Dietary salt initiates redox signaling between endothelium and vascular smooth muscle through NADPH oxidase 4

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Prevention of phenotype switching of vascular smooth muscle cells is an important determinant of normal vascular physiology. Hydrogen peroxide (H₂O₂) promotes osteogenic differentiation of vascular smooth muscle cells through expression of Runt related transcription factor 2 (Runx2). In this study, an increase in dietary NaCl increased endothelial H₂O₂ generation through NOX4, a NAD(P)H oxidase. The production of H₂O₂ was sufficient to increase Runx2, osteopontin and osteocalcin in adjacent vascular smooth muscle cells from control littermate mice but was inhibited in mice lacking endothelial Nox4. A vascular smooth muscle cell culture model confirmed the direct involvement of the activation of protein kinase B (Akt) with inactivation of FoxO1 and FoxO3a observed in the control mice on the high NaCl diet. The present study also showed a reduction of catalase activity in aortas during high NaCl intake. The findings demonstrated an interesting cell-cell communication in the vascular wall that was initiated with H₂O₂ production by endothelium and was regulated by dietary NaCl intake. A better understanding of how dietary salt intake alters vascular biology may improve treatment of vascular disease that involves activation of Runx2.

1. Introduction

Vascular smooth muscle cells (VSMC) may undergo significant phenotypic change in association with arterial diseases [1]. Osteoblastic differentiation of VSMC develops with upregulation of Runt related transcription factor 2 (Runx2), the key transcription factor of the Runx family [2]. Expression of Runx2, also known as Core-Binding Factor Subunit Alpha-1 (CBF-alpha-1), has been identified in calcified human vascular tissue specimens, but not in normal vessels [3–5]. By regulating the expression of alkaline phosphatase and bone matrix protein genes, including osteocalcin, osteopontin, collagen type I, and bone sialoprotein [6,7], Runx2 is an essential and sufficient regulator of vascular calcification [8,9] and aortic fibrosis and stiffness [10]. Onset of these vascular changes predicts development of heart failure, myocardial infarction, stroke, and chronic kidney disease [11–17]. Understanding those mechanisms that increase Runx2 and produce osteogenic differentiation of VSMC is therefore an important goal of study.

Ingestion of certain dietary electrolytes, especially sodium and potassium, regulate arterial structure and function. The endothelium may be considered a ‘first responder’ to the vascular changes resulting from dietary NaCl and potassium intake. Ambient concentrations of potassium regulated endothelial cell function by modifying cell signaling mediated by protein kinase B (Akt) and Phosphatase and tensin homolog (PTEN) [2]. A prior study also demonstrated that dietary potassium...
regulated the VSMC expression of Runx2 and development of calcification and arterarial stiffness in ApoE-deficient mice [18]. Along with potassium, studies have shown that dietary NaCl intake had profound effects on endothelial function in rodents [2,19–23]. The dietary content of NaCl determined aortic stiffness in humans [24,25], but there are gaps in understanding how these vascular changes are regulated. Dietary NaCl activated endothelial cell signaling pathways reminiscent of potassium, studies have shown that dietary NaCl intake had profound cation and arterial stiffness in ApoE-deficient mice [18]. Along with regulated the VSMC expression of Runx2 and development of calcification.

Hydrogen peroxide (H$_2$O$_2$) has gained attention as an important molecule involved in a several signaling pathways that impact cardiovascular physiology, providing adaptation to changes in the environment in normal and pathological states [29,30]. While H$_2$O$_2$ may be derived from several cellular sources [30], the NAD(P)H oxidase family is an important source of superoxide and H$_2$O$_2$. NOX4 is a unique NAD(P)H oxidase that is more evolutionarily distant from the other NOX enzymes, preferentially produces H$_2$O$_2$, is thought to be constitutively active [31–33], and is the major catalytic component of endothelial NAD(P)H oxidase [34]. In renal proximal tubule epithelium, H$_2$O$_2$ promotes cell signaling events that involved phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), Forkhead O class 3a (FoxO3a), and Sirtuin 1; the resultant changes in the activities of these molecules downregulated catalase, a critically important antioxidant enzyme [35]. Another recent study showed that H$_2$O$_2$ promoted the phosphorylation of Peroxisomal Biogenesis Factor 14 (Pex14), preventing transport of catalase into peroxisomes [36]. By decreasing catalase and preventing the proper cellular localization of this enzyme, these H$_2$O$_2$-dependent events enhanced the other cellular effects of H$_2$O$_2$.

