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ORIGINAL RESEARCH

Endoplasmic Reticulum Chemical Chaperone 3-Hydroxy-2-Naphthoic Acid Reduces Angiotensin II-Induced Vascular Remodeling and Hypertension In Vivo and Protein Synthesis In Vitro

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BACKGROUND: Investigations into alternative treatments for hypertension are necessary because current treatments cannot fully reduce the risk for the development of cardiovascular diseases. Chronic activation of unfolded protein response attributable to the endoplasmic reticulum stress has been proposed as a potential therapeutic target for hypertension and associated vascular remodeling. Triggered by the accumulation of misfolded proteins, chronic unfolded protein response leads to downstream signaling of cellular inflammation and dysfunction. Here, we have tested our hypothesis that a novel chemical chaperone, 3-hydroxy-2-naphthoic acid (3HNA) can attenuate angiotensin II (AngII)-induced vascular remodeling and hypertension.

METHODS AND RESULTS: Mice were infused with AngII for 2 weeks to induce vascular remodeling and hypertension with or without 3HNA treatment. We found that injections of 3HNA prevented hypertension and increase in heart weight body weight ratio induced by AngII infusion. Histological assessment revealed that 3HNA treatment prevented vascular medial thickening as well as perivascular fibrosis in response to AngII infusion. In cultured vascular smooth muscle cells, 3HNA attenuated enhancement in protein synthesis induced by AngII. In vascular adventitial fibroblasts, 3HNA prevented induction of unfolded protein response markers.

CONCLUSIONS: We present evidence that a chemical chaperone 3HNA prevents vascular remodeling and hypertension in mice with AngII infusion, and 3HNA further prevents increase in protein synthesis in AngII-stimulated vascular smooth muscle cells. Using 3HNA may represent a novel therapy for hypertension with multiple benefits by preserving protein homeostasis under cardiovascular stress.

Key Words: angiotensin II ■ ER stress ■ hypertension ■ vascular fibrosis

Hypertension is a major risk factor for cardiovascular diseases.[1] Interventions against the renin angiotensin system are mainstream anti-hypertensive treatments with evidenced cardiovascular disease protection.[2] However, ideally treated patients with hypertension still show significant risk for cardiovascular disease development.[3] Alternative drugs to reduce pathological vascular remodeling in hypertension may aid in breaking the cycle of increased blood pressure leading to structural changes in arteries, thus preventing end-organ damage.[4]

Vascular remodeling associated with hypertension includes vascular medial thickening and adventitial fibrosis.[5] Angiotensin II (AngII), a main peptide hormone

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CLINICAL PERSPECTIVE

What Is New?

- We demonstrated that a novel small molecule chemical chaperone 3-hydroxy-2-naphthoic acid prevented hypertension and cardiovascular remodeling, including vascular medial thickening, adventitial fibrosis, and cardiac hypertrophy, in mice with angiotensin II infusion.
- We found that 3-hydroxy-2-naphthoic acid attenuated enhancement in protein synthesis in vascular smooth muscle cells exposed to angiotensin II in vitro.

What Are the Clinical Implications?

- Our study demonstrating the cardiovascular protective effects of 3-hydroxy-2-naphthoic acid in an animal model of hypertension suggests a potential clinical application of the compound in patients with hypertension.
- Our findings suggest a novel strategy to use an endoplasmic reticulum stress inhibitor 3-hydroxy-2-naphthoic acid against hypertensive cardiovascular remodeling by halting enhanced protein synthesis.

Nonstandard Abbreviation and Acronyms

3HNA	3-hydroxy-2-naphthoic acid
4-PBA	4-phenylbutyric acid
AngII	angiotensin II
ATF6	activated transcription factor 6
CHOP	C/EBP-homologous protein
eIF2α	eukaryotic initiation factor 2 α
ER	endoplasmic reticulum
GRP78	78-kDa glucose-regulated protein
IRE1α	inositol requiring enzyme 1α
UPR	unfolded protein response
VSMC	vascular smooth muscle cells

in the renin angiotensin system, has been shown to regulate a myriad of signaling pathways that contribute to hypertension and associated vascular remodeling by activating its receptor angiotensin type-1 (AT1).[6] One such regulatory pathway that is disturbed in hypertension is cellular protein homeostasis "proteostasis," which requires the coordination of protein folding, degradation, and trafficking.[7] Disturbances in the proteostasis lead to accumulation of misfolded or unfolded proteins in endoplasmic reticulum (ER), termed ER stress.[8] An ER chaperone, GRP78 (78-kDa glucose-regulated protein), is a master ER stress sensor. Under ER stress, GRP78 releases its specific binding partners, IRE1α (inositol requiring enzyme 1α), PERK (ER-resident eukaryotic initiation factor 2 α [eIF2α] kinase), and ATF6 (activated transcription factor 6) to activate 3 unfolded protein response (UPR) pathways. Activated IRE1α uses its ribonuclease domain to cleave transcription factor XBP1 (X-Box binding protein 1) into its active form (XBP1s) to induce ER chaperones, protein folding enzymes, and ER-associated degradation components. Activated PERK phosphorylates and inactivates eIF2α and thereby attenuates translational initiation of general mRNAs. Released and subsequently cleaved ATF6 also acts as a nuclear transcriptional factor and activates ER stress response element present in the promoters of genes encoding ER chaperones.[9] While UPR is an evolutionally conserved adaptive stress response, chronic UPR activation leads to inflammation and cellular dysfunction.[8,9]

Chronic UPR activation has been found in animal models of hypertension.[10–13] Rodents infused with AngII displayed increase in blood pressure and cardiovascular remodeling that were associated with UPR. Chemical chaperones, 4-phenylbutyric acid (4-PBA) and taurine-conjugated ursodeoxycholic acid, blunted these pathological cardiovascular responses.[10,12,13] In spontaneous hypertensive rats, 4-PBA and taurine-conjugated ursodeoxycholic acid also attenuated hypertension.[11] These data support the hypothesis that ER stress promotes hypertension and associated cardiovascular pathology. However, 4-PBA and taurine-conjugated ursodeoxycholic acid have pleiotropic effects beyond mitigation of ER stress.[14,15] Further exploration of drug therapies to target ER stress pathway may aid in better treatment in hypertension.

