MicroRNA-181b Improves Glucose Homeostasis and Insulin Sensitivity by Regulating Endothelial Function in White Adipose Tissue

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ABSTRACT

Rationale: The pathogenesis of insulin resistance involves dysregulated gene expression and function in multiple cell types including endothelial cells (ECs). Posttranscriptional mechanisms such as microRNA-mediated regulation of gene expression could affect insulin action by modulating EC function.

Objective: To determine whether microRNA-181b (miR-181b) affects the pathogenesis of insulin resistance by regulating EC function in white adipose tissue during obesity.

Methods and Results: MiR-181b expression was reduced in adipose tissue ECs of obese mice, and rescue of miR-181b expression improved glucose homeostasis and insulin sensitivity. Systemic intravenous delivery of miR-181b robustly accumulated in adipose tissue ECs, enhanced insulin-mediated Akt phosphorylation at Ser473, and reduced endothelial dysfunction, an effect that shifted macrophage polarization towards an M2 anti-inflammatory phenotype in epididymal white adipose tissue (eWAT). These effects were associated with increased eNOS and FoxO1 phosphorylation as well as nitric oxide activity in eWAT. In contrast, miR-181b did not affect insulin-stimulated Akt phosphorylation in liver and skeletal muscle. Bioinformatics and gene profiling approaches revealed that PHLPP2, a phosphatase that dephosphorylates Akt at Ser473, is a novel target of miR-181b. Knockdown of PHLPP2 increased Akt phosphorylation at Ser473 in ECs, and ‘phenocopied’ miR-181b’s effects on glucose homeostasis, insulin sensitivity, and inflammation of eWAT in vivo. Finally, ECs from diabetic subjects exhibited increased PHLPP2 expression.

Conclusions: Our data underscore the importance of adipose tissue EC function in controlling the development of insulin resistance. Delivery of miR-181b or PHLPP2 inhibitors may represent a new therapeutic approach to ameliorate insulin resistance by improving adipose tissue endothelial Akt-eNOS-NO signaling.

Keywords: microRNA-181b, insulin resistance, phosphatase, endothelial cells, adipose tissue, obesity.

Nonstandard Abbreviations and Acronyms:

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<td>HFD</td>
<td>high-fat diet</td>
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<td>MiRNA</td>
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<td>miR-181b</td>
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<td>3'UTR</td>
<td>3’-untranslated region</td>
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<td>PBMCs</td>
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<td>NS-m</td>
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<td>181b-m</td>
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<td>HUVECs</td>
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<td>GTT</td>
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<td>ITT</td>
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<td>Ewat</td>
<td>epididymal white adipose tissue</td>
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<td>PHLPP2</td>
<td>PH domain and leucine rich repeat protein phosphatase 2</td>
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INTRODUCTION

Adipose tissue dysfunction, characterized by low grade inflammation, is considered to play a primary role in obesity-associated insulin resistance, which predisposes the majority of obese patients to the development of important chronic metabolic diseases including type 2 diabetes and cardiovascular diseases. Excessive caloric intake may expose tissues such as white adipose tissue to super-physiological levels of metabolic substrates and promote the development of low-grade inflammation. Inflamed white adipose tissue contains a range of leukocyte subsets including monocytes that preferentially differentiate toward M1 macrophages, and are associated with increased expression of proinflammatory cytokines. Accumulating studies support the concept that chronic inflammation in white adipose tissue is critically involved in the pathogenesis of obesity-associated insulin resistance. White adipose tissue is comprised of multiple cell types including not only adipocytes and leukocytes, but also endothelial cells. Cellular interactions emanating from white adipose tissue may control local and systemic homeostasis of cardiometabolic function. However, our understanding of the basic mechanisms linking EC dysfunction with the adipocyte response in insulin resistant states remains incompletely understood.

EC dysfunction is a common feature of type 2 diabetes and cardiovascular disease, and a hallmark of insulin resistance. The reciprocal relationships between insulin resistance and endothelial dysfunction are experimentally and clinically established. In both rodents and primates, EC activation is an early event that occurs prior to or in parallel with the development of impaired insulin signaling. ECs of inflamed adipose tissue from obese subjects have adverse effects on insulin signaling in adipocytes such as reduced expression of phospho-Akt at Ser473, increased endoplasmic reticulum stress, and release of inflammatory mediators. On the other hand, EC function is subjected to the regulation by insulin-mediated signaling. For example, transient activation of endothelial PI3K/Akt signaling inhibits the expression of adhesion molecules involved in leukocyte rolling and adhesion to the vascular luminal wall. Insulin resistance also leads to EC dysfunction through increased circulating free fatty acids and hyperglycemia. Indeed, ECs from visceral adipose tissue of obese mice or human subjects exhibit a marked inflammatory state with increased expression of chemokines, cytokines, and adhesion molecules. Several studies have shown insulin signaling in ECs protect endothelial function and attenuates the progression of atherosclerosis. However, it remains unknown whether enhancing endothelial insulin signaling in adipose tissue may improve systemic insulin resistance.

Akt phosphorylation regulates many fundamental biological processes such as the insulin signaling cascade. Dysregulation of Akt phosphorylation often is involved in heart disease and diabetes. Full Akt activity depends on the phosphorylation of residues Thr308 and Ser473, which can be repressed by the lipid phosphatase – PTEN and the protein phosphatase – PH domain leucine-rich repeat phosphatases isoform 2 (PHLPP2). PHLPP2 inactivates Akt signaling by specifically dephosphorylating Ser473 but not Thr308 of Akt. It remains unknown whether altering the expression of PHLPP2 in ECs regulates Akt signaling, downstream substrates, insulin sensitivity, and glucose homeostasis.

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs, which post-transcriptionally regulate gene expression by promoting mRNA degradation and/or inhibiting translation. It has been reported that miRNAs are differentially expressed in adipose tissue between lean and obese mice, as well as lean and obese human subjects. We have shown that microRNA-181b (miR-181b) ameliorates NF-κB-mediated EC activation and vascular inflammation in mouse models of endotoxemia and atherosclerosis. However, it remains unknown whether: 1) miR-181b expression is dysregulated in ECs of adipose tissue, and 2) increasing miR-181b expression in adipose tissue will ameliorate obesity-associated insulin resistance and inflammatory responses.
In this study, we examined the expression of miR-181b in adipose tissue ECs of obese mice and the role of miR-181b and PHLPP2 in modulating glucose homeostasis and insulin sensitivity. Our findings reveal that miR-181b improves insulin signaling and reduces inflammation and EC dysfunction in white adipose tissue by targeting endothelial PHLPP2 without altering hepatic steatosis or lipid profiles.

METHODS

Methods are provided in the Online Supplement.

RESULTS

MiR-181b is reduced by diabetic stimuli in endothelial cells.

Differential expression of miRNAs has been observed in adipose tissue from lean and obese mice as well as humans, although miRNA expression in adipose tissue ECs has not been previously explored. To examine the expression of miR-181b, an anti-inflammatory microRNA that we previously identified in the macrovasculature, ECs or adipocytes were isolated as described from eWAT of C57BL/6 mice fed a 60% HFD for 0, 3, 7, or 14 days followed by qPCR analysis (Figure 1A). We found miR-181b is the most dominantly expressed among miR-181 family members in adipose tissue ECs as demonstrated that its expression is 3.6-fold higher than that of miR-181a. MiR-181b expression was reduced by 33% and 36% after HFD for 7 and 14 days, respectively, while the expression of miR-181a and miR-181c was reduced by 53% and 67% after HFD for 14 days (Figure 1A). In contrast, the expression of miR-181 family members was not significantly changed in adipocytes after HFD for the indicated days (Figure 1A), and miR-181b expression was not reduced in liver or skeletal muscle ECs (Online Figure I A). However, miR-181b expression was reduced by 24%, 32%, 31%, and 33% in ECs in vitro after treatment with glucose, PMA, TNF-α, or palmitate (Figure 1B). In addition, we examined miR-181b expression in ECs of eWAT over the period of 12-weeks of HFD. MiR-181b expression was decreased by 42%, 84% and 66% in ECs isolated from eWAT of mice fed a HFD for 2, 6, and 12 weeks, respectively (Online Figure I B). Although the ratios of phosphorylated Akt / total Akt were not changed under basal conditions (Online Figure I C), insulin responsiveness was blunted as indicated by reduced phosphorylation of Akt in ECs isolated from eWAT of mice fed on HFD at 2 weeks (Online Figure I D). The expression of VCAM-1, ICAM-1, and E-selectin were increased in ECs from eWAT of mice fed a HFD for 2 weeks compared to chow-fed mice (Online Figure I E). These data demonstrate that the reduction of miR-181b in ECs is an early event during the development of insulin resistance, which was associated with ongoing inflammatory responses.

