Materials and Methods

Study subjects

Consecutive obese men and women (BMI ≥ 35 kg/m^2, age ≥ 18 years) with severe long-standing obesity enrolled in the Boston Medical Center Bariatric Surgery Program were recruited into the study. Samples of subcutaneous and visceral adipose tissue were both collected intraoperatively from the lower abdominal wall and greater omentum, respectively, during planned bariatric surgery as previously described[1-3]. Each subject provided one biopsy specimen from the subcutaneous depot and one specimen from the visceral depot (paired samples). We also recruited a cohort of non-obese subjects (BMI 18 to ≤ 30 kg/m^2, age ≥ 18 years) who similarly provided subcutaneous and visceral fat samples during elective abdominal surgery such as hernia repair. Subjects with unstable medical conditions such as active coronary syndromes, congestive heart failure, systemic infection, acute illness, malignancy or pregnancy were excluded. The study was approved by the Boston University Medical Center Institutional Review Board, and written consent was obtained from all participants.

Anthropometric, biochemical, and vascular measures

During a single pre-surgical outpatient visit, clinical characteristics including blood pressure, height, weight, BMI, and waist circumference were measured, and cardiovascular risk factors recorded. Fasting blood was taken via an antecubital vein for biochemical parameters including lipids, glucose, insulin, glycosylated hemoglobin (HbA1c), high-sensitivity CRP (hs-CRP), homeostasis model assessment (HOMA) as the index of insulin sensitivity. All biochemical analyses were performed by the Boston Medical Center clinical chemistry laboratory. Brachial artery flow-mediated (FMD) vasodilation as a measure of peripheral arterial endothelial function was performed pre-operatively during a fasting state using a standardized method of ultrasound using a Toshiba Powervision 6000 system (Toshiba Medical USA, Tustin, CA) as previously described[1, 4,5].

Endothelial cell isolation from adipose tissue

Subcutaneous and visceral fat tissue samples collected during surgery were placed immediately in cold DMEM (catalogue # 11885-084, Gibco life technology, Grand Island, NY) supplemented with penicillin, and streptomycin and 0.5% serum. Tissue was cut into small pieces, minced and digested in collagenase I (catalogue # C130, 1 mg/ml, Sigma-Aldrich, St. Louis, MO) for 1 hour in a 37°C water bath at 100 rpm rotation. To remove undigested tissue, cells were passed through 100-uM filter, and then centrifuged at 600 rpm at 4°C for 10 minutes to separate adipocytes (top layer). Red blood cells were lysed using 1 X RBC lysis buffer (Catalogue # WL1000, R&D Systems, Minneapolis, MN) and remaining cells passed through 40-uM filter and washed with DMEM. Cells were labeled with CD31 microbeads (catalogue # 130-092-545, Miltenyi Biotech, Auburn, CA) before being loaded into the auto MACS Pro Separator. Isolated CD31 positive endothelial cells were plated on fibronectin (catalogue # NC0702888, Fisher Scientific, Pittsburg, PA) coated 4 and 8-well chamber slides (catalogue # 354104 and 354108BD, BD Bioscience, San Jose, CA). Cells were allowed to settle for 4 hours and serum starved (0.5% serum media) overnight.

Insulin stimulation of isolated endothelial cells and adipose tissue

After overnight starvation, endothelial cells were treated with vehicle (control) or 100 nM insulin (Sigma Aldrich, St. Louis, MO) for 30 minutes. Slides were then fixed in 4% paraformaldehyde and stored in -80^{o}C for quantitative immunofluorescence staining. Separately, freshly collected adipose tissue from both subcutaneous and visceral depots were cut into 1-2 mm pieces and serum starved (0.5% serum) in EBM-2 media without growth factors (catalogue # CC5036, Lonza, Hopkinton, MA) for 5 hours. Tissue was then treated with vehicle (control) or 100nM insulin for 30 minutes and frozen

in liquid nitrogen stored at -80C for protein analysis.

