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Nox4 and soluble epoxide hydrolase synergistically mediate homocysteine-induced inflammation in vascular smooth muscle cells

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ABSTRACT

Background: Hyperhomocysteinemia leads to a vascular smooth muscle cell (VSMC) inflammatory response. Meanwhile, Nox4 dependent reactive oxygen species (ROS) signaling and soluble epoxide hydrolase (sEH)/epoxyeicosatrienoic acids (EETs) are both involved in vascular inflammation. Herein, we hypothesized that Nox4 and soluble epoxide hydrolase cross regulated during homocysteine-induced VSMC inflammation.

Methods and results: In cultured VSMCs, the expression of the inflammatory factors VCAM1 and ICAM1 was measured by real-time PCR and Western blotting, while supernatant MCP1 was measured by ELISA. Upon VSMC stimulation with 50 μM homocysteine, we observed the VCAM1 and ICAM1 mRNA levels were increased by 1.15 and 1.0 folds, respectively. The MCP1 levels in the supernatant of cultured VSMCs treated with 100 μM increased to 1.76 folds. As expected, homocysteine induced Nox4 expression and Nox4-dependent ROS generation. The sEH expression was also upregulated in the presence of homocysteine in a dose-dependent manner. Furthermore, we knocked down Nox4 with siRNA. Knockdown of Nox4 decreased ROS generation and homocysteine-induced sEH expression. Overexpression of Nox4 with an adenovirus stimulated sEH expression. Similarly, knockdown or chemical inhibition of sEH blunted the upregulation of Nox4 by homocysteine. In vivo, in homocysteine-fed mice, concomitant upregulation of Nox4 and sEH was associated with increased VCAM1 and ICAM1 expression in the aortic wall.

Conclusions: The inflammatory response induced by homocysteine in VSMCs was accompanied by Nox4 and sEH upregulation. Nox4 and soluble epoxide hydrolase synergistically contribute to homocysteine-induced inflammation.

1. Introduction

Hyperhomocysteinemia (HHcy) has been reported to be a potent risk factor for cardiovascular diseases and is related to vascular smooth muscle cell (VSMC) proliferation and inflammation responses [1,2]. Vascular inflammation promotes VSMCs to proliferate and leads to vessel wall remodeling [3,4]. Accumulating data from laboratory and clinical studies have shown that homocysteine (Hcy)-induced inflammation plays a pivotal role in atherogenesis and atherosclerosis [5]. In addition to the levels of inflammatory cytokines, monocyte-endothelial cell adhesion and C-reactive protein (CRP) levels are increased.

Reactive oxygen species (ROS) are important mediators of inflammatory processes. NADPH oxidases are the major sources of ROS in vascular cells during pathological vascular conditions. Distinct from other Nox members, Nox4 generates H_2O_2 and is constitutively active. We have previously shown that Nox4 mediates VSMC inflammation and thus enhances neointimal hyperplasia and atherogenesis [6,7]. The correlation between Hcy and Nox4 has been determined in numerous types of cells, including endothelial cells [8], adventitia fibroblasts [9,10] and cardiac cells [11]. However, the underlying mechanism of Nox4 regulation by Hcy has not been fully elucidated.

Epoxyeicosatrienoic acids (EETs) are important vasoactive molecules whose formation from arachidonic acid is catalyzed by cytochrome P450 (CYP) epoxygenases. EETs can hyperpolarize and relax VSMCs and exert similar effects as endothelium-derived hyperpolarizing factors. EETs are enzymatically hydrolyzed by soluble epoxide hydrolase (sEH) into their respective diols. EET bioavailability is

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therefore thought to be determined by sEH activity. sEH has been reported to be expressed in many cardiovascular tissues, including endothelial cells, VSMCs and cardiomyocytes. sEH has attracted great interest as a potential cardiovascular therapeutic target for hypertension, atherosclerosis [12], stroke [13] and ischemic heart disease [14]. Interestingly, Hcy regulates sEH in vascular endothelial cells [15]. However, whether this regulation is established in VSMCs remains unknown. Furthermore, it is intriguing whether sEH/EET pathways and ROS pathways convene in homocysteine-induced inflammation.

Herein, we hypothesized that cross-regulation might exist in VSMCs and contribute synergistically to Hcy-induced inflammation. The present study aimed to determine the activation of ROS and sEH/EET signaling in the presence of Hcy. Regulation between Nox4 and sEH was also explored as a further mechanism to explain Hcy-induced inflammation.

2. Methods

2.1. Materials and agents

The agent 14,15-EET was purchased from Cayman Chemical (Ann Arbor, MI, USA). Homocysteine and folic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental animals

Male C57BL/6 J mice were obtained at 8 weeks of age. The mice were fed a normal diet (ND) of mouse chow supplemented with or without 1.8 g/L DL-homocysteine daily prepared in drinking water for 16 weeks. All animal experimental procedures were approved by the Committee for Animal Research of the Third Military Medical University (Army Medical University) and were carried out in accordance with National Institutes of Health guidelines (United States).

2.3. Cell culture

Rat VSMCs were cultured as we described previously [16]. Briefly, primary VSMCs were cultured in DMEM supplemented with 10% FBS (Gibco, US). VSMCs were incubated at 37 °C in 5% CO_2. VSMC experiments were performed with VSMCs between passages 3 and 8.

