Proteomic analysis of differentially expressed proteins in endothelial cells under low shear stress

Peile Ren
Xinjiang Medical University
Yiwei Zhao
Xinjiang Medical University
Pan Yang
Xinjiang Medical University
Fengxu Yu (yuluchuan@163.com)
Southwest Medical University
Yongmei Nie

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Abstract

Shear stress (SS) affects the morphology, migration, differentiation, and proliferation of endothelial cells, and regulates protein expression. The objective of this study was to perform a proteomic analysis of human umbilical vein endothelial cells (HUVECs) under low SS. HUVEC lines were cultured on glass slides (test group n=30; control group n=30) and transferred to a parallel-plate flow chamber. The test group was subjected to low SS at 4.58 dyne/cm2 for 2 hours, and the control group was maintained under static conditions. Approximately 2×107 cells were collected from each group and analyzed by two-dimensional electrophoresis. The proteins from each spot were identified by mass spectrometry. The biological functions and processes associated with the identified proteins were evaluated using the NCBInr, SwissProt, and DAVID databases. The protein expression of 14-3-3 proteins, annexin I, annexin II, zyxin, filamin A, filamin B, Ras-related nuclear protein (Ran), and talin were assessed by Western blotting. A total of 234 proteins were analyzed; 14 proteins were upregulated and 78 were downregulated in the test group compared with the control group. The expression of 14-3-3 proteins, annexin I, annexin II, zyxin, filamin A, and filamin B was significantly decreased, whereas the expression of Ran and talin was significantly increased in the test group. It is known that SS induces the remodeling of endothelial cells by modulating protein expression. However, the mechanisms underlying this modulation are unknown. The present results provide evidence that SS changes the protein profile of HUVECs.

Introduction

Vascular endothelial cells (VECs) are located in the inner lining of blood vessels and serve as an interface between blood flow and the vascular wall. VECs are sensitive to changes in blood flow shear stress, blood pressure, inflammatory signals, and hormone levels in the circulation. Furthermore, VECs help maintain homeostasis and play a critical role in pathophysiological processes[1]. The expression of several genes is regulated by laminar shear stress in endothelial cells; these genes are involved in cell proliferation and differentiation, cell-cell adhesion, maintenance of vascular tone, as well as physiological and pathological processes, including inflammation and immune regulation[2]. High shear stress (>15 dyne/cm^2) upregulates the expression of vasodilators, growth factors, fibrinolytic components, and antioxidants and downregulates the expression of vasoconstrictors, growth factors, inflammatory mediators, and adhesion factors to protect endothelial cells (ECs) from damage and prevent atherosclerosis[3, 4]. Low shear stress (±4 dyne/cm^2) increases the response of ECs to chemical substances, including oxidized low-density lipoprotein and tumor necrosis factor (TNF), which promote cell proliferation and platelet adhesion, and ultimately results in the dysfunction of ECs[4, 5] and atherosclerosis[2]. Atheromas typically occur in vascular bends and bifurcations, where shear stress is

low and uneven. Low shear stress and oscillatory shear stress have been shown to promote atherosclerosis in animal models. The alteration in hemodynamics and its effects on cell rheology may play a significant role in the pathogenesis of atherosclerosis; however, the regulation of protein expression following shear stress is not fully known.

Proteomics has advanced rapidly in recent years. In this study, we investigated the proteomic profile of HUVECs that were subjected to either shear stress or static conditions. The proteomic analysis identified differentially expressed proteins associated with mechanotransduction of low shear stress. The current results provide evidence that shear stress changes the protein profile of HUVECs.

Materials And Methods

Instruments and reagents

The instruments used included a flow chamber system (185.0 mm × 94.5 mm × 0.8 mm; Chengdu Ximu Subsidiary), centrifuge (Thermo, USA), cell lysis instrument (Ningbo Toshiba Cell Ultrasonic Splitter), isoelectric focusing instrument, electrophoresis apparatus (Bio-Rad), and a mass spectrometer (Thermo-QE, USA). The reagents used were M199 medium, PBS buffer, trypsin, double antibody/green streptomycin mixture (Hyclone, USA), fetal bovine serum (Gibco, USA), human fibronectin (Corning, USA), dithiothreitol, and iodoacetamide (Sigma). Images were acquired and analyzed using PDQuest software (Bio-Rad).