Current evidence supported a direct role for H$_2$O$_2$ in the expression of Runx2 and subsequent osteogenic differentiation of VSMC [8]. We generated a mouse model that lacked Nox4 in endothelium (VE-Cad-Cre$^{+/Nox4^{-/}}$) genotype, and compared with their littermate (VE-Cad-Cre$^{+/Nox4^{-/}}$) controls. Our studies revealed that a high-salt diet increased endothelial NOX4, which produced amounts of H$_2$O$_2$ sufficient to promote in adjacent VSMC redox signaling events that increased Runx2 and reduced catalase. The findings supported a dietary salt intake-mediated endothelial-VSMC crosstalk that featured redox signaling and produced Runx2 in adjacent VSMC.

2. Material and methods

2.1. Commercial reagents

Recombinant human transforming growth factor-beta 1 protein (TGF-β1) was obtained commercially (Cat# 240B, R&D Systems, Inc., Minneapolis, MN). LY294002 (Cat# 15447-36-6, Selleckchem, Houston, TX), a pharmacological inhibitor of phosphatidylinositol 3-kinase (PI3K) [37], was dissolved in 0.001% DMSO final and used at a concentration of 50 μM. The solvent alone served as the corresponding vehicle controls. Antibodies directed against Akt (pan) (C67E7, Cat# 4691); p-Akt (Ser473) (D9E, Cat# 9271); FoxO1 (C29H4, Cat# 2880); and rabbit anti-smooth muscle actin, anti-SMA, ab5694 (D Systems, Inc., Woburn, MA) were obtained from a commercial vendor (Cell Signaling Technology Inc., Danvers, MA). Experiments also used commercial antibodies directed against FoxO1 (Cat# NB100-2312, Novus Biologicals, Centennial CO); p-FoxO1 (ser256) (Cat# NB100-81927, Novus Biologicals), Runx2 (Cat# ab23981, Abcam Inc., Cambridge, MA), Runx2 (C-12) (Cat# sc-390715, Santa Cruz Biotechnology Inc., Dallas, TX); P2A (clone ID6, Cat# 05–421, Millipore, Temecula, CA), osteopontin (Cat# ab283656, Abcam Inc., Cambridge, MA), osteocalcin (Cat# ab133612, Abcam Inc., Cambridge, MA), and GAPDH (Cat# Ab8245; Abcam Inc., Cambridge, MA), which served as a loading normalization control. Secondary antibodies used for western analyses included Alexa Fluor AffiniPure 680-conjugated goat anti-rabbit antibody (Cat# 712-625-150); goat anti-mouse IgG (Cat# 115-625-146); Alexa Fluor 790-conjugated goat anti-rabbit antibody (Cat# 111-655-144); and goat anti-mouse IgG (Cat#115-655-146); all were obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.

2.2. Animal and tissue preparation

This study was carried out in accordance with the recommendations in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved the project. Details on the generation of mice used in this study were included in the Supplement. Studies were conducted using 52 ten-week-old male endothelial Nox4$^{-/-}$ mice (VE-Cad-Cre$^{+/Nox4^{-/}}$) and littermate controls (VE-Cad-Cre$^{+/Nox4^{-/-}}$).

The protocol that was followed has been standardized in our laboratory [28]. The mice were housed under standard conditions and given formulated diets (AIN-76A, Dyets, Inc., Bethlehem, PA) that contained 0.3% or 4.0% NaCl. Four groups of mice were studied: VE-Cad-Cre$^{+/Nox4^{-/}}$ (endothelial Nox4$^{-/-}$) mice received either 0.3% or 4% NaCl diet; littermate VE-Cad-Cre$^{+/Nox4^{-/-}}$ mice received either 0.3% or 4% NaCl diet. Blood pressure was recorded and analyzed using DSI radiotelemetric system (DSI, Saint Paul, MN) as previously described [28]. Briefly, mice were anesthetized using isoflurane. Left carotid artery was exposed. The catheter of a PA-C10 transmitter was inserted into the carotid artery reached to aorta, and the transmitter body was placed at the left flank subcutaneously. The mice were allowed to recover for one week before experiments. Blood pressure of both strains of mice was monitored during 0.3% NaCl diet intake as baseline, then switched to 4% NaCl diet intake for 28 days.

On the 28th day of the study, mice were sacrificed and aortic tissues were harvested. Parts of the tissues were homogenized and sonicated in Pierce RIPA buffer (Cat# 89901, Thermo Fisher Scientific Pierce Protein Research Products, Rockford, IL) with Halt Protease & Phosphatase Inhibitor Cocktail (Cat# 1861284, Thermo Fisher Scientific Pierce Protein Research Products, Rockford, IL). Total soluble protein concentration in lysates was determined using a bicinchoninic acid assay (BCA) kit (Cat# 23227, BCA Protein Assay Reagent Kit, Thermo Fisher Scientific Pierce Protein Research Products, Rockford, IL).

