## Supplement 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 Adiponectin knockout mice ($APN^{-/-}$) (Background Strain: C57BL/6J) were from Dr. William P. Fay' laboratory. All of these mice were maintained on a normal rodent chow diet. Male, 20-35g m $Lepr^{db}$ and $APN^{-/-}$, 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.[1]

### Measurement of Glycemic Status

Non-fasting blood glucose levels were measured by OneTouch Ultramini glucometer (LifeScan). Non-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 450 nm. Insulin resistance was determined by the homeostasis model assessment; HOMA-IR using the following formula:[2]

$$\text{HOMA-IR} = ((\text{non-fasting glucose [mmol/L]}) \times (\text{non-fasting insulin [mU/L]}))/22.5.$$

**Treatment with Adiponectin, TNFα Neutralization, or Recombinant TNFα**

At 12-16 weeks of age, Lepr$^{db}$ mice were treated with the recombinant murine globular adiponectin (30 μg/day, s.c. twice daily for 10 days, PeproTech). The neutralizing antibody to TNFα is 2E2 monoclonal antibody (2E2 MAb. 94021402, NCI Biological Resources Branch). The Lepr$^{db}$ mice received the neutralizing anti-TNFα (anti-TNF, 0.625 mg/ml/kg/day, i.p. for 10 days).[3] m Lepr$^{db}$ control mice received murine recombinant TNFα (10 μg/day, i.p. for 3 days, R&D). After treatment, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Blood was obtained from vena cava, heart and aorta were excised for functional study preparation.

**Functional Assessment of Isolated Coronary Arterioles**

The techniques for identification and isolation of coronary microvessels were described in detail previously.[3] Coronary arterioles (40 to 100 μm in diameter) from mouse heart were carefully dissected for *in vitro* study. To determine whether adiponectin plays a role in vascular dysfunction in type 2 diabetes, vasodilation to endothelium-dependent vasodilator acetylcholine (ACh, 0.1 nmol/L to 10 μmol/L), endothelium-independent vasodilator sodium nitroprusside (SNP, 0.1 nmol/L to 10 μmol/L), or flow-induced dilation (NO-mediated, endothelial-dependent, but agonist-independent; 4 to 60 cm $H_2O$) were assessed in isolated coronary arterioles in m Lepr$^{db}$, Lepr$^{db}$ and Lepr$^{db}$ mice treated with adiponectin. At the end of each experiment, the vessel was relaxed with 100 μmol/L SNP to obtain its maximal diameter at 60 cm $H_2O$ intraluminal pressure. All diameter changes in response to agonists were normalized to the vasodilation in response to 100 μmol/L SNP and expressed as a percentage of maximal dilation.

**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 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 phenylephrine (PE). Dose-response curve was obtained by cumulative addition of ACh (1 nmol/L to 10 μmol/L) and 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. [4] The contribution of NO in vasorelaxation was assessed by incubating the vessels with NOS (eNOS and neuronal NOS) inhibitor $N^G$-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L for 20min).[5]

**Protein Expression by Western Blot Analyses**

Coronary arterioles (4-6 vessels per sample) or aortas were homogenized in lysis buffer (Cellytic™ MT Mammalian Tissue Lysis/Extraction Reagent, Sigma). Protein concentrations were assessed with a BCA™ Protein Assay Kit (Pierce) and samples were separated by SDS-PAGE and transferred to PVDF membranes. TNFα, IκBα, phospho-IκBα (Santa Cruz), Adiponectin (R&D), AdipoR1 and AdipoR2 (Alpha Diagnostics), and NFκB (Abcam) were determined. 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). Housekeeping gene β-actin was used as the loading control and we validated that no significant variation was detected between

control and experimental groups as well as across samples.[3-4] The relative amounts of protein expression in various groups were quantified and normalized to those of the corresponding m $Lepr^{db}$ control, which were set to a value of 1.0.[3]

**Immunofluorescence Staining**

Immunohistochemistry was used to identify and localize proteins in sections of vessels or myocardial tissue. Hearts or aortas were embedded in OCT and sectioned at 5 μm. Slides were incubated with blocking solution (10% donkey serum in PBS). Primary antibodies for adiponectin (goat polyclonal, R&D, AF1119), and endothelial cell marker, von Willebrand factor (rabbit polyclonal, Abcam), or smooth muscle α-actin (rabbit polyclonal, Abcam) or fibroblast (rat monoclonal, Novus Biologicals) were used for sequential double immunofluorescence staining. Secondary fluorescent antibodies were either FITC or Texas Red conjugated. For negative controls, primary antibodies were replaced with IgG-isotype controls at the same concentration. Sections were finally mounted in an anti-fading agent (Slowfade gold with DAPI, Invitrogen). Slides were observed and analyzed using a fluorescence microscope with a 40×objective (IX81, Olympus).[6]

