Materials and Methods

Cell culture and SILAC

HUVEC cells (of human endothelial origin, with passage number between 3 and 8) and BAEC (of bovine aortic endothelial origin, with passage number at 3 to 4) were grown in EGM-2 medium (Lonza). For SILAC, cells were grown in reconstituted medium containing either "light" ($^{12}C_6$ L-Lysine and $^{12}C_6$ L-Arginine) or "heavy" ($^{13}C_6$ L-Lysine and $^{13}C_6$ L-Arginine) stable isotope labeled amino acids (Thermo). After the incorporation efficiency was verified (>6 passages in reconstituted medium), equal amounts of protein extracts from labeled cells were combined, then subjected to a biotin switch assay, in-gel digestion, and mass spectrometry analysis. For assaying palmitoylated proteins regulated by insulin, the "heavy" labeled cells were treated with insulin (100 ng/ml) for 6 h. 93T cells (human embryonic fibroblast cells used for lentivirus generation) and COS7 cells (monkey fibroblast cells used for expression and mutagenesis studies) were cultured in DMEM.

Assays for protein S-palmitoylation

S-palmitoylation was detected using a modified biotin switch assay [1]. Cells were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) in the presence of 20 mM NEM (Sigma). Following sonication on ice, homogenates were mixed with Triton-X-100 (1.7%), rotated (4^{o}C) for 1 h, then treated with chloroform/methanol (1:4). Proteins were solubilized in 4% SDS with 10 mM NEM at 37^{o}C for 30 min, then serially precipitated to remove residual NEM. Each sample was then aliquoted into two tubes and resuspended in buffers containing HPDP-biotin (1 mM, Thermo) and Triton X-100 (0.2%) in the presence or absence of hydroxylamine (NH_2OH, 0.6M). After 1 h, proteins were precipitated twice with chloroform/methanol, then solubilized in buffers containing both 0.1% SDS and streptavidin-agarose beads. After 1.5 h at room temperature, biotin-selected proteins were purified. Protein palmitoylation was independently confirmed using biosynthetic labeling [2-3]. Cultured cells were metabolically labeled with [^{3}H]-palmitic acid, then the target proteins were immunoprecipitated, separated by SDS-PAGE and subjected to autoradiography. Duplicate gels were treated with hydroxylamine to confirming thioester dependence of palmitoylation.

Proteomic analysis by mass spectrometry

Samples were resolved by SDS-PAGE followed by Coomassie blue staining. Visualized lanes were separated into 10 fractions, and in-gel trypsin digestion was performed before analysis by LC/MS/MS. For nanoHPLC-ESI-MS/MS, mass spectrometric analyses were performed on a LTQ-Orbitrap (Thermo) instrument. Samples were loaded with an autosampler onto a 15 cm Magic C18 column (5 μm particles, 300 Å pores, Michrom Bioresources) packed into a PicoFrit tip (New Objective) and analyzed with a nanoLC-2D plus HPLC (Eksigent). Analytical gradients were from 0-80% organic phase (95% acetonitrile, 0.1% formic acid) over 60 min. Aqueous phase composition was 2% acetonitrile, 0.1% formic acid. Eluent was routed into a PV-550 nanospray ion source (New Objective). The LTQ-Orbitrap was operated in a data-dependent mode with the precursor scan over the range *m/z* 350-2000, followed by twenty MS2 scans using parent ions selected from the MS1 scan. The Orbitrap AGC target was set to 1E06, and the MS2 AGC target was 1E04 with maximum injection times of 300 ms and 500 ms, respectively. For tandem MS, the LTQ isolation width was 2 Da, the normalized collision energy 30%, and the activation time 10 ms. Raw data were submitted to Mascot Server 2.0 and searched against the SwissProt database. Results were quantified by analyzing the mascot "dat" file and its respective thermo "raw" file with the home-brewed program (VC++) "SILACLTQ".

Cloning, expression, and mutagenesis of PAFAH1b3

A cDNA encoding full-length mouse PAFAH1b3 (NM_008776.2) was obtained from Origene. This fragment was sequenced and subcloned into an expression vector (pKH3) with an N-terminal 3x HA tag. The HA-tagged PAFAH1b3 was expressed in COS7 cells using Lipofectamine (Invitrogen). To generate mutant forms of PAFAH1b3, cysteine to serine

substitutions (at residues 56 and/or 206) were generated using a site-directed mutagenesis kit (Stratagene). Wild type or mutant PAFAH1b3 (along with RFP co-transfection) expression plasmids were transfected in COS7 cells or 293T cells using Lipofectamine 2000 (Invitrogen).

