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Atheroprotective laminar flow inhibits Hippo pathway effector YAP in endothelial cells

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Abstract

Atherosclerosis is a mechanobiology-related disease that preferentially develops in the aortic arch (AA) and arterial branches which are exposed to disturbed/turbulent blood flow (DF), but less in thoracic aorta (TA) where the flow pattern is steady laminar flow (LF). Increasing evidence supports that steady laminar flow 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 regulates 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 ApoE^{-/-} mice and human ECs, LF decreased the levels of nuclear YAP proteins and YAP target gene expression (CTGF and Cyr61) through promoting Hippo kinases LATS1/2-dependent YAP (Ser127) phosphorylation. Functionally, we revealed that YAP depletion in ECs phenocopying LF responses, reduced the expression of cell cycle gene cyclin A1 (CCNA1) and pro-inflammatory 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.

Keywords

Laminar flow; Hippo/YAP pathway; Endothelial cells; Atherosclerosis

Introduction

Atherosclerosis associated cardiovascular disease is the leading cause of death worldwide^{1–3}. In atherosclerotic patients and athero-susceptible rodents, atherosclerosis

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preferentially develops in the aortic arch (AA) and arterial branches where the flow pattern is disturbed/turbulent blood 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, while 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 tissue homeostasis, thus controlling organ size and tissue regeneration ⁸. YAP serves as a downstream effector as well as transcriptional co-activator in the Hippo pathway to regulate tissue growth by controlling the expression of many genes such as Cyr61 ⁹ (Cystein-rich protein 61, also known as CCN1) and CTGF ¹⁰ (connective tissue growth factor, also known as CCN2), 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 LATS1/2 and other kinases leads to its cytoplasmic retention, thereby inhibiting YAP transactivation ⁸. Therefore, strategies that inhibit YAP activation has 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 mechano-signal exerted by different blood flow pattern remains elusive. Here, we hypothesized that, in 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

ApoE^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and fed a normal chow diet till three-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. In brief, three-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 USPU/ml heparin from left ventricle for 5 min, followed by perfusion of pre-chilled 4%

paraformaldehyde (PFA) in PBS (pH 7.4) for 10 min. Subsequently, after adipose tissues were removed, the whole aorta was dissected from thoracic aorta 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 h at room temperature. Next, aorta segments were incubated with rat anti-VE-Cadherin (also known as CD144, BD Bioscience, 1:100, #555289), rabbit anti-YAP (Cell Signal Tech, 1:100, #8418) antibody in the antibody dilution buffer (3%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 h 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 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 endothelial cell (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^{15, 16}, with minor modifications. Briefly, mice were sacrificed by CO₂ inhalation according to University of Rochester IACUC protocol and pressure perfused with saline containing heparin (40 USPU/ml) via the left ventricle. Aortic arch and thoracic aorta were then isolated and carefully cleaned of peri-adventitial fat. The aorta lumen was quickly (1 second) flushed with 250 µl of QIAzol lysis reagent (QIAGEN) using a 25^{1/2}-gauge syringe in a 35 mm petri dish. The volume of QIAzol lysis reagent is optimized by a previous study showing the smallest amount of smooth muscle cell contamination. The intimal eluate was then collected for intimal RNA isolation. To precipitate RNA from small quantity samples, 3 µg RNase-free glycogen (#R0561, Fermentas) was used as a carrier to aqueous phase before isopropanol precipitation. To prepare RNA from media smooth muscle cells, after peeling off the adventitia, the media layer of artery was snap-frozen in liquid nitrogen, then cut into small pieces and homogenized in 1 ml QIAzol lysis reagent by using Precellys® Minilys Bead Homogenizer (Bertin Technologies, France). A panel of marker genes for endothelial cells (Platelet Endothelial Cell Adhesion Molecule-1, PECAM1) and smooth muscle cells (α-Smooth Muscle Actin, α-SMA) was used to determine the enrichment of endothelial RNA from the preparation by RT-PCR (supplemental Figure S1). Intimal RNAs from this method yield ~150 ng total RNA.

