ESC European Society of Cardiology

Cardiovascular Research (2018) **114**, 1016–1028
doi:10.1093/cvr/cvy051

HDAC4 regulates vascular inflammation via activation of autophagy

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Received 9 January 2018; revised 10 February 2018; editorial decision 14 February 2018; accepted 23 February 2018; online publish-ahead-of-print 24 February 2018

Time for primary review: 17 days

Aims Angiotensin II (Ang II) causes vascular inflammation, leading to vascular endothelial cell dysfunction, and is associated with the development of cardiovascular diseases. Therefore, interventions in inflammation may contribute to the reduction of cardiovascular diseases. Here, we aim to demonstrate that HDAC4, one of class IIa family histone de-acetylases (HDACs) members, promotes autophagy-dependent vascular inflammation.

Methods and results By loss-of-function approaches, our study provides the first evidence that HDAC4 mediates Ang II-induced vascular inflammation *in vitro* and *in vivo*. In response to the Ang II, HDAC4 expression is up-regulated rapidly, with increased autophagic flux and inflammatory mediators in vascular endothelial cells (VECs). In turn, HDAC4 deficiency suppresses activation of autophagy, leading to reduced inflammation in Ang II-induced VECs. Consistently, using autophagy inhibitor or silencing LC3-II also alleviates vascular inflammation. Furthermore, HDAC4 regulates autophagy via facilitating transcription factor forkhead box O3a (FoxO3a) de-acetylation, thereby to increase its transcriptional activity. Loss of HDAC4 in VECs results in inhibition of FoxO3a de-acetylation to block its transcriptional activity, leading to downregulation of the downstream FoxO3a target, and hence reduces autophagy and vascular inflammation. FoxO3a silencing using siRNA approach significantly inhibits activation of autophagy. Finally, knockdown of HDAC4 in Ang II-infused mouse models ameliorates vascular inflammation, suggesting that inhibitor of HDAC4 may be potential therapeutics for vascular diseases associated with inflammation.

Conclusion These results suggest that HDAC4-mediated FoxO3a acetylation regulates Ang II-induced autophagy activation, which in turn plays an essential role in causing vascular inflammation.

Keywords Vascular inflammation • Angiotensin II • HDAC4 • Autophagy • De-acetylation

1. Introduction

Endothelial cells play a key role in the vascular homeostasis and several aspects of vascular biology, including the permeability barrier function, inflammation, and angiogenesis.[1] Inflammation localized in vascular tissue is an important contributor to the pathophysiology of cardiovascular diseases including atherosclerosis and hypertension.[2–4] Angiotensin II (Ang II) is a major mediator of the renin-angiotensin system, associated with cardiovascular diseases.[5,6] Ang II leads to the overexpression of inflammation-related genes such as cellular adhesion molecules [e.g. vascular adhesion molecule-1 (VCAM-1)], inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines [e.g. interleukin-6 (IL-6)], thus conferring an inflammatory phenotype that contributes to endothelial dysfunction.[7–9] Therefore, the inhibition of Ang II-induced inflammation may provide benefits to cardiovascular diseases.

Epigenetic mechanisms contribute to diverse physiological systems and pathological process.[10] Histone acetyltransferases (HATs) and histone de-acetylases (HDACs) control a wide array of biological processes by epigenetically regulating accessibility of transcription factors to the gene promoter by acetylation or de-acetylation of the histone.[11] Up to now, HDACs have been classified into four groups termed class I HDACs (HDAC1, 2, 3, and 8), class II HDACs (HDAC4, 5, 6, 7, 9, and 10), class III HDACs, and class IV HDAC (HDAC11).[12] Among them, it was reported that HDAC4 plays important roles in mediating cardiovascular diseases. For example, in cultured cardiomyocytes, HDAC4 is regulated by hypertrophic agonists[13] and HDAC4 controls neointimal hyperplasia via stimulating proliferation and migration of vascular smooth

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muscle cells (VSMCs).[14] Considering the importance of HDAC4 in cardiovascular diseases, the role of HDAC4 in vascular inflammation needs to be investigated.

Autophagy, is an important homeostatic process that is responsible for degrading intracellular organelles and protein aggregates,[15] and has received greater attention due to its implications in pathological processes, including tumorigenesis, ageing, and neurodegeneration.[16,17] Recent studies have revealed that in the cardiovascular system, autophagy exerts critical pathophysiological roles in controlling endothelial cell function, vascular tone, inflammation, proliferation, angiogenesis, and vascular remodelling.[18] In certain circumstances, autophagy plays a protective role via clearing the damaged and unhealthy organelles. However, the massive and persistent activation of autophagy may contribute to excessive cell inflammation and pathological angiogenesis.[19,20] In addition, recent reports demonstrate that multiple forms of stress, including pressure overload, chronic ischaemia, and ischaemia-reperfusion provoke an increase in autophagic activity in cardiovascular diseases, thereby activated autophagy contributes to disease pathogenesis.[21] FoxO3a is an isoform of transcription factor forkhead box class O (FoxO) family, and can regulate autophagy in skeletal and cardiac muscles by activating genes that are involved in autophagosome formation.[22] Thus, considering that autophagy is involved in cardiovascular system, protectively or detrimentally, meanwhile, HDAC4 regulates cardiovascular diseases,[23–25] we hypothesize that maladaptive autophagy is HDAC4 dependent and contributes to the further development of vascular inflammatory. We further posit that HDAC4 facilitating FoxO3a de-acetylation to mediate autophagy plays significant roles in Ang II-induced vascular inflammatory.

2. Methods

2.1 Materials

Reagent sources were as follows: angiotension (Ang II) was from Sigma-Aldrich (St. Louis, MO, USA). 3-methyladenine (3-MA), LY294002 (LY), and tasquinimod (Taq) was purchased from Selleckchem (Selleck, USA). Mithramycin (Sp-1 inhibitor) was purchased from Calbiochem (San Diego, CA). Antibodies were obtained from the following commercial sources: VCAM-1 was purchased from Proteintech (Proteintech, USA); IL-6 and iNOS were purchased from Epitomics (Burlingame, CA); HDAC4, FoxO3a, LC3-II, Atg5, and Beclin 1 were purchased from Cell Signalling Biotechnology (Danvers, MA, USA); COX2, HDAC4, and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Acetyl Lysine polyclonal antibody was purchased from BioVision (BioVision Incorporated, CA, USA).

2.2 Animal studies

All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and were approved by Institutional Animal Care and Use Committee (IACUC), School of Pharmacy, Fudan University, China.

C57BL/6 mice (6–8 weeks of age) were randomly assigned to one of four groups: The sham control group, Ang II infusion group, shRNA HDAC4 group (HDAC4 KD), and 3-MA group. Briefly, the mice were anesthetized under isoflurane (induction: 3–4%, maintenance: 2–3%; in 0.2 L/min medical air and 0.2 L/min O_2), followed by subcutaneous implantation of an Alzet mini-osmotic pump (Model 2004, ALZA Scientific Products, Mountain View, CA, USA) containing only saline (control group), or Ang II dissolved in saline. Mice were then continuously infused with saline or Ang II (1.5 mg/kg/d). One week after mini-osmotic pump, in HDAC4 KD group, these mice were injected via tail vein with purified lentiviral particles (1×10^9 MOI) that carry short hairpin RNA (shRNA) targeting HDAC4 every 7 days until the end of the experiment. The 3-MA treatment group was given a daily intraperitoneal injection (*i.p.*) injection of 3-MA (20 mg/kg/day) until the end of the experiment on day 28. 3-MA was dissolved in dimethyl sulfoxide (DMSO) and then diluted with phosphate-buffered saline (PBS) to the final concentration. Ang II infusion group was injected via tail vein with entiviral vector (shMock).

After 4 weeks, mice were anesthetized with sodium pentobarbital (60 mg/kg) and the thoracic aorta of each mouse was cut into two sections. One section was stored at –80°C for protein extraction. The other section was fixed with 4% paraformaldehyde overnight, and was then embedded in paraffin for immunofluorescence staining.

