Characterization of Extracellular Signal-Regulated Kinase 5 Levels in Human Umbilical Vein Endothelial Cells Exposed to Disturbed and Uniform Flow

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Abstract

Keywords

- pulsatile uniform flow
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- disturbed flow
- laminar flow
- uniform flow

Extracellular signal-regulated kinase 5 (ERK5) has been reported to regulate endothelial cell integrity and protect from vascular dysfunction under continuous laminar flow. However, the effect of flow on ERK5 levels has not been determined. Confluent human umbilical vein endothelial cells (HUVECs) were seeded on fibronectin coated glass slides and serum starved for 2 hours with 1% fetal bovine serum (FBS). HUVECs were then exposed to to and fro flow (TFF), pulsatile forward flow (PFF), or continuous laminar flow (CLF) in a parallel plate flow chamber for up to 2 hours. At the end of experiment, cell lysates were prepared and immunoblotted with antibodies to total ERK5. Both CLF and TFF exhibited a decrease in ERK5 after levels after 2-hour exposure. However, the level of ERK5 for PFF remained the same. Disturbed, but not uniform pulsatile, flow decreases ERK5 levels in HUVECs.

There is a strong association between localized atherosclerotic plaque and regions exposed to the disturbed flow found at vessel curvatures, bifurcations, and branches.¹ As an example, the human coronary artery exposed to disturbed flow is vulnerable to atherosclerosis. In contrast, the incidence of atherosclerosis in the internal mammary artery is low due to its uniform blood flow patterns.¹ Endothelial cells (ECs), which form the inner lining of blood vessels are directly exposed to blood flow and hemodynamic forces.² The major mechanical force is fluid shear stress, the frictional force on ECs generated by blood flow. Disturbed flow occurs when the fluid shear stress is not constant in magnitude, direction, or frequency and is associated with dysfunction in ECs, such as altered vasoregulation, activation of inflammatory processes, and compromised barrier function. In contrast, sustained laminar blood flow and unidirectional high shear stress are associated with upregulation of antioxidant and growth-arrest genes in ECs.³

The anti-inflammatory and antiatherosclerotic effects of sustained laminar flow on ECs have been attributed to its effects on mitogen-activated protein kinase (MAPK)

signaling.^{4,5} MAPK signaling is relatively complex and diverse; MAPK can engage in cross-talk with other MAPKs to regulate critical cell functions.⁶ Four distinct, classical MAPK cascades exist within mammalian cells: extracellular signal-regulated kinases (ERK) 1/2, c-Jun NH2-terminal kinases (JNK) 1/2/3, p38MAPK $\alpha/\beta/\gamma/\delta$, and ERK5.⁶ The ERK 1/2, JNK 1/2/3, p38, and ERK5 are activated via various hemodynamic forces, such as cyclic strain⁷ and shear stress. ERK5, the most recent member of MAPK family, has been evidenced to play a vital role in the mechanism of atheroprotective effects under sustained laminar flow which upregulates ERK5 activity.⁵ However, it is not clear if flow affects ERK5 levels. We hypothesized that uniform and disturbed flow would have different effects on ERK5 which may be associated with its atheroprotective or anti-inflammatory properties.

Materials and Methods

Cell Culture

Primary cultures of human umbilical vein endothelial cells (HUVECs) (obtained from the laboratory of Dr. Jordan Pober,

Department of Pathology, Yale School of Medicine) were cultured with M-199 medium enriched with 20% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 10 µg/mL heparin, 50 µg/mL EC growth supplement (BD Biosciences, Bedford, MA), and 100 µg/mL penicillin-streptomycin antibiotic combination, 100 µg/mL L-Glutamine (Life Technologies, Carlsbad, CA) in a 5% CO₂ incubator at 37°C. After reaching confluence, 0.25% trypsin ethylene diamine tetra acetic acid was used for detachment, and passage three to five cells were seeded on fibronectin (BD Biosciences) coated glass slides (7 × 38 mm; Fisher Scientific, Pittsburgh, PA). After reaching confluence, HUVEC were serum starved (SS) in 1% FBS media for 2 hours before experimentation. Positive control samples were reincubated in 20% FBS media for 2 hours after SS.

