Supplemental Methods and Data

Retinol Binding Protein 7 is an Endothelial-Specific PPARγ Cofactor Mediating an Antioxidant Response Through Adiponectin

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Supplemental Experimental Procedures

Metabolic tests

Glucose and insulin tolerance tests were conducted in about 20-week high fat diet (HFD)-fed male and female RBP7-deficient mice and their littermate controls, as well as in age-matched normal diet (ND)-fed littermates. After five hours of fasting, glucose tolerance test was performed, and blood glucose was measured with a commercial glucometer before and 15, 30, 60, and 120 minutes after glucose injection (1 g/kg, IP). After one week recovery, insulin tolerance testing was conducted in the same cohort of mice fasted for five hours. Blood glucose was measured before and 15, 30, 60, and 120 minutes after bolus injection of human regular insulin (0.75 U/kg, IP; Humulin R U-100).

Plasma metabolites

Twenty-week HFD-fed RBP7-deficient and their control mice as well as age-matched ND-fed littermates were fasted overnight. Blood samples were obtained through cardiac puncture, and blood glucose was measured as described above. Plasma was collected from EDTA-treated blood samples via centrifugation (7000 rpm, 5 minutes, 4°C). Plasma RBP4 and adiponectin were determined by ELISA (41-RBPMS-E01, 47-ADPMS-E01, respectively, ALPCO).

Histology

Livers were collected from 20-week HFD-fed RBP7-deficient and their control mice, as well as age-matched ND-fed littermates, and frozen in OCT compound on dry ice. Frozen sections (7 μm) were cut using a cryostat, and stained with oil red O for detection of neutral lipids (k043, Poly Scientific).

Angiotensin II (Ang-II) administration

Ang-II was infused for 2 weeks through an osmotic minipump (Alzet, model 1002) as previously described (1, 2). Briefly, during general anesthesia with isoflurane, a minipump was implanted subcutaneously into the mid-scapular region to administer Ang-II (subpressor or pressor dose at a rate of 120 or 1000 ng/kg/min, respectively) or vehicle (isotonic saline).

Blood pressure (BP) measurements

In HFD-fed obesity model, BP was measured by radiotelemetry (TA11PA-C10, Data Sciences International) as previously described (1, 2). Radiotelemeters were implanted in 6-week HFD-fed RBP7-deficient and their control mice as well as age-matched ND-fed littermates. After one week of recovery, BP was continuously recorded for 7 days (sampling every 5 min for 10-second intervals) at the beginning of each of the following points: 8-week, 12-week, 16-week, and 20-week HFD-fed period. Data were collected and stored using Dataquest ART. For each mouse, data was averaged hourly, and corresponding times across 7 days were averaged to create a single composite 24-hour tracing. In Ang-II-administered model, BP was measured by tail cuff (Visitech 2000 System). Mice were trained for 2 consecutive weeks before 1-week baseline BP measurement, following by osmotic minipump implantation and BP measurement for 2 more consecutive weeks. Data was averaged every 5 days to obtain the mean systolic BP for each mouse.

Reactive oxygen species (ROS) detection by dihydroethidium (DHE) fluorescence

ROS production in carotid arteries was evaluated using DHE staining (Sigma-Aldrich Biochemical) as described (2). Carotid arteries were embedded in OCT compound, and frozen sections (10 μm thick) were incubated for 15 minutes at room temperature in PBS (1X) containing 8 μmol/L DHE. Images were visualized with a confocal microscope (Zeiss LSM710) at an excitation/emission of 488/568 nm, and analysed using ImageJ software.

Immortalized mouse lung endothelial cell (MLEC) culture

MLECs were a kind gift from Dr. Brandon S. Davies (Department of Biochemistry, the University of Iowa) and were described previously (3-5). MLECs were cultured in MCDB-131 complete medium with antibiotics (VEC Technologies). To determine whether RBP7 is a PPARγ-dependent gene in MLECs, the cells were seeded in 6-well cell culture plate and treated with different dose of rosiglitazone (Rosi, 0.1 μM and 1.0 μM) for 24 hour in the presence or absence of PPARγ antagonist GW9662 (10 μM). Then, MLECs were collected for conducting real-time quantitative RT-PCR as described below.

Western blotting

MLECs were infected with adenovirus-mediated transduction (multiplicity of infection = 10) of GFP (AdGFP) or PPARγ (AdPPARγ) for 24 hours, and then cells were treated with rosiglitazone (10 μM) or vehicle dimethyl sulfoxide (DMSO) for another 24 hours. In another cohort, MLECs were infected with different doses of AdGFP or Ad-PPARγ (multiplicity of infection = 20, 50, and 100) for 72 hours. In the experiment determining the bioactivity of adiponectin, MLECs were plated in a 6-well plate and starved overnight. When they reached about 80% confluence, MLECs were treated with different dose of adiponectin (5 μg/ml, 15 μg/ml) for 15 min, which has been shown to increase the phosphorylation of endothelial nitric oxide synthase (eNOS) (6). Insulin (100 nM) treatment was used as positive control. Following above treatments, MLECs were homogenized in a lysis buffer containing: 50 mmol/L Tris Cl buffer, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA (pH 7.5), 1% w/v Na deoxycholic acid, 1% v/v NP-40 and 0.1% v/v SDS, with protease inhibitors (Roche) with or without phosphatase inhibitors (Roche). Protein lysates were separated by SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). The immunoblots were incubated with monoclonal anti-PPARγ (C26H12, Cell Signaling) and

