Endoplasmatic Reticulum Stress Contributes to Aortic Stiffening via Proapoptotic and Fibrotic Signaling Mechanisms

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Abstract—Vascular smooth muscle cell apoptosis and collagen synthesis contribute to aortic stiffening. A cellular signaling mechanism contributing to apoptotic and fibrotic events is endoplasmic reticulum (ER) stress. In this study, we tested the hypothesis that induction of ER stress in a normotensive rat would cause profibrotic and apoptotic signaling, thereby contributing to aortic stiffening. Furthermore, we hypothesized that inhibition of ER stress in an angiotensin II (Ang II) model of hypertension would improve aortic stiffening. Induction of ER stress with tunicamycin in normotensive Sprague-Dawley rats (10 μg/kg per day, osmotic pump, 28 days) caused an increase in systolic blood pressure (mm Hg; 160±5) compared with vehicle-treated (127±3) or tunicamycin-treated rats that were cotreated with ER stress inhibitor 4-phenylbutyric acid (100 mg/kg per day, 28 days, [124±6]). There was an increase in aortic apoptosis (fold; 3.0±0.3), collagen content (1.4±0.1), and fibrosis (2.0±0.1) in the tunicamycin-treated rats compared with vehicle-treated rats. Inhibition of ER stress in male Sprague-Dawley rats given Ang II (60 ng/min, osmotic pump, 28 days) and treated with either tauroursodeoxycholic acid or phenylbutyric acid (100 mg/kg per day, IP, 28 days) led to a 20 mm Hg decrease in blood pressure with either inhibitor compared with Ang II treatment alone. Aortic apoptosis, increased collagen content, and fibrosis in Ang II–treated rats were attenuated with ER stress inhibition. We conclude that ER stress is a new signaling mechanism that contributes to aortic stiffening via promoting apoptosis and fibrosis.

Key Words: apoptosis ■ fibrosis ■ muscle, smooth, vascular ■ vascular stiffness

Aortic stiffening is associated with increased vascular smooth muscle cell (VSMC) proliferation, migration, and apoptosis, as well as increased fibrosis. Although cell proliferation and migration have been extensively studied, apoptosis is becoming recognized as playing a major role in vascular stiffening. Apoptosis within the aortic wall is critical in determining aortic structure, and although beneficial in the early stages of stiffening, it later becomes detrimental. An interplay exists between apoptotic VSMCs and collagen synthesis in which apoptotic VSMCs have been shown to promote collagen synthesis. Increased collagen deposition and synthesis are critical contributors to aortic fibrosis. Recent evidence suggests ER stress is involved in cardiac damage via an increase in apoptosis and fibrosis in hypertensive mice. Indeed, in osteoblasts and gingival fibroblasts, ER stress has been shown to trigger collagen synthesis; however, it is unknown whether ER stress can increase VSMC collagen synthesis, thereby contributing to aortic fibrosis.

Therefore, the hypothesis of this study was ER stress contributes to aortic stiffening via increasing VSMC apoptosis and collagen synthesis. To elucidate the role of ER stress, a drug that induces ER stress, tunicamycin (TM), should cause a profibrotic-like phenotype by increasing aortic VSMC apoptosis and collagen synthesis. Consequently, inhibition of ER stress through the use of chemical chaperones, tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (PBA) that work by increasing ER folding capacity in an angiotensin

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II (Ang II) model of hypertension, will attenuate apoptosis and collagen synthesis, and improve aortic function.

Methods

Experimental Animals

Male Sprague-Dawley rats (12 weeks old, Harlan Laboratories) were used in these studies. They were maintained on a 12:12 hour light-dark cycle with both rat chow and water ad libitum. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were reviewed and approved by the Institutional Animal Care and Use Committee of the Georgia Regents University.

