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Histone deacetylase 6 inhibitor tubastatin A attenuates angiotensin II-induced hypertension by preventing cystathionine γ-lyase protein degradation

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ABSTRACT

Cystathionine γ-lyase (CSEγ) is a hydrogen sulfide (H_2S)-producing enzyme. Endothelial H_2S production can mediate vasodilatory effects, contributing to the alleviation of hypertension (high blood pressure). Recent studies have suggested a role of histone deacetylase 6 (HDAC6) in hypertension, although its underlying mechanisms are poorly understood. Here, we addressed the potential regulation of CSEγ by HDAC6 in angiotensin II (AngII)-induced hypertension and its molecular details focusing on CSEγ posttranslational modification. Treatment of mice with a selective HDAC6 inhibitor tubastatin A (TubA) alleviated high blood pressure and vasoconstriction induced by AngII. Cotreatment of the aorta and human aortic endothelial cells with TubA recovered AngII-mediated decreased H_2S levels. AngII treatment upregulated HDAC6 mRNA and protein expression, but conversely downregulated CSEγ protein. Notably, potent HDAC6 inhibitors and HDAC6 siRNA as well as a proteasomal inhibitor increased CSEγ protein levels and blocked the downregulatory effect of AngII on CSEγ. In contrast, other HDAC isoforms-specific inhibitors and siRNAs did not show such blocking effects. Transfected CSEγ protein levels were also reciprocally regulated by AngII and TubA, and were reduced by wild-type, but not by deacetylase-deficient, HDAC6. Moreover, TubA significantly increased both protein stability and K73 acetylation level of CSEγ. Consistent with these results, AngII induced CSEγ ubiquitination and degradation, which was inhibited by TubA. Our results indicate that AngII promoted HDAC6-dependent deacetylation of CSEγ at K73 residue, leading to its ubiquitin-mediated proteolysis, which underlies AngII-induced hypertension. Overall, this study suggests that upregulation of CSEγ and H_2S through HDAC6 inhibition may be considered as a valid strategy for preventing the progression of hypertension.

Abbreviations: ACh, acetylcholine; AngII, angiotensin II; CHX, cycloheximide; CSEγ, cystathionine γ-lyase; HAEC, human aortic endothelial cells; HDAC6, histone deacetylase 6; H_2S, hydrogen sulfide; IP, immunoprecipitation; NO, nitric oxide; qRT-PCR, quantitative real-time reverse transcription PCR; SAHA, suberoylanilide hydroxamic acid; SNP, sodium nitroprusside; TSA, trichostatin A; TubA, tubastatin A

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1. Introduction

Hypertension, also termed high blood pressure, is the most common cause of the death and high prevalence of diseases of the elderly population. More than 1 billion people worldwide live with hypertension, with the associated medical costs increasing every year [1]. In particular, chronic hypertension is recognized as a high-risk factor for cardiovascular disorders including stroke and heart failure [2,3]. Numerous studies have identified that several factors including vasculature, sympathetic nervous system, renin-angiotensin-aldosterone system, and immune system are critically involved in the control of blood pressure [3–6]. However, specific roles of these factors in blood pressure regulation and pathophysiological mechanisms of hypertension are not yet fully appreciated.

Hydrogen sulfide (H_2S), one of several gasotransmitters such as nitric oxide (NO) and carbon monoxide, has emerged as a physiological vasodilator helping to reduce high blood pressure [7,8]. It has also been demonstrated that H_2S mediates anti-inflammatory and anti-oxidative responses as well as protein S-sulfhydration, and suppresses apoptotic cell death and leukocyte-endothelial cell adhesion [8–10]. Cystathionine γ-lyase (CSEγ) is considered to be the major enzyme responsible for H_2S generation in endothelial cells [11,12]. Genetic depletion of CSEγ results in marked hypertension and impaired endothelial function [11], and caused atherosclerosis [13]. Moreover, accumulating evidence suggests that CSEγ-dependent H_2S generation plays an important role in regulating blood pressure and endothelium-dependent vasodilation, thereby contributing to cardioprotection and maintenance of vascular function and integrity [7,11–15]. In addition, CSEγ undergoes crosstalk with endothelial NO synthase (eNOS) to produce NO, which functions as another inducer of vasculature relaxation [16].

Several studies have shown a role for histone deacetylase 6 (HDAC6) in the pathophysiology of hypertension-related vascular diseases. Apparently, HDAC6 inactivation via its inhibitors and/or gene knockdown ameliorates hypertension and exerts cardioprotective effects [17–20]. Thus, HDAC6 is considered as a promising target for drug development of cardiovascular diseases. HDAC6 belongs to the class IIb HDAC family and is distinct from other HDAC family members in that it mediates the protein deacetylation of non-histone substrates such as α-tubulin, heat shock protein 90, and cortactin, which are present in the cytoplasm [21–24].

Angiotensin II (AngII) constitutes a well-known inducer of hypertension and endothelial dysfunction [25,26]. Several studies have shown that HDAC6 participates in AngII-induced renal fibrosis, cardiac dysfunction, and skeletal muscle wasting [18,27]. A recent study reported that the upregulation of HDAC6 expression by an atherogenic factor, oxidized low-density lipoprotein, acted to inhibit CSEγ, resulting in defective vasorelaxation [28]. However, the molecular details regarding how HDAC6 regulates CSEγ remain unknown.

In this study, we addressed the potential regulation of CSEγ protein and H_2S levels by HDAC6 using aortic endothelial cells and mice that were challenged with AngII, as this process has yet to be examined. We also attempted to determine effects of tubastatin A (TubA), a highly selective inhibitor of HDAC6 [17,27,29], on AngII-induced changes in blood pressure and aortic vessel relaxation. Furthermore, we assessed whether HDAC6 utilizes a novel mechanism to modulate the protein stability of CSEγ, i.e., by mediating CSEγ protein deacetylation, which is linked to its proteasomal degradation in response to AngII.

2. Materials and methods

2.1. Chemicals

Most chemicals, including AngII, MG132, chloroquine, cycloheximide (CHX), acetylcholine (ACh), sodium nitroprusside (SNP), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from HyClone (Logan, UT, USA). Lipofectamine 2000, Lipofectamine RNAiMAX, Opti-MEM I, and TRIzol reagent were purchased from Thermo Fisher Scientific Life Sciences (Waltham, MA, USA). HDAC inhibitors including TubA, nicotinamide, Scriptaid, suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), and MGCD0103 were obtained from Selleckchem (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO).

2.2. AngII-infused hypertensive mice and TubA injection

The 11-week-old C57BL/6 N male mice were purchased from Orient Bio (Seongnam, Korea). The experimental procedures for the handling of mice were approved by the Laboratory Animal Research Center of Ajou University Medical Center (approval ID: 2016-0065). The hypertensive mouse model was generated following the previously described methods of AngII infusion using osmotic pumps [25–27]. After anesthesia with forane solution, micro-osmotic pumps (Alzet model 1004) were subcutaneously implanted into the back of the mice, allowing continuous delivery of AngII (1 mg kg^{-1} day^{-1}) for 4 weeks. For accurate comparison, control group mice also underwent the same surgery without the pump insertion. The AngII-treated groups were intraperitoneally injected with either phosphate-buffered saline or TubA (50 mg kg^{-1} day^{-1}).

2.3. Force tension myography

Myographic measurements were performed according to the previously described procedures [28]. In brief, the aorta was isolated from the hypertensive mice and placed in cold Krebs-Ringer bicarbonate buffer containing 99.0 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11.1 mM glucose, and 20 mM Na-HEPES (pH 7.35–7.45). The aorta was carefully cleaned of perivascular fat, cut into 2-mm long ring segments, and immersed in a bath filled with constantly oxygenated Krebs buffer at 37 °C without damage to the endothelium. Aortic rings were connected to a transducer and a micromanipulator and were passively stretched to an optimal resting tension using the micromanipulator. The isometric contractions of arterial rings were measured by using the wire myograph system (model 620 DMT, Danish Myo Technology, Aarhus, Denmark). The aortic rings were treated with 60 mM KCl and the passive stretch was repeated after a wash with Krebs buffer, and then equilibrated for 60 min. The vessels were pre-constricted with phenylephrine (1 μM) for 15 min. We then monitored dose-dependent (0.1 nM–10 μM) responses with ACh and then with SNP after the washes and re-equilibrium.