polyclonal anti-RBP7 (14541-1-AP, Proteintech), P-T172-AMPKα (2535, Cell Signaling), and total AMPKα (2532, Cell Signaling). GAPDH or actin was used as a loading control.

Endothelial cell isolation

The method was modified according to a previous report (7). Male and female RBP7-deficient and their control mice (8-12 weeks) were utilized, and were perfused with 20 mL heparinized DPBS (1X) through left ventricle following euthanasia with CO_2. The mouse kidney, lung and aorta were isolated, cleared of lymph nodes and skeletal muscle and minced in 1 mL digestion buffer containing collagenase A (1 mg/mL, Roche), collagenase B (1 mg/mL, Roche), and DNase I (100 µg/mL, Roche). Minced tissues were digested by incubation at 37 °C for 20 minutes before being passed through a 70 µm cell strainer to yield single cell suspension (Falcon). Cells were centrifuged for 5 minutes at room temperature (300 x g). The pellets were resuspended in 80 µL Magnetic Activated Cell Sorting (MACS) buffer in DPBS (1X) containing 1% bovine serum albumin (BSA) and 0.5% NaN3, and 20 µL cardiac endothelial cell isolation cocktail (130-104-183, Miltenyi Biotec). These cell suspensions were incubated for 15 minutes at 4°C in the dark, and then washed with 2 mL of MACS buffer and centrifuged at 300 x g for 5 minutes at 4°C. The supernatant was discarded and the cell pellets were resuspended in 500 µL MACS buffer. Endothelial cells were isolated using positive selection method on autoMACS (Miltenyi Biotec, Gladbach, Germany), centrifuged (800 rpm, 10 min, 4°C), and pellets were collected for RNA isolation using the Trizol method.

For PPARγ ligand Rosi-incubated experiments, carotid arteries from RBP7-deficient and their control mice were cleared of adipose tissue and adventitia layer and blood, followed by incubation with Rosi (10 uM) or vehicle DMSO for 24 hrs. Then, endothelial cells were collected as described above to detect gene expression.

Quantitative Real-Time PCR

To detect the gene expression in HFD-fed mice, the carotid artery or aorta was isolated with adipose tissue and adventitia layer and blood removed, and snap-frozen in liquid nitrogen. To detect gene expression in Rosi-incubated carotid artery, the carotid artery was prepared as described above, and incubated with Rosi or vehicle DMSO for 24 hrs followed with/without endothelial cell isolation. RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA). Total RNA was prepared using a RNA mini kit (Invitrogen) according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop spectrophotometer with an OD260/OD280 ratio of greater than 1.9. Total RNA was reverse transcribed using SuperScript III (Invitrogen) and qPCR was conducted using Taqman Gene Expression Assays (Applied Biosystems) or SYBR green (Invitrogen). The assay numbers for TaqMan were as follows: Mm00458145_m1 (mouse RBP7), Mm00456425_m1 (mouse adiponectin), Mm00445878_m1 (mouse FABP4), Mm01287743_m1 (mouse Nox2), Mm00479246_m1 (mouse Nox4), Mm00437992_m1 (mouse Catalase), Mm01344233_g1 (mouse SOD1), Mm01313000_m1 (mouse SOD2), Mm00448831_s1 (mouse SOD3), Mm00435217_m1 (mouse NOS3), Mm01242576_m1 (mouse CD31), Mm01291334_mH (mouse adiponectin receptor 1), Mm01184032_m1 (mouse adiponectin receptor 2), Mm00433972_m1 (mouse Hoxc9), Mm00840165_g1 (mouse Fgf21), Mm01235642_g1 (mouse Cited1), and Mm00441899_m1 (mouse Cd137). The primer sequences for SYBR green were as follows: mouse adiponectin (CCCAAGGGAACTTGTGCAGGTTGGATG, GTTGGTATCATGGTAGAGAAGAAAGCC), mouse GAPDH (CATGGCCTTCCGTGTTCCTA, GCGGCACGTCAGATCCA). The individual amount of mRNA was calculated after normalizing to its corresponding β-actin or GAPDH using the $2^{-\Delta\Delta CT}$ method, as previously described (2).

