Forkhead Box O-1 Modulation Improves Endothelial Insulin Resistance in Human Obesity

Shakun Karki, Melissa G. Farb, Doan T.M. Ngo, Samantha Myers, Vishwajeet Puri, Naomi M. Hamburg, Brian Carmine, Donald T. Hess, Noyan Gokce

Objective—Increased visceral adiposity has been closely linked to insulin resistance, endothelial dysfunction, and cardiometabolic disease in obesity, but pathophysiological mechanisms are poorly understood. We sought to investigate mechanisms of vascular insulin resistance by characterizing depot-specific insulin responses and gain evidence that altered functionality of transcription factor forkhead box O-1 (FOXO-1) may play an important role in obesity-related endothelial dysfunction.

Approach and Results—We intraoperatively collected paired subcutaneous and visceral adipose tissue samples from 56 severely obese (body mass index, 43±7 kg/m²) and 14 nonobese subjects during planned surgical operations, and characterized depot-specific insulin-mediated responses using Western blot and quantitative immunofluorescence techniques. Insulin signaling via phosphorylation of FOXO-1 and consequent endothelial nitric oxide synthase stimulation was selectively impaired in the visceral compared with subcutaneous adipose tissue and endothelial cells of obese subjects. In contrast, tissue actions of insulin were preserved in nonobese individuals. Pharmacological antagonism with AS1842856 and biological silencing using small interfering RNA-mediated FOXO-1 knockdown reversed insulin resistance and restored endothelial nitric oxide synthase activation in the obese.

Conclusions—We observed profound endothelial insulin resistance in the visceral adipose tissue of obese humans which improved with FOXO-1 inhibition. FOXO-1 modulation may represent a novel therapeutic target to diminish vascular insulin resistance. In addition, characterization of endothelial insulin resistance in the adipose microenvironment may provide clues to mechanisms of systemic disease in human obesity. (Arterioscler Thromb Vasc Biol. 2015;35:1498-1506. DOI: 10.1161/ATVBAHA.114.305139.)

Key Words: FOXO1 protein, human insulin nitric oxide synthase type III obesity

Obesity and its associated cardiometabolic complications have developed into major healthcare problems worldwide. Regional adiposity with central accumulation of ectopic visceral fat, in particular, has been closely associated with insulin resistance, endothelial dysfunction, and cardiovascular disease. Although insulin resistance generally implies diminished actions of insulin in mediating glucose uptake, transport, and storage, insulin also exerts important physiological actions on the vasculature that regulate metabolism and blood flow via activation of endothelial nitric oxide synthase (eNOS) and endothelial nitric oxide (NO) production. Impaired insulin signaling in the vasculature has been shown to promote vascular inflammation, vasoconstriction, and progression of atherosclerotic plaques. Although little is known about mechanisms of vascular insulin resistance, experimental models suggest that transcription factor forkhead box O-1 (FOXO-1) may be a potential key mediator involved in the pathogenic process. Experimental studies show that FOXO-1 downregulates eNOS protein expression, and conversely endothelial ablation of FOXO-1 blunts atherosclerosis in animal models. However, the role of FOXO-1 in the human vasculature and its regulation of eNOS bioaction in adipose tissue and obesity-related disease are completely unknown. In this study, we aimed to characterize the function of FOXO-1 in the pathophysiology of endothelial insulin resistance in human obesity, examine adipose depot-specific responses, and to differentiate findings in obese and nonobese individuals.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results

Study Population
A total of 56 obese (body mass index, 43±7 kg/m²) and 14 nonobese (body mass index, 25.7±2 kg/m²) subjects

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From the Evans Department of Medicine and Whitaker Cardiovascular Institute (S.K., M.G.F., D.T.M.N., S.M., V.P., N.M.H., N.G.) and Department of General Surgery (B.C., D.T.H.), Boston University School of Medicine, MA.
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Correspondence to Noyan Gokce, MD, Boston Medical Center, 88 E Newton St, D-8, Cardiology Section, Boston, MA 02118. E-mail Noyan.Gokce@bmc.org
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were recruited. As displayed in Table 1, obese individuals had higher body mass index, waist circumference, plasma insulin, homeostasis model assessment, hemoglobin A1C, high-sensitivity C-reactive protein, and cardiac risk factors.

