Coronary and Aortic Endothelial Function Affected by Feedback Between Adiponectin and Tumor Necrosis Factor \( \alpha \) in Type 2 Diabetic Mice

Hanrui Zhang, Yoonjung Park, Cuihua Zhang

Objective—To verify that adiponectin and tumor necrosis factor (TNF)-\( \alpha \) reciprocally regulate their expression, thereby synergistically affecting both coronary and aortic endothelial dysfunction in type 2 diabetic mice.

Methods and Results—We examined endothelium-dependent and endothelium-independent vasodilation/vasorelaxation of coronary arterioles and aortas in control mice, diabetic mice (Lepr\( ^{db} \)), and Lepr\( ^{db} \) treated with adiponectin or neutralizing antibody to TNF-\( \alpha \) (anti–TNF-\( \alpha \)). Endothelium-dependent vasodilation to acetylcholine in both coronary arterioles and aortas was blunted in Lepr\( ^{db} \) compared with control mice. Endothelium-independent vasodilation to sodium nitroprusside was comparable. Adiponectin and anti–TNF-\( \alpha \) improved acetylcholine-induced vasodilation of coronary arterioles and aortas in Lepr\( ^{db} \) without affecting dilator response to sodium nitroprusside. Adiponectin protein expression was significantly reduced, and TNF-\( \alpha \)-protein expression was significantly greater, in coronary arterioles and aortas of Lepr\( ^{db} \) compared with control mice. Immunofluorescence staining results indicate that adiponectin was colocalized with endothelial cells. Anti–TNF-\( \alpha \) treatment upregulated adiponectin protein expression in Lepr\( ^{db} \) coronary arterioles and aortas. Adiponectin administration reduced TNF-\( \alpha \) protein expression in Lepr\( ^{db} \). Although adiponectin receptor 1 protein expression in coronary arterioles and aortas was similar between control and diabetic mice, adiponectin receptor 2 protein expression was significantly reduced in Lepr\( ^{db} \). Both adiponectin and anti–TNF-\( \alpha \) inhibited \( \kappa B \) phosphorylation and nuclear factor \( \kappa B \) protein expression in Lepr\( ^{db} \), suggesting that adiponectin and TNF-\( \alpha \) signaling may converge on nuclear factor \( \kappa B \) to reciprocally regulate their expression.

Conclusion—A reciprocal suppression occurs between adiponectin and TNF-\( \alpha \) that fundamentally affects the regulation of coronary and aortic endothelial function in type 2 diabetic mice. (Arterioscler Thromb Vasc Biol. 2010;30:2156-2163.)

Key Words: coronary circulation • cytokines • reactive oxygen species • vasodilation

The growing epidemic of cardiovascular disease in developed countries is closely associated with increased prevalence of obesity and type 2 diabetes mellitus.\(^1\,^2\) Much of the recent work on obesity\(^3\) has highlighted the key role of adipose tissue as an endocrine organ that secretes several factors, termed adipokines, that mediate many of the vascular and metabolic complications of obesity. An adverse adipokine expression profile, characterized by diminished production of protective factors (eg, adiponectin) and increased detrimental adipokines (eg, tumor necrosis factor [TNF]-\( \alpha \)), has been suggested in obese and type 2 diabetic patients.\(^4\,^7\)

Adiponectin, also known as ACRP30 or AdipoQ, is an adipokine that is secreted from adipocytes.\(^8\) Adiponectin plays an important role in the regulation of glucose and lipid metabolism.\(^9\) Paradoxically, the serum concentration of adiponectin is decreased in obese and type 2 diabetic patients despite increased adiposity.\(^10\) In contrast, plasma TNF-\( \alpha \) levels are elevated in such patients, suggesting that there may be an imbalance between the production of adiponectin and TNF-\( \alpha \) in obesity.\(^11\) In 3T3-L1 adipocytes, decreased adiponectin mRNA levels by TNF-\( \alpha \) were partially recovered by treatment with a c-JUN N-terminal kinase inhibitor, suggesting that the c-JUN N-terminal kinase signaling pathway, activated by TNF-\( \alpha \), is involved in the regulation of adiponectin expression in adipocytes.\(^7\) Another study\(^12\) shows that adiponectin decreases leptin-induced TNF-\( \alpha \)-expression by murine macrophages through suppression of phosphorylation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase pathways. Thus, there may be a reciprocal association between adiponectin and TNF-\( \alpha \).