Recently, using a chemical library screening, 3-hydroxy-2-naphthoic acid (3HNA) has been identified as a chemical chaperone that can mitigate ER stress and UPR.[16] In a diabetic mouse model, 3HNA treatment significantly improved insulin sensitivity that was associated with reduced UPR in the liver.[17] Accordingly, by using AngII treatment in mice to recapitulate hypertensive vascular remodeling, we have tested the hypothesis that 3HNA treatment will mitigate vascular remodeling and hypertension in mice induced by 2 weeks infusion of AngII. We also explored the potential molecular mechanisms using vascular cells in culture.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. All experimental mice were bred in an Association for Assessment and Accreditation of Laboratory Animal Care international accredited animal facility at the Temple University under National Institutes

of Health guidelines for care and use of laboratory animals and housed with free access to standard rodent chow and water. All animal procedures were performed with prior approval of the Temple University Institutional Animal Care and Use Committee (No. 4940). All animal studies complied with the Animal Research: Reporting of In Vivo Experiments guidelines.

Animal Protocols

Eight- to ten-week-old male C57BL/6 mice were bred inhouse or obtained from the Jackson Laboratory. In all experimental models, mice were randomized (8 animals per group), and researchers were blinded to the treatments. For AngII-induced animal model of hypertension, mice were anesthetized by isoflurane inhalation, and osmotic mini-pumps (Alzet micro-osmotic mini pump) containing AngII were implanted subcutaneously into the dorsum of the mouse. AngII (4006473, Bachem) was delivered at a rate of 1 μg/kg per minute for 2 weeks; 3HNA sodium salt (H0284, TCI America) was solubilized in saline at 50 mg/mL, and 3HNA sodium salt[17] (100 mg/kg per day) or vehicle (saline 50 μL) were administered by intraperitoneal injection, every other day for 14 days, starting 90 minutes before pump implantation. It has been demonstrated that half-maximal inhibitory concentration of 3HNA attenuating UPR is 40 to 50 times lower than 4-PBA,[17] which was used at 1 g/kg per day with intraperitoneal injection in mice with AngII infusion.[10] Accordingly, a pilot experiment comparing the effects of 3HNA sodium salt (100 or 50 mg/kg every other day intraperitoneal injection) with vehicle saline on vascular remodeling upon AngII infusion for 2 weeks was performed. The 100 mg/kg protocol was chosen based on the consistent suppression of aortic medial thickening.

Blood Pressure Assessment

Blood pressure was assessed by a tail-cuff method as previously described.[18] Briefly, mice were acclimated in restraints for 5 minutes on heated platform before recordings using CODA Noninvasive Blood Pressure System (Kent Scientific); 15 to 25 recordings were obtained, and of those, the last 10 recordings were averaged for each mouse. Measurements with SD values >30 were excluded. Measurements were taken for at least 5 days before osmotic minipump implantation to familiarize mice to device, followed by day 0, 8, and 14 recordings.

Histology

Masson trichrome staining and Sirius Red staining were performed with aorta and heart samples as previously reported.[19] In brief, extracted aortas and hearts were fixed in 10% formalin overnight, dehydrated in 70% ethanol at 4 °C for 24 hours, then processed and embedded in wax. For cardiac histology, serial cross-sections (5 μm) were stained in Sirius Red (Electron Microscopy Sciences, Hatfield PA). To assess cardiac perivascular fibrosis, the value of fibrosis area was subtracted from vessel area and divided by the true area of the vessel. True area was calculated by outer medial perimeter[2] divided by 4π. The value generated was the area of the vessel in true circular form. To assess cardiac vascular hypertrophy, the value of vascular medial area was divided by the true area of the vessel. In total, 3 vessels per animal (4–6 animals per condition) were analyzed. To evaluate medial hypertrophy of aortas, Masson trichrome staining was performed to distinguish medial area from adventitia in thoracic aortas. Briefly, after deparaffinization and rehydration, sections were incubated with Bouin fluid for 1 hour at 56 °C. Sections were washed 3 times in distilled water for 3 minutes per wash and then incubated with hematoxylin and eosin solution for 7.5 minutes followed by washing in distilled water for 30 seconds. Sections were then incubated with Biebrich Scarlet-Acid Fuchsin solution for 5 minutes. After incubation with phosphotungstic-phophomolybdic acid solution for 5 minutes, sections were stained with Aniline Blue for 5 minutes. They were then dehydrated and penetrated with ethanol and xylene. Medial thickness was quantified by measurements of 4 randomly selected locations per slide; 3 representative vascular images were analyzed per sample.

