwich enzyme-linked immunosorbent assay (ELISA; Cayman Chemical, MI) according to the manufacturer instructions.

NADPH oxidase activity assay
Superoxide anion levels generated by NADPH oxidase activity were measured in lysates of aorta and MRA using lucigenin chemiluminescence. Briefly, lysates were prepared in a sucrose buffer
containing KH2PO4 50 mM, EGTA 1 mM, sucrose 150 mM; pH=7.0 and the “Complete-C mini” protease inhibitor cocktail (Roche Diagnostics, IN) in a Tissue Dounce homogenizer on the ice, and aliquots of the homogenates were used immediately. To start the assay, a volume of 100 µL of each lysate was used in a total volume of 1 mL PBS buffer preheated at 37°C, containing lucigenin (5 µM) and NADPH (100 µM). Blank samples were prepared using 100 µL of sucrose buffer. Lucigenin activity was measured every 30 seconds for 10 min in a luminometer (Turner biosystem 20/20, single tube luminometer) or till enzymatic activity reached a plateau. The NADPH oxidase activity was performed in the presence or absence of a direct inhibitor of NOS (L-NAME) or an inhibitor of NADPH oxidase (Apocynin). Data are expressed as % of the area under the curve of relative light units (RLU) normalized to protein content (µg protein) compared to WT.

**eNOS ELISA**

Total eNOS were determined in all groups by an ELISA Kit according to the manufacturer's guidelines (Mouse-eNOS kit, Qayee-Bio, China).

**Reverse Transcription Polymerase Chain Reaction Real-Time Assay**

ER stress markers (CHOP, BIP, ATF6), NOX isoforms (Nox 2 and 4) and STIM1 mRNA levels were determined in MRA as previously described³,⁴.

**Drugs**

Phenylephrine hydrochloride, acetylcholine, NADPH, Apocynin and angiotensin II were obtained from Sigma-Aldrich. U46619, Tunicamycin, and SB431542 were obtained from Tocris Bioscience. The gp91-ds-tat was obtained from AnaSpec, Inc. Stock solutions of drugs were prepared in ultrapure water, stored at -20°C and appropriate dilutions were made on the day of the experiment.

**Statistical analysis**

Data are expressed as mean ± SEM. Concentration-response curves were analyzed using the GraphPad Prism 4.0 software (GraphPad, USA) and adjusted to a logistic equation. Statistical calculations for significant differences were performed using Student’s t-test, one-way followed by Post-Hoc test or two-way ANOVA as appropriate. Comparisons are considered significant when p< 0.05.

Hypertension

↑STIM1

Heart

↑Fibrosis  ↑ER stress  ↑Hypertrophy

Heart damage

Vessel

↑ER stress

↑NADPH oxidase (MRA)

↑TGFβ (Aorta)

↓cGMP  ↓eNOS

Endothelial Dysfunction

Graphic abstract