ESM Methods

Animals

Adult male Sprague-Dawley rats weighing about 250 g (Charles River Laboratories, Wilmington, MA, USA) were housed at ~22 °C with a 12-h light/dark cycle and fed ad libitum. Rats were randomly assigned to either a HFD (60% fat, 20% protein, and 20% carbohydrate; product # D12492, Research Diets, New Brunswick, NJ, USA) or a control diet (CHOW) for 1 week as described before (1). Rats were euthanized by CO_2. The aorta was quickly dissected and put into 5% CO_2 in O_2-bubbled modified Krebs buffer. The surrounding fascia was carefully removed and the vessel then divided into small segments and cut open. After stabilization at 37 ^{o}C, with 95% O_2-5% CO_2 for 1 hour, these aortic segments were incubated with 50 nmol/l FITC-insulin (Sigma-Aldrich, St. Louis, MO) for 30 min with or without the pre-treatment with 10 μmol/l of the Src inhibitor PP1(Enzo Life Science, Farmingdale, NY, USA) for 30 min. The ECs were harvested using pre-coated coverslips and processed for immunocytochemical staining as described before (1). The study procedure was approved by the Animal Care and Use Committee of the University of Virginia.

Serum analyses

Arterial serum glucose concentrations were measured using Cayman's Glucose Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, Michigan, USA). Serum insulin (Mercodia AB, Uppsala, Sweden) and triacylglycerol (TG) (Cayman Chemical) concentrations were measured using ELISA assays.

Cell culture

These experiments were performed as previously described (1;2). Briefly, bovine aortic

endothelial cells (bAECs) or rat aortic endothelial cells (rAECs) (passage numbers 2-8) were incubated in serum-free basal medium for 6 or 24 hours (see figure legends) and then treated with either 50 nmol/l FITC-insulin or 10 nmol/l regular insulin (Humulin R, Eli Lilly, Indianapolis, Indiana, USA) for 30 min at 37^{o}C . For treatment with genistein (50 μmol/l) or wortmannin (100 nmol/l) (both from Sigma-Aldrich) or PP1 (10 μmol/l), these reagents were added to cell incubations 30 min prior to insulin addition. TNFα (20 ng/ml) or IL6 (20 ng/ml) (Sigma-Aldrich) were added to cell incubations 24 hours prior to addition of insulin. Cells were then processed for either immunocytochemical staining or Western blotting.

Small interfering RNA (siRNA) design and transfection

The siRNA knockdown of the insulin receptor was carried out as described previously (1;3;4). Briefly, specific siRNA duplexes directed against the target sequence 5′ -GCACAGACCATTTGAGAAA-3′ , which corresponds to bases 2732 – 2750 from the open reading frame of rat insulin receptor mRNA, was purchased from Dharmacon, Inc. (Lafayette, CO, USA) along with a scrambled control siRNA (siCONTROL Non-Targeting siRNA no. 5). The rAECs were seeded and transfected with either the siRNA duplex against insulin receptor or scrambled siRNA (as control) to a final concentration of 40 nM using Oligofectamine (Invitrogen, Carlsbad, CA, USA) when cells reached approximately 30–50% confluence. Seventy-two hours after transfection, cells were either lysed for Western blotting or serum starved followed by treatment with 50 nmol/l insulin for 30 min for immunocytochemical staining (see below).

DNA constructs and transfection

Plasmids encoding FLAG -tagged wild-type caveolin-1 and caveolin-1Y14F were obtained from Dr. Martin A. Schwartz (5;6). The plasmid DNA was purified by EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA, USA). The proper orientation and sequence were verified by sequencing. The bAECs were grown to approximately 70% confluence and then transfected with 0.4 μg of total DNA using Lipofectamin2000 according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA). Forty-eight hours after transfection, cells were serum-starved for 6 h followed by incubation with 50 nmol/l FITC-insulin for 30 min as described above.