2.3 Culture of primary vascular cells and treatment

Rat primary endothelial cells (RAECs) were isolated from male rats according to the method described previously.[26] Male Sprague-Dawley rats, 80–100 g, were anesthetized with sodium pentobarbital (40 mg/kg, Sigma), the isolated cells were cultured in DMEM supplemented with 20% foetal bovine serum, 1% penicillin–streptomycin, at 37°C in a 95% air/5% CO_2 incubator. The cells passages of 3–6 were used for all of the experiments performed. The identity of the RAECs was confirmed by immunofluorescence staining by CD31 antibody. The different batch of RAECs was used in each *in vitro* assay. Drugs were used at the following final concentrations: Ang II at 2 μM; 3-MA at 5 mM; Taq at 10 μM; LY294002 at 10 μM.

Rat primary VSMCs were isolated from the abdominal aortic arteries of rats and identified following the same protocol as described previously.[27] The cells passages of 3–7 were used for all of the experiments performed.

2.4 Small interfering RNA (siRNA) transfection

HDAC4 siRNA (5'-GCUAUGACGAUGGGAACUUTT-3'), LC3-II siRNA (5'-GCUUCGAACAAAGAGUGGATT-3'), FoxO3a siRNA (5'-CCCAGAUCUACGAGUGGAUTT-3'), and control siRNA were produced by GenePharma (Shanghai, China). To introduce siRNA into cells, the cells were plated on 6-well plates at 30% to 50% confluence before transfection. Individual siRNA (at 25 to 50 nM), Lipofectamine RNAiMAX, and Opti-MEM was mixed and incubated at room temperature for 5 min. siRNA-lipofectamine RNAiMAX complexes were added to cells for 24 h and the medium was replaced by fresh serum DMEM medium after transfection. The efficiency of gene knockdown was verified by western blot 72 h post-transfection.

2.5 Lentivirus generation and infection

The specific Short Hairpin RNA (shRNA) HDAC4 was gift from Prof. Zeng (Shandong University, Jinan, China). Lentivirus generation and infection were performed according our described previously.[28] In brief, lentivirus was generated in HEK293T after transfection with lipofectamine 2000 (ThermoFisher Scientific, Shanghai, China) and plasmids. We collected culture media 48 h after transfection and added fresh media to the transfected cells and collected them 24 h later again. The two collections of media were combined and virus particles were pelleted by

ultracentrifugation (25 000 rpm, 4°C, 2 h; Beckman SW 28 rotor). Virus particles were then resuspended with PBS, aliquoted and flash frozen.

2.6 mCherry-GFP-LC3 assay

Autophagy was visualized in RAECs by transfection of the adenovirus expressing mCherry-GFP-LC3B fusion protein (Ad-mCherry-GFP-LC3B, Biotime Biotechnology, Jiangsu, China) and followed by analysis with a fluorescence microscopy (Zeiss LSM780, Carl Zeiss). Autophagy was induced with 2 μM Ang II for 48 h in RAECs in the presence of Taq or silencing HDAC4.

2.7 RNA isolation, cDNA synthesis, and qRT-PCR

Total RNA was isolated by TRIzol Reagent (Takara, TaKaRa Biotechnology, Dalian, China). Total RNA (2 μg) of each sample was reverse-transcribed into cDNA and amplified using a PrimeScriptTM1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's directions. PCR was conducted using the Taq polymerase (Takara). See Supplementary material online, *Table S1* for the primer sequences.

2.8 Western blotting

Cell and tissue proteins lysed in RIPA buffer (Pierce, Rockford, IL, USA) containing protease inhibitor cocktail (Sigma, St Louis, USA). Whole lysates samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to the nitrocellulose membrane. Membranes were incubated with the specified primary antibodies and HRP-conjugated secondary antibodies detected by the Supersignal West Dura Extended Duration Substrate kit (Pierce). In some cases, western blotting was performed with fluorophore-conjugated secondary antibodies, and detection and analysis were performed with the Odyssey imaging system (LI-COR).

2.9 Co-immunoprecipitation

Following different treatments, RAECs or vascular tissues were lysed with the RIPA buffer, the debris was removed by centrifugation at 10 000 g for 10 min. Aliquots (500–1000 μg) of lysates were incubated with rabbit-IgG antibody or Acetyl Lysine (Biotin) Polyclonal antibody (BioVision) at 4°C for 1 h, then were incubated with 20 μL of suspended volume of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) at 4°C on a rocker platform overnight. Immunoprecipitates were collected by centrifugation at 2500 rpm for 5 min at 4°C. The pellet was washed four times with 1.0 mL RIPA buffer, each time repeating centrifugation step above. After final wash, supernatant was discarded and pellet was re-suspended in 40 μL of 1 × electrophoresis sample buffer, then were analysed by SDS-PAGE and autoradiography.

2.10 Chromatin immunoprecipitation PCR (ChIP-PCR)

ChIP assays of RAECs induced with Ang II (2 μM) for 1 h or 48 h were performed using EZ-ChIP™ Chromatin immune-precipitation kit (Millipore) according to the manufacturer's instructions. Antibody for FoxO3a was used for chromatin immunoprecipitation (ChIP). Primers for the ChIP assay were designed in the promoter regions of *MAP1LC3B* (LC3-II) and *Atg5*. Sequences of primers for the ChIP assay were as Supplementary material online, *Table S2*.

2.11 Immunofluorescence staining

The arterial sections were blocked and incubated overnight with primary antibodies at 4°C. RAECs were cultured on coverslips placed in cell cultured plate. Following different treatments, cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.2% Triton X-100 in PBS 10 min. Next, the slides were blocked in PBS with 10% goat serum for 1 h at room temperature, and incubated overnight with primary antibodies at 4°C. Appropriate secondary antibodies were incubated for 1 h at 37°C. The nuclei were stained with DAPI. The images were captured by using a fluorescence microscope (Zeiss LSM780, Carl Zeiss).

2.12 Statistical analysis

Experimental results were expressed as means ± SEM. Differences of means were analysed by using one-way ANOVA with the Turkey–Kramer *post hoc* test for multiple groups and unpaired Students *t*-test for two groups. Probability values, $P < 0.05$ were considered significant.

3. Results

3.1 Ang II induces autophagy and inflammation in RAECs with enhanced HDAC4 expression

RAECs were treated with Ang II (2 μM) for 24, 48, and 72 h, protein expression was shown in *Figure 1A*. Ang II treatment for all three time periods markedly increased inflammatory mediators such as COX2 and VCAM-1 expression. Furthermore, HDAC4 up-regulation was also visible at the mRNA and protein level (*Figure 1A* and *B*). Autophagy is an important mechanism during the regulation of cell homeostatic process. LC3-II, Atg5 and Beclin1 are also used as markers of autophagy. Thus, to further examine whether Ang II treatment induced autophagy, we measured the LC3-II, Atg5 as well as Beclin 1 expression following Ang II treatment. Notably, the protein levels of LC3-II, Beclin1, and Atg5 appeared to increase after treatment with Ang II in a time-dependent manner in RAECs (*Figure 1A*). Using immunofluorescence double staining we found that HDAC4 and LC3-II were consistently up-regulated (*Figure 1C*). To determine if Ang II-treated cells activated autophagy, we used red/green fluorescence expressing a tandem-tagged mCherry-GFP-LC3B fusion protein, which allows for analysis of autophagy due to loss GFP but retention of mCherry fluorescence in lysosomes.[29] Ang II treated cells exhibited numerous GFP-LC3 dots (*Figure 1D*), consistent with the activation of autophagy. These results showed Ang II induced autophagy and inflammation with enhanced HDAC4 expression in RAECs.

To address whether such association exists in animals, mice were subjected to a 28-day infusion of vehicle or Ang II, and the aorta vessels were assessed for HDAC4 and autophagy. Strikingly, the abundance of HDAC4 at the mRNA and protein level was dramatically increased in response to Ang II induction (*Figure 1E* and *F*). This increase was concomitant with the up-regulation of some classic markers of autophagy and inflammation (*Figure 1F*). Immunofluorescence staining showed that the expression of HDAC4 in vascular wall was increased in Ang II-infused mice (*Figure 1G*). All these results supported the idea that HDAC4 and autophagy was associated with vascular inflammation not only in cell model but also in animal model.