**mRNA Expression of Adiponectin and TNFα by Real-time Polymerase Chain Reaction**

We have used a quantitative real time RT-PCR technique to analyze mRNA expression of adiponectin and TNFα in mouse aortas, using the iCycler iQ5 Real-Time PCR Detection System (BioRad). Total RNA was isolated with RNeasy Fibrious Tissue Mini RNA Isolation Kit (Qiagen) and was reverse transcribed using Superscript III RT (Invitrogen). Primers were designed with the use of Primer3 (v.0.4.0).[7] Efficiency of the PCR reaction was determined using dilution series of a standard vascular sample. Quantification was performed using the $2^{-\Delta\Delta CT}$ method as reported.[6, 8] The mRNA levels of the various genes tested were normalized to

housekeeping gene β-actin, used as an internal control in all experiments. Results are presented as fold change of transcripts for adiponectin or TNFα in $Lepr^{db}$ mice and $Lepr^{db}$ mice treated with adiponectin or anti-TNFα, compared with the mean transcript abundance in aortas of m $Lepr^{db}$ mice (defined as 1.0).

**Serum Concentration of TNFα and Adiponectin**

Serum TNFα level was measured with the use of a commercial kit, BIO-Plex cytokine assay (Bio-Rad Laboratories,Hercules, Calif) as previously reported.[3] TNFα concentrations were automatically calculated by BIO-Plex Manager software with the use of a standard curve derived from a recombinant cytokine standard. Values were expressed as pictograms per milliliter. Serum adiponectin level was determined by a commercially available ELISA kit (ALPCO). Values were expressed as micrograms per milliliter.

**Data Analysis**

All data were presented as mean±SEM except as specifically stated. Statistical comparisons under various treatments were performed with one-way ANOVA, and intergroup differences were tested with LSD 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$^{db}$+adiponectin | Lepr$^{db}$+anti-TNF |
|---|---|---|---|---|
| Body Weight, g | 25.54±0.62 | 45.18±0.47* | 45.11±0.89* | 45.03±1.08* |
| Abdominal Girth, cm | 8.56±0.12 | 12.56±0.30* | 12.75±0.33* | 12.64±0.27* |
| Blood Glucose, mg/dl (non-Fasting) | 156.25±8.06 | 529.63±11.58* | 515.75±18.15* | 523.38±20.83* |
| Insulin, ng/ml (non-Fasting) | 2.83±0.77 | 6.46±0.70* | 6.93±0.24* | 7.03±0.11* |
| HOMA | 29.78±7.66 | 234.31±22.39* | 250.47±18.34* | 244.59±9.32* |
| Adiponectin (μg/ml) | 9.54±0.89 | 5.43±0.37* | 8.88±0.85$^{\#}$ | 5.24±0.29* |
| TNFα (pg/ml) | 9.01±1.43 | 33.51±2.92* | 28.83±2.82* | 10.49±1.56$^{\#}$ |

**Table II. – Log $EC_{50}$ and $E_{max}$ Values for ACh Concentration-Response Curves**

| | -Log $EC_{50}$ | $E_{max}$ |
|---|---|---|
| m Lepr$^{db}$ | 7.71±0.06 | 87.12±2.42 |
| Lepr$^{db}$ | 7.10±0.10* | 73.31±1.74* |
| Lepr$^{db}$ + adiponectin | 7.32±0.05* | 83.13±1.62$^{\#}$ |
| Lepr$^{db}$ + anti-TNF | 7.21±0.04* | 83.04±1.79$^{\#}$ |
| m Lepr$^{db}$ + TNF | 7.70±0.09$^{\#}$ | 78.12±1.24* |

