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PHOSPHOTYROSINE PROTEIN DYNAMICS IN CELL MEMBRANE RAFTS OF SPHINGOSINE-1-PHOSPHATE-STIMULATED HUMAN ENDOTHELIUM: ROLE IN BARRIER ENHANCEMENT

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Abstract

Sphingosine-1-phosphate (S1P), a lipid growth factor, is critical to the maintenance and enhancement of vascular barrier function via processes highly dependent upon cell membrane raft-mediated signaling events. Anti-phosphotyrosine 2 dimensional gel electrophoresis (2-DE) immunoblots confirmed that disruption of membrane rafts formation (via methyl- β -cyclodextrin) inhibits S1P-induced protein tyrosine phosphorylation. To explore S1P-induced dynamic changes in membrane rafts, we used 2-D techniques to define proteins within detergent-resistant cell membrane rafts which are differentially expressed in S1P-challenged (13M, 5 min) human pulmonary artery endothelial cells (EC), with 57 protein spots exhibiting >3-fold change. S1P-induced the recruitment of over 20 cell membrane raft proteins exhibiting increasing levels of tyrosine phosphorylation including known barrier-regulatory proteins such as focal adhesion kinase (FAK), cortactin, p85 α phosphatidylinositol 3-kinase (p85 α PI3K), myosin light chain kinase (nmMLCK), filamin A/C, and the non-receptor tyrosine kinase, c-Abl. Reduced expression of either FAK, MLCK, cortactin, filamin A or filamin C by siRNA transfection significantly attenuated S1P-induced EC barrier enhancement. Furthermore, S1P induced cell membrane raft components, p-caveolin-1 and glycosphingolipid (GM1), to the plasma membrane and enhanced co-localization of membrane rafts with p-caveolin-1 and p-nmMLCK. These results suggest that S1P induces both the tyrosine phosphorylation and recruitment of key actin cytoskeletal proteins to membrane rafts, resulting in enhanced human EC barrier function.

Keywords

endothelial cells; cell membrane rafts; 2-DE; Mass spectrometry; sphingosine 1-phosphate (S1P)

1. Introduction

Cell membrane rafts are dynamic assemblies of proteins and lipids within the plasma membrane which are enriched in sphingolipids and cholesterol and have been implicated in dynamic membrane signaling and trafficking [1–3]. In response to diverse extracellular stimuli, a variety of proteins are selectively enriched in cell membrane rafts, including G-protein-coupled receptors (GPCR), proteins involved in cell signaling, non-receptor tyrosine kinases (i.e. Src family kinases) and their downstream targets. Thus, within these dynamically assembled cell membrane raft domains, extracellular stimuli are converted into intracellular signaling events [4–6]. The ganglioside GM1 is a common component of membrane raft microdomains. In addition, a subset of membrane raft microdomains contains

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a specialized scaffolding protein called caveolin-1 [7–9]. The colocalization of gangliosides, caveolin-1, and signaling kinases in membrane rafts has been described in many cell types as mediating signal transduction [9–11]. Recent evidence suggests that phospho-caveolin-1 (pCav-1) plays an important role in cell adhesion and migration [10–12]. Sphingosine 1-phosphate (S1P), a bioactive phospholipid growth factor, induces various cellular responses including cell proliferation, cytoskeleton remodeling, and cytokine secretion via G-protein coupled receptors, S1P_{1–5} [13–15]. Our previous studies demonstrated that S1P plays a critical role in vascular barrier regulation via S1P receptor 1 (S1P₁) ligation and recruitment of signal components to the cell membrane rafts of human pulmonary artery endothelial cells [16, 17]. Disruption of the liquid-ordered phase, by removal of cholesterol with methyl- β -cyclodextrin (MBCD), inhibits S1P-induced barrier enhancement [16]

Our initial study utilized quantitative iTRAQ/mass spectrometry proteomic strategies and investigated cell membrane rafts within human lung ECs and identified >200 proteins in cell membrane rafts. Only two proteins, however, were specifically and differentially recruited into cell membrane rafts following S1P treatment [18]. These results highlight the challenges in examination of cell membrane raft dynamics which include the low abundance of cell membrane raft proteins in relation to other cellular membranes, the extensive spectrum of protein expression level and the difficulty in protein resolution. Since cell membrane rafts are important in regulation of a wide range of biological processes, many techniques have been used to improve cell membrane raft sample preparation and facilitate their efficient identification, characterization and quantitation [18–21]. Two-dimensional gel electrophoresis (2-DE) has been used for quantitative proteomics analysis and represents a powerful proteomics approach when combined with mass spectrometry (MS) [19, 20, 22], despite difficulties with solubilization of protein mixtures for isoelectric focusing (IEF), a critical issue for high performance 2-DE.

The present study demonstrates for the first time that S1P-induced protein tyrosine phosphorylation is dependent on membrane rafts formation as detected by 2-D phosphotyrosine western blots. To explore S1P-induced dynamic changes in membrane rafts, we performed protein quantitative analysis on 2D gel images revealing 57 protein spots with a >3-fold significant change after S1P compared to controls. As endothelial cell integrity and barrier function are linked to the stability of cell adhesion junctional complexes which are highly modulated by protein tyrosine phosphorylation of junctional components [23–25], we assessed phosphotyrosine levels in cell membrane raft fractions (reflecting activity of protein tyrosine kinases and phosphatases). Using 2-DE immunoblotting with anti-phosphotyrosine antibody, a powerful technique for visualizing post-translational modifications of complexly modulated proteins [19, 26, 27], we determined that S1P markedly increases the tyrosine phosphorylation of over 20 cell membrane raft proteins including a number of known barrier-regulatory proteins. Silencing (siRNA) the expression of lipid raft-recruited proteins, such as FAK, MLCK, cortactin, filamin A or filamin C, attenuated S1P-induced EC barrier enhancement. In addition, the colocalization of GM1, a cell membrane raft marker and tyrosine phosphoprotein were examined by immunofluorescence assay. Consistent with previous observations [9, 11, 28, 29], S1P induced p-caveolin-1 and glycosphingolipid (GM1) components of cell membrane rafts to the plasma membrane and enhanced co-localization of membrane rafts with p-caveolin-1 (Try14) and phospho-nmMLCK. The results indicate that S1P induces targeted recruitment and tyrosine phosphorylation of actin cytoskeletal proteins to cell membrane rafts which are essential for S1P-mediated signaling pathways and enhanced human lung endothelial cell barrier function.