Endothelial cell culture and treatment

HUVEC line EA. hy926 was purchased from Shanghai Cell Bank. These cells were routinely subcultured and cultured in an incubator (37 °C and 5% CO_2) in M199 complete medium containing 10% fetal bovine serum and 1% double antibody. Third-generation cells were used for shear stress treatment. A slide (75 mm × 25 mm) with 1 mL fibronectin (FN) was incubated at 37 °C for 30 min. Trypsin-digested cells were then seeded on the treated slides at a cell concentration of 1×10^6/mL. After cell confluence, the slides were transferred to a parallel-plate flow chamber system (Fig. 1), and shear stress was applied (4.58

dyne/cm^2) for 2 hr. After treatment, the slides were removed from the chamber, and the adhered cells were trypsinized, counted, and stored at −80°C until further use.

Two-dimensional electrophoresis

The cell suspensions were centrifuged and washed three times with PBS (room temperature, 1000*g*, 5 min for each wash). The cells were transferred to 1.5-mL Eppendorf tubes, and the residual PBS was collected. After that, 500 μL of bidirectional electrophoretic lysis buffer was then added to the cell suspension. The cells were lysed by sonication with fifteen 15-s pulses at 30-s intervals at a temperature of 4 °C and power output of 15%. The lysates were centrifuged at 40,000*g* for 1 h, the supernatant was collected, and protein was quantified using the Bradford method. The samples were aliquoted and stored at −80 ℃ until further use. Samples were rehydrated into 24-cm pH 3–10 IPG strips (Bio-Rad) at −20 °C for 18 hr. The hydrated strips were transferred to the isoelectric focusing tray; the cathodic and anodic ends of the strip were placed on the respective cathodic and anodic ends of the tray. After that, 3 mL of mineral oil was added to each strip, and isoelectric focusing was performed (Table 1). The strip was removed from the tray and sequentially treated with dithiothreitol for 15 min and iodoacetamide for 15 min. The strips were subjected to 12% SDS-PAGE. After electrophoresis, the strips were removed, stained with Coomassie brilliant blue, destained, and washed. Images were subsequently acquired using the PDQuest software. Ten distinct protein spots were subjected to enzymatic hydrolysis and analyzed using a Q Exactive mass spectrometer.

Image processing and data analysis

Ten different spots were selected for mass spectrometry analysis. The structure, function, and biological processes of DEPs were evaluated using the databases NCBInr; SwissProt; Database for Annotation, Visualization, and Integrated Discovery (DAVID); and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING).

Western blotting

The slides were removed from the parallel-plate flow chamber, washed twice with cold PBS, and dried. The adhered cells were detached in PBS using a scraper. The cell suspension was transferred to 1.5-mL sterile Eppendorf tubes and centrifuged at high speed for 3–5 min. PBS was then removed. The protein

lysate was added to the test tubes and incubated on ice for 30 min with agitation at 10-min intervals. The test tubes were centrifuged at 12,000 rpm for 20 min at 4°C, and then the supernatant was transferred to a new sterile test tube. The protein concentration was measured using the Price BCA Protein Assay kit according to the manufacturer's instructions. In brief, 30 mg of protein was isolated on a 12% SDS-PAGE gel and transferred onto a polyvinyl difluoride membrane overnight at 4 °C. The membrane was blocked with 5% skim milk powder for 6 hr. The membrane was then incubated with a primary antibody (1:1000) overnight at 4 °C, washed three times (10 min for each wash) with TBTS at room temperature, incubated with a secondary antibody (1:1000) at room temperature in the dark for 2 hr under agitation, and then washed three times with TBST (10 min for each wash) and once with distilled water for 5 min each. After this step, the membrane was incubated with a color development solution (1:1) for 3 min. Images were acquired using a chemiluminescence imager (Bio-Rad). The detected proteins were 14-3-3 (25–32 kDa), annexin (37 kDa), annexin II (37 kDa), zyxin (61 kDa), filamin A (FLNA) (286 kDa), filamin B (FLNB) (286 kDa), Ras-related nuclear protein (Ran) (24 kDa), talin (270 kDa), and GAPDH (internal control [37 kDa]).

Statistical analysis

The results are expressed as mean ± SD (n=3). Comparisons were made using a one-way analysis of variance. All statistical analyses were performed with SPSS software version 17.0. P-values of less than 0.05 were considered significant.

Results

Determination of total protein concentration

The protein concentration of the cell lysates was determined by the Bradford method using 1 mg/mL bovine serum albumin as the standard. The concentration in the test and control group was 4.935 and 4.89 mg/mL, respectively.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed on the cell lysates. This method detected 79 protein spots in the low shear stress group (Fig. 2A) and 189 specific protein spots in the control group (Fig. 2B).