2.4. Quantitative PCR

Total RNA was extracted from cultured VSMCs and converted to complementary DNA. Real-time qPCR was performed using synthetic TaqMan gene-specific primers (Applied Biosystems, CA, USA). GAPDH expression was used as an internal control. The following cycling conditions were used: denaturation, annealing, and extension at 95 °C, 55 °C and 72 °C for 30 s, 30 s, and 1 min, respectively, for 40 cycles.

2.5. Small interfering RNA (siRNA) transfection

VSMCs were plated in 6-well plates, and siRNA was transfected into cells at approximately 70% confluence. siRNAs against Nox4 (sc-61,887, Santa Cruz Biotech) or Ephx2 (encoding sEH, Thermo Fisher, Cat. #194734) at a concentration of 50 nM were transfected with Lipofectamine RNAiMAX. After 72 h of transfection, the VSMCs were collected for further protein or mRNA evaluation.

2.6. Western Blot analysis

VSMCs were lysed, and the proteins were subjected to SDS-PAGE and immunoblotted with antibodies to detect Nox4 (OriGene, NJ, USA), sEH (Santa Cruz, CA, USA), vascular cell adhesion molecule-1 (VCAM1, Santa Cruz, CA, USA), intercellular adhesion molecule 1 (ICAM1, Santa Cruz, CA, USA), and GAPDH (Cell Signaling Technology, MA, USA) as a loading control. The protein bands were visualized with an ECL system (Pierce). Densitometry analysis was performed using NIH ImageJ software.

2.7. Immunohistochemistry

Tissues were embedded in paraffin and sectioned. After blocking with 20% goat serum, aorta slices were subjected to antibodies against Nox4, sEH, VCAM1 and ICAM1 overnight at 4 °C. An EnVision/HRP Kit was used for development, and the specimens were counterstained with hematoxylin.

2.8. ELISA

The concentrations of MCP-1 in culture medium were determined using an ELISA kit (R&D Systems, Minneapolis, MN) per the manufacturer's instructions. Briefly, VSMC medium was replaced with phenol red-free culture medium before the assays were started. The medium was collected after the indicated treatment. The standard solution or 50 μL of medium was added to each well, and the wells were then incubated with an HRP-conjugated secondary antibody (50 μL/well) for 2 h at 37 °C. After stopping the enzymatic reaction, the absorbance was measured at 450 nm with a microplate reader.

2.9. Amplex Red assay for H_2O_2 production

Amplex Red was used to measure H_2O_2 production in VSMCs. After treatment overnight with or without homocysteine (50 μM or 100 μM), the VSMCs were washed with phenol red-free DMEM and then incubated with phenol red-free DMEM containing Amplex Red and horseradish peroxidase as we described previously [7]. The reaction was carried out for 45 min, and subsequent fluorescence readings were made in a 96-well plate (excitation 570 nm, emission 580 nm).

2.10. Statistical analysis

The values are expressed as the means ± SEM. Student's *t*-test was used to compare data between groups. One-way analysis of variance was used to analyze the differences among groups. Statistical significance was defined as $p < .05$.

3. Results

3.1. Homocysteine induced the VSMC inflammatory response

First, we examined the inflammation in VSMCs upon homocysteine stimulation. Consistent with previously published literature [17], homocysteine at 50 μM increased the mRNA levels of VCAM1 and ICAM1 by 1.15 and 1.0 fold, respectively (Fig. 1A). The protein levels of VCAM1 and ICAM1 were also detected under different homocysteine treatments. The protein expression of VCAM1 and ICAM1 was increased in a dose-dependent manner (Fig. 1B &C). In addition, we measured MCP1 levels in the cell culture supernatant. MCP1 was also significantly enhanced in the presence of homocysteine (Fig. 1D). These results indicated that homocysteine could trigger the inflammatory response in cultured VSMCs.

3.2. Homocysteine upregulated Nox4 expression and led to oxidative stress

Oxidative stress is one of the common pathways leading to the inflammatory response. Nox4 expression was determined in homocysteine-treated VSMCs. Both the mRNA and protein levels were increased markedly in the homocysteine-treated VSMCs compared with the control VSMCs (Fig. 2A-C). Hydrogen peroxide, the specific ROS generated by Nox4, was similarly elevated in the homocysteine-treated VSMCs compared with the control VSMCs (Fig. 2D). Therefore,