Immunofluorescent Staining

Immunofluorescent staining was performed as previously reported.[20] The formalin fixed 5-μm thick aorta sections were cut via microtome onto charged slides that were then incubated at 37 °C overnight. To perform immunofluorescent staining, slides were washed in xylene to remove wax, then rehydrated in decreasing levels of ethanol water, boiled in sodium citrate followed by PBS washing. Blocking and permeabilization was performed simultaneously with 5% normal goat serum and 1% BSA in PBS containing 0.3% TritonX-100 for 1 hour at room temperature, followed by washes in PBS with 0.03% TritonX-100. Aorta sections were then incubated with 1:500 dilution of primary antibodies (IRE1α phosphorylated at Ser724, MBS9610512, MyBioSource, San Diego, CA, and α-smooth muscle actin, NBP2-33006, Novus Biologicals, Centennial, CO) overnight at 4 °C. Upon washing in PBS with 0.03% Triton-X100, the sections were incubated in AlexaFluor 488 anti-rabbit immunoglobulin G (IgG) (Invitrogen, A11034) or AlexaFluor 594 anti-mouse IgG (Invitrogen, A11005) diluted 1:1000 at room temperature for 2 hours and subsequently

incubated with autofluorescent quenching reagent TrueVIEW (Vector Laboratories SP-8400) to reduce autofluorescence. Slides were mounted with antifade medium Vectashield containing DAPI (Vector Laboratories H-1200-10), dried overnight, and images were obtained at 40× on EVOS2100 microscope. Mean fluorescence intensity of IRE1α phosphorylated at Ser724 merged with α-smooth muscle actin positive area was quantified in ImageJ.

Cell Culture

Vascular smooth muscle cells (VSMCs) were prepared from thoracic aorta of male Sprague–Dawley rats using the explant method as previously described.[21] VSMCs were subcultured in DMEM with the addition of 10% fetal bovine serum, penicillin, and streptomycin. Primary cultured adventitial fibroblasts were obtained from 12-week-old male Sprague–Dawley rat thoracic aortas with the modified explant method.[22] In brief, aorta sediments are placed on culture plate with adventitia side to the bottom plastic in the presence of growth media, 100 mg/dL D-glucose DMEM with 1 mmol/L sodium pyruvate (10-014-CV; Corning, Glendale, AZ), 100 IU penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum supplementation (970685-085; VWR International, Radnor, PA). Upon microscopic confirmation of fibroblasts, tissue was removed. We confirmed a fibroblast marker, S100A4 expression in these fibroblasts.[22] Subcultured fibroblasts were supplemented with growth media twice a week and passaged every week. Cells from passage 3 to 8 were used for the experiments. For experiments, subconfluent (80%–90% area occupancy with original seeding number of 1 to 2×10^5 cells per well) cells in 6-well plates were starved with serum free media for 2 days. For the experimental treatments, the media was replaced with fresh media with 0.5 to 1 mmol/L 3HNA[16] (TCI America H0281) dissolved in DMSO (0.1% final) or vehicle DMSO (0.1% final) 30 minutes before the stimulation with tunicamycin (Research Product International T22500, 2 μg/mL) for the duration of 24 hours. To avoid phenotypic alteration by freezing and stocking, VSMCs and fibroblasts were replaced with a new isolation every 2 to 3 months, and passages 2 to 9 were used in the experiments.

Immunoblotting

Protein samples from cell lysates (≈50 μg) were subjected to SDS-PAGE gel electrophoresis, transferred to a nitrocellulose membrane, and subjected to immunoblotting as previously described.[23] Briefly, 10% acrylamide/bisacrylamide resolving gels were used and transferred onto 0.45-μm nitrocellulose membranes overnight at 30 V. Membranes were blocked for 1 hour in 5% non-fat dry milk in tris-buffered saline. Protein detection was performed using primary antibodies incubated in tris-buffered saline-Tween 20 (0.01% Tween 20) overnight at 4 °C with gentle rocking. Secondary antibody incubation (100 ng/mL, IRDye 800CW Goat anti-Rabbit IgG 926-32211; LI-COR, Lincoln, NE, IRDye 680RD Goat anti-Rabbit IgG 926-68071; LI-COR, IRDye 800CW Goat anti-Mouse IgG 926-32210; LI-COR, and IRDye 680RD Goat anti-Mouse IgG 926-68070; LI-COR) followed for ≈1 hour at room temperature with subsequent detection and analyses with the LI-COR Odyssey system and the Image Studio software (LI-COR). Stripping and reprobing were performed to detect expression of distinct proteins in each independent experiment. For the stripping, the membrane was incubated with a buffer containing 100 mmol/L 2-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris–HCl pH6.7 at 50 °C for 30 minutes with agitation. For normalization, we have used GAPDH expression as well as β-actin expression. For comparison, protein expression in cells treated with vehicle was set as 1.

Protein Synthesis

Analysis of new protein synthesis in VSMCs was performed using an adapted Surface Sensing of Translation assay protocol as previously described.[24] Briefly, VSMCs were serum starved for 2 days to synchronize cell cycle and subsequently incubated with 500 μmol/L 3HNA (TCI America H0281) dissolved in DMSO (0.1% final) for 30 minutes before AngII stimulation for 6 hours. At conclusion of the experiment, cells were pulsed with 5 μmol/L puromycin (P7255, Sigma) for 30 minutes. Cells were washed twice with PBS, harvested, and cell lysates prepared for immunoblotting with a puromycin antibody diluted at 1:10000 (MABE343, Sigma). GAPDH expression in the cell lysate in each independent experiment was evaluated by reprobing of the membrane with GAPDH antibody and used for normalization.

Primary Antibodies

Primary antibodies used in this study were ATF6 (100 ng/mL, NBP1-40256, Novus Biologicals), CHOP (C/EBP-homologous protein) (1:10.000, 2895, Cell Signaling, Danvers, MA), GRP78 (70 ng/mL, 11587-1-AP, Proteintech, Rosemont, IL), IRE1α phosphorylated at Ser724 (200 ng/mL, MBS9610512, MyBioSource, San Diego, CA), IRE1α (40 ng/mL, sc-390960, Santa Cruz Biotechnology, Dallas, TX), eIF2α phosphorylated at Ser51 (1:10.000, 3398, Cell Signaling), eIF2α (1:10.000, 2103, Cell Signaling), GAPDH (100 ng/mL, MAB374; Sigma-Aldrich, St. Louis, MO), and β-actin (6.1 ng/mL, 4970; Cell Signaling Technology).