**Table III. Primer Sequences Used for Real-Time RT-PCR.**

| Name | Accession # | Sequence | Target Length |
|---|---|---|---|
| TNFα s | NM_013693 | GTCCCCAAAGGGATGAGAAG | 134 |
| TNFα as | NM_013693 | CACTTGGTGGTTTGCTACGA | 134 |
| Adiponectin s | NM_009605 | AGGTTGGATGGCAGGC | 129 |
| Adiponectin as | NM_009605 | GTCTCACCCTTAGGACCAAGAA | 129 |
| β-actin s | NM_007393 | GCTCTTTTCCAGCCTTCCTT | 168 |
| β -actin as | NM_007393 | CTTCTGCATCCTGTCAGCAA | 168 |

**Figure I.**

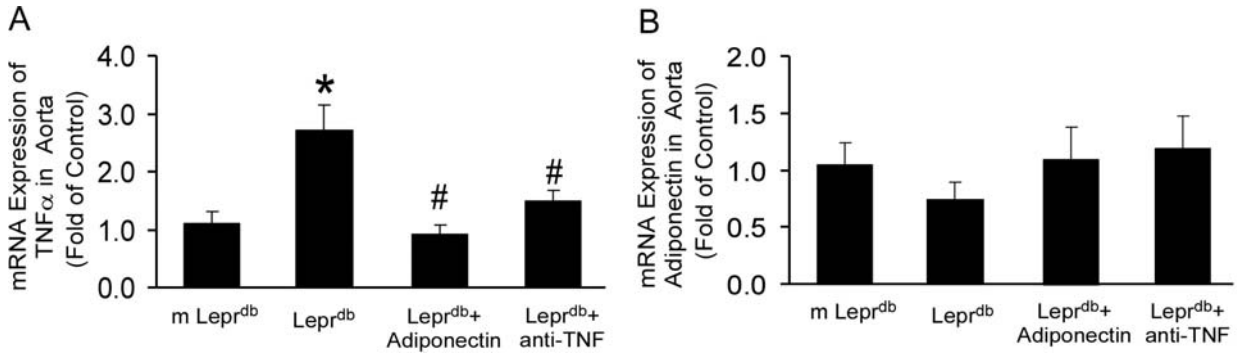


**Figure II.**

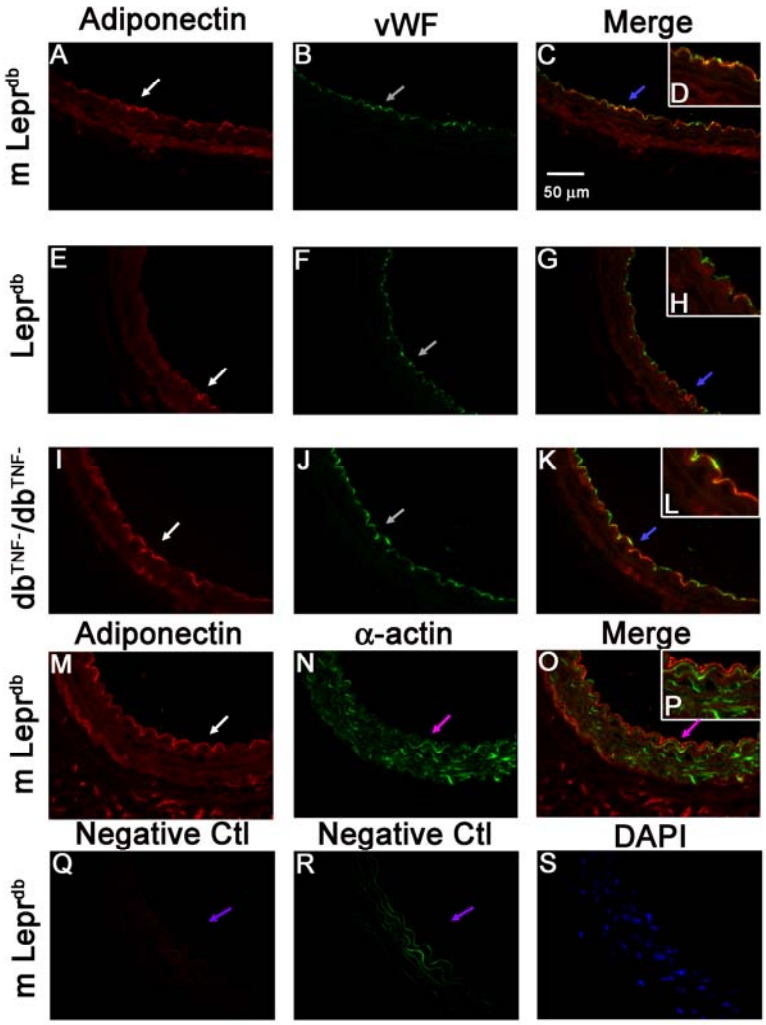

**Figure III.**

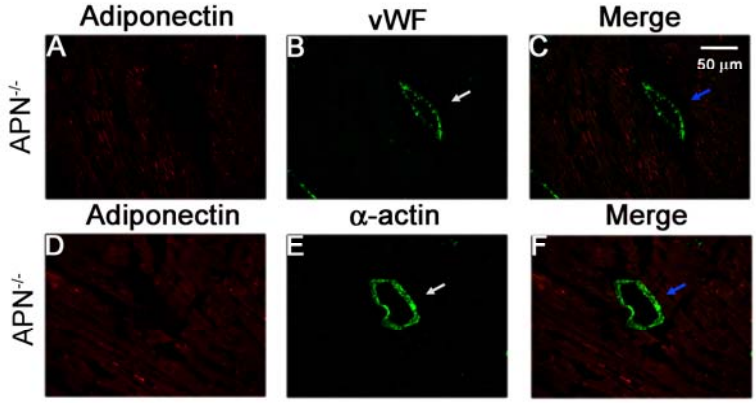


**Figure IV.**

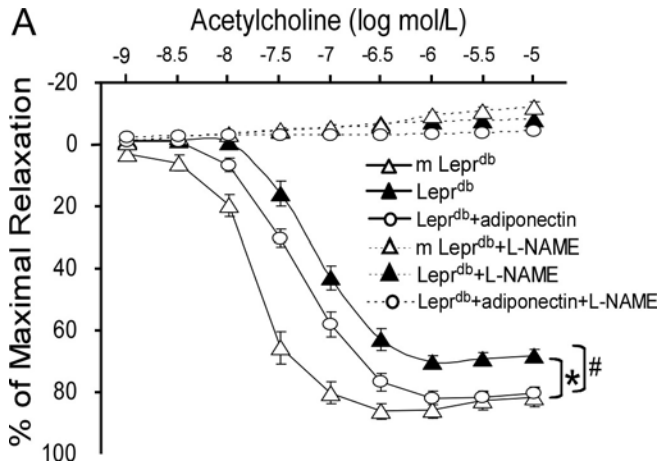


**Table I. Baseline Plasma Parameters**

Body weight and abdominal girth were higher in Lepr$^{db}$ vs. m Lepr$^{db}$. Non-fasting blood glucose level, plasma insulin level and HOMA-IR were elevated in Lepr$^{db}$. Adiponetin or anti-TNFα treatment did not affect the above parameters in Lepr$^{db}$. Serum adiponectin level was