RNA Sequencing (RNA-Seq)

Paired-end reads (100-bp) from the Illumina HiSeq 2500 were aligned to the mouse transcriptome (UCSC known genes, build mm10) with the Kallisto program. The quality of the reads was assessed using the FastQC program (version 0.11.2). For all samples, more than 98% of the reads were of high quality (phred score > 20). Differential expression was assessed using the Kallisto companion program Sleuth. The gene list was then filtered by p-value (< 0.05), fold change (≥ 1.5), and baseline level of expression (average Transcripts per Million or TPM ≥ 0.1). Data from the RNA-Seq experiment have been deposited at the NCBI Gene Expression Omnibus (accession GSE88706).

Identification of PPARγ target genes

We used expression data and genome-wide chromatin immunoprecipitation (ChIP) studies to search for potential PPARγ target genes. A gene was considered to be a potential target if the following conditions were satisfied: 1) the PPARγ binding site was located within the gene (e.g., intronic) or in the intergenic regions (upstream or downstream) between the gene and its neighboring genes, and 2) the gene was expressed in the cell type used in the corresponding ChIP experiments. Datasets from adipocytes and macrophages, two important sites for PPARγ action, were examined. For adipocytes, an RNA-Seq dataset from 3T3-L1 adipocytes (NCBI-GEO, GSE64757) was used to define the set of adipocyte expressed genes. The corresponding ChIP datasets were from either mouse 3T3-LI adipocytes (8, 9) or from human adipocytes (GSE59703). For the human dataset, PPARγ binding site locations in the human genome were mapped to the mouse genome using the liftover utility. For macrophages, microarray dataset GSE15998 (NCBI-GEO) was used to determine macrophage-expressed genes, and ChIP dataset GSE21314 (NCBI-GEO) was used to examine PPARγ binding.

Identification of Endothelial genes

To identify genes that exhibit enriched expression in endothelial cells, we analyzed microarray dataset GSE3239 (NCBI-GEO). This dataset contained 26 smooth muscle samples and 16 endothelial cell samples, all from human. Differential expression analysis was performed online using the GEO2R utility at NCBI. Ve-cadherin (CDH5), a gene with known endothelial-specific expression, was enriched by 156 fold in this dataset. As an additional measure of endothelial specificity, we used published data (10) that used the TRAP (translating ribosome affinity purification) method to enrich for endothelial-expressed genes.

To assess absolute levels of endothelial gene expression, we used RNA-Seq datasets from human and mouse. For the human data, we used two independent datasets. The first is from a detailed analysis by Hart et al. (11) using data on human umbilical vein endothelial cells (HUVECs) available from the Encode project. The second dataset, also from the Encode project, is from human thoracic aortic endothelial cells (Encode Accession ENCSR000CUK). For the mouse data, we used dataset GSE52564 (NCBI-GEO). In this study, Tie2-EGFP transgenic mouse and Fluorescence Activated Cell Sorting was used to enrich for endothelial cells in the brain. Each of these datasets was associated with high expression of known endothelial marker genes including CDH5 and NOS3.

Chemicals

Ang II, acetylcholine (ACh), sodium nitroprusside (SNP), papaverine, Tempol, superoxide dismutase–polyethylene glycol (PEG-SOD), polyethylene-glycolated catalase (PEG-catalase), hydrogen peroxide (H_2O_2), glucose, recombinant mouse adiponectin protein, and compound C were obtained from Sigma-Aldrich Biochemical; and all of these reagents were dissolved in saline except for adiponectin in 1% BSA and compound C in dimethyl sulfoxide (DMSO). U46619 (Cayman) was dissolved in ethanol with subsequent dilutions made in saline.

Recombinant human insulin (Eli Lilly) was dissolved in saline. H89 (Calbiochem) and rosiglitazone (Cayman) and GW9662 (Cayman) were dissolved in DMSO.

Supplemental References

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Supplemental Figures and Legends