2. Materials and Methods

2.1. Chemicals, reagents and cell culture

Protease inhibitor cocktails were purchased from Roche. Phosphatase inhibitors (Na_3VO_4 , NaF and okadaic acid), S1P, sucrose, fetal bovine serum (FBS), α -cyano-4-hydroxycinnamic acid (CHCA), SDS, colloidal blue gel stain kit, MBCD and antibodies including c-Src and phospho-Src (Tyr⁴¹⁶) were purchased from Sigma Aldrich (Saint Louis, MI). Urea, thiourea, CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfate), dithiothreitol (DTT), HPLC-grade acetonitrile, Triton X-100, trifluoroacetic acid (TFA), formic acid (FA), acetic acid and iodoacetamide were obtained from Fisher Scientific (Illinois, USA). Water was obtained from a Milli-Q Plus purification system (Millipore, Bedford, MA). All 2-DE gel IPG buffer and dry strips were purchased from GE Healthcare (Piscataway, NJ). Novex pre-cast zoom gels, Sypro Ruby protein gel stains, anti-mouse-Alexa Fluor 594 and anti-rabbit-Alexa Fluor 488 were purchased from Invitrogen (Carlsbad, CA). Criterion precast 2D gels, SDS PAGE running buffer and transfer buffer were obtained from Bio-Rad Laboratories (Hercules, CA). Mass Spectrometry grade trypsin was obtained from Promega (Madison, WI). The following antibodies were utilized for this study: anti-FAK, anti-caveolin-1, anti-flotillin-1, anti-filamin-A, anti-MLCK, anti-phospho-MLCK (Tyr⁴⁷¹), anti-c-Abl, anti-phospho-phosphatidylinositol 3-kinase (p85 α PI3K, Tyr⁵⁰⁸) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-cortactin (Tyr⁴²¹) (Chemicon, Temecula, CA), anti-phospho-FAK (Tyr^{576/577}) and anti-pCav-1 (Tyr¹⁴) (Cell Signaling, Beverly, MA), anti-cortactin and anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), anti-filamin-C and anti-S1P₁ receptor (Affinity Bioreagents, Golden, CO), monoclonal asialo GM1 antibody (US biological, Swampscott, MA). Secondary horseradish peroxidase (HRP)-labeled antibodies were purchased from Pierce (Rockford, IL).

EBM-2 culture medium was obtained from Lonza (East Rutherford, NJ, USA). Human pulmonary artery ECs, passages between 5 and 8 were grown to contact-inhibited monolayers with typical cobblestone morphology in EGM-2 complete media with 10% FBS, 100 units/ml penicillin and streptomycin in a 37 °C incubator under 5% CO₂, 95% air atmosphere [17].

2.2. Isolation of detergent resistant membrane raft proteins

Raft proteins were obtained by buoyant-density fractionation over a discontinuous optiprep-density gradient as we have described previously [30]. HPAECs (5.6×10^8) were scraped in PBS, centrifuged at 2000 rpm at 4°C and lysed with 0.2 mL of TN solution [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, protease inhibitors, 10% sucrose, 1% Triton X-100] for 30 min on ice. Triton X-100-insoluble materials were then mixed with 0.6 mL of cold 60% Optiprep™ and overlaid with 0.6 mL of 40%, 30%, and 20% Optiprep™ in TN solution. The top 0–20% discontinuous gradients were centrifuged at 35,000 rpm in SW60 rotor for 12 h at 4°C and pellets were collected.

2.3. Sample preparation and two dimensional electrophoresis (2-DE)

Cell membrane rafts pellets from ECs were first pre-solubilized in 50 μ l optimized buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT) at 37°C water bath for 5 min, and then centrifuged at $1000 \times g$ for 5 min at room temperature to separate soluble and insoluble fractions. Protein concentrations were determined by the Bio-Rad Bradford Assay. Small aliquots of soluble and insoluble fractions were diluted by adding SDS sample buffer and run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-caveolin-1, anti-S1P₁ receptor (Figure 1). After addition of 40 mM Tris-HCl and 0.5 % IPG buffer 3–10 carrier ampholytes/0.0002% bromophenol blue, 2-DE was carried as follows. Samples were equally loading on an IPG strip (Immobiline 7-cm or 11-cm Dry strip 3–10, Amersham

Biosciences). The strips were passively rehydrated overnight for 12 h followed by isoelectric focusing steps of 500 Vhr, 1,000 Vhr, and 8,000 or 14,000 Vhr using the IPGphor IEF system (Amersham Biosciences). After focusing, IPG strips were equilibrated for 25 min with gentle shaking in 5 mL equilibration solution containing 50 mM Tris-Cl buffer, 6 M urea, 1% DTT, 30% glycerol, 2% SDS, and a trace of bromophenol blue. The second dimension separation was run using XCell Surelock mini-cell system (Invitrogen) or the Criterion Cell system (Bio-Rad Laboratories) in 1.5-mm 4–20% gels. After electrophoresis, gels were fixed and stained using Sypro Ruby or colloidal CBB G-250 (Sigma). For MBCD treatment experiment, the 2-DE was performed in a similar way, with exception that HPAECs (5.6×10^8) was treated with three different conditions: S1P (1 μ M, 5min), MBCD (5 mM, 2h) followed with S1P (1 μ M, 5min) at 37°C and an appropriate carrier control after serum starvation. The cells were scraped in PBS, centrifuged at 2000 rpm at 4°C and lysed in 2-DE rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT and 0.5 % IPG buffer 3–10 carrier ampholytes). 2-DE immunoblots of proteins phosphotyrosine were carried after protein concentrations determined by the Bio-Rad Bradford Assay.

2.4. 2-DE image scanning and analysis

The gels were stained post electrophoresis with Sypro Ruby protein stain (Molecular Probes) as previously described [31]. The Molecular Imager PharosFX Plus system (Bio-Rad Laboratories) with excitation at 532 nm and emission filter of 610 nm BP30 was used to scan the gels. The data were then imported into the PDQuest v 7.4.0 software (Bio-Rad Laboratories) and background subtraction, filtering algorithms, automatic spot detection, spot matching processes and normalization were performed as described by Marengo *et al.* [32]. Final manual editing was performed on all spots that were automatically matched by the respective software to eliminate any spots that had been incorrectly matched by the software from the evaluation. The correlation coefficient of cell membrane raft proteins was calculated between control and S1P treatment samples using PDQuest (Bio-Rad Laboratories). In this study, three sets of Sypro Ruby stained gels from independent experiments samples were used to perform quantitative analysis.

2.5. Protein in-gel digestion and protein identification

2-DE gels stained with colloidal CBB were scanned using Molecular Imager PharosFX Plus system (Bio-Rad Laboratories). Protein patterns of scanned 2-D images were detected and matched automatically by PDQuest (Bio-Rad Laboratories). Spots of interest were excised from the gel and destained with 50% v/v methanol and 10% v/v acetic acid. Samples were washed twice with 25 mM ammonium bicarbonate (ABC) and 100% acetonitrile (ACN), and reduced and alkylation using 10 mM DTT and 100 mM iodoacetamide and digested in situ using 20 ng/ μ l modified trypsin. The digested peptides were extracted with 5% FA and 50% ACN. The resulting digests were analyzed by mass spectrometry (MALDI TOF/TOF using an Applied Biosystems ABI 4700 and nanospray LC-MS/MS using Thermo Finnigan FT-LTQ). The acquired MS and MS/MS data were searched against the National Center of Biotechnology Information non-redundant (NCBI nr) database with human species restriction for protein identification by using GPS Explorer™ Software version 3.5 with the integrated Mascot™ version 2.1 searching engine. Database search parameters include 2 missed cleavages, variable modifications: oxidation of methionines and carbamidomethylation of cysteines, 50 ppm tolerance for precursor ions and 1.0 Da tolerance for fragment ions.

