Supplemental Material

Supplemental Methods

Animal Models

The procedures followed were in accordance with approved guidelines set by the Laboratory Animal Care Committee at the University of Missouri. Heterozygote control mice (m $Lepr^{db}$) (Background Strain: C57BLKS/J), homozygote type 2 diabetic mice ($Lepr^{db}$) (Background Strain: C57BLKS/J) and $Lepr^{db}$ null for TNFα (db^{TNF-}/db^{TNF-}) (Background Strain: C57BL/6J) were purchased from Jackson Laboratory and maintained on a normal rodent chow diet. Male, 20-35g m $Lepr^{db}$, 40-60 g $Lepr^{db}$ and db^{TNF-}/db^{TNF-} mice of either sex were used in this study. The cross (db^{TNF-}/db^{TNF-}) of $Lepr^{db}$ with TNFα knockout mice is heterozygous for $Lepr^{db}$ and homozygous for TNF knockout mice ($TNF^{-/-}$). These db^{TNF-}/db^{TNF-} mice show the phenotypes of hyperglycemia and obesity, the diabetic phenotype that is consistent with the penetrance of the leptin receptor mutation. The obese mice from the second round of breeding of $Lepr^{db}$ and $TNF^{-/-}$ were used in experimentation. At the age of 10 weeks, m $Lepr^{db}$ and $Lepr^{db}$ mice were either treated with resveratrol (20 mg/kg/d, Cayman Chemical) or vehicle (0.5% methylcellulose) orally for 4 weeks.[1] After treatment, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), blood was obtained from vena cava, and aortas were excised for functional study preparation.

Measurement of Glycemic Status

Fasting blood glucose levels were measured by OneTouch Ultramini glucometer (LifeScan) after overnight fasting. Fasting plasma insulin level was measured with the use of a commercial kit, Insulin (Mouse) Ultrasensitive EIA (ALPCO Diagnostics) by spectrophotometry (Multiskan MCC, Fisher Scientific) at 550 nm. Insulin resistance was determined by the homeostasis model assessment; HOMA-IR using the following formula:[2]

HOMA-IR= ((non-fasting glucose [mmol/L]) × (non-fasting insulin [mU/L]))/22.5.

Insulin Tolerance Test

The tail was nicked with a fresh razor blade and OneTouch Ultramini glucometer was used to measure baseline blood glucose after overnight fasting. 1.0 unit per kg body weight of diluted porcine insulin (Sigma) was injected into the intraperitoneal cavity. Blood glucose was sampled from the tail of each mouse by gently massaging a small drop of blood onto the glucometer strip at 0 (baseline), 15, 30, 60, 90 minutes following insulin injection.[3]

Serum Concentration of TNFα

Serum TNFα level was measured with the use of a commercial kit, BIO-Plex cytokine assay (Bio-Rad) as previously reported.[4]

mRNA Expression of TNFα by Real-time Polymerase Chain Reaction

We have used a quantitative real time RT-PCR technique to analyze mRNA expression of TNFα in mouse aortas, using the Strategen MX3000 as reported.[5, 6] Total RNA was isolated with Mini RNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript III RT (Invitrogen). Efficiency of the PCR reaction was determined using dilution series of a standard vascular sample. Quantification was performed using the efficiency-corrected ΔΔCT method as reported.[5, 6] Fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel. The housekeeping gene β-actin was used for internal normalization. Results are presented as fold change of transcripts for TNFα in $Lepr^{db}$ mice, and resveratrol treated mice, compared with the mean transcript abundance in aortas of m $Lepr^{db}$ mice (defined as 1.0).