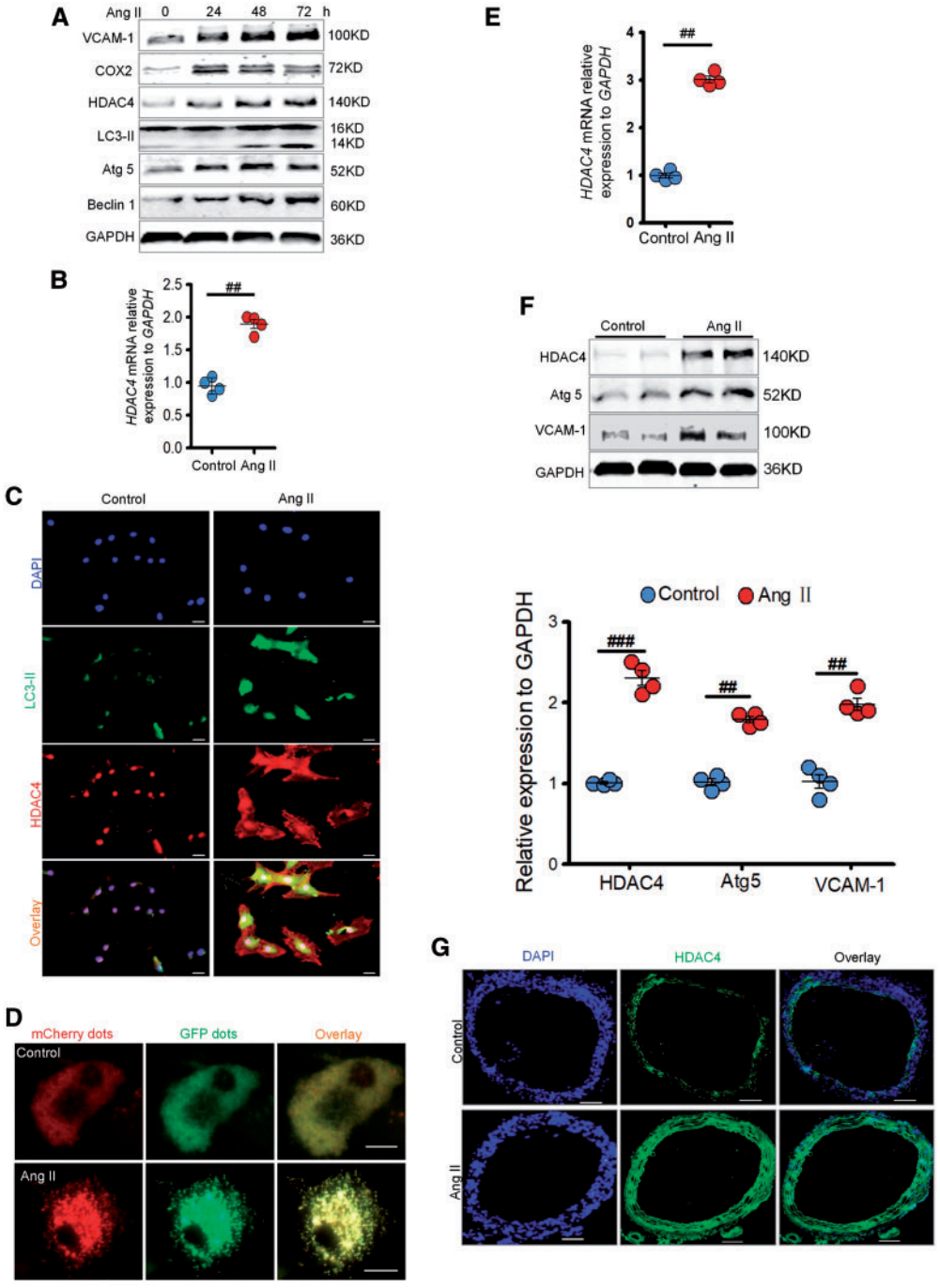

Figure 1 Ang II induces autophagy in cultured RAECs and mice with enhanced HDAC4 expression. (*A*) RAECs were treated with Ang II (2 μM) for the indicated times, immunoblot analysed for HDAC4, VCAM-1, COX2, LC3- II, Atg5, and Beclin 1. Experiments were repeated at least three times to obtain reproducible results. (*B*) RAECs were treated with Ang II (2 μM) for 48 h, mRNA level of HDAC4 by qRT-PCR in control and Ang II-treated cells, HDAC4 expression was normalized against GAPDH expression. Data are presented as mean ± SEM of four independent experiments, $^{\#\#}P < 0.01$. (*C*) RAECs were treated with 2 μM Ang II for 48 h, and were double immunostained with anti-LC3B antibodies (green), HDAC4 (red), and nuclei were stained with DAPI (blue) and analysed by fluorescence microscopy, scale bars: 50 μm. (*D*) RAECs were treated with 2 μM Ang II for 48 h, representative images of fluorescent punctate foci of GFP-LC3, scale bars: 20 μm. (*E–G*) The mice were subjected to a 28-day infusion of vehicle or Ang II (1.5 mg/kg/day). mRNA level of HDAC4 by qRT-PCR in aortic vessels from seven control and Ang II-infused mice; Data are presented as mean ± SEM of four experiments in each group; $^{\#\#}P < 0.01$ (*E*). Protein level of HDAC4, VCAM-1, and Atg5 was assessed by Western blot in aortic great vessels in Ang II-infused mice compared to control mice, quantitative data are presented as mean ± SEM of four experiments; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (*F*). The aortic great vessels were immunostained with anti-HDAC4 (green), and nuclei were stained with DAPI (blue) and analysed by fluorescence microscopy, scale bar: 200 μm (*G*).

3.2 Silencing of HDAC4 and inhibitor Taq reverses Ang II-induced RAECs inflammation

As shown in *Figure 1A*, Ang II significantly induced HDAC4 expression in RAECs in a time-dependent manner. To further investigate the role of HDAC4 on RAECs function, gene silencing of HDAC4 by siRNA-HDAC4 was used in this study. We found that Ang II-induced the production of pro-inflammatory mediators, such as IL-6, VCAM-1, COX2, and iNOS were attenuated by HDAC4 knockdown (*Figure 2A*). Moreover, to further determine whether HDAC4 mediates inflammation response, we used Taq, an inhibitor of HDAC4. The pro-inflammatory mediators were significantly decreased in Ang II/Taq-co-treated RAECs, but increased in Ang II-treated cells (*Figure 2B*). Immunofluorescence analysis further confirmed that treatment with siRNA HDAC4 or Taq also resulted in decreased HDAC4 and VCAM-1 protein expression (*Figure 2C*). Our study showed that Ang II induced inflammatory response, which was alleviated by HDAC4 deficiency.

3.3 Inhibition of autophagy reverses Ang II-induced RAECs inflammation

A growing body of studies has suggested that autophagy contributes to endothelial dysfunction in vascular biology, and autophagy is connected to the expression of inflammatory cytokines. As shown in *Figure 1*, Ang II significantly induced autophagy in RAECs in a time-dependent manner. To further investigate the role of autophagy on RAECs function, gene silencing of LC3-II by siRNA was used. We found that Ang II-induced production of pro-inflammatory mediators was attenuated by LC3-II knockdown compared with siRNA control (*Figure 3A* and *B*). Moreover, we measured the expression of pro-inflammatory mediators following co-treatment with Ang II and the autophagy inhibitor LY294002 or 3-MA. The expression of IL-6, VCAM-1, and COX2 was decreased in LY294002 or 3-MA-treated cells (*Figure 3C* and *D*). These data indicated that inhibition of autophagy reversed the effects of Ang II-induced inflammation in RAECs.

