Loss of Bone Morphogenetic Protein-binding Endothelial Regulator Causes Insulin Resistance

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Accumulating evidence suggests chronic inflammation of metabolic tissues plays a causal role in obesity-induced insulin resistance. Yet, how specific endothelial factors exert impacts in metabolic tissues remains undefined. Bone morphogenetic protein (BMP)–binding endothelial regulator (BMPER) adapts endothelial cells to inflammatory stress in diverse organ microenvironments. Here we demonstrate BMPER is a driver of insulin sensitivity. Inducible knockout (iKO) of BMPER causes hyperinsulinemia, glucose intolerance and insulin resistance without increasing inflammation in metabolic tissues. Interestingly, BMPER can directly activate insulin signaling, which requires its internalization and interaction with Niemann-Pick C1 (NPC1), an integral membrane protein that transports intracellular cholesterol. These results suggest the endocrine function of the vascular endothelium maintains glucose homeostasis. Of potential clinical significance, the delivery of BMPER recombinant protein or its overexpression significantly alleviates insulin resistance and hyperglycemia in high-fat diet (HFD)-fed mice and Lepr<sup>db/db</sup> (db/db) diabetic mice. We conclude that BMPER exhibits therapeutic potential for the treatment of diabetes.

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia and impaired carbohydrates, lipids and protein metabolism resulting from defects in insulin secretion, insulin action or both. More than 100 million U.S. adults are now living with diabetes or prediabetes, a condition that if not treated often leads to type 2 diabetes mellitus (T2DM) within five years<sup>1</sup>. T2DM is the most common form of DM, accounting for 90% to 95% of all diabetic patients and is expected to increase to 693 million by 2045<sup>2</sup>. T2DM is associated with an increased risk of micro- and macrovascular complications, such as diabetic nephropathy, neuropathy, retinopathy and coronary artery disease, which impose profound impacts on the quality of life and health care resources. Current management of diabetes includes lifestyle intervention, routine blood glucose monitoring and pharmacological therapy. However, high costs, variable efficiency and tolerability are important barriers to the use of these pharmacological treatments.
Insulin resistance is a core defect of T2DM and associated with chronic inflammatory responses. During chronic inflammation, macrophage plays a crucial role in obese-induced insulin resistance\(^3,4\). After microphages are infiltrated into metabolic tissues such as obese adipose tissue, their secreted pro-inflammatory cytokines inhibit insulin signaling and result in insulin resistance. Another key player of chronic inflammation is vascular endothelium, which coordinates the action of immune cells through the continual adjustment in its own structure and function, including induction of adhesion molecules and pro-inflammatory cytokines, disruption of its barrier function and angiogenesis\(^5\). Even though endothelial dysfunction and inflammation have been observed during the development of T2DM\(^6,7\), it remains unknown whether endothelial cells (ECs) play a crucial role in the maintenance of glucose homeostasis and how its dysregulation contributes to insulin resistance and diabetes.

BMP pathway is known as an important regulator in the maintenance of endothelial integrity and the induction of vascular inflammatory responses\(^8\). BMPER (also called CV-2) binds BMPs and is an extracellular modulator of BMP signaling pathway\(^9\). We have discovered BMPER plays a pivotal role in BMP-mediated endothelial events and chronic inflammation in the vasculature\(^10-15\). However, the role of BMPER in obesity and insulin resistance has not been studied before.

In this study, we have made a surprising observation that BMPER regulates glucose homeostasis without affecting chronic inflammation. Instead, BMPER is a driver of insulin sensitivity through activating insulin signaling pathway in hepatocytes. This activation requires BMPER endocytosis and interaction with Niemann-Pick C1 (NPC1), an integral membrane protein that transports intracellular cholesterol\(^16,17\). Both whole-body and EC-specific BMPER inducible knockout (iKO) mice display similar glucose defects, including hyperinsulinemia, insulin resistance and glucose intolerance, suggesting vascular endothelium plays a causal role in glucose homeostasis through secreting metabolic regulators. More importantly, the delivery of BMPER recombinant protein or its adeno-associated viral (AAV) particles dramatically alleviates insulin resistance and hyperglycemia in diabetic mice. Our data
reveal that BMPER is a protective regulator of glucose homeostasis and could become a potential therapeutic target for treating diabetes.

Results

Loss of BMPER results in glucose dysregulation. BMPER null mice die at birth due to lung defects\(^\text{12}\), which limits our understanding of BMPER’s functions during pathological conditions at adulthood. To study the role of BMPER in glucose homeostasis, we generated BMPER\(^{\text{flox/flox}}\) mice using CRISPR-cas9 gene editing (Fig. 1a). A BMPER inducible knockout KO (iKO) mouse model was created by crossing BMPER\(^{\text{flox/flox}}\) and CAG-CreER\(^{+/−}\) transgenic mice (Fig. 1a), which allows temporal depletion of BMPER upon tamoxifen injection. BMPER depletion was confirmed in plasma and a variety of tissues of BMPER iKO compared to their littermate control (WT, BMPER\(^{\text{flox/flox}}\); CAG-CreER\(^{+/−}\)) mice (Fig. 1b). Interestingly, BMPER iKO mice displayed hyperinsulinemia and higher homeostasis model assessment for insulin resistance (HOMA-IR) scores than WT mice at four months after tamoxifen injection (Fig. 1c-e, Supplementary Table 1). In addition, BMPER iKO mice were more glucose intolerant and insulin resistant (Fig. 1f-g). To further explore insulin sensitivity, hyperinsulinemic-euglycemic clamp studies were performed. As shown in Fig. 1h-i, the amount of exogenous glucose required for maintaining euglycemia (glucose infusion rate, GIR) and the glucose disposal rate (GDR) were markedly lower in BMPER iKO mice than WT mice. Both the ability of insulin to suppress hepatic glucose production, which reflects hepatic insulin sensitivity, and the insulin-stimulated glucose uptake in skeletal muscle, heart and brown adipose tissue, which reflects insulin sensitivity in peripheral tissues, were significantly decreased in BMPER iKO mice (Fig. 1j-k). We also evaluated the expression of liver genes that reflect glucose output. BMPER depletion led to a significant induction of gluconeogenic enzymes, including G6Pase (glucose-6-phosphatase) and PEPCK (phosphoenolpyruvate carboxykinase), and a lipogenic transcription factor SREBP1 (sterol regulatory element-binding transcription factor 1; Supplementary Fig. 1a). However, no change was observed with the glycolytic enzyme- glucokinase (GK;
Taken together, these results suggest BMPER depletion results in glucose dysregulation through regulating insulin sensitivity, gluconeogenesis and lipogenesis.

