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Original Paper

Endothelial-Specific Overexpression of Histone Deacetylase 2 Protects Mice against Endothelial Dysfunction and Atherosclerosis

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Key Words
Atherosclerosis • Endothelial Dysfunction • HDAC2 (Histone deacetylase 2) • OxLDL (Oxidized low-density lipoprotein) • PCSK9 (Proprotein convertase subtilisin/kexin type 9) • PWV (Pulse wave velocity)

Abstract
Background/Aims: We recently described a novel regulatory role for histone deacetylase 2 (HDAC2) in protecting endothelial cells from oxidized low-density lipoprotein (OxLDL)-induced injury. In this study, we examined the effects of endothelial-specific HDAC2 overexpression on endothelial-dependent vasorelaxation and atherogenesis *in vivo*. ***Methods:*** Endothelial-specific HDAC2-overexpressing transgenic mice (HDAC2-Tg) were generated under control of the Tie2 promoter. An atherosclerosis model was produced by injecting HDAC2-Tg and wild-type (WT) mice with adeno-associated virus encoding a PCSK9 gain-of-function mutant under control of a liver-specific promoter and feeding them a high-fat diet for 12 weeks. Aortic stiffness *in vivo* was determined by measuring pulse wave velocity. Wire myography was used to measure endothelium dependent (acetylcholine) and independent (sodium nitroprusside) relaxation in isolated mice aortas. Atherosclerotic plaque burden in aortas was determined by Oil Red O staining and protein expression was determined by western blotting. ***Results:*** At baseline, HDAC2-Tg mice had normal mean arterial blood pressure (MAP) and body weight, but pulse wave velocity (PWV), an inverse measure of vascular health and stiffness, was decreased, suggesting that their vessels were more compliant. Moreover, basal nitric oxide production

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was enhanced in the vessels of HDAC2-Tg mice as compared to that in WT controls, although no significant differences in acetylcholine (endothelial component)- or sodium nitroprusside (non-endothelial component)-mediated relaxation were observed. However, after exposure to OxLDL, aortas from HDAC2-Tg mice exhibited greater acetylcholine-induced relaxation than did those from WT mice. Thus, endothelial-specific vasodilator production was enhanced despite oxidative injury. Atherosclerosis induction in WT mice led to a significant increase in PWV, but in HDAC2-Tg mice, PWV and MAP remained unchanged. Further, aortic rings from HDAC2-Tg exhibited better endothelial-dependent vascular relaxation than did those from WT mice, but not when treated with nitric oxide synthase inhibitor L-NAME. Finally, plaque burden, determined by Oil red O staining, was significantly increased in WT, but not HDAC2-Tg mice, subjected to the atherogenic model. Deletion of endothelial HDAC2 led to impaired endothelial cell-dependent vascular relaxation and increased PWV, compared with those in littermate controls. ***Conclusion:*** HDAC2 protects against endothelial dysfunction and atherogenesis induced by oxidized lipids. Hence, overexpression or activation of HDAC2 represents a novel therapy for endothelial dysfunction and atherosclerosis. HDAC2-Tg mice provide an opportunity to determine the role of endothelial HDAC2 in vascular endothelial homeostasis.

Introduction

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in the United States and accounts for 40% of all deaths. The vascular endothelium plays a major role in the regulation of vascular homeostasis by modulating vasomotor tone, growth and migration of vascular smooth muscle cells (SMCs), endothelial permeability, and inflammatory cell adhesion. Endothelial dysfunction, as measured by impairment of the aforementioned protective mechanisms, causes abnormalities in the arterial wall [1] and is recognized as the earliest precursor of the atherogenic process. In addition to its well-known role in foam cell formation [2], oxidized low-density lipoprotein (OxLDL) induces pro-atherosclerotic effects in endothelial cells (ECs) by altering cell surface adhesion molecule expression [3, 4], stimulating apoptosis [5, 6], inducing superoxide anion formation [7], and impairing endothelial nitric oxide (NO) and vascular protection [8, 9]. Decreased NO production and activity manifest as impaired vasodilatation and may be the earliest signs of EC dysfunction and atherogenesis [2]. NO is produced by the enzyme endothelial nitric oxide synthase (eNOS), which uses L-arginine as a substrate. L-arginine is also a substrate for arginase (Arg), which converts it to L-ornithine and urea. An established paradigm in NO biology is that Arg reciprocally regulates NOS activity by substrate competition. Thus, Arg activity can effectively inhibit NO-dependent processes by depleting the substrate pool available for NO biosynthesis. Given the critical role of Arg2 in regulation of endothelial function, mechanisms that regulate its expression and activity are of great interest, yet our understanding of these mechanisms is incomplete

We have identified the mechanism that controls the expression of Arg2 in endothelial cells in the context of oxidative injury. Histone deacetylase (HDAC)-2 suppresses transcription of endothelial Arg2, and therefore contributes to maintain proper vascular endothelial homeostasis. Transcriptional control of genes by HDACs may occur by removal of acetyl groups from histones, thereby altering chromatin accessibility, but other mechanisms of action are also known. Global HDAC inhibition results in complex and selective shifts (in both directions) in the transcription of several EC genes, including eNOS [10, 11]. Moreover, our data suggest that this enzyme may indeed be a critical node in the modulation of endothelial health.