Statistical Analysis

The values for each parameter within a group are expressed as the mean ± SEM. Shapiro–Wilk test was used to confirm normality. For comparisons between 4 groups, statistical significance was assessed using 1-way ANOVA followed by Tukey correction. For comparisons between the groups with repeated measurements, 2-way ANOVA (time and treatment including interaction term) was performed using Prism 9.4.1 (GraphPad, San Diego, CA). A P value of <0.05 was considered statistically significant. All n numbers including in vitro data indicated in the Figures or Figure legends were independent repetitions.

RESULTS

AngII-Induced Hypertension and Cardiac Hypertrophy Were Reduced by 3HNA

Figure 1A illustrates chemical structure of 3HNA and the animal protocol. When comparing mice infused with AngII, 3HNA reduced systolic blood pressure (145.0±8.6 versus 119.4±7.6 mm Hg, P<0.01) in C57/BL6 mice on day 14 compared with saline-injected mice (Figure 1B). Cardiac hypertrophy assessed via heart weight to body weight ratio was increased by AngII relative to the control mice (P<0.05), which was attenuated by 3HNA (Figure 1C). Echocardiography also indicated that AngII treatment increased interventricular septal thickness, which was attenuated by 3HNA (Table S1). Therefore, similar to a past report using chemical chaperones such as 4-PBA and taurine-conjugated ursodeoxycholic acid in AngII-infused mice,[10] 3HNA appears to attenuate AngII-induced hypertension and cardiac hypertrophy.

Vascular Hypertrophy and Fibrosis Were Attenuated by 3HNA

Using Masson trichrome staining to assess aortic medial thickness, it was observed that AngII induced significant medial thickening compared with control mice (60.22 versus 38 μm, P<0.0001), which was partially reduced with 3HNA treatment (60.22 versus 48.5 μm, P<0.05) (Figure 2A and 2B). Cardiac vessel histology was analyzed via Sirius Red staining of the hearts (Figure 2C). The coronary artery medial area relative to total vascular area was increased by AngII in control saline-treated mice, which was attenuated by 3HNA treatment (Figure 2D). Furthermore, the adventitial fibrotic area surrounding vessels was significantly increased by AngII infusion and reduced with 3HNA treatment (Figure 2E). These findings are consistent with our prior finding in AngII-infused mice treated with 4-PBA.[13]

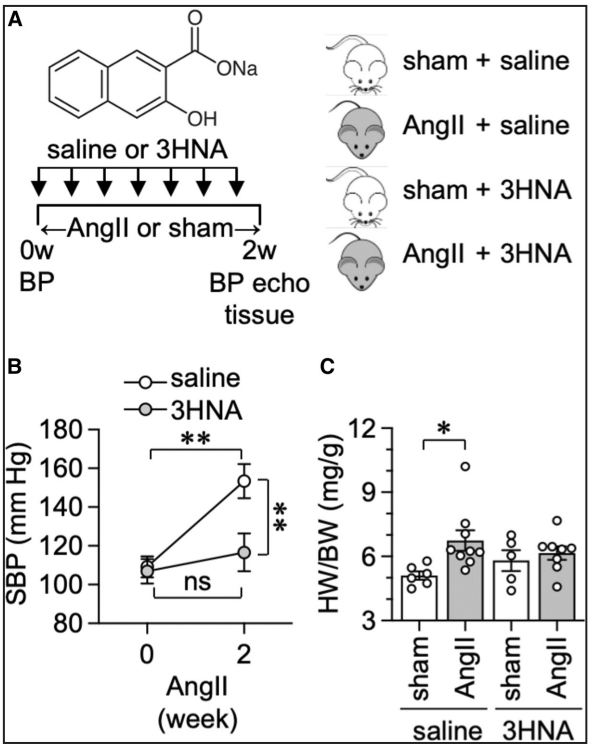


Figure 1. Animal protocol and analyses in blood pressure and cardiac hypertrophy.
A, Three-hydroxy-2-naphthoic acid chemical structure and animal protocol. At 8 weeks of age, male C57BL6 mice were infused with 1 μg/kg per minute angiotensin II via osmotic mini-pump for 2 weeks or sham-operated for osmotic mini-pump implantation. These mice were either given 100 mg/kg 3HNA injection or vehicle (saline 50 μL) injection every other day for the 2 weeks. **B**, Blood pressure analysis. Tail cuff plethysmography was performed to evaluate development of hypertension. Data (mean±SEM) showed systolic blood pressure at day 14. Significance was indicated as **P<0.01 compared with the corresponding controls. **C**, Heart weight body weight ratio was measured for indication of cardiac hypertrophy upon 2-week treatments. Bar graph showed mean±SEM. 3HNA indicates 3-hydroxy-2-naphthoic acid; AngII, angiotensin II; BP, blood pressure; and SBP, systolic blood pressure. *P<0.05 compared with the sham control.

Potential Reduction of Aortic UPR by 3HNA

Aortas collected from mice injected with either saline or 3HNA with and without AngII infusion were subjected to immunofluorescent analysis. While statistically insignificant, there was a trend suggesting induction of a UPR marker, IRE1α phosphorylation by AngII, and reduction by 3HNA (Figure S1).