Functional Assessment of Murine Aortas

After anesthesia, aortas were rapidly excised and rinsed in cold physiological saline solution (PSS) and loose fat and connective tissue were removed. PSS contains 118.99 mM NaCl, 4.69 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4 \bullet 7H_2O$, 2.50 mM $CaCl_2 \bullet 2H_2O$, 14.9 mM $NaHCO_3$, 5.5 mM D-Glucose, and 0.03 mM EDTA. Aortas were maintained in PSS in 95% O_2-5% CO_2 at 37 ºC for the remainder of the experiment. 2 mm of aortic rings were isometrically mounted in a in a myograph (model 610M, DMT, Denmark). After an equilibration period of 45 min, during which an optimal passive tension (15 mN) was applied, aortic rings were precontracted with 1 μmol/L phenolnephrine (PE). Dose-response curve was obtained by cumulative addition of acetylcholine (ACh, 1 nmol/L to 10 μmol/L) and sodium nitroprusside (SNP, 1 nmol/L to 10 μmol/L). Relaxation at each concentration was measured and expressed as the percentage of force generated in response to PE. The contributions of TNFα, NO, NAD(P)H oxidase and $O_2^{\cdot -}$ in vasorelaxation were assessed by incubating the vessels with recombinant TNFα (R&D, 10 ng/ml, 90 min), NO synthase (eNOS and nNOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (100 μmol/L, 20 min),[7, 8] NAD(P)H oxidase inhibitor apocynin (100 μmol/L, 60 min), and TEMPOL (a membrane-permeable superoxide dismutase mimetic, 3 mmol/L, 60 min), respectively.[9]

Protein Expression of TNFα, $gp91^{phox}$, eNOS, phospho-eNOS, nitrotyrosine (N-Tyr), Superoxide Dismutase (SOD)-1, SOD-3, Glutathione Peroxidase (GPx) and Catalase by Western Blot Analyses

Aortas were homogenized in lysis buffer (Celllytic MT Mammalian Tissue Lysis/Extraction Reagent, Sigma). Protein concentrations were assessed with the use of BCA Protein Assay Kit (Pierce). Equal amounts of protein (30 μg for TNFα and 8 μg for other proteins) were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF)

membranes (Pierce). TNFα, gp91^{phox}, eNOS, phospho-eNOS, N-Tyr, SOD-1, SOD-3, GPx and catalase protein expressions were detected by Western Blot with the use of TNFα primary antibody (Santa Cruz, 1:200), gp91^{phox} (BD Bioscience, 1:500), eNOS primary antibody (BD Bioscience, 1:500), phospho-eNOS (Ser1177) primary antibody (Invitrogen, 1:1000), N-Tyr (an indicator for peroxynitrite-mediated tissue injury) primary antibody (Abcam, 1:1000), SOD-1 (Santacruz, 1:200), SOD-3 (Santa Cruz, 1:200), GPx (Santacruz, 1:200), catalase (Abcam, 1:1000). Horseradish peroxidase-conjugated secondary antibodies were used. Signals were visualized by enhanced chemiluminescence (ECL, Santa-Cruz), scanned with a Fuji LAS3000 densitometer and quantified by Multigauge software (Fujifilm). The relative amounts of protein expression were quantified to those of the corresponding m Lepr^{db} control, which were set to a value of 1.0.

NADP(H) Oxidase Activity

NAD(P)H oxidase activity was assayed in homogenized aorta samples using lucigenin-derived chemiluminescence assay as previously reported.[4, 10] $O_2^{\cdot -}$ production was measured in the presence of 5 μmol/L lucigenin. The reaction was started by adding NAD(P)H (100 μmol/L). The relative light units (RLU) of chemiluminescence were read in a luminometer (Fluoroscan Ascent FL, Thermal Scientific). Samples were run in duplicate and the NAD(P)H oxidase activity was normalized to the m Lepr^{db} control group.

Detection of Vascular $O_2^{\cdot -}$ Production by Ethidium Bromide (EB) Fluorescence Assay

Dihydroethidium (DHE), an oxidative fluorescent dye, was used to localize $O_2^{\cdot -}$ production *in situ* as previously reported.[11] In brief, aortas isolated from control and resveratrol-treated mice were transferred to chambers containing PSS and incubated at 37ºC for 60 min. DHE (5 μmol/L) was then added to the PSS and incubated for 15 min, followed by 5 min of

washing in cold PSS to remove the non-intercalated EB molecules. Frozen sections of vessels were visualized by Bio-Rad Radiance 2000 MPl microscope. To determine the role of TNFα and NAD(P)H oxidase in $O_2^{\cdot -}$ production in aortas in type 2 diabetes, the vessels were treated with an NAD(P)H oxidase inhibitor apocynin (100 μmol/L, 60 min) or recombinant TNFα (R&D, 10 ng/ml, 90 min). The specificity of $O_2^{\cdot -}$ production was examined in the presence of the $O_2^{\cdot -}$ scavenger TEMPOL (a membrane-permeable superoxide dismutase mimetic, 1 mmol/L, 60 min). Negative control was performed without adding DHE in the staining procedure.