Ten protein spots were selected, subjected to enzymatic digestion, and analyzed by mass spectrometry (Figs. 2C-D). The analysis indicated that the test and control group presented 234 and 92 DEPs, respectively, with a fold-change 2.

Functional analysis of DEPs using the DAVID database and interaction network analysis

The main biological processes identified in the DAVID database were metabolic and catabolic processes, apoptosis, programmed cell death, protein transport, assembly and transport of macromolecular complexes, cytoskeletal composition, RNA processing and splicing, mRNA processing and metabolism, protein folding, and cellular stress response (Figs. 3-4). The main pathways identified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were cell cycle, neurotrophin signaling, hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy, cardiac muscle contraction, Hippo signaling, and PI3K-Akt signaling (Fig. 5).

Analysis of DEPs under low shear stress

Eight DEPs identified by mass spectrometry were selected for Western blot analysis. The results showed that 14-3-3 proteins, annexin I, annexin II, zyxin, FLNA, and FLNB were downregulated ($n=3$, $p<0.05$) whereas Ran and talin were upregulated ($n=3$, $p<0.05$) in the treated group compared to the control group (Fig. 6).

Discussion

Molecular and cellular studies have demonstrated that ECs regulate vascular tone and blood vessel growth by secreting vasoactive substances with multiple functions, including the prevention of thrombosis. Other studies have shown that endothelial dysfunction is a critical event in early cardiovascular disease (CVD) and is closely related to the occurrence and development of atherosclerosis, hypertension, and heart failure[6].

In the cardiovascular system, mechanical factors play an important role in regulating vascular pathophysiology. ECs are subjected to different types of shear stress caused by the tangential friction force in blood flow, such as laminar shear stress and disturbance flow, which can affect endothelial

function[7, 8]. Under normal conditions, high laminar shear stress can increase the expression of genes with hypoplastic, anti-inflammatory, and anti-thrombotic effects that can consequently aid in preventing atherosclerosis[3]. In contrast, low laminar shear stress or disturbance flow (and other blood flow changes) induces the expression of pro-inflammatory genes and promotes cell proliferation and apoptosis[4, 9]. Studies have shown that shear stress achieves the pathophysiological regulation of angiogenesis by modulating endothelial genes[2]. The present results confirmed that the expression of fractalkine on ECs was highest at low shear stress (4.58 dyne/cm^2) for 2 hr. Fractalkine can promote the formation and development of atherosclerotic plaques[10]. Therefore, we intended to use 4.58 dyne/cm^2 as a low shear stress value, and proteomics to analyze DEPs in VECs under low laminar shear stress.

The proteomics analysis identified 78 downregulated proteins with a fold-change 2 in the low shear stress group compared with the control. The DEPs identified using DAVID are involved in several biological processes, including metabolic processes, protein localization, apoptosis, programmed cell death, protein transport, assembly and transport of macromolecular complexes, energy metabolism, cytoskeletal composition, RNA processing and splicing, mRNA processing and metabolism, protein folding, cellular stress response, and translation. The most enriched pathways in the KEGG analysis were cell cycle, neurotrophin signaling, HCM, dilated cardiomyopathy, myocardial contraction, Hippo signaling, and PI3K-Akt signaling. The identification of CVD-related proteins in these pathways provides evidence that the development of CVD is induced by low shear stress.

The results of Western blotting showed that the expression of 14-3-3 proteins was significantly reduced after 2-hr low shear stress. These proteins are highly conserved in eukaryotic organisms and play different roles in regulatory processes, including cell growth, apoptosis, signal transduction, protein transport, and stress response. The subtype 14-3-3σ is an epithelial marker found in the epithelium. In recent years, the study of 14-3-3 proteins and tumors has become a popular topic. Studies have shown that 14-3-3 proteins are also involved in the TNF-a-mediated protection of VECs[11]. In this study, six TNF subtypes (β, γ, ε, σ, ζ, and η) (encoded by YWHAB, YWHAG, YWHAE, YWHAZ, YWHAQ, and YWHAH, respectively) were significantly downregulated. This result is consistent with those of mass spectrometry analysis and suggest that 14-3-3 proteins may be associated with cell adhesion and apoptosis, and other functions under low shear stress conditions.

