Endoplasmic Reticulum Stress Is Involved in Cardiac Damage and Vascular Endothelial Dysfunction in Hypertensive Mice

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Objective—Cardiac damage and vascular dysfunction are major causes of morbidity and mortality in hypertension. In the present study, we explored the beneficial therapeutic effect of endoplasmic reticulum (ER) stress inhibition on cardiac damage and vascular dysfunction in hypertension.

Methods and Results—Mice were infused with angiotensin II (400 ng/kg per minute) with or without ER stress inhibitors (taurine-conjugated ursodeoxycholic acid and 4-phenylbutyric acid) for 2 weeks. Mice infused with angiotensin II displayed an increase in blood pressure, cardiac hypertrophy and fibrosis associated with enhanced collagen I content, transforming growth factor-β1 (TGF-β1) activity, and ER stress markers, which were blunted after ER stress inhibition. Hypertension induced ER stress in aorta and mesenteric resistance arteries (MRA), enhanced TGF-β1 activity in aorta but not in MRA, and reduced endothelial NO synthase phosphorylation and endothelium-dependent relaxation (EDR) in aorta and MRA. The inhibition of ER stress significantly reduced TGF-β1 activity, enhanced endothelial NO synthase phosphorylation, and improved EDR. The inhibition of TGF-β1 pathway improved EDR in aorta but not in MRA, whereas the reduction in reactive oxygen species levels ameliorated EDR in MRA only. Infusion of tunicamycin in control mice induced ER stress in aorta and MRA, and reduced EDR by a TGF-β1–dependent mechanism in aorta and reactive oxygen species–dependent mechanism in MRA.


Key Words: cardiac hypertrophy ■ endoplasmic reticulum stress ■ endothelial NO synthase ■ fibrosis ■ hypertension ■ oxidative stress ■ transforming growth factor-β1 ■ vascular reactivity

Emerging evidence from experimental and clinical research indicates that endoplasmic reticulum (ER) stress is involved in cardiovascular diseases such as cardiac hypertrophy, heart failure, atherosclerosis, and ischemic heart disease.1–3 Although the clinical management of hypertension has advanced substantially, cardiovascular disease still constitutes a major and increasing health burden worldwide. Although treatments for hypertension have progressed, the development of novel therapies for patients with vascular complications and cardiac damage in hypertension remains a major research goal. These thoughts are supported by the observation that impaired macro- and microcirculatory heart function predicts poor outcome for patients with cardiovascular disease.4

The ER is an organelle in which membrane-bound proteins are folded into their final 3-dimensional structures where lipids and sterols are synthesized and where free calcium is stored. Various types of cellular stress (ischemia, hypoxia, gene mutation, oxidative stress, and increased protein synthesis) lead to impairment of ER function, creating a state collectively termed ER stress that leads to the activation of a complex signaling network called the unfolded protein response.5–4 The unfolded protein response is activated in the cell by 3 main signaling pathways: (1) inositol-requiring protein 1 activation, (2) the protein kinase RNA–like ER kinase activation, and (3) the activating transcription factor 6 (ATF6).9

Recent report suggests that ER stress is involved in cardiac remodeling in hypertensive animals.10 It was also shown that the inhibition of ER stress attenuates cardiovascular remodeling in aldosterone salt-treated rats.11 These observations suggest that ER stress is an important factor in cardiovascular homeostasis. The significance of ER stress and its role in vascular dysfunction and cardiac damage in hypertension

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are important questions that have remained unanswered. Therefore, the aim of the present study was to determine the role of ER stress in vascular endothelial dysfunction and cardiac damage in angiotensin II (ang II)–induced hypertension model.

**Materials and Methods**

An extensive description of materials and methods can be found in the online-only Data Supplement. A brief summary is given below.

**General Protocol in Mice**

One hundred mice (C57BL/6J, 8-week-old males) were purchased from Jackson Laboratories (Bar Harbor, ME).

**Cardiac Fibrosis**

Transverse sections of left ventricle were stained with the collagen-specific stain Sirius red (Sigma-Aldrich, St. Louis, MO).

**Vascular Reactivity**

**In Vivo Experiments**

Aorta and mesenteric resistance arteries (MRA) were mounted in a small vessel dual-chamber myograph for measurement of isometric tension.12,13 After preconstriction with phenylephrine (10^{-4} mol/L) and steady maximal contraction, cumulative concentration response curves were obtained for acetylcholine (1×10^{-8} to 3×10^{-5} mol/L) and sodium nitroprusside (1×10^{-8} to 3×10^{-5} mol/L).

