Wall shear stress promotes intimal hyperplasia through the paracrine H₂O₂-mediated NOX-AKT-SVV axis

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ABSTRACT

Aims: Oscillatory wall shear stress (WSS)-linked oxidative stress promotes intimal hyperplasia (IH) development, but the underlying mechanisms are not completely understood.

Materials and methods: We used an in vivo rabbit carotid arterial stenosis model representing different levels of WSS and found that WSS was increased at 1 month with 50% stenosis and was accompanied by VSMCs proliferation and interstitial collagen accumulation. Increased WSS promoted the expression of NOX, AKT, and survivin (SVV) and the proliferation/migration of VSMCs and reduced apoptosis.

Key findings: Our in vitro study suggested that H₂O₂ promoted proliferation and migration while suppressing apoptosis in cultured human umbilical vascular endothelial cells.

Significance: We demonstrated that the elevation of WSS promotes VSMC proliferation and migration through the H₂O₂-mediated NOX-AKT-SVV axis, thereby accelerating IH development.

1. Introduction

Accumulating evidence has suggested that hemodynamic changes are a common event in the pathophysiology of many vascular diseases including atherosclerosis and restenosis after vascular surgical and nonsurgical procedures such as percutaneous coronary intervention [1]. In the pathogenesis of vascular diseases, vascular endothelial cells (VECs) can transform mechanical stimulations, such as wall shear stress (WSS), into a biological signal that promotes proliferative, migratory and anti-apoptotic activities of vascular smooth muscle cells (VSMC) [2,3]. These pathological events ultimately lead to intimal hyperplasia (IH) and vascular stenosis [4]. Previously studies showed that WSS inhibited inflammatory progression and suppressed IH [5]; however, recent studies have shown that WSS fluctuation could significantly accelerate IH development [6], which could counteract the anti-hyperplasia caused by high WSS. In addition, multiple factors/signaling pathways are involved in WSS-linked IH [7]. One of these factors is oxidative stress, which is an imbalance between endogenous antioxidants and oxidants in favor of the latter, which was shown to be activated by oscillatory WSS [8] and to significantly contribute to the proliferation and migration of VSMCs as well as to driving an atherosclerotic phenotype [9].

Generally, oxidative stress occurs as a result of the persistent production of reactive oxygen species (ROS), which was reported to act as the second messenger that changes the biological activity of VSMCs [10]. A large proportion of ROS were generated from mitochondrial enzymes, such as NADPH oxidases (NOX) and Xanthine oxidase, and were divided into three major types: O₂⁻, H₂O₂, and OH⁻ [11]. Among these free radicals, H₂O₂ can freely cross multiple cell membranes and has a longer half-life than the others. This functional feature permits H₂O₂ to regulate adjacent cells through paracrine passage [12], thereby inducing disease phenotypes in tissues. Mechanistically, H₂O₂ activates a multitude of downstream molecules and signaling pathways. For instance, H₂O₂ potentiated the activity of NOX [13], which magnifies its biological effects such as proliferation and migration. Additionally, H₂O₂ was shown to activate kinase signaling, including that for MAPK, ERK, and AKT [14–18]. In addition, proteomic studies discovered that survivin (SVV) (also known as Birc5), a member of the inhibitor of apoptosis gene family, participated in controlling multiple signaling pathways of tumor cells, including apoptosis, proliferation, invasion, protein modification and signal transduction [19]. Although SVV was shown to be closely related to tumor progression [20], studies indicated that it was not the tumor-specific gene [21]. Our previous study revealed that SVV, a key factor in the AKT pathway, regulated the proliferation, migration and anti-apoptosis of vascular endothelial cells.

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However, whether WSS activates the NOX-AKT-SVV axis through paracrine H$_2$O$_2$ during the pathogenesis of IH remains unclear.

The present study utilized rabbit carotid artery stenosis models that exhibited different levels of WSS and investigated the link between WSS levels and paracrine H$_2$O$_2$-induced IH and stenosis. Our findings demonstrate an additional molecular basis for the existing mechanisms underpinning the development of phenotypic changes in vessels induced by WSS.