The present results demonstrate that low shear stress decreases the expression of annexins I and II. Annexin I is a calcium- and phospholipid-binding protein involved in various biological processes, including cellular differentiation, proliferation, apoptosis, and migration[12], and suppresses and resolves inflammation predominantly by targeting neutrophils[13]. Evidence is accumulating that neutrophils play fundamental roles in atherogenesis and contribute to the initiation, progression, and destabilization of atherosclerotic plaques [14-17]. Moreover, annexin I has been shown to modulate apoptosis, efferocytosis, and macrophage polarization, which are dysregulated in atherosclerosis[18]. Annexin II is expressed in several cell types, including tumor cells, ECs, macrophages, and mononuclear cells. Annexin II binds to the p50 subunit of NF-κB in a calcium-independent manner, and the annexin II–p50 complex is translocated into the nucleus. Furthermore, annexin II increases the transcriptional activity of NF-κB in both the resting and activated state, and upregulates the transcription of several target genes downstream of NF-κB, including interleukin-6[19] and NF-κB genes, which play an essential role in the inflammatory response. However, the biological function of intracellular annexin II and the mechanism underlying the production thereof are not fully elucidated.

Zyxin is predominantly localized to cell adhesion sites. Mechanisms by which zyxin affects gene expression have been proposed[20], and this protein is a potential therapeutic target to affect the onset of endothelial cell phenotype. Zyxin is usually associated with focal adhesions and stress fibers, and plays a major role in stretch-induced endothelial gene expression [21, 22]. Mechanical forces mobilize zyxin from focal adhesions to actin filaments and regulate cytoskeletal reinforcement [23]. Furthermore, zyxin regulates cAMP agonist-induced Weibel–Palade body exocytosis in cultured human primary ECs, and zyxin deletion impairs vascular hemostasis and thrombosis[24]. The present results demonstrate that zyxin protein expression was significantly downregulated, suggesting that low shear stress affected zyxin production.

Filamins are involved in cytoskeletal formation and play a crucial role in cell migration, adhesion, proliferation, signal transduction, and gene therapy[25]. FLNA is an actin-binding protein that helps maintain cell integrity and is critical for cell migration[26]. FLNB is widely distributed across species and is the second most abundant actin filament protein in humans, being highly expressed in normal endothelial cells. Studies have shown that the endothelial cells of FLNA-deficient mice are structurally disordered, and the migration ability of endothelial cells in mice lacking FLNB is altered. FLNA and FLNB

are expressed in endothelial cells and play an essential role in vascular development[27]. In this study, mass spectrometry analysis demonstrated that FLNB expression was downregulated under low shear stress conditions, indicating that the stimulation of the physical condition can change the migration ability of endothelial cells.

The Ran family is unique in the Ras-GTPase superfamily and helps regulate nuclear import and export[27, 28]. Ran is activated in the cytoplasm and nucleus and acts as a molecular switch that cycles between the GDP- and GTP-bound state[29]. The deregulation of Ran-dependent processes has been implicated in the development of severe diseases such as cancer and neurodegenerative disorders[30]. Our results show that the expression of Ran was substantially increased by low shear stress. However, the function of Ran in vascular disease and Ran regulation were unclear until recently. Talin is a large focal adhesion protein that links intracellular networks with the extracellular matrix (ECM) via the cytoskeleton and integrins. Talin binds and recruits cytoskeletal proteins involved in mechanotransduction[31, 32]. The dysregulation of talin activators may lead to disease states in which aberrant integrin activation and mechanotransduction promote changes in cell spreading, migration, and survival[33]. The present results indicate that talin expression was upregulated in ECs under mechanotransduction.

In summary, the proteomic analysis demonstrated that the expression of VEC proteins changed under low shear stress conditions. These results may help elucidate the mechanism by which shear stress affects VECs.

Declarations

Acknowledgements

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Competing interests

The authors declare that there are no conflicts of interest associated with this study.

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Table

Table 1. Isoelectric focusing program settings.

Program	Power (volts)	Speed	Time (hours)	Effect
S1	100	Stp	0.5	Desalting
S2	250	Stp	0.5	Desalting
S3	500	Stp	0.5	Desalting
S4	1000	Grp	1.0	Desalting
S4	3000	Grp	2.0	Boosting
S5	6000	Stp	2.0	Boosting
S5	8000	Stp	2.0	Boosting
S6	8000	Stp	60,000 (v h)	Focusing
S7	500	Fast	24	Maintenance

