ORIGINAL RESEARCH

VPO1 Modulates Vascular Smooth Muscle Cell Phenotypic Switch by Activating Extracellular Signal-regulated Kinase 1/2 (ERK 1/2) in Abdominal Aortic Aneurysms

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***Background*—**Hydrogen peroxide (H_2O_2) is a critical molecular signal in the development of abdominal aortic aneurysm (AAA) formation. Vascular peroxidase 1 (VPO1) catalyzes the production of hypochlorous acid (HOCl) from H_2O_2 and significantly enhances oxidative stress. The switch from a contractile phenotype to a synthetic one in vascular smooth muscle cells (VSMCs) is driven by reactive oxygen species and is recognized as an early and important event in AAA formation. This study aims to determine if VPO1 plays a critical role in the development of AAA by regulating VSMC phenotypic switch.

***Methods and Results*—**VPO1 is upregulated in human and elastase-induced mouse aneurysmal tissues compared with healthy control tissues. Additionally, KLF4, a nuclear transcriptional factor, is upregulated in aneurysmatic tissues along with a concomitant downregulation of differentiated smooth muscle cell markers and an increase of synthetic phenotypic markers, indicating VSMC phenotypic switch in these diseased tissues. In cultured VSMCs from rat abdominal aorta, H_2O_2 treatment significantly increases VPO1 expression and HOCl levels as well as VSMC phenotypic switch. In support of these findings, depletion of VPO1 significantly attenuates the effects of H_2O_2 and HOCl treatment. Furthermore, HOCl treatment promotes VSMC phenotypic switch and ERK1/2 phosphorylation. Pretreatment with U0126 (a specific inhibitor of ERK1/2) significantly attenuates HOCl-induced VSMC phenotypic switch.

***Conclusions*—**Our results demonstrate that VPO1 modulates VSMC phenotypic switch through the H_2O_2/VPO1/HOCl/ERK1/2 signaling pathway and plays a key role in the development of AAA. Our findings also implicate VPO1 as a novel signaling node that mediates VSMC phenotypic switch and plays a key role in the development of AAA.

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Key Words: abdominal aortic aneurysm • hydrogen peroxide • oxidative stress

Abdominal aortic aneurysm (AAA) is characterized by a progressive enlargement of the aorta and weakening of the aortic wall, which increases the risk of acute aortic rupture.[1] During the progression of AAA, vascular smooth muscle cells (VSMCs) switch from a contractile to a synthetic phenotype. The phenotypic switch is characterized by VSMC dedifferentiation and migration into the neointima. VSMC phenotypic switch is an important first step in AAA formation and is marked by a loss of smooth muscle cell (SMC) gene expression, an increase in matrix metalloproteinases (MMPs)

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Clinical Perspective

What Is New?

- Vascular peroxidase 1 (VPO1) is highly expressed in human and mouse aneurysmal tissues.
- VPO1 promotes vascular smooth muscle cell phenotypic switch.
- Vascular smooth muscle cell phenotypic switch and abdominal aortic aneurysm formation is mediated via the H_2O_2/VPO1/ hypochlorous acid/extracellular signal-regulated kinase 1/2 (ERK 1/2) signaling pathway.

What Are the Clinical Implications?

- VPO1 plays a vital role in cardiovascular disease and this study uncovers that a new role for VPO1 is implicated in abdominal aortic aneurysm formation.
- Oxidative stress plays a critical role in abdominal aortic aneurysm formation, and this study demonstrates that VPO1 promotes vascular smooth muscle cell phenotypic switch during abdominal aortic aneurysm formation by catalyzing H_2O_2 to produce HOCl, which is a stronger oxidant that aggravates oxidative stress.

and extracellular matrix synthesis, as well as enhanced VSMC proliferation.[2,3] The precise mechanism for VSMC phenotypic switch, however, is not well understood.

Oxidative stress plays a critical role in AAA formation and has also been implicated in regulating VSMC phenotypic switch.[4] Hydrogen peroxide (H_2O_2), an important reactive oxygen species, acts as a key signaling molecule that modulates VSMCs growth, differentiation, and migration.[5] Overexpression of the H_2O_2 scavenger catalase in VSMCs attenuates vascular wall damage and blocks AAA formation.[6]

Vascular peroxidase 1 (VPO1), a member of the heme-containing peroxidase family, is highly expressed in the cardiovascular tissues, especially VSMCs.[7,8] In the presence of chloride, VPO1 catalyzes the formation of hypochlorous acid (HOCl) from H_2O_2 and enhances oxidative stress.[9,10] Our previous studies have demonstrated that HOCl derived from VPO1 contributes to the proliferation, migration, and calcification of VSMCs.[7,11] The role of VPO1 in AAA formation and VSMC phenotypic switch, however, was previously unreported. Here we report a novel role for VPO1 in AAA formation and the underlying mechanism by which VPO1 mediates VSMC phenotypic switch.

Materials and Methods

The data, analytic methods and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. Material will be available from Xiangya Hospital of Central South University (Changsha, China), which is responsible for maintaining availability upon request to the corresponding author.

Human Tissue Collection

The collection of human tissue samples was approved by the Ethics Committee of the Xiangya Hospital of Central South University, and informed consent was obtained from all participants. Abdominal aortic aneurysm tissues (AAA, n=18) were obtained from patients who underwent open surgical operations for abdominal aortic repair, and the characteristics of aneurysm patients were described in Table S1. Human normal aortas (n=7) were collected as control group in this study. Five normal abdominal aortic tissues were obtained from people who were liver or kidney transplantation donors at Xiangya Hospital of Central South University (CSU) with informed consent from participants and/or their legal guardian/s. In addition, 2 normal aortic tissues were obtained from the ascending aortas during coronary artery bypass grafting procedures from the proximal aortotomy as control aortas. These tissues were collected and flushed with saline and immediately fixed in 4% paraformaldehyde for histology and immunohistochemistry.

AAA Murine Model

All animal procedures were approved by Xiangya Medical School, Central South University Institutional Animal Care and Use Committee. The study conformed to the *Guide for the Care and Use of the Chinese Association for Laboratory Animal Science Policy*. C57BL/6J mice aged 7- to 8-weeks-old (Central South University Laboratory Animal Division, China) that weighed between 15 and 20 g were assigned randomly to either control (control, n=5) or experimental (AAA, n=5) groups. AAA was induced by infiltrating the adventitial surface with pancreatic elastase (PPE; specific activity, 6 U/mg protein; catalog #E1250, Sigma-Aldrich, USA), as described previously.[12] Briefly, mice were anesthetized with sodium pentobarbital (0.5%, 50 mg/kg, intraperitoneal). A laparotomy was performed and the abdominal aorta extending below the left renal vein to the iliac bifurcation was identified. The abdominal aorta was isolated in situ after retroperitoneal reflection and was infiltrated with either 10 μL of PPE (AAA, 68.68 U/mL) or 0.9% sodium chloride (sham) for 10 minutes. After elastase exposure, the abdominal contents were irrigated with normal saline (0.9%) and the fascial layers and skin were closed. Mice were euthanized at 14 days by intraperitoneal injection of pentobarbital and perfused at physiological pressure with PBS followed by 4% paraformaldehyde. Abdominal aortas were excised using microscissors and imaged in PBS to avoid collapse of the vasculature and to

obtain accurate external diameter measurements. Specimens were then prepared for histological analysis.

Histology and Immunohistochemistry

Murine and human aortas were fixed in 4% paraformaldehyde embedded in paraffin. Four -micrometers thick cross sections were prepared for hematoxylin and eosin stain and Elastica-van Gieson staining for morphological assessment. For immunohistochemistry, rabbit anti-VPO1 antibody VPO1 (5 μg/mL; EMD Millipore, USA), rabbit anti-3-Cl-tyr antibody (2.5 μg/mL; Cell Science, USA), mouse anti-KLF4 antibody (5 μg/mL; Abcam, UK), rabbit anti-α—SMA and SM-22α antibodies (1.25 μg/mL; Sigma, USA), and rabbit anti-MMP-2 antibody (2.5 μg/mL; Abcam, UK) were used. Paraffin sections were rehydrated and endogenous peroxidase activity was blocked for 30 minutes in methanol containing 0.3% hydrogen peroxide. Five percent normal goat serum (Sigma-Aldrich, St. Louis, MO, USA) was incubated for 30 minutes at room temperature to block non-specific background staining. Primary antibodies were incubated at 4°C overnight, followed by 60 minutes in biotinylated secondary antibody (2 μg/mL; Abcam, England). All specimens were counterstained with hematoxylin staining solution (Beyotime Institute of Biotechnology, China). Sections were scanned using OLYMPUS CX41 and Leica Application Suite 4.0 software. Morphological analysis and collateral degree was determined.

