Effect of Pulsatile and Continuous Flow on Yes-Associated Protein

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Int J Angiol 2014;23:183–186.

Abstract

Yes-associated protein (YAP) is a mechanosignaling protein that relays mechanical information to the nucleus by changing its level of phosphorylation. We hypothesize that different flow patterns show differential effect on phosphorylated YAP (pYAP) (S127) and total YAP and could be responsible for flow dependent localization of atherosclerosis. Confluent human umbilical vein endothelial cells (HUVECs) seeded on fibronectin-coated glass slides were exposed to continuous forward flow (CFF) and pulsatile forward flow (PFF) using a parallel plate flow chamber system for 30 minutes. Cell lysates were prepared and immunoblotted to detect the levels of phosphorylated YAP and total YAP. HUVECs exposed to both PFF and CFF showed a mild decrease in the levels of both pYAP (S127) and total YAP. While the levels of pYAP (S127) decreased to 87.85 and 85.21% of static control with PFF and CFF, respectively, the levels of total YAP significantly decreased to 91.31 and 92.27% of static control. No significant difference was seen between CFF and PFF on their effect on pYAP (S127), but both conditions resulted in a significant decrease in total YAP at 30 minutes. The results of this experiment show that the possible effect of different types of flow on YAP is not induced before 30 minutes. Experiments exposing endothelial cells to various types of flow for longer duration of time could help to elucidate the role of YAP in the pathogenesis of atherosclerosis.

Keywords

- atherosclerosis
- yes-associated protein
- endothelial cells
- pulsatile flow
- continuous flow
- mechanosignaling

Although the risk factors for atherosclerosis are quite variable, localization of atherosclerotic lesions in the arterial tree is surprisingly constant. Atherosclerotic plaques show an increased predilection to areas with complex geometry such as branch points, arches, and bifurcations. Several studies have shown that the pattern of flow in straight portion of the arteries is different from areas of complex geometry.^{1,2} While the flow in straight portions of arteries is steady, laminar, and unidirectional, flow in areas of complex geometry is low in magnitude and often changes direction.³ Although there is evidence that these differences in flow patterns could be responsible for the increased predilection of atherosclerosis to these areas, how these changes are being sensed and transduced to the nucleus is poorly understood.⁴

Yes-associated protein (YAP) is a recently discovered homolog of Yorkie protein in *Drosophila melanogaster* which functions as a transcriptional co-activator for transcription enhancer activator domain family member 2 (TEAD II) group of genes that regulate cellular growth.⁵ Several studies show that YAP could be involved in the pathogenesis of several diseases including cancers of the breast, pancreas, and liver.^{6–8} Some investigators postulate that YAP is a mechanosignaling molecule that transmits mechanical information to the nucleus by detecting changes in actin cytoskeleton induced by changes in shape of the cell.⁹

Previous studies have shown that cell shape changes differently with different types of flow. While endothelial cells (ECs) exposed to static conditions were uniformly

polygonal and randomly aligned, cells exposed to laminar flow aligned in the direction of flow and elongated.¹⁰ We hypothesized that the differences in shape of ECs induced by different types of flow would result in differential effect on the phosphorylation of YAP and could be responsible for the flow dependent localization of atherosclerosis. The purpose of this study was to evaluate the level of phosphorylation of YAP (Serine 127) induced by continuous and pulsatile forward laminar flow.

Methods

Primary cultures of human umbilical vein endothelial cells (HUVECs) (obtained from Dr. Pober's laboratory, Yale University School of Medicine) were cultured with Medium-199 enriched with 20% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 50 µg/mL of endothelial cell growth supplement (BD Biosciences, Bedford, MA), 10 µg/mL of heparin, and 100 µg/mL each of penicillin and streptomycin in an incubator maintained at 37°C and 5% CO₂. Cells were trypsinized with 0.25% trypsin ethylenediaminetetraacetic acid for detachment after reaching confluence and were seeded on 0.025 mg/mL fibronectin (BD Biosciences, San Jose, CA) coated glass slides (75 × 38 mm; Fischer Scientific, Pittsburgh, PA) and were grown till confluence.

