Materials and Methods

Cell culture and shear stress experiments

HUVECs were cultured in medium M199 supplemented with 15% fetal bovine serum, 3 ng/mL β-EC growth factor, 4 U/mL heparin, and 100 U/mL penicillin-streptomycin. MEFs and HEK293 cells were cultured in DMEM containing 10% FBS. For shear stress experiments, the flow rate was 12 dyne/cm^2. The oscillatory stimulator was connected to the circulating system to generate a magnitude of 4 dyne/cm^2 with oscillation at 1 Hz.

Antibodies and reagents

The antibodies used were anti-cortactin and anti-acetyl-cortactin (Millipore); anti-pan-AMPK, anti-phospho-AMPK Thr-172, and anti-eNOS (Cell Signaling Technology); anti-phospho-eNOS 633/635, 1177/1179 and anti-flot-2 (BD Transduction Laboratory). Decetylation-deficient mimic (9KQ) and deacetylation mimic (9KR) mutants were described.[1] Rhodamine phalloidin was purchased from Cytoskeleton, Inc. Site-direct mutagenesis was used to generate phospho-deficient cortactin T401A and phospho-mimic T401D, T401A/9K/Q, and T401D/9K/R mutants. Recombinant adenovirus (Ad)-AMPK-CA and Ad-SIRT1 expressing a constitutively active form of AMPKα2 and full-length SIRT1 were described previously.[2]

In vitro kinase assay

AMPK kinase assays were conducted in 50 μl reaction buffer containing 50 mM Hepes, 0.38 mM AMP, 0.38 mM ATP, 9 mM $MgCl_2$, 11 pM AMPKα, and 2 μg of recombinant cortactin at 37 °C for 1 hr. Reaction mixtures were then resolved by SDS-PAGE followed by Coomassie blue staining or autoradiography. Identical reaction conditions were used for assays with synthetic peptides as substrates. Radioactivity of the peptide substrates was determined by a standard protocol.

Nano-LC-MS/MS tandem mass spectrometry

Kinase reaction mixtures were resolved by 10% SDS-PAGE. The bands containing cortactin were excised and proteins were reduced in-gel, alkylated with iodoacetamide, and digested with trypsin at 37°C. Peptides were extracted with 5% acetic acid in H_2O, then 5% acetic acid in CH_3CN/H_2O (1:1, vol/vol). Online LC-MS/MS analysis was performed with an LTQ-Orbitrap Velos mass spectrometer coupled with an EASY n-LCII HPLC system and a nanoelectrospray ionization source for peptide sequencing and phosphorylation identification. The LC-MS/MS data were analyzed by Mascot Server 2.2 (Matrix Science) for the mass-to-charge (m/z) ratio data files against the National Center for Biotechnology Information database.

siRNA knockdown and transient transfection

Transient transfection of siRNA involved use of lipofectamine RNAiMAX (Invitrogen). HUVECs at 70% confluence were transfected with AMPKα1 and AMPKα2 siRNA (10 nM, Qiagen SI02622235 and SI02758595), SIRT1 siRNA (20 nM, Qiagen, SI00098434), cortactin siRNA (20 nM, Qiagen SI02661960), or scramble RNA (20 nM) in Opti-MEM (Gibco). HEK293 cells were transiently transfected with 1 μg of respective DNA plasmid with 2 μL Lipofectamine 2000 (Invitrogen) per 10^6 cells. At 24 hr after transfection, cells were infected with Ad-AMPK or Ad-SIRT1 for another 48 hr before use.

Immunoblotting, immunoprecipitation, NO bioavailability, and RT-qPCR

Cell lysates or mouse aortic extracts were resolved by 10% SDS-PAGE. Immunoblotting and immunoprecipitation were performed with standard methods. NO production was assessed as the accumulated nitrite (NO^{2-}) in cell culture media by nitrite/nitrate fluorometric assay (Cayman Chemicals) or Griess reagent (Sigma). Total RNA was isolated with TRIzol reagent (Invitrogen). Reverse transcription involved use of SuperScript II reverse transcriptase (Invitrogen). RT-qPCR involved iQ SYBR Green supermix (Bio-Rad) on the iCycler real-time PCR detection system (Bio-Rad); primers are listed in Supplemental Table 3.