Adipose-derived adipokines actively participate in the regulation of vascular function (ie, TNF-\( \alpha \) contributes to the impairment of coronary and aortic vascular function in type 2 diabetic mice).\(^13\,^14\) However, the following issues have not been resolved: (1) the role of adiponectin in regulating coronary and aortic vascular function in type 2 diabetic mice.
and (2) whether there is a reciprocal association between adiponectin and TNF-α. Thus, the goal of this study was to examine the nature and mechanisms of putative reciprocal suppressive effects between adiponectin and TNF-α in coronary microvessels and aortas in type 2 diabetic mice and how this reciprocal regulation can contribute to the pathogenesis of diabetes-associated vascular dysfunction.

Methods

Animals

The procedures followed were in accordance with approved guidelines by the Institutional Animal Care and Use Committee at the University of Missouri. Heterozygote control mice (m Lepr<sup>db</sup>) (background strain: C57BLKS/J), and Lepr<sup>db</sup> null for TNF-α (dbTNF<sup>H11002</sup>/H11002<sup>/H9262</sup>) (background strain: C57BL/6J) were purchased from The Jackson Laboratory, Bar Harbor, Me; and adiponectin knockout (APN<sup>H11002</sup>/H11002<sup>/H9251</sup>) mice (background strain: C57BL/6J) were obtained from a laboratory. All of these mice were maintained on a normal rodent chow diet. Male mice, 20 to 35 g Lepr<sup>db</sup> and APN<sup>H11002</sup>/H11002<sup>/H9251</sup> were assessed in isolated coronary arterioles in mm H2O, were assessed in isolated coronary arterioles in mm H2O, were assessed in isolated coronary arterioles in mm H2O, were assessed in isolated coronary arterioles in mm H2O; or flow-induced dilation (NO<sup>IX81</sup>). These dbTNF<sup>H11002</sup>/H9251<sup>/H9262</sup> mice show the phenotypes of hyperglycemia and obesity in the diabetic phenotype that is consistent with the presence of the leptin receptor mutation. The obese mice from the second round of breeding of Lepr<sup>db</sup> and TNF<sup>−/−</sup> were used in experimentation.13

Treatment With Adiponectin, TNF-α Neutralization, or Recombinant TNF-α

At the age of 12 to 16 weeks, Lepr<sup>db</sup> mice were treated with the recombinant murine globular adiponectin, 30 μg/d SC twice daily for 10 days. The neutralizing antibody to TNF-α is 2E2 monoclonal antibody. Lepr<sup>db</sup> mice received the neutralizing anti–TNF-α, 0.625 mg/mL per kilogram per day IP for 10 days.13 m Lepr<sup>db</sup> received murine recombinant TNF-α, 10 μg/d IP for 3 days.

Functional Assessment of Isolated Coronary Arterioles

The techniques for identification and isolation of coronary microvessels were described in detail previously.13 Coronary arterioles, 4- to 6 vessels per sample, or aortas were homogenized in lysis buffer (Cellytic MT Mammalian Tissue Lysis/Extraction Reagent). Protein concentrations were assessed with a kit (BCA Protein Assay Kit), and samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The levels of TNF-α, IkBα, phosphorylated IkBα, adiponectin, adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), and nuclear factor (NF)κB were determined. Signals were visualized by enhanced chemiluminescence and quantified by film imaging (Fuji) software.13 Housekeeping gene β-actin was used as the loading control, and we validated that no significant variation was detected between control and experimental groups and across samples.13,14 The relative amounts of protein expression in various groups were quantified and normalized to those of the corresponding m Lepr<sup>db</sup> control, which were set at 1.0.13

Immunofluorescence Staining

Immunohistochemistry was used to identify and localize proteins in sections of vessels or myocardial tissue. Hearts or aortas were embedded in optimal cutting temperature and sectioned at 5 μm. Slides were incubated with blocking solution (10% donkey serum in PBS). Primary antibodies for adiponectin (goat polyclonal), endothelial cell marker, von Willebrand factor (rabbit polyclonal), smooth muscle α-actin (rabbit polyclonal), or fibroblast (rat monoclonal) were used for sequential double immunofluorescence staining. Secondary fluorescent antibodies were either fluorescein isothiocyanate 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 antifading agent (SlowFade gold with 4′,6-diamidino-2-phenylindole). Slides were observed and analyzed using a fluorescence microscope with a ×40 objective (model IX81).16

Data Analysis

All data are presented as mean ± SEM except as specifically stated. Statistical comparisons under various treatments were performed with 1-way ANOVA, and intergroup differences were tested with a lysogenic acid diethyamide inequality. P<0.05 was considered significant. The supplemental data (available online at http://atvb.ahajournals.org) provide further details.