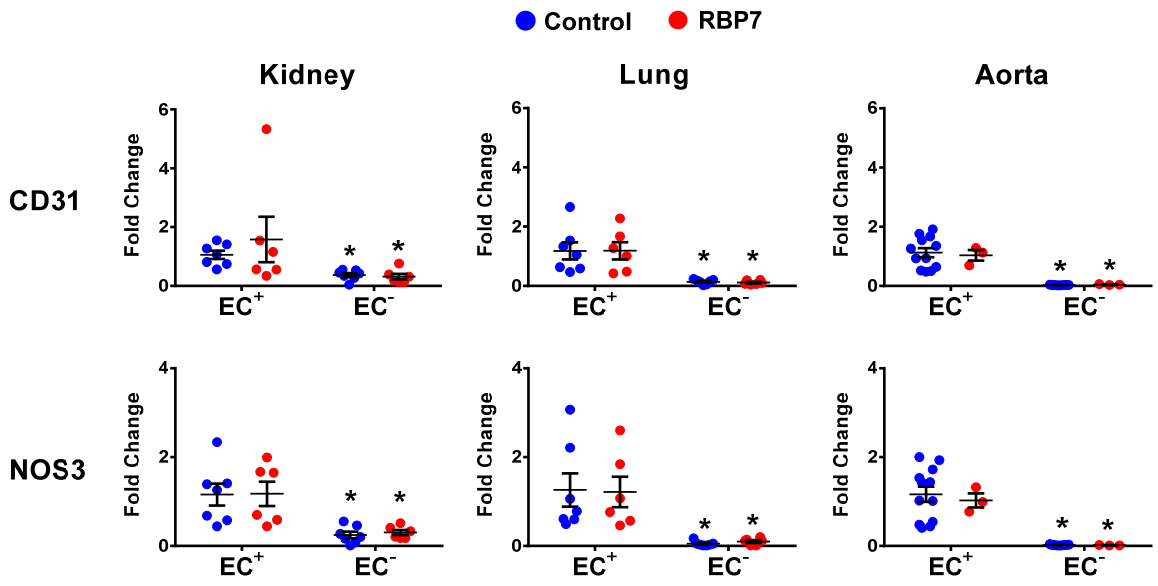

Figure S1. Gene expression of NOS3 and CD31 in endothelial (EC^+) and non-endothelial (EC^-) cells from kidney (N=6-7), lung (N=6-7), and aorta (3-12) of control and RBP7-deficient mice. Results are means ± SEM. *P<0.05 vs EC^+ by two-way ANOVA.

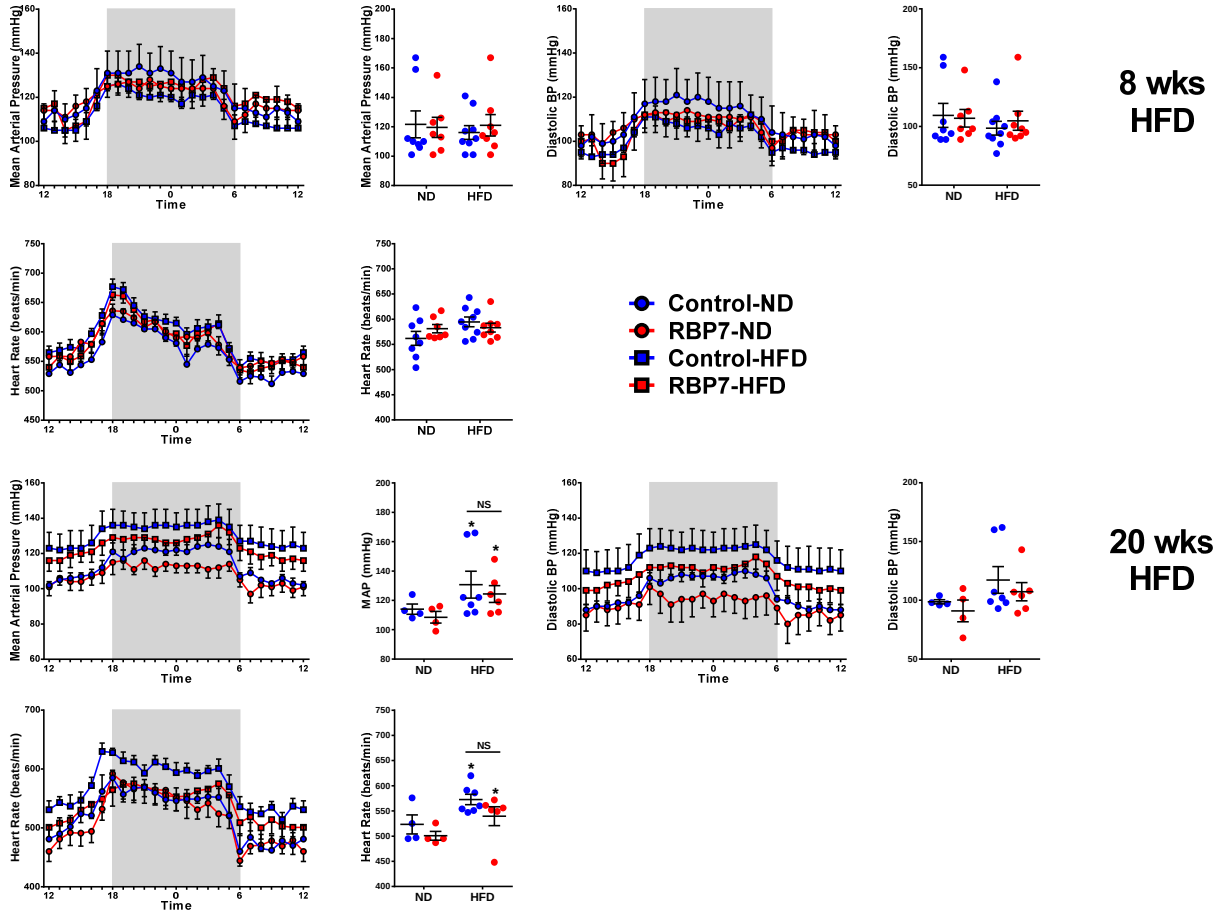

Figure S2. Mean and Diastolic BP and heart rate were measured by radiotelemetry in RBP7-deficient and control mice after either 8-weeks or 20-weeks of normal diet (ND) or high fat diet (HFD)(N=4-9 per group). Summary data for each 24 tracing is provided. *P<0.05 HFD vs ND-fed mice by two-way ANOVA. NS indicates nonsignificant.