Rats were anesthetized via nose cone for minipump implantation with isoflurane at an initial concentration of 5% and then maintained at 2.5% in 100% oxygen. The anesthesia was verified by toe pinch and noting the absence of any physical movement. Osmotic minipumps (28 days, model 2004, Alzet Co) were implanted subcutaneously below the neck. For pharmacological induction of ER stress, animals were divided into 3 groups: control group receiving sham surgery (n=9); angiotensin II group receiving (Ang II 60 ng/min, osmotic pump) and tauroursodeoxycholic acid buffered saline (250 μL/day, IP, n=9); or injections of 4-phenylbutyric acid (PBA, 100 mg/kg per day, IP, 28 days), and these doses were chosen based on previous reports. For pharmacological inhibition of ER stress in vivo, animals were divided into 4 groups: control group receiving sham surgery and injections of saline (vehicle, IP, 28 days; n=10); and 3 groups receiving TM (10 μg/kg per day, 28 days, osmotic pump, n=11) and saline (250 μL, IP, 28 days), or injections of 4-phenylbutyric acid (PBA, 100 mg/kg per day, IP, 28 days), and these doses were chosen based on previous reports. For pharmacological inhibition of ER stress in a model of Ang II-induced hypertension, animals were divided into 4 groups: control group receiving sham surgery (n=9); angiotensin II group receiving (Ang II 60 ng/min, osmotic pump) and phosphate buffered saline (250 μL, IP, 28 days, n=9); angiotensin II group receiving (Ang II 60 ng/min, osmotic pump) and tauroursodeoxycholic acid (TUDCA, 100 μg/kg per day, IP, n=9); and angiotensin II group receiving (Ang II 60 ng/min, osmotic pump) and PBA (100 mg/kg per day, IP, n=9) doses that have been reported in the literature to decrease ER stress.

For detailed methods on blood pressure measurements, vascular functionality, Western blot, immunohistochemical studies, serum creatinine measurements, and chemicals, please see the online-only Data Supplement.

Data Analysis

The data are shown as mean±standard error of the mean, and n represents the number of rats used in the experiments. Contractions are noted as changes in force (mN) from baseline. Relaxation is expressed as percentage change from the phenylephrine (PE)-contracted levels. Concentration–response curves were fitted using a nonlinear regression program (GraphPad Prism 5.0; GraphPad Software Inc, San Diego, CA). The length–tension curve was fitted by a linear equation, the slope relates directly to the stiffness. Statistical analysis was performed using 2-way analysis of variance with Bonferroni post hoc analysis to compare the responses between all groups. P values of <0.05 were taken as being statistically significant.

Results

Effect of ER Stress Induction on Blood Pressure

At the end of the treatment, the rats treated with TM had a lower body weight (grams; 315±11) compared with the vehicle (397±4) or TM-treated rats given PBA (398±4.5). Serum creatinine levels were increased in the TM-treated rats (mg/dL; 2.0±0.1) compared with vehicle or TM-treated rats given PBA (0.78±0.2; 0.64±0.1; respectively). The systolic blood pressure was elevated in the TM-treated rats (mmHg; 160±5) compared with vehicle-treated (127±3) or TM rats treated with PBA (124±6).

Effect of ER Stress Induction on ER Stress and Apoptotic Marker Expression in the Aorta

We observed increased expression of ER stress markers: 78 kDa glucose–regulated protein (GRP78), an ER protein chaperone and CCAAT/-enhancer–binding protein homologous protein (CHOP), a proapoptotic transcription factor in the aorta from TM-treated rats compared with the vehicle-treated animals or TM-treated rats given PBA (Figure 1A). Furthermore, prolonged ER stress is widely linked to apoptosis and, in the aorta of TM-treated animals, there was increased expression of cleaved Caspase 12 and Caspase 3, and this was abolished when TM-treated animals were given PBA (Figure 1B).

Effect of ER Stress Induction on Aortic Apoptosis, Fibrosis, and Collagen Content in the Aorta

There was an increase in the number of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive nuclei from aortic sections of the TM-treated rats compared with vehicle-treated rats, which was prevented when TM-treated rats received PBA (Figure 2A). Interestingly, TM treatment caused an increase in collagen content and fibrosis in the aortic sections compared with vehicle-treated rats, which was prevented with PBA administration (Figure 2B and 2C). Subsequently, there was an increase in matrix metalloproteinase-2 (MMP-2) activity in the aorta of the TM-treated rats compared with vehicle or TM-treated rats given PBA (Figure S1 in the online-only data supplement).