Measurement of Hydrogen Peroxide (H_2O_2)

Serum production of H_2O_2 was determined by using QuantiChrom™ peroxide assay kit (BioAssay Systems). Samples were run in duplicate and the concentration was determined by measuring the optical density in each well (microplate reader at 590 nm).

Measurement of Nitrite/Nitrate

Pieces of aorta were homogenized in lysis buffer and supernatants were collected for the quantification of nitrite/nitrate level using amperometric sensors (World Precision Instruments) according to previous publication.[12] Briefly, nitrate was converted to nitrite using Nitralyzer Nitrate to Nitrite Reduction Kit (World Precision Instruments). 2 mm sensor (ISO-NOP) was calibrated by chemical generation of NO at room temperature and NO was generated by the chemical reaction of nitrite with 0.1M H_2SO_4+0.1M KI. The currents (pA) detected by the sensor represent the concentration of nitrite in vessel samples and were normalized to the protein concentration.

Data Analysis

All data were presented as mean±SD except as specifically stated. Statistical comparisons under various treatments were performed with one-way ANOVA, and intergroup differences

were tested with Tukey inequality. BioDataFit 1.02 was used for dose-response analysis**.** Significance was accepted at $P < 0.05$.

Supplemental Results

Table I. Baseline Plasma parameters

Groups	m Lepr^{db}	Lepr^{db}	Lepr^{db}+RSV	m Lepr^{db}+RSV
Body Weight, g	24.54±0.35	44.18±0.34*	43.65±0.47*	24.42±0.39$^{\#}$
Abdominal Girth, cm	8.44±0.12	12.44±0.30*	12.23±0.25*	8.19±0.14$^{\#}$
Blood Glucose, mg/dl (Fasting)	150.50±5.55	407.13±7.27*	403.19±8.77*	145.19±3.84$^{\#}$
Insulin, ng/ml (Fasting)	0.48±0.04	3.00±0.65*	2.38±0.67*	1.02±0.23$^{\#}$
HOMA-IR	5.09±0.36	63.77±16.22*	67.04±9.35*	11.34±2.23$^{\#}$
TNFα, pg/ml	7.74±1.67	25.33±7.82*	20.25±2.70*	10.49±3.11

Table II. EC_{50} and E_{max} values for ACh concentration-response curves

	-Log EC50	E_{max}
m Lepr^{db}	7.7±0.2	85.6±6.3
Lepr^{db}	7.1±0.2*	70.1±5.4*
Lepr^{db}+RSV	7.5±0.2*$^{\#}$	79.4±5.6$^{\#}$
m Lepr^{db}+RSV	7.7±0.1$^{\#}$	87.0±3.9$^{\#}$
db^{TNF-}/db^{TNF-}	7.0±0.3*	92.4±5.0*$^{\#}$
m Lepr^{db}+TNF	7.4±0.1*$^{\#}$	82.2±10.7$^{\#}$
Lepr^{db}+RSV+TNF	7.1±0.3*^	79.4±6.0$^{\#}$
Lepr^{db}+Apocynin	7.0±0.1*	90.6±3.1$^{\#}$
Lepr^{db}+TEMPOL	7.3±0.2*	85.3±2.6$^{\#}$