3.4 Silencing of HDAC4 and inhibitor Taq reverses Ang II-induced autophagy in RAECs

In order to analyse a potential role of HDAC4 in an autophagy-mediated inflammation in RAECs, HDAC4 was silenced by siRNA transfection, RAECs followed by a treatment with Ang II, siRNA clearly attenuated HDAC4 protein levels (*Figure 4A*). Intriguingly, knockdown of HDAC4 could block the Ang II-induced increase in Beclin 1, Atg5, and LC3-II (*Figure 4A*). Immunofluorescence double staining also showed that the co-expressions of HDAC4 and LC3-II upon Ang II induction disappeared when knockdown of HDAC4 (*Figure 4B*). Besides, Ad-GFP-LC3 punctate formation assays further confirmed that HDAC4 knockdown inhibited Ang II-induced autophagy activation in RAECs (*Figure 4C*).

Next, to further clarify the role of HDAC4 on activating autophagy, we used HDAC4 inhibitor Taq to examine the beneficial effect after the onset of autophagy. As shown in *Figure 4D*, HDAC4 inhibitor Taq decreased autophagy markers Beclin 1, LC3-II, and Atg5 levels in RAECs under Ang II treatment. Additionally, Ad-GFP-LC3 punctate formation further confirmed that Taq inhibited Ang II-induced autophagy in RAECs (*Figure 4E*). All these results supported the idea that HDAC4 deficiency could alleviate activation of autophagy in Ang II-induced RAECs.

3.5 Knockdown of HDAC4 ameliorates Ang II-induced vascular inflammation *in vivo*

VSMCs are also a dominant cellular constituent of arteries and a critical determinant of vascular disease. VSMCs were treated with Ang II (2 μM) for the indicated time, HDAC4, and the inflammatory mediator VCAM-1 expression were markedly increased, with up-regulated autophagy makers LC3-II and Atg5 (*Figure 5A*). Furthermore, using siRNA-HDAC4 to silence HDAC4 expression, Ang II-induced production of pro-inflammatory mediators, such as IL-6, VCAM-1, and iNOS, were attenuated by HDAC4 knockdown, whilst Atg 5 expression also decreased (*Figure 5B*). These results showed that Ang II induced HDAC4 expression in RAECs as well as VSMCs.

To address the role of HDAC4 and autophagy in Ang II-induced vascular inflammation *in vivo*, lentivirus-mediated knockdown of HDAC4 and autophagy inhibitor 3-MA were applied in mice model. Mice were subjected to Ang II-infusion, followed by treatment of lentivirus encoding control or HDAC4 short hairpin RNA (KD HDAC4) (*Figure 5C*) or 3-MA. As shown in *Figure 5D*, Ang II-induced VCAM-1, IL-6, and iNOS expression were attenuated by HDAC4 knockdown, as well as attenuated by 3-MA. Importantly, HDAC4 knockdown was accompanied by decreased expression of autophagy markers including Beclin 1, Atg5, LC3-II, which was further confirmed by the fact that gene silencing of HDAC4 prevented the onset of autophagy after Ang II-infusion mice (*Figure 5D*). Immunofluorescence double staining analysis also showed HDAC4 knockdown was accompanied by decreased expression of LC3-II (*Figure 5E*). Furthermore, immunofluorescence analysis of the cross-sectional area of blood vessels clarified that 3-MA could down-regulate IL-6 protein expression level (*Figure 5F*), and the increased pro-inflammatory mediator iNOS protein was inhibited after gene silencing of HDAC4 (*Figure 5G*), which further confirmed the fact that gene silencing of HDAC4 ameliorated Ang II-induced vascular inflammation at least partially by mediating autophagy.

3.6 FoxO3a is required for Ang II-induced autophagy and HDAC4 controls acetylation of transcription factor FoxO3a

It is reported that Sp-1, a zinc finger protein belonged to the Specificity Factor (Sp) family of transcriptional factors, could mediate HDAC4 expression.[30] To investigate mechanisms that might control expression of HDAC4, we treated cells with mithramycin, a Sp-1 inhibitor. We found that Ang II-induced HDAC4 expression was suppressed by mithramycin in concentration-dependent manner, whilst mithramycin also inhibited the expression of VCAM-1 induced by Ang II (*Figure 6A*). These data suggested that transcriptional factor Sp-1 was involved in Ang II-induced HDAC4 expression in RAECs.

To evaluate whether FoxO3a, a major isoform of the FoxO family, is required for Ang II-induced autophagy, RAECs were transduced with FoxO3a siRNA. Knockdown of FoxO3a decreased LC3-II, Atg5, and Beclin 1 expression in the presence of Ang II, indicating that endogenous FoxO3a is required for Ang II-induced autophagy (*Figure 6B*). FoxO3a acetylation was determined by Western blot with anti-FoxO3a antibody after immunoprecipitation with anti-acetyl-lysine antibody. Treatment of Ang II did not affect protein levels of FoxO3a at various times (*Figure 6C*); however, FoxO3a acetylation was significantly decreased by Ang II treatment for 48 h (*Figure 6D*). Meanwhile, FoxO3a acetylation of vascular tissues from Ang II-infusion mice was significantly decreased, compared with control mice (*Figure 6D*). Also, we performed Western blot using anti-FoxO3a antibody after immunoprecipitation using anti-acetyl-lysine