Although studies in cell culture and isolated vessels support the notion that HDAC2 protects endothelium, no *in vivo* data have illuminated its function. Our current study utilized recently generated endothelial-specific HDAC2-overexpressing mice and a novel strategy for inducing atherogenesis and endothelial dysfunction to ascertain whether HDAC2 is protective of the endothelium.

Materials and Methods

Animals

All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee at The Johns Hopkins University School of Medicine. Apolipoprotein E deleted (*ApoE*$^{-/-}$) mice were purchased from Jackson Laboratories. HDAC2 transgenic (HDAC2-Tg) mice were generated at The Transgenic Core Laboratory at Johns Hopkins University by pronuclear microinjecting of embryos (BAC C57BL/6J) with linearized Tie2 promoter-driven HDAC2 plasmid. Human HDAC2 was cloned into pSPTg.T2FpAXK (gift from Dr. Thomas Sato; Addgene plasmid #35962 ; http://n2t.net/addgene:35962 ; RRID:Addgene_35962) at a NotI and HindIII site. This construct contains an upstream Tie2 promoter and a downstream Tie2 enhancer (Supplementary Fig. S1, upper panel – for all supplementary material see www.cellphysiolbiochem.com). To facilitate the cloning, the HindIII site present in the open reading frame of HDAC2 plasmid was subjected to a silent mutation (GCT to GCG) (Supplementary Fig. S1A). Using an HDAC activity assay kit (Promega), we compared HDAC activity between WT HDAC2- and mutant HDAC2-expressing 293 HEK cells and found no significant differences (Supplementary Fig. S1B). Finally SalI-digested DNA fragments (Supplementary Fig. SIC) were injected into the pronuclei of B6SJLF2 mouse embryos (The Transgenic Core Laboratory, Johns Hopkins University) [12]. Endothelial-specific HDAC2 KO mice (HDAC2ecKO) was created by crossing floxed HDAC2 mice and VE-Cadherin controlled Cre-recombinase mice purchased from Jackson (#17968 B6:129-Tg(Cdh5-Cre)1Spe/J).

Reagents

OxLDL was purchased from Intracel Co. (Frederick, MD). Y-27632 and O-phenanthroline were purchased from Calbiochem (Darmstat, Germany). Unless otherwise stated, all other reagents were obtained from Sigma (St. Louis, MO). eNOS (sc-376542) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX), HDAC2 polyclonal antibody was purchased from Abgent (Cat#AP9762), and GAPDH antibody was purchased from Proteintech.

Atherosclerosis model

Male C57BL/6 wild-type (WT) and HDAC2-Tg mice, 8-12 weeks old, were injected with adeno-associated virus (AAV) encoding PCSK9 gain-of-function mutant (pAAV/D377Y-mPCSK9) or PBS and were fed a high-fat rodent diet containing 1.25% cholesterol (Research Diet, New Brunswick NJ, USA) for 12 weeks. Age-matched WT control mice (C57BL/6) were fed a normal diet. After 12 weeks, mice were euthanized and aortic tissue was used in the experiments described. pAAV/D377Y-mPCSK9 was a gift from Jacob Bentzon (Addgene plasmid #58376 ; http://n2t.net/addgene:58376 ; RRID:Addgene_58376) [13].

Preparation of aorta

Heparin was administered 1 hour before mice were sacrificed. The animals were euthanized and the aorta from aortic root to the bifurcation of iliac arteries was dissected and immersed in Krebs solution containing (in mM: 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, and 11.1 glucose). The vessels were carefully cleaned free of connective tissues.

Western blotting

Intact aortas isolated from WT C57Bl/6 or HDAC2-Tg mice with were cleaned to remove excess tissue/fat, longitudinally cut to expose the endothelium, and incubated in cold RIPA lysis buffer (20 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 1% sodium deoxycholate, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 μg/mL leupeptin, and a 1:1000 diluted protease inhibitor cocktail from Sigma) on ice for 1 hour. Samples were vortexed briefly every 10 minutes. Then, after the remaining tissue was discarded, the samples were sonicated briefly (4 times) at power level 4 and centrifuged for 30 minutes at 16,000 rpm and 4°C. Protein in the supernatant was quantified with a protein quantification assay kit (Bio-Rad, Hercules, CA). Twenty micrograms of protein per sample were combined with 2x Laemmli sample buffer (Bio-Rad), boiled for 5 minutes at 95°C, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was performed by transferring proteins from the gel onto a polyvinylidene difluoride membrane. Protein bands were visualized by using horseradish peroxidase-conjugated secondary antibodies (Invitrogen).