MiR-181b improves glucose tolerance and insulin sensitivity in a mouse model of diet-induced diabetes.

Our prior observations indicate that miR-181b serves as an anti-inflammatory regulator in the macrovasculature. Based upon the reduced expression of miR-181b in the microvasculature of white adipose tissue, we hypothesize that rescue of miR-181b expression may delay the progression of inflammation and insulin resistance, and improve insulin sensitivity. To examine the effect of miR-181b systemic delivery on glucose homeostasis and insulin sensitivity, C57BL/6 mice were fed a 60% HFD for 12 weeks. After 6 weeks HFD, mice were treated with miR-181b mimics (181b-m) or miRNA negative control (NS-m) for 6 weeks (twice a week, i.v. 1nmol/injection) (Figure 2A). Insulin Tolerance Tests (ITT) and Glucose Tolerance Tests (GTT) were performed at week 5 and 6, respectively, after miR-181b treatment (Figure 2A). The body weights were significantly increased in HFD-fed mice, which were independent of miRNA treatments (Figure 2B). However, miR-181b treatment markedly improved glucose
tolerance (Figure 2C and 2D) and insulin sensitivity (Figure 2E and 2F) compared with NS-m treatment. MiR-181b reduced the area under curves for ITT and GTT by 57% and 38%, respectively, compared to control mice (Figure 2D and 2F). These beneficial effects occurred independent of any changes in lipid profiles, fat mass, or plasma levels of insulin and free fatty acids (Online Table I). Taken together, these data demonstrate that miR-181b delivery is able to improve glucose homeostasis and insulin sensitivity.

**MiR-181b reduces inflammation in epididymal white adipose tissue.**

We have shown that miR-181b ameliorates NF-κB-mediated EC activation and vascular inflammation in mouse models of endotoxemia and atherosclerosis.³³,³⁴ This prompted us to examine the effect of miR-181b delivery on inflammation and EC dysfunction in obese mice. First, paraffin sections of eWAT or liver were stained for the macrophage marker Mac2. Macrophage content indicated by Mac2 staining was significantly increased in eWAT of mice after 12-week of HFD. However, macrophage accumulation in eWAT of miR-181b-treated mice was reduced by 60% compared to NS ctrl-treated mice (Figure 3A). The macrophage content in liver was not reduced by miR-181b delivery. Second, macrophage M1 and M2 markers were examined by qPCR. MiR-181b delivery reduced the expression of the macrophage M1 markers $\text{Nfia}$, $\text{Il-1b}$, and $\text{Il12}$ by 48%, 51%, 39%, respectively, and increased the expression of M2 markers $\text{Mrc2}$, $\text{Mgl2}$, $\text{Fizz1}$, $\text{Yml}$ by 43%, 33%, 60%, and 21%, respectively, in eWAT; while it exerted minimal effects on M1 and M2 markers of macrophage in liver (Figure 3B). Third, we observed that miR-181b delivery reduced ICAM-1 and VCAM-1 expression in eWAT from obese mice (Figure 3C), suggesting that EC dysfunction in eWAT in obese mice was ameliorated by miR-181b delivery. These effects of miR-181b treatment are associated with a 1.5-fold overexpression of miR-181b in eWAT and ~15-fold overexpression in ECs of eWAT (Online Figure II A). Systemic delivery of miR-181b could lead to more pronounced exogenous miR-181b expression in specific cell types within eWAT such as adipocytes or adipose tissue ECs. To test this, the expression of miR-181b in eWAT or ECs isolated from eWAT was examined after three consecutive injections of miRNA negative control (NS-m) or miR-181b. Three daily injections of miR-181b result in 105-fold expression of miR-181b in adipose tissue ECs, and 29-fold expression in eWAT (Online Figure II B). These data suggest that systemic delivery of miR-181b leads to predominant accumulation of exogenous miR-181b in adipose tissue ECs within eWAT, and reduces EC activation, macrophage accumulation, and inflammatory phenotype in white adipose tissue.

**MiR-181b does not directly inhibit the cell-intrinsic capacity of monocytes/macrophages to migrate, proliferate, or be activated.**

Macrophage infiltration, proliferation, and activation are all involved in the pathogenesis of obesity-induced insulin resistance.³⁵,³⁶ Reduced macrophage content in eWAT could result from the direct effect of miR-181b on migratory and proliferative ability of monocytes/macrophages or by directly reducing EC activation and dysfunction. To explore any direct effects of miR-181b on monocyte migration, adoptive transfer of monocytes was conducted as previously described.⁴⁰ Monocytes were isolated from HFD-fed mice treated with miR-181b or miRNA negative control (NS-m), labeled with PKH26, and injected into HFD-fed obese mice. Stromal vascular fractions were isolated from eWAT of the recipient mice two days later, and subjected to FACS analysis.⁴¹ As shown in Figure 4A, miR-181b overexpression (25-fold, data not shown) in monocytes does not significantly inhibit their migration into eWAT revealed by the percentage of PKH26-positive cells among CD11b and F4/80 double-positive cells [NS-m: (22.1 ± 5.1)%; 181b-m: (19.2 ± 4.0)%]. To examine the effect of miR-181b on macrophage proliferation, staining for Ki67 (cell division marker), F4/80 (macrophage marker), and DAPI (nuclear marker) was performed on paraffin sections of eWAT from mice. The percentages of proliferating macrophages are (13.9 ± 1.9)% and (12.5 ± 1.1)% in NS-m or miR-181b treated mice respectively, suggesting systemic delivery of miR-181b did not affect macrophage proliferation in eWAT (Figure 4B). We previously showed that miR-181b does not inhibit NF-κB activation in macrophages and NF-κB target gene expression in PBMCs.³³ Consistently, miR-181b did not affect TNF-α, IL-1β, and COX-2 gene expression in PBMCs isolated from
insulin-resistant mice (Figure 4C). Collectively, these data indicate that miR-181b does not directly regulate cell-intrinsic monocytes/macrophage function including migration, proliferation, and activation.

**MiR-181b expression promotes glucose uptake in adipocytes in a paracrine manner.**

Since miR-181b does not directly regulate cell-intrinsic functions of monocytes/macrophages, the protective effects of miR-181b delivery on insulin signaling in eWAT may result from the direct effects of miR-181b on adipocytes or ECs. To assess this, glucose uptake experiments were performed in differentiated, mature 3T3-L1 adipocytes. Consistent with our hypothesis, miR-181b overexpression in 3T3-L1 adipocytes did not promote glucose uptake (Figure 5A). ECs of inflamed adipose tissue may have adverse effects on insulin signaling in adipocytes, possibly via paracrine effects. Since miR-181b attenuates endothelial inflammation in adipose tissues (Figure 3C), we reasoned that miR-181b improves insulin signaling in a paracrine manner by exerting protective effects in ECs. Indeed, the conditioned medium (Supe) from ECs overexpressing miR-181b markedly improved glucose uptake in adipocytes (Figure 5B). These data suggest that miR-181b overexpression in ECs may improve glucose uptake via paracrine mechanisms with adipocytes.