reduced in Lepr$^{db}$, while TNFα level was increased in Lepr$^{db}$. Globular adiponectin treatment increased serum adiponectin level without significantly affecting serum TNFα level. Anti-TNFα (anti-TNF) treatment diminished serum TNFα level without changing serum adiponectin level. Data were shown as mean±SEM. n=4-8 mice. *P<0.05 vs. m Lepr$^{db}$, #p<0.05 vs. Lepr$^{db}$.

**Table II. –Log $EC_{50}$ and $E_{max}$ Values for ACh Concentration-Response Curves**

The –log $EC_{50}$ was significantly lower in $Lepr^{db}$. $Lepr^{db}$ treated with adiponectin or anti-TNFα (anti-TNF) and m $Lepr^{db}$ treated with recombinant TNFα (TNF) showed no changes in –log $EC_{50}$. The $E_{max}$ for ACh-induced vasorelaxation was decreased in $Lepr^{db}$. Adiponectin and anti-TNFα treatment increased $E_{max}$ in $Lepr^{db}$. m $Lepr^{db}$ treated with recombinant TNFα showed lower $E_{max}$ compared with that of m $Lepr^{db}$. Data were shown as mean±SEM. n=4-10 mice. *P<0.05 vs. m $Lepr^{db}$, #p<0.05 vs. $Lepr^{db}$.