Inflammation is not observed in BMPER iKO mice. Given that vascular inflammation also disrupts function of metabolic tissues such as liver and WAT\textsuperscript{18-20}, we examined whether BMPER depletion resulted in an inflammatory response in these tissues. In the liver of BMPER iKO and WT mice, the induction of the inflammatory cytokine IL1\textbeta\textsuperscript{2} (Supplementary Fig. 1b) was not changed while others- IL6 and TNF\alpha were not detectable (data not shown). Their levels did not change in WAT either (Supplementary Fig. 1c). These results suggest that inflammation does not contribute to the disruption of glucose homeostasis by BMPER depletion.

BMPs play a role in obesity through regulating adipogenesis and energy storage partitioning\textsuperscript{21-24}, we asked whether BMPER also regulates body weight through modulating BMP signaling. Surprisingly, there was no body weight difference between BMPER iKO and WT mice (Supplementary Table 1). Further CLAMS (comprehensive lab animal monitoring system) studies demonstrated a decrease in respiration exchange ratio (RER), a mild increase in physical activity and a non-significant trend of increase in food intake of BMPER iKO mice compared to WT mice (Supplementary Fig. 2). In addition, we observed no significant changes with the expression of BMPs and BMP receptor 2 (BMPR2) in the liver of BMPER iKO mice compared to WT mice (Supplementary Fig. 1d). It suggests, unlike BMPs, BMPER does not play a significant role in body weight control.

Given that BMPER iKO mice spontaneously developed hyperinsulinemia, insulin resistance and glucose intolerance without weight gain (Fig. 1c-k, Supplementary Table 1), we hypothesized BMPER iKO mice are more sensitive to the metabolic effects of high-fat diet (HFD). Following eight weeks of HFD feeding, BMPER iKO and WT mice gained similar weight and displayed comparable glucose and insulin levels (Supplementary Fig. 3a-c). However, BMPER iKO mice exhibited more severe glucose intolerance and insulin resistance than WT mice (Supplementary Fig. 3d-e). These observations suggest BMPER depletion exacerbates obesity-induced insulin resistance and glucose intolerance.
Loss of BMPER in ECs results in glucose dysregulation. Our previous studies\textsuperscript{10-15} suggest that BMPER is highly expressed in vascular endothelial cells (ECs). To determine whether EC is an important source for BMPER production, we generated endothelium-specific BMPER inducible knockout (eKO) mice by breeding BMPER\textsuperscript{floox/floox} and Cdh5-CreER\textsuperscript{+/-} mice. BMPER eKO mice, similarly to its global depletion (Fig. 1), displayed hyperinsulinemia, glucose intolerance and insulin resistance without weight difference compared to their littermate control (eWT, BMPER\textsuperscript{floox/floox}; Cdh5-CreER\textsuperscript{+/-}) mice (Supplementary Fig. 4). BMPER depletion in ECs also resulted in hepatic and peripheral insulin resistance (Supplementary Fig. 5). These data suggest EC is an important source for BMPER in periphery.

BMPER plasma level decreases in humans with metabolic syndrome. Since BMPER serum level was lower in BMPER iKO mice that spontaneously developed hyperinsulinemia and insulin resistance, we examined whether there is an association between BMPER level and insulin resistance. To this end, we observed that BMPER plasma level was reduced by $\sim$50\% in humans with metabolic syndrome (MS)\textsuperscript{25}, a complication often coupled with insulin resistance and increased risk for T2DM\textsuperscript{26}, compared to healthy individuals (Fig. 2a, b). In addition, BMPER levels negatively correlated with body weight and plasma levels of insulin and triglycerides (TG; Fig. 2c-f), suggesting an association between decreased BMPER levels and conventional serum markers of insulin resistance. Similarly, a $\sim$70\% decrease of BMPER plasma level was observed in high-fat diet (HFD)-fed mice (Fig. 2g-h). These results suggest that BMPER plasma level is negatively regulated by metabolic stress.

BMPER activates insulin signaling pathway through IR. To understand the underlying mechanism by which BMPER regulates insulin sensitivity and glucose tolerance, we focused on its impact on the canonical insulin signaling pathway that governs glucose output\textsuperscript{27,28}. As expected, we observed BMPER
increased activation of insulin signaling pathway in the liver, skeletal muscle and heart, indicated by the phosphorylation of insulin receptor substrate1 (IRS1) and AKT (Fig. 3a). To determine whether BMPER regulates insulin signaling through modulating BMP signaling pathway, we used the BMPR2 kinase-dead (BMPR2-KD) mutant construct that inhibited BMP2-induced Smad 1, 5, 8 phosphorylation in primary hepatocytes (Supplementary Fig. 6a). However, BMPR2-KD mutant failed to inhibit BMPER-promoted IRS1 phosphorylation (Fig. 3b), suggesting BMPR2 is not required for the activation of IRS1 by BMPER. On the other hand, IRS1 phosphorylation induced by BMPER or insulin was blocked in IR-depleted hepatocytes isolated from IR-iKO mice (Fig. 3c). Thus, our data suggest that IR, but not BMP signaling, is required for BMPER activation of insulin signaling.