2.3. Histology and immunofluorescence microscopy of aorta

A portion of the aortic tissue (n = 8 animals in each group) was fixed in 4% paraformaldehyde overnight and cryopreserved in 20% sucrose. Embedded tissue was sectioned at 5-μm thickness and underwent standard antigen retrieval methods that included pretreatment with 70% ethanol at –20 °C for 10 min. Autofluorescence was reduced by incubation in 50 mM ammonium chloride in PBS for 15 min. Nonspecific staining in the sections was blocked by incubation in 2% normal horse serum in PBS for 1 h, followed by incubation with specific primary antibodies (mouse anti-Runx2 antibody, D130-3, MBL International, Woburn, MA; and rabbit anti-smooth muscle actin, anti-SMA, ab5694, Abcam Inc., Cambridge, MA) overnight at 4 °C. The sections were washed and incubated with the respective secondary antibodies conjugated with Alexa Fluor 488 (green, Invitrogen) and Alexa Fluor 594 (red, Invitrogen). Counterstaining of the nucleus was achieved by mounting sections with hardset mounting media containing DAPI (blue, Vector Laboratories). Negative controls by omission of primary antibody were included in each experiment. Images were acquired using a Leica DM6000 epifluorescence microscope (Leica Microsystems, Bannockburn, IL).
2.4. Dose-dependent effect of TGF-β1 on H₂O₂ production by endothelial cells

Rat aortic endothelial cells and the EC growth medium MCDB-131C were purchased from VEC Technologies (Cat# RAE1-T/75, Cat# MCDB-131C). Human umbilical vein endothelial cells (HUVEC) (Cat# CRL-1730, ATCC, Manassas, VA) were grown in complete F-12K medium (Cat# 30–2004, ATCC) with 10% serum, 0.1 mg/ml of heparin, Sigma, Cat# H3393 and Endothelial Cell Growth Supplement (Cat# CB-40006, Fisher Scientific, Middletown, VA). Cells were seeded in a 24 well plate. Before treatment with TGF-β1, (Cat# 240B, R&D Systems, Inc., Minneapolis, MN), confluent cells were fasted were fasted for 6 h in EC growth medium, or in F-12K medium without serum. Cells were treated with supplemental TGF-β1 at 0, 0.5, 1, 3, 10, 20 ng/ml for 16 h. After treatment, cells were washed with PBS, and incubated with Amplex® Red reaction mixture (300 μl/well, 0.1 U horseradish peroxidase in 1x reaction buffer) for 1 h. The reaction mixtures were collected and fluorescence determined using an excitation wavelength of 535 nm and emission detected at 590 nm (Spectramax M2e Microplate Reader; Molecular Devices, Sunnyvale, CA). Live cells were counted from the same well with a Scepter Handheld Automated cell Counter (Cat#, PHCC00000, Millipore Corp. Billerica, MA).

2.5. Human smooth muscle cell culture

Human aortic smooth muscle cells were obtained commercially (Cat# 354-05A, Sigma-Aldrich Corp. St Louis, MO). Monolayers of VSMC were grown on 6-well plates or 100 mm dishes (Corning-Costar, Cat# 3812, Clear, EMBL-EBI, Cambridge, UK) that contained 0 mM or 0.4 mM H₂O₂ for an additional 24 h. Cells were then harvested by gentle scraping and centrifugation at 300×g for 10 min at 4°C and resuspended in PBS. Cell lysates were obtained for analysis of catalase activity, PP2a activity, Runx2 ELISA, total protein concentration, or western blotting for Akt, p-Akt, p-FoxO1, p-FoxO3a, p-FoxO1, p-FoxO1, catalase, Runx2, and GAPDH.

2.7. Quantification of H₂O₂

H₂O₂ was quantified using a kit (Amplex® Red Hydrogen Peroxide/ Peroxidase Assay Kit, Molecular Probes), following the protocol provided by the manufacturer. Medium or serum samples, which were diluted 1:10 in reaction buffer, and standards were mixed with the working solution and incubated at room temperature for 30 min, protected from light. Fluorescence was excited at 535 nm and emission detected at 590 nm (Spectramax M2e Microplate Reader; Molecular Devices, Sunnyvale, CA). All samples were tested in duplicate and averaged.