Effect of ER Stress Inhibition on Aortic Contraction, Relaxation, and Compliance

To assess ER stress induction on functional changes in the aorta vascular contractility, relaxation and compliance were assessed.
Maximum contraction to PE was reduced in aortic segments from TM-treated animals (mN; 16±1.2) compared with vehicle (24±2.3), and this was prevented with PBA treatment (22±2.1; Figure 3A). Maximum relaxation to acetylcholine (ACh) in aortic segments from TM-treated rats was impaired (% relaxation; 31.5±4.5) compared with vehicle-treated rats (8.1±6.3) and TM-treated rats receiving PBA (11.1±5.2; Figure 3B). As a measurement of aortic compliance, a length–tension curve was performed. A leftward shift in the maximum tension development after increasing the length of the aortic ring was seen in the aorta from TM-treated rats (mN; 84.4±3.5) compared with vehicle-treated rats (61.2±1.1) rats, which was attenuated when TM-treated rats were given PBA (68.5±2.7; Figure 3C).

Effect of ER Stress Inhibition on Blood Pressure in the Ang II–Induced Hypertensive Rat

No significant differences were observed in body weight among groups (grams; vehicle, 407±8; Ang II, 390±8; Ang II TUDCA, 392±7; Ang II PBA, 396±5). Serum creatinine levels were increased in the Ang II–treated rats (mg/dL; 1.79±0.1) compared with vehicle-treated, TUDCA, or PBA-treated Ang II rats (0.77±0.06; 0.61±0.1; 0.78±0.1; respectively). The systolic blood pressure was elevated in the Ang II–treated group (mm Hg; 186.5±3) compared with vehicle-treated group (125±5). Additionally, a decrease in systolic blood pressure was seen in the TUDCA (165±3)-treated and PBA (167±4)-treated Ang II rats.

Effect of ER Stress Inhibition on ER Stress and Apoptotic Marker Expression in the Ang II–Induced Hypertensive Rats

Increased expression of ER stress markers, GRP78 and CHOP, in the aorta from Ang II alone–treated rats was attenuated in the aorta from Ang II groups receiving either TUDCA or PBA (Figure 4A). Increased expression of aortic cleaved Caspase 12 and Caspase 3 in the Ang II alone–treated rats was abolished in aorta from the Ang II rats treated with either TUDCA or PBA (Figure 4B).

Figure 2. Induction of endoplasmic reticulum (ER) stress increased Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive nuclei, collagen content, and fibrosis in the aorta. A, Top, Representative TUNEL staining of aortic sections (100× magnification). Bottom, Analysis of positive TUNEL-stained cells per field of view. B, Top, Representative Sirius Red staining of aortic sections (40× magnification). Bottom, Analysis of % Sirius Red stain per total area. C, Top, Representative Masson’s Trichrome staining of aortic sections (40x magnification). Bottom, Analysis of percentage of Masson’s Trichrome stain per total area. *P<0.05 vs vehicle-treated rats. n=4 to 10.

Figure 3. Induction of endoplasmic reticulum stress decreases vasoconstriction, impairs relaxation, and causes loss of compliance in the aorta. Concentration–response curves were performed in aortic rings to A, phenylephrine (PE, 1 nmol/L–10 μmol/L) or B, acetylcholine (ACh, 1 nmol/L–10 μmol/L). C, A length–tension curve was performed in aortic rings. *P<0.05 vs vehicle-treated rats. n=4 to 8.
Effect of ER Stress Inhibition on Aortic Apoptosis, Fibrosis, and Collagen Content in the Ang II–Induced Hypertensive Rats

There was an increase in the number of TUNEL-positive nuclei from aortic sections of the Ang II alone–treated rats compared with vehicle-treated rats (Figure 5A). Treatment with either TUDCA or PBA significantly decreased the number of TUNEL-positive nuclei in Ang II–infused rats. As depicted in Figure 5B and 5C, Ang II caused an increase in aortic collagen content and fibrosis compared with vehicle-treated rats, which was attenuated in the aorta from Ang II rats treated with either TUDCA or PBA. There was an increase in MMP-2 activity in the aorta from Ang II–treated rats compared with sham, which was prevented by treatment with either TUDCA or PBA (Figure S2).