Figure I. Insulin Tolerance Test

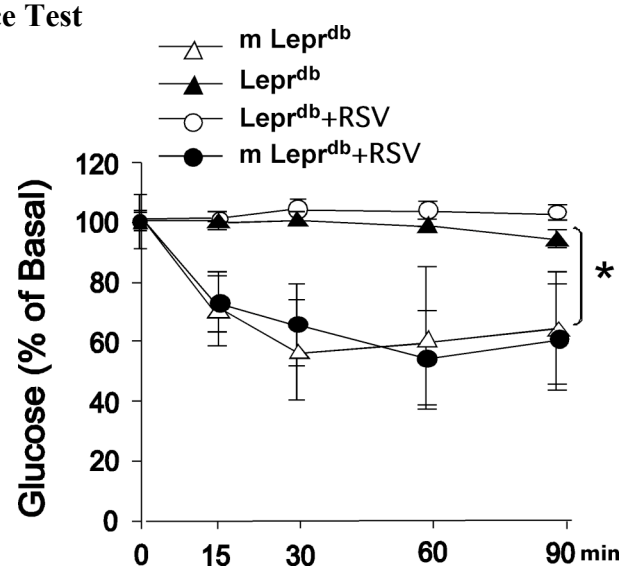

Figure II

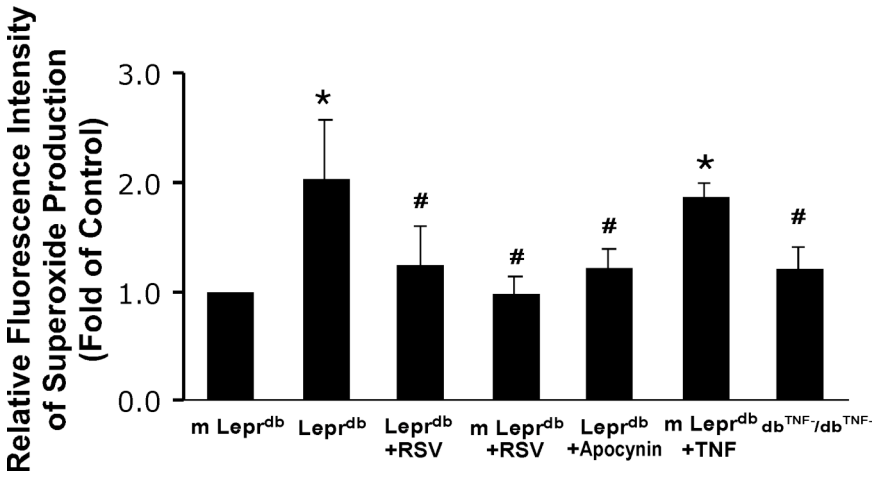

Figure III

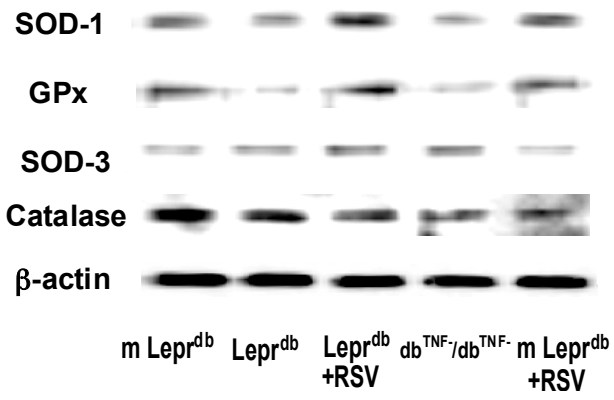

Figure IV

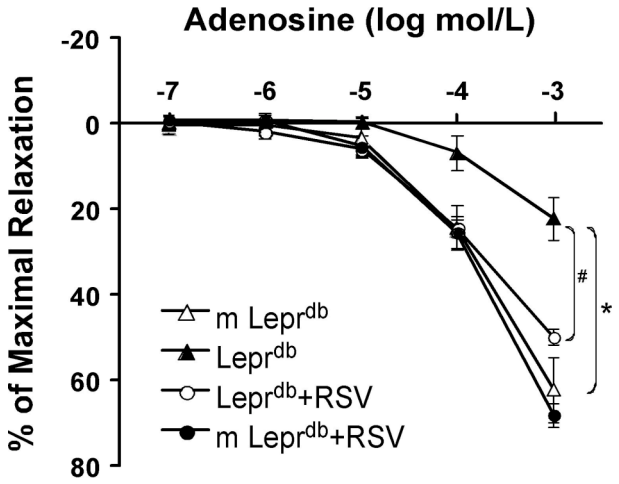

Table I. Baseline Plasma parameters

Body weight and abdominal girth were higher in $Lepr^{db}$ vs. m $Lepr^{db}$. Fasting blood glucose level, plasma insulin level and HOMA-IR were elevated in $Lepr^{db}$. Resveratrol (RSV) treatment did not affect the above parameters in both $Lepr^{db}$ and m $Lepr^{db}$ (n=8-16 mice). Serum level of TNFα was elevated in $Lepr^{db}$, but resveratrol did not significantly decrease serum TNFα level (n=4 mice). Data were shown as mean±SEM. *P<0.05 vs. m $Lepr^{db}$, #p<0.05 vs. $Lepr^{db}$

Table II. EC_{50} and E_{max} values for ACh concentration-response curves

The EC_{50} was significantly higher in $Lepr^{db}$. Resveratrol-treated $Lepr^{db}$ showed decreased EC_{50}. db^{TNF-}/db^{TNF-} and $Lepr^{db}$ treated with apocynin or TEMPOL showed no changes in EC_{50} compared with $Lepr^{db}$. The E_{max} for ACh-induced vasorelaxation was decreased in $Lepr^{db}$, and resveratrol treatment slightly increased E_{max}. db^{TNF-}/db^{TNF-} and $Lepr^{db}$ treated with apocynin or TEMPOL showed significantly increased E_{max} compared with $Lepr^{db}$. Data were shown as mean±SD, n=4-12 mice. *P<0.05 vs. m $Lepr^{db}$, #p<0.05 vs. $Lepr^{db}$, ^ p<0.05 vs. $Lepr^{db}$ treated with resveratrol.

Figure Legends

Figure I. To test insulin tolerance, insulin was injected (1.0 U/kg, i.p.) after overnight fasting and blood samples were taken for glucose determinations at 0, 15, 30, 60, and 90 minutes. Insulin tolerance test revealed that $Lepr^{db}$ had impaired insulin sensitivity although there was no