**BMPER-activated insulin signaling requires NPC1 and BMPER internalization.** To further understand how BMPER increased insulin signaling, we tested whether BMPER binds IR. However, we failed to observe the direct interaction of BMPER and recombinant IR, indicating BMPER regulates insulin pathway via unknown mediators. By performing co-immunoprecipitation-combined with liquid chromatography-mass spectrometry proteomic techniques, we identified NPC1, an endosome and lysosome-residing membrane protein\textsuperscript{16}, as an interacting protein of BMPER (Supplementary Fig. 6b, Table 2). NPC1 loss of function mutants develop devastating lysosomal cholesterol accumulation\textsuperscript{29,30}. NPC1 also influences insulin signaling in adipocytes and NPC disease mouse models\textsuperscript{31,32}. However, its exact role in insulin action remains unclear. Therefore, we tested the notion that NPC1 is required for BMPER to regulate insulin signaling. We confirmed NPC1 and BMPER were in the same complex and BMPER co-localized with endogenous NPC1 mainly in vesicles inside of hepatocytes (Fig. 4a-b). In addition, NPC1 knockdown markedly blocked BMPER, but not insulin, induced IRS1 phosphorylation (Fig. 4c-d), suggesting NPC1 specifically mediates BMPER-promoted insulin signaling. By performing immunoprecipitation studies with membrane fractions purified from hepatocytes, we discovered that BMPER was associated with endogenous IR \textit{in vivo} and NPC1 knockdown markedly blocked the
BMPER and IR complex formation in hepatocytes (Fig. 3e, Supplementary Fig. 6c). These observations suggest NPC1 is required for BMPER to interact with IR and promote insulin signaling. Our previous studies demonstrate BMPER can be internalized into endothelial cells. In hepatocytes, BMPER was also internalized and the internalized BMPER was degraded with a half-life at 38.45 min during a chase period with cold media (Supplementary Fig. 6d). Its internalization was inhibited by chlorpromazine (CPM), an inhibitor of clathrin-dependent endocytosis but not by methyl-β-cyclodextrin (MCD), an inhibitor of the caveolin-dependent endocytosis (Fig. 3f-g). In addition, CPM abolished IRS1 phosphorylation induced by BMPER (Fig. 3h, Supplementary Fig. 6e), suggesting BMPER internalization is required for IRS1 activation.

**BMPER supplementation improves glucose homeostasis in diabetic mice.** Since BMPER depletion resulted in defective glucose metabolism, we hypothesized that BMPER supplementation improves glucose homeostasis in diabetic mice. We injected adeno-associated virus (AAV) of BMPER, or AAV-GFP as the control, into mice and then fed them with HFD for eight weeks. We observed AAV-BMPER injection recovered BMPER plasma level in HFD-fed mice back to the level seen in control chow-fed mice (Fig. 5a) and had no impacts on appetite and weight gain (Supplementary Fig. 7a-b). However, plasma insulin and glucose levels were decreased in AAV-BMPER-injected mice compared to AAV-GFP group (Fig. 5b-c, Supplementary Fig. 7c-d). Moreover, responses of glucose clearance and insulin sensitivity were improved in AAV-BMPER- injected mice following HFD feeding, although no difference was observed in CC-fed mice (Fig. 5d, Supplementary Fig. 7e-f).

We also tested whether BMPER alleviates insulin resistance in a Lepr<sup>db/db</sup> (db/db) monogenic mouse model of severe diabetes. BMPER plasma level was markedly lower than wild-type (C57BL/6) mice and AAV-BMPER injection recovered BMPER level in db/db mice (Fig. 5e). We observed no differences in food intake and body weight between AAV-BMPER and AAV-GFP- injected mice (Supplementary Fig. 8a-b). However, AAV-BMPER injection led to the normalization of
hyperinsulinemia and hyperglycemia (Fig. 5f-g, Supplementary Fig. 8c-d) and improved glucose clearance and insulin sensitivity in db/db mice (Fig. 5h). Polyuria and glucosuria are important hallmark clinical features of T2DM. We observed dramatic decreases in urinary glucose and albumin levels and urine volume in AAV-BMPER- injected db/db mice (Fig. 5i-k).

The liver-specific IR knockout mice display severe insulin resistance and defects in insulin clearance. Our signaling studies suggest that IR is required for BMPER-promoted insulin signaling (Fig. 3c). Therefore, we tested whether AAV-BMPER could improve glucose response in IR-iKO mice that developed insulin resistance and glucose intolerance (Fig. 5l). Although AAV-BMPER improved glucose response in diet-induced obese (DIO) and db/db mice (Fig. 5a-k), it failed to improve insulin resistance or glucose clearance in IR-iKO mice (Fig. 5l). It suggests that BMPER protects glucose homeostasis through an IR-dependent mechanism.

We also tested the feasibility of delivering recombinant BMPER protein in vivo. Following the injection of recombinant BMPER into HFD-fed mice, we detected significant normalization of hyperinsulinemia and improved glucose and insulin tolerance responses (Fig. 6). Taken together, our results suggest BMPER supplementation through AAV or recombinant protein delivery can improve T2DM.

Discussion

Taken together, our data unravel an unexpected role for BMPER in glucose homeostasis and indicate BMPER supplementation (i.e. gene therapy or recombinant protein delivery) can be a potential approach to treat T2DM and insulin resistance. Notably, the requirement of NPC1 and endocytosis for BMPER’s action suggests a new perspective for transactivating insulin signaling. Although the exact role for NPC1 in BMPER IR complex formation and downstream insulin signaling events remains to be further characterized, our data strongly support that NPC1 can directly impact insulin signaling pathway through recruiting BMPER to IR.
The endothelium, by virtue of its location, tightly controls metabolic exchange between the circulation and surrounding tissues. Endothelial dysfunction and inflammation have been observed during the development of T2DM and insulin resistance\textsuperscript{6,7}, however, it remains a question how important endothelial cells are in the maintenance of metabolic homeostasis and how its dysregulation contributes to metabolic dyshomeostasis. Here, our data indicate BMPER is downregulated in humans with metabolic syndrome and DIO mice (Fig. 2), suggesting endothelial secretory function might also be disrupted during metabolic stress conditions and contribute to the progression of T2DM and insulin resistance.

**Methods**

**Reagents and antibodies**

All the chemicals and antibodies are listed in the main reagent table (Supplementary Table 3).