Mechanical Stress Exposure

SS HUVECs were exposed to shear stress for 2 hours under different flow conditions utilizing a parallel plate flow chamber system (Cytodyne, San Diego, CA) as previously described.^{8,9} Flow of the perfusion medium was regulated by a computer-controlled syringe pump (PHD 2000 and PHD Ultra Programmable; Harvard Apparatus, Holliston, MA). To generate pulsatile forward flow (PFF), an automated switch clamp (Auto-Fill valve box; Harvard Apparatus, Holliston, MA) is placed between the syringe pump and the flow chamber, and between the syringe pump and the culture medium reservoir. Synchronous activation of both switch clamps with the cycle allows unidirectional flow. In the to and fro flow (TFF) model, the switch clamp was not used and thus creating a form of disturbed flow. The flow chambers were directly attached to the flow loop circuit including the flow reservoir, which enabled culture medium in the flow chamber to be exchanged by every to and fro impulse. Approximately, one-tenth of the culture media in the flow chamber was exchanged at least every 1 second. The shear stresses were 60 cycle per minute (cpm) of PFF, that is, a forward square wave impulse for 0.5 second alternating with no flow for 0.5 second; 60 cpm of TFF, that is, a forward square wave impulse for 0.5 second alternating with a backward square wave impulse for 0.5 second. Continuous laminar flow (CLF) setup is perfusion medium circulated in norprene tubing (Masterflex, Court Vernon Hills, IL) using a roller pump (Masterflex, Court Vernon Hills, IL) to parallel plate flow chambers placed in the circuit. Small pulsations produced by the roller pump were dampened by increasing length of the tubing. The magnitude of shear was kept constant at 14 dyne/cm² for all flow conditions.

Immunoblot for Extracellular Signal Regulated Kinase 5

After experimentation, cells were washed in ice-cold PBS (Hyclone, South Logan, UT) twice and scraped in RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% sodium dodecyl sulfate (SDS), 2 mM sodium orthovanadate, cocktail of phosphatase inhibitors I and II (EMD4Bioscience, San Diego, CA), and protease inhibitor (Roche Applied Science, Indianapolis, IN). Cell lysates were centrifuged for 15,000 g for 10 minutes, and supernatants were collected. Protein content

was measured by Bio-Rad protein assay system (Bio-Rad, Hercules, CA). Laemmli sample buffer was added to equal amounts of each sample, and samples were boiled for 5 minutes. Samples were resolved on 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. The membranes were probed with ERK5 (Cell Signaling Technology, Danvers, MA) as the primary antibody and rabbit secondary antibody (Cell Signaling Technology, Danvers, MA) with washing, as suggested by the manufacture and detected using chemiluminescence (Lumiglo, Cell Signaling Technology, Danvers, MA). Membranes were developed on X-ray films and quantification of bands was performed by densitometric analysis using ImageJ software (<http://rsbweb.nih.gov/ij/>). Densitometric data were expressed as the fold induction compared with the static control level.

Statistics

Calculation of the densitometric mean ± standard error of the mean of multiple experiments and statistical analysis utilizing a two-tailed *t*-test was performed using SPSS (IBM, Armonk, NY) and a "*p*" value of <0.05 was considered significant.

Results

► **Fig. 1** shows representative immunoblots from HUVEC SS for 2 hours or 4 hours, or exposed to CLF, PFF, and TFF for 2 hours after a 2-hour SS period. We observed that exposure to CLF resulted in a significant 35% reduction of ERK5 levels. Under disturbed pulsatile flow conditions (TFF), HUVECs also demonstrated a significant decrease in ERK5 (39% reduction compared with static). However, HUVEC exposed to uniform PFF did not show significant reduction in ERK5 levels compared with static 4-hour control cells nor incubation of static SS HUVEC with 20% FBS media for 2 hours.

► **Fig. 2** shows representative light microscopy photographs of HUVEC under the various conditions. PFF and CLF cultures showed maintenance of confluence of cells while HUVEC exposed to TFF demonstrated significant detachment after 2 hours.

Discussion

Artherosclerosis is a disease that is defined by the deposition of fatty plaques on the inner walls of arteries. A strong correlation has been established between localized atherosclerotic plaque and regions exposed to disturbed flow found at vessel curvatures, bifurcations, and branches.¹ Regions exposed to disturbed flow are also exposed to different magnitudes of hemodynamic forces, such as shear stress, where they have effects on endothelial and vascular biology. Regions of the vasculature where shear stress is low are more likely to develop atherosclerosis.¹⁰ In contrast, ECs exposed to unidirectional flow, especially high laminar shear, have been reported to express atheroprotective genes.¹¹

ERK5 or BMK1, is the most recently identified member of the MAPK family. It consists of an aminoterminal kinase domain, with a relatively large carboxyl-terminal of unique

Fig. 1 ERK5 levels in HUVEC exposed to different flow conditions. (A) Immunoblot of ERK5 exposed to 2 hours SS (lane 1), 4 hours SS (lane 2), 2 hours SS followed by 2 hours TFF (lane 3) and 2 hours SS followed by 2 hours of 20% FBS (lane 4). The corresponding actin loading blots are at the bottom. There was a significant decrease in ERK5 in HUVEC exposed to TFF. (B) Immunoblot of ERK5 exposed to 4 hours SS (lane 1), 2 hours SS followed by 2 hours PFF (lane 2) and 2 hours SS followed by 2 hours CLF (lane 3). The corresponding actin loading blots are at the bottom. There was no change in ERK5 in HUVEC exposed to PFF while HUVEC exposed to CLF had a decrease. (C) Densitometric arbitrary units (based on five separate experiments) standardized to 4 hours SS (static) condition. There was significant decrease in ERK5 levels in CLF and TFF. However, there was no significant ERK5 change in PFF conditions. CLF, continuous laminar flow; ERK5, extracellular signal-regulated kinase 5; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; PFF, pulsatile forward flow; SS, serum starved; TFF, to and fro flow.