2.4. Blood pressure measurement

Systolic, diastolic, and mean blood pressure of the hypertensive mice were measured prior to and 2 or 4 weeks following insertion of AngII osmotic pumps by using the non-invasive CODA tail-cuff blood pressure monitor system (Kent Scientific, Torrington, CT, USA). The volume pressure recording cuff was placed on the tail and blood pressure measurements were performed on a heated platform set at 37 °C. The animals were trained in a holder every day for 2 weeks and maintained in a quiet and dark place. The average of ten readings was used for the analysis.

2.5. Cell cultures and treatments

Human aortic endothelial cells (HAEC) were purchased from ScienCell Research Laboratories (San Diego, CA, USA) and grown in an endothelial cell medium containing a growth supplement, 5% FBS, and penicillin/streptomycin according to the supplier's instructions. HEK293 cells purchased from the American Type Culture Collection (Manassas, VA, USA) were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. The cell cultures were maintained at

37 °C in a humidified atmosphere of 5% CO_2 and 95% air and were routinely subcultured at 2- or 3-day intervals. For sample preparations, equal numbers of cells were plated into culture dishes at a density of 5×10^4 cells/cm^2 overnight and treated with a 100 nM concentration of AngII for 12 h or for the indicated times. In the case of pretreatment with TubA (5 μM) or other HDAC inhibitors, cells were incubated in the presence and absence of inhibitor for 1 h prior to AngII treatment.

2.6. H_2S measurement

Levels of H_2S gas were determined by the agar trapping method according to the previously described protocols [30]. In brief, 1% agar layers containing zinc acetate (45 mM) and NaOH (3 M) were prepared in overturned T-25 flasks. Cells or aortas were placed into the opposite side of the agar layer and then incubated with AngII and/or TubA in the presence of cysteine (3 mM) for 12 h. After removal of culture media, H_2S trapped as zinc sulfide in zinc-agar layer was converted into methylene blue through sequential chemical reaction with 2 ml of *N,N*-dimethyl-*p*-phenylenediamine chloride (40 mM in 7.2 M HCl) and 0.4 ml of $FeCl_3$ (30 mM in 1.2 M HCl). Aliquots (0.2 ml) of the reaction mixtures were transferred into a 96-well and absorbance at 670 nm was measured using a microplate reader (model VMAX, Molecular Devices, San Jose, CA, USA). Similarly, a standard curve was obtained with serially diluted (up to 250 μM) sodium hydrosulfide (NaHS) as a H_2S donor without cells.

2.7. Antibodies and plasmids

Myc (#sc-40), β-actin (#sc-1616), and CSEγ (#sc-374249) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies to HDAC6 (#7558), HDAC2 (#5113), acetylated-lysine (#9441), and acetyl-α-tubulin (Lys40, #12152) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies to HDAC10 (#ab18971, Abcam, Cambridge, MA, USA), HA (#MMS-101R, Covance, Princeton, NJ, USA), FLAG (#F1804, Sigma-Aldrich), α-tubulin (#T5168, Sigma-Aldrich), and CSEγ (#12217-1-AP, Proteintech, Rosemont, IL, USA) were commercially purchased. Expression plasmids of pcDNA-HDAC6-FLAG (#30482), pcDNA-HDAC6.DC-FLAG (#30483), and pRK5-HA-ubiquitin (#17608) were purchased from Addgene (Cambridge, MA, USA). A plasmid of CSEγ-Myc (#RC231191) was obtained from OriGene Technologies (Rockville, MD, USA). The K73R mutation was introduced into the CSEγ-Myc template using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. All plasmids were purified using an EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA, USA).

2.8. Transfection and gene knockdown

HEK293 cells were transfected with expression plasmids of HDAC6, CSEγ, and/or ubiquitin, or corresponding empty vectors as controls using Lipofectamine 2000 for 1 day. For gene knockdown of HDACs, double-stranded small interfering RNA (siRNA) targeting HDAC2 (#sc-29345, Santa Cruz Biotechnology), HDAC6 (5′-AGA CCU AAU CGU GGG ACU GC-3′), HDAC10 (5′-UCA CUG CAC UUG GGA AGC UCC UGU A-3′), or an *AccuTarget* Negative Control siRNA (#SN-1002) from Bioneer (Daejeon, Korea) were mixed with Lipofectamine RNAiMAX in Opti-MEM I, and the mixtures were added to cells for 2 days. Similarly, CSEγ siRNA (5′-GGU UAU UUA UCC UGG GCU GUU-3′) from Bioneer was used for CSEγ knockdown.

2.9. Western blotting and immunoprecipitation (IP)

Cell lysate preparation, protein quantification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, and IP were performed as described previously [31,32]. The lysis buffer was freshly supplemented with *N*-ethylmaleimide (10 mM) for the detection of ubiquitinated CSEγ. For IP of endogenous CSEγ and transfected CSEγ-Myc, cell lysates (1.0–1.5 mg) were incubated with 5 μg of anti-CSEγ, anti-Myc mouse monoclonal antibody, or normal mouse IgG (Santa Cruz Biotechnology) as a negative control for 4 h at 4 °C. The immune complexes were captured with 25 μl of Protein A/G PLUS-Agarose IP reagent (Santa Cruz Biotechnology) for an additional 2 h, and washed 5 times with the lysis buffer.

Table 1
qRT-PCR primers used in this study.

Gene	Sequence
HDAC1	Forward, 5'-TCAAGCCGGTCATGTCCAAA-3'
	Reverse, 5'-CCTCCCAGCATCAGCATAGG-3'
HDAC2	Forward, 5'-TGAGATTCCCAATGAGTTGCCA-3'
	Reverse, 5'-GGGTTGCTGAGCTGTTCTGA-3'
HDAC3	Forward, 5'-TGTGATCGATTGGGCTGCTT-3'
	Reverse, 5'-GGTCCAGATACTGGCGTGAG-3'
HDAC4	Forward, 5'-AGGCTCAGACTTGCGAGAAC-3'
	Reverse, 5'-ATCTGGTCTCTTTTCGGCGG-3'
HDAC5	Forward, 5'-CAACTCACACCTCACTGCCT-3'
	Reverse, 5'-CACACGTTCACCCGTCACTA-3'
HDAC6	Forward, 5'-ATCCGGAGGGTCCTTATCGT-3'
	Reverse, 5'-GTGGTGGACCAGTTAGAGGC-3'
HDAC7	Forward, 5'-AGTCCTTAATGACCACCGAGC-3'
	Reverse, 5'-GGCTGACCTCTTGATCACCT-3'
HDAC8	Forward, 5'-GCTGCGGAACGGTTTTAAGC-3'
	Reverse, 5'-CATACTGGCCCGTTTGGGG-3'
HDAC9	Forward, 5'-CTTTGCCTCAGAGCACGTTG-3'
	Reverse, 5'-CGAAAGCAGTTTGTTCATGTGG-3'
HDAC10	Forward, 5'-AGAACTCCACACCCCAGCTA-3'
	Reverse, 5'-GGATGGCACTGCAACATCTTC-3'
GAPDH	Forward, 5'-AAAATCAAGTGGGGCGATGC-3'
	Reverse, 5'-AGGAGGCATTGCTGATGATCT-3'

2.10. Quantitative real-time PCR

cDNA was synthesized from total RNA (1 μg) purified using TRIzol reagent, and then quantitative real-time reverse transcription PCR (qRT-PCR) was performed on a Rotor-Gene 6000 thermocycler (Corbett Research, Sydney, Australia) using a KAPA SYBR FAST Universal 2X qRT-PCR Master Mix (Kapa Biosystems, Woburn, MA, USA) [33]. The specific primers of HDACs (Table 1) were obtained from Cosmo Genetech (Seoul, Korea). PCR reaction parameters were as follows: 1 cycle of 95 °C for 3 min, followed by 40 cycles each comprising three steps of 95 °C for 3 s, 55 °C for 15 s, and 72 °C for 30 s. All PCR samples were prepared in triplicate and the relative mRNA expression levels were normalized to GAPDH and determined by the $2^{-\Delta\Delta Ct}$ method.

2.11. Cell viability assay

Cell viability was measured using the EZ-CYTOX assay kit (Daeil Lab Service, Seoul, Republic of Korea) following the manufacturer's protocol. In brief, HAEC seeded into 96-well plates (10,000 cells/well) were incubated with the EZ-CYTOX reagent (20 μl) for 6 h, then absorbance of water-soluble tetrazolium salt formed was measured at 450 nm using a microplate reader (Molecular Devices, San Jose, CA, USA).