Cell culture and flow experiments

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cords and cultured in Medium 200 (Cascade Biologics, Portland, OR) containing 5% FBS and low serum growth supplement (LSGS) (Invitrogen)¹⁴. HUVECs were collected in accordance with the University of Rochester human subjects review board procedures that prescribe to the Declaration of Helsinki. Human coronary artery endothelial

cells (HCAECs) obtained from normal human coronary artery were purchased from Cell Applications Inc. (San Diego, CA). HCAECs were cultured in MesoEndo Cell Growth Media supplemented with Growth Supplement (all from Cell Applications Inc.) plus 5% fetal bovine serum (FBS, Invitrogen). For flow experiments, confluent HUVECs monolayer were changed with fresh complete media before exposure to laminar flow¹⁷ or disturbed flow¹⁸ in a 60 mm or 100 mm cone and plate viscometer. HUVECs and HCAECs at passage 2 to 5 were used for all the experiments.

RNAi experiments

Silencer[®] Select human LATS1 (#s17392), LATS2 (#s25503), and negative control (#4390844) siRNAs were purchased from Thermo Fisher Scientific. SMARTpool siGENOME YAP1 siRNA (#M-012200) and siGENOME Non-Targeting siRNA Pool (#D-001206-13) were purchased from GE Dharmacon. HUVECs at 80% confluence in 60-mm dishes were used for transfection using Lipofectamine 2000 as previously described¹⁴. Four hours after adding the Lipofectamine 2000-siRNA (20 nM) complex, medium was changed with M200 complete medium and continued the culture for 48 hours before treatment.

Immunofluorescent staining in cultured cells

Immunofluorescent staining was performed as previously described¹⁹. In brief, after flow treatment, cells were fixed in 4% PFA (Electron Microscopy Sciences) for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature, and blocked with 10% normal goat serum (Invitrogen) containing 0.5% Tween-20 for 1 h at room temperature. Then cells were stained with rabbit anti-YAP (Cell Signal Tech, 1:100, #8418) and mouse anti-VE-Cadherin (BD Bioscience, 1:100, #555661) overnight at 4°C in the blocking solution. Cells were then rinsed with 0.5% Tween-20 in PBS 3 times and incubated with Alexa Fluor 488 conjugate goat anti-mouse and Alexa Fluor 546 conjugate goat anti-rabbit secondary antibodies (Invitrogen, 1:1,000 dilution) for 1 h at room temperature. After another 3 rinses with PBS, slides mounted in the ProLong Gold-antifade Mounting Media with DAPI, images were acquired using a laser-scanning confocal microscope as mentioned above.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from cultured human ECs and indicated mouse tissues using an RNeasy Mini kit (Qiagen) and Trizol Lysis Reagent (Invitrogen), respectively. RNA concentration and purity were determined by Nanodrop2000 Spectrophotometer (Thermo Scientific). For reverse transcription, total RNA was converted into first strand complementary DNA (cDNA) in a 20 µl reaction using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. Regular RT-PCR was performed using 2X GoTaq Green Master Mix (Promega). Reaction products were separated in 1% agarose gel and visualized with Image Lab 5.1 software (Bio-rad). Quantitative real-time PCR was then performed with a Bio-Rad CFX96 Touch Real-Time PCR Detection System or Bio-Rad iQ5 real-time PCR thermal cycler, using iQ SYBR Green Supermix (Bio-Rad) for relative mRNA quantification. The sequences of all the primers used were listed in Supplemental Table S1 and Table S2. The comparative cycle threshold

(Ct) method (2^{-Ct})¹⁹ was used to determine the relative mRNA expression of target genes after normalization to housekeeping gene GAPDH or β -actin. For determining YAP mRNA distribution in different tissues, relative YAP mRNA to GAPDH level in skeletal muscle was set at 1.0.

Western blot analysis

Cells were harvested in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β -Glycerolphosphate, 50 mM NaF, 1 mM Na_3VO_4 and supplemented with protease inhibitor cocktail (#P8340, Sigma). After centrifugation at 4 °C (14, 000 rpm) for 20 min, protein extract supernatant was collected for western blot analysis as essentially described previously¹⁴. The primary antibodies used in this study were listed in Supplemental Table S3. Images were visualized by using an Odyssey Infrared Imaging System (LI-COR).

