MAPK signaling mediates low shear stress-induced oxidative damage in human umbilical vein endothelial cells in vitro

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INTRODUCTION

Atherosclerotic lesions occur preferentially in the arterial branches, bifurcations and curvatures where shear stress is low [1, 2]. Shear stress, a tangential friction generated by blood flow, exerts a variety of effects on endothelial function [2, 3]. Several studies reported that distribution of shear stress influenced the location of atherosclerotic plaques due to the differential expression of endothelial nitric oxide synthase (eNOS) [4, 5]. As a key enzyme in regulating nitric oxide (NO) release, eNOS has several phosphorylation sites, among which Thr495 negatively controls NO bioactivity [6].

Mitogen-activated protein kinase (MAPK) signaling pathway is ubiquitous in cells. Of these protein kinases, P38 MAPK, and c-Jun response to various stresses such as excessive oxidize and inflammatory factors [7, 8], while ERK is noted for its role in regulating cell growth [8, 9]. MAPK is also identified to suppress eNOS-Ser1177 and leads to oxidative stress [10, 11]. Oxidative stress is crucial in promoting atherosclerosis and is characterized by excessive production of reactive oxygen species (ROS) and impaired NO release. Enhanced ROS production lowers NO bioactivity and increases cell apoptosis to cause endothelial dysfunction, endothelial injury, and potentially atherosclerosis [12, 13]. In this study, we aimed to investigate the role MAPK in low shear stress (LSS)-induced oxidative damage.

MATERIALS AND METHODS

Materials

The parallel-plate flow chamber was provided by Shanghai Medical Instrumentation College (Shanghai, China). Human umbilical vein endothelial cells (HUVECs) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The protease inhibitor cocktail, dimethyl sulfoxide (DMSO), gelatin, SB202190, PD98059, SP600125 and 4′, 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Horseradish peroxidase-coupled secondary antibody and primary antibodies against eNOS-Thr495 and total eNOS, pi-P38 and P38, pi-ERK 1/2 and ERK 1/2, pi-c-Jun and c-Jun were purchased from Cell Signaling Technology (Beverly, MA, USA). The primary antibody against GAPDH was from Kangchen (Shanghai, China). MitoSOX was supplied by Life Technologies (Grand Island, NY, USA). TUNEL kit and PhosSTOP tables were the products of Roche Applied Science (Mannheim, Germany).
Cell culture

HUVECs were cultured in 1640 medium supplemented with 10% fetal bovine serum (FBS, HyClone) in an incubator with 5% CO₂. When grown to 90% confluency, the cells were trypsinized, harvested, resuspended and then seeded to a 0.1% gelatin-coated glass slide. The monolayer cells grown on the glass were used for subsequent experiments.

Shear stress study

LSS was simulated using a parallel-plate flow chamber that produced a continuous flow by sandwiching a silicon gasket between two stainless steel plates with a cover slip sink in the base plate. The flow chamber height was 0.56 mm with a pump rate of 60 min⁻¹. The level of shear stress was adjusted by modulating the after-loading flow and automatically calculated using pressure transducers. The shear stress used in this study was controlled at 2 dynes/cm².

Detection of ROS

MitoSOX is a fluorescent probe that selectively detects superoxide in the mitochondria. The glass slide mounted with monolayer cells was loaded into the flow chamber, and the cells were subjected to LSS for 60 min followed by treatment with MitoSOX for 20 min to examine the levels of ROS under light-protected conditions. The cell nuclei were labeled with DAPI.

TUNEL assay for cell apoptosis

HUVECs exposed to LSS for different time lengths were fixed in 4% paraformaldehyde and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100. After being blocked with 3% H₂O₂ for 10 min, the cells were incubated with TUNEL reaction mixture at room temperature for 1.5 h. Following a briefly washing, convert-POD and substrate solution were added and the cell nuclei were stained with hematoxylin.

Western blot analysis

The monolayer cells on a glass slide were exposed to LSS for different time lengths with serum-free 1640 medium as the cycling fluid; in the inhibition groups, the cells were pre-incubated with different inhibitors prior to LSS exposure. After the flow experiment, the cells were gently washed twice with ice-cold PBS and lysed in a cocktail of RIPA buffer, protease inhibitor and phosphatase inhibitor. After 20 min on ice, the cells were scraped and then the cell lysates were centrifuged at 12,000×g for 15 min at 4 ºC. The protein concentrations were quantified by bicinchoninic acid assay according to the manufacturer’s instructions. Briefly, 60 µg of protein was separated by a SDS-PAGE gel and transferred to a PVDF membrane which was then incubated overnight at 4 ºC with appropriate primary antibodies. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After a second wash, the membranes were developed using enhanced chemiluminescence substrate and the band intensities were analyzed using Image J software (National Institutes of Health, Bethesda, USA).

Statistical analysis

The continuous variables are expressed as Mean ± SD. Student’s t-test was used to analyze the data between two groups and ANOVA utilized to compare the data from more than two groups. A P value less than 0.05 was considered to denote a significant difference.