**Mice**

BMPER\textsuperscript{flox;flox} mice were generated with standard CRISPR-cas9 gene editing technique. C57BL/6 embryos were microinjected with Cas9 protein, each guided RNA and a circular donor vector. Designed guide RNAs targeted intron 3 and intron 4 of the bmper gene for insertion of loxP sites flanking Exon 4 (Fig. 1a). Guide RNAs were evaluated in vitro using purified Cas9 and PCR-amplified target region. Functional guide RNAs were identified and used for model generation. Two founder animals were identified by specific PCR assays as positive for both 5’ and 3’ loxP site insertions. PCR assays spanning each loxP site followed by sequencing was also performed to confirm the loxP sequences. Spanning PCR assays across both loxP sites was performed to confirm cis orientation. Further validation to rule out random integration of the donor vector was performed, including long PCR assays across 5’
and 3’ homology arms and PCR assays specific to the donor vector backbone. In addition, off-target analysis was performed on the original founders and no hits in any of the potential off-target sites screened were identified. Male chimeras were mated to wild-type C57BL/6 females to establish an isogenic line, and then backcrossed to C57BL/6 mice to obtain BMPER\(^{\text{flox/flox}}\) mice on C57BL/6 background. All experiments were conducted on the resulting C57BL/6 background. Genomic DNA of BMPER\(^{\text{flox/flox}}\) mice was isolated as described previously\(^{12}\) and subjected for standard PCR assays to identify wild-type and targeted alleles. A PCR assay has been developed to genotype the pups. PCR samples were denatured in 95°C for 2 min, and then subjected to 35 cycles of three-step amplification, a 30-s 94°C denaturation, 30-s 50°C annealing, and 1-min 72°C extension step. A 740-bp product represents the wild-type allele and an 820-bp product (primers BMPER-SeqF and BMPER-SeqR) indicates the target allele. PCR primers: BMPER-SeqF, 5’- CGCACCCCTCTAA CCTGTTCAGAC-3’; BMPER-SeqR, 5’_AGAACCACTGTTTTGCTCCAAGC-3’. The B6.Cg-Tg (CAG-cre/Esr1*) 5Amc/J (CAG-CreER\(^{+/c}\)) mice were obtained from Jackson Laboratories. The Cdh5 (PAC)-CreERT2 (Cdh5-CreER\(^{+/c}\)) mice were generously provided by Dr. Ralf H. Adams\(^{34}\). All mice were housed on a 12-hour light/dark cycle, with food and water \textit{ad libitum}. All experimental procedures on mice were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee for the Use of Animals in Research at Baylor College of Medicine.

We used the mating of BMPER\(^{\text{flox/flox}}\) and CAG-CreER\(^{+/c}\) or BMPER\(^{\text{flox/flox}}\) and Cdh5-CreER\(^{+/c}\) mice to generate the BMPER\(^{\text{flox/flox}}\), CAG-CreER\(^{+/c}\) (WT or iKO) mice or BMPER\(^{\text{flox/flox}}\); Cdh5-CreER\(^{+/c}\) (eWT or eKO) male mice for control chow (CC, 14.7% calories from fat) or high-fat diet (HFD, 60% calories from fat)- induced diabetes studies. In addition, IR-iKO and their littermate control IR-WT mice were generated from the mating of IR\(^{\text{flox/flox}}\) and CAG-CreER\(^{+/c}\) mice followed by tamoxifen injection. Blood serum was obtained before and after they were fed with different diets. Primary hepatocytes were
isolated from 5~8 weeks mice. For insulin signaling experiments, mice were injected with insulin at 0.5 U/kg (for CC fed mice) or 1.0 U/kg (for HFD and db/db mice) and blood glucose levels were monitored. For adeno-associated virus (AAV)-transduced experiments, C57BL/6 and db/db mice at five weeks old were injected intravenously with the AAV-GFP or AAV-BMPER respectively at the titer at $\sim 10^{12}$ per 25 g mice. C57BL/6 mice were then fed CC or HFD and db/db mice were fed CC as indicated. Metabolic studies were performed with these mice and tissues and serum were collected for further analysis.

**Subjects**

Plasma samples were obtained from the study\textsuperscript{25} that was approved by the Institutional Review Board of Baylor College of Medicine. Male and female volunteers were recruited at the Center for Cardiometabolic Disease Prevention at Baylor College of Medicine, Houston, Texas, or by advertisement.

**Cell lines and primary cells**

HEK293 cells were grown in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin, 68.6 mol/L streptomycin). Primary hepatocytes were isolated using collagenase perfusion method from C57BL/6 mice based on previously published paper with modifications\textsuperscript{35}. Briefly, the liver from 5 ~8 weeks old mice was perfused through the inferior vena cava with 7 ml pre-warmed (37°C) liver perfusion medium at 1 ml/min. Then, the liver was constantly digested with 5 ml pre-warmed (37°C) collagenase (1mg/ml) at 1 ml/min. The perfused liver was chopped gently in DMEM and centrifuged at 72 g for 2 minutes. Cells were washed with 20 ml hepatocyte wash medium and purified with 20 ml 45% (v/v) Percoll solution at 72 g for 2 min. Cell viability and number were measured through Trypan blue exclusion and manual counting. Hepatocytes were cultured in William's E medium containing 5% FBS, 100 units/ ml of penicillin and streptomycin, and primary hepatocyte maintenance supplements at 37°C in a 5% CO$_2$ incubator. For NPC1 depleted cells, primary hepatocytes were transduced with
lentiviral NPC1 shRNA or control virus for 3 days. For signaling experiment, hepatocytes were starved overnight and treated with insulin at 100 nM and BMPER at 100 nM for 1 hour in all the experiments.

**Analysis of endocrine hormones and metabolites**

For fasting blood serum, mice were fasted 4 hours or overnight and then blood was collected through submandibular bleeding using a lancet. Plasma and urinary values for glucose were measured with a mouse endocrine multiplex assay, and insulin and albumin with ELISA kits.

**Glucose/insulin tolerance tests**

Glucose tolerance tests (GTTs) and Insulin tolerance tests (ITTs) were performed following our established protocol\(^3^6\). Briefly, GTTs were performed after an overnight (for CC and HFD-fed mice or \(db/db\) mice) fasting. Blood glucose was measured before and 15, 30, 60, 120 min after an *i.p.* glucose injection (1 g/kg) with a Freestyle Glucose Monitoring System (Abbott Laboratories). ITTs were performed after 4 hours fasting. Blood glucose was measured before and indicated time periods after an *i.p.* insulin injection.