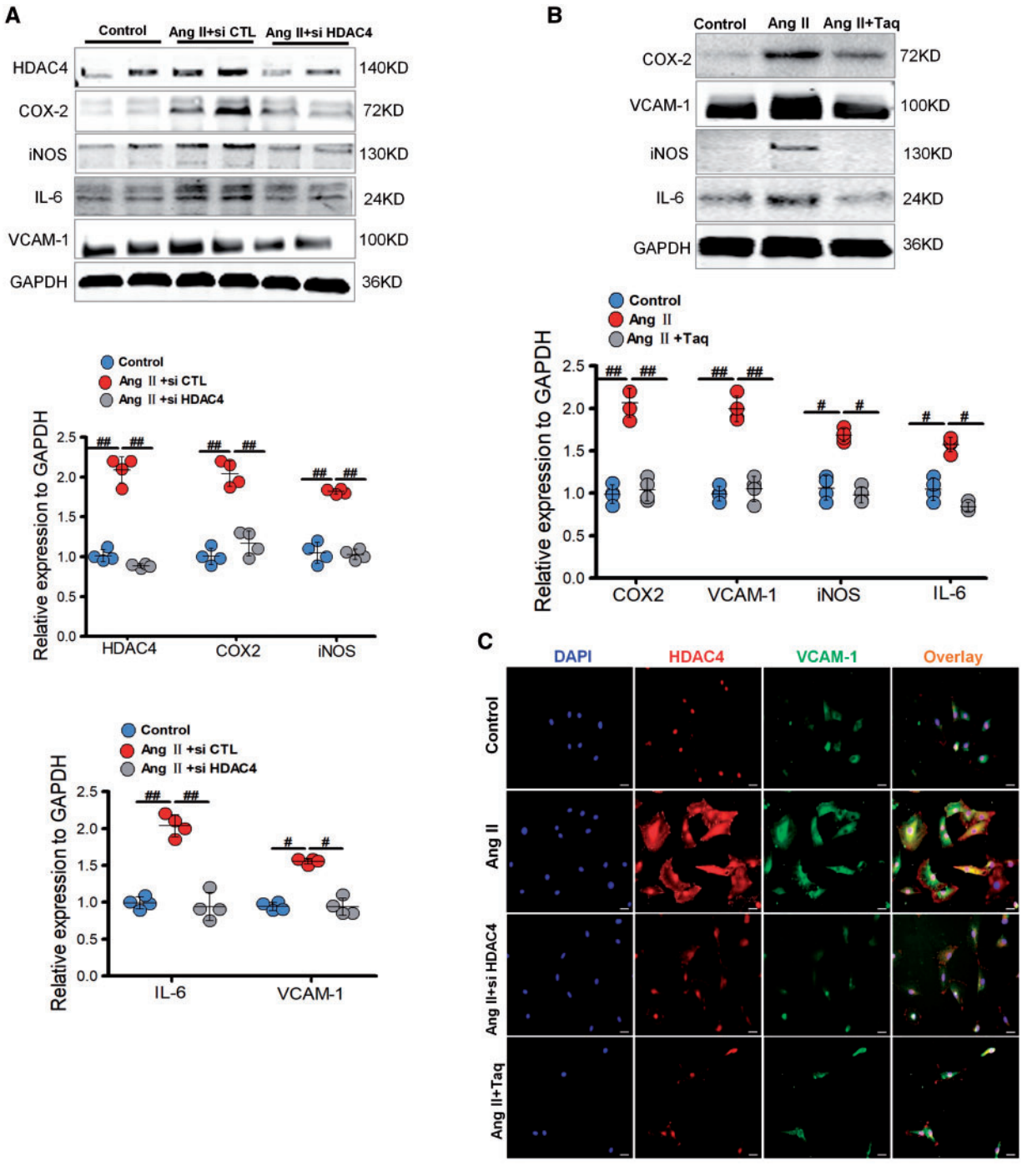

Figure 2 Inhibition of HDAC4 reduces inflammation response in Ang II-induced RAECs. (*A*) Effects of HDAC4 knockdown on Ang II-induced inflammation mediators protein. RAECs were transfected with control (si CTL) or HDAC4 small interfering RNA (si HDAC4), then were treated with 2 μM Ang II for 48 h. Expression of HDAC4, COX2, iNOS, IL-6, and VCAM-1 was determined by Western blotting; Data are presented as mean ± SEM of four independent experiments; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. (*B*) Effects of HDAC4 inhibitor tasquinimod (Taq) on Ang II-induced inflammation mediators protein. After RAECs were pretreated for 1 h with 10 μM Taq, and then were treated with 2 μM Ang II for 48 h. Expression of COX2, iNOS, IL-6, VCAM-1 was determined by Western blotting; Data are presented as mean ± SEM of four independent experiments; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. (*C*) After RAECs were pretreated with 10 μM Taq or transfected with siRNA HDAC4, and were treated with 2 μM Ang II for 48 h. The cells were double immunostained with anti-VCAM-1 antibodies (green), HDAC4 (red), and nuclei were stained with DAPI (blue) and analysed by fluorescence microscopy; scale bars: 50 μm.

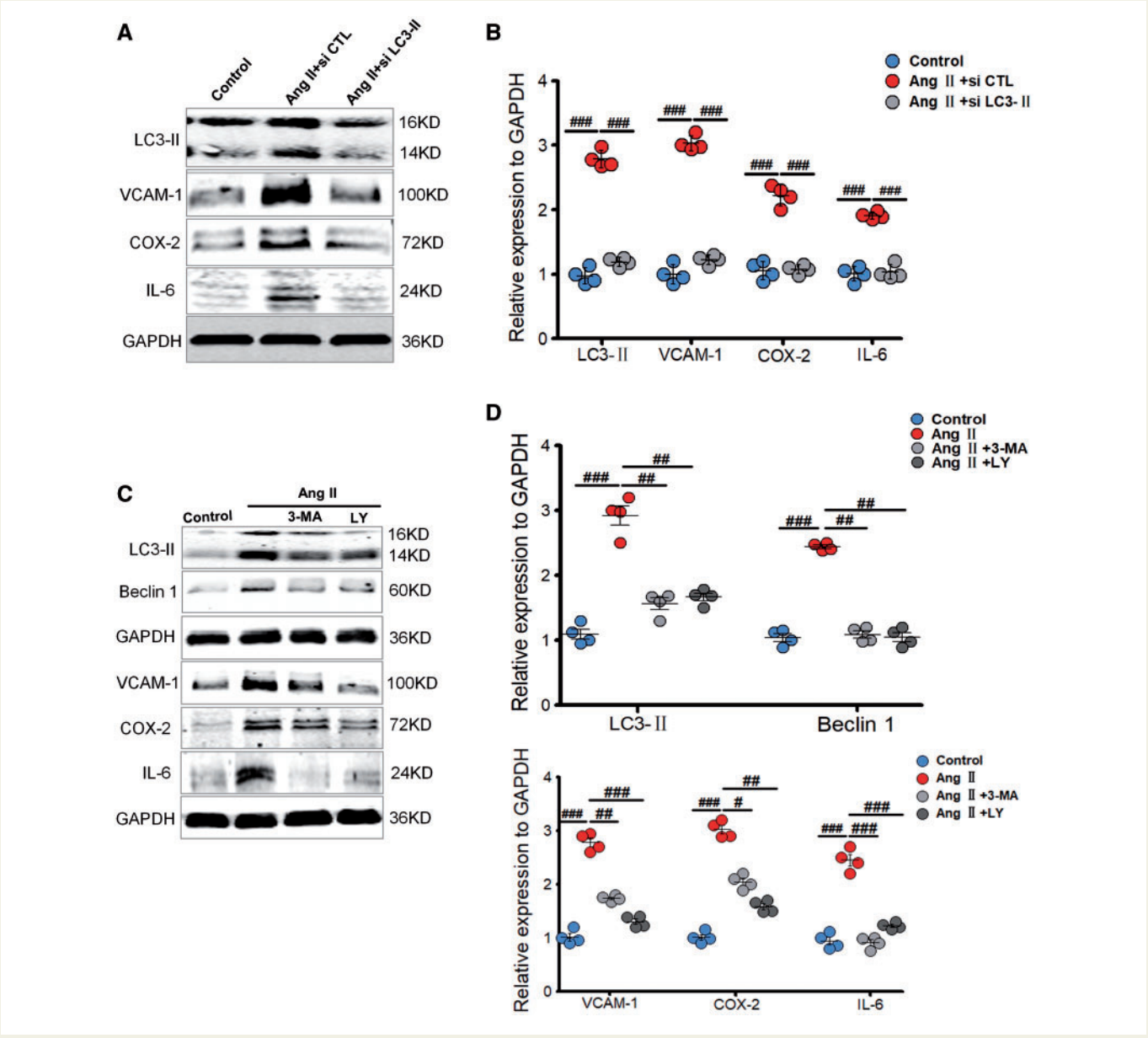

Figure 3 Inhibition of autophagy reduces inflammation response in Ang II-induced RAECs. (*A*, *B*) Effects of LC3-II knockdown on Ang II-induced inflammation mediators protein. After RAECs were transfected with control (si CTL) or LC3-II small interfering RNA (si LC3-II), they were treated with 2 μM Ang II for 48 h. Expression of LC3-II, COX2, IL-6, and VCAM-1 was determined by Western blotting. Representative immunoblots were presented (*A*); and quantified in (*B*). (*C*, *D*) Effects of autophagy inhibitor 3-methyladenine (3-MA) and LY294002 (LY) on Ang II-induced inflammation mediators protein. After RAECs were pretreated with 3-MA or LY for 1 h, they were treated with 2 μM Ang II for 48 h. Expression of LC3-II, Beclin 1, COX2, IL-6, and VCAM-1 was determined by Western blotting. Representative immunoblots were presented (*C*); Scatter plots showed quantitative analysis (*D*). All data are presented as mean ± SEM of four independent experiments; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

antibody in transfected RAECs with siRNA HDAC4. Results showed that FoxO3a acetylation was increased by HDAC4 knockdown (*Figure 6E*).

As previous studies indicated that FoxO1 acetylation directly disrupts its ability to bind DNA,[31,32] we examined the association of FoxO3a with autophagy gene promoters. Ang II treatment resulted in increased ChIP of endogenous FoxO3a with the *MAP1LC3B* (LC3-II) and *Atg5* promoters for various time (*Figure 6F*), consistent with increased FoxO3a acetylation and loss of DNA binding.

4. Discussion

Several reports demonstrated that HDACs are unique and attractive therapeutic targets in clinical research. In this study, we demonstrated that HDAC4 deficiency was associated with a reduced expression of pro-inflammatory markers, which correlated with attenuation of autophagy in Ang II-induced RAECs. In addition, we found that HDAC4 knockdown attenuated cell autophagy in Ang II-stimulated RAECs mainly through up-regulating acetylation-modified FoxO3a to inhibit autophagy.