Western blotting

Western blotting was performed as described previously (4;6). Briefly, loaded samples were electrophoresed on a 15% polyacrylamide gel or a 4–20% precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes. The membranes were incubated with antibody against phospho-caveolin-1 (Tyr^{14}) (Cell Signaling Technology, Danvers, MA, USA, 1: 1000) overnight at 4^{o}C. This was followed by incubation with species-specific secondary antibodies coupled to horseradish peroxidase (Amersham Life Sciences, Pittsburgh, PA, USA, 1:5000). After stripping with a stripping buffer (Pierce, Rockford, IL, USA) for 15 min, the membranes were re-probed with a primary antibody against caveolin-1 (Santa Cruz Biotechnology, Dallas, Texas, USA, 1:500) and β-actin or GAPDH (Sigma-Aldrich, 1:5000), respectively. The primary antibodies used to detect phospho-Src (Tyr^{416}) (1:1000) and c-Src (1:200) or insulin receptor (1:200) were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz), respectively. The developed films were scanned using a densitometer and quantified using ImageQuant 5.0 software.

Immunocytochemistry

The double-staining protocols were as described previously (2;7). The following primary antibodies were used: mouse monoclonal antibody against phospho-caveolin-1 (1:50); rabbit polyclonal (1:100) anti-fluorescein (FITC) (Molecular Probes, Grand Island, NY, USA); mouse monoclonal anti- FLAG (Sigma-Aldrich). The following secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used: donkey anti-rabbit IgG conjugated to Cy3; donkey anti-mouse IgG conjugated to Cy2. The cells were cover-slipped with the anti-fade mounting medium with DAPI.

Imaging

The immunocytochemical labeling was examined using a confocal microscope as described previously (1). During image acquisition (single optical section) the individual microscopic field was selected to include similar numbers of cells but was otherwise random. To quantify fluorescence intensity, the images from randomly selected microscopic fields were outlined and the integrated fluorescence intensities were measured using Image J software. The fluorescent intensity of DAPI measured in the same microscopic fields was used as cell number-loading control for the measurement of fluorescent intensity of whole microscopic field in each fluorescent channel. In the DNA construct transfection study (figure 5B-D) in which only a proportion of cells were transfected by the FLAG-tagged constructs, individual cells (either the transfected cells as labeled by anti-FLAG or non-transfected cells) were outlined by polygonal method and the integrated fluorescence intensities were measured. Digital images were processed identically with Adobe Photoshop.

Reference

1. Wang H, Wang AX, Aylor K, Barrett EJ (2013) Nitric oxide directly promotes vascular endothelial insulin transport. Diabetes 62: 4030-4042

2. Wang H, Wang AX, Liu Z, Barrett EJ (2008) Insulin signaling stimulates insulin transport by bovine aortic endothelial cells. Diabetes 57: 540-547

3. Wang H, Wang AX, Barrett EJ (2012) Insulin-induced endothelial cell cortical actin filament remodeling: a requirement for trans-endothelial insulin transport. Mol.Endocrinol. 26: 1327-1338

4. Wang H, Wang AX, Liu Z, Chai W, Barrett EJ (2009) The trafficking/interaction of eNOS and caveolin-1 induced by insulin modulates endothelial nitric oxide production. Mol.Endocrinol.2009.Oct.;23(10):1613.-23.Epub.2009.Jul.16. 23: 1613-1623

5. del Pozo MA, Balasubramanian N, Alderson NB, et al (2005) Phospho-caveolin-1 mediates integrin-regulated membrane domain internalization. Nat.Cell Biol.2005.Sep.;7.(9.):901.-8.Epub.2005.Aug.21. 7: 901-908

6. Wang H, Wang AX, Barrett EJ (2011) Caveolin-1 is required for vascular endothelial insulin uptake. Am.J.Physiol Endocrinol.Metab.2011.Jan.;300.(1):E134.-44.Epub.2010.Oct. 300: E134-E144

7. Wang H, Liu Z, Li G, Barrett EJ (2006) The vascular endothelial cell mediates insulin transport into skeletal muscle. Am.J.Physiol Endocrinol.Metab 291: E323-E332