On-line nanospray separation of the tryptic peptides was accomplished with a 15 cm X 0.75 mm, 3.5 μ m reverse phase column C-18 (Agilent Technologies, Wilmington, DE) connected to a Dionex Ultimate 3000 two-dimensional microcapillary HPLC system and LTQ-FT (Thermo Finnigan) tandem mass spectrometer equipped with nanospray interface. A

gradient LC method used mobile phases A (95:5:0.1 water: ACN: FA) and B (95:5:0.1 CAN : water : FA) at a flow rate of 250 nL/min for 55min. Survey full-scan MS spectra with 2 microscans (m/z 400–1,800) were acquired in the FT-ICR cell with mass resolution of 100,000 at m/z 400 (after accumulation to a target value of 2×10^6 ions in the linear ion trap), the five most intense ions in each survey scan were sequentially fragmented in the ion trap by collision-induced dissociation (CID) using an isolation width of 2.5 and relative collision energy of 35%, dynamic exclusion was utilized with no repeat counts, and with an exclusion duration of 60 sec. MS/MS spectra from FT-LTQ were analyzed by the SEQUEST (University of Washington, licensed to Thermo Finnigan) searching program in the BioWorks 3.3 software suite or and Mascot version 2.1 on an in-house server (Matrix Science). The NCBI database of human species was used to run protein searches. General protein identification was based on at least two unique peptides, the ΔCh threshold was 0.1 regardless of charge state and the X_{corr} scores with the thresholds at >2.50 for 3+ charge; >2.00 for 2+ charge; >1.50 for 1+ charge; and peptide probability P -value < 0.001 . The representative parameters for the Sequest search included missed cleavage sites 2; peptide precursor ions tolerance ± 5 ppm; fragment ion tolerance ± 0.8 Da [33, 34]. Criteria for protein identifications by Mascot algorithm included using score threshold achieved $p < 0.05$ and required having a minimum of 2 or more peptide matches. The individual MS/MS spectrum with expectation value < 0.05 and best ion score (based on MS/MS spectra) were accepted [35].

2.6. Construction and transfection of siRNA against MLCK, filamin-A and filamin-C

The siRNA sequence targeting human MLCK, cortactin, FAK, filamin-A and filamin-C were generated using mRNA sequences from Gen-Bank™ (gi: 7239695, gi: 20357555, gi: 182394, gi: 116063572, gi: 116805321). Sequences containing less than 50% G/C content were chosen if available. Specific target sequences were then aligned to the human genome database in a BLAST search to eliminate sequences with significant homology to other human genes. Scramble sequences were also aligned to the human genome database in a BLAST search to determine no significant homology to any known human gene. For each mRNA (or scramble), two targets were identified. Specifically, MLCK target sequence 1 (5'-AAGATGCTGGCTCCCATTACC-3'), MLCK target sequence 2 (5'-AAAGCCCGGACCAGGACAGT-3'), cortactin target sequence 1 (5'-AATGCCTGGAAATTCCTCATT-3'), cortactin target sequence 2 (5'-AAACAGAAATTCGTGAACAGC-3'), FAK target sequence 1 (5'-AAAGAAGTCTAACTATGAAGT-3'), FAK target sequence 2 (5'-AAGTCTAACTATGAAGTATTA-3'), filamin-A target sequence 1 (5'-AAAATGAGTAGCTCCCACTCT-3'), filamin-A target sequence 2 (5'-AAATGCAGCTTGAGAACGTGT-3'), filamin-C target sequence 1 (5'-AACTCCGCCAAATGAAGCTG-3'), filamin-C target sequence 2 (5'-AAATGAAGCTGGAGAACGTGT-3'), scrambled sequence 1 (5'-AAGAGAAATCGAAACCGAAAA-3') and scramble sequence 2 (5'-AAGAACCAATTAAGCGCAAG-3') were used. Sense and antisense oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). For construction of the siRNA, a transcription-based kit from Ambion was used (Silencer™ siRNA construction kit). EC were then transfected with siRNA using siPORTamine™ as transfection reagent (Ambion, TX) according to the protocol provided by Ambion. Cells (~40% confluent) were serum starved for 1 hour followed by incubated with 3 μ M (1.5 μ M of each siRNA) of target siRNA (or scramble siRNA or no siRNA) for 6 h in serum-free media. Media with serum was then added (1% serum final concentration) for 42 h before biochemical experiments and/or functional assays were conducted.

2.7. SDS-PAGE and Western immunoblot analysis

One and two dimensional electrophoreses were performed on triton-insoluble pelleted proteins. For one dimensional SDS-PAGE (1-DE) gel, cell membrane raft proteins were dissolved in a nonreducing loading buffer containing 1% SDS, 25 mM Tris, pH 6.8, and separated by SDS-PAGE according to Laemmli [36] on 4–20% (w/v) acrylamide gel. Proteins separated by 1-DE or 2-DE were transferred to PVDF membrane for immunoblot analysis. After blocking for 1 h at room temperature membranes were incubated overnight at 4 °C in primary antibody, washed three times for 5 min in TBS-Tween 20 (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20), incubated for 1 h at room temperature in secondary antibody (dilution 1:2500 horseradish peroxidase conjugated donkey anti-rabbit or anti-mouse in TBS-Tween 20, 5% BSA powder) (Pierce), and finally washed three times for 5 min with TBS-Tween 20. Blots were developed with ECL Pico western blotting detection kit (Pierce). In some cases, relative intensity for immunoreactive bands in cell membrane rafts was generated using QuantityOne™ software (Bio-Rad). Primary antibodies used were caveolin-1, pCav-1 (Tyr¹⁴) and flotilin-1, phosphotyrosine, c-Src and phospho-Src (Tyr⁴¹⁶), cortactin and phospho-cortactin (Tyr⁴²¹), Phospho-FAK (Tyr^{576/577}), c-Abl, filamin-A, filamin-C, MLCK and phospho-p85αPI3K (Tyr⁵⁰⁸).

2.8. Measurement of human lung trans-endothelial cell electrical resistance (TER)

ECs were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes and TER measurements were performed using an electrical cell substrate impedance sensing system obtained from Applied Biophysics (Troy, NY, USA) as we have previously described in detail [17]. TER values from each microelectrode were pooled at discrete time points and plotted vs. time as the mean ±SE.

2.9. Immunofluorescence assay and confocal microscopic imaging

Confluent HPAECs grown on 25mm circular cover glass were either treated with S1P (1μM) for 5 min or left untreated (Control), washed with cold PBS (pH 7.4), fixed with 4% paraformaldehyde and blocked with 1% BSA and 1% normal donkey serum in PBS for 30 minutes. Cells were then washed in PBS, and probed with mouse monoclonal anti-GM1 ganglioside antibody (1:50) in 1% BSA/1% normal donkey serum/PBS, followed by anti-mouse Alexa-conjugated secondary antibodies in 1% BSA/1% normal donkey serum/PBS at room temperature for 30 minutes followed by washing in PBS. Cells were then probed with specific rabbit phosphoprotein antibodies (1:50 dilution), p-cortactin (Tyr⁴²¹), p-FAK (Tyr^{576/577}), p-MLCK (Tyr⁴⁷¹) and pCav-1 (Tyr¹⁴), respectively followed by secondary Alexa-conjugated antibodies and visualized using a Nikon Eclipse TE 300 microscope and Sony Digital Photo camera DKC 5000. Images were recorded and saved in Adobe Photoshop.

Z-series images of cells were acquired with a Leica TCS SP5 confocal microscope with 488 nm Ar and 561nm He/Ne laser lines scanning at 8000 Hz and a line average setting of 16. All images were background subtracted then smoothed with a Kalman stack filter using ImageJ software (<http://rsb.info.nih.gov/ij>, Wayne Rasband, NIH). Colocalization images were created with the intensity correlation analysis method developed by Li et al [37] and written into a plug in application incorporated into ImageJ by Tony Collins (http://www.macbiophotonics.ca/imagej/colour_analysis.htm#coloc_ica, McMaster University). In the colocalization images, each pixel with a signal represents the positive covariance of green and red signals rising above the average fluorescence intensity of the original green and red images.

2.10. Bioinformatics and statistical analysis

The theoretical pI and molecular mass (M_r) values of proteins were defined by using Swiss-Prot & TrEMBL protein database (<http://us.expasy.org/sprot/>) with protein function and subcellular location annotation from this protein database. A paired student's T-test method was used, which assigned statistical significance to the differences between the control and S1P treatment cell membrane raft sample.