Genotyping and RT-PCR

Genomic DNA from ear pinches was isolated with the DNeasy blood and tissue kit (Qiagen). RT-PCR was carried out with Go Tag DNA polymerase (Promega) in a conventional Bio-Rad PCR machine. The following primer sets were used for genotyping. For HDAC2-Tg, Forward: 5′-GTCCTCATCGCATACCATAC-3′, Reverse: 5′-GCTTCATGGGATGACCCTGTC-3′. For HDAC2$^{fl/fl}$ mice, Forward: 5′TGGTATGTGCATTTGGGAGA-3′, Reverse: 5′-ATTTCACAGCCCCAGCTAAGA-3′. For VeCadherin controlled Cre mice, Forward: 5′-GACGCCACACTGGGTCTTCATCAGT-3′, Reverse: 5′-GCATCAGCTAGCAGCAGCAGGTCCAACT-3′. IL-6 internal control primers were Forward: 5′-TACACCAAAATTTGCCTGCATTACCGC-3′ and Reverse: 5′-TTTCCATGAGTGAACGAACCTGGT-3′.

Noninvasive pulse wave velocity (PWV) measurements

A high-frequency, high-resolution Doppler spectrum analyzer (DSPW, Indus Instruments, Houston, TX, USA) was used. Mice were anesthetized with 1.5% isoflurane and placed in a supine position on a heated pad equipped with echocardiogram (EKG) capability. The animals were allowed to stabilize to a physiologic heart rate before a 20 MHz probe was used to measure the descending aortic and abdominal aortic flow velocities. The time from the R wave of the EKG to the start of pulse waveform for each measurement location was calculated by using a real-time signal acquisition and spectrum analyzer system.

Endothelial function (vasoreactivity)

Mouse aorta was isolated and cleaned in ice-cold Krebs-Ringer-bicarbonate solution containing the following (in mM: 118.3 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.2 KH_2 PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, and 11.1 dextrose). The aorta was immersed in a bath filled with constantly oxygenated Krebs buffer at 37°C. Equal size thoracic aortic rings (2mm) were mounted using a microscope, ensuring no damage to the smooth muscle or endothelium. One end of the aortic rings was connected to a transducer, and the other to a micromanipulator. Aorta was passively stretched to an optimal resting tension using the micromanipulator, after which a dose of 60 mM KCl was administered, and repeated after a wash with Krebs buffer. After these washes, all vessels were allowed to equilibrate for 20–30 min in the presence of indomethacin (3μM). Phenylephrine (1 μM) was administered to induce vasoconstriction. A dose- dependent response (1nM to 10 μM), with the muscarinic agonist, ACH or nitric oxide donor, SNP, was then performed as necessary. Relaxation responses were calculated as a percentage of tension following pre-constriction. Sigmoidal dose-response curves were fitted to data with the minimum constrained to 0. Two to four rings were isolated from each animal and the number of animals in each group (n) was 6.

Measurements of aortic NO production

Aortic strips were isolated from 10-week-old male HDAC2-Tg and WT C57Bl/6 mice, pinned down on silastic with the endothelial side up, and exposed in the dark to 5 μmol/L DAF2-DA for 2 hours. The aortic strips were then washed with clean PBS for three times and fluorescence intensity at excitation (max) of 495 nm and emission (max) of 515 nm was measured over a period of 30 minutes. Images were acquired with a NikonTE-200 epifluorescence microscope. To confirm that NO was produced by eNOS, the NOS inhibitor NMLA was used. Rates of NO were calculated as the slope of the fluorescence measured over time. Where noted in the text, a wire was used to denude the endothelium from the aorta.

Gross pathological assessment of plaque

Aorta, from aortic root to iliac artery bifurcation, was carefully dissected, perfused with Krebs solution, and fixed with 4% paraformaldehyde overnight. The aorta was opened longitudinally and pinned onto a wax surface by microneedles. Images of the submerged vessels were captured with a digital camera. The lipid-rich intraluminal lesions were stained with Oil red O (Sigma). Digitized images were transferred to a computer and analyzed with NIH Java Image (ImageJ, version 1.42n). The amount of aortic atheroma in each animal was measured as percent lesion area per total area of the aorta.

Adenoviral-mediated gene delivery in vivo

For injection of adenoviruses *in vivo*, mice were anesthetized with ketamine (100 mg/kg), xylazine (10 mg/kg), and atropine (9 mg/kg) constituted in 100μl via the intraperitoneal route. Once anesthetized, animals were placed on a heated pad at 37±0.5°C to prevent hypothermia and dilate the tail vein. Mice were

monitored during anesthesia for the presence of breathing and respiration. Adenovirus particles ($3x10^{10}$ GC) encoding HDAC2 or empty vector were injected into the tail vein in a final volume of 100μL PBS. Care was taken to prevent introduction of air bubbles into the vein. We repeated the adenoviral injection two more times on alternate days (3 total doses). We monitored animals for 4 hours post-injection to observe any pain/distress and then returned them to the vivarium.