Cell Culture and Transfection

Animal protocols were approved by the Animal Care and Use Committee of the Xiangya Hospital, Central South University (Changsha, China). Primary vascular smooth muscle cells (VSMCs) were isolated by auto-growth of explant culture from the abdominal aortas of 8-week-old male Sprague–Dawley rats as previously described.[13] Briefly, rat abdominal aortas were removed and washed with PBS. Intima and inner two-thirds of media were carefully dissected from the vessels, cut into pieces (≈1 mm^3) and carefully seeded onto the poly-L-lysine coated flask. To get a firm attachment of tissue pieces, the flask was incubated upside-down for 1 hour and then Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 20% fetal bovine serum (FBS; Gibco), 100 μg/mL penicillin and 100 μg/mL streptomycin was slowly added. Cells were allowed to auto-grow for 1 week and then passaged until enough cells were obtained. VSMCs were identified by immunofluorescence with an anti-alpha smooth muscle cell (α-SMA) antibody (1.25 μg/mL; Sigma, USA). VSMCs were used at passage 5 to 8 for secondary culture. Before experiment, VSMCs were serum starved for 24 hours, followed by incubation with H_2O_2 (100 μmol/L), HOCl (10 μmol/L) or vehicle control in serum-free medium.

Rat small interfering RNA (siRNA) against VPO1 and KLF4 and negative controls (sequences can be found in Table S2) were synthesized and purchased from RiboBio Co Ltd (Guangzhou, China). VSMCs were seeded in 6-well plates at 50% to 70% confluence, cells were washed with PBS and followed by serum starvation for 24 hours, and double transfected with siRNA against VPO1 (50 nmol) or negative control siRNA (50 nmol), and transfected with siRNA against KLF4 (50 nmol) or negative control siRNA (50 nmol). Transfection was achieved using the ribo FECTTM CP Transfection Kit (RiboBio Co Ltd, China) following the manufacturer's protocol.

Western Blot

Cells were lysed with RIPA lysis buffer (Beyotime, China) for 30 minutes. Subsequently, protein concentrations were measured using BCA Protein Assay kit (Beyotime, Jiangsu, China) and proteins (50–100 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Polyvinylidene fluoride (PVDF) membranes with 0.45 μm of pores (Millipore, MA, USA). The membranes were blocked in 5% non-fat dry milk for 1 hour, followed by incubation with primary antibodies overnight at 4°C. Primary antibodies for rabbit anti-VPO1 (5 μg/mL; EMD Millipore, USA), rabbit anti-3-Cl-tyr (5 μg/mL; Cell Science, USA), mouse anti-KLF4 (1.25 μg/mL; Abcam, UK), rabbit anti-α-SMA and SM-22α (1 μg/mL; Sigma, USA), rabbit anti-MMP-2 (1 μg/mL; Abcam, UK), and rabbit anti-ERK1/2/p-ERK1/2 (1 μg/mL; Sigma-Aldrich, USA) were used. The membranes were then washed and incubated with secondary antibodies (normal rabbit immunoglobulin G and normal mouse immunoglobulin G, Beyotime, Jiangsu, China) conjugated to horseradish peroxidase for 1 hour. Finally, membranes were washed with 0.1% Tween diluted in Tris-buffered saline and treated with Super Signal West Pico Chemiluminescent Substrate (PIERCE, Rockford IL, USA). The membranes were stripped with stripping buffer (Beyotime, China) and reblotted with glyceraldehyde-3-phosphate dehydrogenase (mouse anti-GAPDH antibody; 0.2 μg/mL; Abcam, UK). The bands were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Multi-Analyst version 1.1, Bio-Rad Gel DOC1000).

Measuring SMC Migration by Trans-Well Assay

For the Trans-well migration assays, transfected VSMCs (4×10^4 cells) were seeded on the top chamber of Trans-well chambers (24-well insert plate, 8 μm pore-size, Corning Inc, USA) using 0.2 mL of medium without serum, and 0.6 mL of medium with 10% fetal bovine serum were added to the lower chamber as a chemoattractant. After a 24 hours incubation

with H_2O_2 treatment, non-migrating cells in the upper surface of the membrane were removed with a cotton swab, and the cells that penetrated the lower chamber were fixed and stained with crystal violet. The number of cells migrated into the membrane was counted with a microscope (CX41, Olympus, Japan) in 5 randomly selected fields.

Measuring SMC Migration by Wound Scratch Assay

Wound scratch assay is a well-accepted assay for measuring SMC migration. To further confirm the migration ability, wound scratch assay is performed. And 5×10^4 VSMCs were seeded into 6-well plates. When the cell confluence reached ≈80% at 24 hours after transfection, VSMCs were serum starved for 24 hours. Starved VSMCs were stimulated with either H_2O_2 (100 μmol/L) or vehicle control for another 24 hours in serum-free medium. Scratch wounds were made by scraping the cell layer across each culture plate using a sterile micropipette tip. After the detached cells were flushed with PBS and removed, the medium was replaced with serum-free medium to inhibit cell proliferation and the cells were treated with H_2O_2. Images were captured microscopically at 0 and 24 hours and analyzed using Image-Pro Plus (version 6.0) to evaluate the percentages of recovered areas.

Immunofluorescence Microscopy

VSMCs were seeded on glass coverslips and fixed using 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were then rinsed 3 times in PBS and subsequently blocked and permeabilized in 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) for 120 minutes at room temperature. Cover slips were then rinsed and incubated with mouse anti-KLF4 antibody (5 μg/mL; Abcam, UK), rabbit anti-α-SMA and SM-22α antibodies (1.25 μg/mL; Sigma, USA) overnight at 4°C. Cover slips were then rinsed with PBS and incubated with antibodies labeled with Alexa Fluor dye with a maximum excitation at 488 nm (green; rabbit or mouse; 5 μg/mL; Abcam, UK) or for red with Alexa Fluor 594 nm (red; rabbit or mouse; 5 μg/mL; Abcam, UK) for 1 hour at 37°C in the dark. Finally, cover slips were incubated in DAPI (5 μg/mL; 4′, 6-diamidino-2-phenylindole; Sigma-Aldrich). Images were acquired using confocal microscopy (DM14000B, Leica, Germany).

Hypochlorous Acid Measurement

Measurement of 3-cholorotyrosine (3-Cl-tyr), a chlorination product of HOCl reaction with tyrosine, was used to compare relative levels of HOCl production.[14] 3-Cl-tyr levels were evaluated by Western blot. After blotting for 3-chlorotyrosine, 3 immunospecific bands of molecular weight 43, 55, and 72 kDa were visualized.[15,16]

Statistical Analysis

All data were presented as mean±SD. Statistical significance was determined using either one-way ANOVA or a 2 sample *t* test where data were normally distributed. Due to the relatively small number of animals the non-parametric Mann–Whitney test was used to examine statistical significance in this study. A 2-tailed value of $P<0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism 6.0 and SPSS 14.0.

Results

VPO1 Expression is Upregulated in Human and Murine Abdominal Aortic Aneurysmal Tissues

To elucidate the potential role of VPO1 in aneurysm development, aneurysmal tissue samples obtained from AAA patients and animal models were examined for VPO1 expression. VPO1 expression is significantly increased in human aneurysmal aortas compared with healthy aortas (Figure 1A). To better study the role of VPO1, we established a porcine pancreatic elastase (PPE)—induced mice model of AAA. In our model, aortas infiltrated by PPE are significantly dilated compared with saline-infused control aortas (1.566±0.093 mm versus 0.502±0.031 mm; $P<0.01$; Figure S1). Consistent with our findings in human AAA samples, VPO1 expression is also significantly elevated in the tunica media of PPE-induced AAA mice.

Histological examination of human aneurysmal samples and PPE-induced AAA tissue reveals significant dilation and distortion of the normal architecture as well as thinning of the media. Additionally, the levels of 3-chlorotyrosine (3-Cl-tyr; a product of HOCl induced-protein modification) are significantly increased in both human and mouse aneurysmal tissues (Figure 1A).