**Hyperinsulinemic-euglycemic clamp studies**

The hyperinsulinemic-euglycemic clamp studies were performed in unrestrained mice using the insulin clamp technique (with constant insulin dose) in combination with \[^3\text{H}\] glucose and \[^{14}\text{C}\]2-deoxyglucose following our established protocol. In summary, mice were cannulated as described previously\(^3^7\) and allowed to recover for 4 to 7 days before the clamp. After an overnight fasting, mice received a primed dose of \[^3\text{H}\]glucose (10 μCi) and then a constant rate intravenous infusion (0.1 μCi/min) of \[^3\text{H}\]glucose using a syringe infusion pump for 90 minutes. Blood samples were collected for the determination of basal glucose production. After 90 minutes, mice were primed with a bolus injection of insulin followed by a 2-hour continuous insulin infusion. Simultaneously, 25% glucose was infused at an adjusted rate to
maintain the blood glucose level at 100-140 mg/dl. Blood glucose concentration was determined every 10 minutes by a glucometer. At the end of a 120-minute period, blood was collected for the measurements of hepatic glucose production and peripheral glucose disposal rates. For tissue specific uptake, we inject 2-deoxy-D-[1,\textsuperscript{14}C] glucose (10 \( \mu \)Ci) into bolus during hyperinsulinemic-euglycemic clamp at 45 min before the end of the clamps and collect blood sample at 5, 10, 15, 25, 35 and 45 minutes. At the end of the clamp, mouse tissues were harvested for the evaluation of glucose uptake. Glucose uptake in different tissues was calculated from plasma 2-[\textsuperscript{14}C] deoxyglucose profile and tissue content of [\textsuperscript{14}C] glucose-6 phosphate.

**Indirect calorimetry and metabolic cage studies**

This assay was performed following our established protocol\textsuperscript{36} with minor modification. Mice were individually housed in metabolic chambers maintained at 20–22\(^\circ\)C on a 12-h light/dark cycle. Metabolic measurements (oxygen consumption, food and water intake, locomotor activity) were recorded using an Oxymax/CLAMS (Columbus Instruments) open-circuit indirect calorimetry system. Food and water intake were also monitored. To determine the amount of urinary albumin excretion, individual mice were separated in a metabolic cage, where urine was collected and measured for 24 h. The urinary volume and its glucose and albumin concentration was determined.

**Membrane fractionation**

Membrane fractionation was performed following our published protocol with small modification\textsuperscript{15}. Briefly, tissue or cells were washed twice with cold PBS and suspended in buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA, 1 mM DTT, protease inhibitor). The mixture was homogenized and incubated on ice for 10 min, and then centrifuged at 4\(^\circ\)C at 10,000 g for 3 min. The supernatant was collected and further centrifuged at 100,000 g at 4 \(^\circ\)C for 1 hour. The cloudy pellet was washed and dissolved in the lysis buffer as the membrane fraction, which was used for further analysis.
Immunoblotting and immunoprecipitation

Immunoblotting experiments were performed based on our previously published paper\(^\text{38}\). Briefly, cells were harvested in lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol and protease and phosphatase inhibitors) and clarified by centrifugation at 8000 g for 5 min. Equal amounts of protein were loaded on the SDS-PAGE gel and subjected for Western blotting. For endogenous immunoprecipitation (IP) experiments, protein A/G Plus-agarose was used to pull down antibody complexes following our established methods\(^\text{13}\). For transient transfection, HEK293 cells were transfected with Flag-tagged NPC1, GFP-tagged BMPER plasmid or both together with Lipofectamine 2000. Two days later, cell lysates were harvested and IPed with anti-Flag resin and precipitates were blotted with anti-GFP and anti-flag antibodies. For endogenous IP, primary hepatocytes were transduced with lentiviral control or NPC1 shRNA, respectively. 3 days later, purified flag-tagged BMPER protein was added to the medium for 30 min. Cells were then washed with cold PBS and cross-linked with DSP at 4°C for 2 hours. Cells were harvested and membrane fractions were used for IP experiments.

Gene expression analysis (real-time PCR)

Total RNAs were reversely transcribed into cDNAs with iScript\textsuperscript{TM} cDNA synthesis kit. The specific pairs of primers used for the real-time PCR are listed in Supplementary Table 4 (designed by Universal ProbeLibrary Assay Design Center tool from Roche, Indianapolis, IN). The real-time PCR was performed with FastStart Universal Probe Master mix, specific primers and probes for each gene (Universal ProbeLibrary Probes #19 for G6Pase, #49 for PEPCK, #58 for GK, #77 for SREBP1, #38 for IL1β, #6 for IL6, #25 for TNFα, #64 for β-Actin, #79 for BMPER, #20 for BMP2, #63 for BMP4, #22 for BMP6, #1 for BMP7, #38 for BMP9, #67 for BMPR2) in Roche Lightcycler 480 PCR machine. Reaction mixtures were incubated at 95°C for 10 min followed by 55 cycles at 95°C for 10 sec and 60°C for 30 sec. β-Actin was used as the housekeeping gene.
Quantification and statistical analysis

No statistical methods were used to predetermine the sample size. No randomization was used as all mice used were genetically defined, inbred mice. Statistical data were drawn from normally distributed group with similar variance between groups. Data are shown as the mean ± SEM. Differences were analyzed with Student’s t-test, 1-way or 2-way ANOVA and followed by a Fisher’s LSD test unless otherwise specifically stated. Values of $P \leq 0.05$ were considered statistically significant.