RESULTS

Morphology of LSS-exposed cells

Routinely cultured HUVECs presented with a spindle-shaped morphology, aligned longitudinally in parallel. The cells exposed to LSS-treated showed membrane disruption and nuclear condensation (Fig.1).

LSS induced cell oxidative damage

ROS is an indicator of oxidative stress and oxidatively induced cell apoptosis leads to deposition of oxidized low-density lipoprotein. After exposure to LSS for 60 min, the accumulated ROS showed a marked increase and obvious cell apoptosis occurred as compared to the cells in normal culture condition (Fig.2).
Fig. 2. LSS increases oxidative damage in HUVECs. **A**: Representative images of ROS-positive cells (red), and the cell nuclei were stained with DAPI; **B**: Apoptotic cells detected by TUNEL staining as confirmed by DAPI staining (C); **D, E**: Bar diagram showing quantitative data of ROS-positive cells and apoptotic cells, respectively. *P*<0.05 vs LSS 0 min (n=5). Scale bar=100 µm.
**LSS activated MAPK signaling**

The MAPK family is noted for its significant role in cellular homeostasis. We assayed the changes in P38, ERK and c-Jun in the cells exposed to LSS for different time lengths. As shown in Fig.3, LSS promoted the activation of P38, ERK and c-Jun in a time-dependent manner, suggesting an association between MAPK and LSS-induced cellular injury.

**ERK inhibition deactivated eNOS-Thr495**

Different inhibitors of MAPK were used to explore the upstream regulator of eNOS-Thr495. PD98059, other than SB202190 and SP600125, suppressed LSS-evoked eNOS-Thr495 (Fig.4). The antioxidant effect of ERK inhibition was further confirmed by measuring intracellular superoxide dismutase (SOD). The results demonstrated that ERK-activated eNOS-Thr495 was involved in LSS-induced oxidative stress.

**DISCUSSION**

Fluid shear stress exhibits various effects on cells [14]. Physiological shear stress is considered anti-atherosclerosis, but a low level of shear stress promotes the formation and progression of atherosclerosis [4, 14, 15]. In this study, we found that LSS enhanced ROS and cell apoptosis and increased cytotoxin in cultured HUVECs [16]. Oxidative stress is vital in promoting atherosclerosis and features an excessive superoxide production [17]. Accumulated ROS disturbs the biological activities of NO and promotes cell apoptosis [18, 19], thus leading to a disruption of endothelial integrity and the deposition of lipid [20, 21].

We demonstrated that P38, ERK and c-Jun were associated with endothelial dysfunction in LSS-treated cells. Specifically, ERK other than P38 or c-Jun was responsible for decreased NO level and hence impaired SOD activity in LSS-exposed cells, and inhibition of P38 or c-Jun did not affect the expression of eNOS-Thr495. This result indicated that LSS-induced oxidative stress might be attributed to the suppression of ERK/eNOS-Thr495.

There are several limitations in this study. Atherosclerosis is a chronic disease and a prolonged LSS exposure time in vivo should be applied to better simulate the condition. As cellular signaling pathways constitute complicated, interacting networks, we can not exclude significant factors other than MAPK involved in LSS-induced cell damages, such as PI3K/PKD/eNOS-Ser1177 and PKA/eNOS-Ser633, which were reported to regulate endothelium function under high shear stress [22-24], and matrix metalloprotease-9, which plays a crucial role in shear stress-induced cell migration and proliferation [24].

**REFERENCES**


摘要: 目的 观察体外模拟低剪切力对血管内皮细胞产生的氧化应激性损伤并探讨其可能机制。方法 用平行板流动腔装置于体外模拟低剪切力作用于体外培养的人脐静脉内皮细胞60 min后,用MitoSOX检测线粒体内活性氧簇(ROS)含量,用TUNEL及DAPI染色标记凋亡的细胞。低剪切力作用不同时间后,用Western blotting检测内皮型一氧化氮合酶(eNOS),P38,细胞外调节蛋白激酶(ERK)及c-Jun及其磷酸化蛋白的表达水平,并分别用P38,ERK和c-Jun的抑制剂作用细胞后检查eNOS的负性调节位点Thr495的表达。结果 体外模拟低剪切力可以明显诱导血管内皮细胞的氧化应激性损伤,呈时间依赖性激活eNOS、P38,ERK及c-Jun蛋白的磷酸化,但对总蛋白的合成无影响。ERK抑制剂可有效抑制eNOS-Thr495的活化并逆转LSS诱导的细胞内超氧化物歧化酶(SOD)减少。结论 体外模拟低剪切力诱导的细胞氧化应激性损伤是促分裂素原活化蛋白激酶(MAPK)信号通路活化有关,ERK/eNOS的活化参与低剪切力调节的氧化应激。

关键词: 剪切力; 内皮细胞; 动脉粥样硬化; MAPK信号通路; 氧化应激