Lentivirus shRNA knockdown

PAFAH1b3 or other candidate genes were knocked down in HUVEC cells using a lentiviral-based shRNA strategy (Open Biosystems). To prepare lentivirus, 293T cells were transfected with packaging vectors along with an expression plasmid containing shRNA sequences for the scrambled control or for the specific target gene. Two days later, viruses in the supernatants were collected and filtered, then used to infect HUVEC cells (10 μg/ml polybrene). Infected HUVECs were selected with puromycin (2 μg/ml) for 3 days.

Immunoprecipitation and Western blotting

For IP, cells were lysed in buffer (50 mM Tris HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.25% Na deoxycholate, 2 mM NaVO, and protease inhibitor) and mixed with protein A-sepharose beads and HA antibody (or control serum). After overnight incubation at 4^{o}C, immunoprecipitates were washed and analyzed. For blotting, antibodies detected eNOS (BD Bioscience), Cav1 (Santa Cruz), PAFAH1b3 (Abcam), Actin (Sigma), Akt (Cell Signaling), and HA (Santa Cruz).

Cell migration and angiogenesis assays

Migration/invasion capacity was assessed in a transwell assay. HUVECs were serum starved before the treatment with insulin or VEGF. Inserts with an 8 μm pore size were precoated with 0.2% gelatin. HUVECs were trypsinized and 200 μl of cell suspension (1×10^5 cells) was added in triplicate to transwells. Media with 0.5% FBS was added to the lower wells containing insulin or vehicle. After 4 h, HUVECs that had migrated to the underside of the membrane were fixed. Cells remaining on the upper side of the membrane were removed with cotton tips. Migrated cells were stained with DAPI, photographed, and counted. A similar approach was used with 293T cells transfected either with wild type or mutant PAFAH1b3 or vector alone; cells were co-transfected with an RFP expression vector to allow counting using immunofluorescence microscopy.

A correlate of the angiogenic response was assayed by in vitro tube formation [4]. Matrigel (BD Bioscience) was diluted 1:2 with media without growth factors, added to 96-well plates, and allowed to gel for 1 h at 37^{o} C before seeding. HUVECs (2×10^4 cells per well) were suspended in media with 0.5% FBS. The cell suspension was then stimulated with insulin or recombinant VEGF in the absence or presence of 2-bromopalmitate (2-BP, 20 μM, Sigma), and added to the Matrigel surface. After 6 h, cells were labeled with Calcein AM (Sigma) and photographed using immunofluorescence microscopy. Tube formation, defined as the cellular extension linking cell masses or branch points, was quantified in triplicate wells by image processing.

Isolation of detergent-resistant membranes (DRMs)

To isolate DRMs, cells or tissues were lysed in MES buffer (10 mM MES, 150 mM NaCl, pH=6.5) containing protease inhibitors and incubated with 1% Triton X-100 on ice for 30 min. The homogenates were then adjusted to 40% sucrose and placed at the bottom of tubes with sucrose gradient layers (5% and 30%) on the top. The tubes were then centrifuged at 39000 rpm, 4^{o} C for 16-20 h. The fractions were collected from top to bottom and assayed by Western blotting.

Experimental diabetes

To induce insulin-deficiency, streptozotocin (150 mg/kg) or vehicle was administered to 2-month old C57BL/6 male mice, and animals with serum glucose >300 mg/dl were studied.

Statistics

For proteomics, standard deviations (Table II and IV) and P values (analyzed by one-sample t-test, Table III) are shown. In order to examine differential regulation by insulin, the peptide SILAC ratios of each protein were compared to the constant value 1 (a theoretical number indicating the value of an unchanged protein). P values were then calculated using the one-sample t-test to determine if the level of palmitoylation for that individual protein was altered by insulin (significantly different from 1).

For multiple comparisons in migration and tube formation assays, data were analyzed by two way ANOVA and Bonferroni posttests, or by one way ANOVA and Tukey's multiple comparison test. Data are expressed as mean $\pm$ SEM. $P<0.05$ was considered significant (indicated by *).

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