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DISCLOSURES

None.
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Figure 1. BMPER depletion causes mice to develop hyperinsulinemia, glucose intolerance and insulin resistance. (a) The generation of the BMPER iKO mouse model. The gRNA-guided CRISPR/Cas9 strategy was used for targeted deletion of bmper gene. (b) BMPER depletion was examined with Western blotting. (c-e) Fasted insulin and glucose, HOMA-IR. (f-g) Glucose and insulin tolerance tests. (h) Glucose infusion rate (GIR). (i) Glucose disposal rate (GDR). (j-k) Hepatic glucose production (HGP; j) and glucose uptake in peripheral tissues (k) were analyzed with hyperinsulinemic-euglycemic clamps. GM, gastrocnemius muscle; BAT, brown adipose tissue; WAT, white adipose tissue.
WT, BMPER^flox/flox^; CAG-CreER^+/−^ iKO, BMPER^flox/flox^; CAG-CreER^+/−^ n=4 (b), 8 (c-g) and 7 (h-k). *, P< 0.05. **, P< 0.01. ***, P< 0.001. ****, P< 0.0001. NS, not significant.
Figure 2. BMPER plasma level decreases in metabolic syndrome patients and DIO mice. (a-b) BMPER plasma levels were decreased in metabolic syndrome (MS) patients. (c-f) BMPER plasma level was associated with body weight (BW), insulin and triglyceride (TG) plasma levels in MS patients. Correlation was computed for Pearson correlation coefficients with an assumption of Gaussian distribution. (g-h) BMPER plasma level was decreased in HFD-fed mice. n=11 (b-f) and 8 (h). ***, P< 0.001. ****, P< 0.0001.
**Figure 3.** BMPER promotes insulin signaling through IR but not BMPR2. (a) BMPER (B, 1 hour) or insulin (Ins, 0.5 hour) was injected (i.p.) into mice. Tissues, including liver, GM (gastrocnemius muscle) and Hrt (heart), were used for Western blotting. (b) Hepatocytes were transduced with adenovirus of flag-tagged BMPR2 full-length (FL) or kinase-dead mutant (KD). Cells were then treated with BMPER (1 hr) and harvested for Western blotting. (c) Hepatocytes were isolated from IR-iKO and their littermate control (IR-WT) mice. Cells were treated with insulin (Ins, 0.5 hour) or BMPER (B, 1 hour) and harvested for Western blotting. IR-WT, IR^{flox/flox}, CAG-CreER^{-/-}. IR-iKO, IR^{flox/flox}, CAG-CreER^{+/+}. 
Figure 4. BMPER promotes insulin signaling through NPC1 and endocytosis. (a) Co-immunoprecipitation of GFP-tagged BMPER and flag-tagged NPC1 in HEK293 cells. (b) Hepatocytes were treated with flag-tagged BMPER for 30 min and staining of BMPER and endogenous NPC1 was performed. (c-d) Hepatocytes were transduced with NPC1 shRNA lentivirus and then treated with flag-tagged BMPER (B, 1 hr) or insulin (Ins, 30 min). Western blotting was performed and band intensity was quantified (d). (e) Hepatocytes were transduced with NPC1 shRNA lentivirus and then treated with flag-tagged BMPER (B, 1 hr). Membrane fractions were separated and then subjected for immunoprecipitation with flag antibody. (f-g) Hepatocytes were treated with chlorpromazine (CPM, 50 µM) or methyl-β-cyclodextrin (MCD, 10 mM). 30 minutes later, cells were pulsed with flag-BMPER (100 nM) for 1 hour and cell lysates were used for Western blotting. Internalized BMPER was...
quantified in (g). (h) Hepatocytes were treated with CPM for 30 min, and then treated with BMPER for the detection of IRS1 phosphorylation. n=3 (d, g). *, P < 0.05. ***, P < 0.001. ****, P < 0.0001. NS, not significant.
Figure 5. AAV-BMPER improves glucose responses in diabetic mice. (a) BMPER plasma levels in C57BL/6 (WT), AAV-GFP or AAV-BMPER (B)- injected mice that were fed HFD or CC diet. (b-c) Fasted insulin and glucose. (d) Glucose and insulin tolerance tests. (e) BMPER plasma levels in C57BL/6 (WT), AAV-GFP or AAV-BMPER (B)- injected db/db mice. (f-g) Fasted insulin and glucose. (h) Glucose and insulin tolerance tests. (i) Urinary glucose levels. (j) Urinary albumin levels. (k) Urine volume. (l) Glucose and insulin tolerance tests. IR-WT, IR^{flox/flox}; CAG-CreER^{+/-}. IR-iKO, IR^{flox/flox}; CAG-CreER^{+/-}. n=3 (a), 8 (b-d), 5 for the AAV-GFP and 7 for AAV-BMPER group (e-k) and 4 (l). *, P<0.05. **, P<0.01. ***, P<0.001. ****, P<0.0001.
Figure 6. Recombinant BMPER protein improves glucose responses in DIO mice. C57BL/6 mice were fed HFD or control chow (CC) for 4 weeks and then injected with recombinant BMPER protein at 0.1 mg/kg/mouse every other day for 6 weeks. Metabolic parameters were measured, including (a) Body weight. (b) Fasted insulin. (c) Fasted glucose. (d) Glucose tolerance tests. (e) Insulin tolerance tests. n=5 for each group. *, P<0.05. **, P<0.01. ****, P<0.0001. NS, not significant.
**Supplementary Table 1. Metabolic parameters.**

| Parameters | WT          | iKO          |
|------------|-------------|--------------|
| BW (g)     | 32.25 ± 0.86 | 32.18 ± 1.05<sup>(NS) </sup> |
| Glucose (mg/dl) |            |              |
| Fasted     | 139.0 ± 4.79 | 150.0 ± 3.81<sup>(NS) </sup> |
| Fed        | 174.7 ± 6.12 | 176.4 ± 8.134<sup>(NS) </sup> |
| Insulin (ng/ml) |        |              |
| Fasted     | 1.31 ± 0.13  | 2.80 ± 0.22<sup>c</sup> |
| Fed        | 2.81 ± 0.20  | 16.77 ± 0.42<sup>d</sup> |
| TG (mg/dl) | 73.12 ± 4.25 | 85.78 ± 3.30<sup>a</sup> |
| FFA (mEq/l) | 0.78 ± 0.10  | 0.78 ± 0.05<sup>(NS)</sup> |

NS, not significant.

<sup>a</sup> P<0.05, <sup>b</sup> P<0.01, <sup>c</sup> P<0.001, <sup>d</sup> P<0.0001

WT, BMPER<sup>floxflox</sup>;CAG-CreER<sup>−/−</sup>.  

iKO, BMPER<sup>floxflox</sup>;CAG-CreER<sup>+/−</sup>.  