**Adipose Depot-Specific Insulin-Mediated eNOS Activation**

As shown in Figure 1A, insulin stimulation significantly increased eNOS phosphorylation at activation site serine 1177 in the subcutaneous fat of obese subjects, which was blunted in the visceral depot. In contrast, phospho-eNOS (p-eNOS) increased significantly in both visceral and subcutaneous tissue in nonobese individuals (Figure 1B), and the same pattern was evident for phospho-AKT expression (Figure 1 in the online-only Data Supplement). No interdepot difference in total basal eNOS protein expression was observed. Basal phosphorylation of eNOS was significantly higher in the visceral adipose depot from obese compared with nonobese individuals (P<0.05), but we found no difference in their subcutaneous depot (P=0.15). In addition, insulin stimulation did not promote differential activation of eNOS at alternative site serine 633 which has been shown to be a protein kinase-A site (Figure II in the online-only Data Supplement).20

**Isolated Endothelial Cell Responses**

Consistent with whole adipose tissue findings, endothelial cells isolated from fat tissues of obese subjects displayed impaired p-eNOS to insulin in the visceral compared with subcutaneous depot as displayed in Figure 2A. This corresponded with reduced NO production in endothelial cells from the visceral compared with subcutaneous depot (Figure IIIA and IIIB in the online-only Data Supplement). Conversely, eNOS activation was preserved in both depots in nonobese subjects (Figure 2B). Representative quantitative immunofluorescence images of adipose endothelial cells from obese subjects are displayed in Figure 2C illustrating cellular insulin resistance in visceral fat. Total eNOS expression was similar in endothelial cells from both depots (Figure IVA in the online-only Data Supplement).

**Insulin Effects on FOXO-1**

Impairment in phosphorylation and inactivation of FOXO-1 at serine 256 is implicated as a potential mechanism of insulin resistance in experimental models. In obese subjects, insulin induced FOXO-1 phosphorylation in subcutaneous fat which was blunted in the visceral depot (Figure 3A), whereas total FOXO-1 protein was similar in both regions (Figure 1VB in the online-only Data Supplement). In nonobese subjects, p-FOXO-1 to insulin was preserved in all depots (Figure 3B). Consistent with above data, we observed selective impairment in FOXO-1 signaling in endothelial cells isolated from visceral fat in only the obese (Figure 4A and 4B).

**Effect of Pharmacological and Biological FOXO-1 Inhibition**

We examined whether FOXO-1 antagonism augments eNOS in visceral fat using specific inhibitor AS1842856. To confirm drug bioactivity, we demonstrated diminished pyruvate dehydrogenase kinase-4 expression, a known downstream target of FOXO-1,21 within 24 hours of pharmacological treatment (P<0.01; Figure 5A). We observed upregulation of basal eNOS expression (Figure VA in the online-only Data Supplement) and markedly improved (>3-fold) insulin-mediated p-eNOS in visceral fat in the presence of AS1842856 as displayed in Figure 5B. When we examined the specificity of this effect for endothelial cells, we observed significant improvement in insulin sensitivity (Figure 5C) that was also associated with increased basal eNOS protein (Figure VB in the online-only Data Supplement). Further supporting the functional relevance of the observed changes, AS1842856 treatment increased insulin-mediated NO production by endothelial cells from the visceral depot (Figure IIIB in the online-only Data Supplement) but had no significant effect on subcutaneous endothelial cells where insulin responses were already relatively preserved (Figure IIIA in the online-only Data Supplement). Moreover, FOXO-1 inhibition had
no significant effect on p-eNOS in the subcutaneous adipose depot of the obese (Figure VI in the online-only Data Supplement).