Endothelial cell quantitative immunofluorescence

We quantified phosphorylation of FOXO-1 at serine 256 (p-FOXO-1), and eNOS at serine 1177 (p-eNOS) in response to insulin stimulation. Total eNOS and FOXO-1 were also quantified. Briefly, fixed samples were rehydrated with 50 mmol/L glycine (Sigma-Aldrich St. Louis, MO), permeabilized with 0.1% Triton-X and blocked with 0.5% bovine serum albumin (BSA). Slides were incubated for an hour at 37°C with primary antibodies against p-FOXO-1 at serine 256 (1:100 dilution, catalogue #ab26651, Abcam, Cambridge, MA), FOXO-1 (1:100 dilution, Cell Signaling, Danvers, MA), p-eNOS at serine 1177 (1:200 dilution, catalogue #ab75639, Millipore, Billerica, MA), eNOS (1:200 dilution, catalogue # PA3-031A, Thermo Scientific, Rockford, IL), and von Willebrand factor (vWF, 1:300 dilution, catalogue #M0616, Dako Carpinteria, CA) to select endothelial cells. We used analogous Alexa Fluor-488 and Alexa Fluor-594 antibodies (1:200 dilution, Invitrogen, Carlsbad, CA) for the secondary antibodies. Cells were mounted under glass coverslips with Vectasheild (catalogue #H1500, Vector Laboratories, Burlingame, CA) containing DAPI to identify nuclei. Slides were imaged using a fluorescent microscope (x20 magnification, Nikon Eclipse TE2000-E) and digital images were captured using a Photometric CoolSnap HQ2 Camera (Photometrics, Tucson, AZ). Exposure time was kept constant and fluorescent intensity (corrected for background fluorescence) was quantified by NIS Elements AR Software (Nikon Instruments Inc, Melville, NY). Fluorescence intensity was quantified in 20 cells from each depot/subject and averaged. To control for batch-to-batch staining variability, fluorescence intensity for each sample was normalized to the intensity of human aortic endothelial cells (HAEC) staining performed simultaneously. Data are expressed in arbitrary units (a.u.) calculated by dividing the average fluorescence intensity of the subject sample by the intensity of the HAEC sample multiplied by 100, as previously described and validated[2].

Endothelial cell nitric oxide production

Endothelial nitric oxide (NO) production was measured using a sensitive and specific fluorescent probe by utilizing the membrane permeable probe 4,5-diaminofluorescein diacetate (DAF-2DA) as described previously[6, 7]. Briefly, isolated endothelial cells were grown for 2-3 days and serum starved as described above, then subsequently treated with vehicle (control) or 100 nM insulin and NO probe DAF-2DA (Enzo, Framingdale, NY) for 30-minutes. For the last 10 minutes of the experiment, Hoechst (Molecular probes, Grand Island, NY) was added for nuclear staining. For the FOXO-1 inhibitor study, cells were treated with AS184256 prior to DAF-2DA treatment, as described above. Slides were imaged using a fluorescent microscope (x20 magnification, Nikon Eclipse TE2000-E) and digital images were captured using a Photometric CoolSnap HQ2 Camera (Photometrics, Tucson, AZ). Exposure time was kept constant and fluorescent intensity (corrected for background fluorescence) was quantified by NIS Elements AR Software (Nikon Instruments Inc, Melville, NY). Fluorescence intensity was quantified in 20 cells from each depot/subject and averaged as described above.

Pharmacological and biological inhibition of FOXO-1.