To quantify the effect of catalase knockdown on cell content of H₂O₂, 48 h after transfection using either siRNA that targeted catalase or nontargeting siRNA, VSMC were then incubated in serum-free medium that contained 0 mM or 0.4 mM H₂O₂ for 5, 10, 15, 20, and 30 min. At each time point, medium was removed and cells were washed with PBS, then incubated with Amplex® Red reaction mixture (150 μl/well, 0.1 U horseradish peroxidase in 1x reaction buffer) for 1 h. Fifty μl of the reaction mixture was collected for each assay and fluorescence determined using an excitation wavelength of 535 nm and emission detected at 590 nm (Spectramax M2e Microplate Reader; Molecular Devices, Sunnyvale, CA). Live cells were counted from the same well with a Scepter Handheld Automated cell Counter (Cat#, PHCC00000, Millipore Corp. Billerica, MA).

2.8. Western blot analyses

Tissue and cell lysates (20–60 μg total protein) were boiled for 5 min in Laemmli buffer and separated using 7–12% SDS-PAGE (Cat# 17061044 Bio-Rad Laboratories, Hercules, CA), before electrophoretic transfer onto PVDF membranes. The membranes were blocked in 5% nonfat milk and then probed with an antibody (diluted 1:1000) that recognized specifically catalase, FoxO1, pFoxO1 (ser256); FoxO3a, pFoxO3a (S253), Akt (pan), pAkt (S473), Runx2, PP2A, osteopontin, osteocalcin, and GAPDH (diluted 1:5000). After washes, the blots were incubated for 1 h at room temperature with Alexa Fluor 680 or 790 conjugated AffiniPure anti-rabbit or anti-mouse secondary antibody (1:10,000 dilution). The bands were detected using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), and densitometric analysis was performed using Image Studio Software (LI-COR Biosciences, Lincoln, NE).

2.9. Quantification of human Runx related transcription Factor2 (Runx2)

Cell Lysates levels of Runx2 were also determined using ELISA (Cat# MB3452519, MyBioSource, Inc. San Diego, CA), following the protocol provided by the manufacturer. Collected cells were washed and resuspended in PBS and then ultrasonicated. Cell lysates were centrifuged at 1500×g for 10 min to remove cellular debris. 100 μl of cell lysates and a dilution of each standard were added to an antibody-coated 96-well plate and incubated at 37°C for 2 h 100 μl of Detection Reagent A were added into each well. The plate was incubated at 37°C for 1 h. After washing, 100 μl of Detection Reagent B was added into each well. The plate was incubated at 37°C for 1 h, followed by substrate and stop solution. Runx2 levels were quantified using a colorimetric plate reader (Spectramax M2e Microplate Reader; Molecular Devices, Sunnyvale, CA).
2.10. Determination of catalase activity

Catalase activity was quantified using a commercially available kit (Catalase Colorimetric Activity Assay Kit, Cat# EIACATC, Invitrogen by Thermo Fisher Scientific, Frederick, MD). VSMC at 1 × 10⁶ were resuspended in cold 1X assay buffer and homogenized on ice by sonication, following the protocol provided by the manufacturer. The reaction included hydrogen peroxide, substrate, and HRP solution. Colorimetric absorbance was read at 560 nm. (Spectramax M2e Microplate Reader; Molecular Devices, Sunnyvale, CA). All samples were tested in duplicate and averaged.

2.11. Protein phosphatase 2 (PP2a) activity assay

PP2a activity was assayed using a PP2A Immunoprecipitation Phosphatase Assay Kit, (Cat# EIAPATC, Invitrogen by Thermo Fisher Scientific, Frederick, MD). VSMC at 2 × 10⁶ were resuspended in 0.3 ml of phosphatase extraction buffer, containing 20 mM imidazole HCl, 2 mM EGTA, pH 7.0, with 10 μg/ml each of aprotinin, leupeptin, and pepstatin, and 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride. The cells were homogenized and centrifuged at 2000 × g for 5 min. Total protein concentration of the supernatants was determined using a BCA Protein Assay Reagent Kit (Cat# 23227, Pierce). Protein samples were denatured in 6× SDS PAGE loading buffer and separated by SDS PAGE. The proteins were transferred to a nitrocellulose membrane (Millipore) and blocked with 5% skimmed milk. The membrane was incubated with 4 μg of anti-PP2a, C subunit antibody (clone 1D6, Cat# 05–421, Millipore) and 40 μl of Protein A agarose slurry for 2 h at 4 °C. Following washes, phosphatase substrate was resuspended in cold 1X assay buffer and homogenized on ice by sonication. Colorimetric absorbance was read at 405 nm using a microplate reader (Spectramax M2e Microplate Reader; Molecular Devices, Sunnyvale, CA). Absorbance was compared to the standard curve to determine PP2a activity. Along with determination of PP2a activity, western analysis of the immunoprecipitates was performed using the same antibody that was directed against the catalytic subunit of anti-PP2a.