2.12. Statistical analysis

All experiments were performed independently at least three times with similar results. Band intensities of western blots were measured using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). Data shown in the graphs are presented as the means ± S.E.M. The statistical significance of the data was determined by one-way analysis of variance with multiple comparison tests using GraphPad Prism 7 software (La Jolla, CA, USA).

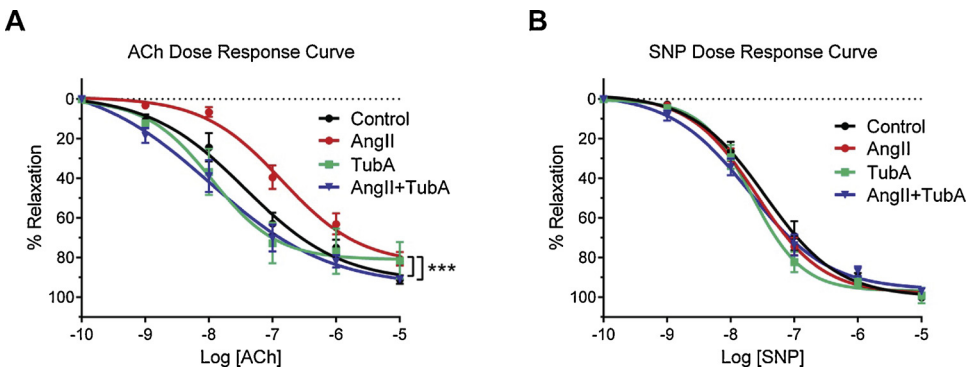

Fig. 1. Increased endothelium-dependent vasorelaxation by TubA in AngII-treated mice. Thoracic aortas were isolated from mice infused with AngII via osmotic pump and/or TubA via peritoneal injection for 4 weeks, then were processed for vascular reactivity measurements using wire myography. The dose-dependent responses to ACh (A) and SNP (B) were monitored in the indicated four mouse groups: control (n = 8), AngII (n = 8), TubA (n = 4), and AngII + TubA (n = 8). Three to four aortic rings per mouse were used. The group data were analyzed by logistic dose-response curves with four parameters using Graphpad Prism 7 software. The relaxation responses were prepared by nonlinear regression (curve fit) method and calculated as a percentage of tension after pre-construction using 1 μM phenylephrine. Statistical significance of the indicated data sets was determined by least square fit and extra sum-of-squares F test. Values represent the means ± SEM. AngII vs. control, ***$p < 0.001$. AngII vs. AngII + TubA, ***$p < 0.001$.

3. Results

3.1. Inhibition of HDAC6 by TubA alleviates AngII-induced vascular endothelial dysfunction

To evaluate the role of HDAC6 in AngII-induced vascular constriction, we monitored vascular reactivity using force tension myography in *ex vivo* aorta rings isolated from AngII- and/or TubA-treated mice. AngII and TubA were chronically infused for up to 4 weeks via osmotic pump and peritoneal injection, respectively. ACh stimulates endothelial NO production and is commonly used to characterize endothelial-dependent relaxation [28]. In the control group, ACh (0.1 nM–10 μM) dose-dependently induced the relaxation of aortic rings that were pre-constricted with 1 μM phenylephrine (Fig. 1A). The ACh-induced relaxation was significantly reduced in the aorta rings from the AngII-infused group (Fig. 1A), confirming the impairment of vascular endothelial function by AngII. Conversely, however, the presence of TubA, an HDAC6-specific inhibitor, blocked the AngII effects, leading to improved vasorelaxation (Fig. 1A). The maximal difference between AngII and AngII plus TubA groups was observed at the ACh concentration range from 10 to 100 nM. TubA alone also showed mild increases in the relaxation mediated by ACh compared to that of the control. In contrast, endothelium-independent relaxation of aorta rings, as assessed by dose-dependent responses to the exogenous NO donor SNP [28], was largely unaffected by AngII and/or TubA (Fig. 1B). These results supported that HDAC6 inhibition exhibits a potential role in suppressing the endothelium-dependent vasoconstriction induced by AngII.

3.2. HDAC6 inhibition by TubA antagonizes AngII-mediated effects on hypertension and H_2S production

To examine whether HDAC6 inhibition by TubA could modulate AngII-induced hypertension, we performed tail-cuff measurements of blood pressure in the *in vivo* AngII-induced hypertensive mice that were used for the myography measurements (Fig. 1). As expected, systolic (Fig. 2A), diastolic (Fig. 2B), and mean (Fig. 2C) blood pressure of mouse tails were elevated at 2 and 4 weeks after delivery of AngII. In contrast, the elevation in blood pressure mediated by AngII was returned to baseline levels in the mouse group co-administrated with TubA (Fig. 2A–C). The counterbalancing effect of TubA indicated that HDAC6 inhibition contributes to attenuate AngII-induced hypertension.

We next examined whether AngII and TubA could affect H_2S production based on the observation that H_2S serves as a vasorelaxant and exhibits a blood pressure-lowering effect [7,14]. We measured H_2S production by employing an agar trap method in combination with an *in situ* methylene blue assay that has been demonstrated to successfully and sensitively collect and detect H_2S generated from cultured cells [30]. We prepared isolated murine aortas and then treated these with AngII for 12 h. Our results showed that whereas H_2S levels were decreased by AngII in aorta samples, 1 h pretreatment with TubA abolished the AngII effect, leading to recovery of H_2S levels (Fig. 2D). We also obtained similar results with HAEC under the same experimental conditions (Fig. 2E). These results suggested that the antihypertensive activity of TubA is related to its effect on H_2S production. CSEγ is a major H_2S-producing enzyme. As control experiments, the effects of CSEγ knockdown or overexpression on H_2S production were tested by using the agar trap method. As expected, H_2S levels were decreased by siRNA-mediated CSEγ knockdown (Fig. 2F) and increased in the cells overexpressing CSEγ-Myc (Fig. 2G), supporting the specificity of the H_2S measurements.

3.3. AngII reciprocally regulates HDAC6 and CSEγ protein levels

We further examined the possibility that AngII might induce changes in HDAC6 and CSEγ protein expression levels in cultured HAEC. Notably, HDAC6 protein levels increased in a time-dependent manner up to 12 h after treatment with AngII (Fig. 3A, B). Conversely, AngII treatment showed time-dependent decreases in CSEγ protein levels under the same conditions (Fig. 3A, C). We also tested mouse aortas prepared in the same manner for the myographic measurements as described in Fig. 1. The opposite regulation of HDAC6 (Fig. 3D, E) and CSEγ (Fig. 3D, F) by AngII was also found in the aorta samples that were prepared 2 or 4 weeks after AngII treatment.

3.4. HDAC6 inhibitors enhance CSEγ protein levels

As HDAC6 and CSEγ proteins were differentially regulated by AngII, we addressed a potential causal relationship between the two protein changes. For this, we tested TubA and other chemicals such as Scriptaid and SAHA, which have been widely used as potent HDAC6 inhibitors [34–36], for their effects on CSEγ protein levels. Nicotinamide, an inhibitor of the structurally unrelated sirtuin family of HDACs [37], and MGCD0103, an inhibitor of class I HDAC [38], were included for comparison. Pretreatment of HAEC with TubA, Scriptaid, or SAHA inhibited the downregulation effect of AngII on CSEγ, resulting in relatively high CSEγ protein levels (Fig. 4A). In contrast, nicotinamide and MGCD0103 were much less effective in mediating such inhibitory effects than the tested HDAC6 inhibitors (Fig. 4A). Treatment of HAEC with TSA (a classical HDAC inhibitor) [36], SAHA, or Scriptaid alone also enhanced basal CSEγ protein levels (Fig. 4B). Similarly, protein levels of CSEγ increased in a dose-dependent manner after TubA treatment in HAEC (Fig. 4C). These results indicated that HDAC6 plays a role in negatively regulating CSEγ protein levels in resting and AngII-treated cells.