To exclude nonspecific off-target drug effects, we used complementary small interfering RNA methods to selectively diminish FOXO-1 activity in visceral fat which produced the desired decline in FOXO-1 compared with control as shown in Figure 6A. This was associated with significant increase in basal eNOS (Figure VC in the online-only Data Supplement) and insulin-induced p-eNOS at Ser 1177 (Figure 6B), in similar magnitude to that observed with drug treatment. Based on our cumulative findings, a proposed schematic summary diagram of impaired insulin signaling in visceral obesity is displayed in Figure 7. Finally, we observed a significant positive correlation between p-eNOS expression in visceral adipose endothelial cells and brachial arterial endothelium-dependent flow–mediated vasodilation prompting speculation for a pathophysiological adipose-systemic connection (Figure VII in the online-only Data Supplement).

Discussion

We describe, for the first time, evidence of profound endothelial insulin resistance in the visceral adipose tissue of obese humans. We demonstrate diminished agonist–mediated activation of eNOS, representing a key early pathogenic step in the development of endothelial dysfunction and atherogenesis, which was associated with impaired FOXO-1 signaling. Both targeted pharmacological inhibition and biological small interfering RNA–mediated knockdown of FOXO-1 restored insulin sensitivity in human visceral adipose tissue. Our findings suggest that FOXO-1 modulation may represent a novel mechanism and potential therapeutic target to diminish vascular insulin resistance in obesity.

In addition to a wide range of metabolic actions, insulin modulates vascular function in part via stimulation of vascular NO production that controls vasodilation and arterial pressure. Insulin regulates blood flow through activation of eNOS in vascular cells by binding to the IRS-1 receptor with subsequent Akt-mediated phosphorylation and activation of eNOS at Ser 1177. Obese individuals with insulin resistance exhibit endothelial dysfunction, and functional impairment in this pathway at the level of the endothelium is implicated in mechanisms of cardiovascular disease. In animals, endothelium-specific deletion of the insulin receptor impairs eNOS bioavailability, promotes atherogenesis, and links to whole-body insulin resistance, hypertension, and ischemia. Recent work from our laboratory demonstrated impaired insulin-stimulated eNOS phosphorylation, inflammation, and vasodilator dysfunction of endothelial cells isolated from the vascular wall of obese diabetics. Compelling evidence is thus mounting that supports a mechanistic link between vascular insulin
resistance and arterial disease and suggests that improving vascular insulin sensitivity may represent a therapeutic target.

Clinical data consistently link degree of visceral adiposity burden to cardiometabolic risk, presumably owing to forced ectopic expansion and maladaptive remodeling of visceral fat, which leads to dysregulated release of proatherogenic adipocytokines that are implicated in the pathogenesis of systemic disease.30 In contrast, clinical ramifications of subcutaneous expansion have been mixed with some reports even suggesting it as a favorable metabolic sink.31,32 Published data support the widely accepted notion that the visceral adipose tissue microenvironment, compared with subcutaneous fat, exhibits proinflammatory, pro-oxidant, and antiangiogenic properties associated with severe endothelial dysfunction.33–35 Our novel finding of preserved insulin sensitivity in the visceral fat of nonobese subjects was remarkable and suggests that a pathogenic phenotype is not necessarily inherent to all visceral fat per se, but develops as a consequence of obesity. Although few clinical studies have been able to specifically examine tissue pathophysiology given the difficult nature of obtaining invasive intra-abdominal biopsies from healthy lean subjects, at least 1 study reported normal microvasculature in the visceral fat of lean individuals which exhibited dysfunction only in obese conditions as in our findings.36

Mechanisms underlying these associations remain relatively ill-defined from a molecular perspective. In this study, we focused on FOXO-1 because no prior study has examined its role in the pathogenesis of insulin resistance in vascular cells of obese humans. In experimental models, FOXO-1 has been shown to regulate eNOS by reducing its mRNA and protein expression via transcriptional repression.15,19,37 Mimicking a hyperglycemic insulin-resistant state via exposure of cultured cells to high glucose activates FOXO-1 and blunts eNOS.38 Animal models also suggest that endothelium-specific deletion of FOXO-1 delays progression of atherosclerosis and its knockdown improves eNOS bioavailability.15 In our present study, we newly demonstrate that FOXO-1 expressed in human adipose tissue plays a significant role in mediating endothelial insulin resistance. To gain evidence for potential therapeutic modulation, we demonstrated that FOXO-1 inhibition using pharmacological antagonism improved eNOS protein expression and insulin-mediated phosphorylation at serine 1177, the commonly reported major

