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Expression of synaptopodin in endothelial cells exposed to laminar shear stress and its role in endothelial wound healing

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ABSTRACT

We examined the hypothesis that certain actin binding proteins might be upregulated by laminar shear stress (LSS) and could contribute to endothelial wound healing. Analysis of mRNA expression profiles of human umbilical vein endothelial cells under static and LSS-exposed conditions provided a list of LSS-induced actin binding proteins including synaptopodin (SYNPO) whose endothelial expression has not been previously reported. Additional studies demonstrated that SYNPO is a key mediator of endothelial wound healing because small interfering RNA-mediated suppression of SYNPO attenuated wound closure under LSS whereas overexpression of exogenous SYNPO enhanced endothelial wound closure in the absence of LSS. This study suggests that LSS-induced actin binding proteins including SYNPO may play a critical role in the endothelial wound healing stimulated by LSS.
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1. Introduction

Since the vascular endothelium is constantly exposed to blood flow, endothelial cell physiology is affected by various physical factors generated by the interaction between the blood stream and blood vessels. The straight regions of the arteries are usually protected from atherosclerotic lesion formation whereas the arterial branch points, curvatures, and bifurcations are very prone to atherogenesis [1,2]. Laminar shear stress (LSS) resulting from fast and uni-directional blood flows at straight regions of blood vessels can provide anti-atherogenic effects by enhancing endothelial nitric oxide production [3,4], and by inhibiting endothelial apoptosis [5–7] and inflammation [8,9].

Endothelial wounding injuries occur after various pathogenic events such as vein bypass graft failure, ischemia, coarctation, and mechanical trauma [10]. Wound healing after injury is a highly coordinated process that involves inflammation, cell migration and proliferation, matrix deposition, and remodeling [11,12]. Incomplete wound repair can cause severe disabilities of endothelium. Implicating the important role of LSS in endothelial wound healing, the regions of arteries where blood flow is slow or disturbed show higher permeabilities to macromolecules and higher susceptibilities to atherosclerosis [13]. LSS has been shown to enhance endothelial wound healing *in vitro* [14–16] and *in vivo* [17,18]. We previously reported that LSS induces the expression of aquaporin 1 (AQP1) [4], a molecular water channel protein in plasma membranes [19], and that AQP1 plays a critical role in endothelial cell migration and wound healing stimulated by LSS [20].

As major components of the cytoskeleton, actin and actin binding proteins can play a key role in the morphogenesis and migration of endothelial cells in wounded blood vessels [21]. Thus, it was hypothesized that certain actin-binding proteins might be expressionally upregulated by LSS and could promote endothelial wound healing under LSS-exposed conditions. To test this hypothesis, we analyzed endothelial cell gene expression affected by LSS using the cDNA microarray method, and identified a list of LSS-induced actin binding proteins. This list includes synaptopodin (SYNPO) whose mRNA expression showed the biggest fold increase by LSS.

SYNPO is a dual F-actin/α-actinin binding protein [22], and it elongates and bundles F-actin through interactions with α-actinin [23]. SYNPO plays a key role in the structure formation and function of dynamic cell compartments such as foot process of podocytes in the kidney and spinal apparatus of neurons in the brain [22,23]. In podocytes, SYNPO regulates stress fiber formation and cell motility due to inhibition of RhoA and Nck proteasomal degradation [24,25]. SYNPO is also reported as a component of the tension-induced autophagy pathway essential for mechanotransduction

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[26]. To our knowledge, the expression and function of SYNPO in endothelial cells have not been reported previously. Thus, in the present study, we examined the endothelial expression and function of SYNPO with respect to wound healing stimulated by LSS.

2. Materials and methods

2.1. Cell culture and LSS treatment

Human umbilical vein endothelial cells (HUVECs) obtained from Clonetics Cambrex (Rockland, ME, USA) were cultured in EBM-2 medium containing endothelial growth supplements (Clonetics Cambrex), 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B) on 0.2% gelatin-coated 100 mm-tissue culture dishes (BD Biosciences, San Jose, CA, USA) at 37 °C and 5% CO_2. Cells were exposed to steady LSS at 12 dyn cm^{-2} by using a Teflon cone (0.5° cone angle) which was mounted onto a culture dish and rotated at 320 rpm, as previously described [4,9]. Control cells were kept under static conditions for the same period.

2.2. The cDNA microarray analysis

Total cellular RNA was extracted from the static and LSS-exposed HUVECs using the RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's instruction, and analyzed by the cDNA microarray method using GeneChip® HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA). Details of experimental conditions and analytical procedure are described in previous studies [4]. The complete datasets were deposited in the Gene Expression Omnibus database [Accession Number, GSE13712].