Chemical Chaperone 3HNA Attenuated ER Stress in Vascular Fibroblasts

To test whether 3HNA acts as a small molecule chaperone in vascular cells, vascular adventitial fibroblasts were exposed to an ER stressor tunicamycin in

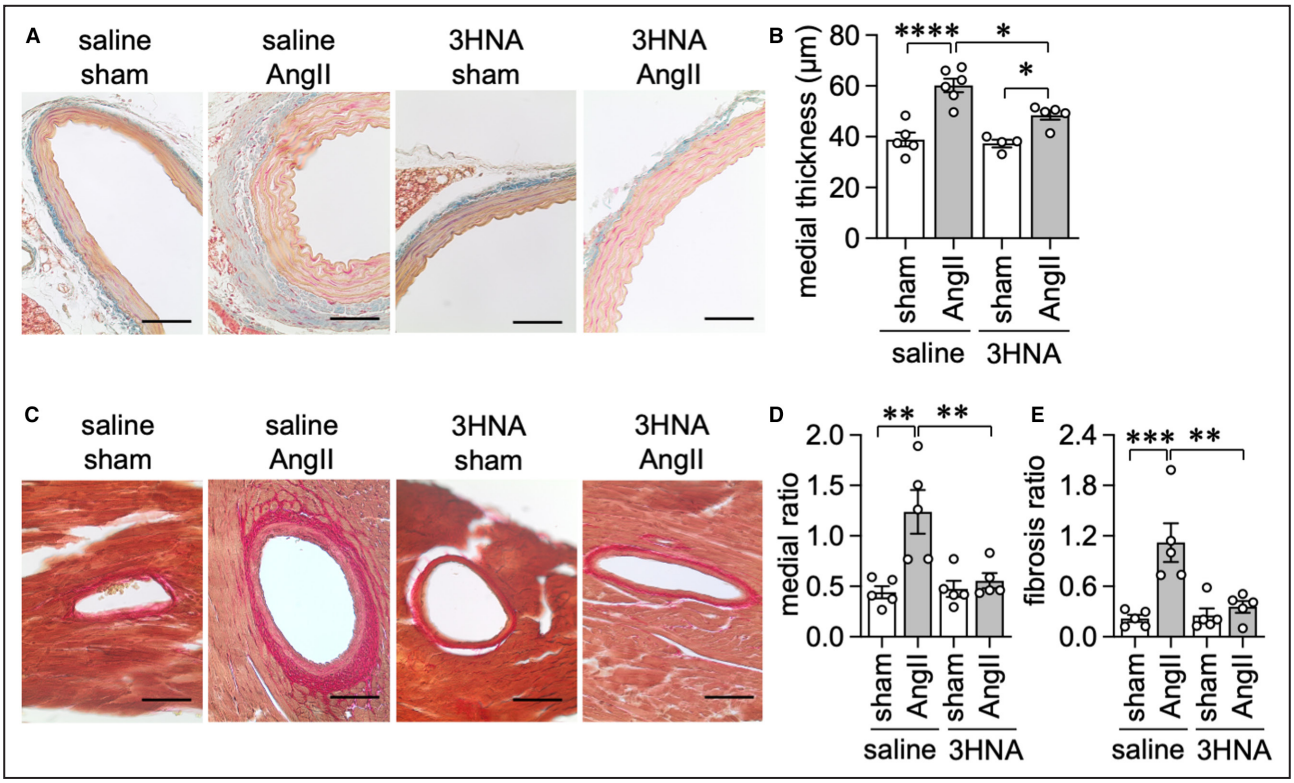


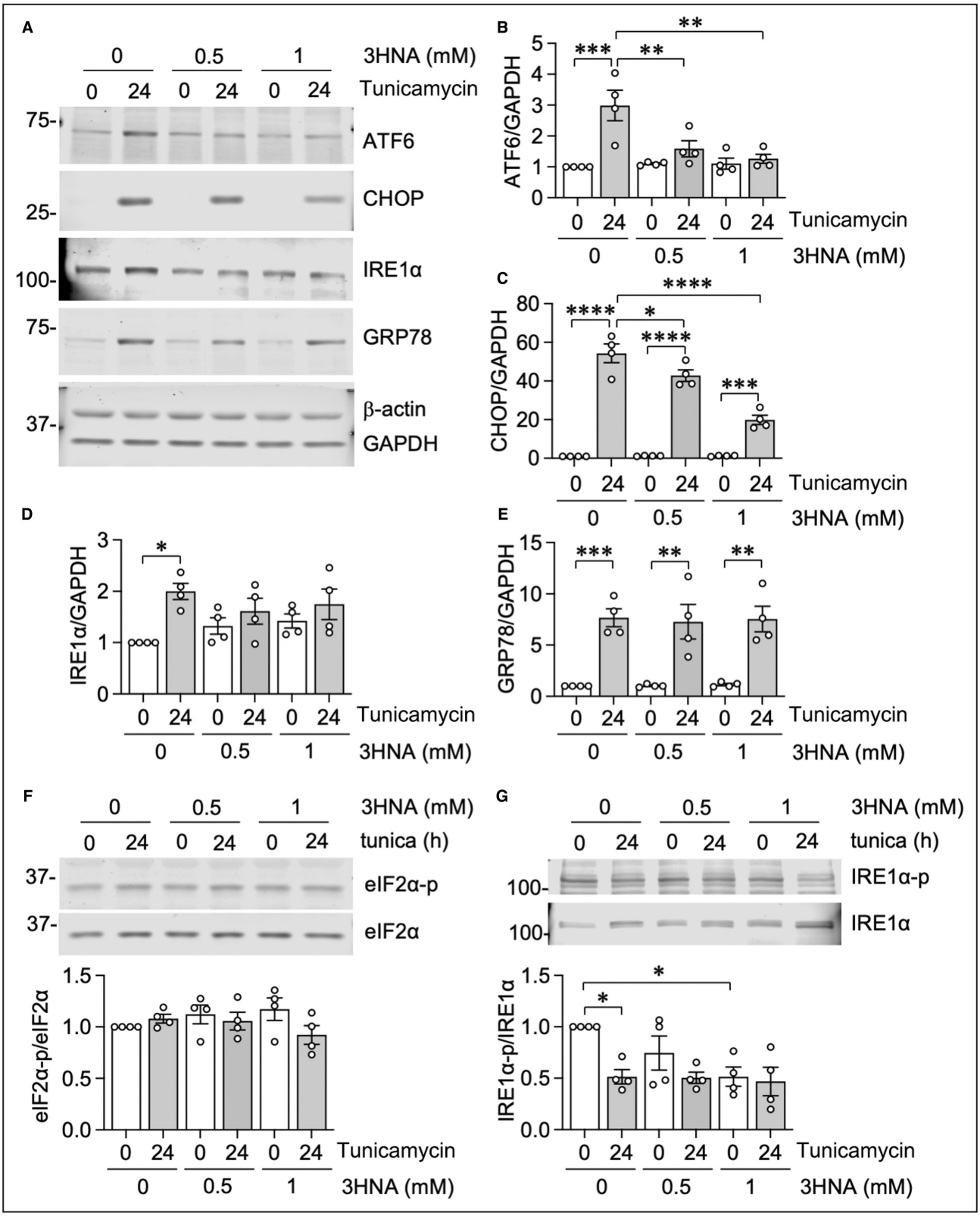
Figure 2. Angiotensin II-induced vascular remodeling was attenuated by treatment with 3-hydroxy-2-naphthoic acid.
Mice treated with 100 mg/kg 3-hydroxy-2-naphthoic acid every other day or vehicle saline were infused with 1 μg/kg per minute angiotensin II via osmotic mini-pump for 2 weeks or sham-operated as a control. **A**, Upon Masson trichrome staining, medial thickness of ascending aortas was compared. **B**, Aortic medial thickness was quantified. **C** through **E**, Upon Sirius Red staining (**C**), ratio of medial area per true vessel area (**D**) and ratio of Sirius Red positive perivascular fibrosis area per true vessel area (**E**) were calculated to indicate vascular medial thickening and perivascular fibrosis, respectively. Bar graph showed mean±SEM. 3HNA indicates 3-hydroxy-2-naphthoic acid; and AngII, angiotensin II. Significance was indicated as ***P*<0.01, ****P*<0.001, and *****P*<0.0001. Scale bar indicates 50 μm.

the presence or absence of 3HNA (Figure 3A); 0.5 to 1 mmol/L 3HNA almost completely attenuated induction of cleaved ATF6 by tunicamycin (Figure 3B and Figure S2A), and 0.5 to 1 mmol/L 3HNA partially attenuated induction of CHOP by tunicamycin (Figure 3C and Figure S2B). In addition, when data were normalized with GAPDH, induction of IRE1α by tunicamycin was not observed in the presence of 3HNA (Figure 3D and Figure S2C). In contrast, induction of GRP78 was unaltered by 3HNA (Figure 3E and Figure S2D). Phosphorylation ratio of eIF2α was unchanged regardless of the treatment at the time point tested (Figure 3F). Phosphorylation ratio of IRE1α was decreased by tunicamycin because of increase in total protein expression; 1 mmol/L but not 0.5 mmol/L 3HNA reduced basal phosphorylation ratio of IRE1α (Figure 3G).