Effect of ER Stress Inhibition on Aortic Contraction, Relaxation, and Compliance

Maximum contraction to PE was enhanced in aortic segments from Ang II alone–treated rats (mN; 34.9±3.1) compared with vehicle (27.2±1.6) and normalized in the Ang II rats treated with either TUDCA (26.9±2.2) or PBA (24.3±1.9; Figure 6A).
Maximum relaxation to ACh in aortic segments from Ang II alone–treated rats (% relaxation; 33.2±2.5) was significantly reduced compared with sham rats (4.9±4.1, Figure 6B). Treatment with either TUDCA (13.6±3.8) or PBA (21.6±3.6) improved ACh-induced vascular relaxation. A shift to the left in maximum tension development was seen in the Ang II alone–treated rats (E_max mN; 95.6±6.6) compared with sham (60.0±4.5) rats, which was attenuated in the Ang II rats treated with either TUDCA (77.8±3.1) or PBA (70.1±3.8; Figure 6C).

Discussion

The purpose of this study was to investigate the role of ER stress in the signaling mechanisms that occur in the aorta, which leads to VSMC apoptosis and fibrosis. We observed that (1) ER stress induction with TM leads to elevated blood pressure in rats, (2) TM induced increased aortic ER stress, apoptosis, collagen deposition, and fibrosis and impairment of vascular function, (3) ER stress occurs in the aorta of Ang II–induced hypertensive rats, and (4) ER stress inhibition decreases blood pressure, aortic apoptosis, collagen content, and fibrosis, whereas improving vascular function in an Ang II model of hypertension.

The administration of TM led to an elevation in blood pressure; moreover, the systemic treatment with chemical chaperones TUDCA or PBA decreased blood pressure in the Ang II hypertensive rat model. This presents a question of how these chemicals are acting systemically to cause changes in blood pressure. When directly infused into the subfornical organ, Ang II or ER stress inducer thapsigargin can cause ER stress, increased sympathetic outflow and hypertension, which could be prevented with TUDCA.16 Therefore, the systemic treatments in the present study may be acting directly through the subfornical organ to alter blood pressure. Furthermore, the role of the kidneys during the development of hypertension has identified and characterized by heightened nephron damage and tubular apoptosis. Rats treated with TM had higher serum creatinine levels, a characteristic of impaired renal function compared with sham or TM plus PBA–treated rats. The Ang II–treated rats receiving either TUDCA or PBA had normalized levels of serum creatinine compared with Ang II alone–treated rats, suggesting an attenuation of renal dysfunction in these rats. In this case, the systemic treatments could be altering kidney function and therefore affecting blood pressure. Lowering blood pressure could have consequences on aortic stiffening; however, in the spontaneously hypertensive rat, minoxidil, a vasodilatory agent that lowered blood pressure did not ameliorate changes in the collagen content in the aorta of the spontaneously hypertensive rat, suggesting that prevention of vascular fibrosis may be independent of blood pressure–lowering effects.

ER stress–mediated apoptosis of VSMCs contributes to the disease pathology of many cardiovascular diseases.17,18 After transverse aortic constriction in mice, prolonged ER stress in cardiomyocytes initiated apoptotic signaling that leads to heart failure19 and, in human coronary arteries, increased ER stress and VSMC apoptosis contribute to plaque vulnerability associated with acute coronary syndrome.20 However, the role of ER stress–induced VSMC apoptosis and how it might contribute to aortic stiffening are still unclear. In this study, prolonged treatment with ER stress inducer, TM, caused increased aortic ER stress, apoptotic proteins, and aortic TUNEL–positive nuclei. Several studies have reported that Ang II causes VSMC apoptosis and as a consequence can lead to vascular dysfunction, stiffening, and fibrosis.21 Here, we found that the Ang II–induced hypertensive rat has increased aortic ER stress marker expression, proapoptotic proteins, and TUNEL–positive nuclei, and cotreatment with chemical chaperones, TUDCA, and PBA prevented this from occurring.

