Supplemental Data for

Soluble (Pro) Renin Receptor Treats Metabolic Syndrome in Mice with Diet-Induced Obesity via Interaction with PPARγ

Supplemental Methods

Hematocrit measurement. Hematocrit (Hct) was measured in lean, DIO and DIO+sPRR-His groups. The sphenous vein was punctured using a 23-gauge needle, and one drop of blood (~5-10 µl) was collected by using a 10 µl capillary glass (Idaho Technology, Salt Lake City, UT). One side of the tube was sealed with Hemato-Seal and then centrifuged for 2 min in a Thermo IEC MICRO-MB microcentrifuge machine.

Glucose tolerance test (GTT) and intraperitoneal insulin tolerance test (ITT). After 2 wk of sPRR-His infusion, an intraperitoneal ITT was performed on mice after an 8-h fast, and blood samples were drawn at different times following insulin injection (0.75 U/kg ip). For the GTT, mice were fasted for 8 h, and blood samples were taken from the tail vein after the 1 g/kg glucose injection. Blood glucose concentrations were measured by using a glucometer (OneTouch).

Comprehensive lab animal monitoring system metabolic chamber experiments. Each mouse was measured individually in a resting state for 24 h at 23°C in the presence of food and water by using a computer-controlled, comprehensive lab animal monitoring system (Columbus Instruments, Columbus, OH). Body weight, food intake, water intake, O_2 consumption, CO_2 production, heat production, and respiration exchange ratio were measured automatically.

Plasma volume determination. Under general anesthesia with isoflurane (2 mL/min), FITC-dextran 500000-conjugate (FITC-d, 2 mg/100 g 46947–100MG-F, Sigma) was injected into the jugular vein. Seven minutes later, blood was withdrawn from the vena cava. Plasma was separated by centrifugation of the blood at 4,000 rpm for 10 min in the dark. Fluorescence levels were measured at an excitation wavelength of 485 nm and emission wavelength of 520 nm (Synergy Neo2 Hybrid Multi-Mode Reader, BioTek Instruments), and the FITC-d concentration per milliliter of plasma was calculated based on a standard curve generated by serial dilution of the 2 mg/mL FITC-d solution. The standard curve was linear and highly reproducible. The plasma volume data were shown as relative values normalized by body weight.

GFR measurement. Mice were injected with fluorescein isothiocyanate-sinistrin (Mannheim Pharma and Diagnostics, Mannheim, Germany) retro-orbitally (7.5 mg/100 g body weight). The NIC-Kidney (Mannheim Pharma and Diagnostics, Mannheim, Germany) was used to detect fluorescence in the skin on the shaved back over 1 h. GFR was calculated based on the kinetics of fluorescence decay.

Cell culture and differentiation. Mouse 3T3-L1 pre-adipocytes (ATCC, Manassas, VA) were cultured with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% donor calf serum in an atmosphere of 10% CO_2 at 37°C. Two days after the 3T3-L1 fibroblasts had reached confluence, differentiation was induced by treating the cells with DMEM containing 4 μg/mL dexamethasone, 0.5 mM 3-isobutyl1-methylxanthine, 200 nM insulin (Sigma), and 10% fetal bovine serum (FBS) for 48 h. Cells were fed DMEM supplemented with 10% FBS every other day and used as mature 3T3-L1 adipocytes on day 8 after the induction of differentiation.

2-Deoxyglucose (2-DG) uptake assay. The mature 3T3-L1 adipocytes or rat aorta smooth muscle cell line (VSMC) were starved for 12 h and then treated with sPRR-His (10 nM), PF (10μM), anti-PRR antibody (1.5 μg/mL), and rosiglitazone (1 μM) alone or as combination treatment for 24 h or 30 min. Glucose uptake was then assessed by using a 2-DG Uptake Assay Kit according to the manufacturer′s instructions (Abcam, ab136955). Briefly, cells were starved in serum-free medium overnight and then in Krebs-Ringer Phosphate-HEPES buffer with 2% bovine serum albumin for 40 min. After insulin stimulation, the glucose analog 2-DG was added to cells and the accumulated 2-DG6P was oxidized to generate NADPH, which resulted in oxidation of a substrate. The oxidized substrate could then be detected at optical density = 412 nm.