**Hypertrophy**

Hearts were harvested and then atrium and all epicardial fat were removed. Cardiac hypertrophy was calculated using heart weight/body weight ratios.

**Active Transforming Growth Factor-β1 Level**

Active transforming growth factor-β1 (TGF-β1) level was measured in heart, aorta, and MRA tissue lysates using TGF-β1 immunoassay (Quantikine, R&D systems, Minneapolis, MN) as previously described.14

**Western Blot Analysis**

Western blot analysis for endothelial NO synthase (eNOS), eukaryotic translation initiation factor 2α, TGF-β1, GRP78 (1:1000 dilution; Cell Signaling Technology, Inc), collagen I, P-Smad2/3, and Smad1/2/3 (1:250 dilution; Santa Cruz Biotechnology, Inc) was performed in lysates of aorta, MRA, and heart as previously described.15,16

**Immunofluorescence**

Immunostaining was performed for C/EBP homologous protein (CHOP) in paraffin sections of aorta and MRA using specific antibodies, anti-CHOP (1:200, Santa Cruz Biotechnology, Inc).

**Immunohistochemistry**

Aorta, MRA, and hearts were fixed in 4% paraformaldehyde followed by zinc-saturated formalin for immunohistochemical studies. Immunostaining was performed as previously described.17

**Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay**

Four-micrometer paraffin sections from heart were mounted using Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc). The images were captured using a deconvolution fluorescent scope.

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**ER Stress and Hypertension-Induced Pathology**

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

CHOP, ATF4, ATF6, and TGF-β1 mRNA levels were determined in heart, aorta, and MRA from all groups, whereas nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 (Nox1), Nox2, and Nox4 mRNA levels were studied only in vessels.

**Nitrite Levels**

The amount of nitrites, the end product of NO metabolism, was measured in plasma samples by the Griess reaction.

**Cardiac Fibrosis**

Chronic ang II infusion induces cardiac fibrosis associated with increased active caspase-3, cardiomyocyte size, and apoptotic cells (Figure 2A and 2C–2E). In addition, collagen type I expression and mRNA levels of ATF4, CHOP, and ATF6 were also increased (Figure 2B, 2G, and 2H; Figure IIA in the online-only Data Supplement). All these parameters were significantly reduced with ER stress inhibitors (Figure 2A–2E, 2G, and 2H; Figure IIA in the online-only Data Supplement). The mRNA level of TGF-β1 in heart was similar in all groups (Figure 2I) whereas active TGF-β1 level, and total and phosphorylated Smad2/3 were increased in hearts from hypertensive mice compared with Sham and hypertensive mice treated with ER stress inhibitors (Figure 2F and 2J).

**ER Stress and TGF-β1 in Aorta and MRA**

Ang II–induced hypertension increased ATF4, CHOP mRNA levels, and phosphorylated eukaryotic translation initiation factor 2α levels, which were reduced with ER stress inhibition in aorta and MRA (Figure 3A–3C, 3I–3K; Figure IIB and IIC in the online-only Data Supplement). We observed a different pattern in mRNA level, and total and active form of TGF-β1 between aorta and MRA. Thus, TGF-β1 mRNA and protein levels, its active form, and phosphorylated Smad2/3 were augmented in aorta from hypertensive mice and reduced with ER stress inhibitors (Figure 3D–3H), whereas no changes were observed in MRA in all groups of mice (Figure 3I–3P).

**ER Stress and Vascular Reactivity in Hypertension**

To determine the role of ER stress in vascular endothelial dysfunction in hypertension, we examined endothelium-dependent relaxation (EDR) and endothelium-independent...
relaxation in aorta and MRA. Ang II–induced hypertension attenuated EDR in aorta and MRA, and was significantly improved in hypertensive mice treated with Tudca and PBA compared with untreated hypertensive mice (Figure 4A and 4I). Endothelium-independent relaxation was similar in hypertensive mice treated with and without ER stress inhibitors (Figure 4B and 4J). We did not observe any change in EDR and endothelium-independent relaxation in aorta and MRA from Sham mice treated with Tudca or PBA (Figure 1A, IC, ID, and IF in the online-only Data Supplement).

The inhibition of eNOS with L-NAME reduced EDR in hypertensive mice (Figure 4C and 4K) and Sham mice (Figure 1B and IE in the online-only Data Supplement). Ang II–induced hypertension reduced eNOS phosphorylation in aorta and MRA, which was restored after ER stress inhibition. In Sham mice treated with Tudca and PBA, we did not observe any effect on eNOS phosphorylation in aorta and MRA. Total eNOS expression was similar in all groups of mice (Figure 4E and 4M). In addition, nitrite levels were reduced in plasma from hypertensive mice and restored after ER stress inhibition (Figure IID in the online-only Data Supplement).