3. Results

3.1. S1P-induced increases in levels of phosphotyrosine proteins is dependent on membrane raft formation

Our previous studies have demonstrated that maximal barrier enhancement is observed with 1 μ M S1P peaking at 10–20 min and sustained for several hours [17]. Exposure of endothelial cells to S1P produces rapid and significant translocation of cortactin from the cytoplasm to a peripheral cortical distribution within 5 min [38]. Therefore, 1 μ M of S1P for 5 min was chosen to interrogate S1P signaling pathways in this study. The cholesterol-disrupting drug, MBCD, inhibits S1P-induced barrier enhancement [16] and tyrosine phosphorylation (Figure 1). These results confirm that S1P is critical to the maintenance and enhancement of pulmonary vascular barrier function via processes highly dependent upon cell membrane raft-mediated signaling events and suggest that dynamic changes in tyrosine kinase/phosphatase activities are essential for S1P-mediated EC barrier regulation.

3.2. Solubilization of endothelial cell membrane raft proteins

The combination of 2-DE and mass spectrometry (MS) is a powerful proteomics approach [19, 20, 22] but limited by challenges associated with solubilization of proteins mixtures for isoelectric focusing (IEF). Figure 2 indicates significant EC membrane raft proteins solubilization with western immunoblots reflecting the presence of caveolin-1 and S1P1 receptor (S1P₁) only in the soluble fraction and S1P (1 μ M, 5 min) significantly inducing S1P₁ membrane rafts recruitment. Serving as cell membrane raft biomarkers, flotillin-1 and caveolin-1 (Figure 2B) showing in both control and S1P-challenged EC (1 μ M, 5min) indicates that we have successfully isolated membrane rafts. Quantitation of western blots demonstrates that the absence of significant recruitment of flotillin-1 and caveolin-1 levels between control and S1P-challenged EC (Figure 2C). However, there are obvious caveolin-1 distribution changes between control and S1P by using 2-DE western blots. The reason for this change in distribution is that S1P promotes caveolin-1 phosphorylation (Figure 2C and Figure 8).

3.3. Differential 2-DE analysis

In order to assess S1P-mediated alterations in cell membrane raft protein profiles, three sets of 50 μ g membrane raft protein mixtures were generated from unchallenged and S1P-stimulated EC (1 μ M, 5 min) and subsequently applied on 2-DE and analytical gels which were stained by Sypro Ruby. Figure 3A is a master synthetic gel image containing the Gaussian spots. Compared with cell membrane rafts isolated from control, vehicle-challenged EC, 57 out of the 177 protein spots identified in cell membrane rafts after S1P treatment exhibited >3-fold significant change (marked with blue circles and $p < 0.05$) (Figure 3A). These included 18 newly recruited proteins (marked with red triangles) and 7 proteins which were now excluded from the cell membrane raft by S1P challenge (marked with green crosses). The correlation coefficient of cell membrane raft proteins between control and S1P treatment samples is 0.744 using PDQuest (Bio-Rad Laboratories) (Figure 3B) indicating that the majority of cell membrane raft proteins remain unchanged. Corresponding to spots number (Figure 3C), the identified proteins are listed on Table 1.

3.4. Visualization and description of post-translation modification changes

Dynamic changes in tyrosine kinase/phosphatase activity following S1P treatment are key to EC barrier regulation [16, 17, 24, 39]. 2-DE is a successful technique for identification and visualization of post-translation modifications including changes in tyrosine phosphorylation [19, 26, 27]. We next characterized changes in tyrosine phosphoprotein content in cell membrane rafts via two dimensional western blotting with anti-phosphotyrosine antisera. These studies demonstrated a S1P-mediated increase in tyrosine phosphorylation of > 20 membrane raft proteins marked by blue circles (Figure 4A) (PDQuest software, Bio-Rad laboratory). 2-DE Western immunoblots evaluation indicated that p60Src kinase (MW: 60 KDa, theoretical pI: 7.10), cortactin (MW: 82 KDa, theoretical pI: 6.19), phosphorylated cortactin (Tyr⁴²¹), phosphorylated FAK (Tyr^{576/577}) (MW: 120 KDa, theoretical pI: 6.19) and the tyrosine kinase, c-Abl (MW: 128 KDa, theoretical pI: 8.31), are recruited into S1P-induced cell membrane raft fractions whereas phosphorylated Src (Tyr⁴¹⁶) showed decreased levels in S1P-challenged membrane rafts compared with control samples (Figures 4 panel B) which had been confirmed by independent 1-DE immunoblots performed in triplicate (Figure 4C).

3.5. Protein identification

To analyze proteins exhibiting differential expression following S1P challenge, protein spots were excised from visualized 2-DE gels, stained with colloidal CBB, and in gel digestion performed with peptides analyzed by mass spectrometry (MALDI TOF/TOF and Nanospray LC-MS/MS). Table 1 demonstrates the list of identified protein spots enriched in cell membrane raft fractions relative to the plasma membrane. Representative spectra and protein spots images, including those for myosin light chain kinase (MLCK) and vitronectin, were identified and are shown in Figures 5 panel A and panel B with recruitment images of MLCK and vitronectin demonstrated in S1P-treated cell membrane raft samples (comparison to actin).

To confirm results of mass spectrometry, 1-DE western immunoblots were performed (Figure 6) which demonstrated that filamin-A, filamin-C, phosphorylated-MLCK, MLCK and phosphorylated p85αPI3K were selectively increased in S1P-induced cell membrane raft fractions compared to the cell membrane raft marker, caveolin-1, which was unchanged. These results suggest that recruitment of filamin-A, filamin-C and MLCK to cell membrane raft fractions may be mechanistically associated with S1P-mediated signaling to the endothelial cytoskeleton required for human endothelial cell barrier regulation.

3.6. Role of MLCK, filamin, cortactin and FAK in S1P-mediated membrane raft signaling and barrier regulation

Our previous studies demonstrated that S1P ligation of the S1P₁ localized in cell membrane rafts plays a critical role in EC barrier regulation [16, 17]. To directly address the functional effects of MLCK, filamin-A, filamin-C, cortactin and FAK recruitment to cell membrane rafts in S1P-mediated cell membrane raft signaling and barrier regulation, we generated siRNA constructs which selectively down-regulate expression of filamin-A, filamin-C, MLCK, FAK, or cortactin proteins by > 90% (Figures 7A & 7B) followed by TER measurements of EC barrier function *in vitro* (transendothelial cell electrical resistance). These studies revealed that reduction in expression of filamin-A, filamin-C, MLCK, FAK or cortactin significantly attenuated the well recognized S1P-induced EC barrier enhancement (Figures 7C & 7D) we have previously described [17, 38]. These results strongly suggest that S1P-induced EC barrier enhancement requires cytoskeletal regulatory protein (i.e. filamin-A, filamin-C, MLCK, FAK, cortactin) recruitment into detergent-resistant cell membrane rafts.