Statistics

Data were expressed as means $\pm$ SEM. Statistical analysis was performed using GraphPad Prism software 5.0 (GraphPad, La Jolla, CA). Student's t test and one-way analysis of variance (ANOVA) with post hoc Bonferroni tests were used for comparisons between two groups and multiple comparisons, respectively. A *p* value less than 0.05 was considered statistically significant.

Results

YAP is expressed in murine EC-enriched tissues and human ECs

We first characterized the expression of YAP in murine tissues and human ECs. We found that, in normal C57BL/6J mice, YAP mRNA was ubiquitously expressed in murine heart, lung, liver, kidney, aorta, skeletal muscle, spleen and brain (Figure 1A). Noticeably, more abundance of YAP gene expression was observed in EC-enriched tissues, lung and aorta in particular. Using Western blot analysis, we detected comparable level of YAP protein expression in cultured HUVECs and HCAECs (Figure 1B).

YAP nuclear localization and activity were decreased in atheroprotective thoracic aorta

To delineate the potential role of YAP in atherosclerosis, we analyzed subcellular localization of YAP in the endothelium at different regions of aorta from ApoE^{-/-} mice fed a normal chow diet. In the atheroprone aortic arch (AA) of ApoE^{-/-} mice, we observed significant YAP nuclear immunostaining in the endothelium (Figure 2A). In contrast, thoracic aorta (TA) area showed cytoplasmic YAP immunostaining but decreased YAP nuclear immunostaining compared that observed in AA region. CTGF is a well-characterized YAP direct target gene¹⁰ that plays a pro-angiogenic²⁰ and pro-atherosclerotic role²¹ in the pathogenesis of atherosclerosis. Cyr61 is another YAP target gene⁹ that is involved in inflammation and atherosclerosis²²⁻²⁴. We, therefore, compared CTGF and Cyr61 mRNA expression in AA and TA regions. Our data showed decreased CTGF and Cyr61 mRNA expression in the intimal lysate of TA from ApoE^{-/-} mice (Figure 2B). This is consistent with a previous study showing decreased CTGF-positive ECs in down-stream regions of the luminal surface of human atherosclerotic vessels, compared with up-stream regions²⁵.

Laminar flow decreased YAP nuclear localization and YAP target gene expression in cultured human ECs

ECs exposed to LF increase the expression of flow-responsive genes including krüppel-like Factor 2 (KLF2), endothelial nitric oxide synthase (eNOS), and thrombomodulin (TM) (Supplemental Figure S2A). We found that, in confluent culture of HUVECs in the complete media, YAP nuclear expression is at constitutively high levels. ECs under LF aligned in the direction of flow (evidenced by VE-cadherin staining) and showed an elongated morphology (Figure 3A). HUVECs exposed to LF displayed decreased nuclear YAP staining (Figure 3A), compared with that in cells under static conditions. By examining the YAP downstream genes, we found that CTGF and Cyr61 were significantly downregulated by LF in ECs (Figure 3B). LF also decreased mRNA expression of another atherosclerosis-related YAP target gene, Birc5²⁶ (Baculoviral IAP repeat containing 5, also called Survivin) (Supplemental Figure S2B), suggesting that LF decreases YAP transcriptional activity. However, when we compared the effect of DF-treated and static cells, we did not observe significant increase in YAP nuclear staining, YAP phosphorylation, or protein expression of CTGF and Cyr61 (Supplemental Figure S3).

Laminar flow decreases YAP phosphorylation dependent of LATS1/2

YAP can shuttle between cell nucleus and the cytoplasm in a phosphorylation-dependent manner⁸. Specifically, upon phosphorylation at Ser127 by LATS1/2 kinases or other kinases, YAP translocates to the cytoplasm, where it is sequestered through association with 14-3-3 proteins, leading to decreased YAP activity⁸. We observed that LF markedly induced the phosphorylation of YAP at S127 in a time-dependent manner (Figure 4A). Because YAP can be directly phosphorylated (at Ser127) by Hippo kinase LATS1/2, we next determined whether LF-mediated YAP phosphorylation is LATS1/2-dependent. Our data show that LATS1/2 depletion by siRNA almost abolished LF-mediated YAP phosphorylation (Figure 4B), indicating a critical role of LATS1/2 in mediating LF-induced YAP phosphorylation.