Lipid raft isolation and analysis

Cells were lysed and the homogenate was mixed with an equal volume (1 ml) of MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl, 500 mM Na_2CO_3) containing 80% sucrose. A volume of 6 ml of MES-buffered saline containing 30% sucrose was layered on top, then 4 ml MES-buffered saline containing 5% sucrose. Gradients were centrifuged for 16 hr at 30,000 rpm in an SW41 rotor. Tubes were fractionated into twelve 1 ml fractions.

Immunostaining and fluorescence microscopy

ECs were fixed in 4% paraformaldehyde for 10 min at 4°C and subjected to permeabilization. Triton X-100 (0.1%) was added to PBS to permeate the cell membrane at 4 °C for 10 min. For en face staining, mouse aortas were perfused, isolated, and fixed. After blocking in 2.5% BSA for 20 min, then primary antibody for 1 hr, specimens were incubated for 2 hr with Alex-488 (anti-mouse)- or Alex-568 (anti-rabbit)-conjugated secondary antibody. Rhodamine phalloidin was used to stain F-actin. An Olympus FV100 confocal microscope was used to acquire the fluorescent images.

Flow-induced vessel dilation and assessment of atherosclerosis

The animal protocols were approved by University of California San Diego Institutional Animal Care and Use Committee. $ApoE^{-/-}$ mice (B6.129P2-*Apoe*^{tm1Unc}/J, stock number 002052) were from the Jackson Laboratory. The $cortactin^{+/-}$ mouse line (in C57BL/6j background) was obtained from Dr. Steven Dudek (University of Illinois, Chicago).[3] For the flow-induced vessel dilation experiment, 8- to 10-week-old male $cortactin^{+/-}$ mice and their $cortactin^{+/+}$ littermates were used to evaluate the flow-induced vasodilation as described.[4] Cerebellar microvessels were dissected and cannulated with the perfusion chamber connected to the SoftEdge Acquisition Subsystem (Living Systems, Burlington, VT). The vessels were perfused with a buffer containing 130 mM NaCl, 10 mM HEPES, 6 mM glucose, 4 mM KCl, 4 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 0.025 mM EDTA, pH 7.4. Phenylephrine (1 μM) was used to obtain maximal vasoconstriction, followed by flow perfusion at 400 μl/min. The external diameter of the vessels was monitored and recorded as percentage of vasodilation. For atherosclerosis experiments, $ApoE^{-/-}$ mice were bred with a $cortactin^{+/-}$ line to generate $cortactin^{+/-}$ mice with an ApoE-null background. Eight-week-old male $ApoE^{-/-}$/$cortactin^{+/+}$ mice and their $ApoE^{-/-}$ /$cortactin^{+/-}$ littermates were fed a high-fat, high-cholesterol Paigen diet containing 15% fat, 1.25% cholesterol, and 0.5% sodium cholate (Harlan Teklad) ad libitum for 10 weeks. Animals were then killed and aortas isolated. Aortic specimens were stained with Oil-red O to assess lesion severity and distribution.

Transmission electron microscopy

Mice were scarified and perfused through the left ventricle with PBS followed by fixative (2% paraformaldehyde, 2% glutaraldehyde in PBS, pH 7.4). The aortas were isolated and kept in

fixative overnight. Samples were then washed 3 times with 0.15 M sodium cacodylate buffer and fixed with 2% OsO_4 followed by 0.8% potassium ferrocyanide in 0.15 M sodium cacodylate buffer for 1 hr. The specimens were stained overnight in 2% uranyl acetate, dehydrated, and embedded into Durcupan resin. Ultra-thin sections (60-70 nm) were stained with 2% uranyl acetate and statured lead citrate solution, and examined under a JEOL-1200EX transmission electron microscope at an accelerating voltage of 80 kV. TEM was performed at the National Center for Microscopy and Imaging Research, University of California, San Diego.

Statistical analysis

Data are expressed as mean ± SEM (n=3 unless otherwise noted). For parametric data that were normally distributed, Student *t* test was used to analyze differences among groups. For nonparametric data, the Mann-Whitney U test with the exact method was used to analyze differences between 2 groups. $P<0.05$ was considered statistically significant.

References

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