Preparation of luciferase constructs. Genomic DNA was extracted from rat tail by using a Tissue DNA Kit (D3396-01; Promega). A 2,016-bp fragment of the 5′ flanking region of the *PRR* gene (GenBank accession no. NM_001007091; 1,941 ± 75 bp) was amplified from the rat genomic DNA by PCR and subcloned to the pGL3-Luc Basic reporter vector (Promega) by using NheI and BgIII restriction sites (termed pGL3-PRR-Luc). The *PRR* promoter contains two putative PRREs at positions -834 to -828 bp (AGGTCA) and -318 to -312 bp (GGTGCA). To mutate these two putative PPREs, we performed PCR with the following two primer sets: primer set 1, forward primer: 5′-CATCTTTCATTTTCATCAGCTGGG-3′ and reverse primer: 5′-CCCAGCTGATGAAAATGAAAGATG-3′; primer set 2, forward primer: 5′-GGGAGGGATTTGCAAGATCGGG-3′ and reverse primer: 5′-CCCGATCTTGCAAATCCCTCCC-3′. After that, the *PRR* promoter with the putative PRRE sites at positions -834 to -828 bp and -318 to -312 bp were mutated to TTTTCA and TTTGCA,

respectively. These PCR products were subcloned to the pGL3-Luc Basic reporter vector to generate −834/-828Δ pGL3-PRR-Luc and -318/-312Δ pGL3-PRR-Luc. The identity of these constructs was validated by sequencing.

Luciferase assay. The mature 3T3-L1 adipocytes were transfected with pGL3-PRR-Luc or -834/-828Δ pGL3-PRR-Luc or -318/-312Δ pGL3-PRR-Luc or empty vector by using HiPerFect Transfection Reagent (catalog no. 301702; Qiagen). After 72 h, all cells were starved for 12 h; the transfected cells were then treated for 24 h with rosiglitazone (1 μM). The vehicle-treated group served as a control. The luciferase activities were measured by using a luciferase assay system (Promega), and luminescence was detected by using an illuminometer (BMG FLUOstar OPTIMA).

Figure S1.

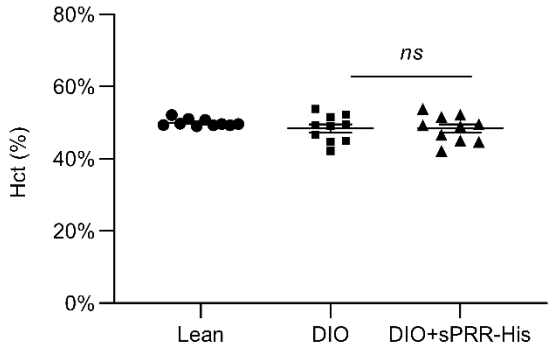

Figure S1. Effect of sPRR-His on Hct in DIO mice (n =10). Statistical significance was determined by using ANOVA with the Bonferroni test for multiple comparisons. Data are mean ± SE.

Figure S2.

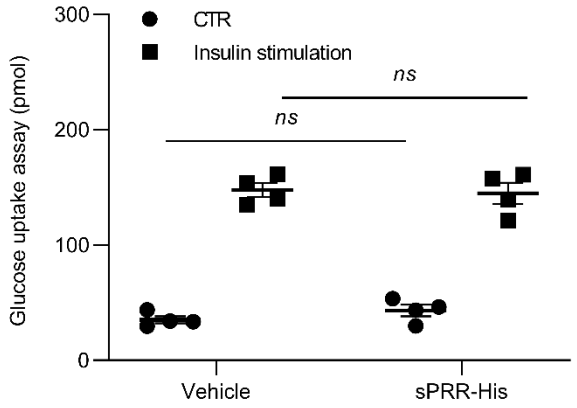

Figure S2. Effect of short-term sPRR-His treatment on basal and insulin-induced glucose uptake in VSMCs (n=4). Statistical significance was determined by using ANOVA with the Bonferroni test for multiple comparisons. Data are mean ± SE.

Figure S3.

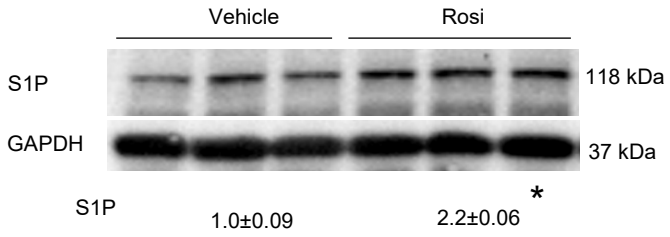

Figure S3. The effect of Rosiglitazone (Rosi) on S1P protein expression in 3T3-L1 adipocytes (n=3, repeat 3 times). The cells were treated for 24 h with vehicle or Rosi, followed by immunoblotting analysis of S1P. The same samples were run on a separate gel for detecting GAPDH. The densitometry values were shown underneath the blot. * means $p<0.05$ vs. Vehicle group. Statistical significance was determined by using unpaired student's t-test. Data are mean ± SE.