2.12. Statistics

All data including those represented graphically were expressed as mean ± SEM. For multiple group comparisons, either one-way analysis of variance (ANOVA) or two-way ANOVA, followed by Tukey’s multiple comparisons test, was performed using Prism, version 9.2.0. Two-way ANOVA partitioned the overall variance of the outcome variable into three components, plus a residual (or error) term. It computed variants for the null hypotheses that each factor had no effect on the response, as well as P values for the null hypotheses that each factor had no effect on the response. Where appropriate, P values that tested the null hypotheses were provided in the figure legends, while significance demonstrated through subsequent post-hoc testing was illustrated with asterisks within the figure. P < 0.05 was considered statistically significant.

3. Results

3.1. Endothelium produced H₂O₂ in response to TGF-β1 in vitro and increased dietary salt intake in vivo

Consistent with our prior finding that increased dietary NaCl intake activates endothelial TGF-β1 signaling, which was upstream of NOX4 [28], overnight incubation of human umbilical vein endothelial cells (HUVEC) in medium containing supplemental TGF-β1 produced a dose-dependent increase in H₂O₂ release (Fig. 1A). Primary cultures of aortic endothelial cells also demonstrated this dose-dependent response to the addition of TGF-β1 into the medium (Supplemental Fig. 1). These findings were also compatible with studies from other investigators showing that TGF-β1 increased production of H₂O₂ through NOX4 in arterial beds [42–46]. While on a diet that contained 0.3% NaCl, arterial beds [42–46]. While on a diet that contained 0.3% NaCl,
endothelial Nox4−/− and littermate (VE-Cad-Cre/Nox4fl/fl) control mice demonstrated similar concentrations of H2O2 in the serum (Fig. 1B). When fed the diet containing 4.0% NaCl, serum levels of H2O2 increased (P < 0.05) in the littermate controls, when compared to the other three groups, but did not increase (P > 0.05) in the endothelial Nox4−/− mice. These studies identified endothelial NOX4 as the source of increased H2O2 levels during increased NaCl intake. This effect was independent of changes in arterial pressures, since telemetry-monitored blood pressures did not differ between the VE-Cad-Cre/Nox4fl/fl and VE-Cad-Cre−/Nox4fl/fl mice during ingestion of the 4% NaCl diet (Fig. 2).
3.2. Endothelium-Nox4−/− mice were protected from NaCl-induced activation of Runx2 in VSMC

Because of the known inhibitory effects of H₂O₂ and other reactive species on catalase protein levels [35] and activity [47–49], initial studies determined catalase activity in aortic lysates from mice on the low salt (0.3%) or high salt (4.0%) diet for 30 days. Aorta catalase activity levels did not differ (P > 0.05) between endothelial Nox4−/− mice and littermate controls on the 0.3% NaCl diet. In the 4.0% NaCl groups, catalase activity fell in both strains but to a greater (P < 0.05) extent in the littermate control mice (Fig. 3A). In a similar fashion, Western analysis of the relative content of catalase in the aorta showed a pattern like the catalase activity findings (Fig. 3B). Western analysis of aortic lysates from endothelial Nox4−/− and littermate control mice given either a low salt (0.3%) or high salt (4.0%) diet for 30 days showed similar amounts of Runx2 in the low salt groups; however, aortic lysates of endothelial Nox4−/− mice contained less (P < 0.05) Runx2 in the high salt groups (Fig. 4A). With the increase in dietary salt intake, two bone matrix proteins—osteocalcin and osteopontin—that were regulated by Runx2 [6,7] also increased in the littermate controls, but were lower in the endothelial Nox4−/− mice (Fig. 4B and C). Consistent with the western analyses, immunohistochemistry of aortas from endothelial

![Diagram](https://example.com/diagram.png)
Nox4−/− and littermate control mice demonstrated minimal expression of Runx2 when maintained on 0.3% NaCl. Runx2 was identified in VSMC of littermate control mice on the 4.0% NaCl diet for 30 days. In contrast, Runx2 was not identified in VSMC of endothelial Nox4−/− mice on the 4.0% NaCl diet (Fig. 5).