**MiR-181b delivery improves insulin signaling by increasing Akt phosphorylation in epididymal white adipose tissue.**

Because Akt phosphorylation is a central event in insulin signaling cascade, we examined the expression of phospho-Akt (Ser473 and Thr308) in eWAT, skeletal muscle, and liver. HFD mice were treated with miR-181b or NS ctrl mimics in an analogous manner as Figure 2 and stimulated in the presence or absence of insulin for an additional 10 min prior to tissue harvest. In insulin-stimulated miR-181b-treated mice, phospho-Akt (Ser473) was significantly increased 2.2-fold in eWAT, whereas there were no significant differences in skeletal muscle or liver (Figure 6A). Phospho-Akt (Thr-308) was not changed by miR-181b delivery compared with controls in all the tissues examined. Moreover, the effect of miR-181b delivery on upstream insulin signaling was examined in eWAT. Since inflammatory stimuli can blunt insulin action by affecting the phosphorylation of mediators in upstream insulin signaling such as insulin receptor beta (InR-β) and insulin receptor substrate 1 (IRS1), their phosphorylation status was examined by immunoprecipitation assays from eWAT lysates (Figure 6B). MiR-181b delivery did not affect the expression of phospho-InR-β (Tyr1162) and phospho-IRS1 (Ser307) in eWAT, suggesting upstream insulin signaling was not changed by miR-181b. Both FoxOs and eNOS are important molecules mediating insulin signaling events downstream of phospho-Akt in ECs. We found phospho-FoxO1 (Ser 256) is increased up to 2.0-fold in eWAT of miR-181b treated mice compared with controls in response to insulin (Figure 6B). Consistently, miR-181b overexpression increased Akt phosphorylation at Ser473 and reduced nuclear accumulation of FoxO1 at 10 min after insulin stimulation in ECs (Figure 6C). Similarly, miR-181b increased phospho-eNOS (Ser1176) by 1.7-fold (Figure 6D) and nitric oxide (NO) activity by 1.5-fold (Online Figure III) in eWAT compared to controls after insulin stimulation. In a separate experiment, obese mice were treated with miR-181b using the same dosing regimen as outlined in Figure 2, and ECs were isolated from eWATs to examine the effects of miR-181b delivery on insulin signaling and inflammation. Consistent with the effects of miR-181b on insulin signaling and inflammation in eWATs, we found miR-181b increased the phosphorylation of Akt by ~1.6-fold (Figure 6E) and reduced ICAM-1 expression by 43% in ECs isolated from eWATs (Online Figure IV). These data indicate that miR-181b delivery reduced insulin resistance and inflammation in ECs within eWATs. We also examined the expression of genes involved in thermogenesis and hepatic glucose production. The mRNA expression of UCP-1, PRDM16, and PGC-1α genes were not changed in brown fat and eWAT by miR-181b, suggesting that the thermogenic program is not affected by miR-181b delivery (Online Figure V A). To exclude any effects of miR-181b on hepatic glucose production, we examined miR-181b delivery on the expression of key metabolic enzymes involved in maintaining hepatic glucose homeostasis in liver from mice described in Figure 2. MiR-181b had no effect on mRNA expression for GS, GK, PK, FBP1, G6P and PEPCK.
Finally, miR-181b delivery had no effect on regulating lipid homeostasis in liver revealed by Oil Red O staining (Online Figure V C). These data suggest that systemic delivery of miR-181b improves glucose homeostasis and insulin sensitivity in a tissue-specific manner by promoting Akt phosphorylation and sensitizing insulin action in eWAT independent of any effects on insulin signaling in the liver and skeletal muscle, or lipid accumulation in the liver.

The reduction of endogenous miR-181b by inflammatory stimuli such as TNF-α could affect insulin signaling in ECs. Indeed, TNF-α treatment reduced insulin-induced Akt phosphorylation in ECs (Online Figure VI A) as previously described.49 MiR-181b inhibition reduced insulin-induced Akt phosphorylation, which was potentiated in the presence of TNF-α treatment (Online Figure VI A). Furthermore, miR-181b overexpression rescued the reduction of insulin-induced Akt phosphorylation by TNF-α treatment (Online Figure VI B). The data imply that the early reduction of endogenous miR-181b in ECs of eWAT is likely involved in the pathogenesis of endothelial insulin resistance in eWATs during the development of obesity.

**MiR-181b targets PHLPP2.**

MiR-181b could improve insulin signaling by directly regulating a target intrinsic to this pathway, or by suppressing target genes of other inter-related signaling pathways. Our previous studies demonstrated that miR-181b can repress importin-α3, a protein involved in the nuclear translocation of NF-κB (p65/p50), by binding to its 3’-UTR.33, 34 MiR-181b could inhibit adipose tissue inflammation by reducing importin-α3 expression in ECs. We examined NF-κB p65 nuclear accumulation in adipose tissue ECs. Surprisingly, nuclear p65 expression was not significantly induced by HFD-feeding, and miR-181b delivery did not reduce p65 nuclear accumulation in adipose tissue ECs (Online Figure VII). A combined strategy using bioinformatics and microarray gene chip profiling was taken to identify potential miR-181b direct targets. Genes reduced by miR-181b overexpression identified by microarray gene chip analysis (GEO database accession no. GSE35030)34 that are also predicted as direct targets of miR-181b by different algorithms will be of interest as potential miR-181b targets. Six algorithms predicted 1280 mouse and human orthologous genes as miR-181b direct targets (Figure 7A). Among 4190 genes identified by microarray gene chip assay with more than 1.2-fold reduction by miR-181b overexpression, 306 genes were also found in the list of 1280 genes predicted by *in silico* algorithms50 as miR-181b direct targets (Figure 7A). Gene ontology analysis of these 306 genes revealed that the targets of miR-181b encode regulators of biological processes such as protein amino acid phosphorylation, phosphorylation, intracellular signaling cascade, protein serine/threonine kinase activity, among others (Figure 7B). We focused on phosphatases since miR-181b likely promotes Akt phosphorylation at Serine 473 by reducing the expression of a phosphatase. Several serine/threonine phosphatases have been shown to regulate insulin signaling, and are involved in the pathogenesis of insulin resistance.29, 51, 52 Among the combined list of 306 genes was Pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) isozyme2 (PHLPP2) which can directly de-phosphorylate and inactivate Akt.28, 29 Indeed, we found miR-181b reduced PHLPP2 protein expression in ECs (Figure 7C). To verify that miR-181b directly targets PHLPP2, we performed Argonaute2 (AGO2) micro-ribonucleoprotein IP (miRNP-IP) studies to assess whether PHLPP2 mRNA is enriched in the RNA-induced silencing complex (RISC) following miR-181b overexpression. An approximately 4-fold enrichment of PHLPP2 mRNA was observed after AGO2 miRNP-IP in the presence of miR-181b, as compared with that with the miRNA-negative control (Figure 7D). Furthermore, miR-181b reduced the PHLPP2 3’-UTR activity by 54% (Figure 7E). To show the specificity of miR-181b on PHLPP2 3’UTR, the seed sequence in pcDNA3.1-miR-181b construct was mutated. MiR-181b mutant lost the ability to inhibit the activity of PHLPP2 3’UTR as shown in Figure 7E. Importantly, pharmacological inhibition55 or siRNA knockdown of PHLPP2 promoted insulin-induced Akt phosphorylation in ECs (Figure 7F). To examine whether miR-181b targets PHLPP2 in vivo, PHLPP2 expression was examined in lysates of eWAT, skeletal muscle, and liver from obese mice treated with miR-181b (twice a week for 6 weeks). Systemic delivery of miR-181b reduced PHLPP2 protein expression in eWAT, but not in skeletal...
Because PHLPP2 expression is significantly lower in liver (nearly undetectable) than in eWAT and skeletal muscle, PHLPP2 may play a minimal role in regulating insulin signaling in liver (Online Figure VIII B). Moreover, miR-181b treatment (twice a week for 6 weeks) reduced PHLPP2 expression by 55% in ECs isolated from eWAT of obese mice (Figure 7H). These results identify PHLPP2 as a *bona fide* direct target of miR-181b. PHLPP2 expression was significantly increased in ECs of eWAT from mice fed a HFD for 2, 6, or 12 weeks (Online Figure VIII C), suggesting a counter-regulation of the miR-181b target PHLPP2 during the development of insulin resistance. In addition to PHLPP2, we examined the effects of miR-181b on other potential phosphatases including INPP5E, PPM1A, CTDSPL, and PPAR2B, which are predicted as miR-181b targets among 306 genes. MiR-181b overexpression reduced the expression of CTDSPL and PPAP2B by 41% and 56%, respectively, in HUVECs (Online Figure VIII D). In contrast, miR-181b overexpression had no effects on the expression of INPP5E and PPM1A. However, siRNA-mediated knockdown of CTDSPL or PPAP2B did not promote the phosphorylation of Akt at Serine 473 in response to insulin in HUVECs (Online Figure VIII E), suggesting CTDSPL and PPAR2B did not mediate miR-181b’s effect on insulin-induced Akt phosphorylation. It has been reported that miR-181b reduces IGF-1R expression in tumor cells, and inhibits VEGF-induced PI3K-Akt signaling; 54 in contrast, in T cells it may reduce PTEN expression, thereby promoting PI3K-Akt signaling. 55 Therefore, we examined whether miR-181b regulates Akt phosphorylation by reducing IGF-1R and PTEN expression in ECs. As shown in Online Figure VIII F, miR-181b overexpression did not reduce PTEN and IGF-1R expression in ECs, and also had no effect on PTEN expression in eWAT. These data suggest a cell-specific regulation of PTEN and IGF-1R expression by miR-181b. Collectively, these results identify PHLPP2 as a direct target of miR-181b that may mediate miR-181b’s effects on the insulin signaling pathway.