3.7. Analysis of signaling proteins in EC membrane rafts after S1P treatment

To further confirm the data from immunoblots (Figures 4 and 6), immunocytochemistry was performed to determine the co-localization of cell membrane rafts and associated phosphoproteins by using asialo GM1 monoclonal antibody and specific tyrosine phosphorylation antibodies. Gangliosides GM1 has been generally regarded as an authentic raft molecule and asialo GM1 noted to be an altered GM1 form which contributes to formation of tighter cell membrane raft clusters [7, 8, 40–42]. In this study, asialo GM1 is referred to as GM1. S1P treatment induced the accumulation of caveolin-1 (Try14) and GM1, components of cell membrane rafts, within the plasma membrane (Figure 8A) and enhanced co-localization of cell membrane rafts with pCav-1 and p-MLCK (Try 471) (Figure 8B) in plasma membrane. Significant co-localization of cell membrane rafts and p-MLCK was observed by confocal microscopy (Figure 8C) and was especially prominent in the plasma membrane after S1P. Furthermore, S1P also induced co-localization of cell membrane rafts with p-FAK and p-cortactin in plasma membrane (Zhao, J. et al. unpublished data). These results are consistent with our biochemical studies (Figures 4 and 6) and suggest that increased levels of tyrosine phosphoproteins and their accumulation within cell membrane rafts play a critical role in S1P-induced signaling transduction in ECs.

4. Discussion

Cell membrane rafts are a heterogeneous group of lipid microdomains consisting of cholesterol and sphingolipids such as ceramide, sphingomyelin and various gangliosides (GM1, GM2 and GM3) in plasma membranes [43–45] and concentrate signaling molecules to promote regulated activation by relevant stimuli [46–49]. Consistent with a critical role for cell membrane raft proteins in cellular signal transduction, more than 50% of current drug targets belong to this class [19, 50, 51]. Cell membrane raft proteins within the endothelium are particularly attractive as targets as the vascular endothelium serves as a semi-permeable cellular barrier between circulating vascular contents and the interstitial space of all organs. Endothelial cell (EC) barrier dysfunction results in the increased vascular permeability observed in inflammation, tumor angiogenesis, and atherosclerosis. Maintenance of this vascular barrier integrity represents a critical physiological process for preservation of organ function. We have previously defined the therapeutic promise and utility of the phospholipid angiogenic growth factor, S1P, as an agent for reducing EC permeability *in vitro* and *in vivo* [15–17]. The molecular mechanisms contributing to S1P-mediated barrier regulation are mediated via profound cytoskeletal reorganization initiated by cell surface receptor-mediated G protein activation and downstream signaling involving the Rho family of small GTPases [52–54] and cell membrane raft microdomains [55]. Disruption of cell membrane raft formation, removes a critical platform for concentrating signaling molecules within the cell membrane and affects S1P-induced phosphotyrosine modification (Figure 1) resulting in inhibition of S1P-induced barrier enhancement and cell-cell adhesion [55]. Detailed information about these critical protein interaction networks obtained via proteomic approaches is crucial for understanding biological processes and may provide opportunities for disease intervention by interrupting or inducing key signaling pathways. The current study shows that S1P activates signaling molecules including tyrosine kinases and actin-binding proteins which are recruited to EC plasma membrane, where tyrosine kinase/phosphatase activities exhibit dynamic changes essential for S1P-mediated EC barrier regulation. Lipid rafts serve as platforms to concentrate signaling components and other molecules which participate in S1P-induced barrier enhancement and cell-cell adhesion.

2-DE is a traditional approach used for quantitative proteomics analysis and can provide simple recognition and comparisons of protein abundance and visualization of post-translational modifications [26, 27, 56]. In response to S1P (1 μ M, 5min), we identified 57

protein spots present in EC cell membrane rafts that demonstrate significant changes including 18 newly recruited proteins as well as loss 7 proteins from the cell membrane raft fraction (Figure 3). Newly recruited cell membrane rafts proteins in response to S1P include the nonmuscle myosin light chain kinase (nmMLCK) and the actin-binding protein filamin (Table 1 and Figure 6). Furthermore, reductions in expression of either protein significantly attenuate S1P-mediated TER elevation (Figures 7C). These findings support our prior work the role of filamin in EC barrier regulation [57] and that nmMLCK and cortactin are essential for S1P-induced EC barrier enhancement via regulation of cytoskeletal rearrangement within the cell periphery and at interactions with cell junction components [38]. The observation that a number of these cytoskeletal regulatory proteins are recruited to membrane microdomains following S1P challenge strongly suggests an important and functional role within membrane microdomain structures.

The current study presents several novel observations including S1P-dependent effects on the total protein content and tyrosine phosphorylation status of cortactin (Tyr⁴²¹), c-Src (Tyr⁴¹⁶) and FAK (Tyr^{576/577}) as well as the actin-binding tyrosine kinase, c-Abl within cell membrane rafts (Figure 4). Tyrosine kinases occupy key positions in the mechanism controlling cell responses mediated through various cell surface receptors, which use tyrosine phosphorylation to transduce extracellular signals. The stability of many adhesion sites is regulated by protein tyrosine phosphorylation of cell adhesion molecules and their associated components, with high levels of phosphorylation promoting disassembly [23–25]. We observed that increased recruitment of cortactin to these lipid microdomains accompanied by increased tyrosine phosphorylation of the protein in S1P-induced EC cell membrane rafts (Figure 4) are of interest given that both the tight physical linkage of cortactin with MLCK as well as EC barrier regulatory effects of these two proteins are dependent upon the tyrosine phosphorylation status of each protein [38]. These results further demonstrate that S1P induces increased cortactin tyrosine phosphorylation, an event now determined as taking place within cell membrane raft domains. In addition, c-Abl is an actin-binding tyrosine kinase which participates as a regulatory molecule in cytoskeleton remodeling during cell differentiation, cell division and cell adhesion [58–60]. Ongoing studies in our laboratory are currently examining the role of c-Abl in S1P-induced EC barrier function.

We have previously reviewed the importance of focal adhesions and the focal adhesion kinase FAK in EC barrier regulation [61]. Consistent with our prior work, in the current study, we verified involvement of FAK activities in S1P-induced EC barrier function with reductions in FAK expression inhibiting S1P-induced TER responses. The identification of FAK in cell membrane rafts in association with S1P-induced FAK phosphorylation [Y⁵⁷⁶] (Figure 4 & 7D) represent novel findings. In order to determine the tyrosine kinases responsible for FAK phosphorylation in S1P-induced EC barrier function, we examined pp60^{c-src} activities and noted decreased phosphotyrosine pp60^{c-src} levels in S1P-induced cell membrane raft fractions (Figure 4). Previous studies have indicated that Src family kinases, and pp60(c-src) in particular, have a central role in regulating protein dynamics at cell-matrix interfaces, both during early stages of interaction and in mature focal contacts [62, 63]. Although there is a role for pp60^{c-src} in the establishment of matrix adhesions, the molecular mechanism underlying these events remains unclear. Particularly intriguing is the apparent inconsistency between the role of pp60^{c-src} in focal contact assembly and the destructive effect of the deregulated pp60^{v-src} on cell-matrix adhesion [62]. It is interesting to note the significantly increased levels of total pp60^{c-src} but in the context of decreased phosphorylation of pp60^{c-src} in S1P-induced cell membrane rafts, results consistent with prior reports that activation of Src promotes cytoskeletal-mediated EC barrier disruption [64, 65].

Caveolae, one subset of cell membrane rafts, are formed by polymerization of caveolins and association with cholesterol-rich cell membrane rafts domains [46, 49]. Caveolae have incredible abundance in endothelial cells and have been implicated in EC migration, proliferation, adhesion, endocytosis, cholesterol and calcium regulation, and signal transduction [30, 66, 67]. In agreement with previous observations [7, 8, 28, 29], the current study demonstrated the association of pCav-1 and ganglioside GM1 in the cell membrane rafts of HPAECs, which exhibit dramatically elevated the colocalization at the plasma membrane with S1P treatment (Figure 8A) and indicating that pCav-1 involves S1P-induced cell adhesion and migration bioprocess [10–12]. In response to S1P stimulation, tyrosine phosphorylation proteins accumulation to cell membrane raft on plasma membrane (Figure 4) indicate that S1P-induced cells have more signaling molecules (Figures 3A & 6), more tyrosine kinase activity, or less inhibitory phosphatase activity associated with cell membrane rafts (Figure 8A–C). All of these scenarios are likely to increase signal transduction capacity.