YAP depletion phenocopies flow response to decrease cyclin A1 and CCL2 expression

To evaluate the potential functional involvement of YAP in regulating LF-mediated atheroprotective effects, we analyzed gene expression of cell-cycle related cyclin A1 (CCNA1) and pro-inflammatory CCL2 (also known as MCP-1) in YAP depleted and LF-treated ECs. Our data suggests that both YAP depletion and LF treatment significantly reduced gene expression of CCNA1 and CCL2 (Figure 5A–D), suggesting that LF-mediated YAP inhibition may contribute to the transition of ECs into a quiescent and anti-inflammatory phenotype.

Discussion

Atherosclerosis preferentially develops in the aortic arch and arterial branches, but less in thoracic aorta⁷. Understanding the molecular mechanisms underlying flow-pattern dependent regulation of atherosclerosis is valuable for designing anti-atherosclerotic therapeutic strategies. The main findings of this study include: 1) YAP nuclear localization and activity are decreased in atheroprotective LF area of diseased mouse aorta; 2) LF decreases YAP activity by promoting YAP phosphorylation at Ser127 as well as consequent

downregulation of YAP target genes (CTGF, Cyr61, and Birc5); 3) YAP depletion and LF treatment significantly reduced gene expression of CCNA1 and CCL2. These results provide a mechanistic pathway linking mechanical force stimulus to gene transcription. In this pathway, YAP may serve as a molecular “switch” toggling DF atheropromoting effects and LF atheroprotective actions based upon its subcellular localization and target gene expression (see schematic model in Figure 5E). These findings also provide insight into the mechanisms by which LF confers atheroprotection, and facilitate the development of new therapeutic approaches, such as specifically inhibiting YAP activity, to limit atherosclerosis.

The Hippo/YAP pathway plays an important role in organ size control, tumorigenesis and angiogenesis. Recent studies have shown that mechanical strain regulates the Hippo/YAP pathway through E-cadherin and JNK-dependent pathways^{11, 12}. In contrast to mechanical strain, in this study, we found that fluid shear stress generated by LF diminishes YAP nuclear translocation in endothelium of mouse TA and cultured human ECs. Mechanistically, we found that LF stimulated LATS1/2 dependent YAP Ser127 phosphorylation in cultured ECs. Our studies do not exclude the possibilities for the involvement of other kinases, including Akt and AMP-activated protein kinase (AMPK). A recent study has shown that the PI3K/Akt pathway is involved in VE-cadherin-dependent YAP phosphorylation in ECs²⁷. It is plausible that VE-cadherin/Akt pathway may be responsible for LF-induced YAP phosphorylation since we and others have previously demonstrated that LF potently stimulates the PI3K/Akt pathway^{28–31}. AMPK may also play a role in promoting YAP phosphorylation at Ser127, and other sites, Ser94 and Ser61 in particular, since three independent research groups recently reported glucose deprivation induced energy stress activates LATS kinase and AMPK activation followed by YAP phosphorylation and inactivation^{32–34}, and it has been shown that LF increases AMPK activity³⁵. Further studies are needed to define the specific signaling pathways involved in flow regulation of YAP phosphorylation.

Extensive studies have revealed a critical role of Hippo/YAP pathway in cancer biology. However, the precise role of the Hippo/YAP pathway in vascular biology and atherosclerosis has not been well explored. An elegant study by Wang et al³⁶ has recently shown that YAP expression is significantly increased after carotid artery injury, and deletion of YAP attenuates injury-induced neointima formation, which represent an early phase of atherosclerosis. In agreement with this finding, a more recent study has shown that YAP and TAZ are essential for smooth muscle cell proliferation which can be inhibited by YAP inhibitor verteporfin as well as agents that elevate intracellular cyclic AMP levels³⁷. Our present study found that atheroprotective LF triggered YAP phosphorylation and decrease YAP nuclear expression, consequently decreased the expression of mechanosensory YAP target genes (CTGF and Cyr61 etc)^{38, 39} in ECs. Our data are consistent with a recent RNA-sequencing study performed in LF-treated HUVECs, showing downregulation of multiple YAP target genes (CTGF, Cyr61, Birc5, angiopoietin-2 (ANGPT2) and ankyrin repeat domain 1 (ANKRD1)) as well as TEAD3 by LF⁴⁰.