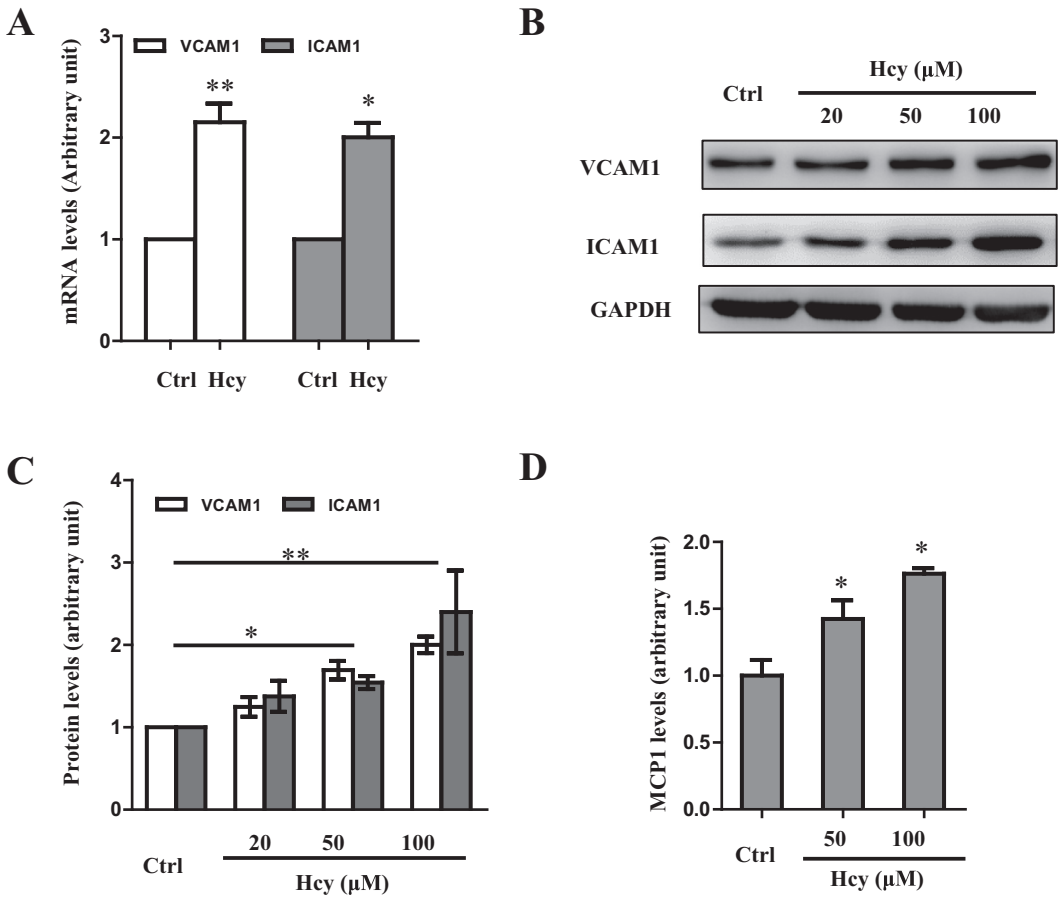

Fig. 1. Homocysteine induced an inflammatory response in VSMCs. A. mRNA levels of ICAM1 and VCAM1 in VSMCs cultured in 0.2% FBS DMEM in the presence of 50 μM homocysteine overnight. *$p < .05$ vs. control, **$p < .01$ vs. control, $n = 3$. B. Representative Western blots of homogenates of cultured VSMCs treated with 20, 50 and 100 μM homocysteine. C. The protein levels of ICAM1 and VCAM1 were quantified by densitometry and normalized to those of GAPDH. *$p < .05$ vs. control, **$p < .01$ s. control, $n = 3$. D. MCP1 levels in the supernatant of cultured VSMCs treated with 50 and 100 μM homocysteine. *$p < .05$ vs. control, n = 3.

homocysteine induced Nox4-associated ROS generation.

3.3. Homocysteine promoted sEH expression

sEH functions to enzymatically hydrolyze epoxyeicosatrienoic acids and has been reported to participate in inflammation. We tested whether homocysteine regulates sEH in VSMCs. The PCR and Western blot results revealed that homocysteine dose-dependently and time-dependently upregulated the mRNA and protein levels of sEH (Fig. 3A&B).

3.4. Nox4 mediated homocysteine-induced sEH expression

Nox4 has been reported to be involved in sEH expression in hyperlipidemic mice [6]; however, whether this regulation exists in the context of homocysteine remains unknown. Nox4 knockdown was achieved with a specific siRNA. In VSMCs transfected with Nox4 siRNA, sEH expression was blunted at both the mRNA and protein levels (Fig. 4A-C). PEG-catalase, which can degrade the H_2O_2 generated by Nox4, was also used to determine the regulation between Nox4 and sEH. Consistently, PEG-catalase treatment decreased the levels of sEH mRNA and protein (Fig. 4D &E). On the other hand, aberrant overexpression of Nox4 with an adenoviral vector further strengthened the expression of sEH in the presence of homocysteine (Fig. 4G).

3.5. sEH cross-regulated homocysteine-induced Nox4 expression

sEH inhibition has been reported to exert antioxidative effects [18]. To further detect whether sEH affected Nox4 expression, sEH was knocked down in homocysteine-treated VSMCs. Nox4 mRNA and protein expression was significantly blocked in the group transfected with the siRNA against sEH (Fig. 5A&B). H_2O_2 levels were lower when sEH was knocked down (Fig. 5C). Compared to DMSO, the sEH chemical inhibitor ADPU also suppressed H_2O_2 production (Fig. 5C). Likewise, Nox4 expression was suppressed when sEH was either knocked down or inhibited by ADPU in homocysteine-treated VSMCs (Fig. 5D). It is speculated that sEH acts, at least in part, through Nox4-dependent H_2O_2 signaling. Furthermore, inhibition of sEH-mediated EET hydrolysis has various cardiovascular protective effects, including anti-inflammatory and antioxidative effects [19,20]. We used 14,15-EET to determine the underlying mechanisms of the antioxidative effect of sEH inhibition. Compared to treatment with DMSO, treatment with 14,15-EET suppressed Nox4 expression and corresponding H_2O_2 production (Fig. 5E&F). These results revealed that sEH regulated homocysteine-induced Nox4 expression.