Statistical methods

All statistical analyses were carried out with Prism 8 for Mac (GraphPad Software Inc., San Diego, CA) and with Microsoft Excel version 16.41 (Microsoft, Redmond, WA) statistical analysis software. The results are expressed as mean and standard error (mean ± SEM). One-way analysis of variance (ANOVA) and the Bonferroni post-hoc test for multiple-comparison were used to compare all experimental data sets, groups, and pairs of data sets. A value of $p < 0.05$ was considered statistically significant. Myograph data were analyzed by using a general linear model with group as a factor and dose as a repeated measure in STATA VERSION 15 software (STATA Corporation, College Station, TX). Post-hoc tests were applied for each dose using a linear combination based on this model.

Results

Aortas from atherosclerotic mice express lower levels of HDAC2, and tail vein injection of HDAC2 adenovirus improves vascular endothelial function

Many converging lines of evidence suggest that enhanced expression of HDAC2 may protect EC function. HDAC2 expression was decreased in ECs of atherosclerotic plaques of human coronary arteries *in situ* [14], and cigarette smoke downregulates HDAC2 abundance, a process that contributes to steroid resistance and enhanced lung inflammation [15-17]. Because vascular endothelial dysfunction is an early etiologic event in the development of atherosclerosis and HDAC2 has previously been shown to play a role in endothelial dysfunction [17, 18], we investigated whether HDAC2 expression is modulated in the blood vessels of the two most widely used mouse models of atherosclerosis—apolipoprotein E deleted (*ApoE*$^{-/-}$) [19] and liver-targeted PCSK9 mutant overexpressing mice [13] fed with a high-fat diet (HFD). Indeed, HDAC2 protein levels were substantially lower in aortas from both mouse models of atherosclerosis than in aortas from C57BL/6 control mice (Fig. 1, A-D). Increased HDAC2 expression achieved via adenoviral mediated delivery in *ApoE*$^{-/-}$ mice markedly improved endothelium-dependent vasorelaxation as compared to that in mice injected with a GFP control adenovirus (Supplementary Fig. S2A). Endothelium-dependent vasorelaxation

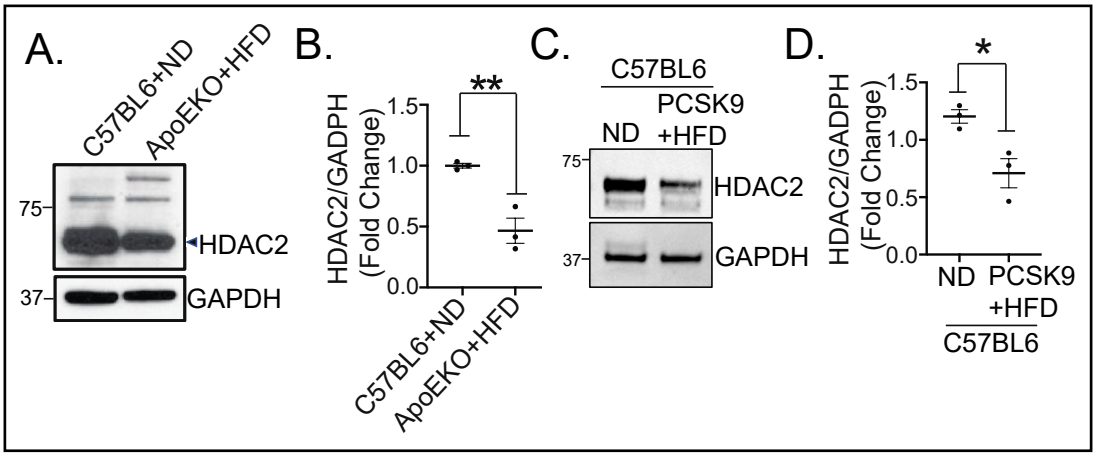

Fig. 1. HDAC2 expression is downregulated in atherosclerotic blood vessels. Isolated aortas from (A, B) ApoE knockout (KO) mice and (C, D) PCSK9 mutant-injected mice fed with normal diet (ND) or high-fat diet (HFD) were subjected to immunoblotting for HDAC2 and GAPDH. *p<0.05, n=3.

occurs via an acetylcholine response, which enhances EC production of NO. Endothelium-independent relaxation (in which SNP acts directly on and relaxes SMCs) did not differ between WT and HDAC2-Tg mice (Supplementary Fig. S2B). Further, the expression of HDAC2 in aortas from HDAC2-injected mice remained higher than that in WT aortas for 10 weeks as shown by western blot analysis of aortic lysates after the completion of high-fat diet regimen (Supplementary Fig. S2C).