2. Methods and materials

2.1. Animal model

Eighty-four female New Zealand rabbits (4–6 years old) were provided by the Experimental Research Center at Chongqing Medical University. The rabbit carotid artery stenosis model was generated as previously detailed with slight modifications [24]. Briefly, the diameter of the internal carotid artery of each rabbit was measured by color Doppler ultrasound. The artery sheath was chosen according to the size of the carotid artery. The carotid artery was ligated via suture with a ring contraction. An artery sheath was placed and the size of carotid artery was controlled at 0%, 30%, 50% and 70%, respectively. The inner diameter and blood flow velocity were assessed by color Doppler ultrasound at day 1, as well as at 1, 2 and 3 months, respectively, after surgery, which reflected the variation in WSS at different degrees of vascular stenosis. All experiments were performed in accordance with relevant guidelines and regulations. All animal procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Chongqing Medical University (Chongqing, China).

2.2. Cell culture

Human umbilical vascular endothelial cells (HUVECs) and human arterial smooth muscle cells (HASMCs) were obtained from CHI Scientific (Maynard, MA, USA) and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium ( Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO$_2$.

2.3. Measurement of lateral pressure of carotid artery and blood lipids

A YPJ01 pressure transducer was placed at the carotid stenosis segment through the carotid artery narrow root. A multi-channel physiological monitor dynamically recorded the change in the carotid artery wall pressure. Peripheral blood lipids were measured enzymatically as previously reported.

2.4. Hematoxylin and Eosin (H&E) staining

Specimens of the carotid artery from different degrees of carotid arterial stenosis were collected at 1 day, as well as 1, 2, and 3 months, respectively, fixed in 10% neutral formaldehyde, embedded in paraffin, and then sliced at 4 μm thickness. After hydration, H&E staining was performed to reveal the degree of arterial IH, to assess the hyperplasia and WSS strength and to determine the sensitive period.

2.5. Measurement of ROS in vivo

In brief, the fresh carotid arteries were isolated after 24 h, 48 h, and 72 h respectively, embedded in tissue-freezing OCT compound, and then cut into 4-μm-thick sections. The cultured VSMCs were seeded on glass slides. Dihydroethidium (DHE), dissolved in dimethyl sulfoxide (40 μmol/l), was added to the tissue sections and cultured VSMCs and incubated at 37 °C for 45 min in the dark. After 3 washes with PBS, the sections/glass slides were placed under an inverted fluorescence microscope (Leica, German), and the fluorescence intensity was analyzed with Image-Pro Plus 6.0 (USA).

2.6. Immunofluorescence staining

Double immunofluorescence staining was performed using a rat anti-rabbit SVV antibody (1:100; 4 °C overnight; Wanlei, China) and a mouse anti-rabbit Nox2 antibody (1:100; 4 °C overnight; BOSTER, China) followed by incubation with appropriate secondary antibodies at room temperature for 1 h (1:200; Goat Anti-Rat IgG/Alexa Fluor®594, Abcam, USA, 1:100 Goat Anti-mouse/Alexa Fluor®488, Bios, Beijing, China). Nox2 and SVV staining were imaged (10×) using a Zeiss Axio A1 microscope (Carl Zeiss, Jena, Germany) and quantified.

2.7. VSMC proliferation assay

VSMCs were co-cultured with H$_2$O$_2$ (100 μM), DPI or H$_2$O$_2$ + DPI for 48 h, followed by incubation with Edu (50 μM/1 Edu) for 2 h. Thereafter, VSMCs were fixed with 4% paraformaldehyde for 30 min at room temperature, washed, and incubated with glycine for 5 min. After that, VSMCs were incubated with 0.5% Triton-100 for 10 min. After washing with PBS, the cells were treated with PBS containing 100 μl Apollo and incubated in the dark for 30 min. Hoechst 33342 was used for nuclear counterstaining. The fluorescence was viewed under fluorescence microscope (Axio Observer Z1, ZEISS, Germany).