**PHLPP2 knockdown improves glucose tolerance and insulin sensitivity in diet-induced diabetic mice.**

To examine whether PHLPP2 knockdown will phenocopy miR-181b’s effects to improve glucose homeostasis, insulin sensitivity, and reduce adipose tissue dysfunction in vivo, C57BL/6 obese mice were therapeutically treated with PHLPP2 siRNAs or negative control siRNAs for 6 weeks (twice a week, i.v. 1nmol/injection) (Figure 8A). PHLPP2 siRNAs injection did not affect the body weights of mice compared with control siRNAs (Figure 8B). However, GTT and ITT studies revealed that PHLPP2 knockdown significantly improved glucose tolerance (Figure 8C and 8D) and insulin sensitivity (Figure 8E and 8F) by 19% and 20%, respectively, compared to mice treated with control siRNAs controls (Figure 8D and 8F). The effect of PHLPP2 knockdown on ITT and GTT was associated with a ~1.5-fold increase of Akt phosphorylation at Ser473 in eWAT but not in liver (Figure 8G). Macrophage content indicated by Mac-2 staining was also reduced by 58% in eWAT of PHLPP2 siRNA treated mice (Figure 8H). In a separate experiment, three daily injections of PHLPP2 siRNA lead to a 72% and 12% reduction of PHLPP2 mRNA expression in adipose tissue ECs and adipocytes, respectively, suggesting that the majority of systemically delivered siRNAs accumulate in adipose tissue ECs (Figure 8I). Interestingly, PHLPP2 expression revealed by immunostaining was increased by 33% in ECs isolated from diabetic patients compared with control subjects (Figure 8J and Online Figure IX). PHLPP2 inhibition may represent a new therapeutic approach to reduce insulin resistance by improving vascular EC function within eWAT. These data suggest that knockdown of PHLPP2 improves glucose tolerance and insulin sensitivity, reduces adipose tissue endothelial inflammation, and inhibits macrophage accumulation.
DISCUSSION

A number of miRNAs have been identified as regulators of obesity-induced insulin resistance. For example, miR-802, miR-143, and miR-103/7 all regulate glucose metabolism and insulin sensitivity in obesity. It remains unknown whether miRNAs affect insulin sensitivity by regulating EC function within eWAT. In the present study, we have discovered that: (1) the expression of miR-181b is reduced in white adipose tissue ECs of HFD mice, which can be rescued by miR-181b systemic delivery; (2) miR-181b delivery improves glucose homeostasis and insulin sensitivity associated with reduced EC activation, macrophage infiltration, and inflammatory phenotype in eWAT; (3) miR-181b had no direct effects on regulating monocyte/macrophage activation, proliferation, or recruitment in vivo; (3) miR-181b targets PHLPP2, a phosphatase that dephosphorylates Akt at Ser473 in ECs; and (4) siRNA-mediated knockdown of PHLPP2 phenocopies miR-181b's effect on glucose homeostasis, insulin sensitivity, and eWAT macrophage accumulation. We demonstrated that enhanced insulin signaling in adipose ECs exerts beneficial effects and promotes glucose uptake in adipocytes in a paracrine manner, and importantly, improves systemic glucose homeostasis and insulin sensitivity without altering hepatic steatosis or lipid profiles.

Several studies have been performed by other groups to uncover the causal relationships among vascular endothelial function, inflammation, and metabolic insulin resistance. For example, vascular inflammation (increased phosphorylation of IκBα and ICAM-1 expression) and insulin resistance (reduced phosphorylation of Akt and eNOS) are detectable in aortas of mice fed a HFD for 1 week, which precedes the onset of peripheral insulin resistance in liver, skeletal muscle, and adipose tissue. Another group showed systemic insulin resistance occurred in mice fed a HFD for three days revealed by GTT and hyperinsulinemic-euglycemic clamp studies. However, the relationship between EC function and endothelial insulin resistance within eWAT remains unclear. We examined Akt phosphorylation in ECs from eWATs of chow- and HFD-fed mice for 2 weeks. Our data suggest that ECs within eWAT display an insulin-resistant and inflammatory phenotype in mice after 2-week HFD (Online Figure I D and E). The reduction of miR-181b in ECs of eWAT was detected as early as 1 week after HFD (Figure 1A), suggesting that the reduction of miR-181b in ECs and associated endothelial activation was an early event during the development of insulin resistance.

Endothelial eNOS-NO signaling is impaired in ECs of visceral fat in obese patients. Activation of eNOS by insulin through Akt in ECs is important for limiting obesity-induced insulin resistance and inflammation. The production of NO by activated eNOS inhibits NF-κB activity, decreases cytokine-induced endothelial activation, and shifts macrophage polarization towards an M2 anti-inflammatory phenotype. MiR-181b delivery increased the phosphorylation of Akt and eNOS, increased NO activity (Online Figure III), and decreased FoxO activity and ICAM-1 expression in eWATs (Figure 6). In ECs of eWATs, miR-181b also increased Akt phosphorylation and decreased ICAM-1 expression (Figure 6E and Online Figure IV). In contrast, miR-181b delivery had no intrinsic effects on macrophage activation, infiltration, and proliferation (Figure 4). However, miR-181b delivery reduced macrophage accumulation and shifted M1 macrophages to an M2 anti-inflammatory phenotype in eWATs (Figure 3A). These data indicate that miR-181b improved insulin resistance in eWATs by primarily improving endothelial Akt-eNOS-NO signaling through targeting PHLPP2, an effect reducing endothelial ICAM-1 expression, EC-leukocyte interactions, and favorably shifting M1 to M2 macrophages thereby generating an anti-inflammatory milieu in eWAT. This anti-inflammatory milieu in eWATs generated by miR-181b may also promote glucose uptake in adipocytes through a paracrine manner (Figure 5).
Our results demonstrate that miR-181b delivery improves insulin signaling in epididymal fat but not in liver or skeletal muscle. There are at least two reasons that may account for this tissue-specific effect: 1) miRNAs may confer target and functional specificity in different cell types or tissues. MiR-181b may target PHLPP2 in eWAT but not liver and skeletal muscle (Figure 7G and Online Figure VIII A); 2) tissue- and cell-specific accumulation of exogenous miR-181b. Although miR-181b delivery leads to its highest overexpression in liver, exogenous miR-181b is enriched higher in adipose ECs than in liver or skeletal muscle ECs (Online Figure II B). A fenestrated, discontinuous endothelium in liver may cause this differential accumulation of exogenous miR-181b. Furthermore, expression of the miR-181b target PHLPP2 is minimally expressed in liver compared to eWAT, a finding that may account for the lack of miR-181b’s effects on insulin signaling in liver. Interestingly, improved insulin signaling in liver ECs potentiated hepatic insulin resistance, suggesting potential phenotype functional differences of liver ECs vs eWAT ECs.

NF-κB mediated vascular inflammation participates in the pathogenesis of obesity-associated insulin resistance. We previously demonstrated that miR-181b reduces acute and chronic vascular inflammation by targeting endothelial importin-α3 and NF-κB signaling. While we anticipated that miR-181b could improve insulin sensitivity by reducing NF-κB-mediated EC activation and inflammation; surprisingly, we found that nuclear accumulation of NF-κB p65 is not significantly induced in white adipose tissue ECs after 12 weeks HFD, and miR-181b delivery had no inhibitory effect (Online Figure VII), suggesting that miR-181b targets different gene(s) than importin-α3 in adipose tissue ECs. Indeed, using a combination of bioinformatics and gene microarray profiling studies, our studies revealed that miR-181b directly targets PHLPP2 in ECs. As miRNAs are known to regulate several targets, our study cannot rule out the possibility that miR-181b targets additional gene(s) that mediate its beneficial effects on insulin sensitivity and glucose homeostasis.