Endothelial-specific HDAC2-overexpressing transgenic mice (HDAC2-Tg)

Because adenoviral-mediated gene delivery is not specific to a particular vascular compartment, vascular protection achieved by adenoviral-mediated HDAC2 overexpression cannot be solely attributed to the endothelial layer. To circumvent this issue, we generated an endothelial-specific HDAC2-overexpressing transgenic mouse (HDAC2-Tg) under the control of the Tie2 promoter. Representative genotyping of the pups is shown in Supplementary Fig. S2D. The founder HDAC2-overexpressing mice appeared to have a normal phenotype, with the exception of large litter sizes during the backcrossing of F0 with F1. Isolated aortas from HDAC2-Tg mice expressed significantly higher levels of HDAC2 than did those from C57BL6 mice (Fig. 2A). This increased HDAC2 expression was limited to ECs, as the denudation of endothelium completely abolished the difference in HDAC2 levels between aortas from WT and HDAC2-Tg mice (Supplementary Fig. S3A). Further, to assess whether off-target activation of Tie2 in myeloid cells of HDAC2-Tg mice during development affects the expression of HDAC2 levels in mature mice, we compared the levels of HDAC2 expression in bone marrow cell lysates from 8-week-old WT and HDAC2-Tg mice and found them to be non-significantly different (Supplementary Fig. S3B).

Endothelial-specific HDAC2-overexpressing transgenic mice are protected against vascular endothelial dysfunction and atherosclerosis

The HDAC2-Tg founder pups had a healthier vascular profile than their non-transgenic littermates. PWV, an inverse measure of vascular health and stiffness, was lower in the HDAC2-Tg mice, suggesting that the vessels were more compliant than those of WT mice—a function of either actively increased vascular relaxation or decreased passive vessel stiffness (Supplementary Fig. S4A). Further, OxLDL exposure produced a significant leftward shift of the endothelial-dependent vasorelaxation curve in aortas from HDAC2-Tg mice as compared to that of aortas from WT mice (Supplementary Fig. S4B). Moreover, basal NO production

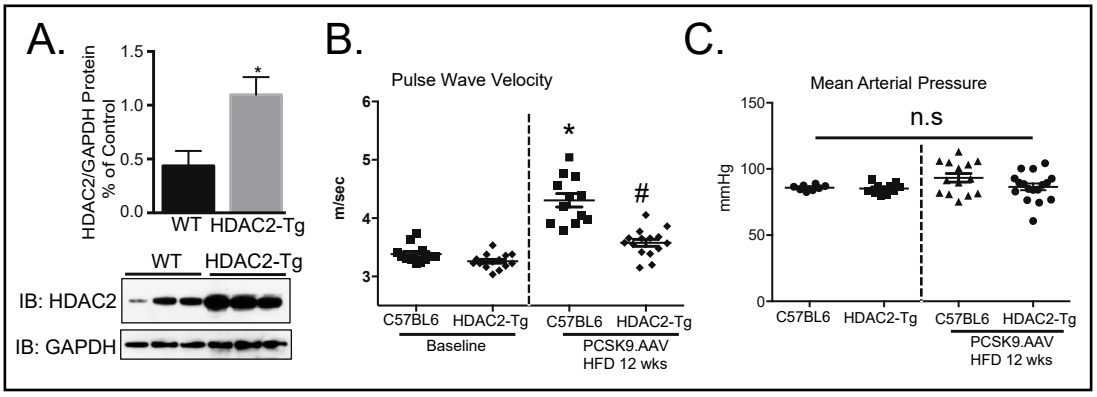

Fig. 2. EC-specific HDAC2 overexpression attenuates elevated vascular stiffness in atherogenic mice. (A) Aortas from wild-type (WT, C57BL/6 control) and HDAC2-Tg (Tie2 promoter) mice were immunoblotted (Ib) with HDAC2 and GAPDH antibodies. *p<0.05, n=3. (B, C) C57BL/6 and HDAC2-Tg mice were induced to develop atherosclerosis via liver-specific overexpression of PCSK9 and a high-fat diet. (B) Pulse wave velocity was higher in the C57BL/6 WT mice than in the HDAC2-Tg mice. *p<0.05 vs C57BL6 baseline, #p<0.05 vs C57BL/6 PCSK9AAV, n=15. (C) Mean arterial pressure did not differ between the C57BL/6 and HDAC2-Tg mice at baseline or after the atherogenic regimen. ns, not significant.

was enhanced in the vessels of HDAC2-Tg mice as compared to that in WT controls (Supplementary Fig. S4C). This finding suggests enhanced endothelial-specific vasodilator production despite oxidative injury.

Mice are inherently resistant to developing atherosclerosis. To determine whether targeted overexpression of HDAC2 in vascular endothelium protects mice from endothelial dysfunction and atherogenesis, we induced atherogenesis in WT and HDAC2-Tg mice by a single injection of PCSK9 gain-of-function mutant AAV and a 12-week HFD diet (1.25% cholesterol, 0% cholate) [13]. This approach produced robust expression of the mutant PCSK9 within a week. Mice that overexpressed the mutant PCSK9 did not express low-density lipoprotein receptor protein but exhibited a robust spike in non-high-density lipoprotein cholesterol (Supplementary Fig. S5, A-C). PWV rose in control but not HDAC2-Tg animals (Fig. 2B), indicating that intrinsic changes in the aortic vessel wall contribute to the increased aortic stiffness. Nevertheless, mean arterial pressure was not altered in either mouse strain (Fig. 2C). Further, aortic rings from HDAC2-Tg mice exhibited better acetylcholine-induced vasorelaxation than did WT control mice after the HFD and PCSK9 mutant transduction (Fig. 3A). No differences in SNP-induced vascular relaxation were observed (Fig. 3B). NO synthase inhibitor L-NAME abolished acetylcholine-mediated relaxation in both groups, highlighting the contribution of eNOS (Fig. 3C). These findings strongly implicate HDAC2 as an endothelial protectant.