2.8. Flow cytometry

HASMCs were co-cultured with H$_2$O$_2$ (100 μM), DPI, an inhibitor for H$_2$O$_2$, or H$_2$O$_2$ + DPI for 48 h. Thereafter, cells were stained with Annexin V: PE (Apoptosis Detection Kit I, BD Biosciences, Franklin Lakes, NJ, USA) based on the manufacturer’s instructions and analyzed by flow cytometry (BD, Franklin Lakes, NJ, USA).

2.9. VSMC migration assay

HASMCs migration was determined using a Boyden chamber assay. HASMCs were seeded onto the upper surface of an 8-μm pore-sized chamber containing the culture medium in the absence or presence of H$_2$O$_2$. After 48 h of incubation, those cells that migrated to the lower surface of the filter were fixed with methanol and stained with 1% crystal violet. The number of stained cells from 4 randomly chosen fields was scored in triplicate.

2.10. Western blot

Whole cell lysates were purified from cells or tissues using lysis buffer (pH 7.4, 150 mM NaCl, 50 μl Tris–HCl, 2 mM EDTA, 1% NP-40), and the protein concentration was determined using the Bradford assay. Equivalent amounts of total proteins from different samples were subjected to SDS-PAGE gel and transferred onto the nitrocellulose membranes. The membranes were then incubated with primary polyclonal antibodies targeting SVV, cyclin D3, cyclin E2, cyclin B1, CDK4, CDK2, CDC2, caspase-3, caspase-8, caspase-9, and bcl-2 (BOSTER, Bios Antibody, Wanleibio, China) (1:1000) at 4 °C overnight. Following incubation with the secondary antibody (goat anti-rabbit IgG, Santa Cruz Biotech, Santa Cruz, CA, USA) at room temperature for 2 h, the signals were visualized by chemiluminescence (UVP, Upland, USA) based on the manufacturer’s instructions.

2.11. Statistical analysis

Data are presented as the means ± standard deviation (SD) from at least three independent experiments. Significant differences between groups were determined by a one-way analysis of variance with either
the least significant difference t-test or multi-logistic analysis using SPSS 20.0 (IBM, Armonk, NY, USA). p < 0.05 was considered statistically significant.

3. Results

3.1. Correlation between WSS and the degree of carotid arterial stenosis

We employed circumferential strain to establish rabbit carotid arterial stenosis models. Ultrasound measurement of blood vessel diameter (Fig. 1E) and flow velocity (Fig. 1F) was performed at 1 day, as well as 1, 2 and 3 months, respectively, after the model establishment. WSS was calculated based on the formula $WSS = \frac{8\eta v_{\text{mean}}}{d}$. The flow velocity had no significant difference when stenosis was < 50% (Fig. 1F), but WSS was substantially higher in the carotid arteries with 50% and 70% stenosis at each time point (Fig. 1G, p < 0.01).

3.2. Comparable circumferential strain and blood lipids between the groups with different stenosis degrees

To rule out the involvement of circumferential strain and blood lipids in our investigation, we measured the circumferential strain and checked blood lipid levels in the peripheral blood in each rabbit of all groups. The results indicated that these parameters were comparable between these groups (Fig. 2).

3.3. Characterization of IH in carotid arteries with different degrees of stenosis

At the time points of 1 day and 1, 2 and 3 months after surgery, the constricted carotid arteries were isolated and analyzed using H&E staining (Fig. 3A). Quantification of intima-media thickness from the indicated groups at each time was also performed (Fig. 3B). Our results indicated that WSS with a stenosis > 50% induced IH in the carotid arteries at 1 month compared with groups with 0% and 30% stenosis (p < 0.01).