VSMC Phenotypic Switch Occurs in Both Human AAA and PPE-Induced Mouse AAA Model Tissues

VSCM phenotypic switch plays an important role in the pathogenesis of AAA.[2] Thus, we examined human aorta and PPE-induced mouse AAA model tissues for synthetic SMCs. The switch from a contractile to synthetic phenotype is characterized by a coordinated downregulation of differentiated SMC markers (SM-22α, SM-MHC, and α-SMA) whereas KLF4 and MMPs are upregulated. By histological analysis, we find SMC-specific markers (α-SMA, SM-22α) are significantly

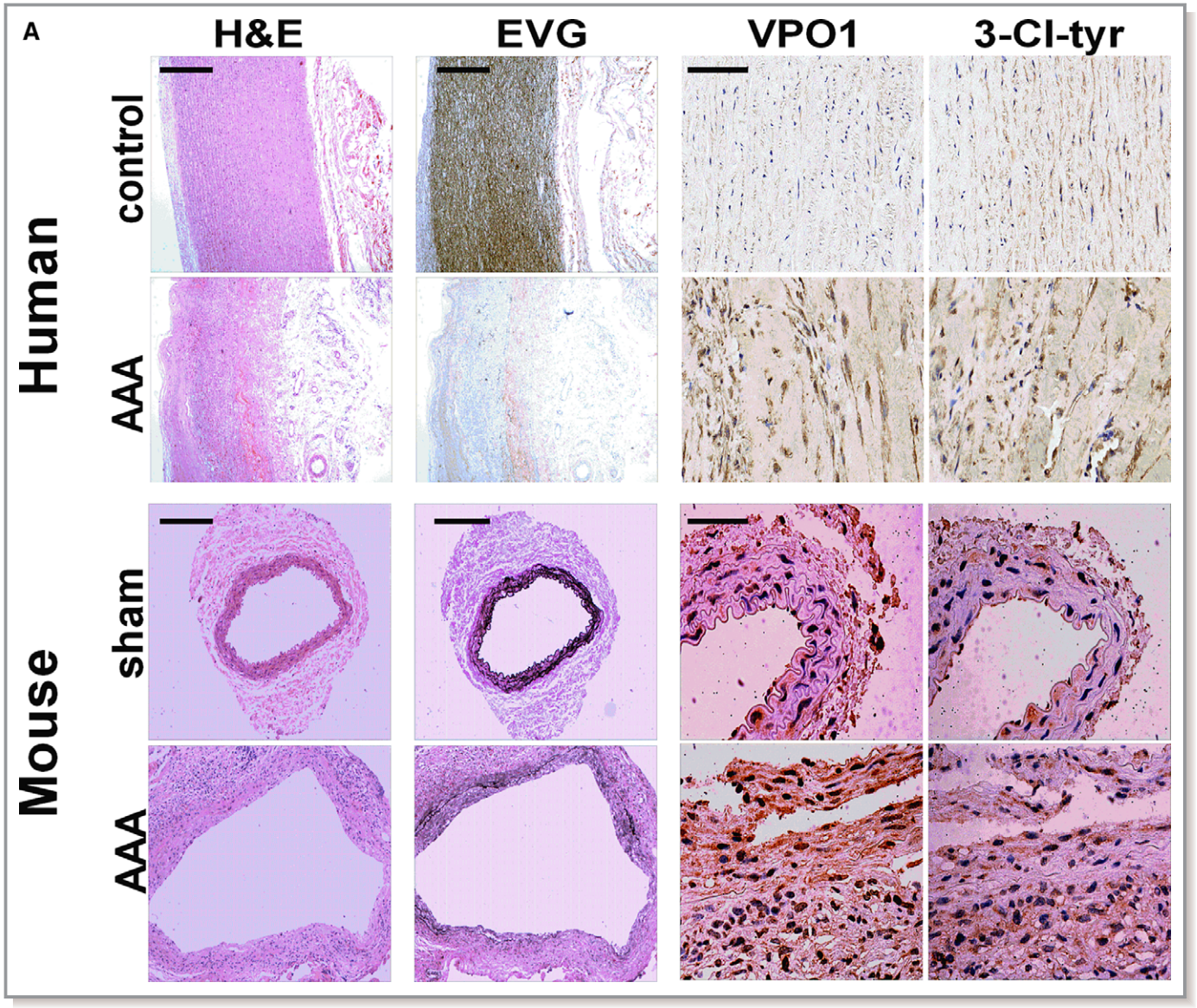

Figure 1. VPO1 is upregulated in both human and mice aneurysm tissues and VSMC phenotypic switch occurs during AAA formation. A, Representative hematoxylin and eosin stain and Elastica-van Gieson staining of human and mouse abdominal aortas (Human: Scale bar=200 μm; Mouse: Scale bar=100 μm), and immunohistochemical staining of VPO1 and 3-Cl-tyr. B, Immunohistochemical staining of KLF4, α-SMA, SM-22α, and MMP-2 in aneurysm tissues, Scale bar=50 μm. 3-Cl-tyr indicates 3-cholorotyrosine; AAA, abdominal aortic aneurysm; EVG, Elastica-van Gieson; H&E, hematoxylin and eosin; KLF4, Krüppel-like factor 4; MMP-2, matrix metalloproteinase-2; VPO1, Vascular peroxidase 1.

decreased in human and murine AAA tissues, and KLF4 and MMP-2 expression are significantly upregulated (Figure 1B). These findings are consistent with previous reports that VSMC phenotypic switch occurs during AAA formation.

VPO1 Depletion Blocks the Loss of VSMC Contractile Phenotype Induced by H_2O_2

To study the role of VPO1 in VSMC phenotypic switch, H_2O_2 was used to induce VSMC phenotypic switch in primary rat abdominal aortic VSMCs (confirmed by α-SMA expression, Figure S2) with VPO1 depletion (VPO1 expression was analyzed using real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot, as shown in Figure S3, and the primer sequences used for the RT-PCR analysis are available in Table S3). VPO1 expression in VSMCs increases in response to H_2O_2 in a dose- and time-dependent manner (Figure S4). The strongest induction of VPO1 occurs after 24 hours of 100 μmol/L H_2O_2 treatment. In response to 100 μmol/L H_2O_2 treatment, α-SMA and SM-22α expression,

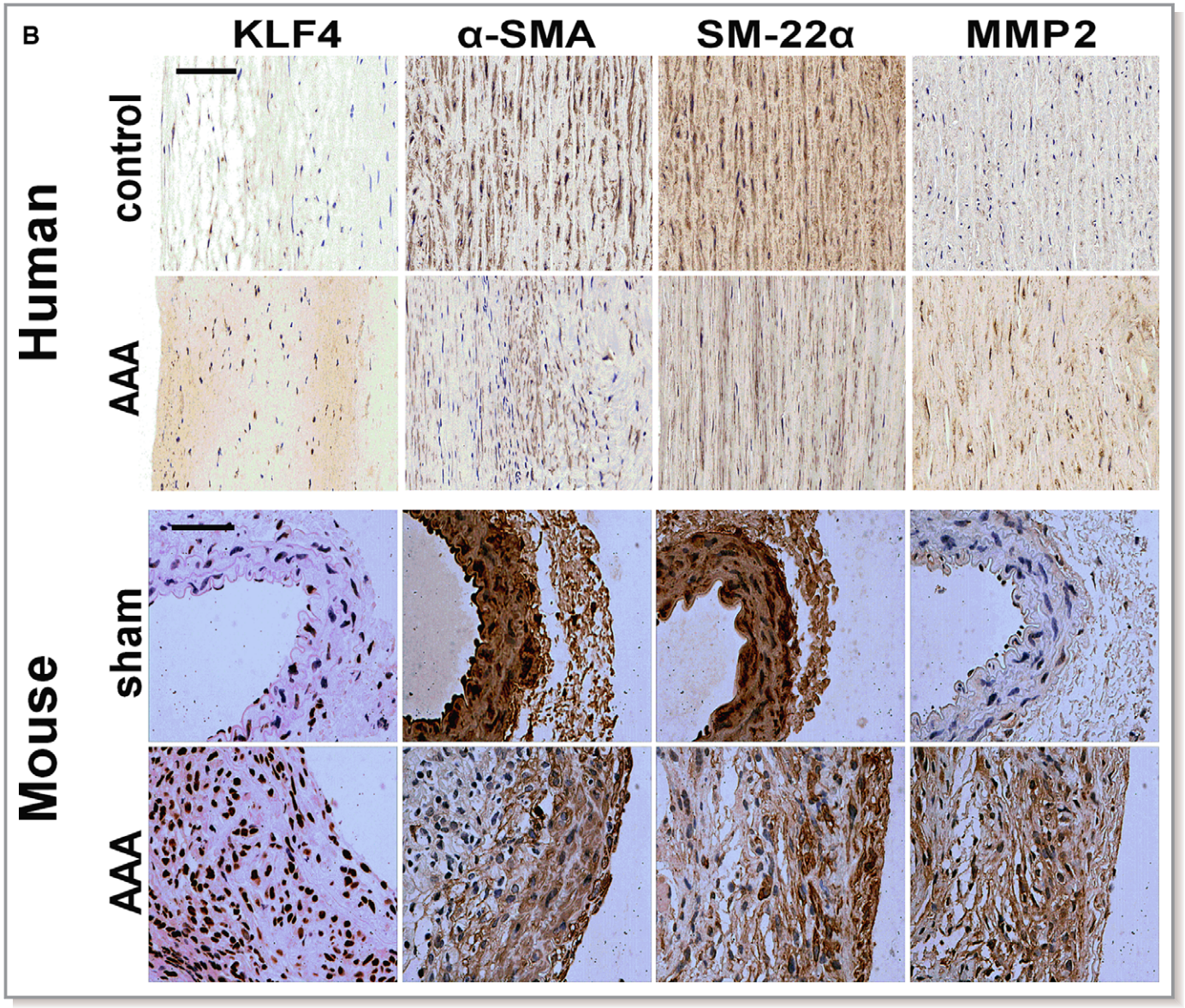

Figure 1. Continued

markers of contractile VSMCs, decrease and KLF4 expression increases. Depletion of VPO1 by siRNA blocks the H_2O_2-induced decrease of α-SMA and SM-22α expression and also attenuates the H_2O_2-induced increase in KLF4 expression (Figure 2A, 2C, and 2E). These results were also confirmed by immunofluorescence staining (Figure 2B, 2D, and 2F). Together, these results indicate that VPO1 plays an important role in H_2O_2-induced VSMC phenotypic switch by regulating KLF4, α-SMA, and SM-22α expression.