3.3. Dietary NaCl-induced activation of Akt, FoxO1, and FoxO3a was prevented in aortas of endothelial Nox4−/− mice

Western analyses of aortic lysates of endothelial Nox4−/− and littermate control mice on 0.3% NaCl and 4.0% NaCl were studied for activity levels of Akt, FoxO1 and FoxO3a; both FoxO1 and FoxO3a have been shown to be phosphorylation targets of Akt [50–52]. When fed the 4.0% NaCl diet, aortic lysates of littermate control mice contained increased levels of phosphorylated Akt and both FoxO1 and FoxO3a, but was limited in lysates from endothelial Nox4−/− mice (Fig. 6).

3.4. Inhibition of Akt prevented H2O2-mediated changes in pFoxO1, pFoxO3, Runx2, and catalase in VSMC

To provide additional mechanistic findings, human aortic smooth muscle cells (VSMC) were studied in vitro. VSMC cells were incubated initially for 30 min in medium containing either LY294002, a pharmacological inhibitor of phosphatidylinositol 3-kinase (PI3K) [37], or PBS control. H2O2 0.4 mM, or vehicle alone was then added to the medium and the incubation continued overnight. H2O2 increased pAkt(S473) in VSMC, while LY294002 inhibited the activation of Akt (Fig. 7A). H2O2 increased phosphorylated FoxO1 (pFoxO1(S256)) and FoxO3a (pFoxO3a(S253)) in VSMC, and addition of LY294002 prevented these increases (Fig. 7B and C). Addition of H2O2, 0.4 mM, into medium increased VSMC Runx2, which was prevented by LY294002 (Fig. 7D). Addition of H2O2, 0.4 mM, into medium of VSMC decreased catalase and catalase activity in lysates of VSMC; the addition of LY294002 mitigated the decrease in catalase and catalase activity (Fig. 7E and F). The results demonstrated that a pivotal role of the PI3K/Akt pathway in mediating H2O2-induced phosphorylation of FoxO1 and FoxO3a, increases in Runx2, and decreases in catalase.

3.5. FoxO1 and FoxO3a participated in H2O2-induced changes in Runx2 in VSMC

Addition of H2O2, 0.4 mM, into the medium increased pFoxO1(S256) and pFoxO3a(S253) in VSMC (Fig. 7A and C). Knockdown of FoxO1 and FoxO3a in VSMC using siRNA directed against FoxO1 and FoxO3a, respectively, was confirmed (Fig. 8A and C). Under these conditions, basal Runx2 levels and H2O2-induced increases in Runx2 were augmented (Fig. 8B and D). The data confirmed a role for both FoxO1 and FoxO3a in the regulation of Runx2 in VSMC and were consistent with a prior publication showing the direct involvement of FoxO1/3 in upregulation of Runx2 and associated vascular calcification in smooth muscle-specific PTEN-deficient mice [51].

Fig. 5. Immunohistochemistry analysis of expression of Runx2 in the aorta. The smooth muscle cell layers were identified using antibody to SMA (green color). Runx2 was identified using anti-Runx2 antibody (red color). Nuclei were counterstained with DAPI (blue color). While on the 0.3% NaCl diet, aortas of endothelial Nox4−/− (VE-Cad-Cre+/Nox4fl/fl) and littermate (VE-Cad-Cre−/Nox4fl/fl) mice demonstrated no discernible amounts of Runx2 with immunohistochemistry. When fed a 4.0% NaCl diet, Runx2 increased in vascular smooth muscle (arrows) of the aorta of littermate mice. Bar represented 50 μm. L, lumen. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
3.6. Catalase mitigated H$_2$O$_2$-induced changes in PP2a activity and Runx2 in VSMC

Because of the demonstrated decrease in catalase in VSMC incubated in medium containing H$_2$O$_2$ (0.4 mM), additional experiments were performed to determine the effect of catalase activity on the upregulation of Runx2. Successful knockdown of catalase in VSMC was performed using siRNA targeting catalase and confirmed using western analysis (Fig. 9A). Cells treated with nontargeting siRNA demonstrated the decrease in catalase when incubated in medium containing 0.4 mM H$_2$O$_2$ (Fig. 9A). Catalase activity levels mirrored the western analyses (Fig. 9B). These data were also consistent with the observed effect of H$_2$O$_2$ catalase on the aortas of endothelial Nox4$^{-/-}$ mice and littermate controls. To confirm that the catalase knockdown experiments increased available H$_2$O$_2$, H$_2$O$_2$ accumulation in VSMC pre-treated with siRNA that targeted catalase and nontargeting siRNA was performed over a 30-min period following the addition of H$_2$O$_2$ or vehicle alone into the medium (Fig. 9C). VSMC treated with either the catalase or the nontargeting siRNA demonstrated no differences when treated with vehicle alone. VSMC treated with the nontargeting siRNA and incubated with H$_2$O$_2$ showed significantly higher levels of H$_2$O$_2$, compared with the other three groups.