In our study, epididymal fat mass in miR-181b treated HFD mice was not reduced, an effect suggesting that miR-181b delivery does not reduce the capacity of white adipocytes/adipose tissue to store lipids in the context of over-nutrition. Consistent with this, the level of circulating triglycerides and liver lipid content were not significantly different between conditions. Furthermore, adipocytes incubated with conditioned medium from ECs overexpressing miR-181b exhibited markedly improved insulin resistance, an effect highlighting the potential importance of EC-adipocyte interactions in regulating the insulin resistant state. Our data also suggest that miR-181b is able to reduce adipose tissue EC inflammation, leukocyte accumulation, and improve systemic glucose homeostasis and insulin sensitivity without potentiating the development of hepatic steatosis.

In conclusion, our study in HFD mice demonstrates that miR-181b expression is reduced in response to HFD-induced obesity and that rescue of miR-181b in the microvasculature of eWAT is sufficient to improve glucose homeostasis and insulin sensitivity. MiR-181b delivery decreases inflammation in adipose tissue ECs in eWAT by targeting the phosphatase PHLPP2, an effect that increases phospho-AKT (Ser473) to improve insulin signaling. MiR-181b-mediated effects were selective for ECs of eWAT because miR-181b overexpression in adipocytes did not promote glucose uptake. These data indicate that strategies aimed at improving microvascular EC function in visceral fat in general and restoring miR-181b expression (or inhibition of PHLPP2 expression) in adipose tissue ECs in particular, may provide the basis for the rationale design of novel therapies for insulin resistance and its attendant cardiovascular complications.
ACKNOWLEDGMENTS
We thank Dr. Alexandra Newton (University of California, San Diego) for providing the PHLPP2 inhibitors.

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DISCLOSURES
None.

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FIGURE LEGENDS

Figure 1. MiR-181b is reduced in response to inflammatory stimuli or hyperglycemia in endothelial cells. **A,** C57BL/6 mice were fed a high-fat diet as indicated (0, 3, 7, or 14 days). Endothelial cells (ECs) and adipocytes were isolated from eWAT for qPCR. The expression of miR-181 was normalized to small RNA U6 expression and compared to the expression of miR-181c in mice on chow that was subsequently set to a value of one, n=6 per group. **B,** ECs were cultured in the absence or presence of 30 mM D-glucose for 48 hours, 100 nM PMA for 24 hours, 10 ng/ml TNF-α or 100 μM palmitate for 4 hours and harvested for qPCR of miR-181b, n=3 per group. Data show Mean ± SEM; *, P < 0.05. N.S., non-significant.

Figure 2. Systemic delivery of miR-181b improves glucose tolerance and insulin sensitivity in diet-induced obese mice. **A,** Schema of experimental procedures. C57BL/6J mice were fed a 60% high-fat diet (HFD) for 12 weeks. Six weeks after HFD, mice were treated with miR-181b (181b-m) or miRNA negative control (NS-m) for 6 weeks as indicated (i.v. 0.6 mg/kg). **B,** Body weights over time of mice on chow (injected with vehicle) or HFD (injected with NS-m or miR-181b). **C** and **E,** Blood glucose levels were measured on week 11 for the insulin tolerance test (ITT) and week 12 for the glucose tolerance test (GTT). Values were compared with basal glucose levels which were set as 100% for ITT. **D** and **F,** Area under the curve (AUC) of glucose and insulin tests was quantified. Mean ± SEM, n=7-9 mice per group; *, P<0.05.

Figure 3. Systemic delivery of miR-181b reduces inflammation in epididymal fat. **A,** Paraffin sections of eWAT or liver were stained with Mac2, and the positive areas were quantified. **B,** qPCR of gene expression in epididymal white adipose tissue (eWAT) and liver; results were presented relative to those of miRNA negative control (NS-m) treated mice. **C,** qPCR and western blot analysis of VCAM-1 and ICAM-1 in eWAT. Mean ± SEM, n=6-9; *, P<0.05.

Figure 4. MiR-181b does not inhibit the proliferation, migration, and activation of monocytes/macrophages. **A,** Paraffin sections of epididymal white adipose tissue (eWAT) were stained with F4/80, DAPI and Ki67. The percentages of Ki67 among F4/80 positive cells were calculated. Mean ± SEM, n=7-10 mice per group. **B,** Adoptive transfer of PKH26 labeled monocytes from obese mice overexpressing miRNA negative control (NS-m) or miR-181b, and FACS analysis of PKH26 positive cells among CD11b and F4/80 double positive cells in the eWAT of recipient obese mice. Mean ± SEM, n=4 mice per group. **C,** PBMCs were isolated from NS-m- or miR-181b-treated mice fed on a high-fat diet for qPCR analysis. Data show mean ± SEM, n=6 mice per group.

Figure 5. Glucose uptake in 3T3-L1 adipocytes is promoted in the presence of conditioned medium from endothelial cells overexpressing miR-181b. **A,** Adipocytes were transfected with miRNA negative control (NS-m) or miR-181b and glucose uptake was quantified. **B,** Adipocytes were cultured with conditioned medium from ECs overexpressing NS-m or miR-181b and glucose uptake was quantified. Mean ± SEM, n=4; *, P < 0.05. N.S., non-significant. Supe, supernatant.

Figure 6. MiR-181b delivery improves Akt phosphorylation at Serine 473 in epididymal white adipose tissue. **A,** Western blot analysis of Akt phosphorylation at Serine 473 and Threonine 308, and quantifications of pSer473-Akt vs. total Akt in epididymal white adipose tissue (eWAT) and skeletal muscle (SM), or liver from insulin-stimulated mice, n=6-9. **B,** Lysates of eWAT were used for immunoprecipitation and western blot analysis. **C,** MiRNA negative control (NS-m) or miR-181b-transfected HUVECs were treated with 100 nM insulin for 10 minutes. Western blot analysis of pSer473-Akt in total lysates, and total FoxO1 in nuclear fraction, n=3 independent experiments. **D,** Western blot analysis of eNOS phosphorylation at Serine 1176, and quantifications of pSer1176-eNOS vs. total eNOS in eWAT from insulin-stimulated mice, n=6-9. **E,** Western blot analysis of Akt phosphorylation at Serine 473 in ECs.
isolated from eWAT of obese mice treated with NS-m or miR-181b, n=6 mice / group. All values show Mean ± SEM; *, P < 0.05.

**Figure 7. MiR-181b targets PHLPP2.** A, Bioinformatic approach predicts miR-181b direct targets among genes reduced by miR-181b overexpression in ECs identified by microarray gene chip profiling. FC, fold-change. B, Gene ontology analysis of 306 genes identified in A. C, Western blot analysis of endothelial cells (ECs) transfected with 10 nM miRNA negative control (NS-m) or 181b-m. D, miRNP-IP analysis of enrichment of PHLPP2 mRNA in HUVECs transfected with NS-m or 181b-m, n=2 independent experiments. E, Luciferase reporter assay of PHLPP2 3’UTR in the presence of pcDNA3.1(+), pcDNA3.1(+)-miR-181b, and pcDNA3.1-miR-181b mutant in HUVECs, n=3 independent experiments. F, Pharmacological inhibition or siRNA knockdown of PHLPP2 increases pSer473-Akt in ECs in response to 100 nM insulin at 10 minutes. G, Western blot analysis of PHLPP2 expression in eWAT, n= 3 – 5. H, PHLPP2 expression was detected in ECs isolated from epididymal white adipose tissue (eWAT) of obese mice treated with NS-m or miR-181b using the dosing regimen as outlined in Figure 2, n=6 mice per group. All values show mean ± SEM; *, P < 0.05.

**Figure 8. Systemic delivery of PHLPP2 siRNA improves glucose tolerance and insulin sensitivity in diet-induced obese mice.** A, Schema of experimental procedure. C57BL/6J mice were fed a 60% high-fat diet (HFD) for 12 weeks. Six weeks after HFD, mice were treated with negative control siRNA or PHLPP2 siRNA for the subsequent 6 weeks as indicated (i.v. 0.6 mg/kg). B, Body weights over time of mice injected with control siRNAs or PHLPP2 siRNAs respectively. C and E, Blood glucose levels were measured on week 11 for the insulin tolerance test (ITT) and week 12 for the glucose tolerance test (GTT). Values were compared with basal glucose levels which were set as 100% for ITT. D and F, Area under the curves (AUC) for ITT and GTT were quantified. G, Western blot analysis of Akt and pSer473-Akt in epididymal white adipose tissue (eWAT) and liver. H, Paraffin sections of eWAT were stained with Mac2, and the positive areas were quantified. B–H, n=6-7. I, Mice were injected with negative control or PHLPP2 siRNAs three times on consecutive days, and the expression of PHLPP2 was examined in endothelial cells (ECs) or adipocytes isolated from eWAT, n=3 mice per group. J, Quantification of PHLPP2 protein expression in ECs freshly isolated from control and diabetic subjects, n=11 subjects each group. All data show mean ± SEM; *, P<0.05.
Novelty and Significance

What Is Known?