VPO1 Depletion Inhibits the H_2O_2-Induced Acquisition of a Synthetic Phenotype in VSMCs

VSMCs with synthetic phenotype are highly proliferative and migratory. Migration into the neointima contributes to the progression of AAA. MMPs, especially MMP-2, mediate extracellular matrix degradation and enable VSMCs migration.[17] H_2O_2 induces MMP-2 expression in VSMCs and knockdown of VPO1 attenuates this effect (Figure 3A). Consistent with these findings, VPO1 depletion also blocks VSMC migration as measured by a Trans-well assay and wound scratch assay. VSMCs treated with H_2O_2 have a significantly higher percentage of cells stained with crystal violet, and this effect is attenuated by VPO1 depletion (Figure 3B and 3C). Similarly, VSMCs treated with H_2O_2 has increased migration ability after a wound scratch compared with no H_2O_2 treatment and this effect was blocked by VPO1 depletion (Figure 3D). These results suggest VPO1 enhances in H_2O_2-enhanced VSMCs migration, possibly by regulating MMP-2 expression.

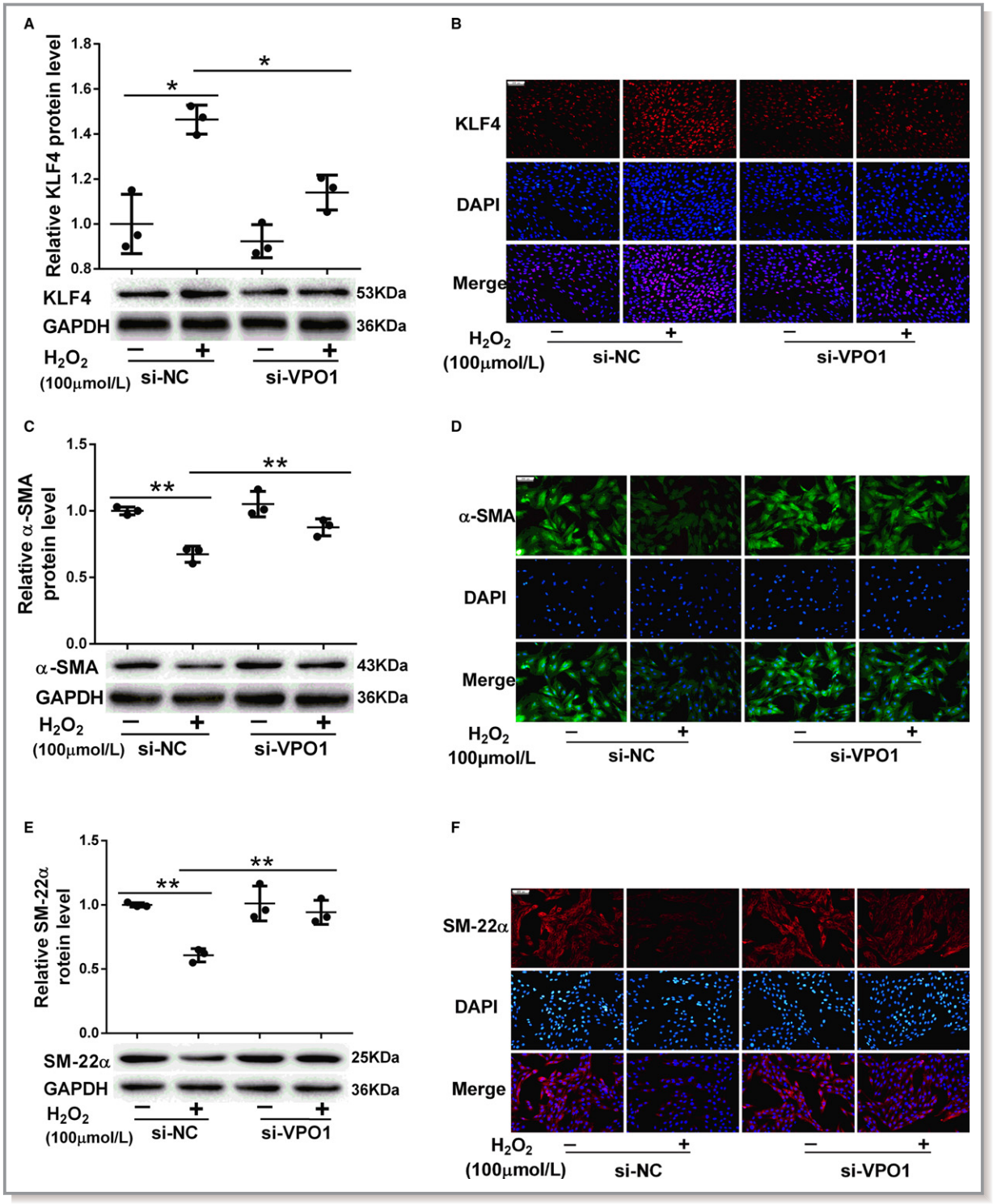

Figure 2. VPO1 depletion blocks the loss of VSMC contractile phenotype induced by H_2O_2. Primary vascular smooth muscle cells (VSMCs) were isolated from the abdominal aortas of Sprague–Dawley rats. VSMCs were used at passage 5 to 8 for secondary culture. VSMCs were serum starved for 24 hours and transfected with either NC-siRNA or VPO1-siRNAs for 24 hours. Transfected VSMCs were stimulated with either H_2O_2 (100 μmol/L) or vehicle control for another 24 hours in serum-free medium. A, C, and E, KLF4, α-SMA, and SM-22α expression were analyzed by Western blot, respectively. B, D, and F, KLF4, α-SMA, SM-22α were stained and imaged by immunofluorescence microscopy. Scale bar=100 μm. Data are presented as mean±SD of 3 independent experiments. $^{**}P<0.01$, $^{*}P<0.05$. DAP1 indicates 4′,6-diamidino-2-phenylindole; H_2O_2, hydrogen peroxide; KLF4, Krüppel-like factor 4; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; si-NC, small interfering negative control small interfering RNA; si-VPO1, small interfering vascular peroxidase 1; VPO1-siRNAs, vascular peroxidase 1 small interfering RNAs.