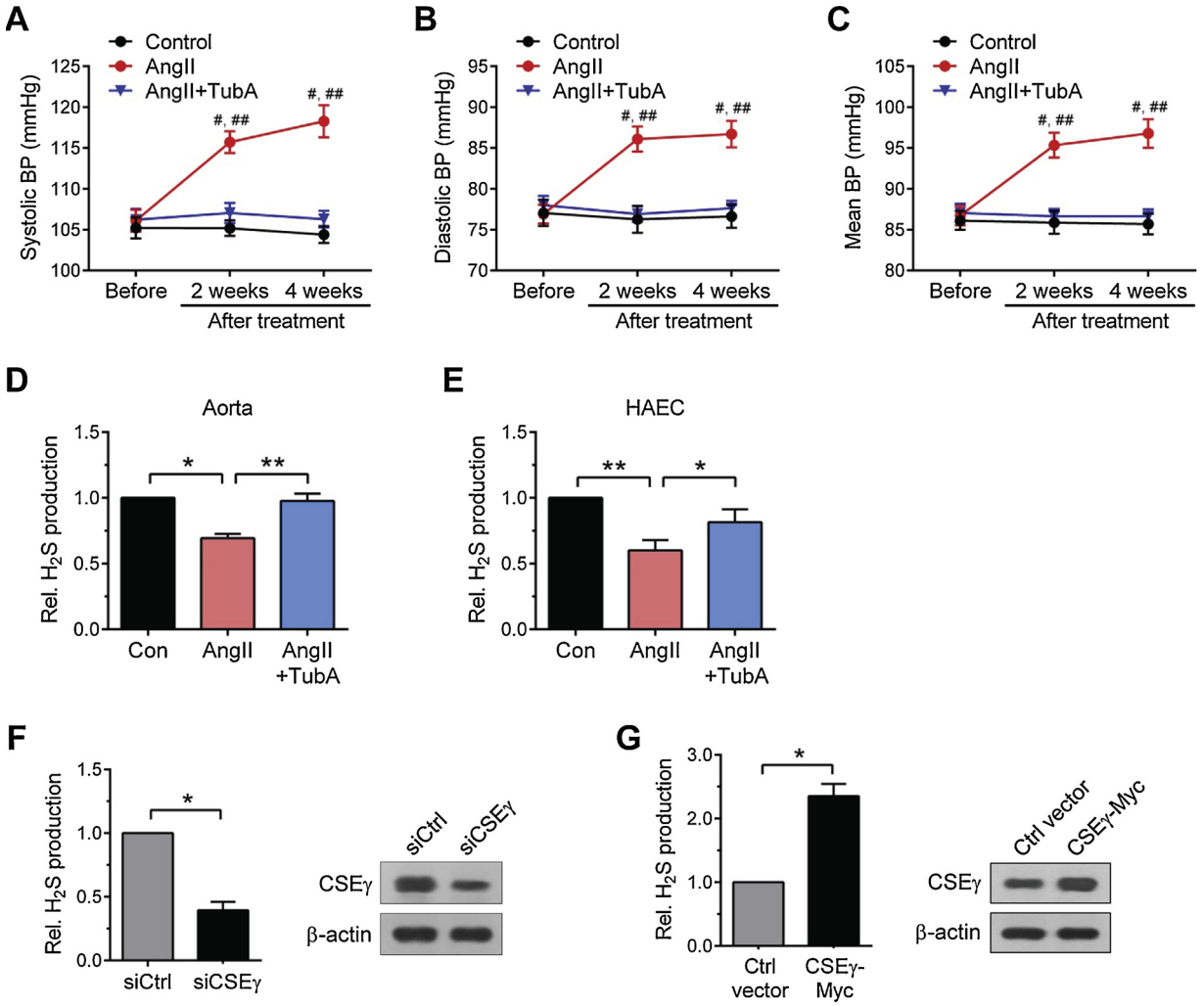

Fig. 2. Opposing effects of AngII and TubA on blood pressure and H_2S production. Changes in systolic (A), diastolic (B), and mean (C) blood pressure were measured by using a tail-cuff monitoring system. The mouse groups of control, AngII, and AngII + TubA (n = 11 each) as described in Fig. 1 were used. Blood pressure was measured prior to and 2 or 4 weeks following the delivery of AngII and/or TubA. Values represent the means ± SEM. $^{\#}p < 0.001$ vs control, $^{\#\#}p < 0.001$ vs AngII + TubA. (D) Isolated mouse thoracic aortas (n = 7) and (E) HAEC (n = 8) were pretreated with DMSO or TubA (5 μM) for 1 h, then incubated with or without 100 nM AngII for 12 h. H_2S production was measured using an agar trapping method in conjunction with an *in situ* methylene blue assay as described in Materials and Methods. H_2S concentration was determined using the linear range of a standard curve generated with NaHS and quantified relative to control. Values represent the means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$. (F) HEK293 cells were transfected with CSEγ siRNA or control siRNA for 2 days. (G) HEK293 cells were transfected with CSEγ-Myc or empty vector for 1 day. (F, G) The resulting cell lysates were analyzed for CSEγ and β-actin (a loading control) protein levels by immunoblotting. H_2S levels were measured by the agar trap method in the same way as in (E) and quantified relative to those in the control condition. Values represent the means ± SEM. $^{*}p < 0.05$.

3.5. *HDAC6 specifically mediates CSEγ downregulation by AngII*

Pan-HDAC inhibitors such as TSA, SAHA, and Scriptaid have broad activities against multiple HDAC isoforms [36,39], raising the possibility that HDAC isoforms other than HDAC6 may also participate in the regulation of CSEγ by AngII. In addition, AngII may upregulate HDAC6 at the transcriptional levels to produce the increased protein levels. Accordingly, we examined whether AngII could affect the transcription of different HDAC isoforms from HDAC1 to HDAC10. qRT-PCR analysis showed that AngII treatment of HEK293 cells significantly upregulated mRNA levels of a class I member HDAC2 and class IIb members HDAC6 and HDAC10 [22] but had relatively mild effects on mRNA levels of the other HDACs (Fig. 5A). *HDAC6* mRNA levels in AngII-treated HAEC were also upregulated for up to 12 h, then slightly declined at 24 h (Fig. 5B).

We then knocked down HDAC2, HDAC6, or HDAC10 using specific siRNAs in HAEC and their knockdown effects were compared with regard to the CSEγ regulation by AngII. siRNA-mediated knockdown efficiency was confirmed by immunoblotting (Fig. 5C–E). We observed that AngII treatment enhanced proteins levels of HDAC2 (Fig. 5D) and HDAC10 (Fig. 5E) as well as HDAC6 (Fig. 5C) in the control knockdown cells, similar to the qRT-PCR results (Fig. 5A). After AngII treatment, CSEγ protein levels in HDAC6 knockdown cells remained relatively high compared to those in control knockdown cells (Fig. 5C). In contrast, however, HDAC2 (Fig. 5D) and HDAC10 (Fig. 5E) knockdown did not produce significant changes in the AngII-induced downregulation of CSEγ protein levels and upregulation of HDAC6 protein levels. Under the same conditions, we measured possible changes in cell viabilities using a method that allows sensitive detection of water-soluble tetrazolium salt formation. As shown in Fig. 5F, AngII and the HDAC siRNAs did not substantially affect cell viability. These results supported that mRNA and protein expression levels of HDAC2, HDAC6, and HDAC10 are upregulated in response to AngII; however, the downregulation effect of AngII on CSEγ is mostly dependent on HDAC6.