To further elucidate the mechanism of vascular endothelial dysfunction in hypertension related to ER stress, we studied the involvement of Nox and TGF-β1 pathways. Our data indicated that inhibition of Nox with apocynin slightly enhanced EDR in aorta but completely restored EDR in MRA, whereas TGF-β1 receptor antagonist significantly enhanced EDR in the aorta but no effect was observed in MRA (Figure 4D and 4L). Ang II–induced hypertension increased Nox activity in both arteries, Nox1 and Nox4 mRNA levels in aorta and MRA, respectively, which were reduced after ER stress inhibition (Figure 4F–4H, 4N–4P). These results were associated with augmented active TGF-β1 levels in aorta from hypertensive mice, which were reduced after ER stress inhibition (Figure 3E and 3G). In MRA, we did not see an increase in active TGF-β1 in all groups of mice (Figure 3M and 3O).

**Oxidative Stress, TGF-β1, and Vascular Reactivity**

To further determine the relationship between ER stress and vascular dysfunction, we performed in vitro studies by incubating isolated aorta and MRA from control mice with ER stress inducer (tunicamycin) for 1 hour. The results showed that tunicamycin significantly reduced EDR in aorta and MRA, which was prevented when aorta was pretreated with Tudca or TGF-β1 pathway inhibition and when MRA was pretreated with Tudca or apocynin (Figure III in the online-only Data Supplement).

In vivo studies showed that tunicamycin injection with and without TGF-β1 pathway inhibition did not increase systolic blood pressure (Figure 5A). Tunicamycin injection increased ER stress marker expression in aorta and MRA, which was reduced only in aorta with TGF-β1 receptor antagonist (SB431542) and 1D11 neutralizing TGF-β1 antibodies (Figure 5B–5E, 5G, and 5H). The mRNA level of TGF-β1 was augmented in aorta and reduced with 1D11 and SB431542 (Figure 5F), but no change was observed in MRA (Figure 5I). Mice injected with tunicamycin showed an attenuated EDR in aorta and MRA (Figure 6A and 6D). The in vivo inhibition of TGF-β1 pathway restored EDR in aorta, whereas only a minor improvement in EDR was observed in MRA (Figure 6A and 6D). In addition, the incubation of MRA with apocynin...
Figure 2. Representative heart sections stained with (A) Sirius red, (C) immunohistochemistry, (D) terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (green arrows indicate the apoptotic cells), and (E) cardiomyocyte area. Quantitative data in all groups, n=6 (A, C, and E). Activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) mRNA levels, normalized to 18S ribosomal RNA (rRNA), in heart (G and H). Western blot analysis and quantitative data for collagen I, T-Smad1/2/3, and P-Smad2/3 in heart (B and F) from all groups, n=5. Transforming growth factor-β1 (TGF-β1) mRNA levels, normalized to 18S rRNA, in heart (I) in all groups, n=6. Active TGF-β1 levels in heart (J) in all groups, n=5. *P<0.05 for HT vs control, control+PBA, control+Tudca, HT+PBA, and HT+Tudca. HT indicates hypertension; PBA, 4-phenylbutyric acid; Tudca, taurine-conjugated ursodeoxycholic acid.
Figure 3. A, B, I, and J, Activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) mRNA levels, normalized to 18S ribosomal RNA (rRNA), in aorta and mesenteric resistance arteries (MRA) in all groups of mice, n=6. C and K, Western blot analysis and quantitative data for eukaryotic translation initiation factor 2α (eIF2α), P-eIF2α in aorta and MRA in all groups of mice, n=5. D and L, Transforming growth factor-β1 (TGF-β1) mRNA levels, normalized to 18S rRNA, in aorta and MRA in all groups of mice, n=6. E, M, G, and O, Active TGF-β1 levels in aorta and MRA in all groups of mice, n=4. F, H, N, and P, Western blot analysis and quantitative data for TGF-β1, T-Smad1/2/3, and P-Smad2/3 in aorta and MRA in all groups, n=5.*P<0.05 for HT vs control, control+PBA, control+Tudca, HT+PBA, and HT+Tudca. HT indicates hypertension; PBA, 4-phenylbutyric acid; Tudca, taurine-conjugated ursodeoxycholic acid.
Figure 4. A, B, C, I, J, and K, Endothelium-dependent and independent relaxation in response to acetylcholine (Ach) and single-nucleotide polymorphism (SNP), respectively, in aorta and mesenteric resistance arteries (MRA) with and without L-NAME in all groups, n=10. E and M, Western blot analysis and quantitative data for P-endothelial NO synthase (eNOS), T-eNOS, and β-actin in aorta and MRA in all groups, n=5. D and L, Endothelium-dependent relaxation in response to ACh with and without apocynin (Apo) and SB431542 in aorta and MRA in all groups of mice, n=5. F and N, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in aorta and MRA in all groups of mice, n=5. G, H, O, and P, NADPH oxidase 1 (Nox1) and Nox4 mRNA levels, normalized to 18S ribosomal RNA (rRNA), in aorta and MRA from all groups, n=6. *P<0.05 for HT vs control, control+PBA, control+Tudca, HT+PBA, and HT+Tudca. HT indicates hypertension; PBA, 4-phenylbutyric acid; Tudca, taurine-conjugated ursodeoxycholic acid.
completely restored EDR, whereas no effect was observed in aorta isolated from tunicamycin-injected mice treated with and without SB431542 or 1D11 (Figure 6B, 6C, 6E, and 6F). The L-NAME completely blocked the EDR in aorta whereas partially in MRA from control mice treated with tunicamycin with and without TGF-β1 pathway inhibition (Figure 6B, 6C, 6E, and 6F).