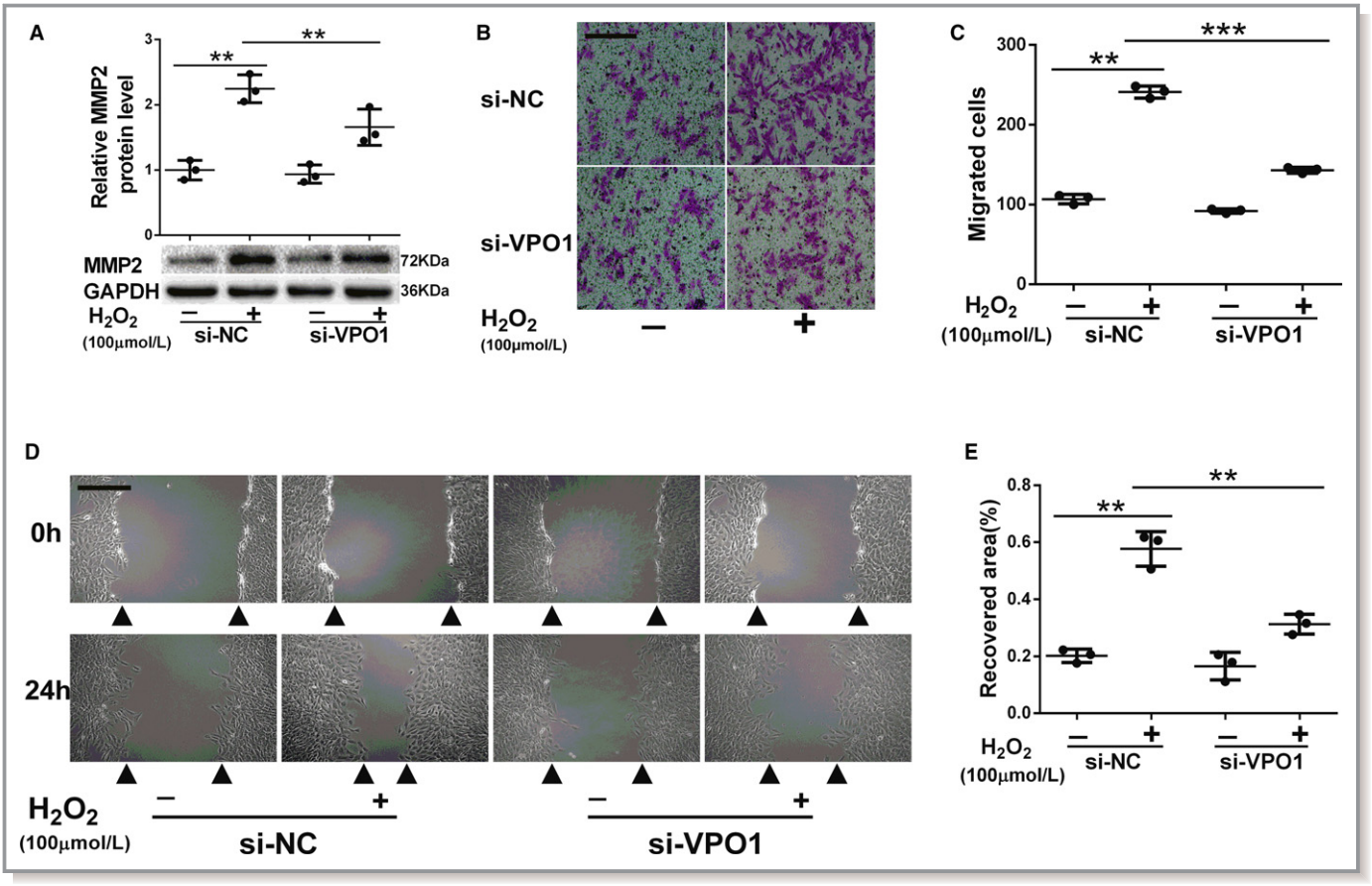

Figure 3. VPO1 depletion inhibits the H_2O_2-induced acquisition of a synthetic phenotype in VSMCs. A, MMP-2 expression was examined by Western blot. B, The migration of VSMCs was analyzed by Trans-well assay. C, The number of migrated cells was counted and analyzed. D, Wound scratch assay was performed to assess VSMC motility and migration. E, The percentage of recovered areas were analyzed. Scale bar=50 μm. Data are presented as mean±SD of 3 independent experiments. $^{**}P<0.01$, $^{***}P<0.001$. H_2O_2 indicates hydrogen peroxide; MMP-2, matrix metalloproteinase-2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; si-NC, small interfering negative control small interfering RNA; si-VPO1, small interfering vascular peroxidase.

VPO1/HOCl Regulates VSMC Phenotypic Switch Induced by H_2O_2

Our group previously reported VPO1 uses H_2O_2 to generate HOCl, a highly reactive oxidant, and enhances oxidative stress in the cardiovascular system.[18,19] To study the effect of HOCl generated by VPO1, we examined the levels of 3-Cl-tyr, a product of HOCl reaction with tyrosine, by Western blot after H_2O_2 treatment in cells with VPO1 depletion. 3-Cl-tyr levels are enhanced by H_2O_2 treatment and knockdown of VPO1 blocks this effect (Figure 4A and 4B). This result suggests that VPO1 is required for generation of HOCl from H_2O_2. To study the effects of HOCl on VSMC phenotypic switch, VSMCs were treated with 10 μmol/L HOCl for 1 hour and protein expression associated with VSMC phenotypic switch were subsequently measured by Western blot. HOCl treatment decreases the markers of differentiated SMCs (α-SMA, SM-22α) and increases the expression of KLF4 and levels of MMP-2 in cultured VSMCs (Figure S5). These findings suggest HOCl plays an important role in regulating SMC phenotypic switch. To examine the effect of KLF4 on HOCl-induced VSMC phenotypic switch, VSMCs were transfected with KLF4 siRNA or control siRNA for 24 hours, KLF4 siRNA efficiently decreased KLF4 at protein levels (Figure S6). Transfected VSMCs with KLF4 siRNA were stimulated with either HOCl (10 μmol/L) or vehicle control for 1 hour and allowed to recover 24 hours in serum-free medium. The proteins expression associated with VSMC phenotypic switch were subsequently measured by Western blot. Knockdown of KLF4 with siRNA attenuated downregulation of smooth muscle gene expression (α-SMA, SM-22α) and upregulation of MMP-2 expression in cultured VSMCs treated by HOCl (Figure 4C and 4D). These findings suggest knockdown of KLF4 reversed HOCl-induced VSMC phenotypic switch.

VPO1/HOCl Promotes H_2O_2-Induced VSMC Phenotypic Switch by Activating ERK1/2

Previous studies have demonstrated ERK1/2 is an important upstream regulator of VSMC phenotypic switch.[20] Thus, we

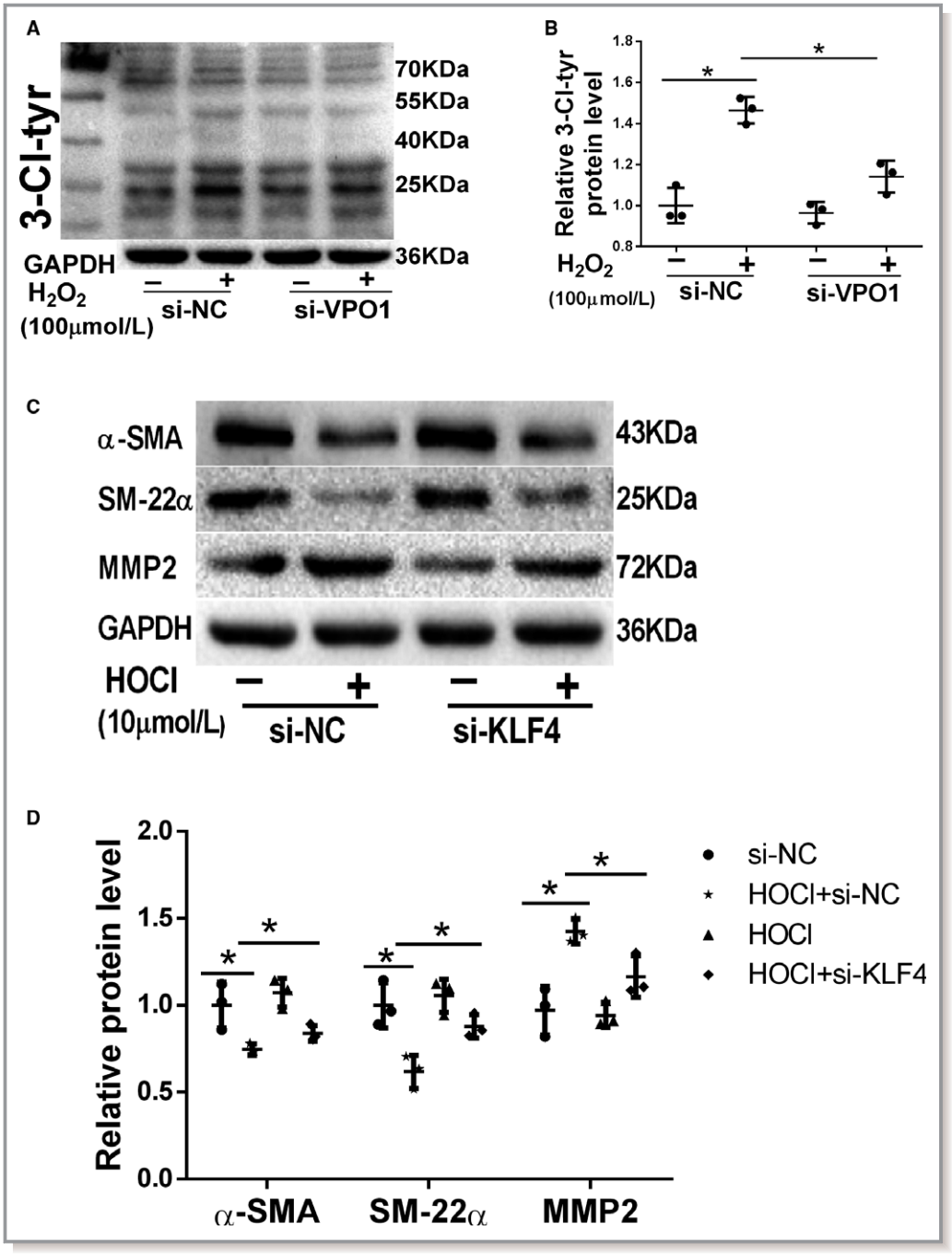

Figure 4. VPO1/HOCl regulates VSMC phenotypic switch induced by H_2O_2. A and B, VSMCs with VPO1 knockdown were treated with H_2O_2 and the expression of 3-Cl-tyr was examined by Western blot. C and D, VSMCs were serum starved for 24 hours and transfected with either NC-siRNA or KLF4-siRNAs for 24 hours, followed by treating with HOCl (10 μmol/L) or vehicle control for 1 hour and allowed to recover 24 hours in serum-free medium. The expression of KLF4, α-SMA, SM-22α, and MMP-2 was examined. Data are presented as mean±SD of 3 independent experiments. **P*<0.05. 3-Cl-tyr indicates 3-cholorotyrosine; MMP-2, matrix metalloproteinase-2; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; si-NC, small interfering negative control small interfering RNA; si-VPO1, small interfering vascular peroxidase; si-KLF4, Krüppel-like factor 4-small interfering RNAs.