3.4. WSS induced oxidative stress in vascular wall and HUVECs

Tissue samples were collected at 24 h, 48 h, and 72 h after model establishment, and 4 μm cryosections were prepared. The level of ROS in each carotid artery was evaluated by DHE staining (Fig. 4A). Our results indicated that the WSS with ≥50% stenosis significantly increased ROS generation after 48 h (p < 0.05) (Fig. 4B). We also examined whether WSS induced ROS production in cultured HUVECs. The pressure of WSS (1.674 ± 0.236 Pa) was simulated to be equivalent to 50% stenosis in vivo and its effect on the generation of $\text{H}_2\text{O}_2$ in HUVECs was detected by the ROS fluorescent probe dihydropyridine after 48 h.

Fig. 1. Correlation of WSS with the degree of carotid arterial stenosis. E, the inner diameter, and F, flow velocity, of different degrees of carotid arterial stenosis at different time points as shown in the upper panels. G is the statistical analysis showing the correlation between WSS and stenosis with 50% and 70% degrees, respectively. * and #, p < 0.05, vs. 0% and 30%.
Fig. 2. Comparable circumferential strain and blood lipids between these groups with different stenosis degrees. Circumferential strain and blood lipids were determined as described in Materials and methods.
The results showed that WSS significantly increased H$_2$O$_2$ generation compared with the control group (Fig. 4D, p < 0.001).

3.5. Characterization of pathological changes of stenotic carotid arteries in vivo

We next characterized the pathological changes of those stenotic carotid arteries by histobiochemistry and immunostaining. Masson staining was used to detect the changes of the intima component in the carotid artery. As shown in Fig. 5A & B, the WSS with 70% stenosis significantly increased the arterial wall elastic fibers (blue) and muscle fibers (pink). Also, WSS significantly induced the expression of α-SMA in neovascular mesangial smooth muscle and microvascular and large vessels, indicating that WSS promoted VSMC proliferation. We also evaluated the expression of NOX2 and SVV by immunostaining. As shown in Fig. 5C, clear staining of Nox2 (green) and SVV (red) was observed in the groups with 50% and 70% stenosis.

3.6. WSS altered the expression of factors involved in NOX2-AKT-SVV signaling, cell apoptosis and cycle progression

Next, Western blots were performed on protein lysates purified from carotid arteries with different WSS at one month after surgery. As shown in Fig. 6A–D, the expression levels of protein implicated in mediating cell cycle progression, such as cyclin D3, CDK4, cyclin E2, CDK2, cyclin B1 and CDC2, were significantly increased in carotid arteries with 50% or 70% stenosis, as were those involved in governing cell apoptosis. Also, the expression of NOX2, PI3K, AKT, SVV, NF-κB, and the invasion-related proteins MMP2, MMP9 and MMP8 were up-regulated in the carotid arteries with 50% and 70% stenosis (p < 0.01). There were no significant differences in the expressions of MMP-8, MMP-2, or MMP-9 between the 50% and 70% groups (Fig. 6E–H, p > 0.05).

3.7. Effects of paracrine H$_2$O$_2$ on proliferation, apoptosis and migration of HASMC

We also evaluated the effects of H$_2$O$_2$ on the proliferation, apoptosis and migration of HASMCs in vitro. HASMCs were cultured in the absence or presence of H$_2$O$_2$ (100 nM) with or without DPI, an inhibitor for H$_2$O$_2$, for 48 h, followed by an analysis of cell proliferation, apoptosis and migration, respectively. EdU staining revealed a significant increase in the cell proliferation in the H$_2$O$_2$+DPI− group compared with the H$_2$O$_2$+DPI+ group (Fig. 7A, *, p < 0.001), while H$_2$O$_2$ treatment substantially decreased cell apoptosis, as revealed by flow cytometry analysis of Annexin V-FITC/propidium iodide-stained cells (Fig. 7B, *, p < 0.001). In addition, H$_2$O$_2$ stimulation greatly enhanced the migration capability of HASMCs (Fig. 7C, *, p < 0.001).