Figure 2. Quantitative immunofluorescence for phospho-endothelial nitric oxide synthase (p-eNOS) in isolated adipose endothelial cells. A, Insulin significantly increased eNOS phosphorylation at serine 1177 in endothelial cells isolated from subcutaneous fat (n=9; *P<0.01), which was blunted in the visceral depot of obese subjects (n=10; P=0.73). B, Insulin significantly induced p-eNOS in both the subcutaneous and visceral depots in nonobese subjects (n=6; *P<0.01). C, Representative immunofluorescence images of isolated endothelial cells demonstrating impaired p-eNOS to insulin in visceral fat. Red color=p-eNOS and blue color=4′,6-diamidino-2-phenylindole. Data are presented as arbitrary units (au), indexed to 1 as the basal condition. Data are presented as mean±SEM.
index of eNOS activation, suggesting effects at both pre and post-translational levels, whereas no effect was seen on serine 633 phosphorylation.20,39 We affirmed our findings with complementary methodology using selective small interfering RNA–mediated FOXO-1 knockdown which confirmed negative regulation of eNOS by FOXO-1. Moreover, increased p-eNOS after FOXO-1 antagonism was associated with functional rise in NO production by vascular endothelial cells. We observed hyperphosphorylation of eNOS under basal conditions in the visceral depot

Figure 3. Adipose depot comparison of insulin-mediated forkhead box O-1 (FOXO-1) phosphorylation in obese and nonobese subjects. A, Insulin significantly increased FOXO-1 phosphorylation at serine 256 in subcutaneous fat (n=12; *P<0.05) but had no effect in the visceral fat of obese subjects (n=13; P=0.63). Representative adipose tissue Western blots with and without insulin stimulation quantified for phosphorylated FOXO-1 at serine 256, total FOXO-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are displayed. B, Insulin significantly induced phospho-FOXO-1 (p-FOXO-1) in both the subcutaneous and visceral depots in nonobese subjects (n=7; *P<0.05). Data are presented as arbitrary units (au), indexed to 1 as the basal condition. Data are presented as mean±SEM. GAPDH=control band.

Figure 4. Quantitative immunofluorescence for phospho-forkhead box O-1 (p-FOXO-1) in isolated adipose endothelial cells. A, Insulin significantly increased FOXO-1 phosphorylation at serine 256 in endothelial cells isolated from subcutaneous fat (n=11; *P<0.01) which was blunted in the visceral depot of obese subjects (n=11; P=0.21). B, Insulin significantly induced p-FOXO-1 in both the subcutaneous and visceral depots in nonobese subjects (n=6; *P<0.01). Data are presented as arbitrary units (au), indexed to 1 as the basal condition. Data are presented as mean±SEM.
of obese subjects, which has been described previously under disease conditions including obesity and likely represents a compensatory mechanism.29 We acknowledge that regulation of FOXO-1 on p-eNOS responses is likely complex, and additional studies are required to further probe these regulatory pathways relevant to disease mechanisms in humans. Collectively, our findings suggest that dysfunctional FOXO-1 signaling plays a role in insulin resistant states, and its antagonism may have a beneficial effect on vascular biology, although no approved drug is yet currently available for clinical investigation.