- Obesity-associated insulin resistance is a major risk factor for cardiovascular disease.
- Endothelial dysfunction contributes significantly to the pathogenesis of obesity-associated insulin resistance.
- We previously showed that the microRNA-181b (miR-181b) inhibits NF-κB-mediated endothelial activation in response to acute (endotoxemia) and chronic (atherosclerosis) inflammation. MiR-181b reduces expression of importin-α3, a protein critical for NF-κB translocation from cytoplasm to nucleus. However, the role of miRNA-181b in obesity-associated insulin resistance has not been examined.

What New Information Does This Article Contribute?

- MiR-181b expression in adipose tissue endothelial cells (ECs) is reduced early after high-fat diet in C57BL/6 mice, coincident with the onset of insulin resistance. Systemic intravenous delivery of miR-181b significantly improves glucose homeostasis and insulin sensitivity.
- MiR-181b reduces adipose EC inflammation, inhibits macrophage infiltration, and shifts M1 to anti-inflammatory M2 macrophages in epididymal white adipose tissue (eWAT). MiR-181b is identified as a new regulator of EC-adipocyte interactions in white adipose tissue, thereby controlling insulin resistance and inflammation.
- Mechanistically, miR-181b targets PHLPP2, a phosphatase that dephosphorylates Akt, leading to increased Akt phosphorylation at Serine 473 and improved EC insulin signaling. PhosphoAkt in turn phosphorylate eNOS and increases nitric oxide activity in eWAT but not liver and skeletal muscle.
- Knockdown of PHLPP2 mimics miR-181b’s effects including increased Akt phosphorylation in ECs and eWAT, reduced macrophage content in eWAT, and improved glucose homeostasis and insulin sensitivity.

Insulin resistance resulting from obesity commonly predisposes to the development of type 2 diabetes mellitus and cardiovascular disease. Chronic low-grade inflammation initiated in adipose tissue is a hallmark of obesity, which has been recognized as a key step in the development of insulin resistance. However, our understanding of adipose tissue EC dysfunction contributing to adipose tissue inflammation and insulin resistance remains inadequate. Results from this study will advance our understanding of miRNA-mediated regulation of adipose tissue EC function in the pathogenesis of adipose tissue insulin resistance and inflammation, which could provide novel and effective therapeutic strategies to manage a range of cardiovascular disease states that are exacerbated by insulin resistance.
FIGURE 3

A. Mac-2 positivity and fold change in eWAT and liver. NS-m 181b-mLean Mac-2 positivity.

B. qPCR (% expression) for ICAM-1 and VCAM-1 in eWAT and liver.

C. % qPCR for ICAM-1 and VCAM-1 in eWAT and liver.
FIGURE 4

A) NS-m 181b-m

Donor mice
HFD

Adoptive
transfer

181b-m

Monocytes
PKH26

Recipient mice
HFD

Monocytes

eWAT

CD11b

F4/80

SSC

PKH26+

PKH26+

22.1 ± 5.1

19.2 ± 4.0

NS-m 181b-m

% PKH26+ / MΦ

% FACS

B) F4/80

DAPI + Ki67

F4/80

DAPI + Ki67

NS-m

NS-m

181b-m

NS-m

181b-m

% Staining

% ki67+ / MΦ

NS-m 181b-m

N. S.

N. S.

C) PBMC TNF-α

PBMC IL-1β

PBMC Cox-2

% qPCR

% qPCR

% qPCR

NS-m 181b-m

NS-m 181b-m

NS-m 181b-m

N. S.

N. S.

N. S.
FIGURE 5

A Glucose uptake

- NS-m
- 181b-m

B Glucose uptake

- Supe (EC + NS-m)
- Supe (EC + 181b-m)

Insulin

- - + +

[^3] 2-DG

0 500 1000 1500

N.S.

Adipocytes

miR-181b

EC

Supe

miR-181b

Adipocytes

*
**A**

6 out of 12 algorithms

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**B**

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**C**

NS-m 181b-m

PHLPP2

1.00 0.55

GAPDH

**D**

Fold enrichment mRNA of PHLPP2

**E**

% 3'UTR activity

**F**

DMSO

PHLPP2 inhibitor

CI siRNA

PHLPP2 siRNA

pAkt

Akt

PHLPP2

**G**

eWAT

NS-m

181b-m

PHLPP2

GAPDH

(100.0 ± 9.3) % *(47.8 ± 15.3) %

**H**

PHLPP2 mRNA in ECs of eWAT

% Expression

NS-m 181b-m
FIGURE 8

A. Injections (i.v. siRNA) 1 Week 7 10 11 12
60% high-fat diet ITT GTT

B. Body weight (g) Weeks 1 3 6 9 12
CTL siRNA PHLPP2 siRNA

C. Glucose (mg/dl) min 0 30 60 90 120
ITT GTT

D. Glucose (mg/dl) min 0 30 60 90 120
ITT (AUC)

E. Glucose (%) min 0 30 60 90 120
ITT

F. Glucose (%) min 0 30 60 90 120
ITT (AUC)

G. p473-Akt Akt p473-Akt Akt

H. Mac-2 Mac-2

I. Fold change pSer473-Akt / Akt

J. PHLPP2 in ECs of diabetic patients

CTL subjects Diabetic patients

PHLPP2 in ECs of diabetic patients
MicroRNA-181b Improves Glucose Homeostasis and Insulin Sensitivity by Regulating Endothelial Function in White Adipose Tissue

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Supplemental Material

MicroRNA-181b improves glucose homeostasis and insulin sensitivity by regulating endothelial function in white adipose tissue

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Reagents and cell culture

Pre-miR™ miRNA precursor molecules negative (non-silencing) control #1 (AM17110) and Hsa-miR-181b-5p Pre-miR™ miRNA precursor (PM12442) were ordered from Ambion. The mature miR-181b sequence is 5'-AACAUUCAUUGCUGUGGUGGGU-3', and its miRBase Accession# is MIMAT0000257. For in vivo studies, oligomers with the same sequence were synthesized on a larger scale by Ambion. Anti-miR miRNA inhibitors-negative control #1 (AM17010), and miR-181b inhibitor (AM12442) were from Ambion. Lipofectamine™ 2000 reagent was from Invitrogen.

Human umbilical vein endothelial cells (HUVECs) (cc-2159) was obtained from Lonza and cultured in endothelial cell growth medium EGM®-2 (cc-4176). Cells were treated with free fatty acid as previously described,1 or other stimuli. Silencer® Select siRNA against INPP5E, PPM1A, CTDSPL, PPAR2B, and PHLPP2 were ordered from Ambion.

Real-time quantitative PCR

Tissues were homogenized using TissueLyser II (QIAGEN). Total RNA was isolated from tissues or cells using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, samples were mixed with chloroform (5:1 v/v sample/chloroform) and centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase containing total RNA was collected, diluted 1:1 (v/v) in isopropanol, and precipitated by centrifugation at 12,000 x g for 15 minutes at 4°C. Pellets were washed with 75% ethanol, dried, and resuspended in nuclease-free water.

QuantiTect Reverse Transcription Kit (QIAGEN) was used to generate cDNA synthesized from 1 μg of total RNA and QuantiFast SYBR Green PCR Kit was used for real-time qPCR with the Mx3000P Real-time PCR system (Stratagene). For primer sequences, refer to Online Table II. Synthesis of stem-loop miRNA cDNAs was achieved by using TaqMan® MicroRNA Reverse Transcription Kit (PN4366596). To detect mature miRNA sequences, TaqMan® MicroRNA Assays hsa-miR-181b (Assay ID 001098), TaqMan® MicroRNA Assays hsa-miR-181a (Assay ID 000480), TaqMan® MicroRNA Assays hsa-miR-181c (Assay ID 000482), U6 snRNA (Assay ID 001973), and TaqMan® Universal PCR Master Mix No AmpErase® UNG (PN4324018) were used. The Delta-Delta CT method with formula: 2^[\(-((\Delta\Delta Ct))\)] was used to calculate relative gene expression values.