structure and function that makes it distinct from other MAPK members.¹² It has been proposed that ERK5 possesses nuclear export activity.¹³ In its unphosphorylated state, ERK5 exists in a folded conformation. The resulting molecular interaction between the N- and C-terminals is considered to either reduce the activity of the nuclear localization signal or to generate a nuclear export signal, causing ERK5 to be sequestered in the cytoplasm.¹⁴ Global ERK5 knockout mice showed embryonic lethality with defective cardiac development, heart, looping, angiogenesis, and vascular maturation.¹⁵ This finding has focused research on ERK5 in relation to all cardiovascular-related diseases to explain physiological and pathologic phenomena.

We used TFF as a model of disturbed flow. The oscillatory flow change is analogous to that seen at regions of high risk for atherosclerotic plaque development, such as the carotid bulb. Oscillatory flow is characterized by time-averaged fluctuations in shear stress during the cardiac cycle due to forward–reverse flow cycles and disrupted flows. We also used PFF, a model of pulsatile forward-directed laminar

flow, with a pulsation frequency of 60 cpm analogous to human heart rate. This model is more representative of normal physiological flow conditions compared with previous nonpulsatile laminar flow systems, such as the CLF model. In this study, we specifically analyzed the effect of TFF, PFF, and CLF on ERK5 regulation in HUVECs. Previous investigations studied shear stress effects on ERK5 using different flow systems.⁸ We used a similar level of shear stress and could demonstrate a decrease in ERK5 in HUVEC exposed to CLF by 2 hours. The novel finding of our study, however, is that there was also a reduction of ERK5 levels under TFF but not with PFF. The mechanism underlying the differential response of HUVEC to disturbed and uniform flows is not clear. We expected that CLF and PFF would have similar effects on HUVEC results because they are both laminar, unidirectional, and had the same magnitude of shear stress and differed only in the presence of pulsatility. However, we have previously shown that there was a difference in expression of tissue factor in HUVEC exposed to CLF and PFF.¹⁶

Fig. 2 (A) Microscopic images of human umbilical vein endothelial cells after exposure to 4 hours serum starved (SS); (B) 2 hours SS + 2 hours to and fro flow (TFF); (C) 2 hours SS + 2 hours continuous laminar flow; and (D) 2 hours SS + 2 hours pulsatile forward flow. There was significant detachment of cells when exposed to disturbed (TFF) flow conditions.

Previous investigations of total protein levels of ERK5 by western blot analysis, after cellular fractionation, showed decreased levels in cytosolic samples and increased in nuclear samples. Also, upon exposure to flow, ERK5 demonstrated multiple bands whereas a single band was evident in static samples.¹⁷ Whether our experiments samples are mostly cytosolic after removing the pellet is yet to be investigated. This observation was also noted in a different study where multiple bands of ERK5 occurred in constitutively active mutants of MEK5 (enzyme responsible for activation of ERK5) in HUVECs compared with control and constitutively inactive mutants of MEK5.¹⁸ This raises the possibility that this experiment's anti-ERK5 antibody is detecting one of the multiple bands of ERK5. However, further investigations are required to validate this possibility.

The significant detachment of cells under TFF conditions is consistent with previous reports that disturbed flow can cause apoptosis. Whether the decrease in ERK5 and the cell detachment are causally related cannot be determined in this study. However, previous studies have confirmed that constitutively active mutants of MEK5 and its display of multiple bands of ERK5 resulted in morphological changes of HUVECs.¹⁸ The cells appeared elongated and cell attachment to fibronectin was largely enhanced. This could explain that the cells remaining in TFF had a common characteristic of strong adherence to fibronectin evidenced by detachment of cells and the elongated morphology of those remaining cells. As for CLF, the cells also appeared

elongated with this similar morphology occurring in all cells but no cell detachment occurred. These similar morphological observations in CLF and TFF with the same trend of ERK5 levels may correspond to a mechanosignaling relationship. This raises the possibility that not only ERK5 can cause morphological changes on cells but induced morphological changes on cells can have an effect on ERK5. Further investigations are required to validate this phenomenon. In addition, adding 20% FBS to HUVEC after 2-hour SS resulted in a no change in ERK5 levels. This may be consistent with its proliferative properties and whether this nonresponsiveness is due to its preservation in non-stress conditions is to be investigated.

In summary, our findings indicate that in contrast to nonpulsatile laminar flow and pulsatile to and fro flow (disturbed flow), HUVECs exposed to pulsatile laminar flow (uniform flow) do not have a significant decrease in ERK5 levels. The different response of HUVECs exposed to disturbed flow than that of HUVECs exposed to uniform flow may provide the basis for understanding the differences in atherosusceptibility of the endothelium to different flow patterns in vivo.

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