During hypertension, changes in the composition of the aortic wall through alterations in extracellular matrix proteins lead to vascular fibrosis and dysfunction.22,23 Interestingly, mice with genetic defects in ER chaperone proteins exhibit impaired collagen synthesis.24 These findings corroborate our findings that after induction of ER stress aortic collagen content, fibrosis and MMP-2 activity were increased. MMPs contribute to vascular fibrosis through the degradation of extracellular matrix proteins and, during Ang II–induced hypertension, MMP-2 has been demonstrated to be a major player.25,26 ER stress inhibition in the Ang II–induced hypertensive rat with either TUDCA or PBA attenuated Ang II–induced increases in aortic collagen levels, collagen content, and fibrosis, as well as MMP-2 activity. Similar studies found that chemical chaperones can decrease collagen deposition and cardiac fibrosis after pressure-overload,27 isoproterenol treatment,28 and in Ang II–infused mice.7

Aortic stiffening, associated enhanced contractility of the vascular smooth muscle, endothelial dysfunction, and a loss of vessel compliance, is an important contributor to heart failure during aging or hypertension.29 Induction of ER stress led to impaired vascular function, as evidenced by decreased contraction to PE, reduced relaxation to ACh, and loss of compliance in the aorta from TM–treated rats. In contrast, the enhanced PE–induced contraction, reduced relaxation to ACh, and loss of compliance were attenuated in the aorta from Ang II rats treated with either TUDCA or PBA compared with Ang II alone–treated rats, demonstrating that inhibition of ER stress improves vascular function.
Perspectives
Aortic stiffening is a negative predictor of negative cardiovascular mortality. During aortic stiffening, changes in vascular smooth muscle cell function and death play an important role. The results suggest that the ER stress response in the aorta could be a new mechanism through which hypertensive stimuli, such as Ang II, mediate aortic stiffening. This study demonstrates that, in the aorta, ER stress activates proapoptotic and profibrotic cellular signaling pathways. Through the elucidation of a signaling pathway linked to aortic apoptosis and fibrosis, a novel mechanism-based therapy can be developed for the treatment of cardiovascular diseases.

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Disclosures
None.

References

Novelty and Significance

What Is New?
- Endoplasmic reticulum stress led to increased vascular smooth muscle cell apoptosis and fibrosis in the aorta.
- Endoplasmic reticulum stress inhibition has beneficial outcomes on aortic stiffening and hypertension.

What Is Relevant?
- Aortic stiffening is an important target during hypertension, and endoplasmic reticulum stress may contribute as a new target for the development of new therapies.

Summary
Induction of endoplasmic reticulum stress leads to increased aortic apoptosis, fibrosis, and stiffening. Ang II causes an increase in pro-apoptotic and fibrotic signaling in the aorta, contributing to stiffening. Administration of inhibitors of endoplasmic reticulum stress in the Ang II-induced hypertensive rat decreased blood pressure and improved aorta function.
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Supplemental Methods

Blood pressure measurement
Systolic blood pressure was measured in nonanesthetized animals by tail cuff plethysmography one day before the end of treatment using a RTBP1001 blood pressure system (Kent Scientific Corporation). An average of the systolic blood pressure from 10 cycles was taken from each animal and then averaged within group.

Vascular Function and Compliance Studies
Animals were euthanized by exsanguination under anesthesia, verified by negative responses to toe-pinches, with isoflurane (5% initial followed by maintenance at 2.5% in 100% oxygen) and the thoracic aorta was rapidly excised and placed in ice-cold physiological salt solution (PSS) containing (mmol/L): NaCl, 130; NaHCO₃, 14.9; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄·7H₂O, 1.18; CaCl₂·2H₂O, 1.56; EDTA, 0.026; glucose, 5.5. In ice-cold PSS, aortic segments were carefully dissected and cleaned of adventitial tissue and cut into 2mM segment rings. The rings were then mounted onto an isometric Mulvany-Halpern myograph (model 610 DMT-USA, Marietta, GA) and recorded by a PowerLab 8/SP data acquisition system (ADInstruments LTD, Colorado Springs, CO) in 37°C PSS with constant bubbling of a 95% O₂ and 5% CO₂ gaseous mixture to maintain the pH at around 7.4-7.5. Aortic rings were then placed at passive force of 30 mN, a tension chosen after optimization with length-tension analysis and allowed to equilibrate for 45 minutes.