sought to determine if VPO1/HOCl promotes H_2O_2-induced VSMC phenotypic switch by activating ERK1/2. H_2O_2 treatment activates ERK1/2 by increasing its phosphorylation and depletion of VPO1 attenuates this phosphorylation (Figure 5A). Additionally, 10 μmol/L HOCl treatment also increases phosphorylation of ERK1/2 (Figure 5B). To study

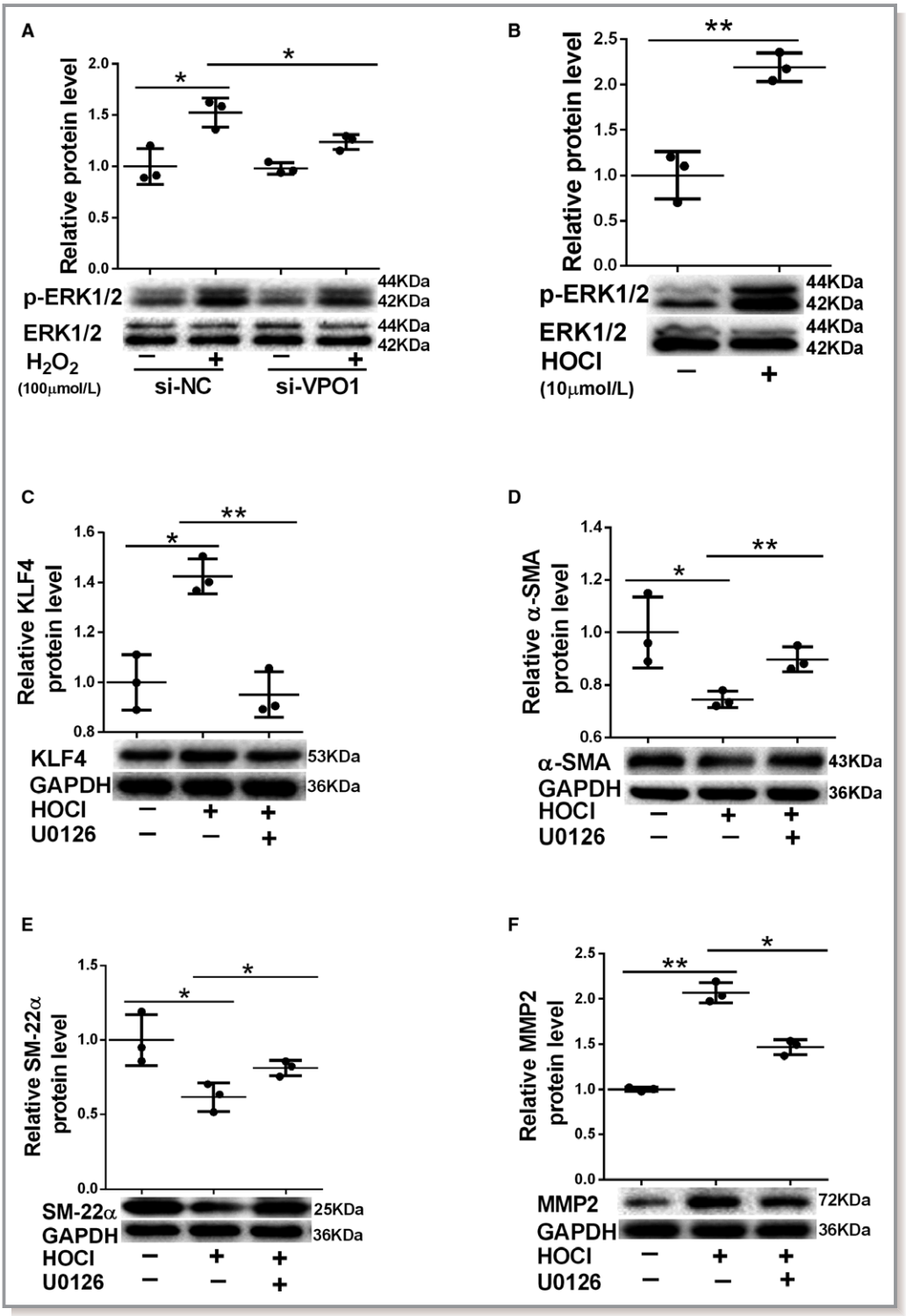

Figure 5. VPO1/HOCl promotes H_2O_2-induced VSMC phenotypic switch by activating ERK1/2. A, The expression of ERK1/2 was determined by Western blot. B, VSMCs were serum starved for 24 hours and treated with HOCl (10 μmol/L) or vehicle control for 1 hour and allowed to recover 24 hours in serum-free medium. The phosphorylation of ERK1/2 was subsequently assessed by Western blot. C through F, VSMCs were pretreated with 10 μmol/L U0126 for 30 minutes followed by HOCl (10 μmol/L) for 1 hour. The expression of MMP-2, KLF4, α-SMA, and SM-22α proteins was then examined by Western blot. Data are presented as mean±SD of 3 independent experiments. $^{**}P<0.01$, $^{*}P<0.05$. H_2O_2 indicates hydrogen peroxide; HOCl, hypochlorous acid; ERK 1/2, extracellular signal-regulated kinase; p-ERK 1/2, phosphorylated ERK 1/2; KLF4, Krüppel-like factor 4; MMP-2, matrix metalloproteinase-2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; si-NC indicates small interfering negative control small interfering RNA; si-VPO1, small interfering vascular peroxidase.

the effects of HOCl-mediated activation of ERK1/2 on VSMC phenotypic switch, KLF4, α-SMA, SM-22α, and MMP-2 levels were examined by Western blot in cells treated with the ERK1/2 inhibitor U0126. HOCl treatment significantly reduces α-SMA and SM-22α, markers of contractile VSMCs, expression and pretreatment with U0126 blocked this effect (Figure 5D and 5E). Consistent with these findings, KLF4 and MMP-2 levels are enhanced by HOCl treatment and this induction is blocked by pretreatment with U0126 (Figure 5C and 5F). Together these results indicate that VPO1/HOCl mediates H_2O_2-induced VSMC phenotypic switch by regulating ERK1/2 phosphorylation.

Discussion

This is the first study to investigate the potential role of VPO1 in the process of AAA formation. The results reveal that: (1) VPO1 is highly expressed in human and mouse aneurysmal tissues, accompanied by VSMC phenotypic switch; (2) VPO1 promotes VSMC phenotypic switch via using H_2O_2 to produce HOCl; (3) VPO1/HOCl promotes H_2O_2-induced VSMC phenotypic switch by activating ERK1/2. Based on these observations, we concluded that VPO1 modulates VSMC phenotypic switch through H_2O_2/VPO1/HOCl/ERK1/2 signaling pathway, which plays a key role in the formation of AAA. Oxidative stress has been described as an important signaling pathway in human AAA development and animal models of AAA.[21,22] The specific identity of reactive oxygen species that is responsible for the development of aortic dilation remains elusive.[4,23,24] Here, we investigate the effect of vascular peroxidase 1 (VPO1), a newly identified heme-containing peroxidase is primarily expressed in cardiovascular system,[25] on VSMC phenotypic switch, which is the early stages of aortic aneurysms formation. Peroxidases can catalyze H_2O_2 to produce hypochlorous acid (HOCl), which is a stronger oxidant that aggravates oxidative stress.[9,10] Though the VPO1 activity is ≈5% to 10% of that of myeloperoxidase (MPO), the total peroxidase activity of VPO1 could be 50- to 100-folds than that of MPO in plasma. In addition, MPO is only expressed in neutrophils and monocytes under inflammatory response and VPO1 is highly expressed in cells of the cardiovascular system,[10] therefore, it may be more significant to explore the role of VPO1 in AAA development. Our previous studies demonstrated that VPO1/HOCl pathway-mediated oxidative stress contributed to hypoxia-induced pulmonary vascular remodeling, angiotensin II-induced VSMC proliferation and