3.6. Homocysteine induced Nox4 and sEH expression and inflammation in the aortas of mice

The cross-regulation between Nox4 and sEH during vascular inflammation was further explored in mice fed homocysteine. In vivo, compared with the normal diet, the diet with homocysteine increased the expression of ICAM1, VCAM1, Nox4, and sEH in the aortic tissues, as shown by immunohistochemical staining and protein blotting (Fig. 6). These data suggest that Nox4 and sEH synergistically regulated aortic inflammation in homocysteine-fed mice.

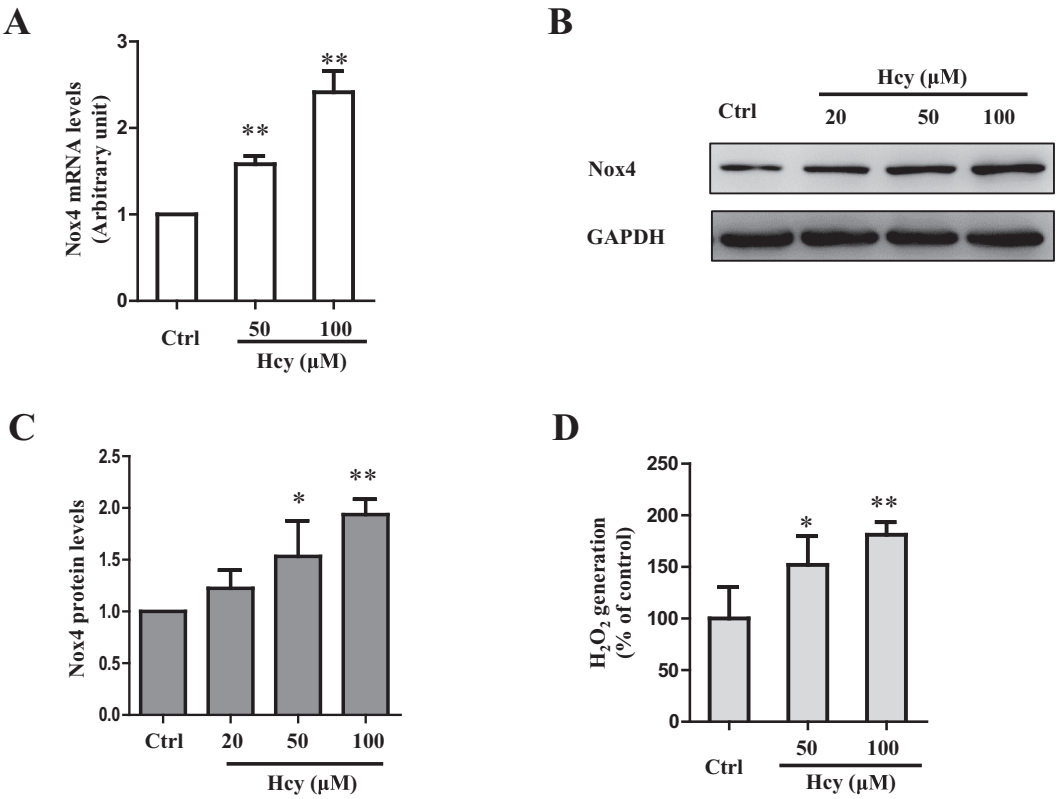

Fig. 2. Nox4- and Nox4-dependent ROS were induced by homocysteine in VSMCs. A. mRNA levels of Nox4 in VSMCs cultured in 0.2% FBS DMEM in the presence of homocysteine at concentrations of 50 and 100 μM overnight. $^{**}p < .01$ vs. control, n = 3. B. Representative Western blots of Nox4 in VSMCs treated with 20, 50 and 100 μM homocysteine. C. Quantitation of the band intensities. $^{*}p < .05$ vs. control, $^{**}p < .01$ vs. control, n = 3. D. H_2O_2 production measured by Amplex Red in VSMCs in the presence or absence of homocysteine. $^{*}p < .05$ vs. control, $^{**}p < .01$ vs. control, $n = 4$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, we found that cross-regulation between Nox4 and sEH expression synergistically promotes the development of arterial inflammation during Hcy-induced vascular pathology.

EETs are endogenous cytochrome P-450 (CYP) metabolites of arachidonic acid. EETs from the endothelium or from VSMCs can regulate vascular function in multiple ways, such as by affecting dilation, proliferation, inflammation and migration. On the other hand, oxidative stress has been comprehensively implicated in vascular remodeling. However, the crosstalk between ROS signaling and CYP450 EETs has rarely been investigated. Herein, we characterized the cross-