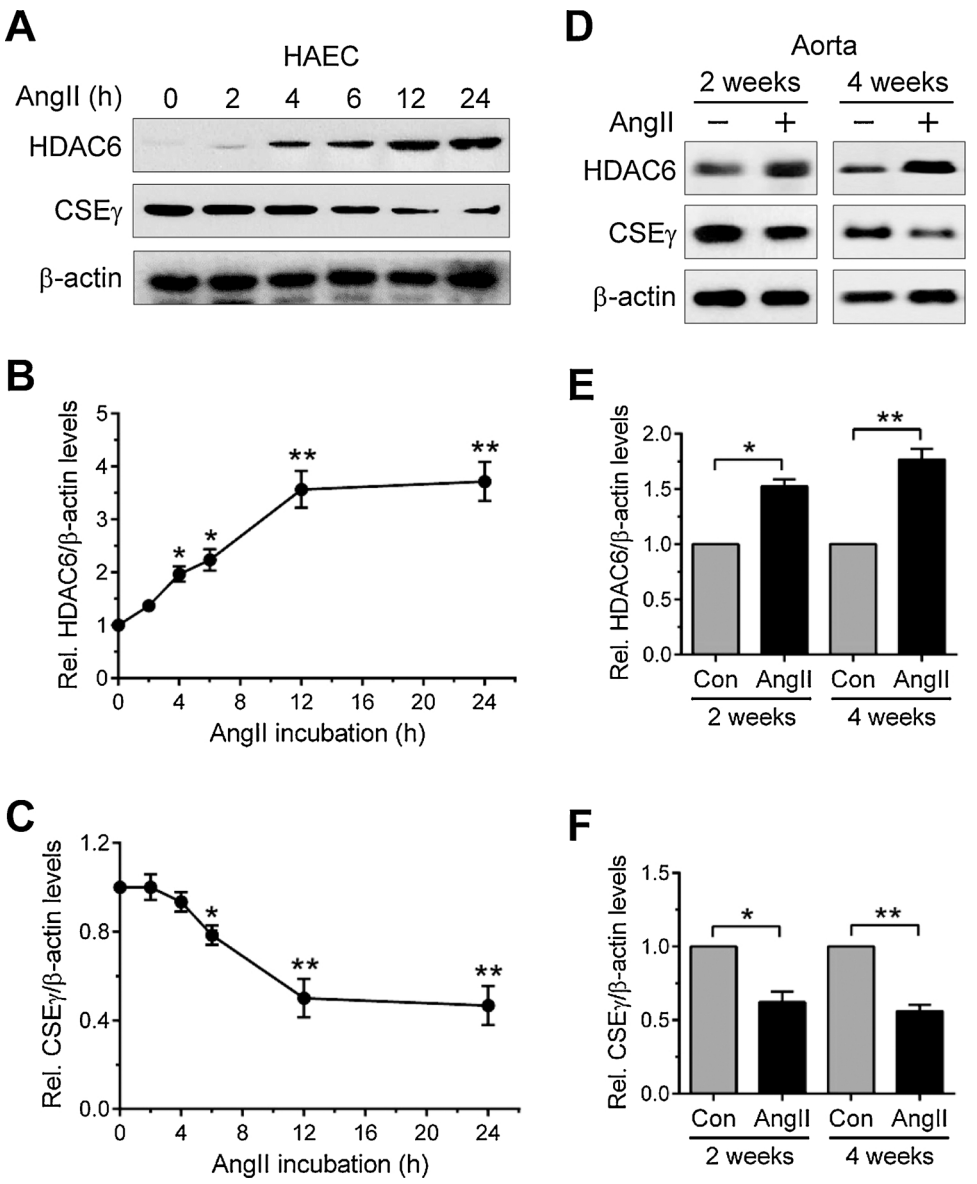

Fig. 3. Differential regulation of HDAC6 and CSEγ proteins by AngII. (A) HAEC were incubated with 100 nM AngII for the indicated times. HDAC6 and CSEγ levels were measured by immunoblotting. The HDAC6 (B) and CSEγ (C) protein levels in (A) were normalized to β-actin (a loading control) levels and quantified relative to those in the zero time condition. Values represent the means ± SEM. $*p < 0.05$, $**p < 0.01$. (D) Mice were administered without or with AngII (1 mg kg^{-1} day^{-1}) for 2 or 4 weeks, after which the aortas were isolated. HDAC6 and CSEγ levels in protein lysates were detected by western blot analysis. β-Actin was included as a loading control. The HDAC6 (E) and CSEγ (F) levels in (D) were normalized to β-actin levels and quantified relative to those in the untreated control. Values represent the means ± SEM. $*p < 0.05$, $**p < 0.01$.

3.6. HDAC6 destabilizes CSEγ in a deacetylase activity-dependent manner, resulting in its proteasomal degradation

We subsequently examined the regulatory effects of AngII and TubA on transfected CSEγ protein. Similar to the endogenous CSEγ protein (Fig. 4A), transiently expressed CSEγ-Myc protein in HEK293 cells was also reduced by AngII, which was inhibited by TubA pretreatment (Fig. 6A). To obtain evidence for the involvement of HDAC6 catalytic activity in these changes, we compared the effect of a deacetylase-deficient (DC) mutant (H216A and H611A) of HDAC6 with that of wild-

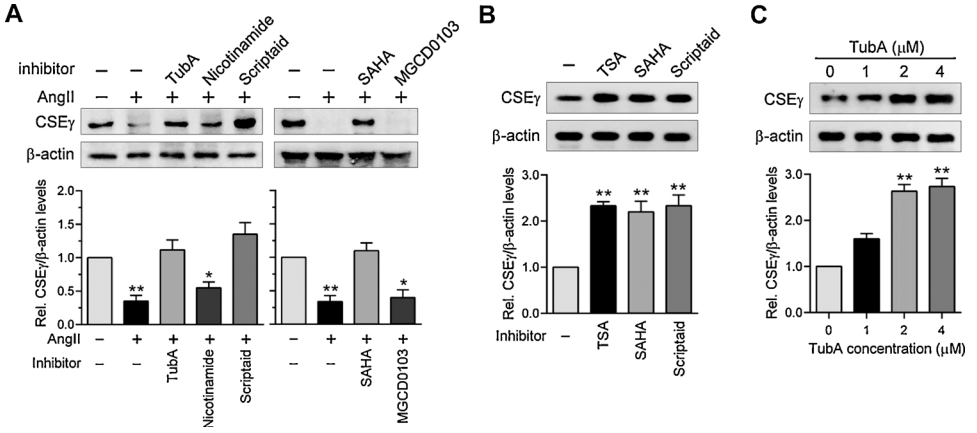

Fig. 4. Effects of HDAC inhibitors on CSEγ protein levels. (A) HAEC were pretreated with or without various HDAC inhibitors, TubA (5 μM), nicotinamide (5 mM), Scriptaid (1 μM), SAHA (1 μM), or MGCD0103 (0.5 μM) for 1 h, then were left untreated or treated with 100 nM AngII for 12 h. HAEC were incubated in the presence and absence of TSA, SAHA, or Scriptaid (1 μM each) for 12 h (B) or with indicated concentrations of TubA for 12 h (C). (A – C) The CSEγ protein levels in cell lysates were detected by western blot analysis, normalized to β-actin (a loading control) levels, and quantified relative to those in the untreated conditions. Values represent the means ± SEM. $*p < 0.05$, $**p < 0.01$.