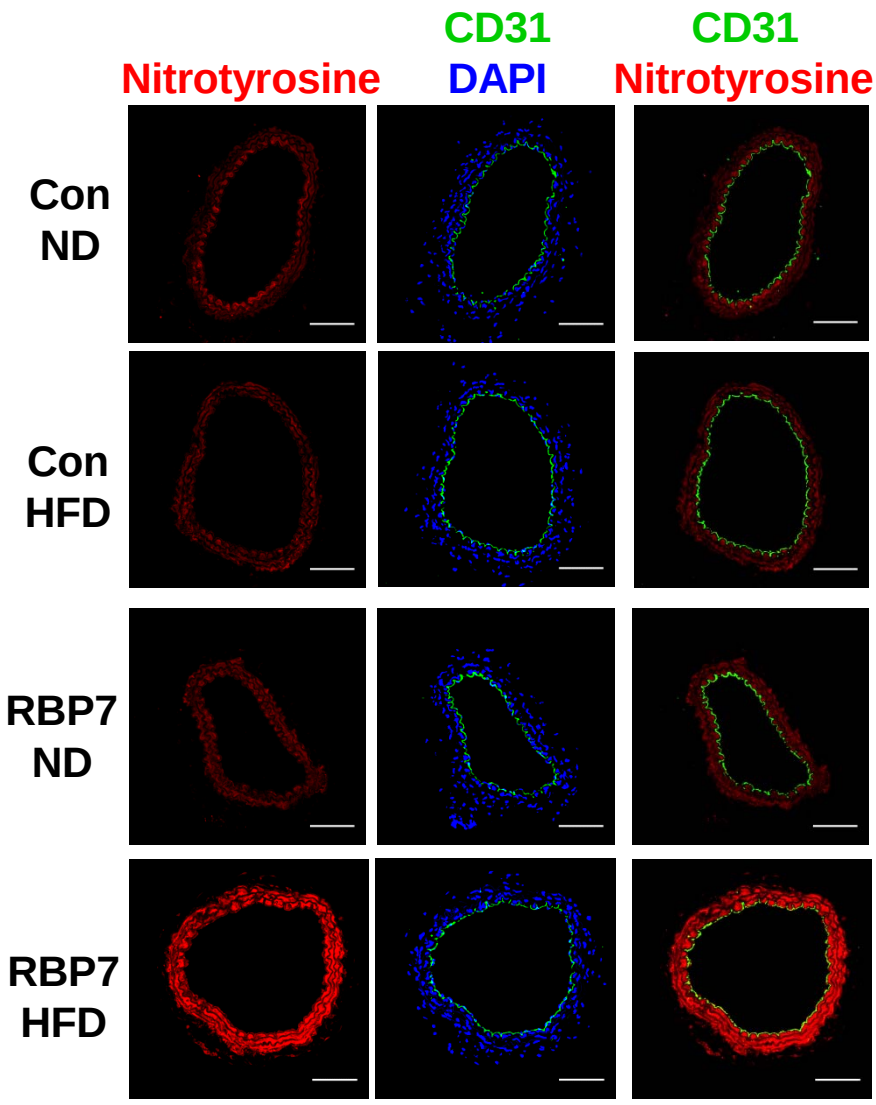

Figure S3. Representative photomicrographs of nitrotyrosine immunofluorescence staining of carotid arteries from normal diet (ND)-fed and high fat diet (HFD)-fed control (Con) and RBP7-deficient mice. Scale bar indicates 100 μm.