Additional experiments included PP2a, a ubiquitous redox-sensitive phosphatase that is present in smooth muscle [54], is sensitive to H$_2$O$_2$ [55, 56], and is upstream of the PI3K/Akt pathway [35]. Immunoprecipitation experiments (Fig. 10A) showed similar amounts of the PP2a C subunit in the lysates, but PP2a activity was reduced in the presence of H$_2$O$_2$ (Fig. 10B). Loss of catalase further reduced PP2a activity in the
samples (Fig. 10B). The anticipated increase in Runx2 by H$_2$O$_2$ was enhanced in samples lacking catalase (Fig. 10C).

4. Discussion

The present studies were the first to demonstrate an interesting redox-mediated communication that developed between the endothelium and VSMC during changes in dietary NaCl intake. Specifically, we demonstrated that an increase in dietary NaCl intake promoted endothelial production of H$_2$O$_2$ by NOX4, an unusual NAD(P)H oxidase that produces only H$_2$O$_2$,[31,32], and thereby increasing Runx2, along with osteopontin and osteocalcin, and reducing catalase in adjacent VSMC of littermate control mice, but not endothelium-Nox4$^{-/-}$ mice. The observations occurred independently of changes in blood
pressure, since telemetry-monitored blood pressures did not differ between the endothelial-Nox4−/− mice and littermate controls. The high NaCl diet also promoted activation of Akt, increased phosphorylation of FoxO1 and FoxO3a, and reduced catalase protein and enzymatic activity in the littermate control mice. To provide mechanistic understanding, a series of in vitro studies with VSMC were performed. Incubation of VSMC overnight in medium containing 0.4 mM H₂O₂ showed similar responses with activation of Akt, increased phosphorylation of FoxO1 and FoxO3a, reduced sirtuin 1 activity, and increased Runx2 and decreased catalase activity. The effect on Runx2 was inhibited by the PI3K/Akt inhibitor, LY294002. Knockdown of FoxO1 and FoxO3a increased basal Runx2 levels. Knockdown of catalase increased Runx2, and an interaction effect (P < 0.05) between H₂O₂ and FoxO3a siRNA on levels of pFoxO3a(S253) was not observed (n = 4 samples in each group; ns, not significant; *P < 0.05; Two-way ANOVA).

Fig. 8. siRNA-mediated knockdown of FoxO1 or FoxO3a increased basal Runx2 levels and H₂O₂-induced increases in Runx2 in VSMC. A, Western blot showed successful knockdown of FoxO1 by FoxO1-specific siRNA. Addition of H₂O₂ into the medium induced the inhibition of FoxO1 (indicated by the increase in pFoxO1(S256)). In these studies, FoxO1 siRNA (P < 0.05), but not H₂O₂ (P = 0.07), affected levels of pFoxO1(S256) in VSMC, but there was an interaction effect (P < 0.05) between H₂O₂ and FoxO1 siRNA on pFoxO1(S256). (n = 4 samples in each group; ns, not significant; *P < 0.05; Two-way ANOVA) B, Knockdown of FoxO1 increased basal expression of Runx2 and increased H₂O₂-mediated increases in Runx2. H₂O₂ (P < 0.05) and FoxO1 siRNA (P < 0.05) affected levels of Runx2 in VSMC and an interaction effect (P < 0.05) between H₂O₂ and FoxO1-specific siRNA on levels of pFoxO1(S256) was observed. (n = 4 samples in each group; *P < 0.05; Two-way ANOVA) C, Western blot showed successful knockdown of FoxO3a by FoxO3a-specific siRNA. Addition of H₂O₂ into the medium induced the inhibition of FoxO3a (indicated by the increase in pFoxO3a(S253)). In these studies, H₂O₂ (P < 0.05) and FoxO3a siRNA (P < 0.05) affected levels of pFoxO3a(S253) in VSMC and an interaction effect (P < 0.05) between H₂O₂ and FoxO3a-specific siRNA on levels of pFoxO3a(S256) was observed. (n = 4 samples in each group; ns, not significant; *P < 0.05; Two-way ANOVA) D, Knockdown of FoxO3a increased basal expression of Runx2 and increased H₂O₂-mediated increases in Runx2. H₂O₂ (P < 0.05) and FoxO3a siRNA (P < 0.05) affected levels of Runx2 in VSMC, but an interaction effect (P > 0.05) between H₂O₂ and FoxO3a siRNA on levels of pFoxO3a(S253) was not observed (n = 4 samples in each group; ns, not significant; *P < 0.05; Two-way ANOVA).
indicating direct involvement of catalase activity in modifying the effects of these redox signaling events. Supported by the in vitro experiments, the reduction in catalase protein in vivo likely contributed to other events known to reduce catalase activity, including cellular mis-localization of catalase [36] and direct inhibition by other reactive species [47–49]. The combined findings supported a dietary NaCl-mediated endothelial cell-VSMC crosstalk that featured redox signaling originating in endothelium and promoting the expression of Runx2 in VSMC (Fig. 11).