Western blot assay

Cells or mouse tissues were lysed in RIPA buffer (Boston BioProducts, Inc.). Protein concentration of tissue extracts or cell lysates was determined by using Pierce BCA Protein Assay Kit (Pierce). Proteins were separated by a 4–15% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) and transferred to a methanol-activated PVDF membrane (Bio-Rad). The membrane was blocked in TBST containing 5% nonfat milk at room temperature for 1 hour and subsequently incubated with primary antibodies overnight at 4 °C. After wash, the membrane was incubated with HRP-conjugated secondary antibodies (Pierce) for 1 hour. Proteins were detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). Primary antibodies used were anti-pan-Akt (#2920, Cell Signaling), anti-phospho-Akt (Ser473; #4060, Cell Signaling), anti-phospho-Akt (Thr308; #2965, Cell Signaling), anti-IRS1 (#2382, Cell Signaling), anti-insulin receptor beta (sc-711, Santa Cruz), anti-phospho-IRS1 (Ser307; 05-1087, EMD Millipore), anti-phospho-insulin receptor beta (Tyr1162/1163; sc-25103, Santa Cruz), anti-FoxO1 (#9454, Cell Signaling), anti-phospho-FoxO1 (Ser256; #9461, Cell Signaling), polyclonal Rabbit Anti-eNOS (610298, BD Transduction Laboratories), anti-phospho-eNOS (Ser1176; #9571, Cell Signaling), anti-PTEN (#9188, Cell Signaling), anti-IGF-1R (#9750, Cell Signaling), anti-GAPDH (#2118, Cell Signaling), anti-ICAM-1 (AF-796, R&D Systems), and anti-PHLPP2
Quantitation was performed using the software Image J. Protein expression was normalized by a loading control.

**Diet-induced obesity, glucose and insulin tolerance tests**

C57BL/6J WT mice were purchased from the Jackson Laboratory. All mice were males and were maintained on a 12-hour light/dark cycle in a pathogen-free animal facility. Mice were kept on a standard chow diet or on a high-fat diet containing 60 kcal% fat (Research Diets, D12492) for 12 weeks. Mice had free access to food and water. Six weeks after HFD, mice were treated with miRNAs or siRNAs for 6 weeks (twice or once a week, i.v. 0.6 mg/kg). Systemic delivery of miRNAs or siRNAs was performed as described in our previous study. ITT and GTT were performed at week 5 and 6 respectively after miR-181b treatment. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at Harvard Medical School, Boston, MA and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For glucose tolerance tests (GTTs), mice were fasted for 12 hours, and then injected i.p. with d-glucose (Sigma, 1.0 g per kg of body weight). Insulin tolerance tests (ITTs) were performed on mice after 6 hours fasting. Recombinant human regular insulin (0.75 U per kg of body weight, Humulin R, Eli Lilly) was given to mice by i.p. injection. Blood glucose levels were measured before injection and at 15, 30, 60, 90, and 120 minutes after glucose or insulin injection using One Touch Ultra glucometer (LifeScan).

** Luciferase activity assay and cell culture transfection**

PHLPP2 3'-UTR sequences were PCR-amplified with specific primers, followed by purification and restriction enzyme digestion. Sequences were cloned into the pMIR-REPORT™-Luciferase vector between SpeI and MluI restriction sites. HUVECs cultured in 12-well plates were transfected in triplicates using Lipofectamine 2000 (Invitrogen) with 200 ng of the final construct per well. The next day, cells were transfected with 10nmol of either a miRNA non-silencing control or miR-181b. Cells were collected 36–48 hours after transfection and assayed using the Luciferase Reporter Assay System (Promega). Cells were co-transfected with pcDNA3.1(+), pcDNA3.1(+)-miR-181b, or pcDNA3.1(+)-miR-181b mutant. The seed sequence of miR-181b was mutated using QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies. Results were normalized to the amounts of protein and expressed relative to the average value of the control.

**Blood chemistry**

Plasma insulin was measured using the Ultra Sensitive Rat Insulin ELISA Kit (90080, Crystal Chem). Mouse Cytokine 32-Plex Discovery Assay was performed by Eve Technologies. Triglyceride levels were determined using Infinity™ Triglycerides Liquid Stable Reagent (Thermo Scientific) as described by the protocol. Total cholesterol was measured using the Infinity™ Cholesterol Reagent (Thermo Scientific). Cholesterol and triglyceride standard were from Pointe Scientific, Inc. Plasma free fatty acid was measured using Free Fatty Acid Quantification Kit (ab65341, Abcam).

**Glucose uptake**

Mouse mature adipocytes were serum-starved for 3–6 hours in DMEM and then incubated with KRH buffer (121 mM NaCl, 5 mM KCl, 0.33 mM CaCl2, 1.2 mM MgSO4, 12 mM Hepes, pH 7.4) with 25 nM insulin at 37 °C for 20 minutes and 2-deoxy-D-[2,6-3H]glucose (0.33 μCi ml⁻¹) for an additional 10 minutes. Uptake was stopped by three rapid washes on ice with KRH containing cytochalasin B (Sigma), the cells were solubilized with KRH buffer containing 0.1% SDS, and radioactivity was measured by liquid scintillation counting.
Histological and immunohistological examinations

For immunohistology, tissues were fixed with neutral buffered 10% formalin solution (HT501128, Sigma), embedded in paraffin wax, cut into sections, and then deparaffinized. Mac-2 staining was performed on the Leica Bond III autostainer. Antigen retrieval was performed using Bond ER1 (AR9961, Leica) for 30 minutes. Sections was incubated with anti-Mac-2 (CL8942AP, Cedarlane Labs) in 1:50,000 dilutions for 30 minutes at RT. Primary antibodies binding to tissue sections was visualized using Bond Polymer Refine Detection kit (DS9800, Leica), and counterstained with hematoxylin. Images were captured by a digital system, and the staining area was measured using computer-assisted image quantification (Image-Pro Plus software, Media Cybernetics). For immunofluorescence staining, antigen retrieval was performed by boiling the slides for 10 minutes in 10mM sodium citrate pH6.0 with a pressure cooker. The sections were then incubated with 1mg/ml sodium borohydride for 5 minutes at room temperature. After three washes with TBS, the sections were incubated with 5% normal donkey serum (Jackson ImmunoResearch Lab Inc.) for 1 hr at room temperature. Slides were then incubated with Rabbit anti-Ki-67 (1:200; RM-9106-S1, Thermo Scientific) and Rat anti-F4/80 (1:100; 14-4801-85, eBioscience) overnight at 4 °C. The slides were washed three times and incubated with Alexa647 conjugated Donkey anti-rabbit and Dylight549 conjugated Donkey anti-rat secondary antibodies. Sections were then washed three times with TBS and counterstained with Hoechst (H21492, Life technology) before mounting with Prolong Gold anti-fade mounting media (P36930, Life technology). Images were acquired on an upright Carl Zeiss LSM 510 confocal microscope equipped with Plan-Neofluar 40×/1.3 oil-immersion objective using the 405 nm diode laser, the 543 nm line of a HeNe543 laser, and the 633 nm line of a HeNe633 laser. Data were analyzed in a blinded fashion, by two independent observers.

Adoptive cell transfer, Isolation of stromal-vascular fraction and ECs, and flow cytometry

Adoptive transfer of monocytes was conducted as previously described. Monocytes were isolated from miR-181b- or control miRNA-treated obese mice, labeled with PKH26 in vitro, and injected into HFD-fed obese mice. Stromal vascular fractions were isolated from eWAT of the recipient mice, and subjected to FACS analysis, or EC isolation. Briefly, the perigonadal adipose tissues were isolated, minced into small pieces with scissors (1 x 2 mm2), digested with collagenase type II and dispase (1mg/ml each in DMEM/F12). Digested tissues were neutralized with DMEM/F12 medium containing 10% FBS, centrifuged at 500 xg for 10 minutes at 4 °C. Anti-Mouse CD11b Alexa Fluor® 488 (53-0112-80, eBioscience) and Anti-Mouse F4/80 APC (17-4801-80, eBioscience) were used for FACS analysis, which was performed on an LSR-II (Beckton-Deckinson, San Diego, CA) and analyzed with BD CellQuest software. For EC isolation, stromal-vascular fractions were incubated with sheep anti-rat IgG Dynabeads coated with PECAM-1 antibodies (557355, BD). The bead-bound cells were collected using a magnet and washed 3 times.