4. Discussion

Using rabbit models with different degrees of carotid arterial stenosis, we found that with a degree of arterial stenosis ≥50%, 1) WSS was sufficient to induce IH and fibrosis in the vascular wall and 2) WSS was sufficient to induce oxidative stress in the vascular wall, accompanied by stimulated paracrine H$_2$O$_2$, resulting in the increased expression of Nox2 and SVV in vascular smooth muscle cells. The in vitro experiments showed that increased H$_2$O$_2$ stimulated proliferation and migration and repressed the apoptosis of cultured VSMCs, all of which were reversed by the H$_2$O$_2$ inhibitor DPI.

A large number of hemodynamic studies have shown that different hemodynamic parameters had direct effects on the activity of signal transduction and the expression of certain endothelial genes [25]. WSS, the drag force per unit area acting on the endothelial cells, activated the intracellular biological signaling pathway and gene expression; it also regulated the cellular events of adjacent VSMCs [26]. Also, elevated WSS was shown to alter the biological behavior of vascular endothelial cells through mediating gene expression [27,28], subsequently promoting the local inflammation of the vascular endothelium and
accelerating the proliferation of arterial intima and atherosclerotic plaque formation [29–31]. During the progression of atherosclerosis, vascular endothelial cells will secrete more substances to promote thrombosis, vasoconstriction and cell proliferation, which was partially mediated by WSS. WSS in smaller arteries is less oscillating and steep spatial gradients are less likely to occur; however, dynamic shear profiles do develop, which may influence flow-induced effects through oxidative signaling. Consistent with the previous reports, the present study also found that WSS increased the level of oxidation in the vascular wall, which was coincident with the increased interstitial collagen accumulation. According to the fluid artery model, a diameter of the vascular wall with no > 50% narrowness did not significantly change the blood speed or pressure [32]. The degree of stenosis at this time is called critical stenosis. At present, surgeons rely on critical stenosis as a reference for surgical intervention. However, according to the formula $WSS = \frac{8 \eta v_{\text{mean}}}{d}$, proposed by Matlung et al. [33], the narrowness of WSS could be high and endometrial hyperplasia and sclerosis could be serious. Thus, using balloon dilatation or stent implantation would not reverse the pathological changes of the diseased artery. Therefore, to clarify the demarcation between IH and stenosis, WSS and its effect time, we obtained the sensitive period of stenosis and IH. We established a rabbit model of carotid arterial stenosis with different degrees of narrowness to represent different levels of WSS and evaluated the IP at each time point. We found that 50% and 70% stenosis significantly induced WSS as well as the above pathological changes in the carotid arteries, accompanied by the abnormal expression a number of genes involved in the cell cycle, apoptosis and migration, including cyclin D3, CDK4, cyclin E2, CDK2, cyclin B1, CDC2, MMP2, CDK2, cyclin B1, CDK2, CDC2, CD2, bcl-2, IMAM-1 and caspase-3, 8, 9. Therefore, 50% stenosis should be a critical point at which WSS may initiate/promote the pathogenesis of vascular diseases.

In the peripheral vascular system, ROS is primarily produced by NOX, which is also one of the downstream mediators for WSS-triggered cellular events [34]. The NOX family comprises seven members, NOX1–7 [35], with NOX1, 2, 4, and 5 being expressed in vascular endothelial cells and smooth muscle cells [36]. H$_2$O$_2$ is one of the ROS, acts as the second messenger, and is extensively implicated in a variety of pathophysiological events of cells [37,38]. For instance, ROS are believed to contribute to endometrial dysfunction, hypersensitivity, inflammatory response and vascular remodeling [39–42]. Indeed, we found that increased WSS potentiated the ROS production in vascular walls in vivo, which was potentially linked to the activation of...
downstream signaling molecules, such as MAPK, HIF-1, AKT, and NFkB, which govern cell differentiation, proliferation, apoptosis, cycle, migration, secretion and gene expression under a variety of physiological and pathophysiological circumstances [43–45]. In our previous studies, we demonstrated that the SVV gene is underexpressed in normal vascular endothelial cells and is involved in regulating the biological activity of endothelial cells. As the strongest anti-apoptotic family member, SVV, is not a tumor-specific gene despite being closely related to tumors. SVV promotes intimal hyperplasia, which is primarily associated with the promotion of vascular smooth muscle cell proliferation and the conversion of macrophage system to form cells. Moreover, our in vitro studies showed that increased H₂O₂ promoted the proliferation, migration and decreased apoptosis of HUVECs. Hence, we believe that H₂O₂ is a downstream effector of WSS that causes pathophysiological changes and gene transcription in vascular walls.