In other experiments, our results showed that mice injected with tunicamycin with and without Tudca did not affect systolic blood pressure or induce cardiac hypertrophy (Figure IVA and IVB in the online-only Data Supplement). However, tunicamycin injection moderately induced cardiac fibrosis that was reduced after ER stress inhibition (Figure IVC in the online-only Data Supplement). The size of cardiomyocytes did not change between groups (data not shown). Nox activity was increased in aorta and MRA from mice injected with tunicamycin, and treatment with Tudca restored the activity (Figure IVD and IVE in the online-only Data Supplement). The EDR was impaired in aorta and MRA from mice injected with tunicamycin, which was restored after ER stress inhibition (Figure VA and VD in the online-only Data Supplement). To determine the mechanism how tunicamycin impairs vascular function, aorta and MRA from mice injected with tunicamycin were incubated with apocynin. The data showed that EDR was restored in MRA, whereas no effect was observed in aorta (Figure VB and VE in the online-only Data Supplement). The L-NAME completely blocked EDR in aorta whereas partially in MRA from control mice injected with tunicamycin with or without Tudca (Figure VVC and VF in the online-only Data Supplement).

To demonstrate the induction of ER stress in vascular endothelial cells, we performed immunostaining, and data indicate the presence of ER stress marker (CHOP) in endothelial cells of aorta and MRA from mice injected with tunicamycin. The induction of CHOP in vascular endothelial cells was blunted in mice injected with tunicamycin and treated with Tudca (Figure VIA and VIB in the online-only Data Supplement).

**Discussion**

Our study demonstrated that ER stress is an important factor in macro- and microvascular pathology and cardiac damage in hypertension. Such evidence first came from in vivo experiments in which hypertensive mice treated with ER stress inhibitors had reduced arterial blood pressure and improved EDR and cardiac damage. We further demonstrated that ER stress inhibition in hypertension differentially improves macrovascular endothelial function by TGF-β1–dependent.

![Figure 5. A, Systolic blood pressure (SBP) measured in all groups, n=10. B and C, Western blotting analysis for GRP78 in aorta and mesenteric resistance arteries (MRA). D, E, F, G, H, and I, Activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and transforming growth factor-β1 (TGF-β1) mRNA levels, normalized to 18S ribosomal RNA (rRNA), in aorta and MRA in all groups of mice, n=6. *P<0.05 for Sham+Tunica vs Sham, Sham+Tunica+1D11, and Sham+Tunica+SB431542.](http://ath.ahajournals.org/content/10/7/1658.full.pdf)
mechanism and microvascular endothelial function by an oxidative stress–dependent mechanism. These results suggest that inhibition of ER stress could be a useful therapeutic strategy to reverse vascular complications and cardiac damage in hypertension.

Vascular pathology and cardiac damage are well characterized in hypertensive animal models and patients. Several mechanisms have been proposed, such as dysregulation of the renin angiotensin system, endothelin-1, cyclooxygenase activity, oxidative stress, calcium channels, immune function, and inflammation. Previous studies established the relationship among ER stress, diabetes mellitus, obesity, and cardiovascular diseases, but the mechanism and the significance of ER stress in hypertension-induced vascular dysfunction and cardiac damage are still unknown.