Figures

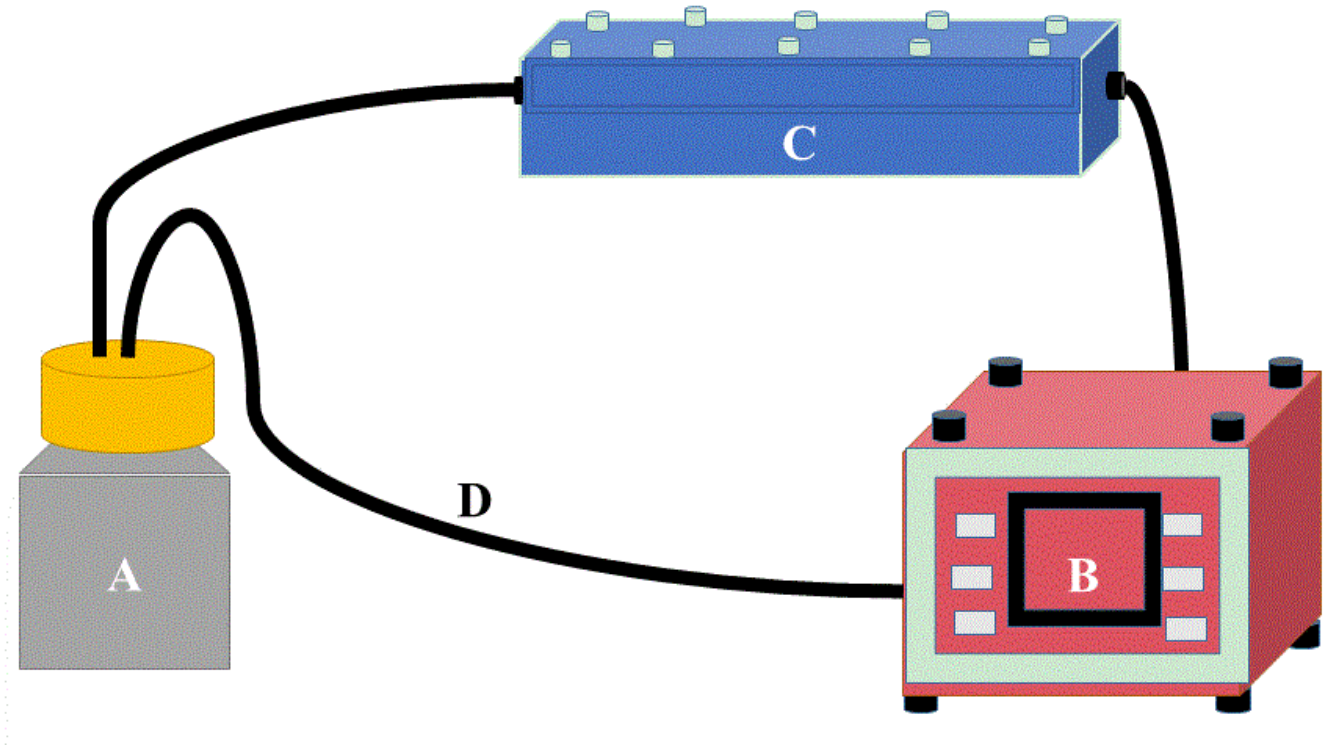

Figure 1

Device used for producing shear stress (A, perfusion vial; B, constant flow pump; C, parallel-plate flow cell; D, connection tube).