Previously, Ardanaz and Pagano [46] reported that H₂O₂ had better paracrine properties than other ROSs and could directly permeate the cell membrane and act on adjacent tissues. In addition, H₂O₂ also activates NOX through a positive feedback mechanism. For instance, in cardiac fibroblasts, H₂O₂ activated Nox4 via PLA₂-dependent arachidonic acid generation [47], leading to more ROS production. In endothelial cells, though much remains to be learned, p47phox is confirmed to be critical in modulating enzymatic activity by interacting with catalytic unit gp91phox (Nox2, Nox for NAD(P)H oxidases, representing a family of novel NAD(P)H oxidases) [48–51]. Studies using deficient mice or inhibitory peptide (gp91ds-tat) targeting Nox2 have established an essential role of Nox2 in producing reactive oxygen species in endothelial cells [50]. However, functions of other newly identified gp91phox homologues (Nox1, Nox4 and Nox5) remain obscure but are under intensive investigation. Since Nox2 is primarily expressed in fibroblasts and vascular cells, we took Nox2 as the main object and found that the increased expression of SVV and Nox2 in the vascular wall was positively correlated with the increased H₂O₂ at 48 h.

Fig. 5. Characterization of in vivo constricted carotid arteries. A & B. Carotid arterial smooth muscle cells and elastic fibers, muscle fiber hyperplasia under the effect of stenosis WSS. C. Co-staining of NOX2 (green) and SVV (red) was performed on specimens prepared from each group as indicated and was visualized under fluorescent microscope (10×). DAPI (blue) was used for nuclear staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
WSS altered the expression of factors involved in NOX2-AKT-SVV signaling, cell apoptosis and cycle progression. A–D, Western blots were performed on protein lysates purified from carotid arteries with different WSS after one month of surgery to detect the expression of proteins as indicated. E–H, statistical analysis of A–D. * and #, p < 0.05, vs. 0% and 30%.
Fig. 7. Effects of paracrine H$_2$O$_2$ on the proliferation, apoptosis and migration of HASMCs. HASMCs were cultured in the absence or presence of H$_2$O$_2$ (100 nM) with or without DPI for 48 h, followed by an analysis of cell proliferation (A), cell apoptosis (B), and cell migration (C). *, p < 0.05 vs. all three other groups.
after WSS. Consistent with the in vivo findings, increased levels of H$_2$O$_2$ in the microenvironment also positively regulated NOX activity and stimulated downstream Akt signaling. Moreover, our studies further confirmed that SVF served as a molecule downstream of NOX/AKT in the pathological process triggered by oscillatory WSS. Therefore, our findings suggested a functional axis, NOX-H$_2$O$_2$-Akt-SVV, through which increased WSS induces pathological changes in the vascular wall.

5. Conclusion

In conclusion, the present study reports that WSS (≥50% stenosis) can promote IH within 1 month through Nox activation-generated ROS and its downstream activation of the AKT-SVV axis in the vascular wall, thereby aggravating IH. We further provided evidence that H$_2$O$_2$ stimulated the proliferation and migration but suppressed the apoptosis of cultured HUVECs. Thus, our findings suggest that the WSS-NOX-H$_2$O$_2$-Akt-SVV axis may represent a prospective target for the prevention and treatment of IH-associated vascular diseases.

Author contribution statement

Haolong Zhang and Xuehu Wang conceived and designed the experiments; Haolong Zhang performed the experiments; Haolong Zhang, Jing Wang and Zhipeng Yang analyzed the data; Yu Zhao and Fangyu Zhu contributed reagents/materials/analysis tools; Haolong Zhang wrote the paper.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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