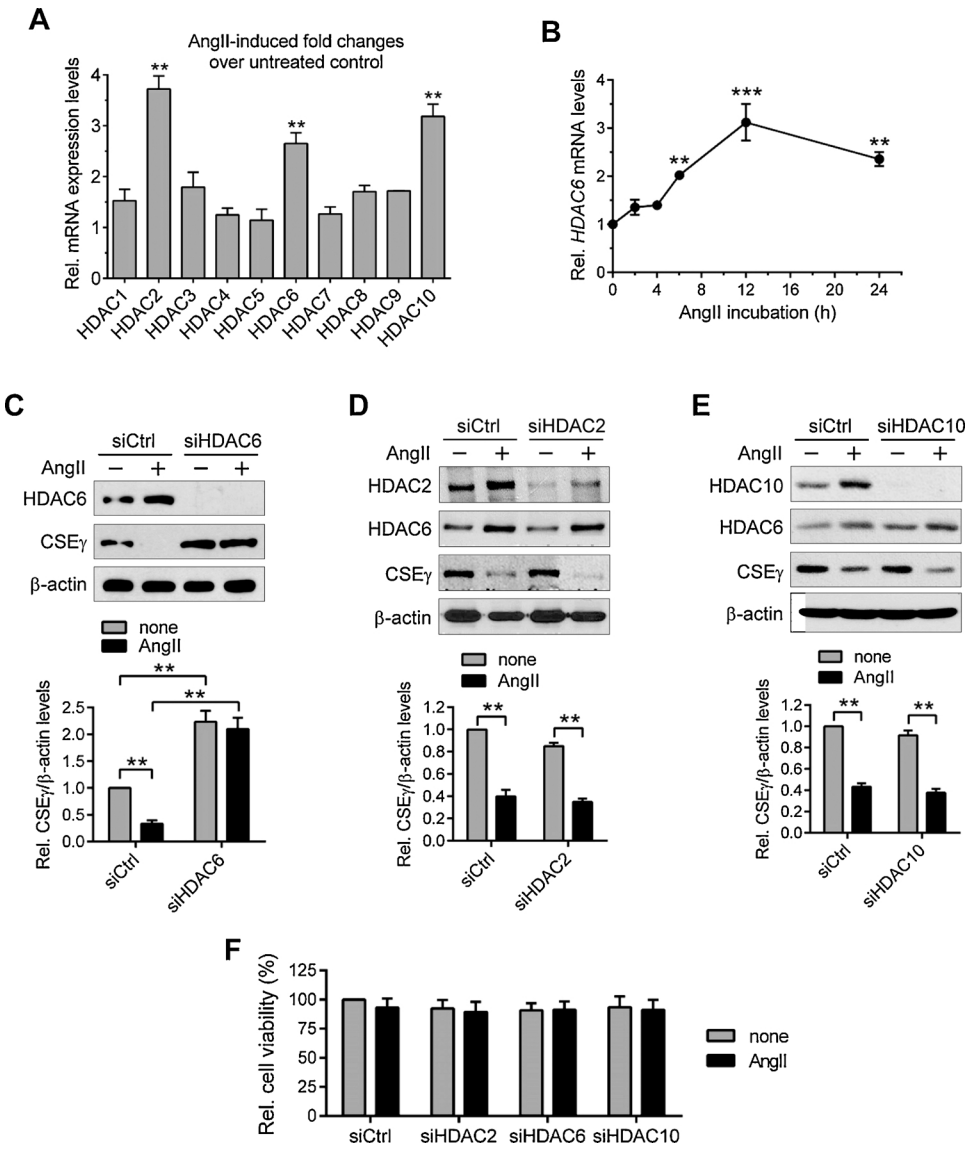

Fig. 5. AngII-induced reduction in CSEγ protein levels is dependent on HDAC6. (A) HEK293 cells were treated with or without 100 nM AngII for 12 h. mRNA levels of different HDAC isoforms (HDAC1 to HDAC10) were measured by qRT-PCR analysis and normalized to *GAPDH* mRNA levels. (B) Time-dependent effect of AngII (100 nM) on *HDAC6* mRNA levels in HAEC were examined as described in (A). (A, B) All transcriptional levels were quantified as fold induction over the levels in untreated control cells. Values represent the means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. HAEC cultures were treated with siRNAs specific for HDAC6 (C), HDAC2 (D), HDAC10 (E), or non-targeting control siRNA (siCtrl) for 48 h, followed by further incubation with or without 100 nM AngII for 12 h. Cell lysates were then analyzed for protein levels of the HDACs, CSEγ, and β-actin (a loading control) by western blotting with the indicated antibodies. (C – E) CSEγ levels were normalized to β-actin levels and quantified relative to those in the untreated control (siCtrl) cells. Values represent the means ± SEM. $^{**}p < 0.01$. (F) Cell viabilities in (C–E) were determined using a cytotoxicity assay kit and quantified relative to those in the untreated control (siCtrl) cells. Values represent the means ± SEM.

type HDAC6 on the CSEγ-Myc downregulation. Overexpression of wild-type HDAC6-FLAG significantly decreased CSEγ-Myc levels, as expected (Fig. 6B). In contrast, HDAC6.DC-FLAG overexpression did not show such a degrading effect on CSEγ-Myc protein (Fig. 6B). Although we could not completely exclude a possibility of HDAC6-dependent transcriptional regulation of CSEγ [28], as transfected CSEγ-Myc is constitutively expressed under the cytomegalovirus promoter independently of endogenous gene expression, these results supported that HDAC6 deacetylase activity can directly regulate CSEγ at the protein level.

To assess the possibility that the increased HDAC6 expression by AngII might induce proteasomal or lysosomal degradation of CSEγ, the effect of AngII on CSEγ was evaluated in the absence and presence of a proteasome inhibitor, MG132, or a lysosome inhibitor, chloroquine. Pretreatment of MG132 strikingly increased CSEγ protein levels in AngII-treated HAEC whereas chloroquine showed a significant but relatively weak effect (Fig. 6C), indicating that the CSE□ downregulation by AngII is mediated mainly through proteasomal degradation and partially through lysosomal degradation. Basal CSEγ was also markedly accumulated by MG132 alone (Fig. 6C), implying a tight control of its protein stability by proteasome activity. We then measured time course of changes in CSEγ protein stability following treatment with CHX, an inhibitor of protein synthesis, in the absence and presence of TubA. CSEγ protein levels declined time-dependently after CHX treatment in both control (DMSO only) and TubA-pretreated HAEC (Fig. 6D). However, TubA pretreatment led to a delayed degradation and relatively high levels of CSEγ during the 4-h incubation period compared to control condition (Fig. 6D). These results further suggested that the HDAC6-dependent destabilization of CSEγ is closely associated with the AngII-induced CSEγ degradation mediated by the proteasome.

3.7. HDAC6 inhibition leads to CSEγ acetylation for suppressing AngII-induced degradative ubiquitination of CSEγ

Next, we sought to delineate the mechanism of how HDAC6 mediates CSEγ degradation. Firstly, we tested whether CSEγ comprises an acetylation substrate. After treatment of HEK293 cells with AngII and/or TubA, CSEγ acetylation was examined via anti-CSEγ IP and immunoblotting using an antibody that detects lysine residues modified by acetylation. α-Tubulin is a well-known substrate that undergoes protein deacetylation by HDAC6 [21]. As expected, the acetylated form of α-tubulin was highly increased by TubA, confirming the specificity of TubA as an HDAC6 inhibitor (Fig. 7A). TubA also greatly enhanced the acetylation levels of CSEγ in the CSEγ IP samples in the absence and presence of AngII (Fig. 7A, B). Changes in CSEγ acetylation levels in HAEC were tested under the same experimental conditions. We found