Exposure to Flow

HUVECs were exposed to flow using a parallel plate flow chamber system (Cytodyne, San Diego, CA) as previously described.^{11,12} In the present experiment, we exposed HUVECs to laminar pulsatile forward flow (PFF) and continuous forward flow (CFF). To generate CFF (→Fig. 1A), perfusion

medium is circulated in Norprene tubing (Masterflex, Court Vernon Hills, IL) using a roller pump (Masterflex) to parallel plate flow chambers placed in the circuit. Small pulsations generated by the roller pump were dampened because of the long length of the tubing. In the circuit for PFF (→Fig. 1B), rate and direction of flow were regulated by a computer-controlled syringe pump (PHD 2000 and PHD Ultra Programmable; Harvard Apparatus, Holliston, MA). An automated switch clamp (Auto-fill valve box; Harvard Apparatus) is placed between the syringe pump and the flow chamber, and between the syringe pump and the culture medium reservoir. Alternate activation of the switch clamps during each cycle allowed unidirectional pulsatile flow. Both CFF and PFF systems were maintained at 37°C and 5% CO₂ balanced air was injected into the perfusion. The magnitude of shear stress produced by either PFF or CFF was kept constant at 14 dynes/cm². Slides kept under static conditions in an incubator maintained at 37°C and 5% CO₂ served as controls.

Western Blot Analysis

HUVECs on glass slides subjected to PFF or CFF for 30 minutes or static conditions were washed twice in ice-cold phosphate buffered saline (Hyclone, South Logan, UT) and lysed in ice-cold radioimmunoprecipitation assay lysis buffer containing 1 mM PMSF, 1% SDS, 2 mM sodium orthovanadate, cocktail of phosphatase inhibitors I and II (EMD4Bioscience, San Diego, CA), and protease inhibitor (Roche Applied Science, Indianapolis, IN). Aliquots of cell lysates containing 20 µg of total protein were mixed with equal volume of Laemmli sample buffer and eluted by boiling for 5 minutes at 95°C, then subjected to 10% SDS-PAGE. The gels were transferred on to polyvinylidene fluoride membrane and probed with anti-

Fig. 1 (A) Schematic of the circuit used for continuous forward flow (CFF). A roller pump was used to circulate perfusion medium located in the reservoir in Norprene food tubing at a flow rate that produces a shear stress of 14 dynes/cm². (B) Schematic showing the circuit used for pulsatile forward flow (PFF). In the first half of the circuit, a roller pump was used to circulate perfusion medium from the bottom to the top of a reservoir. In the other half, an automated syringe pump using a synchronized switch clamp draws and ejects perfusion medium alternatively and this produces a unidirectional pulsatile flow. Parallel plate flow chambers containing human umbilical vein endothelial cells (HUVECs) coated on glass slides were placed in both circuits and exposed to flow for 30 minutes. Five percent CO₂ balanced air was injected from the top of the reservoir and the entire system was maintained at a constant temperature of 37°C.

phospho Serine 127 YAP (#4911, Cell Signaling Technology, Danvers, MA), total YAP (#4912, Cell Signaling Technology), and anti- β -Actin (used as a loading control; #A1978, Sigma, St. Louis, MO) antibodies and detected using chemiluminescence (LumiGLO, Cell Signaling Technology). Membranes were developed on X-ray films and quantification of bands was performed by densitometric analysis using Image J software (<http://rsbweb.nih.gov/ij/>).

Statistics

The densitometric mean $\pm$ standard error of mean of multiple experiments was calculated. Statistical analysis was performed with a two tailed *t*-test using SPSS (IBM, Armonk, NY) and a "*p*" value of ≤ 0.05 was considered significant.

Results

A representative immunoblot showing the change of pYAP (S127) and total YAP with CFF ($\rightarrow$ Fig. 2) and PFF ($\rightarrow$ Fig. 3) is shown at the top and the densitometric means of at least three experiments are shown at the bottom of the figures. After 30 minutes of exposure to either PFF or CFF, a decrease in pYAP (S127) was observed. While the level of pYAP (S127) decreased to 87.85% of static control with PFF ($p = 0.20$), it decreased to 85.21% of static control with CFF ($p = 0.19$). Similar to the change seen in pYAP (S127), a decrease in total YAP was observed after exposure to either CFF or PFF for 30 minutes which was statistically significant, decreasing to

Fig. 2 Effect of continuous flow on phosphorylated yes-associated protein (pYAP) (S127) and total yes associated protein (YAP). A representative immunoblot of three separate experiments is shown on the top. After 30 minutes of exposure to pulsatile forward flow (PFF), levels of both pYAP (S127) and total YAP decreased. Reprobing the membranes with anti-actin antibody showed equal loading protein loading. (**p* value < 0.05 .)

