Atheroprotective laminar flow inhibits Hippo pathway effector YAP in endothelial cells

SUOWEN XU, MARINA KOROLEVA, MEIMEI YIN, and ZHENG GEN JIN

ROCHESTER, NY

Atherosclerosis is a mechanobiology-related disease that preferentially develops in the aortic arch and arterial branches, which are exposed to disturbed/turbulent blood flow but less in thoracic aorta where the flow pattern is steady laminar flow (LF). Increasing evidence supports that steady LF with high shear stress is protective against atherosclerosis. However, the molecular mechanisms of LF-mediated atheroprotection remain incompletely understood. Hippo/YAP (yes-associated protein) pathway senses and effects mechanical cues and has been reported to be a master regulator of cell proliferation, differentiation, and tissue homeostasis. Here, we show that LF inhibits YAP activity in endothelial cells (ECs). We observed that YAP is highly expressed in mouse EC–enriched tissues (lung and aorta) and in human ECs. Furthermore, we found in apolipoprotein E deficient ($ApoE^{-/-}$) mice and human ECs, LF decreased the level of nuclear YAP protein and YAP target gene expression (connective tissue growth factor and cysteine-rich protein 61) through promoting Hippo kinases LATS1/2-dependent YAP (Serine 127) phosphorylation. Functionally, we revealed that YAP depletion in ECs phenocopying LF responses, reduced the expression of cell cycle gene cyclin A1 (*CCNA1*) and proinflammatory gene *CCL2* (*MCP-1*). Taken together, we demonstrate that atheroprotective LF inhibits endothelial YAP activation, which may contribute to LF-mediated ECs quiescence and anti-inflammation. (Translational Research 2016;176:18–28)

Abbreviations: CTGF = connective tissue growth factor; Cyr61 = Cysteine-rich protein 61; DF = disturbed flow; EC = endothelial cells; LATS1/2 = large tumor suppressor kinase 1/2; LF = laminar flow; YAP = Yes-associated protein

INTRODUCTION

Atherosclerosis-associated cardiovascular disease is the leading cause of death worldwide.[1-3] In atherosclerotic patients and athero-susceptible rodents, atherosclerosis preferentially develops in the aortic arch (AA) and arterial branches, where the flow pattern is disturbed flow (DF), but less in straight area (such as thoracic aorta, TA) where the flow pattern is steady laminar flow (LF).[4-7] Generally, endothelial cells (ECs) exposed to LF display a phenotype of cell quiescence and atheroprotection, evidenced by low DNA synthesis, low inflammatory response, low oxidative stress, and low thrombogenicity, whereas DF promotes a phenotype of EC activation, which drives the opposite effects and promotes atherosclerosis.[4] However, the mechanisms of LF atheroprotective action remain incompletely understood.

Like other cell types, ECs also need a pathway to sense their environmental cues to determine cell decisions (being quiescent or keeping proliferating, for example). The Hippo/YAP (yes-associated protein) pathway is a master regulator of cellular proliferation, differentiation, and

Form the Aab Cardiovascular Research Institute, Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY.

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Reprint requests: Zheng Gen Jin, Aab Cardiovascular Research Institute, Department of Medicine, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Box CVRI, Rochester, NY 14642; e-mail: Zheng-gen_Jin@urmc.rochester.edu.

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AT A GLANCE COMMENTARY

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Background

Atherosclerosis preferentially develops at regions of disturbed flow but less in regions of steady laminar flow; however, the detailed mechanisms of laminar flow–mediated atheroprotection remain incompletely understood.