Although our findings may be largely specific to visceral adipose tissue, we observed an interestingly significant correlation between endothelial phenotype in visceral fat and systemic arterial endothelium-dependent vasodilation, which in turn has been shown to correlate with coronary responses and predict future cardiovascular events.40 We have also previously shown agonist-induced p-eNOS in adipose endothelial cells to be a reliable functional and quantifiable cellular readout linked to vasomotor properties of human arterioles.41 As such, pathophysiological mechanisms learned from the adipose microenvironment may provide valuable translational clues to systemic disease, as responses in the adipose vasculature have been shown to correlate with cardiac risk factors and systemic arterial function.42

We emphasize the clinical relevance of elucidating molecular mechanisms of vascular insulin resistance in obesity particularly in relation to weight loss strategies to improve cardiovascular outcomes. Sustained weight loss with medical/dietary intervention is difficult and to date bariatric surgery stands alone as the sole durable weight reduction treatment shown to decrease long-term (>10-year) cardiovascular mortality.43 Although specific mechanisms of risk reduction are unknown, improved survival has been linked primarily to plasma insulin as the key biomarker of clinical response.43 We have similarly recently shown that improved endothelial function with weight loss was directly tied to insulin sensitivity.44 We thus affirm that preservation of insulin signaling may be a key homeostatic mechanism of blood vessels that develops perturbations in obesity, and elucidating disease mechanisms whose origins may lie within the visceral milieu may be highly clinically significant.

There are several limitations to our study. The experimental design was limited to a surgical population undergoing...
planned operations and bariatric subjects were severely obese (class III), thus findings may not be applicable to the general population or lesser degrees of obesity. However, this was counterbalanced by our ability to directly study properties of human visceral adipose tissue which would otherwise be impossible. Although we performed experiments on specimens immediately after surgical biopsy, technical applications may not exactly recapitulate the in vivo physiological environment. We examined interdepot responses but did not specifically compare body mass index categories. Most participants in the study were women, which reflects the general clinical practice nationally and sex differences in populations that seek weight loss treatments.44,45 We focused on serine 1177 phosphorylation site because the primary signal for eNOS stimulation however alternative activation sites may have additional roles. Lastly, the extent to which local insulin resistance in fat contributes to vascular dysfunction and cardiovascular disease systemically in obese states remains unclear.

In conclusion, we demonstrate the presence of endothelial insulin resistance in the visceral fat of obese subjects, which

![Figure 6](image-url) Figure 6. Small interfering RNA (siRNA)-mediated knockdown of forkhead box O-1 (FOXO-1) in the visceral adipose depot of obese individuals. A, FOXO-1 siRNA significantly reduced FOXO-1 protein expression in the visceral fat of obese individuals compared with control siRNA (n=7; \( P < 0.05 \)). B, siRNA-mediated knockdown of FOXO-1 restored activation of endothelial nitric oxide synthase (eNOS) in response to insulin treatment (n=9; \( P < 0.05 \)) compared with control siRNA (n=9; \( P = 0.85 \)). Data represent Western blot analyses quantified as arbitrary units (au), indexed to 1 as the basal condition. Data are presented as mean±SEM. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)=control band. p-eNOS indicates phospho-eNOS.

![Figure 7](image-url) Figure 7. Proposed diagram of impaired insulin signaling in visceral obesity. A, Under physiological conditions, insulin stimulates AKT activation which in turn inactivates forkhead box O-1 (FOXO-1) via phosphorylation, lifting an inhibitory effect of FOXO-1 on endothelial nitric oxide synthase (eNOS). B, In pathological conditions, impaired insulin signaling and blunted eNOS activation promote endothelial dysfunction.
was reversible with FOXO-1 antagonism. FOXO-1 modulation may represent a novel therapeutic target to diminish vascular insulin resistance. With clinical data consistently linking visceral adiposity burden to cardiovascular risk, characterization of cellular derangements in the adipose microenvironment may provide clues to mechanisms of systemic disease.

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Disclosures
None.

References


22. Karki et al. FOXO-1 and Insulin Resistance.


Obesity is a mounting healthcare problem and is associated with cardiometabolic complications. In particular, regional accumulation of visceral fat has been associated with endothelial dysfunction, insulin resistance, and cardiovascular dysfunction. In this study, we used a novel approach to understand endothelial insulin resistance in human obesity comparing subcutaneous and visceral fat and isolated endothelial cells from the same obese individuals, as well as comparing visceral fat and endothelial cells from obese to nonobese subjects. We observe presence of endothelial insulin resistance in visceral fat and endothelial cells of obese subjects which was reversed with FOXO-1 antagonism. FOXO-1 modulation may represent a novel therapeutic target to diminish vascular insulin resistance.