Fig. 3 Effect of pulsatile forward flow (PFF) on phosphorylated yes-associated protein (pYAP) (S127) and total yes-associated protein (YAP). A representative immunoblot of three separate experiments is shown on the top. After 30 minutes of exposure to PFF, levels of both pYAP (S127) and total YAP decreased. Reprobing the membranes with anti-actin antibody showed equal loading protein loading. (**p* value < 0.05 .)

91.31% of static control with PFF ($p = 0.03$) and 92.27% of static control with CFF ($p = 0.05$).

Discussion

Several studies have demonstrated that different flow patterns and the forces associated with them play a major role in vascular morphogenesis, physiology, and pathology.^{13–15} Atherosclerosis, a disease characterized by the deposition of fatty plaques on the inner walls of arteries, has a predilection for areas of complex geometry in arterial tree and this has been attributed to flow disturbances in such areas.¹⁶ While blood flow in straight parts of large arteries is pulsatile, unidirectional, and laminar, flow near arterial bifurcations often changes its direction and is non-laminar.¹⁷ In contrast, blood flow in the peripheral circulation is continuous due to the compliance and elastic recoil of major arteries.¹⁸ Several reports have shown that different types of flow regulate EC turnover and the levels of chemokines in a diverse way. Pulsatile flow increases the levels of nitric oxide (NO) and renders EC in a quiescent state reducing their rate of proliferation.^{19,20} Non-pulsatile flow, on the other hand, induces high rates of endothelial proliferation and produces reactive oxygen species and inflammatory mediators.^{21,22}

YAP is a recently discovered protein that functions as a mechanosignaling agent and a transcriptional co-activator which upon de-phosphorylation has been shown to relay mechanical signals to the nucleus and activate genes related to cellular growth. Decrease in number of actin stress fibers upon compression of cells causes phosphorylation of YAP on Serine 127 resulting in the trapping of YAP in the cytoplasm.²³

On the contrary, an increase in actin stress fibers upon elongation of cells causes de-phosphorylation of YAP which allows YAP to translocate to the nucleus and activate TEAD II genes. We postulated that variations in cell shape produced by different types of flow could be responsible for the specific localization of atherosclerosis. For example, low shear and flow that changes in direction usually seen near branch points and curves of the arterial tree may not produce a significant change in the shape of ECs and, thus, allow the YAP pathway to be active which might be responsible for increased proliferation of ECs and smooth muscle cells (SMCs) by atherogenic stimuli. On the other hand, pulsatile flow seen in straight portions of the arteries by virtue of its high shear stress and the cyclic strain it produces may elongate ECs and SMCs that may cause significant decrease in the size of the cells that inactivates the YAP pathway. This may keep the proliferation of ECs and SMCs at low levels even after atherogenic stimuli.

In the current experiment, we hypothesized that PFF and CFF would have a differential effect on pYAP (S127) and total YAP. Although a statistically significant decrease was observed in the levels of total YAP in HUVECs exposed to PFF or CFF at 30 minutes, a very small, statistically insignificant decrease was seen in the levels of pYAP (S127). The decrease in total YAP observed may be due to phosphorylation at a different site like serine 381 which leads to degradation by ubiquitination.²⁴ A smaller decrease in pYAP (S127) compared with total YAP seen in the current experiment could be due to many reasons. First, although an unknown pathway increases the phosphorylation of YAP at Serine 127, a parallel pathway leading to ubiquitination and destruction of YAP could cancel out the increase seen in YAP. These pathways put together could have resulted in a very small, statistically insignificant, decrease in pYAP (S127) in our experiment. Second, the biological effect of flow on YAP could be time dependent and duration of exposure to flow used in this experiment (30 minutes) could be too short to detect a significant difference between the effect of PFF and CFF on YAP. Moreover, the effect size of the differences in pYAP (S127) expected to see in this experiment may be too small to be detected. The sample size used in this experiment ($n = 3$) may not carry enough power to detect a difference.

In summary, we saw no significant difference in the levels of pYAP (S127) but a significant decline in total YAP in HUVECs exposed to CFF or PFF for 30 minutes. Exposing HUVECs to various types of flow for longer duration and measuring the differences in phosphorylation of YAP at different moieties (like serine 381) might provide a clear insight into how different types of flow could alter levels of pYAP and total YAP and their role in the pathogenesis of atherosclerosis. Future studies directed toward understanding the mechanism by which flow initiates a signal cascade that could result in degradation of total YAP are also warranted.

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