significant difference in the glucose clearance rate between $Lepr^{db}$ and $Lepr^{db}$ treated with resveratrol. The value was normalized to the basal glucose level. Each value represents the mean ± SEM from 6-8 mice. *p<0.05 vs. m $Lepr^{db}$; #p<0.05 vs. $Lepr^{db}$.

Figure II. Quantification of vascular superoxide ($O_2^{\cdot -}$) fluorescence density in aortic rings by dihydroethidium (DHE) staining. Consistent with the representative image in Figure 4A, Both

resveratrol treatment and apocynin incubation significantly reduced $O_2^{\cdot -}$ production, which was greatly elevated in $Lepr^{db}$. db^{TNF-}/db^{TNF-} mice showed decreased $O_2^{\cdot -}$ staining while $O_2^{\cdot -}$ production was elevated in m $Lepr^{db}$ by pre-incubating the vessel with TNFα (10 ng/ml, 90 min). Data were shown as mean±SD, n=4 separate experiments. *P<0.05 vs. m $Lepr^{db}$, #p<0.05 vs. $Lepr^{db}$,

Figure III. Protein expression of superoxide dismutase (SOD)-1, glutathione peroxidase (GPx), SOD-3, and catalase in aortas. Protein expression of SOD-1 and GPx were reduced in $Lepr^{db}$ compared with m $Lepr^{db}$, while SOD-3 and catalase were similar among groups. Resveratrol up-regulated the protein expression of antioxidant enzymes, SOD-1 and GPx. db^{TNF-}/db^{TNF-} did not exhibit increased anti-oxidant enzyme expression vs. $Lepr^{db}$. Data were representative blotting from 3 separate experiments.

Figure IV. Endothelial-dependent vasorelaxation to adenosine was impaired in $Lepr^{db}$ compared with m $Lepr^{db}$. Resveratrol improved adenosine-induced vasorelaxation of $Lepr^{db}$ without affecting that of m $Lepr^{db}$. Data were shown as mean±SEM, n=3 mice. *P<0.05 vs. m $Lepr^{db}$, #p<0.05 vs. $Lepr^{db}$,

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