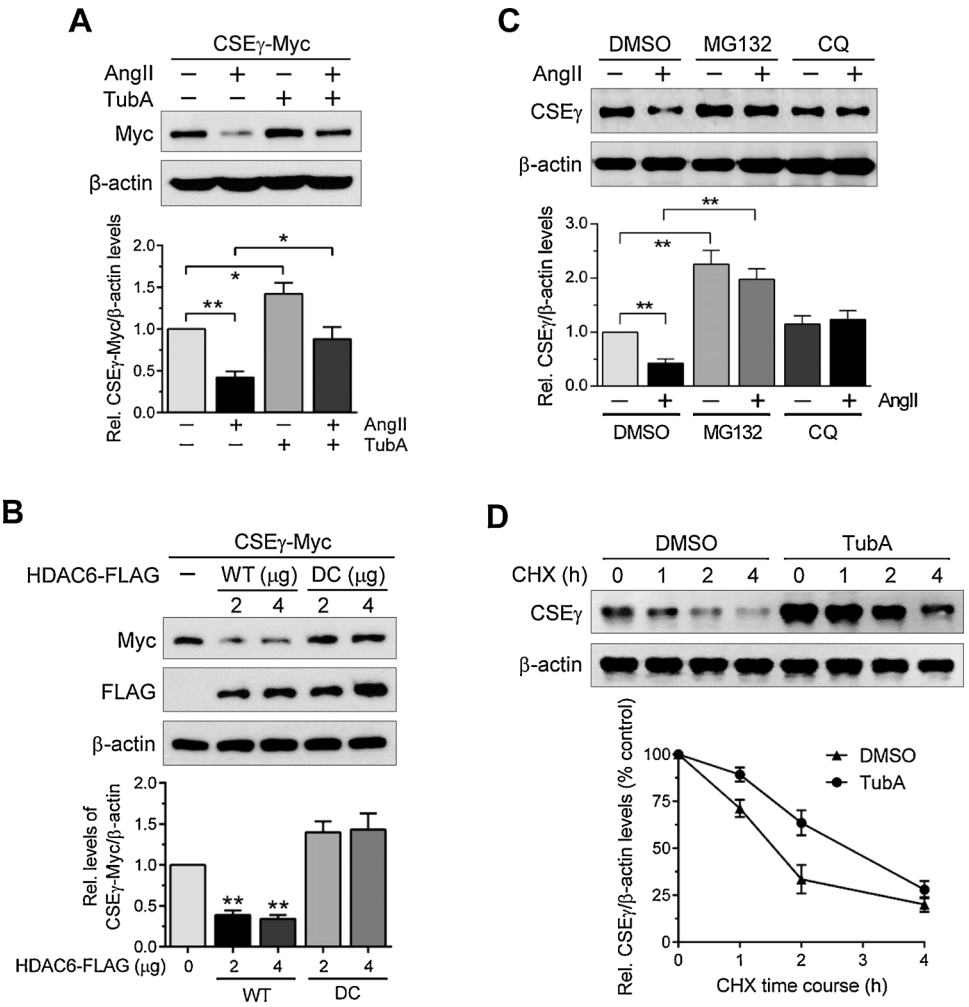

Fig. 6. HDAC6 deacetylase activity mediates proteasomal degradation of CSEγ. (A) HEK293 cells transfected with CSEγ-Myc for 1 day were treated with or without 5 μM TubA for 1 h, then incubated in the presence and absence of AngII (100 nM) for 12 h. (B) HEK293 cells were cotransfected with the indicated amounts of wild-type (WT) or inactive (DC) HDAC6-FLAG together with CSEγ-Myc for 1 day. (C) HAEC pretreated with DMSO (as a vehicle), MG132, or chloroquine (CQ) (10 μM each) for 1 h were incubated in the presence and absence of AngII (100 nM) for 12 h. (D) HAEC were incubated with 10 μM CHX for the indicated times after treatment with DMSO (as a vehicle) or TubA (5 μM) for 1 h. Cell lysates were analyzed for the protein levels of transfected CSEγ-Myc and/or HDAC6-FLAG (A, B), endogenous CSEγ (C, D), and β-actin (a loading control) by western blotting with the indicated antibodies. Protein levels of CSEγ-Myc (A, B) and CSEγ (C, D) were normalized to β-actin levels and quantified relative to those in the untreated control (A, C), the absence of HDAC6-FLAG (B), or the respective zero time condition (D). Values represent the means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

acetylated form of CSEγ in resting condition and the reduction in CSEγ acetylation levels by AngII (Fig. 7C). The acetylation levels of endogenous CSEγ and α-tubulin were also noticeably elevated by TubA irrespective of the presence of AngII (Fig. 7C). Similar results were obtained with anti-Myc IP samples from cotransfection of HEK293 cells with CSEγ -Myc and HDAC6-FLAG. The catalytically inactive HDAC6.DC showed relatively high levels of CSEγ acetylation compared with those of wild-type HDAC6 (Fig. 7D, E). Similarly, wild-type HDAC6, but not HDAC6.DC, reduced acetylation levels of endogenous CSEγ in HAEC (Fig. 7F). These results indicated that HDAC6 induces CSEγ degradation by mediating its deacetylation. In addition, the co-precipitated HDAC6-FLAG in the anti-Myc IP samples suggested a possible interaction between CSEγ and HDAC6 (Fig. 7D). Notably, the interaction between endogenous CSEγ and HDAC6 was induced by AngII in HAEC (Fig. 7G), supporting the physiological relevance of this protein interaction. No immunoreactivity of CSEγ (Fig. 7A, C, F, G) and Myc (Fig. 7D) in the IP samples with control IgG confirmed the specificity of these IP experiments.

In addition, CSEγ underwent proteasomal degradation (Fig. 6C) and its protein levels in the cell lysates were elevated by TubA (Fig. 7A, C) and inactive HDAC6 (Fig. 7D, F), indicating that CSEγ is degraded by HDAC6 in a deacetylation-dependent manner. Based on these results, we then examined whether CSEγ acetylation could affect ubiquitin-mediated degradation. For this, cells cotransfected with CSEγ-Myc and HA-ubiquitin were treated with AngII and/or TubA, then the prepared anti-Myc IP samples were processed for HA immunoblotting. Notably, AngII (100 nM) induced degradative ubiquitination of CSEγ, as shown by the diminished Myc immunoreactivity in both cell lysates and anti-Myc IP samples and by the multiple bands of higher molecular weight than CSEγ-Myc in the anti-Myc immunoprecipitates (Fig. 7H, I). In contrast, however, TubA strongly abrogated the AngII effect, resulting in very low levels of ubiquitinated CSEγ-Myc and its accumulation without being degraded (Fig. 7H, I). Thus, these results suggested that the HDAC6-mediated deacetylation of CSEγ promotes its degradation via ubiquitination.

Previously, a proteomic study identified the protein substrates to undergo ubiquitination and acetylation and their target lysine residues and revealed that the K73 in CSEγ is a main site for such protein modifications [40]. We thus tested the possibility that K73 acetylation levels of CSEγ may be related to regulation of its protein levels by AngII and HDAC6. For this, the point mutant (K73R) of CSEγ-Myc that can be resistant to both acetylation and ubiquitination was generated and compared with its wild-type with regard to acetylation efficiency in the absence and presence of AngII or TubA in HAEC. As shown in Fig. 7J and K, the decreased and increased CSEγ acetylation levels by AngII and TubA, respectively, were observed in the wild-type-expressing cells, whereas the acetylation levels were strongly reduced in the K73R-expressing cells, demonstrating that the K73 residue is a major acetylation site in CSEγ. In addition, the protein levels of CSEγ-Myc K73R in resting conditions were slightly enhanced compared with those of the wild-type and remained largely unchanged upon AngII or TubA treatment (Fig. 7J, K). These results suggested that AngII and HDAC6 exert regulatory effects on CSEγ protein stability likely through the K73 acetylation status of CSEγ.