The chemical chaperone PBA and endogenous bile acids such as TUDCA are known to modulate ER function, stabilize protein conformation, improve ER folding capacity, and facilitate mutant proteins trafficking. It is well established that increased arterial blood pressure is associated with cardiovascular complications, such as cardiac hypertrophy and fibrosis, renal failure, and vascular endothelial dysfunction. In the present study, we demonstrated that ang II–induced hypertension and increased blood glucose level were reduced after ER stress inhibition, indicating that ER stress is an important factor in the development of hypertension and prediabetic state. It is not surprising that the blood glucose level increased after ang II infusion because it is reported that ang II stimulates the degradation of insulin receptor substrate 1. In addition, it has been shown that ER stress inhibition has a beneficial effect in type 1 and type 2 diabetes mellitus in terms of glucose regulation. These results explain the relationship among hypertension, prediabetic stage, and ER stress.

Cardiac hypertrophy and fibrosis are well documented in hypertensive animals and patients. Hypertension-induced cardiac hypertrophy is a progressive event associated with myocardial remodeling characterized by fibrosis and alterations in cardiomyocytes size and function. In our model, ang II–induced cardiac hypertrophy and fibrosis were associated with ER stress induction. Interestingly, ER

Figure 6. A and D, Endothelium-dependent relaxation in response to acetylcholine (Ach) in aorta and mesenteric resistance arteries (MRA) from mice treated with or without TUNICA with and without 1D11 or SB431542 in aorta and MRA, n=5. *P<0.05 for Sham+Tunica vs Sham, Sham+Tunica+1D11, and Sham+Tunica+SB431542. B and E, Endothelium-dependent relaxation in response to ACh in aorta and MRA from mice treated with TUNICA with and without apocynin (Apo), L-NAME, and SB431542 in aorta and MRA, n=5, $P<0.05 for Sham+Tunica+L-NAME and Sham+Tunica+SB431542+L-NAME vs Sham+Tunica+Apo and Sham+Tunica+Apo+SB431542. C and F, Endothelium-dependent relaxation in response to Ach in aorta and MRA from mice treated with TUNICA with and without Apo, L-NAME, and 1D11 in aorta and MRA, n=5. #P<0.05 for Sham+Tunica+L-NAME and Sham+Tunica+1D11+L-NAME vs Sham+Tunica+Apo and Sham+Tunica+Apo+1D11.
stress inhibition blunted ang II–induced cardiac hypertrophy and fibrosis suggesting that ER stress is an important factor in the development of cardiac damage in hypertension. Cardiac fibrosis is a result of the pathological accumulation of extracellular matrix components, particularly collagens I and II.43 Importantly, our data indicate that the increased collagen I expression in hypertensive mice hearts was reduced after ER stress inhibition. It is well known that in hypertension, TGF-β1 stimulates collagen synthesis.44 Our data showed that active TGF-β1 and its downstream signaling Smad2/3 were increased in mice infused with ang II and were reduced after ER stress inhibition. In addition, we observed an increase in the size of cardiomyocytes, which was reduced after ER stress inhibition. The loss of cardiomyocytes by apoptosis has emerged as an important mechanism contributing to myocardial remodeling in response to hemodynamic overload.5,46 Our data indicate that cardiomyocyte apoptosis was increased in hypertensive mice compared with Sham and hypertensive mice treated with ER stress inhibitors.

It is well known that hypertension is associated with vascular endothelial dysfunction.47-48 However, the role of ER stress in vascular endothelial dysfunction in hypertension is still unknown. Ang II–induced hypertension reduced EDR and eNOS phosphorylation and enhanced ER stress in aorta and MRA, and this was associated with an increase in EDR. The inhibition of arterial blood pressure and cardiac damage.

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Disclosures
None.

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SUPPLEMENTAL MATERIALS
MATERIALS AND METHODS

General protocol in mice

All experiments were performed according to the American Guidelines for the Ethical Care of Animals and were approved by Tulane University Health Sciences Center Animal Care and Use Committee. One hundred mice (C57BL/6J, 8 weeks-old males) were purchased from Jackson Laboratories (Bar Harbor, ME), housed in groups of five mice, and maintained at a temperature of 23 °C with 12 h light/dark cycles. Mice were fed on a solid standard diet (Na⁺ content 0.4%) and water. Mice were divided in 6 groups: 1) sham mice (control mice infused with saline, n=10); 2) control mice infused with Ang II (400 ng/kg/min) using subcutaneous mini-osmotic pumps for 2 weeks (HT, n=10); 3) control mice infused with Ang II and daily intra-peritoneal injected with 4-phenyl butyric acid (PBA, 1 g/kg/day) (HT + PBA, n=10); 4) control mice infused with Ang II and daily injected (intra-peritoneal) with taurine-conjugated ursodeoxycholic acid (Tudca, 150 mg/kg/day) (HT+ Tudca, n=10); 5) control mice that received daily injection of PBA (Sham + PBA, n=10); 6) control mice that received daily injection of Tudca for 2 weeks (Sham + Tudca, n=10). The body weight and plasma glucose levels were recorded weekly during the experimental period.