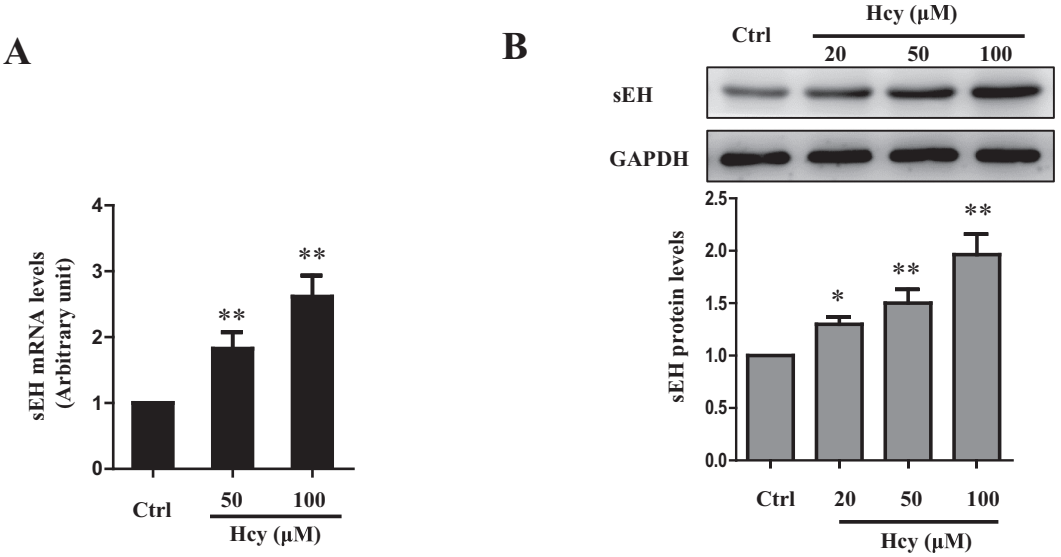

Fig. 3. Homocysteine increased sEH expression. A. mRNA levels of sEH in VSMCs cultured in 0.2% FBS DMEM in the presence of homocysteine at concentrations of 50 and 100 μM overnight. $^{**}p < .01$ vs. control, n = 3. B. Representative Western blots of sEH in VSMCs treated with 20, 50 and 100 μM homocysteine and quantitation of the band intensities. $^{*}p < .05$ vs. control, $^{**}p < .01$ vs. control, $n = 3$.