We next assessed plaque burden in the intact aortas from WT and HDAC2-Tg mice using Oil red O staining. Aortas from control WT mice fed a normal diet showed almost no plaque. The atherogenesis regimen caused a significant increase in plaque staining in WT mice, but this increase was prevented in HDAC2-Tg mice (Fig. 4, A-B).

Endothelial-targeted HDAC2 gene deletion impairs vascular relaxation and augments stiffness

To further understand the role of HDAC2 in endothelial function, we generated endothelial-specific HDAC2 knockout (HDAC2 ecKO) mice under control of the VE-Cadherin promoter with Cre-LoxP-technology (Fig. 5A). Aortas from HDAC2 ecKO mice showed significantly lower expression of HDAC2 than did those of WT mice when lysed under conditions to capture endothelial and SMC proteins (Fig. 5B, C). Notably, HDAC2 expression was significantly higher in ECs than in SMCs (Supplementary Fig. S6, A, B). When compared to littermate controls, HDAC2 ecKO mice had augmented vascular stiffness (increased PWV, Fig. 5D) and impaired EC-dependent vascular relaxation with no changes in SNP-mediated relaxation (Fig. 5E, F).

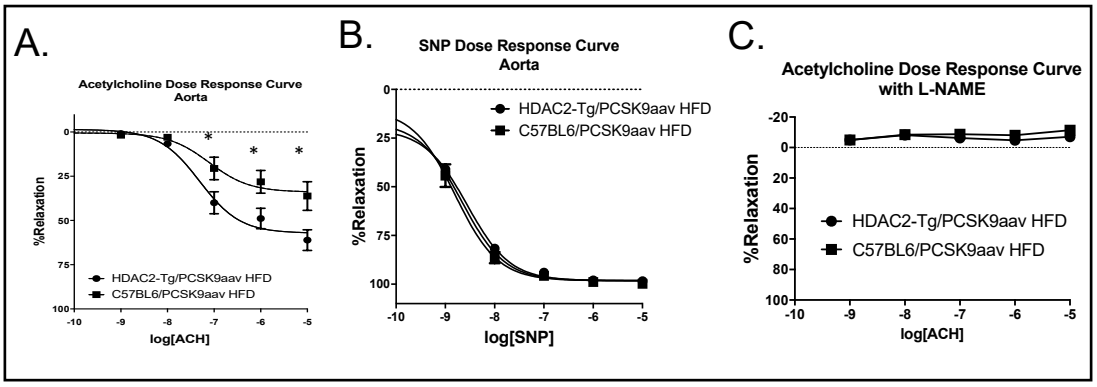

Fig. 3. EC-specific HDAC2 overexpression protects impaired endothelial-dependent vascular relaxation in atherogenic mice. Aortic rings were harvested from PCSK9 AAV-injected C57BL/6 (WT) and HDAC2-Tg mice fed with a high fat diet (HFD). Dose-response effects of acetylcholine (A) and sodium nitroprusside (SNP) (B) on vascular relaxation were determined by wire myography. (C) Acetylcholine dose response was also measured in the presence of 100 μM L-NAME. *p<0.05 vs WT HFD, n=6.