**Significance**
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Supplemental Materials
Supplemental figure I: Insulin-mediated activation of AKT in subcutaneous and visceral fat.

A) Visceral adipose tissue from obese individuals exhibited impaired insulin mediated activation of AKT at serine473 (n=10, p=0.28), whereas AKT phosphorylation was intact in subcutaneous fat (n=10, p<0.001).

B) Insulin mediated p-AKT at serine473 was preserved in both the visceral and subcutaneous fat tissue of non-obese subjects (n=7, p<0.05). Data are presented as arbitrary units (au), indexed to 1 as the basal condition. Data presented as mean ± SEM. GAPDH = control band.
Supplemental figure II: Effect of insulin on Serine633 phosphorylation. Representative immunoblot demonstrating that insulin had no significant effect on phosphorylation of eNOS at Serine633 in either the subcutaneous or visceral depot of obese subjects (n=4). GAPDH = control band.
Supplemental figure III: Effect of FOXO-1 antagonism and insulin on nitric oxide (NO) production in endothelial cells from the subcutaneous and visceral fat of obese individuals.

A) NO production by endothelial cells isolated from the subcutaneous depot significantly increased with insulin stimulation. FOXO-1 antagonism by pharmacological inhibition (AS1842856) did not have any significant added effect (n=6, p<0.05).

B) Endothelial cells from the visceral depot exhibited blunted NO production with insulin stimulation. This impairment was restored with FOXO-1 antagonism (n=6, p<0.05). Data are presented as arbitrary NO fluorescence units (au), indexed to 1 as the basal condition. Data are presented as mean ± SEM.
Supplemental figure IV: Total protein quantification in isolated endothelial cells comparing subcutaneous to visceral depots in obese subjects

A) Total eNOS protein expression was the same in subcutaneous and visceral adipose endothelial cells of obese individuals (n=8, p=0.38). B) Total FOXO-1 endothelial protein expression between depots was also not different (n=13, p=0.31). Data represent quantitative immunofluorescence, presented as arbitrary units (au), indexed to 1 as the subcutaneous depot. Data presented as mean ± SEM.
Supplemental figure V: Effect of FOXO-1 antagonism on basal eNOS expression in the visceral fat of obese individuals.

A) Total eNOS protein expression using western blot in the visceral fat of obese individuals was significantly increased after pharmacological (AS1842856) inhibition of FOXO-1 (n=7, p<0.01).

B) Total eNOS protein expression using immunohistochemistry was significantly increased in isolated endothelial cells from the visceral depot of obese subjects after pharmacological inhibition of FOXO-1 (n=7, p<0.05).

C) Total eNOS protein expression using western blot was significantly increased in visceral adipose tissue after siRNA mediated knockdown of FOXO-1 (n=9, p<0.01). Data are presented as arbitrary units (au), indexed to 1 as the control condition. Data presented as mean ± SEM.
Supplemental Figure VI: FOXO-1 antagonism did not significantly alter Serine1177 phosphorylation of eNOS in the subcutaneous depot of obese subjects. Representative Western immunoblot demonstrating lack of an effect of AS184256 in the subcutaneous depot (n=5). GAPDH= control band.
Supplemental Figure VII: Basal p-eNOS protein expression in visceral adipose endothelial cells correlated significantly with systemic brachial artery endothelium-dependent flow-mediated dilation (FMD) in obese subjects (n=9).
Materials and Methods

Study subjects
Consecutive obese men and women (BMI ≥ 35 kg/m², 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. 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/m², 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.

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°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
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 Vectashield (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.

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. 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 (AS1842856, catalogue #344355, Calbiochem, USA) in 0.5% serum EBM-2 media at 37°C for 18 hours and exposed to conditions with and without insulin for 30 minutes, fixed and stored at -80°C for immunofluorescence staining. Separately, whole adipose tissue was incubated with 50 nM of inhibitor AS1842856 or control at 37°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. 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.
References