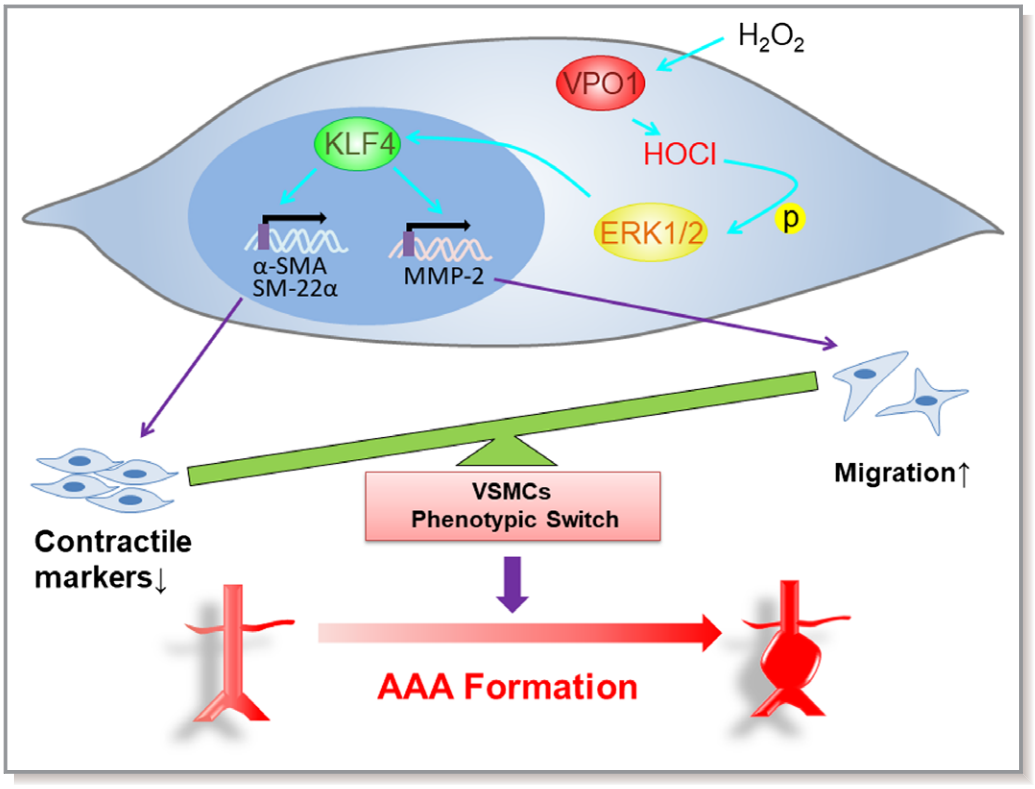

Figure 6. VPO1 signaling in vascular smooth muscle cell. VPO1 modulates vascular smooth muscle cell (VSMC) phenotypic switch through H_2O_2/VPO1/HOCl/ERK1/2 signaling pathway, which characterized by downregulation of SMC differentiation markers, such as SM-22α, α-SMA as well as an increase in migration. AAA indicates abdominal aortic aneurysm; HOCl, hypochlorous acid; KLF4, Krüppel-like factor 4; p, phosphorylated; MMP-2, matrix metalloproteinase-2.

myocardial ischemia-reperfusion injury.[8,11,19] In this study, we find that VPO1 and 3-Cl-tyr (a product of HOCl reacting with tyrosine residues) are significantly increased in human aneurysm tissues compared with healthy aortic tissues. Consistent with these findings, aortic tissues from a porcine pancreatic elastase (PPE)-induced aortic aneurysm mice have significantly increased levels of VPO1 and 3-Cl-try compared with tissues from control animals. These results suggest that VPO1/HOCl may play an important role in AAA formation.

During the early stages of AAA formation, VSMC undergo phenotypic switch which is characterized by downregulation of VSMC differentiation markers, such as SM-22α, α-SMA as well as an increase in proliferation, migration, and synthesis of extracellular matrix proteins.[2,26] In our study, we observed decreased levels of α-SMA and SM-22α by immunohistochemistry in human AAA and PPE-induced AAA tissues. Krüppel-like factor 4 (KLF4), a transcriptional factor, is known to suppress the expression of differentiated SMC maker genes during aneurysm formation.[27,28] Consistent with the literature, we observe an upregulation of KLF4 and a concomitant decrease in VSMC marker expression in human and PPE-induced AAA tissues. A previous study reported that the increased production of reactive oxygen species, especially the generation of H_2O_2, accelerating VSMC phenotypic switch from contractile to synthetic phenotype.[29] To investigate the role of VPO1 on it, in this study, we use H_2O_2 to induce VSMC phenotypic switch in cultured VSMCs. The results show that depletion of VPO1 by siRNA blocks the H_2O_2-induced decrease of α-SMA and SM-22α expression and also attenuates the H_2O_2-induced increase in KLF4 expression. These data suggest that VPO1 may promote the occurrence of VSMC phenotypic switch by regulating SMC gene expression.

Phenotypic switch from contractile to synthetic has been accepted as a prerequisite for VSMC migration.[30] A growing number of reports indicate that proteolytic breakdown of extracellular matrix by MMPs contribute to VSMCs migration.[31] Moreover, mice deficient in MMP-2 are actually resistant to aneurysm formation, indicating that MMP-2 is a causative factor in the pathogenesis of AAA.[17,32] In line with this concept, in our study, we have seen significantly increased MMP-2 levels in aneurysmatic tissue from our human and murine model of PPE-induced AAA. It is well established that the expression of MMPs is modulated by H_2O_2 in vitro.[33] Consistent with previous reports, we find that H_2O_2 promotes MMP-2 expression in cultured VSMCs. We also find that VPO1 depletion inhibits H_2O_2-induced VSMC migration as well as the induction of MMP-2 expression. These results suggest VPO1 mediates H_2O_2-induced migration, possibly by regulating MMP-2 expression.

The MAPK family has been implicated in a variety of changes in VSMC function including decreased SMC gene expression and increased migration, 2 hallmarks of transition of contractile VSMC to the synthetic phenotype.[34] Activation of the ERK and p38MAPK pathways triggered by growth factors such as platelet-derived growth factor, epidermal growth factor, and basic fibroblast growth factor have been shown to be associated with VSMC phenotypic switch and subsequent AAA formation.[34] As reactive oxygen species are a representative MAPK-activating factor, especially the ERK pathway, they are necessary for cell proliferation and migration.[7,8] We hypothesize that VSMC phenotypic switch regulated by VPO1 may be due to HOCl-induced ERK1/2 activation. In the present study, we observed ERK1/2 activation induced by H_2O_2 and this activation is significantly blocked by VPO1 depletion. In addition, HOCl downregulates the differentiated SMC markers SM-22α and SMA-α, induces MMP-2 and KLF4 expression, and enhances phosphorylation of ERK1/2. These effects are blocked by pretreatment with the ERK1/2 inhibitor U0126. Together, these results suggest VPO1 mediates VSMC phenotypic switch by signaling though activation of ERK1/2 (Figure 6).

In summary, our results provide support for the H_2O_2/VPO1/HOCl/ERK1/2 signaling as a critical pathway for VSMC phenotypic switch. Our findings also implicate VPO1 as a novel signaling node that mediates VSMC phenotypic switch and plays a key role in the development of AAA.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Table S1. Patient characteristics.

Characteristics	Control (n=7)	AAA (n=18)	P value
Age, y	67.5 ± 4.2	68.2 ± 6.3	0.095
Male	71.4%	66.7%	0.819
Smoking	42.9%	55.6%	0.568
Hyperlipidemia	57.1%	72.2%	0.468
Hypertension	42.9%	44.4%	0.943
COPD	28.6%	33.3%	0.819
DM	42.9%	50.0%	0.748
CKD	28.6%	22.2%	0.739
FHx	14.3%	11.1%	0.826

COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; CKD, chronic kidney disease; FHx, family history of AAA. Quantitative variables were analyzed using a Two sample t-test for comparisons with two groups and χ^2 test was used for categorical variables. Data were analyzed using SPSS 14.0.

Table S2. siRNAs used for VSMC transfection in the present study.

Target	Sequences
VPO1	5’-GUGGACUUGAAUGGAACAA-3’
	5’-CAAGGAGUUUGUUCUAGAAAU-3’
KLF4	5’-ACCUUGCCUUACACAUGAATT-3’

Table S3. Primers for real-time PCR in the present study.