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Expression of SYNPO at the mRNA level was analyzed by semi-quantitative RT-PCR. RT-PCR was conducted with the GeneAmp® PCR system 9700 (Applied Biosystems, Foster city, CA, USA). The reaction mixture (20 μL) contained Prime RT-PCR Premix (GENET-BIO, Nonsan, Korea), 50–250 ng RNA, and 10 pmol of gene-specific primer sets (Bioneer, Daejeon, Korea). The sequences of the PCR primers were as follows: SYNPO (GeneBank accession number, NM_007286.6, NM_001109971.2, NM_001166203.1, NM_ 001166209.1) 5′-CAG CCG CAA ATC CAT GTT TAC T-3′ (sense) and 5′-GTG TGG CTT GAA GAC TCG ATG A-3′ (antisense); GAPDH (NM_002046.3) 5′-GCC AAA AGG GTC ATC ATC TC-3′ (sense) and 5′-GTA GAG GCA GGG ATG ATG TTC-3′ (antisense). cDNA was synthesized at 42 °C for 30 min followed by heat inactivation of reverse transcriptase at 95 °C for 10 min, and PCR reaction consisted of 32 cycles of 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 72 °C for 30 s (extension) and a final extension at 72 °C for 5 min. The resulting PCR products were analyzed by electrophoresis in 1.0% agarose gel with a DNA Ladder Marker (ELPIS-Biotech, Daejeon, Korea). The gel was stained with ethidium bromide and photographed using a Gel Doc system (BioRad, Hercules, CA, USA).

2.4. Western blot analysis

Whole cell lysates were prepared using a lysis buffer (20 mM Tris–Cl, 2.5 mM EDTA, 1.0% SDS, pH 7.5) supplemented with 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche, Mannheim, Germany) and subjected to Western blot analysis. The lysate samples were diluted in Laemmli sample buffer, heat-denatured at 95 °C for 5 min, electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a BioTrace PVDF membrane (Pall Corporation, Pensacola, FL, USA). The membrane was incubated with a primary antibody overnight at 4 °C and then with a secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. The membrane was incubated with a picoEPD Western Reagent (ELPIS-Biotech) and photographed images were analyzed using the NIH Image program. Primary antibodies used are goat polyclonal SYNPO antibody and rabbit polyclonal AQP1 antibody from Santa Cruz Biotech (Santa Cruz, CA, USA), and mouse monoclonal β-actin antibody from Sigma–Aldrich (St. Louis, MO, USA). Secondary antibodies are donkey anti-goat IgG and donkey anti-rabbit IgG antibodies from Santa Cruz Biotech, and goat anti-mouse IgG antibody from Cell signaling (Danvers, MA, USA). Antibodies were diluted 1:1000 (primary antibodies) or 1:3000 (secondary antibodies) in Tris-buffered saline (TBS) containing 5% skim milk, except for AQP1 antibody that was diluted in TBS containing 3% bovine serum albumin (BSA).

2.5. Immunocytochemistry

HUVECs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, washed with PBS, and then blocked by incubation in PBS containing 5% donkey serum (Jackson ImmunoResearch Laboratories, Bar Harbor, ME, USA), 0.3% Triton X-100 and 0.1% BSA for 1 h. The cells were incubated with goat polyclonal SYNPO antibody or non-immune IgG, diluted 1:100 in PBS overnight at 4 °C, followed by incubation with fluorescein isothiocyanate-conjugated donkey anti-goat IgG antibody diluted 1:200 in PBS for 1 h at room temperature. After extensive rinsing steps, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min to detect nuclei. The slides were mounted under glass coverslips and examined with a confocal microscope (LSM 5 PASCAL, Carl Zeiss, Oberkochen, Germany).

2.6. Small interfering RNAs (siRNAs) and transfection

The siRNA for human SYNPO (#1299001, HSS117638), human AQP1 (#1299001, HSS179896), and a negative control siRNA with scrambled sequences (#12935200) were purchased from Invitrogen (Grand Island, CA, USA). The nucleotide sequences of siRNAs were as follows: SYNPO, 5′-AUA GGU GAG AGG ACA AAG AGC GAG G-3′ (sense) and 5′-CCU CGC UCU UUG UCC UCU CAC CUA U-3′ (antisense); AQP1, 5′-GCC AUC CUC UCA GGC AUC ACC UCC U-3′ (sense) and 5′-AGG AGG UGA UGC CUG AGA GGA UGG C-3′ (antisense). Transfection of HUVECs with the siRNAs was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction. Briefly, the cells were treated with a mixture of 25–75 nM siRNA(s) and 1.25 μL mL^{-1} Lipofectamine RNAiMAX in Opti-MEM for 4 h, and then fed growth medium.

2.7. SYNPO plasmid construct and transfection

The full coding sequence of human SYNPO (GeneBank accession number, BAA82981) was cloned into a modified pEGFP-C2 vector (Clontech, Heidelberg, Germany) using *NdeI/SacI* as cloning sites, as previously described [22]. For plasmid transfection, TrueFect™ (United BioSystems Inc., Rockville, MD) was used. Briefly, cells were treated with 1 μg mL^{-1} of plasmid DNA and 3 μL mL^{-1} of TrueFect in Opti-MEM for 4 h, and then fed fresh culture medium.

2.8. Scratch wound healing assay

Scratch wound healing assay was performed *in vitro* with LSS treatments before and/or after a monolayer of HUVECs were

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