Isolation of fresh human endothelial cells and immunostaining

Isolation of EC from a forearm vein of human subjects was described previously. Briefly, ECs were captured by spring-wires from cannulated forearm veins of human subjects. Wires were cut, and cells were collected from wires by centrifugation in a dissociation buffer. Isolated cells were plated on slides, and fixed for staining. Anti-PHLPP2 (ab153918), anti-vWF (#M061601, Dako), and DAPI were used for staining and quantitative immunofluorescence was used to determine protein expression relative to control staining of cultured human aortic ECs. All human subject protocols were approved by BUMC IRB and subjects provided written informed consent.

Measurement of total nitric oxide levels
To measure total NO production, the concentration of nitrate and nitrite was determined in the lysates of eWAT using a total NO detection kit (ADI-917-020, Enzo Life Sciences). Fat tissues were harvested, immediately flash frozen, and homogenized in a buffer containing 1 mM protease inhibitor cocktail. Nitrate was converted to nitrite using nitrate reductase, and total nitrite was measured according to the manufacturer's instruction.

**Statistical analysis**

Results were expressed as mean ± SEM. We used a paired or unpaired Student t tests as appropriate for statistical comparison between two groups, and ANOVA for the comparison of 3 or more groups. Differences were considered significant when $P<0.05$.

**References**

Online Figure I. A, C57BL/6 mice were fed a HFD as indicated (0, 3, 7, or 14 days). Endothelial cells (ECs) were isolated from liver and skeletal muscle for qPCR. The expression of miR-181b was normalized to small RNA U6 expression and compared to its expression in mice on chow that was subsequently set to a value of one, n=5 – 6 per group. B, C57BL/6 mice were fed a HFD as indicated (0, 2, 6, or 12 weeks). ECs were isolated from epididymal white adipose tissue (eWAT) for qPCR. The expression of miR-181 was normalized to small RNA U6 expression and compared to the expression of miR-181b in mice on chow that was set to a value of 100 %, n=6 –10 per group. C, C57BL/6 mice were fed a HFD for 0 or 2 weeks. ECs were isolated from eWAT for western blot analysis, n=6 – 9 mice per group. D, C57BL/6 mice were fed a HFD for 0 or 2 weeks. ECs were isolated from eWAT for western blot analysis, n=8 – 10 mice per group. Mice were fasted for 6 hours and administered with insulin 0.75 U/kg at 10 minutes before harvesting. E, C57BL/6 mice were fed a HFD for 0 or 2 weeks. ECs were isolated from eWAT for qPCR, n=6 – 9 mice per group. All data show mean ± SEM; *, P < 0.05.
Online Figure II. A, MiR-181b expression was detected in epididymal white adipose tissue (eWAT), skeletal muscle (SM), liver, and endothelial cells (ECs) isolated from eWAT of obese mice treated with NS-m or miR-181b using the dosing regimen as outlined in Figure 2, n=8–10 mice per group. B, Mice were injected with NS-m or 181b-m three times on consecutive days. The expression of miR-181b was examined by qPCR analysis in eWAT and ECs isolated from eWAT, n=3 mice per group. Data show mean ± SEM; *, P < 0.05.
Online Figure III. Nitric oxide activity in epididymal white adipose tissue (eWAT). Obese mice were treated with NS-m or miR-181b using the dosing regimen as outlined in Figure 2 and stimulated with insulin (0.75 U per kg of body weight) before tissue harvesting, n=11 (NS-m) or n=12 (miR-181b) mice per group. Data show mean ± SEM; *, P < 0.05.
Online Figure IV. ICAM-1 expression was detected by qPCR in endothelial cells isolated from epididymal white adipose tissue of obese mice treated with NS-m or miR-181b using the dosing regimen as outlined in Figure 2, n=6 mice per group. Data show mean ± SEM; *, P < 0.05.
Online Figure V

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Liver - Oil Red O

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C

**Online Figure V.** A, qPCR analysis of genes involved in thermogenesis in brown adipose tissue (BAT) and epididymal white adipose tissue (eWAT) from mice described in Figure 2. B, qPCR analysis of gene expression in liver from mice described in Figure 2. GS: Glycogen synthase; GK: Glucokinase; PK: Pyruvate Kinase; G6P: Glucose-6-phosphatase; GP: Glycogen phosphorylase; FBP1: Fructose1,6-biphosphatase; PEPCK: Phosphoenolpyruvate carboxykinase. C, Frozen sections of liver from mice described in Figure 2 were stained with Oil Red O, and positive areas were quantified. All values show mean ± SEM, n=6 – 9 mice per group; *, P < 0.05. N.S., non-significant.
Online Figure VI

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Online Figure VI. **A**, HUVECs were transfected with 50 nM miRNA inhibitor negative control (NS-i), or miR-181b inhibitor (181b-i) for 24 hours. Cells were starved and treated with 10 ng/ml TNF-α or PBS for 8 hours followed by 100 nM insulin for 10 minutes, and harvested for western blot analysis, n=3 independent experiments. **B**, HUVECs were transfected with 10 nM miRNA non-specific negative control (NS-m), or miR-181b (181b-m) for 24 hours. Cells were starved and treated with 10 ng/ml TNF-α or PBS for 8 hours followed by 100 nM insulin for 10 minutes, and harvested for western blot analysis, n=3 independent experiments. Data show mean ± SEM; *, P < 0.05.
Online Figure VII. Paraffin sections of epididymal white adipose tissue (eWAT) from mice described in Figure 2 were stained with antibodies against CD31 and p65, and nuclear accumulation of p65 was quantified in ECs reflecting vehicle (n = 39 cells), NS-m (n= 73 cells) and 181b-m (n= 94 cells). Mean ± SEM, n=6 – 9 mice per group. N.S., non-significant.
Online Figure VIII

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Skeletal muscle

Liver

B

skeletal muscle | eWAT | liver

PHLPP2 | PHLPP2

GAPDH

C

Fold change (qPCR) of PHLPP2 in ECs of eWAT

HFD (weeks)

D

INPP5E mRNA

Fold change (qPCR)

PPM1A mRNA

Fold change (qPCR)

CTDSPL mRNA

Fold change (qPCR)

PPAP2B mRNA

Fold change (qPCR)

E

CtI siRNA | + | − | + | − | − | +

CTDSPL siRNA | − | + | + | + | + | −

Insulin | − | − | + | + | + | +

pSer473-Akt | + | + | + | + | + | +

Akt

F

NS-m | 181b-m | + | − | + | − | − | +

Insulin | − | − | + | + | + | +

PTEN

GAPDH

eWAT

HUVECs

Figure legend is on the next page.
Online Figure VIII. A and B, Western blot analysis of PHLPP2 expression in skeletal muscle, liver, and epididymal white adipose tissue (eWAT). C, C57BL/6 mice were fed a HFD as indicated (0, 2, 6, or 12 weeks). Endothelial cells (ECs) were isolated from eWAT for qPCR. The expression of PHLPP2 was normalized to GAPDH expression and compared to the expression of PHLPP2 in chow-fed mice that was set to a value of 100%, n=6–10 per group. D, HUVECs were transfected with 10 nM miRNA non-specific negative control (NS-m), or miR-181b (181b-m) for 36 hours, and harvested for qPCR analysis, n=3 per group. E, Ctl siRNA, CTDSPL siRNA, PPAP2B siRNA-transfected HUVECs were starved and treated with 100 nM insulin for 10 minutes. Cells were collected for western blot analysis of pSer473-Akt and total Akt. F, PTEN expression was examined in eWAT of obese mice treated with NS-m or miR-181b using the dosing regimen as outlined in Figure 2, n=6 mice per group (upper panel). Western blot analysis of PTEN and IGF-1R in HUVECs transfected with 10 nM miRNA non-specific control or miR-181b (lower panel). Data show mean ± SEM; *, P < 0.05.
Online Figure IX

Online Figure IX. Representative images show PHLPP2 staining in endothelial cells (ECs) freshly isolated from control and diabetic subjects.
Online Table I. Systemic delivery of miR-181b does not affect lipid profile, FFAs, insulin, fat mass, or body weight of obese C57BL/6 mice.

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<td>Body weight</td>
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All values Ctl mimics vs. miR-181b, P=N.S., Mean ± SEM, n=6-10.
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