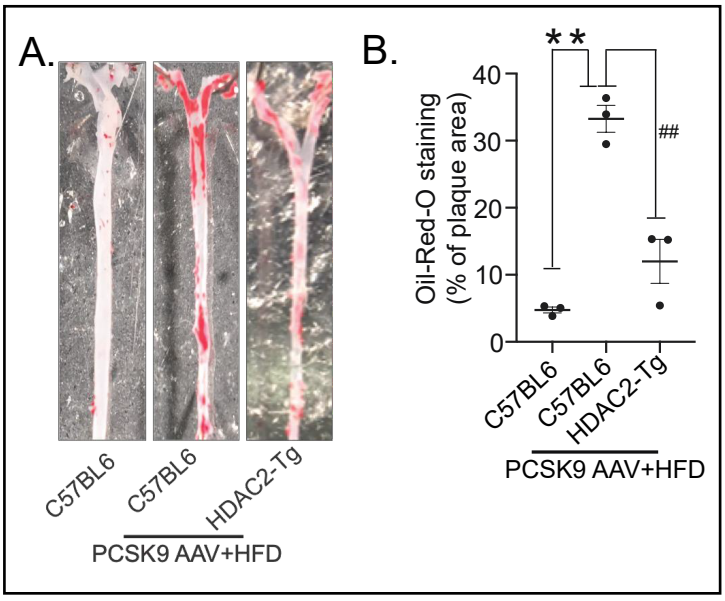

Fig. 4. HDAC2 overexpression in endothelium protects mice from atherosclerotic plaque burden. (A) Atherosclerotic plaques were measured by Oil red O staining in isolated aortas from C57BL/6 and HDAC2-Tg (Tie2) mice that were induced to develop atherosclerosis via liver-specific PCSK9 overexpression and a high-fat diet for 12 weeks. (B) Quantification of plaque from experiment in A. **p<0.05 vs control C57BL/6 mice fed a normal diet; ##p<0.05 vs atherogenic C57BL/6 mice (n=3).

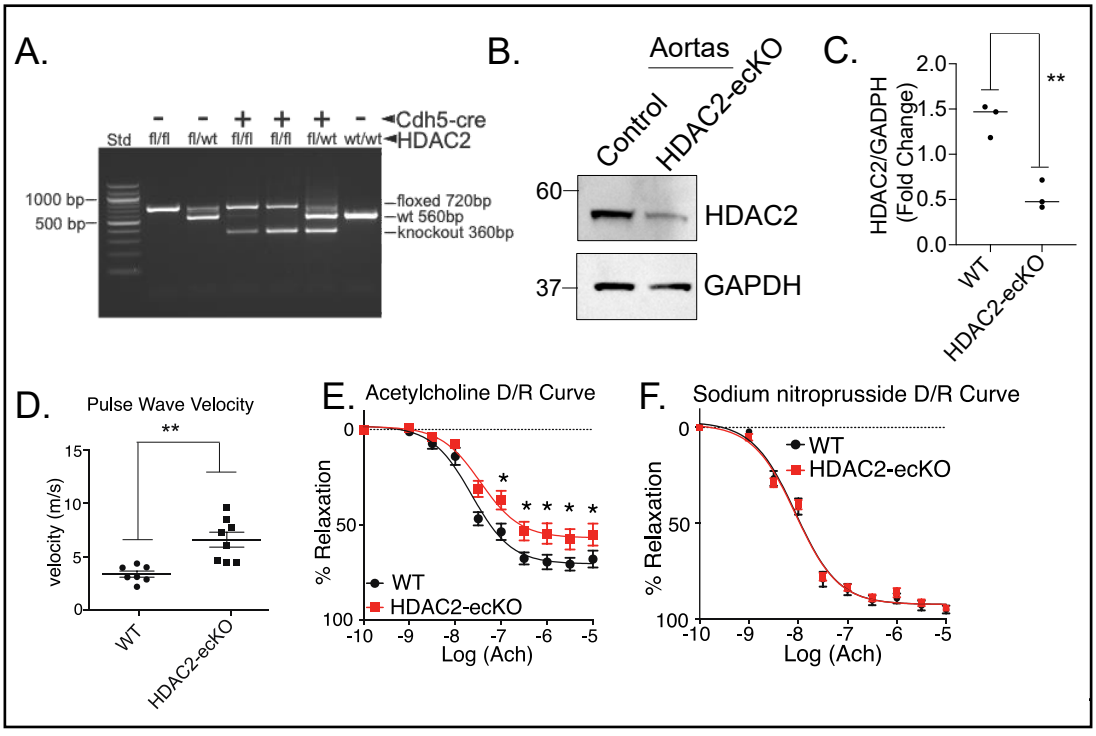

Fig. 5. Vascular function is impaired in endothelial cell-specific HDAC2 knockout mice. (A) Genotyping shows the presence of Cre recombinase under VE-Cadherin (Cdh5) promoter (top) and floxed HDAC2 ($HDAC2^{fl/fl}$) (bottom) in endothelial cell-specific HDAC2 knockout mice (Hdac2-ecKO). (B, C) Intact aortas from HDAC2-ecKO mice were immunoblotted for HDAC2 (**p<0.005 vs wild-type [WT], n=3). (D) Pulse wave velocity in 12–14-week-old HDAC2-ecKO mice and littermate controls (**p<0.005 vs WT, n=8). (E, F) Dose response (D/R) curves show myography of isolated aortic rings from HDAC2-ecKO and littermate control mice in response to acetylcholine and sodium nitroprusside (*p<0.05 vs WT, n=6).

Discussion

The findings highlighted in this paper provide further evidence for the role of HDAC2 in modulating endothelial function *in vivo*. Gain-of-function experiments confirmed that endothelial-specific overexpression of HDAC2 can enhance aortic endothelial NO production, restore endothelial-dependent relaxation in two separate atherogenic models, and prevent the development of vascular stiffness—one of the integrated measures of vascular health *in vivo*. HDAC2 overexpression significantly reduced plaque burden in one of the atherosclerosis models, whereas loss-of-function experiments in endothelial-specific HDAC2 ecKO mice increased vascular stiffness and impaired endothelial-dependent vasorelaxation. Thus both gain- and loss-of-function experiments are highly suggestive of a causative role for HDAC2 in maintaining endothelial health.