Systolic blood pressure (SBP) was measured using the CODA tail-cuff blood pressure system (Kent Scientific Torrington, USA).\(^1\) Arterial blood pressure measurements were performed at the same time of day (between 9 a.m. and 11 a.m.) 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 anaesthetized with isoflurane and blood samples were collected from carotid artery into containing heparin tubes. Then, tissues (heart,
aorta, mesenteric resistance artery) were harvested immediately, placed in PSS solution (composition in mM: NaCl 118; KCl 4.7; CaCl$_2$ 2.5; KH$_2$PO$_4$ 1.2; MgSO$_4$·7H$_2$O 1.2; NaHCO$_3$ 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 other set of experiments, we used 8 weeks-old C57BL/6J male mice divided into five groups: 1) sham group (control received saline, n=10); 2) control group that received intraperitoneal injection of tunicamycin (Tunica, 1 mg/kg, 2 injections/week for two weeks, Sham + Tunica, n=10); 3) control group that received tunicamycin and TGFβ1 receptor antagonist (SB431542, 10 mg/kg; 3 injections/weeks for two weeks) (Sham + Tunica + SB431542, n=10); 4) control group that received tunicamycin and neutralizing TGFβ1 antibody (1D11, 10 mg/kg; 3 injections/weeks for two weeks) (Sham + Tunica + 1D11, n=10); 5) control group that received tunicamycin and Tudca (150 mg/kg/day ) for 2 weeks (Sham + Tunica + Tudca, n=10). Systolic blood pressure was measured weekly during the treatment period. At the end of treatment, mice were anaesthetized with isoflurane and then aorta and mesenteric resistance arteries were immediately harvested and placed in PSS solution for reactivity and biochemistry assays.

**Cardiac Fibrosis**

The atrium was removed from the heart and all the epicardial fat was removed. The right and the left ventricles were separated. Transverse sections of left ventricle were fixed in 4% of formalin, embedded in paraffin and cut into 4 µm thick sections. Slices were stained with the collagen-specific stain 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). The collagen was quantified using Adobe Photoshop CS2 (Microsoft). For each image, the percentage of interstitial fibrosis was determined as the ratio of the collagen surface area with respect to myocardial surface area. Cardiomyocytes cross-sectional area was measured as an average of 30 cardiomyocytes taken from different zones of left ventricle using Image J software (US National Institutes of Health, http://rsb.info.nih.gov/ij/).

**Vascular Reactivity**

*In vivo experiments*

Thoracic aorta and MRA from Sham and hypertensive mice were carefully cleaned of fat and connective tissue and then cut into rings (2 mm in length). Aorta and MRA were mounted in a small vessel dual chamber myograph for measurement of isometric tension.\textsuperscript{2,3} 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 a second 30 min equilibration period, arteries were stimulated with phenylephrine (PE, 10\textsuperscript{-5} M) followed by and (ACh, 10\textsuperscript{-6} M) to assess the function of endothelial cells. After pre-constriction with PE (10\textsuperscript{-4} mol/L) and steady maximal contraction, cumulative concentration response curves were obtained for ACh (1×10\textsuperscript{-8} to 3×10\textsuperscript{-5} mol/L) and SNP (1×10\textsuperscript{-8} to 3×10\textsuperscript{-5} mol/L). The arterial lumen diameter and the contraction in response to PE were similar in all groups of mice.

To determine the role of eNOS and TGFβ1 in the impaired endothelium-dependent relaxation in hypertensive mice, aorta and MRA were incubated with SB431542 (10 μM) for 1h and then endothelium-dependent relaxation was performed after pre-contraction with PE.
The same protocol was used for mice treated with Tunicamycin (1 mg/Kg) and Tunicamycin and SB431542 (10 mg/Kg) or 1D11 (10 mg/kg) or Tudca (150 mg/kg/day). After pre-contraction with PE (10^{-5} M) and the steady maximal contraction, cumulative dose-response curves were obtained for ACh (10^{-8}-10^{-5} M).