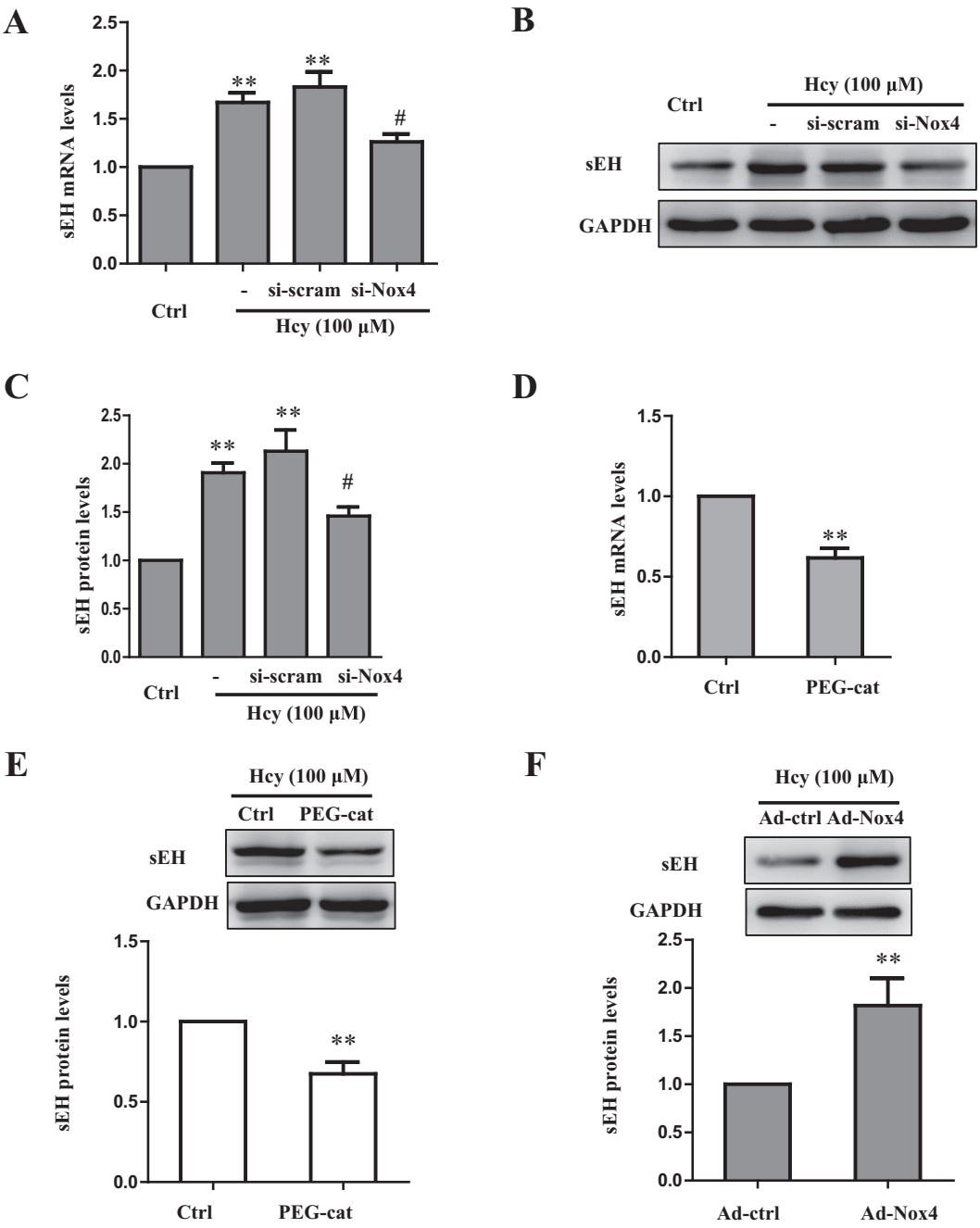

Fig. 4. Nox4 was involved in the upregulation of sEH induced by homocysteine. A. mRNA levels of sEH in VSMCs transfected with scrambled siRNA or siRNA against Nox4 in the presence of homocysteine at a concentration of 100 μM. $^{\#}p < .05$ vs. si-scramble, $^{**}p < .01$ vs. control, n = 3. B. Protein levels of sEH in VSMCs transfected with scrambled siRNA or siRNA against Nox4 in the presence of homocysteine at a concentration of 100 μM. C. Quantification of the sEH western blot results. $^{**}p < .01$ vs. control, $^{\#}p < .05$ vs. si-scramble, n = 3. D. mRNA levels of sEH in VSMCs treated with permeable PEC-catalase in the presence of homocysteine at a concentration of 100 μM. $^{**}p < .01$ vs. control, n = 3. E. Protein levels of sEH in VSMCs treated with permeable PEC-catalase in the presence of homocysteine at a concentration of 100 μM. $^{**}p < .01$ vs. control, n = 3. G. Protein levels of sEH in VSMCs transfected with an adenovirus carrying Nox4 or a control vector in the presence of homocysteine at a concentration of 100 μM. $^{**}p < .01$ vs. control vector, n = 3.

regulation between Nox4 and the EET hydrolase sEH, which worked synergistically during Hcy-triggered VSMC inflammation. This bilateral regulation might form a vicious cycle and accelerate the development of vascular remodeling.

Hcy is widely recognized as an independent risk factor in cardiovascular diseases. Patients with vascular disease, especially atherosclerosis, have significantly elevated plasma Hcy concentrations [15]. Elevation of plasma Hcy is found in 5–10% of the general population and in > 40% of patients with CVDs [21]. Hcy mediates the formation of cardiovascular disease through several different mechanisms, including damage to the endothelium and adverse effects on VSMCs [22]. Reportedly, in VSMCs, Hcy can promote proliferation and migration [23], augmenting VSMC inflammation. Investigation of the effects of Hcy on CRP has revealed that Hcy induces CRP in VSMCs through the ROS-ERK1/2/p38-NF κB pathway [17,24]. In addition, Hcy treatment causes a significant increase in ICAM1 and VCAM1 in endothelial cells [25], but whether this effect exists in VSMCs has not been reported. As VCAM1 and ICAM1 are two target proteins of NF κB, Hcy might presumably induce VCAM1 and ICAM1 transcription. In our study, we confirmed that Hcy promoted VCAM1 and ICAM1 expression.