Taken together, these results indicate that S1P signals are transduced within lipid microdomains through activation of specific protein tyrosine kinases which serve as the driver for subsequent activation of the contractile apparatus or the formation of increased cortical actin and enhanced cytoskeleton-membrane interaction. The tyrosine phosphorylation of key cytoskeletal effectors such as cortactin, MLCK and filamin contributes potentially to this regulation and transiently stimulates EC adherence and focal junction assembly, key to the regulation of endothelial barrier function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

S1P	sphingosine 1-phosphate
HPAEC	human pulmonary artery endothelial cells
ABC	ammonium bicarbonate
MALDI TOF/TOF	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry

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IB: phosphotyrosine

Figure 1. S1P-induced increases in phosphotyrosine protein levels are dependent on membrane rafts formation by 2D phosphotyrosine immunoblots

After serum starvation, HPAECs (5.6×10^8) was treated with three different conditions: S1P (1 μ M, 5min), β -MCD (5 mM, 2h) followed with S1P (1 μ M, 5min) at 37°C and an appropriate carrier control. 2-DE immunoblots of proteins phosphotyrosine were carried after protein concentrations determined by the Bio-Rad Bradford Assay. Experiments were performed in triplicate with highly reproducible findings (representative data shown).

Figure 2. Solubilization analysis of cell membrane rafts in optimized buffer by 1D or 2D immunoblots

Cell membrane raft fractions of HPAECs untreated or treated with 1 μ M S1P, 5 minutes.

Panel A: Cell membrane raft pellets from HPAECs were solubilized in optimized buffer as described in Material and Methods. Small aliquots of soluble (S) and insoluble fractions (IS) were diluted by adding SDS sample buffer and run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-caveolin-1, anti-S1P₁ receptor (S1P₁). S1P₁ quantitative analyses were conducted from three independent 1-DE experiments after normalized by Caveolin-1 and representative data are shown. The asterisks (*) indicates a statistically significant difference ($p < 0.01$) from control values. **Panel B:** 2-DE immunoblots were performed by using anti-caveolin-1 and anti-flotillin-1 antibodies. **Panel C:** 1-DE immunoblots of caveolin-1 and phospho-caveolin-1 (Tyr¹⁴). The asterisks (*) indicates a statistically significant difference ($p < 0.01$) from control values. Experiments were performed in triplicate with highly reproducible findings (representative data shown).

Figure 3. Quantitative analysis of S1P-induced cell membrane raft proteins using 2-DE Cell membrane raft pellets of HPAECs untreated (control) or treated with $1\mu\text{M}$ S1P, 5 minutes (Sample) from three independent experiments were analyzed on 2-DE (see Materials and Methods). Gels were fixed and stained post electrophoresis with Sypro Ruby. Images were taken and analyzed by the PDQuest v 7.4.0 software (see Materials and Methods). **Panel A:** Synthetic master gel image containing the Gaussian spots. After normalization of total protein intensity, master gel image was created. Compared with cell membrane rafts isolated from control, 57 out of 177 protein spots have more than 3-fold changes in cell membrane rafts after S1P treatment ($1\mu\text{M}$, 5 min). Among the 57 protein spots, there are 50 proteins (marked with blue circles) showing increased recruitment to the cell membrane raft including 18 newly recruited proteins (marked with red triangles) and 7 proteins (marked with green cross) excluded from S1P-induced cell membrane raft. **Panel B:** The correlation coefficient (Corr Coeff) of cell membrane raft proteins was calculated between control and S1P treatment samples using PDQuest (Bio-Rad Laboratories). **Panel C:** The real colloidal blue stained gel for protein identification. 100ug cell membrane rafts proteins from S1P-treated HPAECs were separated by their pI on 3–10 linear IPG strip and molecular weight on 4–20% SDS-PAGE gradient gel. Corresponding to spots number, the identified proteins are listed on Table 1.

Figure 4. Characterization of phosphotyrosine changes in S1P-treated cell membrane rafts
Panel A: Identification of tyrosine phosphorylation changes induced by S1P (1 μ M, 5min) using 2DE immunoblots with anti-phosphotyrosine antibody. a: Untreated HPAECs cell membrane raft (control). b: S1P (1 μ M, 5min) treated HPAECs cell membrane rafts. c: Immunoreactive area intensities were calculated using PDQuest software (Bio-Rad laboratories). Differentially expressed tyrosine phosphorylated proteins were marked with blue circles. **Panel B:** phosphotyrosine modification changes of known barrier regulating proteins were analyzed using 2-DE immunoblots with antibodies of src kinase, phospho-src (Tyr⁴¹⁶), cortactin, phospho-cortactin (Tyr⁴²¹), phospho-FAK (Tyr^{576/577}), c-Abl. **Panel C:** Corresponding to the 2-D immunoblots depicted in Panel B, quantitative analysis were conducted from three independent 1-DE experiments and normalized by Caveolin-1. Relative folds change were calculated and representative immunoblot images were shown above the graph. Values shown are the mean $\pm$ SD. * p < 0.05 and *** p < 0.001 represents a significant difference compared with control.

Figure 5. Representative tandem mass nanospray LC-MS/MS Thermo Finnigan FT-LTQ protein identification in the cell membrane raft protein gel

Untreated HPAECs cell membrane raft proteins (control) and S1P (1 μ M, 5min) treated HPAECs cell membrane raft proteins. **Panel A:** Representative 2D and 3D plots of 2-DE protein spots from control and S1P samples. 2DE gels of cell membrane raft proteins from control and S1P-challenged EC were fixed and stained post electrophoresis with Sypro Ruby. The 2D gel images were obtained by using Molecular Imager PharosFX Plus system (Bio-Rad Laboratories). Three dimensional views of interested protein spots were generated by using PDQuest software (Bio-Rad Laboratories). 2D gel protein spot images of MLCK, actin and vitronectin were indicated by black box with three dimensional images below (pointed by black arrows). The recruitment images of myosin light chain kinase (MLCK) and vitronectin are shown in S1P-treated cell membrane raft sample compared to actin.

Panel B: Representative MS/MS spectra for two peptides from MLCK and vitronectin. For each spectrum, y- and b-type fragment ions present in full scan mass spectra enable peptide identification. B-a: the peptide of IIDEDEFELTER (690.34 m/z, +2) from MLCK (1550–1560 amino acid) and B-b: the peptide from vitronectin (198–212 amino acid), DVWGIEGPIDAAFT (823.91 m/z, +2).

20% Optiprep™ Lipid Raft Fractions

Figure 6. Analysis of protein recruitment to cell membrane rafts in S1P-challenged human EC Confluent EC were either untreated (control) or challenged with 1 μ M S1P (5 min) and cell membrane raft fractions were prepared as described in Materials and Methods as the 20% Optiprep™ layer. Immunoblots were performed on the cell membrane raft using anti-filamin-A, anti-filamin-C (A), anti-phosphotyrosine-MLCK, anti-MLCK (B), anti-phosphotyrosine p85 α PI3K and anti-caveolin-1 (C) antibodies. Experiments were performed in triplicate with highly reproducible findings. Relative folds change were calculated and representative immunoblot images were shown above the graph. Values shown are the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ represents a significant difference compared with control.