Notably, CTGF and Cyr61, two immediate-early gene products of the CCN matricellular proteins family, are highly expressed in mouse and human atherosclerotic lesions^{22, 41, 42}. CTGF, in particular, is enriched in advanced human atherosclerotic plaque endothelium,

compared with plaque-free control endothelium⁴³. In human ECs, CTGF can be induced by serum-borne lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P)⁴⁴, two agents that has recently reported to inhibit the Hippo pathway core kinases LATS1/2, thereby results in YAP transactivation⁴⁵. In addition, CTGF and Cyr61 were two important genes in promoting angiogenesis and monocytes adhesion^{22, 46}, therefore, the decreased YAP activation and CTGF, Cyr61 and CCL2 expression may explain our previous findings showing LF inhibits TNF- α induced monocyte adhesion to ECs¹⁷. A recent study performed in YAP-depleted HUVECs indicated that YAP is required for S-phase entry and regulates EC proliferation⁴⁷. Gene array studies revealed that YAP regulates endothelial cell cycle progression by altering the transcription of a wide array of genes involved in DNA homologous recombination and the assembling of replication origins⁴⁷. This finding together with our data (showing YAP depletion and LF treatment decreased CCNA1 expression) reinforced the notion that LF may maintain ECs at a quiescent state via inhibiting YAP activation. We also observe that YAP depletion mimics atheroprotective flow to decrease CCL2 gene expression. These data suggests that YAP inhibition could render ECs quiescent and anti-inflammatory. Genome-wide YAP target genes in ECs remain to be investigated using system biological technologies such as ChIP-sequencing.

From a therapeutic perspective, YAP represents a promising therapeutic target in various human diseases⁴⁸. Potential YAP inhibitors may block either YAP cytoplasmic-nuclear shuttling, YAP proteasomal degradation, or YAP-TEAD interaction⁴⁸. Till now, several agents have been repurposed as YAP inhibitors, including verteporfin⁴⁹, lipid-lowering statins^{50–52}, and tankyrase inhibitors⁵³. Verteporfin has been shown to be a potent YAP inhibitor, which suppresses YAP-TEAD interaction and transcriptional activity⁴⁹. We have unpublished data showing that verteporfin has non-specific effects on endothelial function besides its inhibition of YAP activities. It has also been reported that statins, dasatinib, and pazopanib inhibited the nuclear localization of YAP and its target genes in tumor cells⁵⁰. It remains to be investigated in future studies whether these drugs would inhibit YAP activity in ECs and atherosclerosis development in mice and human. It can be anticipated that by use of high content screening or high throughput screening of FDA-approved drug library or natural compounds library, more and more novel and specific YAP inhibitors will be developed for treating various human diseases in the future. In terms of the vasculature, emerging evidence suggests that YAP is also critical for cardiovascular development and diseases^{36, 37, 47, 54–57}. Whether genetic deletion of YAP in ECs or pharmacological inhibition of YAP activity could prevent the development of atherosclerosis in preclinical settings warrants further studies.

In conclusion, this study demonstrates that Hippo pathway effector YAP senses LF-induced mechanical cues, and that differential YAP activity in endothelium in response to LF versus DF may distinguish between “good” and “bad” flow. This new mechanism provides additional layer of information regarding LF-mediated EC quiescence, anti-inflammation and atheroprotection. Our study also carries implications for genetic and pharmacological inhibition of YAP activity in preventing and treating atherosclerotic vascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

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

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At a Glance Commentary

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 YAP through promoting LAST1/2-dependent YAP phosphorylation. Functionally, we observed that YAP depletion, phenocopies flow response to decrease gene expression of cell cycle gene cyclin A1 (CCNA1), as well as pro-inflammatory CCL2 (or MCP-1). Thus, our study suggests that small molecule YAP inhibitors may be beneficial for the prevention and therapeutic treatment of atherosclerosis.