Stimulation of aortic rings with 120 mmol/L KCl solution was used to assess integrity of rings. The presence of endothelium was assessed by contracting the rings with phenylephrine (PE, 1 µmol/L) followed by acetylcholine (ACh; 10 µmol/L). Concentration-response curves to PE and ACh were performed (1nmol/L to 100 µmol/L) to evaluate vascular contractility and relaxation.

To assess aortic compliance rings (3/treatment group, averaged) were given an initial pre-stretch from zero tension to a stretch of 3 mm in increments of 500 µm at 2 minute intervals. Following the pre-stretch the aortic rings were allowed to equilibrate at zero tension for 15 minutes. A length-tension curve was then performed by increasing the micrometer of the myograph in 500 µm increments every 2 minutes starting from zero tension until the vessel snapped or no further changes in tension were observed.

Western blot analysis:
Proteins (20 µg) were extracted from the thoracic aorta from all experimental groups and separated by electrophoresis on a 10% poly acrylamide gel containing sodium-dodecyl sulfate gel and then transferred onto nitrocellulose membrane. The membranes were then blocked in a 5%- nonfat dry milk in tris-buffered saline solution with Tween 20 (TBS-T) to block any non-specific binding sites for 1 hours at room temperature. Membranes were incubated overnight at
4°C in primary antibodies diluted in a 5% bovine serum albumin solution in TBS-T. The antibodies used for Western blot analysis are as follows: Caspase 3 (1:1000), Caspase 12 (1:500) GRP78 (1:1000) and PDI (1:3000) were from Cell signaling technologies and CHOP (1:200), was from Santa Cruz Biotechnologies.

**Histology**
Aortic ring segments (2mM) were placed in a 4% paraformaldehyde fixing solution (24hrs, 4°C) and then placed in a 70% ethanol solution (4°C). The rings were then paraffin-embedded (5 µM sections) and stained with Masson’s Trichrome stain for fibrosis assessment, Sirius red (0.1% in picric acid, Electron Microscope Services) for collagen content assessment and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL, R & D systems) for apoptosis assessment. Images (5 per ring) for the aortic sections were captured with a 40x objective lens using an Olympus BX60 microscope. Quantitative assessments were performed using ImageJ software (NIH).

**Gelatin Zymography**
To assess MMP-2 activity, 5 µg each of protein from all groups was loaded on an SDS-PAGE gel (0.1% gelatin) and proteins were separated using electrophoresis. The gel was then wash (2.5% Triton X-100, 2 times, 20 minutes). The gels were incubated in zymogram development buffer (1X, 23 hours, 37°C, Biorad) with gentle shaking. Following development the gels were stained with Comassie Brilliant Blue stain (overnight, room temperature, Bio-Rad) with gentle shaking. The gels were destained using Comassie Destain Solution (4 hours, room temperature, Bio-Rad) with gentle shaking. Recombinant MMP-2 standard was run as a positive control. The gels were then digitized and band intensity was quantified by UnScan it software.

**Serum Creatinine Measurement**
Serum was collected from the abdominal aorta from all animals at the end of the treatment period. Blood samples were allowed to clot for 30 minutes at room temperature and then centrifuged for 15 minutes (2,000 x g). The serum layer was then carefully removed and flash frozes in liquid nitrogen and stores at -80°C until ready for testing. Serum creatinine levels were measured according to manufacturer’s specification using a creatinine (serum) assay kit (Cayman Chemical).

**Chemicals**
4-Phenylbutric acid, acetylcholine chloride, phenylephrine hydrochloride and tunicamycin, were purchased from Sigma Aldrich. Angiotensin II was purchase from Phoenix Pharmaceuticals Inc. Tauroursodeoxycholic Acid was purchased from Calbiochem/EMD.
**Supplemental Data**

**S1:** ER stress induction increases MMP2 activity in the aorta. *Top,* Representative image of gelatin zymograph analysis of MMP2 activity. *Bottom,* Densitometry analysis. * p < 0.05 vs. Vehicle (VEH)-treated rats. n = 4-10.

**S2:** ER stress inhibition decreases MMP2 activity in the aorta. *Top,* Representative image of gelatin zymograph analysis of MMP2 activity from all 4 groups. *Bottom,* Densitometry analysis. * P < 0.05 vs. Vehicle (VEH)-treated rats. n = 6-8.