It is clear that as an epigenetic modifier, HDAC2 regulates many genes that modulate endothelial function in response to an atherogenic stimulus. We have previously shown that HDAC2 is a transcriptional regulator of Arg2, a well-studied reciprocal regulator of eNOS that, when upregulated, leads to eNOS uncoupling and contributes to endothelial dysfunction and atherogenesis. Indeed, we have shown that HDAC2 binds to the 5′ untranslated region of the Arg2 gene and acts as a trans-repressor [17]. We and others have reported that activation of the LOX-1 receptor has a critical role in the atherogenic process [20, 21]. Endothelial LOX-1 activation leads to an increase in inflammatory gene production and Arg2 activation, each of which contribute to endothelial dysfuction [20-22]. Dje N'Guessan et al. [14] demonstrated that OxLDL stimulation of ECs leads to increased acetylation of histone protein 4 (H4), decreased expression of HDAC2, and increased transcription of the inflammatory genes IL8 and MCP1—which contribute to atherogenesis [14]. On the other hand, statins can reverse this effect.

It is interesting to speculate whether the protective effects of HDAC2 are cell specific. For example, Kong et al. [23] reported that suppression of HDAC2 in PC12 cells reduces oxidative stress, as evidenced by decreases in Nox2 and Nox4 and an increase in manganese superoxide dismutase (MnSOD). They showed that these effects occurred secondary to an increase in the FOXO3a transcription factor. This finding is important in the context of spinal cord injury, in which ketone B hydroxybutyrate has been shown to reduce oxidative damage by a mechanism that involves inhibition of HDAC2. On the other hand, HDAC2 has recently been shown to regulate miRNA141-3p, itself a negative regulator of ZMPSTE24 [24]. ZMPSTE24, a metalloproteinase, is critical in modulating senescence by processing the nuclear envelope protein prelamin A to lamin A, which is integral to nuclear membrane structure [25]. Indeed suppression of HDAC2 with inhibitors promotes senescence by enhancing acetylation of the miRNA141-3p histone in the promoter region. Thus, in this case, upregulation of HDAC2 would protect against senescence, an important mechanism of impaired endothelial function. In a recent study of human lung microvascular ECs in the context of acute lung injury, Ebenezer et al. [26] suggested that sphingosine1-phosphate–derived long-chain fatty aldehyde may be an epigenetic regulator of pro-inflammatory genes in sepsis-induced lung inflammation. Their findings suggested that HDAC2 inhibition enhances proinflammatory genes such as IL-6 in endothelial cells. Thus, HDAC2 appears to be protective in the context of the endothelium.

In this study, we utilized a new model of atherosclerosis, the PCSK9 AAV model [13]. The expression of PCSK9 D377Y in the liver leads to a marked reduction in expression of the low-density lipoprotein receptor and a subsequent marked increase in serum cholesterol. The HFD fed to the mice significantly exacerbates the hyperlipidemia. The advantage of this approach is that it obviates the need for the lengthy and costly process of generating double knockouts to test gain and loss of function, in this case in the context of HDAC2-Tg and HDAC2 ecKO mice. This model allowed us to efficiently determine that HDAC2 overexpression could be significantly protective in an atherogenic model.

We and others have shown that global PWV is significantly increased in models of atherosclerosis and that attenuation of atherosclerosis or prevention of its progression leads to

a decrease in this metric of global integrated vascular health. For example, arginase inhibition led to a significant decrease in PWV in atherogenic ApoE$^{-/-}$ mice [22]. In HDAC2-Tg mice, we observed a similar phenomenon in that PWV was lower in atherogenic HDAC2-Tg mice than in their WT counterparts. PWV can be altered by either an active or passive change in the vessel wall. It is likely that the enhanced endothelial function (increased NO production) leads to a change in the stiffness of the underlying smooth muscle and in the matrix.

Conclusion

In summary, we have shown that HDAC2 is a critical regulator of endothelial-dependent vasorelaxation and atherosclerosis. This conclusion is supported by our findings that endothelial-specific overexpression is protective, preserves endothelial-dependent vasorelaxation, and attenuates the atherosclerotic plaque load, whereas knockout of HDAC2 has the opposite effect. The mechanism by which HDAC2 provides endothelial protection is likely through its trans co-repressor activity on genes that regulate vascular signaling such as pro-inflammatory genes. The complete range of genes that are influenced by HDAC2 in the endothelium, however, remains to be determined. Nevertheless, it appear that HDAC2 is a critical node in endothelial protection and offers itself as a novel target for the treatment and/or prevention of atherosclerosis. Our demonstration that HDAC inhibition leads to profound endothelial dysfunction, and that increased expression of HDAC2 in isolated murine aortas improves EC function, offers a broadly applicable note of caution for the use of non-specific HDAC inhibitors as pharmacotherapies.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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