Translational Significance

In this study, we demonstrate that atheroprotective laminar flow inhibits the activation of Hippo pathway effector yes-associated protein (YAP) through promoting large tumor suppressor kinase 1/2–dependent YAP phosphorylation. Functionally, we observed that YAP depletion phenocopies flow response to decrease gene expression of cell cycle gene cyclin A1, as well as proinflammatory CCL2 (or MCP-1). Thus, our study suggests that small molecule YAP inhibitors may be beneficial for the prevention and therapeutic treatment of atherosclerosis.

tissue homeostasis, thus controlling organ size and tissue regeneration.[8] YAP serves as a downstream effector as well as transcriptional coactivator in the Hippo pathway to regulate tissue growth by controlling the expression of many genes such as cysteine-rich protein 61 (Cyr61, also known as CCN1)[9] and connective tissue growth factor (CTGF, also known as CCN2),[10] which are involved in promoting cell survival, proliferation, and migration.[8] YAP activity is regulated in a phosphorylation-dependent manner. Phosphorylation of YAP at major site Serine 127 (Ser127) by serine/threonine-protein kinases large tumor suppressor kinase 1/2 (LATS1/2) and other kinases leads to its cytoplasmic retention, thereby inhibiting YAP transactivation.[8] Therefore, strategies that inhibit YAP activation have therapeutic potential of treating various YAP-driven diseases.

Emerging evidence has shown that YAP senses and responds to mechanical cues and relays the signal of mechanical strain[11,12] and cyclic stretch[13] to the nucleus. However, whether YAP transduces mechanosignal exerted by different blood flow pattern remains elusive. Here, we hypothesized that, in endothelial cells (ECs, the innermost cell monolayer that senses blood flow), different patterns of flow shear stress would elicit differential effects on YAP activation, and this YAP-mediated mechanotransduction in ECs could partially explain site-specific atherosclerosis development.

MATERIAL AND METHODS

Mice. Apolipoprotein E deficient ($ApoE^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and fed a normal chow diet till 3-month old. All animal procedures conformed to the Guideline for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Rochester Medical Center.

***En face* immunofluorescent staining of mouse aortic endothelium.** *En face* staining was performed as previously described.[14,15] In brief, 3-month-old male $ApoE^{-/-}$ mice fed a normal chow diet were anesthetized with ketamine/xylazine cocktail (0.13/0.0088 mg/g body weight). Then, the arterial tree was perfused with saline containing 40 United States Pharmacopeia Unit (USPU)/mL heparin from left ventricle for 5 min, followed by perfusion of prechilled 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 10 min. Subsequently, after adipose tissues were removed, the whole aorta was dissected from TA to the heart, cut open longitudinally, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 10% normal goat serum (Invitrogen) in Tris-buffered saline (TBS) containing 2.5% Tween-20 for 1 hour at room temperature. Next, aorta segments were incubated with rat anti-VE-Cadherin (also known as CD144, BD Biosciences, 1:100, #555289), rabbit anti-YAP (Cell Signal Tech, 1:100, #8418) antibody in the antibody dilution buffer (3% bovine serum albumin (BSA) + TBS-2.5% Tween-20) overnight at 4°C. After rinsing with washing solution (TBS containing 2.5% Tween-20) 5 min for 3 times, aortic segments were incubated with Alexa Fluor 488 conjugate goat anti-rat and Alexa Fluor 546 conjugate goat anti-rabbit secondary antibodies (Invitrogen, 1:1,000 dilution) for 1 hour at room temperature. Finally, after another 3 rinses in the washing solution, aortic specimens were gently placed on a glass slide with the luminal side up (under dissection microscope), mounted in the ProLong Gold-antifade Mounting Media with 4′,6-Diamidino-2-Phenylindole (DAPI) (Invitrogen). Mounted slides were cured overnight at room temperature in dark. Aortas were examined by a laser-scanning confocal microscope (FV-1000 mounted on IX81, Olympus) with UPlanFL N 60x Oil lens. The software for taking images was FV10-ASW4.1 from Olympus.

Isolation of EC-enriched RNA from mouse aorta. The isolation of EC-enriched RNA from mouse aorta was performed according to the protocol of intimal RNA isolation from mouse carotid arteries established by Jo's laboratory,[16,17] with minor modifications. Briefly,

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