Figure 7

Figure 7

C

Figure 7

D

Figure 7. Role of cell membrane raft-containing phosphotyrosine and cytoskeletal proteins in S1P-induced endothelial barrier enhancement

Panel A: Immunoblot analysis of siRNA-treated or untreated human EC. Cellular lysates from untransfected (control, no siRNA), scramble siRNA (siRNA that does not target any known human mRNA), filamin A siRNA, filamin C siRNA, MLCK siRNA-transfection were analyzed using immunoblotting with anti-filamin A antibody (a), anti-filamin C antibody (b), anti-MLCK antibody (c) or anti-actin antibody (d) as described in Materials and Methods. **Panel B:** Immunoblot analysis of FAK siRNA or cortactin siRNA treated or untreated human EC. Cellular lysates from untransfected (control, no siRNA), scramble siRNA (siRNA that does not target any known human mRNA), FAK siRNA or cortactin siRNA-transfection were analyzed using immunoblotting with anti-FAK antibody (a), anti-cortactin antibody (b), or anti-actin antibody (c). **Panel C:** EC were plated on gold microelectrodes and treated with scramble siRNA (control) or MLCK siRNA (a) or filamin-A siRNA (b), filamin-C siRNA (c) or both filamin-A siRNA and filamin-C siRNA (d) or FAK siRNA (e) or cortactin siRNA (f) for 48 hours. EC were then serum starved for one hour followed by addition of 1 μ M S1P. The arrow indicates the time of S1P addition. The TER tracing represents pooled data $\pm$ S.E. from three independent experiments as described in Materials and Methods. For Panels C and D, the asterisks (*) indicate a statistically significant difference ($p < 0.05$) from scramble siRNA values.

Figure 8

Figure 8

Figure 8. Immunofluorescence localization of pCav-1 and p-MLCK proteins in HPAECs
 Confluent HPAECs were starved and incubated in presence or absence of S1P (1 μ M) for 5 min. After washed and fixed, the cells were double immunostained with monoclonal antibody GM1 (red) and specific phosphoprotein antibodies (green). Arrowheads point the areas of colocalization. Panel A: Co-localization of pCav-1 and GM1. S1P increased the caveolin-1 phosphorylation on tyrosine 14 and induced the colocalization of pCav-1 and GM1 accumulation to plasma membrane. Panel B&C: Co-localization of pMLCK and GM1. Without S1P treatment, less immunostaining was observed on p-MLCK. S1P elevated GM1 at plasma membrane and the colocalization of p-MLCK and GM1 were highest at area of plasma membrane. A confocal double color merge images of p-MLCK and GM1 is shown on panel C to better demonstrate the colocalization of p-MLCK and GM1.

Table 1

identified from cell membrane raft protein 2-D gels.

Protein Name	No.	Accession number	Method	Coverage	Unique peptides	Peptide sequence	P value
phosphatidylinositol transfer protein, membrane-associated 2	1	gi 24308237	Sequest	1.41%	2		8.82e-04
dynamitin 1	2	gi 56549117	Sequest	3.83%	2		3.55e-04
kelch-like ECH-associated protein 1	3	gi 22027642	Sequest	3.21%	2		3.21e-07
fibronectin 1 isoform 2 preproprotein	4	gi 47132551	Sequest	1.74%	2		6.74e-12
vitronectin [†]	5	gi 88853069	Sequest	3.14%	1	DVWVGIEGPIDAAFTTR	2.92e-10
serum deprivation response protein	6	gi 4759082	Sequest	26.12%	6		9.77e-13
myosin light chain kinase [†]	7	gi 47132561	Sequest&Mascot	10.45%	4		9.0e-14
filamin	8	gi 105990514	Sequest	1.24%	2		9.26e-05
nestin	9	gi 38176300	Sequest	3.82%	4		6.28e-12
ADAM metalloproteinase domain 30	10	gi 31881770	Sequest	3.16%	2		9.50e-04
cold shock domain protein A	12	gi 20070160	Sequest	11.29%	2		7.40e-10
G protein-coupled receptor family	13	gi 40217831	Sequest	6.38%	5		8.90e-04
nucleosome assembly protein 1-like 4;	14	gi 5174613	Sequest	17.87%	5		8.18e-11
laminin, gamma 1	15	gi 9845498	Sequest	16.11%	16		9.28e-12
Vasopressin-activated calcium-mobilizing receptor-1	16	gi 40254446	Sequest	6.39%	3		1.44e-04
fibronectin 1	17	gi 16933542	Sequest	7.26%	10		5.44e-14
tumor rejection antigen (gp96) 1	18	gi 4507677	Sequest&Mascot	30.88%	17		8.24e-13
heat shock 90kDa protein 1, beta	19	gi 20149594	Sequest&Mascot	3.04%	2		1.01e-08
heat shock protein 90kDa alpha (cytosolic), class A member 1	19	gi 40254816	Sequest&Mascot	17.35%	13		3.44e-08
RAS and EF hand domain containing [‡]	20	gi 40255119	Sequest	1.76%	1	QIYDLSMENQKVK	3.51e-04
spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	21	gi 4507191	Sequest	0.97%	2		3.21e-04
alpha-2-HS-glycoprotein	22	gi 4502005	Sequest&Mascot	5.18%	2		9.00e-07
heterogeneous nuclear ribonucleoprotein AB isoform	23	gi 14110404	Sequest&Mascot	25.30%	7		6.76e-07
splicing factor, arginine/serine-rich 10	24	gi 4759098	Sequest&Mascot	17.36%	4		4.95E-11
splicing factor, arginine/serine-rich 5	25	gi 86991438	Sequest&Mascot	25.00%	6		4.05E-09
ubiquitin-conjugating enzyme Cdc34 [‡]	29	gi 16357477	Sequest	3.39%	1	WNPTQNVR	9.99e-04
clathrin, light polypeptide A isoform b	28	gi 6005993	Sequest	10.48%	2		5.97e-05
nascent-polypeptide-associated complex alpha polypeptide	27	gi 5031931	Sequest	13.49%	2		1.58E-11