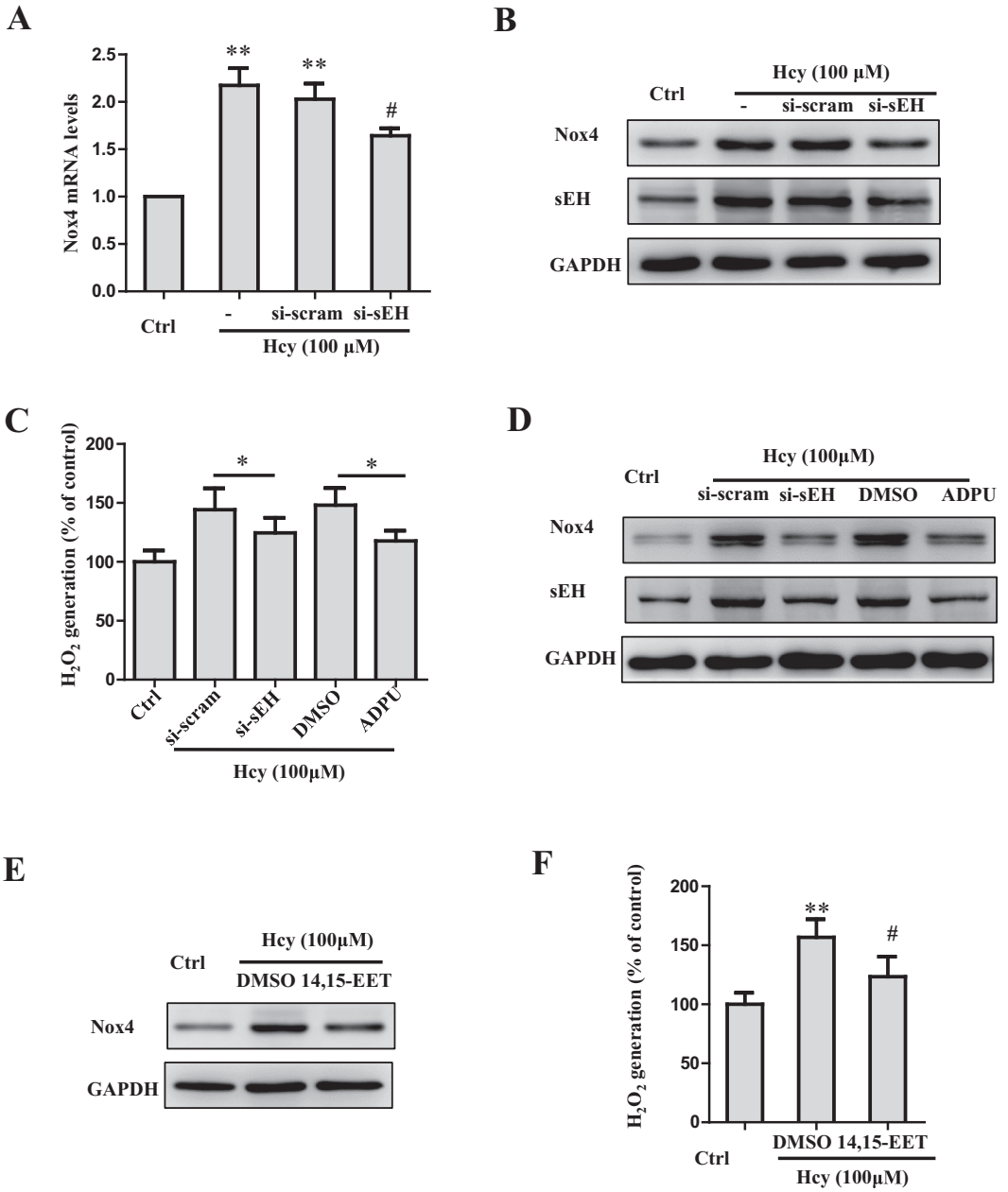

Fig. 5. Homocysteine-induced Nox4 expression and ROS generation was blunted by sEH knockdown or chemical inhibition. A. Nox4 mRNA levels were decreased when sEH was knocked down by siRNA in the presence of homocysteine. $^{**}p < .01$ vs. control, $^{\#}p < .05$ vs. si-scramble, n = 3. B. The protein levels of Nox4 and sEH were determined through Western blot analysis after sEH was knocked down upon stimulation with homocysteine. C. Hydrogen peroxide levels were measured in cultured VSMCs in the presence of homocysteine after sEH was inhibited with either siRNA or the chemical inhibitor ADPU. $^{*}p < .05$ for the indicated comparisons, $n = 6$. D. The protein levels of Nox4 and sEH were determined through Western blot analysis after sEH was inhibited with either siRNA or the chemical inhibitor ADPU. E. Representative Western blot of Nox4 in VSMCs treated with 14,15-EET or DMSO in the presence of 100 μM homocysteine. F. H_2O_2 production measured by Amplex Red in VSMCs in the 14,15-EET or DMSO groups in the presence of 100 μM homocysteine. $^{**}p < .01$ vs. control, $^{\#}p < .05$ vs. DMSO group, n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This observation confirmed that Hcy is a proinflammatory factor, which had been suggested in the study on Hcy-induced modulation of CRP. In addition, other inflammatory markers, such IL-1β, IL-6 and MCP1, have been reported to be elevated in rat VSMCs [26]. These findings together indicate that Hcy-induced inflammation might be a possible mechanism for its adverse vascular effects.

A large body of evidence has linked Hcy to oxidative stress in VSMCs. The proliferation and migration of VSMCs are potentiated by Hcy through ROS generation [27,28]. Neutralization of ROS can blunt the effects of Hcy on VSMCs. Similarly, Hcy-induced inflammation is also ROS dependent [24]. Consistently, we observed total ROS and H_2O_2 accumulation in Hcy-treated VSMCs. Among the sources of ROS, Hcy has been shown to regulate p47phox, Nox1, nox2, and Nox4 in VSMCs [29]. Although Nox1 and Nox2 p47phox have been reported to be involved in neointimal formation, our previous study showed that Nox4 is the predominant isoform of NADPH oxidase in VSMCs [7]. The regulation of Nox4 by Hcy has been established in adventitial fibroblasts [9], endothelial cells [10,30] and endothelial progenitor cells [31]; however, its regulation in VSMCs remains unknown. Statins inhibit Hcy-induced vascular pathological changes involving the Nox4 pathway [31]. In this study, we confirmed that Hcy treatment led to oxidative stress mainly generated by Nox4. Herein, in Hcy-treated