Figure 1. YAP is highly expressed in murine endothelial cell-enriched tissues and human endothelial cells

(A) YAP is expressed in multiple mouse tissues (C57BL/6J mice, 3 month old) as measured by qRT-PCR. Endothelial cell-enriched aortic and lung tissues show enriched expression of YAP mRNA. Relative YAP mRNA to GAPDH level in skeletal (S.K.) muscle was set at 1.0, n = 3.

(B) YAP is expressed in human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs), as measured by Western blot. Tubulin was used as the loading control, n=2.

Figure 2. Differential YAP subcellular localization and expression of YAP-dependent genes CTGF and Cyr61 in endothelium at the atheroprotective area versus the atheroprone area
(A) Decreased nuclear YAP expression in the atheroprotective area of mouse thoracic aorta (TA) isolated from ApoE^{-/-} mice. En face staining was performed to reveal active YAP (red) in the atheroprone area of aortic arch (AA) and the atheroprotective area of TA regions of mouse aorta, VE-cadherin (green) was used to identify endothelial cells. Representative images were shown, n=4, enlarged portion of images was shown as inserts.
(B) Decreased expression of YAP target genes CTGF and Cyr61 in the atheroprotective area of TA isolated from ApoE^{-/-} mice. Intimal RNA was isolated from AA and TA region of ApoE^{-/-} mice, then levels of CTGF and Cyr61 mRNA expression were detected by qRT-PCR using GAPDH as internal control, n=7–8. Data are shown as mean ± SEM. *P<0.05, ***P<0.001 vs AA.

Figure 3. Laminar flow decreased YAP nuclear localization and YAP-dependent genes CTGF and Cyr61 in human endothelial cells

(A) HUVECs were subject to static or laminar flow (LF) for 24 h and then immunostained with YAP (red) and VE-cadherin (green, an endothelial cell marker gene), Representative images were shown, n=3.

(B) HUVECs were subject to laminar flow (LF) for indicated time, and then mRNA was isolated for detecting YAP target genes including CTGF and Cyr61. Data are shown as mean $\pm$ SEM, n=4, *P<0.05, **P<0.01, ***P<0.001 vs control.

Figure 4. Laminar flow inhibits YAP activity by promoting YAP phosphorylation

(A) HUVECs were exposed to laminar flow (LF) for indicated time, then whole cell lysate were collected for detecting the levels of p-YAP (S127), YAP, CTGF, Cyr61 and eNOS. Tubulin was used as the loading control. Representative blots from three independent experiments were shown.

(B) HUVECs were transfected with LAST1/2 siRNAs (20 nM) for 48 h before exposure to LF for 3 h, then whole cell lysate were collected for detecting the levels of p-YAP (S127), YAP, CTGF, Cyr61 and ICAM1. Tubulin was used as the loading control. Representative blots from three independent experiments were shown.

Figure 5. YAP depletion phenocopies flow response to decrease cyclin A1 and CCL2 expression (A–B) HUVECs were transfected with negative control siRNA (siNC) or YAP siRNA for 48 h, then YAP gene and protein expression were analyzed by real time PCR (n=3) and western blot (n=5), respectively. ***P<0.001 vs siNC.

(C–D) HUVECs were transfected with negative control siRNA (siNC) or YAP siRNA for 48 h before analysis of indicated gene expression (n=3). In parallel, HUVECs were exposed to laminar flow for 24 h, before analysis of indicated gene expression, n=3. *P<0.05, **P<0.01, ***P<0.001 vs siNC or static control.

(E) A schematic diagram illustrating the putative role of YAP is laminar flow-mediated atheroprotection. Laminar flow confers atheroprotection by promoting LATS1/2 dependent YAP phosphorylation at Ser127, thereby inhibiting YAP transactivation, and reducing expression of atherosclerosis-related YAP target genes (including CTGF, Cyr61, CCL2, etc).