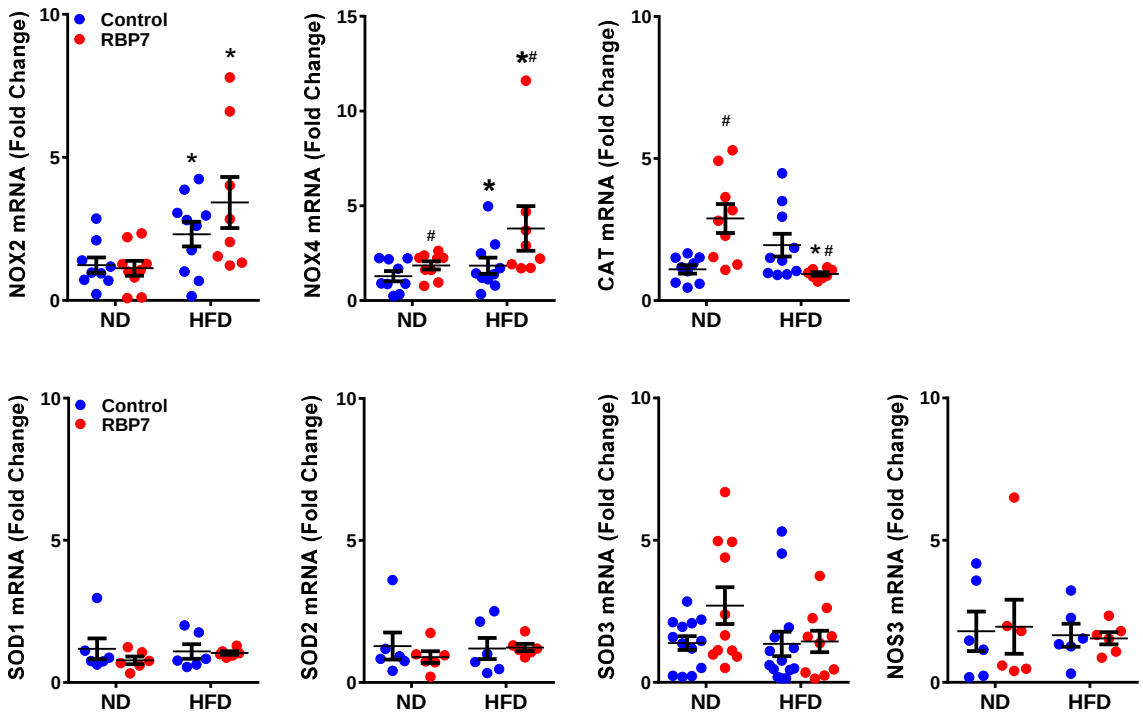

Figure S4. Oxidative stress-related gene expression in carotid arteries from high fat diet (HFD)- and normal diet (ND)-treated RBP7-deficient and their control mice. Results are means ± SEM. $^{*}P<0.05$ HFD vs. ND by genotype; $^{\#}P<0.05$ RBP7 vs control. All tests by two-way ANOVA. N=6-14 each group.

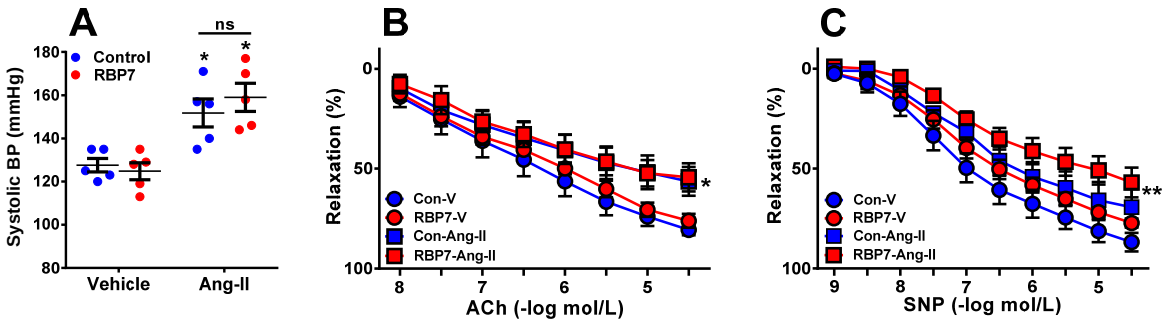

Figure S5. Pressor Response to Ang-II. A) RBP7-deficient and control mice exhibit similar changes in systolic blood pressure (BP) after pressor dose of Ang-II (1000 ng/kg/min for 2 weeks). $^{*}P<0.05$ Ang-II vs. Vehicle by genotype analyzed by two-way ANOVA. N=5 per group. NS indicates nonsignificant. B-C) RBP7-deficient and control mice exhibit similar changes in endothelium-dependent ACh-induced relaxation (B), and endothelium-independent sodium nitroprusside (SNP)-induced relaxation (C). $^{*}P<0.05$ Ang-II vs. Vehicle; $^{**}P<0.05$ RBP7-Ang-II vs. Control-Vehicle. Tested with two-way RM ANOVA. N=5 each group. Results are means ± SEM.