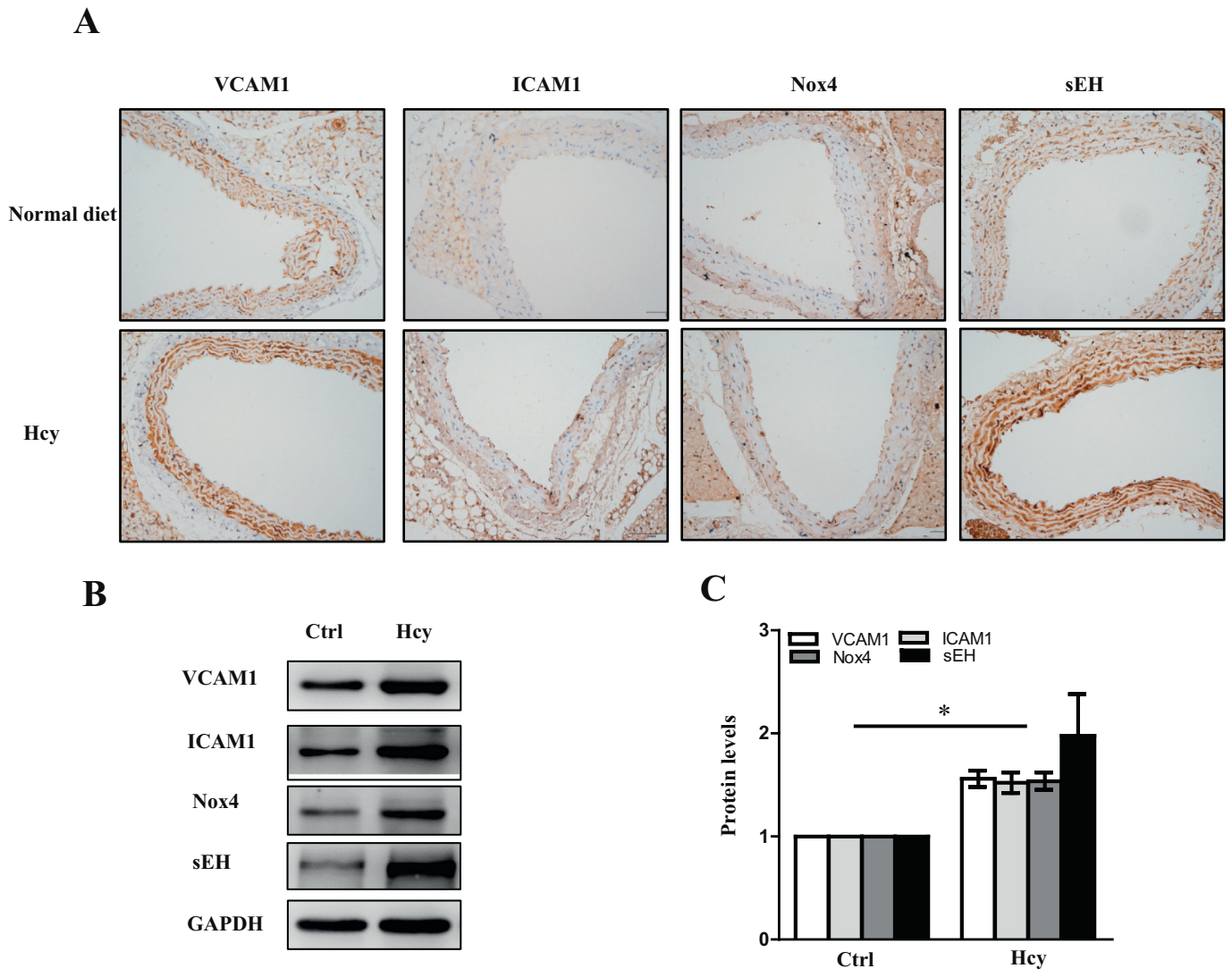

Fig. 6. Homocysteine increased Nox4, ICAM1, VCAM1 and sEH expression in intact aortas. A. Representative immunohistochemical staining of Nox4, ICAM1, VCAM1 and sEH in aortas harvested from normal diet-fed and homocysteine-fed mice. The images were taken at 20× magnification. B. Representative western blot of Nox4, ICAM1, VCAM1 and sEH detected in aortas from mice fed a normal diet or a diet with homocysteine for 16 weeks. C. Quantitation of the western blot results. $^{*}p < .05$ as indicated, n = 3.

VSMCs, Nox4 was upregulated and consequently led to H_2O_2 production and increased oxidative stress. This observation might support the role of Nox4 in VSMCs during Hcy-induced vascular inflammation.

EETs are considered EDHFs and potent endogenous compounds with beneficial vascular actions. The role of EETs in the endothelium has been extensively examined. Endothelial-derived EETs dilate blood vessels by interacting with VSMCs, which exerts antihypertensive action. EETs promote endothelial cell proliferation and migration [32]. Furthermore, EETs stimulated angiogenesis and enhanced neovascularization in a subcutaneous sponge animal model [33]. Importantly, EETs, especially 11,12-EET, have been implicated in preventing the activation of NF κB and VCAM1 in endothelial cells [34]. However, reports of the roles of EETs in VSMCs seem to be inconsistent. Two decades ago, EETs were first reported to be made by the vascular endothelium but not by VSMCs [35]. Recently, with the development of biotechnology, VSMC EETs have been detected, and decreases in these EETs are attributable to sEH-mediated VSMC migration. Given the wide spectrum of sEH effects, considerable interest has arisen in developing methods to enhance the bioavailability of EETs by inhibiting sEH. Previously, sEH inhibition has been found to protect against a wide range of cardiovascular diseases, such as hypertension, coronary stenosis, cardiac fibrosis, atherosclerosis and vascular inflammation [36]. In this study, we established that sEH is regulated by Hcy. Furthermore, we demonstrated that Hcy-induced sEH expression is dependent on Nox4-related ROS. However, sEH expression can also modulate Nox4 expression. The cross-regulation of Nox4 and sEH might partially explain how Hcy induces VSMC inflammation. A vicious cycle of ROS generation and epoxyeicosanoid degradation might form during Hcy-stimulated VSMC inflammation.

Our in vivo study further related Nox4-generated ROS to sEH-catalyzed EET degradation during Hcy-induced vascular inflammation. However, the in vivo study was limited to a phenomenal observation between sEH, Nox4 and inflammation in the vascular wall. The causal relationship could not be established with the current animal experiments. Further investigation using genetically manipulated mice is warranted to explore the regulation between Nox4 and sEH.

In conclusion, our study revealed that Nox4 and sEH are mutually regulated in Hcy-treated VSMCs. Nox4-generated ROS and sEH-hydrolyzed EETs synergistically contribute to the Hcy-induced inflammation response. Our results might reveal a Nox4-sEH vicious cycle as a novel mechanism of Hcy-induced VSMC inflammation that contributes to vascular remodeling. This finding warrants further investigation into

the concomitant use of sEH and Nox4 inhibitors in clinical practice.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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