To determine the role of eNOS and NADPH oxidase in the impaired endothelium-dependent relaxation in hypertensive mice, aorta and MRA were incubated with L-NAME (100 µM) and apocynin (100 µM) for 30 minutes and then endothelium-dependent relaxation was performed after pre-contraction with PE.

- **In vitro experiments**

Thoracic aorta and MRA from control mice were harvested and then carefully cleaned of fat and connective tissue. Aorta and MRA were mounted in a small vessel dual chamber myograph for measurement of isometric tension. Arteries were incubated with Tunicamycin (20 µg/ml) for one hour in the presence or absence of SB431542 (10 µM), Tudca (500 µg/ml) or 1D11 (20 µg/ml). After pre-contraction with PE (10^{-5} M), cumulative dose-response curves were performed for ACh (10^{-8}-10^{-5} M). The role of eNOS and NADPH oxidase activity in EDR were determined as described above.

**Hypertrophy**

Hearts were harvested and then atrium and all epicardial fat were removed. Cardiac hypertrophy was calculated using heart weight/body weight ratios.

**Active TGFβ1 level**
Active TGFβ1 level, which is the cleaved form at the 361 amino acid of TGFβ1, was measured in heart, aorta and MRA tissue lysates using TGFβ1 immunoassay (Quantikine, R&D systems, Minneapolis, USA) as previously described.\(^4\)

**Western blot analysis**

Western blot analysis for eNOS, eIF2-α, TGFβ1, GRP78 (1:1000 dilution, Cell Signaling Technology, Inc, USA), collagen-1, P-Smad2,3 and Smad1,2,3 (1:250 dilution, Santa Cruz Biotechnology, Inc) was performed in lysates of aorta, mesenteric arteries and heart as previously described.\(^5,6\)

**Immunofluorescence**

Aortic and MRA segments were frozen in Tissue Tek OCT embedding medium (Sakura Finetek Europe, The Netherlands). Transverse sections were cut into 5 µm thick sections. After blockade in PBS containing 5 % fetal bovine serum and 0.3 % Triton X-100, sections were incubated with rabbit polyclonal antibody against active TGFβ1 (1:400, LC-1-30; provided by Dr. Kathy Flanders, (National Institutes of Health, Bethesda, MD) overnight at 4 °C. After washing, rings were incubated with the secondary antibody donkey anti-rabbit IgG conjugated to Alexa 594 (Molecular Probes, Invitrogen) at a dilution of 1:500 for 1 h at RT. After washing, slides were mounted with SlowFade Gold antifade reagent with DAPI (Molecular Probes, Invitrogen) and immunofluorescent signals were viewed using a fluorescence microscope Eclipse 55i (x20), Nikon. Immunostaining was performed for CHOP in paraffin sections of aorta and MRA using specific antibodies anti-CHOP (1:200, Santa Cruz Biotechnology, Inc).
Immunohistochemistry.

Aortic, MRA and hearts were fixed in 4% of paraformaldehyde followed by zinc-saturated formalin for immunohistochemical studies. Immunostaining was performed as previously described. 

TUNEL assay

Recombinant Terminal Deoxynucleotidyl transferase (rTdT) mediated nick-end labeling (TUNEL) was performed using the Dead End Fluorometric Tunel System (Promega) according to the manufacturer's guidelines. Four micrometer paraffin sections from heart were fixed in methanol-free paraformaldehyde before and after proteinase K treatment at 20 μg/ml for 8–10 min at room temperature. The sections were incubated with the nucleotide mixture (which included fluorescein-tagged dUTP) and rTdT enzyme for an hour at 37 °C. The slides were mounted using Vectasheild with DAPI (Vector Laboratories, Inc). The images were captured using a deconvolution fluorescent scope.

RT-PCR real-time assay

CHOP, ATF4, ATF6 and TGFβ1 mRNA levels were determined in heart, aorta and mesenteric resistance arteries from all groups, while Nox1, Nox2 and Nox4 mRNA levels were studied only in vessels. Briefly, mRNA from tissues was extracted and reverse-transcribed. Assays-on-Demand (Applied Biosystems) of TaqMan fluorescent real time PCR primers and probes were used for Chop (Mm00492097_m1), Atf4 (Mm00515324_m1), Atf6 (Mm01295317_m1) TGFβ1 (Mm01178820_m1), Nox-1 (Mm00549170_m1), Nox-2
(Mm01287743_m1), Nox-4 (Mm00479246_m1) and 18S rRNA (Hs99999901_s1), which was used to normalize results.