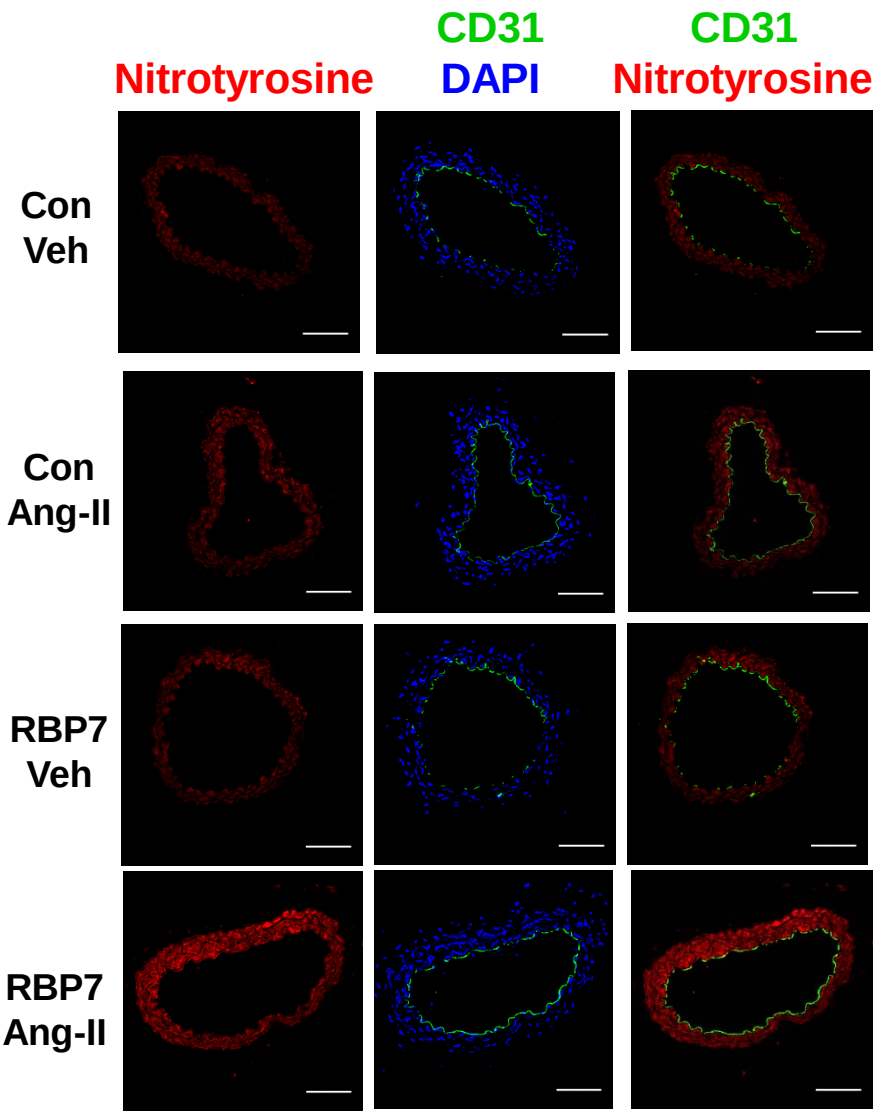

Figure S6. Representative photomicrographs of nitrotyrosine immunofluorescence staining of carotid arteries from control (Con) and RBP7-deficient (RBP7) mice treated with vehicle or subpressor Ang-II. Scale bar indicates 100 μm.

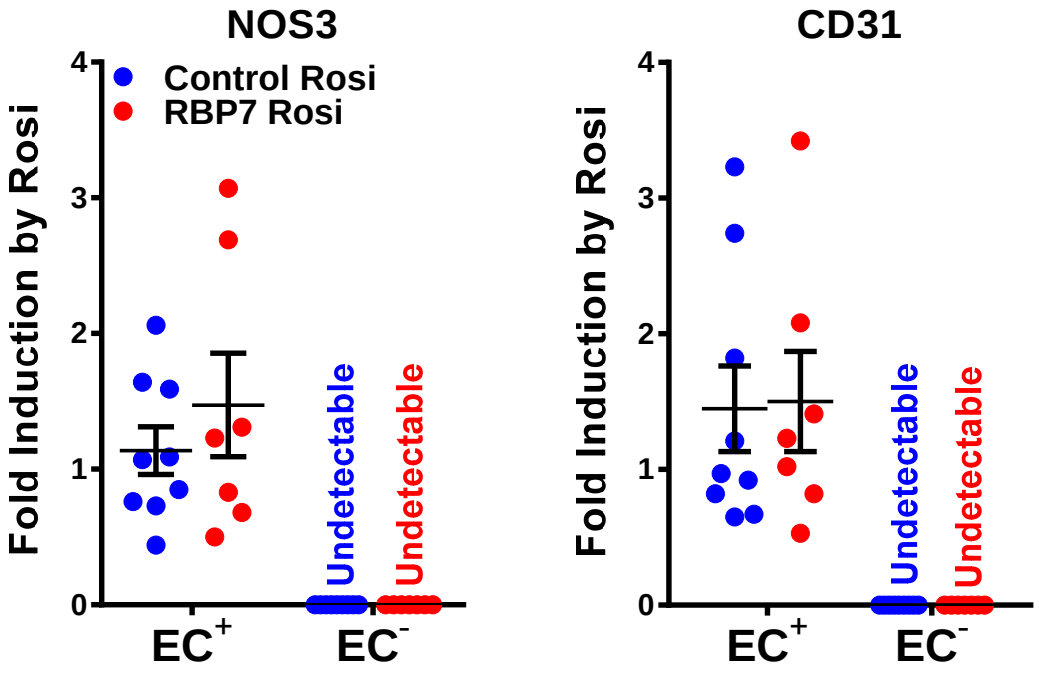

Figure S7. Expression of NOS3 and CD31 in endothelial (EC^+) and non-endothelial (EC^-) cells from rosiglitazone (Rosi)- or DMSO-incubated carotid arteries of control and RBP7-deficient mice. N=7-9 each group. Results are means ± SEM.