Protein Name	No.	Accession number	Method	Coverage	Unique peptides	Peptide sequence	P value
ubiquitin-conjugating enzyme UBC3B	26	gi 22212943	Sequest	21.01%	5		1.88E-10
splicing factor, arginine/serine-rich 7, 35kDa	31	gi 24415994	Sequest&Mascot	25.63%	7		6.20E-10
splicing factor, arginine/serine-rich 1	31	gi 5902076	Sequest&Mascot	29.44%	7		2.88e-08
eukaryotic translation initiation factor 6	32	gi 31563374	Sequest&Mascot	27.35%	5		2.22e-15
ubiquitin B	30	gi 11024714	Sequest&Mascot	44.54%	9		1.64e-06
heterogeneous nuclear ribonucleoprotein A1	33	gi 14043070	Sequest&Mascot	24.38%	6		4.44e-14
actin related protein 2/3 complex subunit 2 [‡]	34	gi 5031599	Sequest&Mascot	3.00%	1	IIIEETLALK	4.40e-04
actin, gamma 1 propeptide	37	gi 4501887	Sequest&Mascot	16.27%	5		3.81e-11
annexin 5; endonexin II; anchorin CII;	35	gi 4502107	Sequest	45.00%	11		6.34e-13
beta actin	36	gi 4501885	Sequest&Mascot	25.87%	7		9.00e-07
acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	38	gi 5453880	Sequest	27.31%	8		3.14e-11
tyrosine 3/tryptophan 5-monoxygenase activation protein, epsilon polypeptide [‡]	40	gi 5803225	Sequest&Mascot	5.10%	1	VAGMDVELTVEER	5.13e-05
ribosomal protein L9	41	gi 15431303	Sequest	47.42%	6		2.16e-06
RNA binding motif protein 8A	42	gi 4826972	Sequest	26.44%	4		2.86e-07
splicing factor, arginine/serine-rich 3	43	gi 4506901	Sequest&Mascot	21.34%	3		1.38e-10
inactive progesterone receptor, 23KDa	47	gi 23308579	Sequest	25.00%	4		2.67e-12
myosin light chain 2	44	gi 94981553	Sequest&Mascot	24.10%	4		8.27e-11
beta-galactosidase binding lectin precursor	46	gi 4504981	Sequest&Mascot	52.59%	6		4.67e-11
enhancer of rudimentary homolog	45	gi 4758302	Sequest&Mascot	47.12%	7		6.92e-12
S100 calcium binding protein A11 [‡]	48	gi 5032057	Sequest&Mascot	8.57%	1	DGYNYTLISK	1.21e-05
barrier to autointegration factor 1 [‡]	49	gi 4502389	Sequest&Mascot	7.78%	1	KDEDLFR	8.46e-04
CD59 antigen p18-20	50	gi 10835165	Sequest&Mascot	15.63%	2		8.77e-07
mitochondrial import receptor Tom22	51	gi 9910382	Sequest&Mascot	28.87%	2		5.98e-10
ribosomal protein P1	52	gi 4506669	Sequest	28.95%	2		3.16e-9
myosin, light polypeptide 6, alkali,	53	gi 88999583	Sequest	73.51%	9		2.22e-16
ribosomal protein P2	54	gi 4506671	Sequest	93.04%	8		1.58e-12
tubulin, alpha 3	55	gi 17986283	Sequest	5.54%	2		4.39e-05
cadherin 13	56	gi 4502719	Sequest	6.45%	3		1.07e-05
fibronectin 1 isoform 1 preproprotein	57	gi 47132557	Sequest	6.18%	12		2.45e-10
drebrin 1	58	gi 18426916	Sequest	31.12%	20		1.44e-14

Protein Name	No.	Accession number	Method	Coverage	Unique peptides	Peptide sequence	P value
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	60	gi 16507237	Sequest	47.71%	26		1.11e-15
heat shock 70kDa protein 8 isoform 1	61	gi 5729877	Sequest	13.62%	7		1.21e-10
heat shock 70kDa protein 1A	62	gi 5123454	Sequest	10.45%	5		4.35e-12
retinoblastoma binding protein 4	63	gi 5032027	Sequest	6.82%	2		8.00e-07
desmin	64	gi 18105050	Sequest	8.32%	5		7.00e-07
peripherin; neurofilament 4 (57kD)	65	gi 21264345	Sequest	6.17%	3		2.00e-08
tubulointerstitial nephritis antigen-like 1	66	gi 11545918	Sequest	14.78%	5		1.0e-9
calreticulin	67	gi 4757900	Sequest	51.80%	19		1.00e-14
nucleosome assembly protein 1-like 1	68	gi 21327708	Sequest	17.14%	5		4.01e-12
polymerase I and transcript release factor	69	gi 42734430	Sequest	38.21%	14		6.00e-15
myosin regulatory light chain MRCL2	70	gi 15809016	Sequest	50.58%	7		6.00e-14
small nuclear ribonucleoprotein polypeptide F	71	gi 4507131	Sequest	24.42%	2		4.00e-11
guanine nucleotide-binding protein,	73	gi 11321585	Sequest	7.94%	3		3.00e-07
SET translocation ; inhibitor-2 of protein phosphatase-2A	74	gi 4506891	Sequest	31.77%	7		2.01e-12
heterogeneous nuclear ribonucleoprotein C isoform a	75	gi 14110428	Sequest	56.11%	20		1.00e-30
cysteine-rich, angiogenic inducer,	76	gi 31542331	Sequest	8.40%	3		7.00e-07
laminin receptor 1	77	gi 9845502	Sequest	45.76%	13		9.00e-14
nucleophosmin 1	78	gi 10835063	Sequest	46.26%	16		1.00e-30
heterogeneous nuclear ribonucleoprote	79	gi 4758544	Sequest	33.45%	12		2.00e-14
ATPase, H+ transporting, lysosoma [‡]	80	gi 19913432	Sequest	3.13%	1	LYPEGLAQLAR.A	1.47e-06
ribosomal protein SA (p40) (34/67 kDa laminin receptor)	81	gi 9845502	Sequest	10.17%	2		1.69e-11
vimentin serine (or cysteine) proteinase	82	gi 4507895	Sequest	68.67%	30		1.00e-30
inhibitor, clade H, member 1 precursor	83	gi 32454741	Sequest	15.31%	5		1.01e-07
lamin B1	84	gi 5031877	Sequest	54.10%	31		1.00e-30
F-actin capping protein alpha-1 subunit	85	gi 5453597	Sequest	45.80%	9		2.00e-13
phosphatidylinositol transfer protein [‡]	86	gi 6912594	Sequest&Mascot	6.64%	1	QLFCWIDKWIDLTMEDIR	4.68e-05
alpha 2 type IV collagen prepropr	87	gi 17986277	Sequest	2.45%	3		2.00e-11
RNA binding motif protein,X-linked	88	gi 56699409	Sequest	8.95%	19		1.00e-07
heterogeneous nuclear ribonucleoprotein A3	89	gi 34740329	Sequest	6.61%	3		6.00e-05
hemopexin [‡]	91	gi 11321561	Sequest	3.25%	1	LYLVQGTQVYVFLTK	1.00e-07
heterogeneous nuclear ribonucleoprotein A2/B1 isoform B1	90	gi 14043072	Sequest	38.24%	14		2.00e-15

Protein Name	No.	Accession number	Method	Coverage	Unique peptides	Peptide sequence	P value
fibrillarin	92	gi 2056465	Sequest	27.10%	6		4.00e-10
annexin A2 isoform 2; lipocortin II	94	gi 4757756	Sequest	30.38%	9		2.00e-09
glyceraldehyde-3-phosphate dehydrogenase	93	gi 7669492	Sequest	12.84%	3		5.00e-10
mitochondrial malate dehydrogenase	95	gi 21735621	Sequest	10.95%	3		3.00e-07
prohibitin 2	96	gi 6005854	Sequest	23.08%	6		2.00e-11
ribosomal protein L7a	97	gi 4506661	Sequest	12.78%	3		5.00e-12
THO complex 4	99	gi 55770864	Sequest	11.28%	2		8.00e-11
voltage-dependent anion channel 1	100	gi 4507879	Sequest	23.32%	6		3.00e-09
voltage-dependent anion channel 3	101	gi 25188179	Sequest	12.10%	3		16.00e-07

Coomassie Blue Colloidal blue stained 2-DE gels of HPAECs cell membrane raft proteins (150 µg) were separated by pI on 3–10 linear 1cm IPG strips followed separation on 4–20% Criterion gel (Bio-Rad Laboratory). Target protein spots were sliced from the gel and digested by modified trypsin. Protein identification was performed on either ABI 4700 Maldi TOF/TOF MS or FT-LTQ and in some cases on both machines dependent on proteins' abundance. Identified protein spots were numbered on gels and their identity is listed.

[†]Representative MS/MS spectrum data with y and b-type ion assignments are provided in figure 4.

^{*}MS/MS fragmentations with y and b-type ion assignments for each single peptide are provided in supplemental figures. No.: Spots numbers.