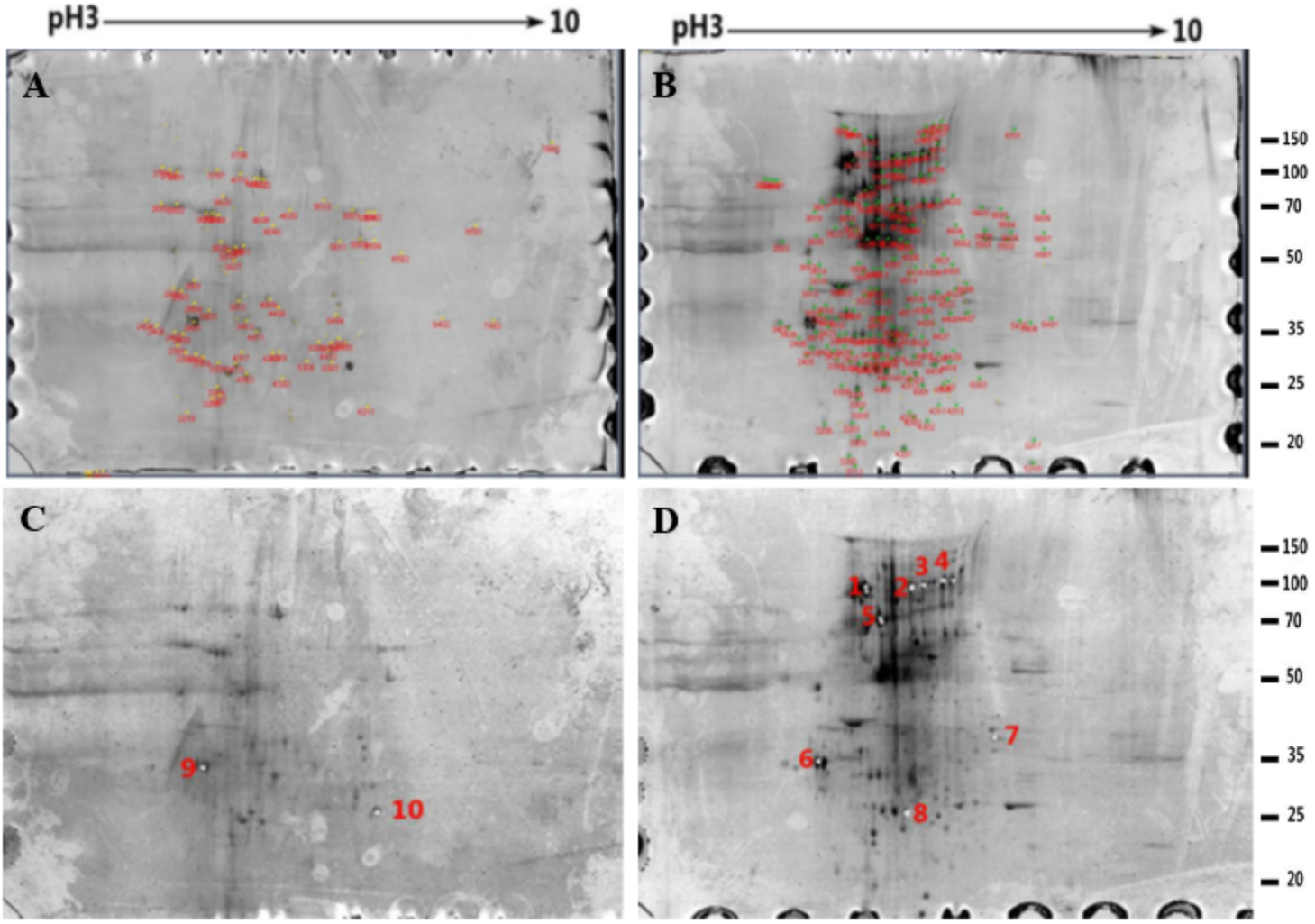

Figure 2

Analysis of differentially-expressed proteins in vascular endothelial cells under shear stress or static conditions. Electrophoretogram of two-dimensional electrophoresis of differentially expressed proteins under shear stress and static conditions. A total of 79 protein spots were identified in the shear stress group (A) and 189 spots were identified in the static control group (B). Of these, ten protein spots were excised and analyzed by mass spectrometry (C, D).