Immediately after isolation, endothelial cells were treated with 50 nM of FOXO-1 inhibitor[8] (AS1842856, catalogue #344355, Calbiochem, USA) in 0.5% serum EBM-2 media at 37^{o}C for 18 hours and exposed to conditions with and without insulin for 30 minutes, fixed and stored at -80^{o}C until immunofluorescence staining. Separately, whole adipose tissue was incubated with 50 nM of inhibitor AS1842856 or control at 37^{o}C for 24 hours, and tissue analyzed for eNOS, PDK4 and GAPDH protein expression. For insulin stimulation adipose experiments, fat tissue was incubated with 50 nM of inhibitor AS184285650 for 24 hours, then stimulated with and without 100 nM insulin for

30 minutes and analyzed for p-eNOS and GAPDH.

Biological adipose tissue transfection experiments with FOXO-1 siRNA were performed using techniques as previously described with minor modifications[9]. Briefly, freshly collected adipose tissue was washed with PBS under sterile conditions and approximately 60-80 mg of tissue cut/minced into 1-2 mm pieces. Tissue explants were suspended in 200 µL Opti-MEM (catalogue # 31985-062, Gibco) with 100 nM FOXO-1 or control siRNA (catalogue #6568 for control siRNA and #6242 for siFOXO-1, Cell Signaling, Cambridge, MA) in electroporation cuvette (0.4 cm, USA scientific) and applied sixteen shocks of 50 V and 950 µF for 30 milliseconds on a Bio-Rad Gene Pulser II system. Tissue was then incubated at 37°C for 55 hours, in EBM-2 media with 5% serum supplemented with 50 µg/mL streptomycin and 50 U/mL penicillin. Media was changed after 2 hours initially and every 24 hours thereafter. At the end of the experiment, tissue was serum starved for 5 hours and treated with 100 nM of insulin or vehicle control for 30 minutes, then processed for protein expression.

Western immunoblot analyses

Proteins were extracted from adipose tissue by homogenization in liquid nitrogen. Ice-cold 1X lysis buffer (Cell Signaling, Danvers, MA) supplemented with protease inhibitor cocktail and phosphatase inhibitor II and III (Sigma Aldrich, St. Louis, MO) were added. Samples were assayed for protein content using Bradford's method. Thirty-five micrograms of protein was subjected to electrophoresis in SDS-polyacrylamide gel under reducing conditions and blotted to a nitrocellulose membrane using the Bio-Rad Transblot Turbo Transfer system. The membranes were blocked in 5% bovine serum albumin 0.1% Tween-20 in TBS for 1 hour at room temperature, and then incubated overnight at 4°C with primary anti-human antibodies (1:500-1000). Membranes were then washed off using TBS and incubated with horseradish peroxidase-conjugated secondary anti-rabbit IgG (R& D System, Minneapolis, MN) for 1 hour at room temperature, immune complexes were detected with the enhanced chemiluminescence ECL detection system (Bio-Rad). Densitometric analysis of bands was performed using ImageQuant™ LAS 4000 biomolecular imaging system (GE Healthcare, Pittsburg, PA). Proteins of interests were FOXO-1 and pFOXO-1 at serine 256 (Cell Signaling and Abcam respectively); eNOS and p-eNOS at serine 1177 (Abcam and Millipore, respectively); and AKT and pAKT at serine 473 (Cell Signaling). Intensity of bands for each protein was normalized to control band GAPDH.

Statistics

Clinical characteristics of subjects were analyzed using SPSS 20.0 and presented as mean ± SD or percentage. Group-differences in clinical characteristics were examined using unpaired t-tests. All other analyses were performed using GraphPad Prism 6.0 software. Differences in protein phosphorylation with insulin stimulation and group difference between subcutaneous and visceral depots were analyzed using paired Students' t-tests. For siRNA experiments, differences in eNOS phosphorylation between basal and insulin stimulation were analyzed using paired Students' t-tests. Linear regression was performed to establish a correlation between basal p-eNOS and FMD in the visceral depot of obese subjects. Group differences in NO production were measured using one-way ANOVA. A value of $p<0.05$ was accepted as statistically significant. Graphic data are presented as mean ± SEM unless otherwise indicated.

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