**Table III. Primer Sequences Used for Real-Time RT-PCR.**

**Figure Legends**

**Figure I.** A, mRNA expression of TNFα was increased in aortas of $Lepr^{db}$. Both adiponectin and anti-TNFα treatment reduced TNFα mRNA expression. B, mRNA expression of adiponectin was not statistically different among m $Lepr^{db}$, $Lepr^{db}$, and $Lepr^{db}$ treated with either adiponectin or anti-TNFα. Data were shown as mean±SEM. n=4 separate experiments. *p<0.05 vs. m $Lepr^{db}$; #p<0.05 vs. $Lepr^{db}$.

**Figure II.** Dual fluorescence combining adiponectin with markers for endothelial cells [von Willebrand factor (vWF)] and vascular smooth muscle (α-actin) with the use of specific primary antibodies followed by fluorescent-labeled secondary antibodies. A, B and C, dual labeling of adiponectin (red) and vWF (green) in control mouse aorta. E, F and G, dual labeling of adiponectin (red) and vWF (green) in $Lepr^{db}$ mouse aorta. I, J and K dual labeling of adiponectin (red) and vWF (green) in $db^{TNF-}/db^{TNF-}$ aorta. The blue arrows in C, G and K show the colocalization of adiponectin and endothelial cells (yellow). The insert in C (D), inserts in G (H) and K (L) show the higher magnification of colocalization pointed by blue arrows in C, G and K.

M, N and O, dual labeling of adiponetin (red) and α-actin (green) in m $Lepr^{db}$ mouse aorta. The pink arrow in O shows the specific α-actin staining with absence of adiponectin staining. Q and R, negative control: the purple arrows show an absence of staining in vessels. S shows nuclear staining with DAPI (blue) in control mice aorta. Data shown are representative of 4 separate experiments.

**Figure III.** The absence of adiponectin staining in adiponectin knockout mice (APN-/-). n=3 separate experiments.

**Figure IV.** Vasorelaxation to ACh in m $Lepr^{db}$, $Lepr^{db}$, and $Lepr^{db}$+adiponectin mice was abolished after incubation of the aortic rings with the nitric oxide synthase inhibitor $N^{G}$-nitro-L-arginine methyl ester (L-NAME, 100 μM for 20 min), which indicated that vasorelaxation of aortas to ACh was nitric oxide-mediated. Data were shown as mean±SEM. n=4-6 mice. *$p<0.05$ vs. m $Lepr^{db}$; #$p<0.05$ vs. $Lepr^{db}$.

**Reference**


1. Yang J, Park Y, Zhang H, Xu X, Laine GA, Dellsperger KC, Zhang C. Feed-forward signaling of TNF-alpha and NF-kappaB via IKK-beta pathway contributes to insulin resistance and coronary arteriolar dysfunction in type 2 diabetic mice. *Am J Physiol Heart Circ Physiol.* 2009;296:H1850-1858.
2. Keskin M, Kurtoglu S, Kendirci M, Atabek ME, Yazici C. Homeostasis model assessment is more reliable than the fasting glucose/insulin ratio and quantitative insulin sensitivity check index for assessing insulin resistance among obese children and adolescents. *Pediatrics.* 2005;115:e500-503.
3. Gao X, Belmadani S, Picchi A, Xu X, Potter BJ, Tewari-Singh N, Capobianco S, Chilian WM, Zhang C. Tumor necrosis factor-alpha induces endothelial dysfunction in Lepr(db) mice. *Circulation.* 2007;115:245-254.
4. Zhang H, Zhang J, Ungvari Z, Zhang C. Resveratrol Improves Endothelial Function. Role of TNF{alpha} and Vascular Oxidative Stress. *Arterioscler Thromb Vasc Biol.* 2009.
5. Chen X, Zhang H, McAfee SR, Zhang C. The Reciprocal Relationship between Adiponectin and LOX-1 in the Regulation of Endothelial Dysfunction in ApoE Knockout Mice. *Am J Physiol Heart Circ Physiol.* 2010.
6. Gao X, Zhang H, Belmadani S, Wu J, Xu X, Elford H, Potter BJ, Zhang C. Role of TNF-{alpha}-induced reactive oxygen species in endothelial dysfunction during reperfusion injury. *Am J Physiol Heart Circ Physiol.* 2008;295:H2242-2249.
7. Shibata R, Sato K, Kumada M, Izumiya Y, Sonoda M, Kihara S, Ouchi N, Walsh K. Adiponectin accumulates in myocardial tissue that has been damaged by ischemia-

reperfusion injury via leakage from the vascular compartment. *Cardiovasc Res.* 2007;74:471-479.

8. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402-408.