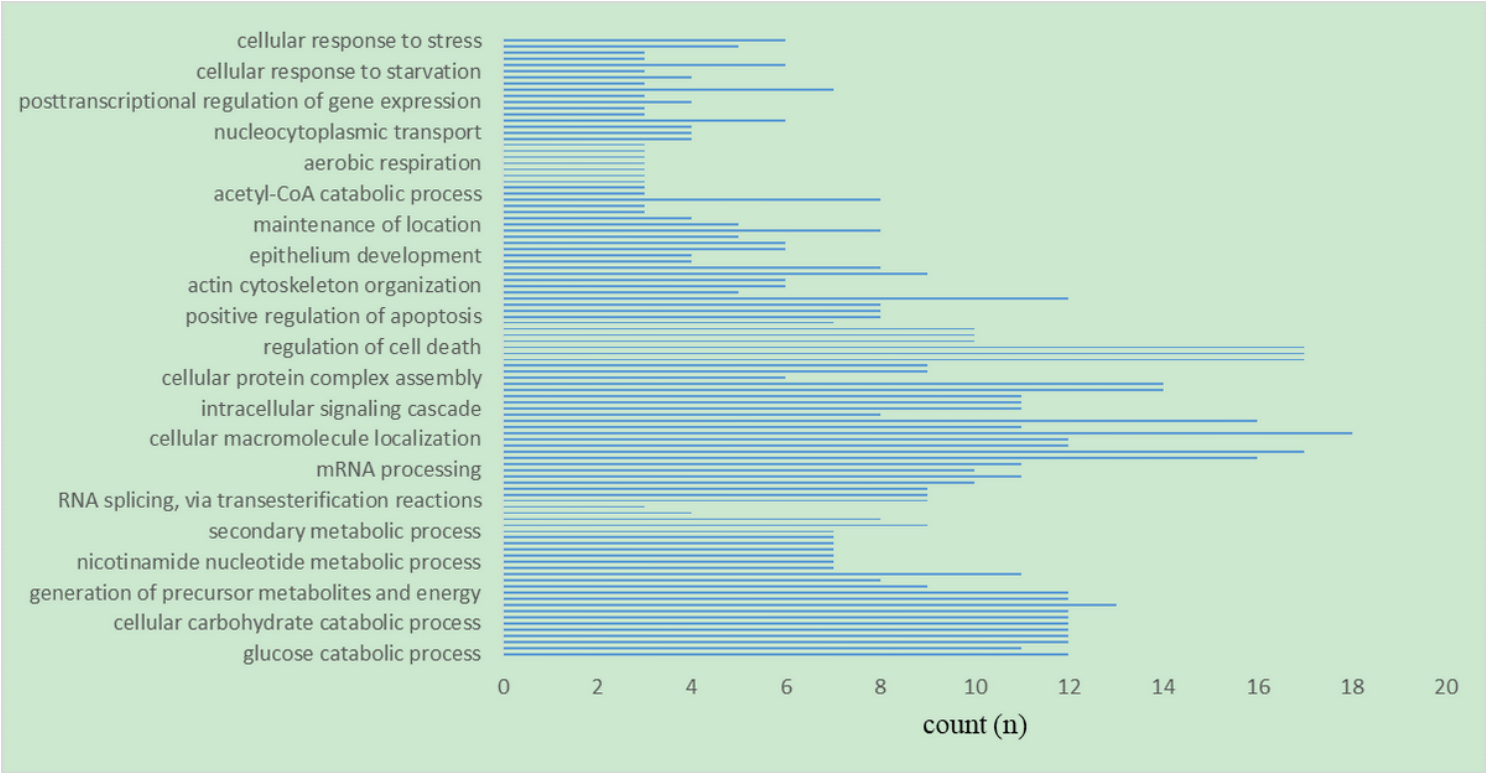

Figure 3

Enriched biological processes.

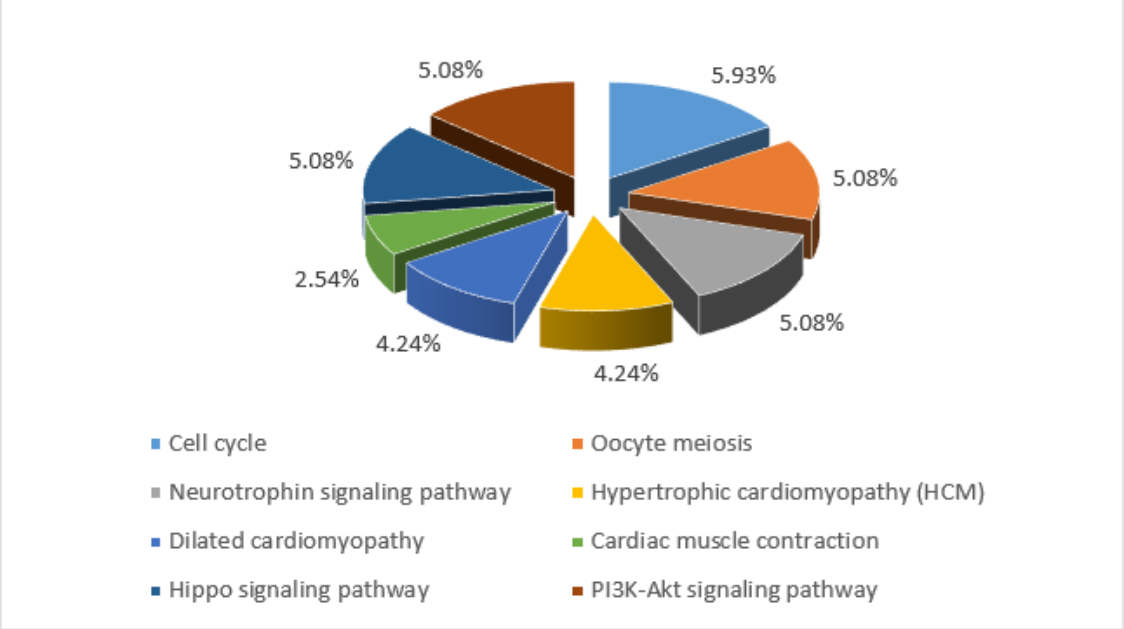

Figure 4

Enriched signaling pathways.