Chemical Chaperone 3HNA Attenuated Protein Synthesis in VSMCs

To test for the effect of 3HNA in cellular protein homeostasis, the rate of protein synthesis in VSMCs was assessed with the Surface Sensing of Translation assay, which evaluates puromycin incorporation into the newly synthesized proteins. Although 3HNA did not alter the basal rate of protein synthesis, the enhancement of

Figure 3. Induction of unfolded protein response markers was attenuated by treatment with 3-hydroxy-2-naphthoic acid in vascular adventitial fibroblasts.
Serum starved adventitial fibroblasts pretreated with 3-hydroxy-2-naphthoic acid-containing media or control vehicle-containing media for 1 hour were treated with 1 μg/mL tunicamycin to induce endoplasmic reticulum stress and unfolded protein response. Immunoblotting was performed with the antibodies as indicated. **A** through **E**, Expression of unfolded protein response markers was examined and quantified. **F** and **G**, Phosphorylation of unfolded protein response markers was examined and quantified. Bar graph showed mean±SEM. 3HNA indicates 3-hydroxy-2-naphthoic acid; ATF6, activated transcription factor 6; CHOP, C/EBP-homologous protein; IRE1α, inositol requiring enzyme 1α; GRP78, 78-kDa glucose-regulated protein; eIF2α-p, eukaryotic initiation factor 2α phosphorylated at Ser51; and IRE1α-p, IRE1α phosphorylated at Ser724. Significance was indicated as **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001.



protein synthesis induced by AngII was not observed in the presence of 3HNA (Figure 4A and 4B). Therefore, in addition to acting as a chemical chaperone, 3HNA seems to prevent stress-mediated protein synthesis in VSMCs.