**Nitrites levels**

The amount of nitrites, the end product of NO metabolism, was measured in plasma samples by the Griess reaction. Optical density at 550 nm wave length was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, Calif). Nitrite concentrations were calculated by comparison with a standard curve of sodium nitrite.

**Drugs**

Phenylephrine hydrochloride, acetylcholine, NADPH, L-NAME, apocynin, PBA and angiotensin II were obtained from Sigma-Aldrich (USA). Tudca was purchased from Calbiochem (US and Canada). SB431542 was obtained from Tocris Bioscience (USA). The 1D11 was kindly provided by Genzyme Corporation. Stock solutions of drugs were prepared in ultrapure water, stored at -20 ºC 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). Statistical calculations for significant differences were performed using Student’s t test, one-way or two-way ANOVA as appropriate. Significance was accepted at p< 0.05.
References supplementary material:


Figure I

A. Aorta relaxation (%)

B. Aorta relaxation (%)

C. Aorta relaxation (%)

D. MRA relaxation (%)

E. MRA relaxation (%)

F. MRA relaxation (%)

- Sham
- Sham + Tudca
- Sham + PBA

- Sham + L-NAME
- Sham + Tudca + L-NAME
- Sham + PBA + L-NAME

- log ACh (M)

- log SNP (M)
Figure III

A. Sham, Sham + Tunica + SB 431542
   - Sham + Tunica
   - Sham + Tunica + Apo
   - Sham + Tunica + Tudca

B. Sham, Sham + Tunica + 1D11
   - Sham + Tunica

C. Sham, Sham + Tunica + SB 431542
   - Sham + Tunica
   - Sham + Tunica + Tudca
   - Sham + Tunica + Apo

D. Sham, Sham + Tunica + 1D11
   - Sham + Tunica

MRA relaxation (%)

log ACh (M)
Figure IV

A

- Sham
- Sham + Tunica + Tudca
- Sham + Tunica

SBP (mmHg)

Time (Weeks)

B

Cardiac hypertrophy (mg/kg)

Sham | Tudca

Sham + Tunica

C

% of Fibrosis

Sham | Sham + Tunica | Tudca

Sham + Tunica

D

NADPH oxidase (RLU) / μg protein (Aorta)

Sham | Tudca

Sham + Tunica

E

NADPH oxidase (RLU) / μg protein (MRA)

Sham | Tudca

Sham + Tunica

Figure IV
Figure VI

A

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Figure VI

B

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MRA (Overlay) MRA (CHOP) MRA (DAPI)
Legends supplementary Figures

Figure I. Endothelium-dependent and independent relaxation in response to ACh and SNP respectively in aorta (A, C) and MRA (D, F) with and without L-NAME (B, E), n=10.

Figure II. ATF6 mRNA levels, normalized to 18S rRNA, in heart, aorta and MRA from all groups, n=6 (A, B, C). Nitrites levels from all groups, n=8 (D). *P<0.05 for HT vs. Control, Control + PBA, Control + Tudca, HT + PBA and HT + Tudca.

Figure III. Endothelium-dependent relaxation in response to ACh in rings incubated with or without Tunica in the presence and absence of Tudca, 1D11, SB 431542 or apocynin (Apo) in aorta (A, B) and MRA (C, D), n=5. *P<0.05 significantly different between Sham + Tunica vs. Sham, Sham + Tunica + Tudca, Sham + Tunica + 1D11, Sham + Tunica + SB 431542 and Sham + Tunica + Apo.

Figure IV. Systolic blood pressure (SBP) (A) and cardiac hypertrophy index (mg/kg) (B) measured in all groups, n=10. Representative heart sections stained with sirius-red and quantitative data in all groups, n=6 (C). NADPH oxidase activity in aorta (D) and MRA (E) from all groups n=8. *P<0.05 for Sham + Tunica vs. Sham, Sham + Tunica + Tudca.

Figure V. Endothelium-dependent relaxation in response to ACh in aorta (A) and MRA (D) from mice injected with or without Tunica and treated with and without Tudca, n=5. Endothelium-dependent relaxation in response to ACh in aorta (B, C) and MRA (E, F) injected with or without Tunica and treated with and without apocynin (Apo), L-NAME and/or Tudca, n=5. *P<0.05 for Sham + Tunica vs. Sham, Sham + Tunica + Tudca.
Figure VI. Representative pictures showing expression of CHOP in aorta (A) and MRA (B) from sham mice injected with or without Tunicamycin (Tunica) and treated with or without Tudca, n=4. Arrows indicate CHOP in the nucleus in aorta and MRA.