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<sup>NS</sup> not significant.
Supplementary Table 2. Mass spectrometry result shows representative proteins that potentially form complex with BMPER.

| Gene ID | GeneSymbol | SRA | PSMs | iBAQ |
|---------|------------|-----|------|------|
| 3939    | LDHA       | S   | 118  | 0.38 |
| 4000    | LMNA       | S   | 100  | 0.17 |
| 8514    | KCNAB2     | S   | 49.2 | 0.12 |
| 4864    | NPC1       | S   | 19.2 | 0.05 |
| 54887   | UHRF1BP1   | S   | 13.5 | 0.02 |

S: high confidence; R: medium confidence; A: weak confidence

PSMs: total normalized peptide hits

iBAQ: total abundance of the gene
Supplementary Table 3. Main reagent table.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| LRP1-CTD            | Sigma  | Cat#L2170  |
| BMPER (Crossveinless-2/CV-2) | R&D Systems | Cat# AF2299 |
| NPC1                | Abcam  | Cat# 124801 |
| IRS-1               | Cell signaling | Cat#2382 |
| phospho-IRS1 (Tyr612) | Millipore | Cat#09-432 |
| Akt                 | Cell signaling | Cat#9272 |
| phospho-Akt (Ser473) | Cell signaling | Cat#9271 |
| phospho-Akt (Thr308) (D25E6) | Cell signaling | Cat#13038 |
| Insulin Receptor β (4B8) | Cell signaling | Cat#3025 |
| β-Actin (C4) -HRP   | Santa Cruz | Cat#sc-47778 |
| Smad1               | Cell signaling | Cat#9743 |
| phospho-Smad1/Smad5/Smad8 (Ser463/465) | EMD Millipore | Cat#AB3848-I |
| Flag antibody       | Sigma-Aldrich | Cat#2426 |
| EZview Red ANTI-FLAG M2 Affinity Gel | Sigma-Aldrich | Cat#F2426 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| 60% high-fat diet (HFD) | Research Diets | Cat#D12492 |
| Insulin             | Novolin R | Cat#0169-1833-11 |
| Mouse BMPER (Crossveinless-2) Protein | R&D Systems | Cat#2299-CV-050 |
| Protein A/G Plus-agarose beads | Santa Cruz | Cat#sc-2003 |
| iScript™ cDNA synthesis kit | Bio-Rad Laboratories | Cat#1708891 |
| Item                                    | Manufacturer          | Cat#       |
|-----------------------------------------|-----------------------|------------|
| iTaq SYBR Green supermix                | Bio-Rad Laboratories  | Cat#1725121|
| TaqMan™ Universal PCR Master Mix       | Thermo Scientific     | Cat#4304437|
| Insulin solution                       | Santa Cruz            | Cat# 11061-68-0 |
| Cell maintenance supplements           | Gibco                 | Cat# A15564|
| Tamoxifen                              | Sigma-Aldrich         | Cat#T5648  |
| Lipofectamine 2000                     | Thermo Scientific     | Cat#11668019|
| Perfusion Medium                       | Thermo Scientific     | Cat#17701038|
| Collagenase                            | Sigma-Aldrich         | Cat#C5168  |
| DMEM                                   | Thermo Scientific     | Cat#11965118|
| William’s E Medium                     | Thermo Scientific     | Cat#12551-032|
| Immobilon-P transfer membrane          | Millipore             | IPVH00010  |
| Wash Medium                            | Thermo Scientific     | Cat#17704-024|
| Percoll                                | GE Healthcare         | Cat#17089101|
| OptiPrep density gradient medium       | Sigma-Aldrich         | Cat#1556   |
| Penicillin & Streptomycin              | Thermo Scientific     | Cat#15140122|
| Fetal bovine serum (FBS)               | Sigma-Aldrich         | Cat#F0926  |
| Horse serum                            | Thermo Scientific     | Cat#16050114|
| Maintenance supplements                | Thermo Scientific     | Cat#CM4000  |
| Chlorpromazine (CPM)                   | Sigma-Aldrich         | Cat#C8138  |
| Methyl-β-cyclodextrin (MCD)             | Sigma-Aldrich         | Cat#M7439  |

**Critical Commercial Assays**

| Item                                    | Manufacturer          | Cat#       |
|-----------------------------------------|-----------------------|------------|
| Insulin ELISA kit                       | Millipore             | Cat#EZRMI-13K|
| RNA purification kit                    | QIAGEN                | Cat#74104  |
| Infinity Glucose kit                    | Thermo Scientific     | Cat#TR15421|
| Albumin ELISA kit                       | Abcam                 | Cat#ab108792|
## Experimental Models: Cell Lines

| Primary hepatocytes | This paper | N/A |
|---------------------|------------|-----|
| HEK293 cells        | ATCC       | Cat#CRL-1573 |

## Experimental Models: Organisms/Strains

| Mouse: B6.Cg-Tg (CAG-cre/ Esr1*) 5Amc/J (CAG-CreER<sup>+</sup>) | Jackson Laboratories | JAX: 004682 |
|--------------------------|----------------------|-------------------|
| Mouse: Cdh5(PAC)-CreERT2 (Cdh5-CreER<sup>+</sup>)                | Dr. Ralf H. Adams from Max Planck Institute for Molecular Biomedicine, Germany | N/A |
| Mouse: C57BL/6            | Jackson Laboratories | JAX: 000664 |
| Mouse: IR<sup>flex</sup>  | Jackson Laboratories | JAX: 006955 |
| Mouse: B6 db             | Jackson Laboratories | JAX: 000697 |