Genes	Primers 5′-3′
Rat GAPDH	FORWARD：AACTTTGGCATTGTGGAAGG
	REVERSE： TGTGAGGGAGATGCTCAGTG
Rat VPO1	FORWARD：GCATAGAATGGTGGGAGAGC
	REVERSE： CACACAGGAGCAGAGGACAA

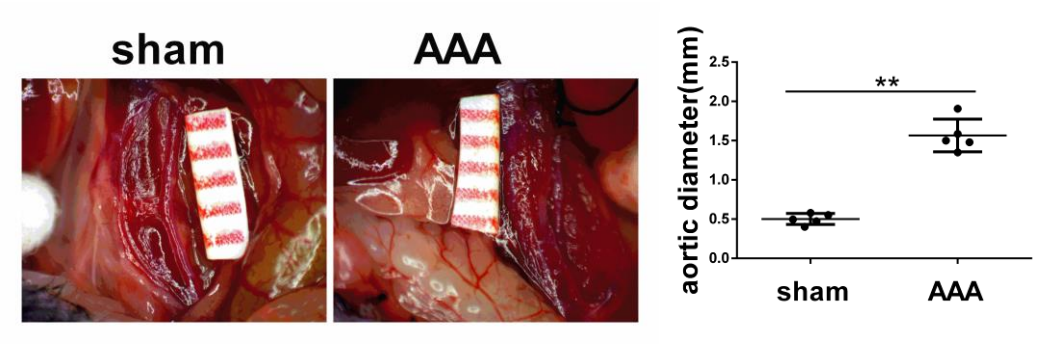

Figure S1. C57BL/6 male mice underwent infrarenal peri-adventitial application of either sodium chloride (sham; n = 5) or porcine pancreatic elastase (PPE; n = 5) for 14 days. Aortas were analyzed by video micrometry. The nonparametric Mann-Whit test was used to examine statistical significance for this figure. Data were presented as mean ± SD (n=5 mice per group), ***P* <0.01. AAA indicates abdominal aortic aneurysm.

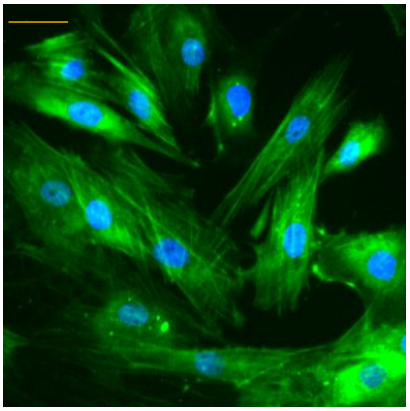

Figure S2. Identification of primary abdominal aorta-derived vascular smooth muscle cells. VSMCs was assayed by immunocytochemistry staining. α-SMA was stained using Alexa Flour (green) and nuclei were stained with 4', 6-Diamidino-2-phenylindole (DAPI, blue). Scale bar = 50 μm.

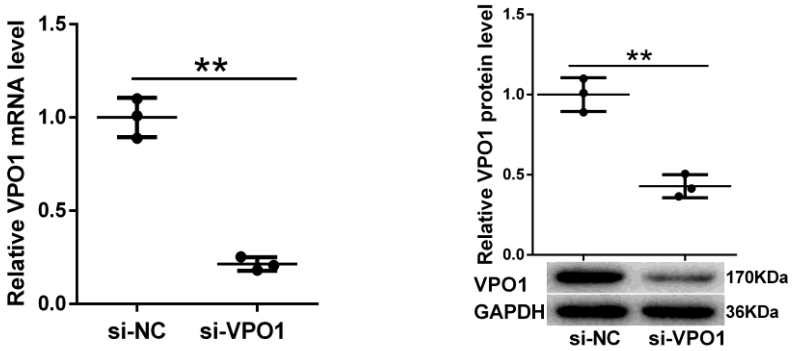

Figure S3. VPO1-siRNA significantly attenuates VPO1 mRNA and protein expression. VSMCs were transfected with either negative control siRNA (NC-siRNA) or VPO1-siRNAs for 24 hours. VPO1 expression was analyzed using real-time PCR and Western blot. Data are expressed as means ± SD of three independent experiments (n=3 per group). ***P* <0.01. si-NC indicates negative control siRNA; si-VPO1, VPO1-siRNAs.

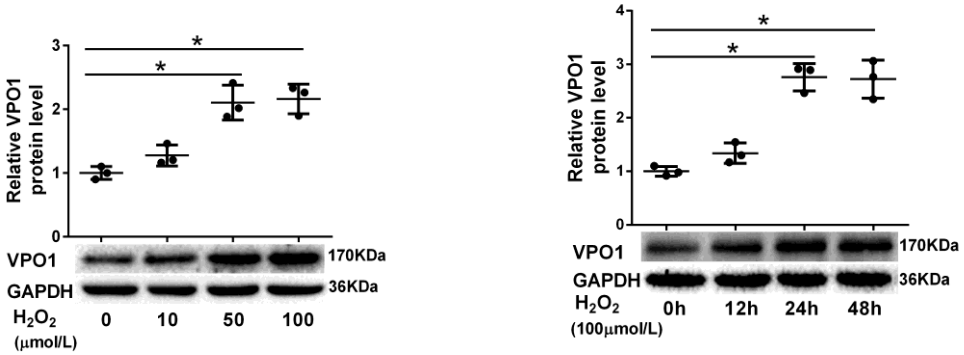

Figure S4. H_2O_2 upregulates the expression of VPO1 in both a dose- (Left) and a time-dependent (Right) manner. Data are presented as mean ± SD of three independent experiments (n=3 per group). *P <0.05.

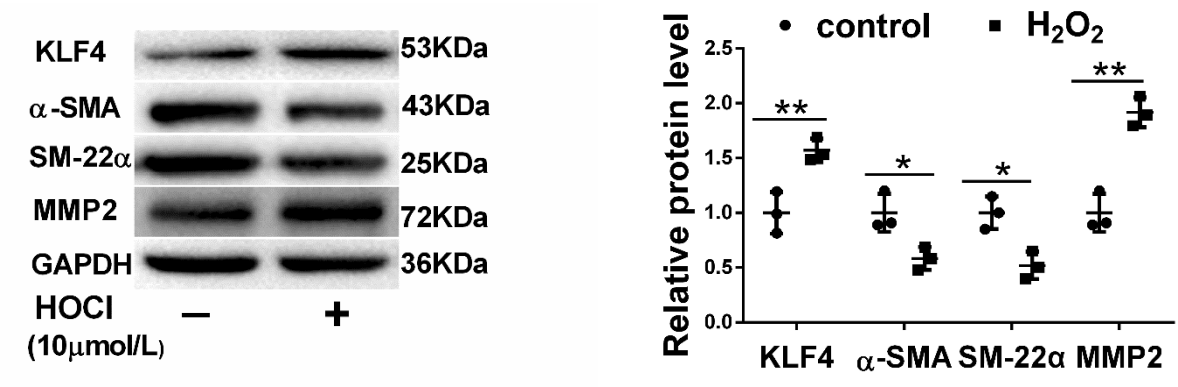

Figure S5. HOCl promotes VSMC phenotypic switch. VSMCs were treated with 10 μmol/L HOCl or vehicle control for 1 hour and allowed to recover 24 hours in serum-free medium. The expression of KLF4, α-SMA, SM-22α, and MMP-2 was examined by Western blot. Data are presented as mean ± SD of three independent experiments (n=3 per group). ***P* < 0.01, **P* <0.05.

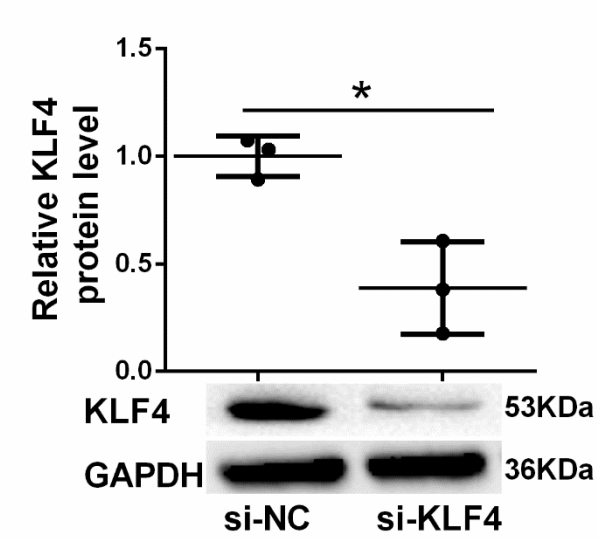

Figure S6. KLF4-siRNA significantly attenuates KLF4 protein expression. VSMCs were transfected with either negative control siRNA (NC-siRNA) or KLF4-siRNAs for 24 hours. KLF4 expression was analyzed using Western blot. Data are expressed as means ± SD of three independent experiments (n=3 per group). *P <0.05. si-NC indicates negative control siRNA; si-KLF4, KLF4-siRNAs.