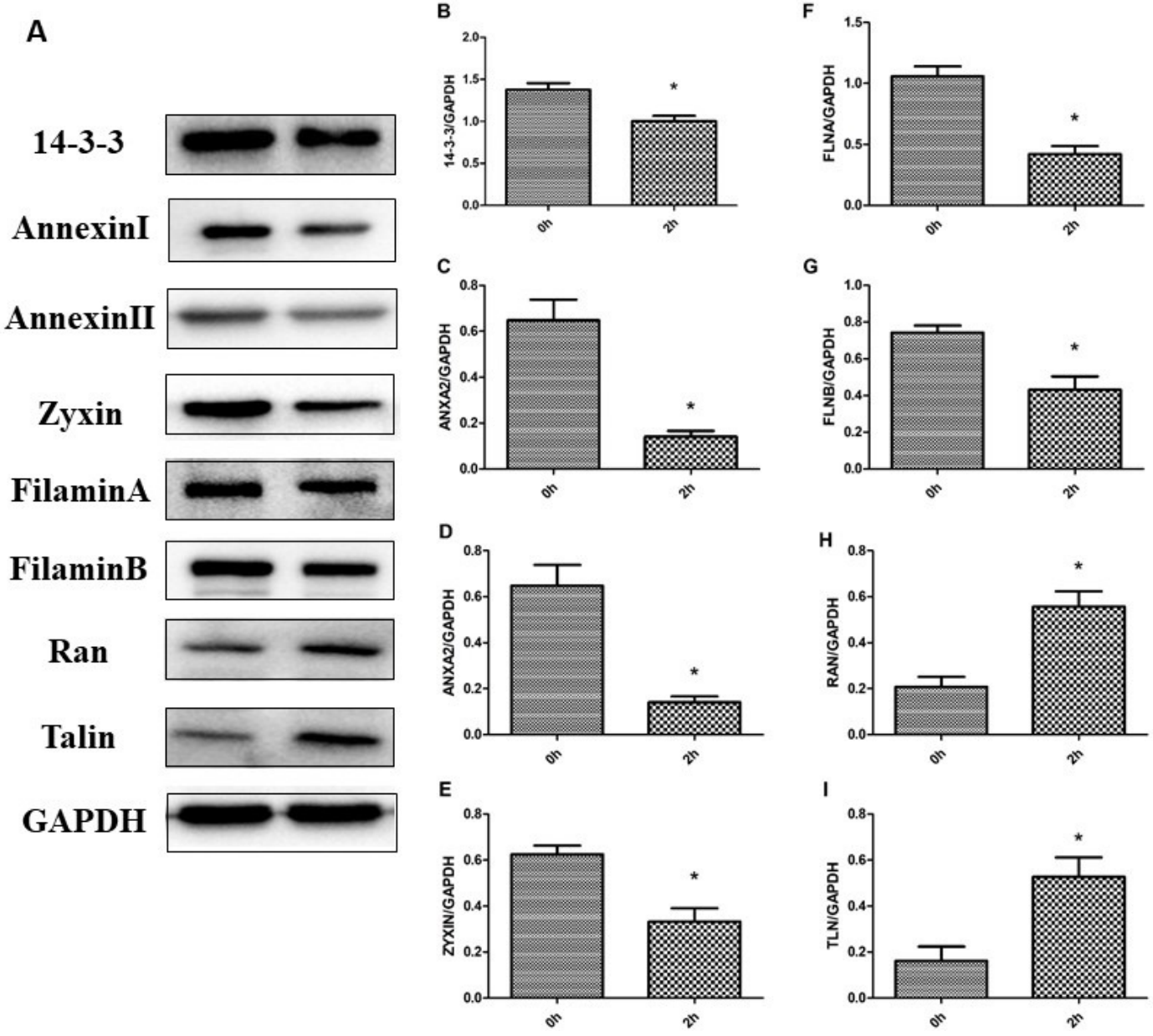

Figure 5

Protein expression after loading shear stress for 2 hr. Protein samples were subjected to immunoblot analysis (A). The proteins levels of 14-3-3 proteins (B), annexin I (C), annexin II (D), zyxin (E), filamin A (F), filamin B (G), Ran (H), and talin (I) were normalized using GAPDH as the internal control. Data are presented as mean ± SEM (N=3). *p<0.05 in the comparison between the shear stress and control group.