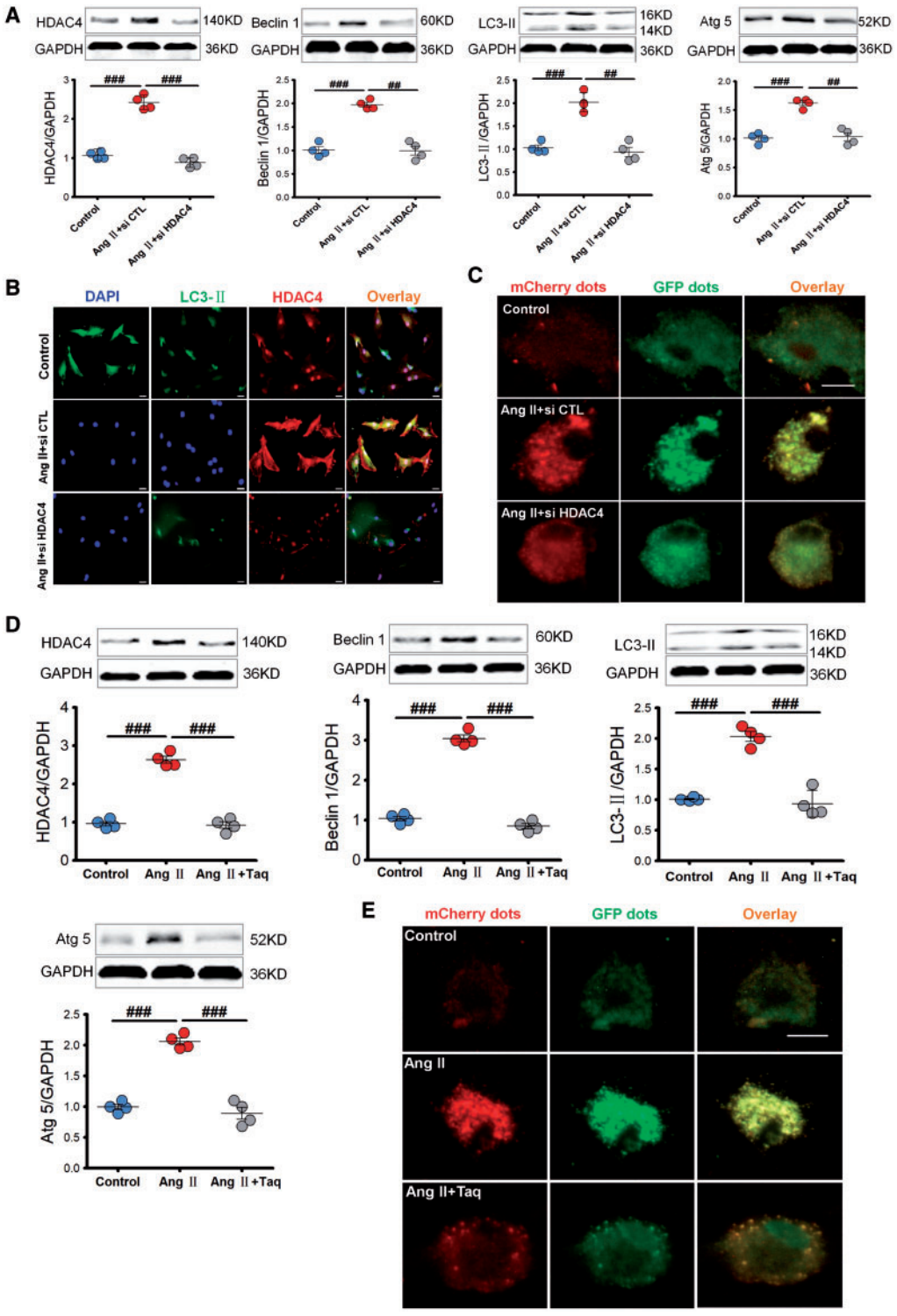

Figure 4 Knockdown of HDAC4 and tasquinimod (Taq) reduces autophagy in Ang II-induced RAECs. (*A*) After RAECs were transfected with control (si CTL) or HDAC4 small interfering RNA (si HDAC4), then were treated with 2 μM Ang II for 48 h. Expression of HDAC4, Beclin 1, LC3-II, and Atg 5 was determined by Western blotting; Quantitative data are presented as mean ± SEM of four independent experiments; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. (*B*) The cells were double immunostained with anti-LC3-II antibodies (green), HDAC4 (red), and nuclei were stained with DAPI (blue) and analysed by fluorescence microscopy; scale bars: 50 μm. (*C*) HDAC4 knockdown reduced punctate foci of GFP-LC3 in Ang II-induced RAECs. Fluorescence microscopy images of punctate foci of GFP-LC3 in the si CTL and siRNA HDAC4 were shown, scale bars: 20 μm. (*D*, *E*) HDAC4 inhibitor tasquinimod (Taq) inhibited Ang II-induced autophagy protein expression. After RAECs were pretreated for 1 h with Taq, then were induced for 48 h with 2 μM Ang II, the expression of HDAC4, Beclin 1, LC3-II, and Atg5 was determined by Western blotting; Data are presented as mean ± SEM of four independent experiments; $^{\#\#\#}P < 0.001$ (*D*). (*E*) HDAC4 inhibitor Taq reduced punctate foci of GFP-LC3 in Ang II-induced RAECs. Fluorescence microscopy images of punctate foci of GFP-LC3 in the absence or presence of Taq were shown, scale bars: 20 μm.