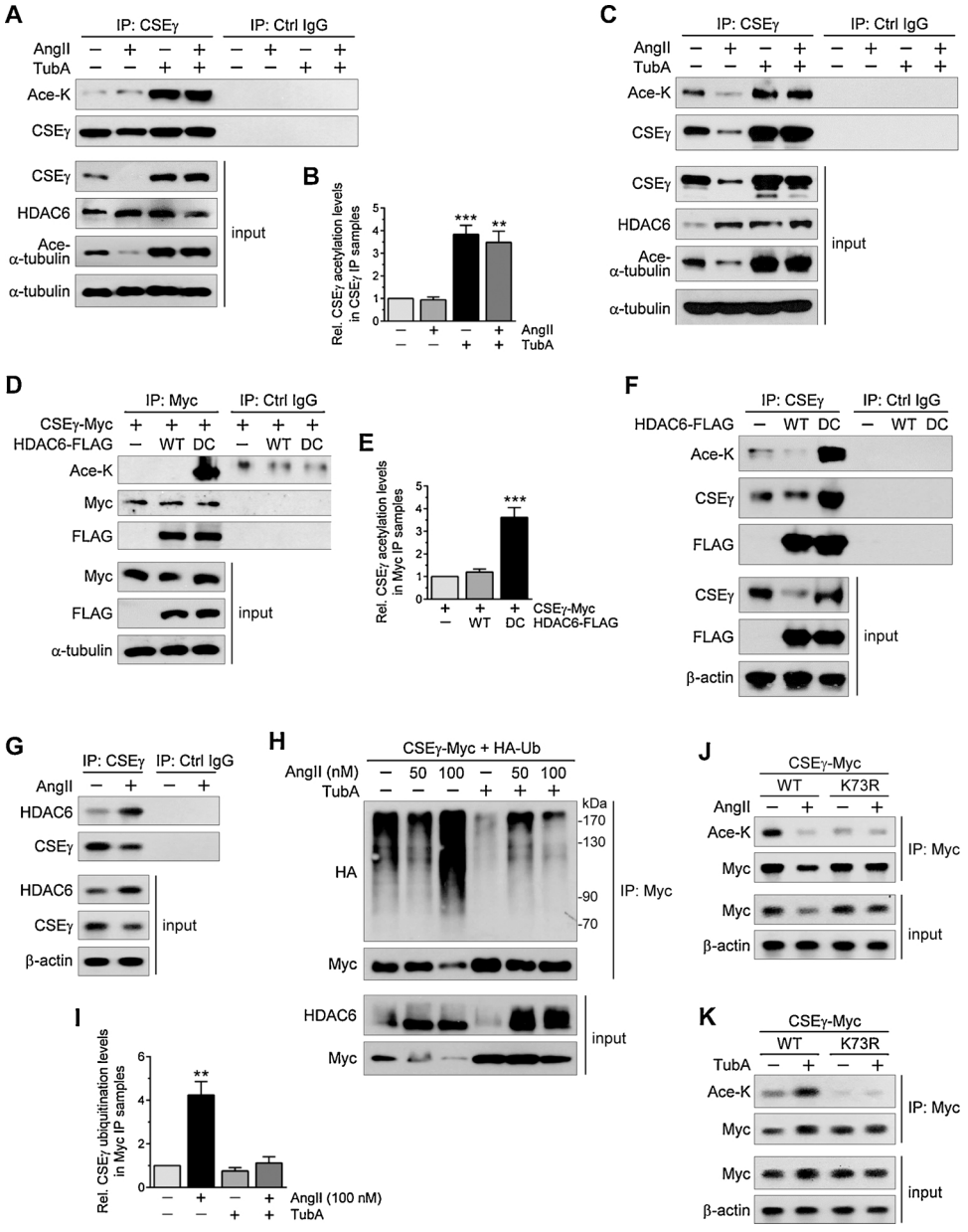

Fig. 7. Enhanced CSEγ acetylation by TubA prevents AngII-induced ubiquitin-dependent degradation of CSEγ. HEK293 cells (A) or HAEC (C) were incubated in the presence and absence of AngII (100 nM) and/or pretreated TubA (5 μM) as described previously, then the resulting cell lysates (input) were processed for anti-CSEγ IP or normal IgG IP as a negative control. The IP products and/or input samples were immunoblotted with the indicated antibodies including the anti-acetylated-lysine (Ace-K) and anti-acetyl-α-tubulin (K40) (Ace-α-tubulin) antibodies. (B) The CSEγ acetylation levels in the CSEγ IP samples in (A) were quantified relative to those in the untreated control. Values represent the means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. (D) HEK293 cells were cotransfected with wild-type (WT) or inactive (DC) HDAC6-FLAG and CSEγ-Myc, as indicated, and then CSEγ-Myc was immunoprecipitated with an antibody to Myc tag. The anti-Myc IP products, normal IgG IP product (a negative control), and/or input samples were analyzed by immunoblotting with the indicated antibodies including the anti-acetylated-lysine (Ace-K) antibody. (E) The CSEγ acetylation levels in the Myc IP samples in (D) were quantified relative to those in the absence of HDAC6-FLAG. Values represent the means ± SEM. $^{***}p < 0.001$. HAEC were transfected with HDAC6-FLAG WT or DC (F) or treated without or with AngII (100 nM) for 12 h (G), and then the CSEγ IP and normal IgG IP products, and/or input samples were subjected to immunoblot analysis with the indicated antibodies. (H) HEK293 cells were treated without or with AngII (50 or 100 nM) and/or TubA in the same manner as in Fig. 6A at 1 day post-transfection of CSEγ-Myc and HA-ubiquitin (Ub). The anti-Myc IP products and input samples were analyzed by immunoblotting with the indicated antibodies. (I) The CSEγ ubiquitination levels in the Myc IP samples in (H) were quantified relative to those in the untreated control. Values represent the means ± SEM. $^{**}p < 0.01$. HAEC were transfected with CSEγ-Myc WT or K73R, and then treated with or without 100 nM AngII for 12 h (J) or 5 μM TubA for 1 h (K). The anti-Myc IP products and input samples were analyzed by immunoblotting with the indicated antibodies.

4. Discussion

In the present study, we showed that TubA significantly improves the endothelium-dependent vascular relaxation that had been impaired by AngII. We also demonstrated that TubA inhibits the rise in blood pressure mediated by AngII. These results indicated that HDAC6 inhibition by TubA provides protection against AngII-induced endothelial dysfunction and hypertension. Moreover, TubA antagonizes the reducing effect of AngII on H_2S production. Our results further showed that AngII and TubA reciprocally regulate protein levels of CSEγ, a H_2S-generating enzyme. The similarity of changes in H_2S and CSEγ levels by AngII and TubA indicated that AngII negatively regulates CSEγ-dependent H_2S production through HDAC6.

Our current data suggested that AngII-induced upregulation of HDAC6 expression is associated with protein downregulation of CSEγ. We demonstrated that HDAC6 induced the deacetylation of CSEγ, which could, in turn, trigger CSEγ ubiquitination and proteasomal degradation. In accordance with these results, TubA significantly increased the levels of acetylated CSEγ, which blocked AngII-induced ubiquitination, thereby protecting CSEγ against degradation. Based on these novel findings, we proposed that HDAC6-mediated CSEγ degradation constitutes an important mechanism underlying the well-established AngII-induced hypertension process and, accordingly, that HDAC6 inhibition by TubA can counteract the hypertensive actions of AngII.

Our results are similar to those of previous studies demonstrating pathologic roles of HDAC6 in hypertension [17–19] and an inhibition of CSEγ function by HDAC6 [28]. H_2S generated by CSEγ is shown to play cytoprotective roles in diverse physiological processes of cardiac, renal, immune, and nervous systems [7,41–44]. It has also been reported that functional inhibition of HDAC6 prevents progressions of endothelial dysfunction, kidney disease, brain infarction, tumor development, and inflammatory response [27,28,45–47]. Thus, our findings suggest that the HDAC6-mediated CSEγ inhibition may be, at least in part, related to those HDAC6-dependent pathophysiological events.

We reveal that CSEγ constitutes an acetylated protein and that its

acetylation levels are reduced by HDAC6 in a deacetylase-dependent manner, indicating CSEγ as an HDAC6 substrate. CSEγ was also identified as a ubiquitinated protein that undergoes degradation by the proteasome. Our evidence supports that CSEγ, once deacetylated by HDAC6, becomes more susceptible to degradation through the ubiquitin-proteasome pathway. Functional inactivation of HDAC6 by TubA, its gene knockdown, or overexpression of an inactive HDAC6 form resulted in enhanced protein levels of CSEγ in both the presence and absence of AngII. Consistent with our results, a previous report showed that pan-HDAC inhibitors including Scriptaid and SAHA, but not MGCD0103 and nicotinamide, elevated CSEγ protein levels in HAEC [28]. These suggested that endothelial CSEγ protein levels are also dynamically controlled by the HDAC6-driven posttranslational modifications under steady state conditions. In addition, the lysosomal pathway was shown to contribute to at least part of the CSEγ degradation by AngII.

Our results show that the enhanced HDAC6 protein levels mediated by AngII likely occur via its transcriptional upregulation. HDAC2 and HDAC10 also underwent increases in mRNA and protein expression levels upon AngII treatment. However, the latter proteins did not exhibit an effect on CSEγ protein levels as strong as that of HDAC6, indicating the specific control of CSEγ by HDAC6. Similar to our data, treatment of HAEC with an oxidized low-density lipoprotein to effect endothelial dysfunction increased mRNA and protein expression levels of HDAC6, which was responsible for the reduced CSEγ expression [28].

Acetylation and ubiquitination comprise common protein modifications that occur on the lysine residues. It has been recognized that HDAC6-driven deacetylation of target substrates could regulate their protein stability through the ubiquitin-proteasome system [48,49]. For example, the deacetylation of heat-shock protein 5 by HDAC6 triggers an E3 ubiquitin ligase GP78-mediated ubiquitination for its proteasomal degradation [50]. In addition, it has also been reported that HDAC6-mediated deacetylation of the Hippo component, tumor suppressor MST1 is involved in its lysosomal degradation via chaperone-mediated autophagy [51]. Among HDAC family members, the class IIb HDAC6 exhibits a unique property in that it contains two catalytic domains. Notably, an earlier report showed that whereas the N-terminal catalytic domain mediated deacetylation of MSH2, a key DNA mismatch repair protein, the central catalytic domain acted as an E3 ubiquitin ligase for proteasomal degradation of the substrate [52]. Although it remains to be further clarified whether acetylation and ubiquitination of CSEγ may occur on the same as yet unidentified lysine residue(s) in a competitive manner or on distinct target lysine residues, our findings suggest that the K73 residue of CSEγ can serve as a potential target site for the HDAC6-mediated deacetylation and also for the ubiquitin-mediated proteolysis. The determination of a relevant E3 ubiquitin ligase responsible for CSEγ ubiquitination is also left unresolved. We assume that the acetylated form of CSEγ is likely to be unfavorable for degradative ubiquitination especially when HDAC6 activity is suppressed. The precise coordination of how HDAC6 and/or certain E3 ubiquitin ligases regulate acetylation and ubiquitination of CSEγ to affect its protein stability remains to be further investigated.

5. Conclusion

In summary, we demonstrated for the first time that AngII increases HDAC6 expression levels, inducing deacetylation of CSEγ, which is then available for ubiquitination and subsequent degradation by the proteasome. Overall, the resulting HDAC6-mediated reduction in CSEγ protein and H_2S levels then contributes, at least in part, to the AngII-induced vasoconstriction and hypertension. In agreement with this, we confirmed the antihypertensive and vasorelaxatory effects of TubA in AngII-infused mice. These findings suggested that HDAC6 acts as a negative regulator of CSEγ and provided novel insight regarding therapeutic strategies to modulate HDAC6 in various hypertensive disorders that are related to the dysregulation of CSEγ and H_2S.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2019.104281.

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