DISCUSSION

Because existing chemical chaperones such as 4-PBA appear to have pleiotropic effects beyond maintaining ER homeostasis, a chemical library screening

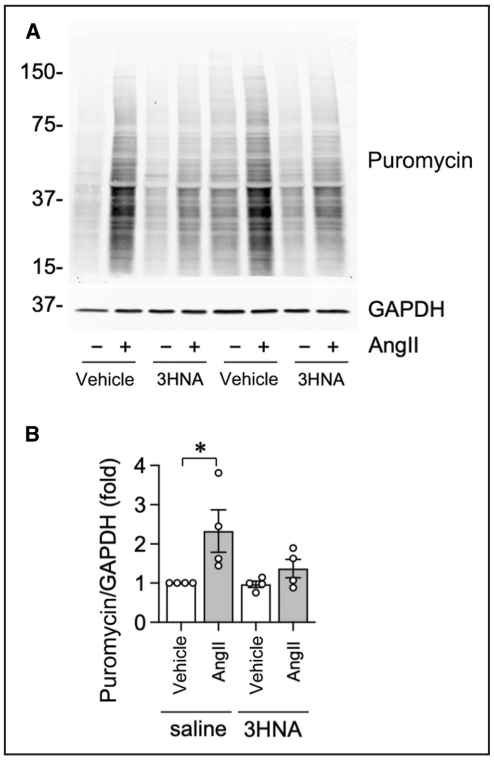


Figure 4. Angiotensin II-induced protein synthesis in vascular smooth muscle cells was attenuated by treatment with 3-hydroxy-2-naphthoic acid.
Serum starved vascular smooth muscle cells pretreated with 0.5 mmol/L 3-hydroxy-2-naphthoic acid-containing media or control vehicle-containing media for 1 hour were treated with 100 nmol/L angiotensin II for 6 hours to enhance protein synthesis. Puromycin was added to the media at a 6-hour time point for 30 minutes. **A**, Immunoblotting was performed with antibodies as indicated. **B**, Puromycin total intensity normalized with GAPDH intensity. Bar graph showed mean±SEM. 3HNA indicates 3-hydroxy-2-naphthoic acid; and AngII, angiotensin II. Significance was indicated as *P<0.05.

has been used to identify more selective ER chaperones. To date, only a limited number of such compounds were identified, including 3HNA,[16] azoramide,[25] IBT21[26] and APC655.[27] Except for IBT21, antidiabetic effects of these compounds have been reported.[17,25,27] However, none of these new chemical chaperones were tested in a model of cardiovascular disease.

Here, several beneficial effects of 3HNA were observed, including suppression of vascular medial thickening, perivascular fibrosis, and hypertension induced by AngII infusion. Rodents have 2 types of AT1 receptor, AT1a and AT1b, in which AT1a activation appears essential for hypertension as well as associated cardiovascular pathology in this AngII infusion model.[28,29] Subsequent studies using conditional AT1a silenced mice further indicated requirements for VSMC[30] and renal proximal tubule[31] AT1a for hypertension and fibroblast[32] AT1a for vascular medial thickening. Attenuation of VSMC ER stress via GRP78 overexpression reduced leukocyte adhesion induced by AngII[21]; 3HNA could reduce vascular fibroblast UPR in this study. These findings suggest that ER stress and UPR in key AT1 expressing cell types such as VSMCs and adventitial fibroblasts are the presumable targets by which 3HNA mitigated hypertension pathology.

Although the potential off-target effects of 3HNA remain to be investigated, it seems important to note cell type differences on the UPR marker response to 3HNA. In tunicamycin-treated HepG2 cells, 3HNA markedly attenuated phosphorylation of IRE1α and induction of CHOP and XBP1s.[16,17] While inhibition of CHOP was in common, 3HNA preferentially attenuated ATF6 induction among other UPR markers in vascular fibroblasts. Less renal fibrosis was reported in mice lacking ATF6.[33] However, cardiac damage in response to ischemic injury was exaggerated in ATF6 knockout mice.[34] In addition, AngII-induced hypertension and cardiac remodeling were significantly mitigated in CHOP knockout mice.[35] Therefore, the role by which each UPR cascade modulates cardiovascular pathology may be context dependent. It is interesting to explore further whether 3HNA prevented AngII-induced vascular remodeling via its inhibition in fibroblast or VSMC ATF6 or CHOP using corresponding conditional knockout animals.

In the present study, we observed that 3HNA also attenuated enhancement in protein synthesis. It has been reported that a protein synthesis inhibitor cycloheximide attenuated UPR in VSMCs and other cell types.[36,37] While we have shown significant translational inhibition by cycloheximide using the puromycin assay in VSMCs,[24] no effect of 3HNA was observed in basal protein synthesis in this study. Therefore, it is unlikely that 3HNA primarily attenuates UPR via inhibition of protein synthesis. The molecular mechanism of the protein synthesis inhibition by 3HNA may involve suppression of UPR-responsive genes in the growth promoting cascade of AngII signal transduction such as induction of A disintegrin and metalloprotease 17 in VSMCs.[13]

Limitations of the study include lack of female animal experiments and only 1 kind of hypertension model used. In vitro AngII stimulation in VSMCs or fibroblasts could be included to confirm ER stress mitigation by 3HNA in closer conditions to hypertension.

CONCLUSIONS

The present study demonstrates the effectiveness of a novel chemical chaperone, 3HNA, in preventing cardiovascular remodeling in a widely used animal model

of hypertension with AngII infusion; 3HNA further attenuated ER stress in vascular fibroblasts and protein synthesis in VSMCs in response to AngII, suggesting multiple benefits of 3HNA by preserving protein homeostasis under cardiovascular stress.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Table S1
Figures S1–S2

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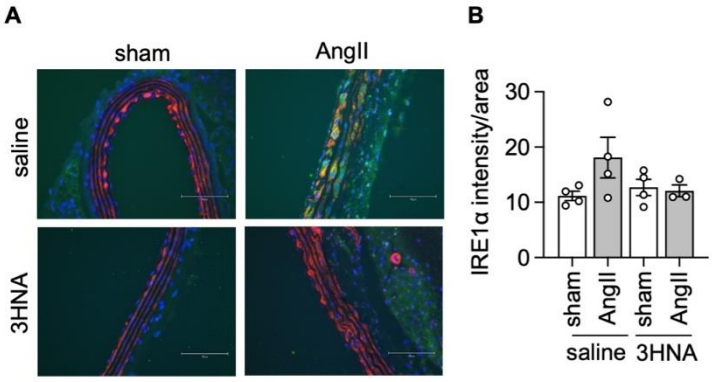
Supplemental Material