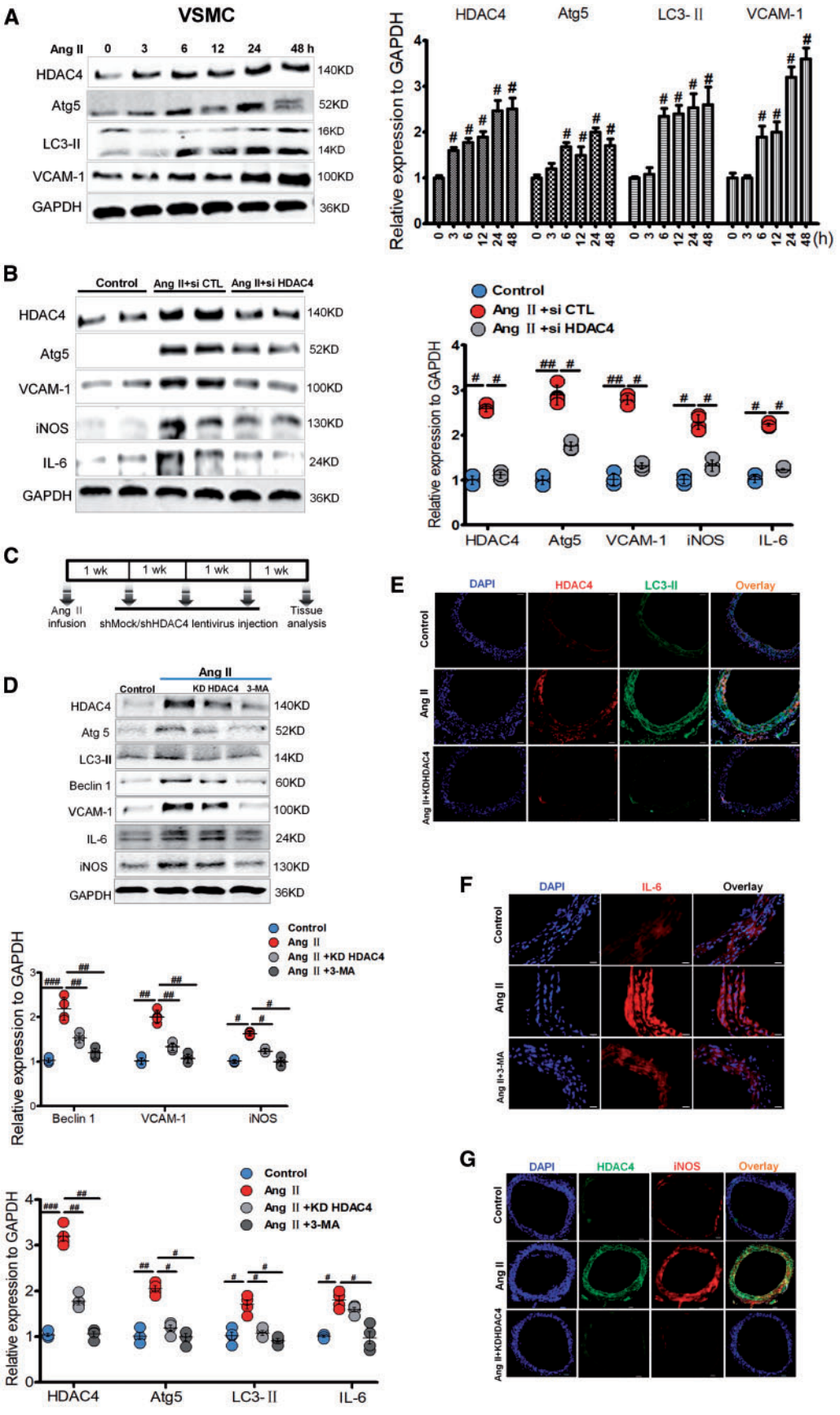

Figure 5 HDAC4 is involved in Ang II-induced VSMC and knockdown HDAC4 or 3-MA prevents vascular inflammation in Ang II-infusion mice. (*A*, *B*) HDAC4 is involved in Ang II-induced VSMCs. VSMCs were treated with Ang II (2 μM) for the indicated times, immunoblot analysed for HDAC4, VCAM-1,

Findings provided strong evidence about the uniquely specific function of HDAC4 in eliciting autophagy in response to vascular inflammation, decreased acetylation FoxO3a enhancing DNA-binding activity and association with Atg5 and LC3-II gene promoters to induce the autophagic process, furthermore, the abnormal occurrence of autophagy contributed to the pathological vascular inflammation in Ang II mediated-cardiovascular diseases. It is suggested that HDAC4 might be at least partly responsible for the vascular inflammation in Ang II infusion mice through eliciting autophagic process.

Ang II, the most potent component of the renin-angiotensin system, plays a central role in cardiovascular diseases.[33] Moreover, Ang II mediates the inflammation-associated cardiomyopathy and carotid arterial remodelling,[34] *et al.* Thus, we used Ang II to induce RAECs inflammation that may provide an approach to make related cardiovascular disorders. HDACs are lysine de-acetylases, which are classified into three main groups based on their homology to yeast proteins, HDAC4 belongs to group II subgroup A of the family.[35] HDACs are emerging as targets for the treatment of several diseases, including cardiac hypertrophy, and heart failure.[36,37] In this study, we showed that HDAC4 is required for Ang II-induced inflammation in RAECs and mice, such that inhibition of HDAC4 activity or knockdown of HDAC4 was capable of preventing or reversing pathological vascular inflammation elicited by Ang II. According to literature, transcriptional factor Sp-1 could mediate HDAC4 expression, here, we also determined up-regulated HDAC4 by Ang II through Sp-1 activation. It is been previously reported that HDAC4 mediates vascular inflammation through ROS-dependent NF-κB activation in TNF-α-induced VSMCs.[25] However, the mechanism by which HDAC4 reduction delays Ang II-induced endothelial inflammation is unknown. Thus, further investigation is required to address this issue.

Autophagy, a self-digestion and dynamic process, is involved in long-lived proteins and dysfunctional organelles degradation.[16,38] Autophagy maintenance of cellular homeostasis is essential for cell survival, and the altered autophagy has been demonstrated in various pathological alterations.[16] Although the beneficial effects of autophagy in the pathological progression of cardiovascular diseases have been demonstrated by some studies, in some cases, pharmacological inhibition of autophagy after some insults has protective effects.[39,40] Our data suggested that the autophagic process was over-activated in RAECs and aortic vessels of mice after Ang II treatment. We expected that the over-activated autophagy after Ang II contributes to the pathological progression of vascular inflammation. And, in the present study we found that 3-MA treatment from the 1st day and end on the 4th weeks post Ang II infusion significantly blocked autophagy activation and improved the inflammation alterations *in vivo*, which further demonstrated this point. Furthermore, knocking down LC3-II diminished Ang II-induced RAECs inflammation and blunted expression of inflammation markers. In aggregate, these data lend support that augmented autophagy is a necessary component of vascular inflammation under Ang II stress conditions. Therefore, an early intervention aimed at the reduction in autophagosomes accumulation has the protective effects after Ang II infusion mice. Importantly, we found that HDAC4 regulated RAECs inflammation by controlling the over-activated autophagy. The inhibition of HDAC4 activity or knockdown of HDAC4 could reduce over-activated autophagy by Ang II, further to inhibit inflammation response.

Based on the above findings, we had a deep thinking about how HDAC4 modulates autophagy. Multiple FoxO family transcription factors have many overlapping functions. Since FoxO3 also stimulates autophagy,[41] FoxO3a may be involved in HDAC4 mediated autophagy in RAECs. Here, we showed that FoxO3a is de-acetylated upon Ang II in HDAC4-dependent manner, further enhancing FoxO DNA-binding activity and associated with autophagy gene promoters, this is consistent with previous studies that FoxO acetylation directly disrupts its ability to bind DNA.[32] FoxO3 remains acetylated, indicating that interaction with HDAC4 is required for FoxO3 to be de-acetylated in the presence of Ang II. Although our studies demonstrated a key role for HDAC4 in the control of FoxO3a acetylation following Ang II in RAEC, FoxO has also been previously shown to be a target of SIRT1 in a number of cell types, particularly defined in muscle and cardiac myocytes.[42,43] Our results suggested that FoxO3a-induced autophagy-related genes including LC3 and Atg5 modulate processes of autophagy. These results were consistent with previous studies that FoxO3 regulated autophagy in skeletal muscle cells by transcriptional activation of genes that were involved in autophagosome formation, including LC3, Atg12l, Atg4, and Beclin1.[41,44]

Perhaps most unexpectedly, the results here suggested that HDAC4 in Ang II treated models actively contributed to the vascular inflammation, which may result from the critical role of FoxO3a in over-activated autophagic process in these vascular inflammation states (see working model in *Figure* 7). Remarkably, shRNA-mediated suppression of HDAC4 function led to a dramatic reduction of inflammation response *in vivo* animal model and *in vitro* cultured cell model. If extended to human studies, these results suggested that small molecules that inhibit HDAC4 may be useful to vascular inflammation, so the potential utility of HDAC4 inhibitors for the treatment of vascular diseases associated inflammation warrants investigation.

LC3-II, and Atg5; Data are presented as mean ± SEM of four independent experiments; $^{\#}P < 0.05$ (*A*). VSMCs were transfected with control (si CTL) or HDAC4 small interfering RNA (si HDAC4), then were treated with 2 μM Ang II for 48 h. Expression of HDAC4, Atg5, iNOS, IL-6, and VCAM-1 was determined by Western blotting; Data are presented as mean ± SEM of four independent experiments; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (*B*). (*C–G*) knockdown HDAC4 or autophagy inhibitor 3-MA prevents vascular inflammation in Ang II-infusion mice. (*C*) Time table for the animal experiment a using shRNA lentivirus for HDAC4 (KD HDAC4) or control shRNA lentivirus (shMock) injection. (*D*) The mice were infused for 28 d with vehicle or Ang II (1.5 mg/kg/day), and were treated by 20 mg/kg/day 3-MA (Ang II + 3-MA) or transfected by shRNA of HDAC4 (Ang II + KD HDAC4). Expression of HDAC4, Beclin 1, LC3-II, Atg5, and VCAM-1 from aortic great vessels was determined by Western blotting, data are presented as mean ± SEM, four samples of each group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. (*E*) HDAC4 knockdown reduced LC3-II expression. The aortic great vessels were double immunostained with anti-HDAC4 (red), LC3-II (green), and nuclei were stained with DAPI (blue) and analysed by fluorescence microscopy, scale bars: 200 μm. (*F*) 3-MA reduced IL-6 expression. The aortic great vessels were immunostained with anti-IL-6 (red) and nuclei were stained with DAPI (blue) and analysed by fluorescence microscopy, scale bars: 100 μm. (*G*) HDAC4 knockdown reduced iNOS expression. The aortic great vessels were double immunostained with anti-HDAC4 (green), iNOS (red), and nuclei were stained with DAPI (blue) and analysed by fluorescence microscopy, scale bars: 200 μm.