Previous research established a link between high NaCl diet and the development of an endothelial cell signaling pathway that followed activation of transforming growth factor-beta (TGF-β) in rats [19,28]. This autacoid pathway involved an ALK5-dependent increase in endothelial NOX4 [28]. In support of these data and prior observations that endothelial cells have been shown to produce H2O2 [42–46], the present studies showed that endothelium produced H2O2 in response to TGF-β1 in vitro. Because serum H2O2 concentration did not increase in the endothelium-Nox4−/− mice during increased NaCl intake, endothelial NOX4 served as the cellular source of this increase in serum H2O2 levels. The release of H2O2 by endothelium permitted function as a diffusible signaling molecule that impacted vascular adaptation during high dietary NaCl intake.

A direct role for H2O2 in the expression of Runx2 and subsequent osteogenic differentiation of VSMC has been identified [8]. However, the mechanism by which H2O2 regulated cellular Runx2 and subsequent osteogenic differentiation was complicated. Mice lacking PTEN in smooth muscle cells demonstrated sustained activation of Akt, upregulation of Runx2 and reductions in poly-ubiquitinated Runx2, and vascular calcification. Akt activation phosphorylated FoxO1/3a to promote nuclear extrusion of these molecules, producing the increase in

Fig. 9. Effect of siRNA-mediated knockdown of catalase on catalase protein, activity, and bioavailable H2O2 in VSMC. A, Western blotting demonstrated successful knockdown of catalase protein by catalase-specific siRNA. Addition of H2O2 into the culture medium decreased (P < 0.05) levels of catalase in VSMC that received nontargeting siRNA. H2O2 (P < 0.05) and catalase siRNA (P < 0.05) affected catalase protein levels in VSMC and an interaction effect (P < 0.05) between H2O2 and catalase siRNA on catalase was observed. (n = 4 samples in each group; ns, not significant; *P < 0.05; Two-way ANOVA) B, Catalase activity followed a similar pattern, with H2O2 (P < 0.05) and catalase siRNA (P < 0.05) affecting catalase activity in VSMC and an interaction effect (P < 0.05) between H2O2 and catalase siRNA on catalase was observed. (n = 8 samples in each group; ns, not significant; *P < 0.05; Two-way ANOVA) C, H2O2 accumulation during vehicle treatment of VSMC pre-treated with siRNA that targeted catalase and VSMC that received nontargeting siRNA did not differ (*P > 0.05). In contrast, incubation of VSMC that received nontargeting siRNA in medium containing H2O2, 0.4 mM, showed significant (**P < 0.05) increases in H2O2 at each time point, compared with the vehicle-treated groups. Incubation in medium containing H2O2, 0.4 mM, of VSMC pre-treated with siRNA that targeted catalase produced significantly higher (***P < 0.05) amounts of H2O2 at each time point, compared with the other three groups in the study. (n = 8 samples in each group; one-way ANOVA).
Runx2 and subsequent osteogenic differentiation of VSMC [51]. In the current study, an increase in dietary NaCl also stimulated increased activity of Akt in lysates of aorta, along with increased phosphorylation of FoxO1 and FoxO3a. The direct involvement of this signaling pathway on Runx2 was strengthened by the in vitro studies that used pharmacological and biological inhibitors of members of this pathway. In addition, the unequivocal involvement of levels of FoxO1 and FoxO3a in determining Runx2 levels in VSMC and an interaction effect (P < 0.05) between H$_2$O$_2$ and catalase siRNA on Runx2 was observed. (n = 4 samples in each group; *P < 0.05; Two-way ANOVA).

5. Conclusions

An increase in dietary salt intake initiated endothelial production of H$_2$O$_2$, uncovering interactions among Akt, FoxO1, FoxO3a, and sirtuin 1 in VSMC. These cellular responses to stress, with Akt serving an important counterregulatory factor by phosphorylating and inactivating the transcriptional activities of FoxO1 and FoxO3a, promoted a phenotypic switch with lowered catalase activity and increased Runx2 in...
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102296.

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