Table S1. **Echo cardiograph at 2 weeks**.

	sham saline	**AngII saline**	**sham 3HNA**	**AngII 3HNA**
IVSd (mm)	0.86 ± 0.07	1.27 ± 0.05*	0.99 ± 0.12	1.06 ± 0.10
IVSs (mm)	1.16 ± 0.15	1.77 ± 0.07*	1.35 ± 0.15	1.47 ± 0.12
LVDd (mm)	3.46 ± 0.14	2.78 ± 0.18	3.40 ± 0.34	3.07 ± 0.16
LVDs (mm)	2.42 ± 0.13	1.61 ± 0.20	2.40 ± 0.28	2.16 ± 0.15
LVPWd (mm)	1.02 ± 0.25	1.54 ± 0.18	1.01 ± 0.17	1.12 ± 0.12
LVPWs (mm)	1.30 ± 0.21	1.92 ± 0.20	1.29 ± 0.09	1.45 ± 0.10
EF (%)	66.29 ± 3.73	75.04 ± 5.72	63.53 ± 2.56	67.95 ± 3.10
FS (%)	35.99 ± 2.79	46.52 ± 5.38	33.69 ± 1.65	37.17 ± 2.51

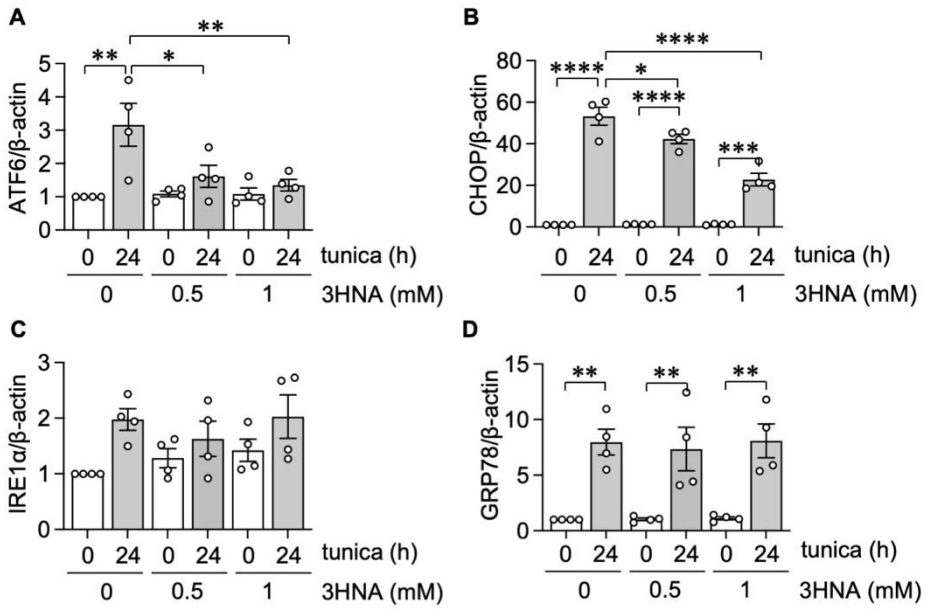
Echo cardiograph was performed to evaluate cardiac wall thickness after (2 weeks) the angiotensin II (AngII) infusion with 3-Hydoxy-2-Naphthoic Acid (3HNA) or control saline treatment. IVSd, interventricular septal thickness in diastole; IVSs, interventricular septal thickness in systole; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVPWd, left ventricular posterior wall thickness in diastole; LVPWs, left ventricular posterior wall thickness in systole; EF, ejection fraction; FS, fractional shortening. n=4 (sham saline), n=8 (AngII saline), n=3 (sham 3HNA), n=8 (AngII 3HNA). *$p<0.05$ compared with the sham saline control.

Figure S1. Effects of 3HNA and AngII on aortic IRE1α phosphorylation.



Mice treated with 100 mg/kg 3-Hydoxy-2-Naphthoic Acid (3HNA) every other day or vehicle saline were infused with 1 μg/kg/min angiotensin II (AngII) via osmotic mini-pump for 2 weeks or sham-operated as a control. **A.** Immunofluorescent staining was performed in aortic sections with antibodies against α-smooth muscle actin (α-SMA) (red) and Ser724 phosphorylated inositol requiring enzyme 1α (IRE1α) (green). **B.** Phosphorylated IRE1α intensity per medial area (colocalizing with α-SMA). 3~4 animals per group were analyzed as indicated with the intensity value averaged from 3 slides per animal. Bar graph showed mean±SEM. Scale bar indicates 75 μm.

Figure S2. Induction of UPR markers was attenuated by treatment with 3HNA in vascular adventitial fibroblasts (normalization with β-actin).



Serum starved adventitial fibroblasts pretreated with 3-Hydoxy-2-Naphthoic Acid (3HNA)-containing media or control vehicle-containing media for 1 hour were treated with 1 μg/mL tunicamycin (tunica) to induce endoplasmic reticulum stress and unfolded protein response (UPR). Immunoblotting was performed (Figure 3A) and expression of UPR markers were quantified. Bar graph showed mean±SEM. Significance was indicated as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. ATF6, activated transcription factor 6; CHOP, C/EBP-homologous protein; IRE1α, inositol requiring enzyme 1α; GRP78, 78-kDa glucose-regulated protein.