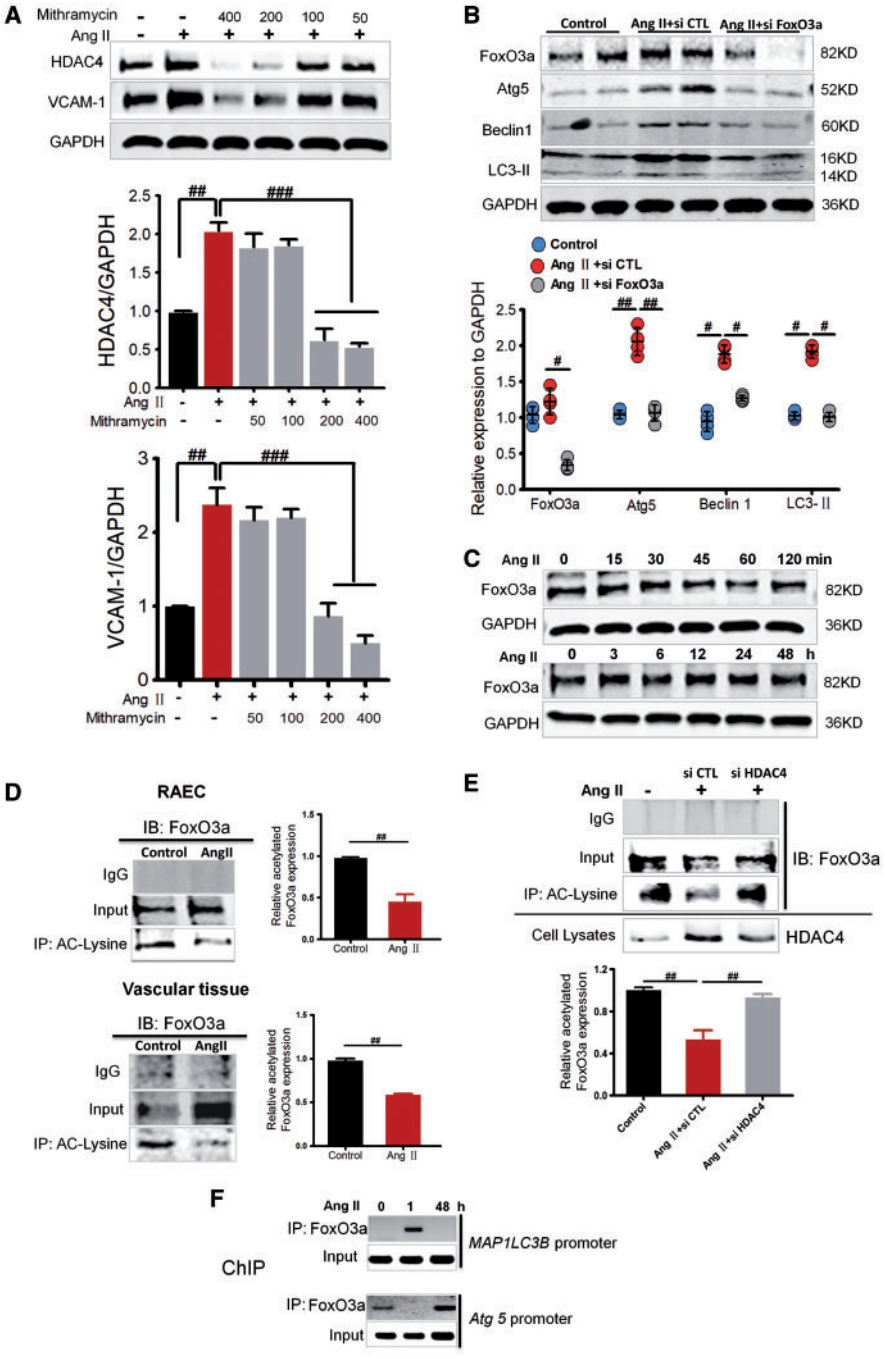

Figure 6 FoxO3a is required for Ang II-induced autophagy and HDAC4 controls FoxO3a acetylation. (*A*) Sp-1 was involved in Ang II-induced HDAC4 expression. After RAECs were pretreated for 1 h with Sp-1 inhibitor mithramycin for indicated concentration, then were induced for 48 h with 2 μM Ang II, the expression of HDAC4, and VCAM-1 was determined by Western blotting. Data are presented as mean ± SEM of four independent experiments; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. (*B*) FoxO3a was required for Ang II-induced autophagy. RAECs transfected with control (si CTL) and FoxO3a siRNA, and were treated with 2 μM Ang II for 48 h and immunoprecipitated with anti FoxO3a, Atg5, and LC3- II antibody. Data are presented as mean ± SEM of four independent experiments; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. (*C*) Treatment of Ang II did not affect protein levels of FoxO3a. RAECs were treated with 2 μM Ang II for the indicated time and immunoprecipitated with anti-FoxO3a antibody. (*D*) RAECs treated with Ang II and Ang II-infused mice decreased FoxO3a acetylation. RAECs were treated with 2 μM Ang II for 48 h, acetylation level of FoxO3a was analysed as described in Materials and Methods. Acetylation level of FoxO3a of the aortic great vessels from control and Ang II-infused mice was analysed. Data are presented as mean ± SEM of four experiments; $^{\#\#}P < 0.01$. (*E*) HDAC4 knockdown recovered Ang II -downregulating FoxO3a acetylation level. RAECs were transfected with Control and HDAC4 siRNA following 2 μM Ang II treatment, and acetyl FoxO3a was analysed as described above. Data are presented as mean ± SEM of four independent experiments; $^{\#\#}P < 0.01$. (*F*) Ang II induced FoxO3a binding to the *MAP1LC3B* or *Atg5* promoter. ChIP analysis assessed on RAEC for FoxO3a on *MAP1LC3B* or *Atg5* promoters following 1 h and 48 h treatment with 2 μM Ang II.

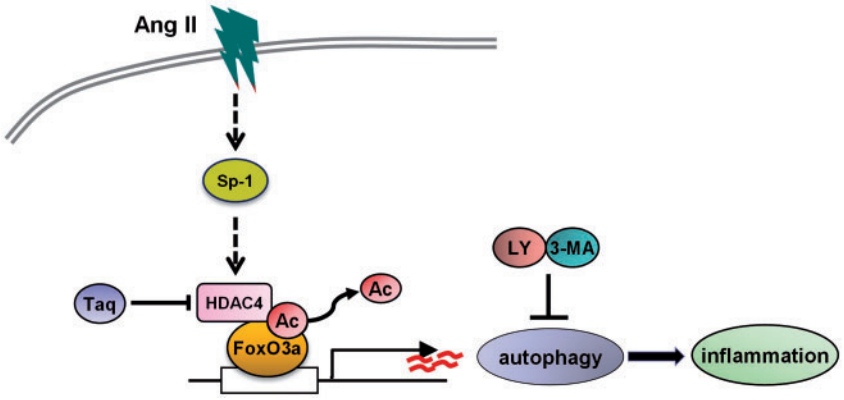

Figure 7 Working model for HDAC4 mediated autophagy in Ang II-induced vascular inflammation. Vascular cells have lower level of HDAC4 protein, after Ang II induced vascular cells or infused mice, HDAC4 protein level increases by Sp-1 modulation, leading to the acute transcriptional induction of autophagy genes via de-acetylation, and activation of FoxO3a transcription factor, subsequently causes vascular inflammation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgement

The authors would like to thank Fan Yi (Shandong University) for gift shRNA HDAC4 plasmid.

Conflict of interest: none declared.

Funding

This work was supported by grants from National Natural Science Foundation of China (No. 81673428; 81330080), a key laboratory program of the Education Commission of Shanghai Municipality (No. ZDSYS14005), and FDCT grant from the Science and Technology Development Fund of Macao (Project code: 039/2016/A and 055/2016/A2).

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