## Recombinant DNA

| Npc1 mouse shRNA plasmid | OriGene               | Cat#TL501501 |
|--------------------------|-----------------------|--------------|
| pCMV-Tag2 vector         | Agilent               | Cat#211172   |
| Scrambled shRNA plasmid  | OriGene               | Cat#TR30021  |
| pLenti-C-Flag-DDK-BMPER   | This paper            | N/A          |
| Ad-CMV-iCre              | Vector Biolabs        | Cat#1045     |
**Supplementary Table 4. Sequence information for qPCR primers.**

| Gene   | Forward primer                     | Reverse primer                     |
|--------|-----------------------------------|-----------------------------------|
| G6Pase | 5'- tcgtcccgatatagctttg-3'         | 5'- gaaagtttcagccacagaa-3'        |
| PEPCK  | 5'- gagttacccattgagggtacat-3'      | 5'- gctgaggcttcatagacaag-3'       |
| GK     | 5'- cagatgtggtgacagacgac-3'       | 5'- gccaggatctgctctacctt-3'       |
| SREBP1 | 5'- acaagattgtggagctcaagac-3'     | 5'- tgcgcaagacagcagatitta-3'      |
| IL1β   | 5'- agttgacggaccccaaaag-3'        | 5'- agctggtgatcctctcatcagg-3'     |
| IL6    | 5'- gctaccaaactggataataacag-3'    | 5'- ccaggtgatagttgtaactccagaa-3'  |
| TNFα   | 5'- ctgtagcccacgtcgtagc-3'         | 5'- ttgatccctgctc-3'              |
| β-Actin| 5'- ccacgctgaaaagatgacc-3'        | 5'- accagagcatacaggagc-3'         |
| BMPER  | 5'- ggtctagccatgtgcctgtc-3'       | 5'- cgcacctgacacagctccag-3'       |
| BMP2   | 5'- agatccttgacgagcact-3'         | 5'- gttcctcagacggttct-3'          |
| BMP4   | 5'- gatctttatctcgctagct-3'        | 5'- tggatgttcctcagatgtc-3'        |
| BMP6   | 5'- actgacgtgctgccagga-3'         | 5'- tgtggagaacactctgc-3'          |
| BMP7   | 5'- cgacacgctcggatacaagt-3'       | 5'- cagcaagaagaggtgcgac-3'        |
| BMP9   | 5'- ggaagctggtggtagatgcc-3'       | 5'- caagtcgctgggagatg-3'          |
| BMPR2  | 5'- gactctcctccgtgacctg-3'        | 5'- gtagcactcgcgtc-3'             |
Supplementary Figure 1. Gene expression changes in liver and WAT tissues of BMPER iKO mice. (a-d) Real-time PCR assays were performed for indicated genes in the liver (a, b, d) and WAT (c) of BMPER iKO and their littermate control (WT) mice. n=5 (a-c) and 7 (d). *, P< 0.05. **, P< 0.01. NS, not significant.
Supplementary Figure 2. CLAMS studies with BMPER iKO mice. Indirect calorimetry studies were performed to evaluate VO$_2$ (a), VCO$_2$ (b), respiratory exchange ratio (RER, c), heat (d), locomotor activity in x axis (XTOT, e) and z axis (ZTOT, f), daily food (g) and water intake (h). n=5 (a-h). *, $P<0.05$. **, $P<0.01$. 
Supplementary Figure 3. BMPER depletion exacerbates glucose responses in HFD-fed mice.

Metabolic studies were performed with BMPER iKO and WT mice following eight weeks of HFD feeding. (a) Body weight. (b-c) Fasted insulin and glucose. (d-e) Glucose and insulin tolerance tests. HFD, high-fat diet. n= 4 (a-e). *, P< 0.05. **, P< 0.01. NS, not significant.
Supplementary Figure 4. BMPER depletion in ECs leads to glucose dysregulation. Metabolic studies were performed with BMPER eKO and eWT mice at four months after tamoxifen injection. (a) Body weight (BW). (b-d) Fasted glucose and insulin, HOMA-IR. (e) Fed insulin. (f-g) Glucose and insulin tolerance tests. eWT, BMPER<sup>flox/flox</sup>; Cdh5-CreER<sup>+/−</sup>. eKO, BMPER<sup>flox/flox</sup>; Cdh5-CreER<sup>+/−</sup>. n=5 (a-g). *, P< 0.05. **, P< 0.01. ***, P< 0.001. NS, not significant.
Supplementary Figure 5. BMPER depletion in ECs leads to insulin resistance. Clamp studies were performed with BMPER eKO and eWT mice at four months after tamoxifen injection. (a) Glucose infusion rate (GIR). (b) Glucose disposal rate (GDR). (c-d) Hepatic glucose production (HGP; c) and glucose uptake in peripheral tissues (d) were analyzed with hyperinsulinemic-euglycemic clamps. GM, gastrocnemius muscle; WAT, white adipose tissue. n=5 (a-d). *, P< 0.05. NS, not significant.
Supplementary Figure 6. The regulation of BMPER-promoted insulin signaling pathway. Hepatocytes were transduced with adenovirus of flag-tagged BMPR2 full length (FL) or kinase-dead mutant (KD). Cells were then treated with BMP2 (4 nM, 1 hr). (b) NPC1 was identified in BMPER-bound complex. HEK293 cells were treated with BMPER (30 nM) and lysates were immunoprecipitated with anti-BMPER antibody and stained with Coomassie blue. Selected bands were subjected for mass spectrometry analysis. (c) Quantitative data for Figure 4e. (d) Hepatocytes were pulsed with Flag-BMPER (100 nM, 7.2 ng) for indicated time periods (left top panel), and then chased at 4°C (left bottom panel). Right panel is graphic representation of normalized intracellular BMPER level from the cold chase. (e) Quantitative data for Figure 4h. n=3 (c, e). ***, P< 0.001. ****, P< 0.0001.
Supplementary Figure 7. AAV-BMPER improves glucose responses in DIO mice. AAV-BMPER or AAV-GFP was injected into C57BL/6 mice and then fed them HFD for eight weeks. (a) Food intake in AAV-GFP or AAV-BMPER (B) injected mice that were fed HFD or CC diet. (b) Body weight. (c-d) Fed insulin and glucose. (e-f) Glucose and insulin tolerance tests. n=6 (a), 8 (b-f). *, P < 0.05. **, P < 0.01. ****, P < 0.0001. NS, not significant.
Supplementary Figure 8. AAV-BMPER improves glucose responses in db/db mice. AAV-BMPER or AAV-GFP was injected into 5-week-old db/db mice and then monitored for eight weeks. (a) Food intake in AAV-GFP or AAV-BMPER (B)- injected db/db mice. (b) Body weight. (c-d) Fed insulin and glucose. n=5 for AAV-GFP and 7 for AAV-BMPER group. *, P< 0.05. NS, not significant.