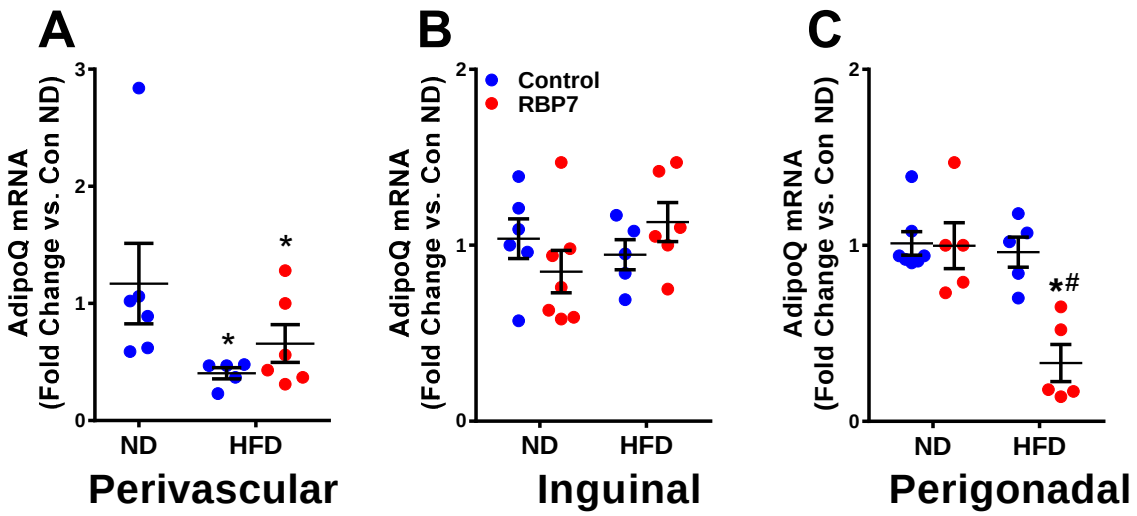

Figure S8. Adiponectin in different adipose tissues in high fat diet (HFD)- and normal diet (ND)-fed control and RBP7-deficient mice. mRNA level of adiponectin in perivascular (aorta) adipose tissue (A), inguinal adipose tissue (B), perigonadal adipose tissue (C). N=5-7 each group. Results are means ± SEM. $^{*}P<0.05$ HFD vs ND; $^{\#}P<0.05$ HFD-fed RBP7 vs HFD-fed control mice by two-way ANOVA.

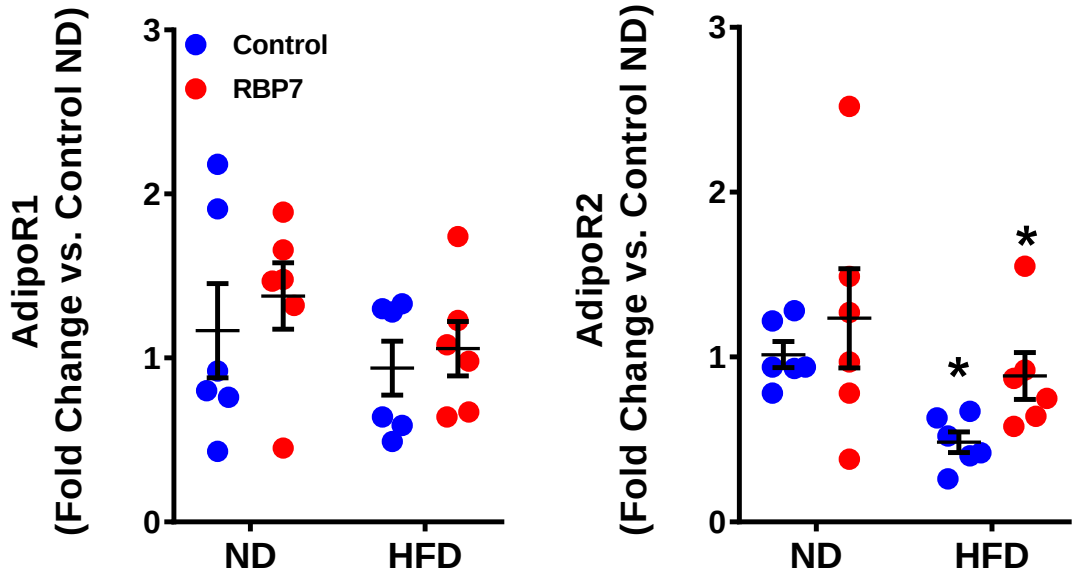

Figure S9. Gene expression of AdipoR1 and AdipoR2 in carotid artery of normal diet (ND) and high fat diet (HFD)-fed control and RBP7-deficient mice. Results are means ± SEM. *P<0.05 vs HFD vs ND by two-way ANOVA. N=6 per group.