Results

Effects of Adiponectin and Anti–TNF-α on Body Weight, Abdominal Girth, Blood Glucose, and Insulin/Insulin Resistance

Body weight and abdominal girth were greater in Lepr<sup>db</sup> versus m Lepr<sup>db</sup>. Nonfasting blood glucose level, plasma insulin level, and homeostasis model assessment of insulin resistance (HOMA-IR) were elevated in Lepr<sup>db</sup>. Adiponectin and anti–TNF-α treatment did not affect the described parameters in Lepr<sup>db</sup> (supplemental Table I).

Role of Adiponectin in Type 2 Diabetes–Induced Coronary Endothelial Dysfunction

Vasodilation to endothelium-dependent vasodilator ACh was impaired in coronary arterioles of Lepr<sup>db</sup> (Figure 1A). Conversely, adiponectin partially restored ACh-induced vasodilation in Lepr<sup>db</sup> (Figure 1A). Moreover, flow-induced vasodilation was diminished in coronary arterioles of Lepr<sup>db</sup> but was rescued by adiponectin (Figure 1B). In contrast, SNP-induced endothelium-independent vasodilation was similar.

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among m Lepr<sup>db</sup>, Lepr<sup>db</sup>, and Lepr<sup>db</sup> treated with adiponectin (Figure 1C).

### Role of Adiponectin and TNF-α in Type 2 Diabetes–Induced Aortic Endothelial Dysfunction

Endothelium-dependent vasorelaxation in response to ACh was significantly impaired in aortas of Lepr<sup>db</sup>. Adiponectin and anti–TNF-α partially restored impaired vasorelaxation (Figure 2A and C and supplemental Table II). Endothelium-independent vasorelaxation to SNP was not statistically different among any of the groups (Figure 2B and D). Recombinant TNF-α treatment impaired aortic function of m Lepr<sup>db</sup> (Figure 2C and supplemental Table II).

### Reciprocal Association Between Adiponectin and TNF-α in Coronary Arterioles and Aortas of Diabetic Mice

Protein expressions of adiponectin and TNF-α from isolated coronary arterioles and aortas were analyzed in m Lepr<sup>db</sup>, Lepr<sup>db</sup>, and Lepr<sup>db</sup> mice treated with anti–TNF-α or adiponectin.
Adiponectin and TNF-α Signaling Converged on NFκB to Reciprocally Regulate Their Expression

In both coronary arterioles and aortas, IκBα (inhibitor of NFκB) expression was decreased in Lepr<sup>db</sup> (Figure 6A and D), whereas phosphorylated IκBα was greatly elevated (Figure 6B and E). Both adiponectin and anti–TNF-α inhibited IκBα phosphorylation (Figure 6B and E) without affecting total IκBα expression (Figure 6A and D). NFκB p65 protein expression was significantly higher in Lepr<sup>db</sup>. Adiponectin and anti–TNF-α decreased NFκB protein expression (Figure 6C and F).

Discussion

To our knowledge, this is the first in vivo evidence for a reciprocal regulation occurring between adiponectin and TNF-α in microcirculation and macrocirculation in type 2 diabetic mice. The circulatory protein adiponectin protects against diabetes-induced coronary arteriolar and aortic vascular dysfunction; this protection involves, at least in part, the downregulation of TNF-α. Furthermore, inhibition of TNF-α is associated with upregulation of adiponectin in microvessels and macrovessels. Taken together, these data suggest that the reciprocal suppression of adiponectin and TNF-α functionally contributes to the regulation of diabetes-associated vascular dysfunction.

Vascular Effects of TNF-α and Adiponectin in Type 2 Diabetes

TNF-α and adiponectin are important adipose-derived factors. TNF-α is a key proinflammatory cytokine mainly secreted by nonfat cells in adipose tissue. It was previously demonstrated that TNF-α contributed to endothelial dysfunc-
Adiponectin in type 2 diabetes by inducing activation of NAD(P)H oxidase and production of reactive oxygen species in both aortas and coronary microcirculation. Adiponectin is a relatively abundant plasma protein specifically secreted by adipocytes. Adiponectin exists in the circulation as a full-length protein and as a putative proteolytic cleavage fragment consisting of the globular C-terminal domain (gAd), which may have enhanced potency. The biological activities of gAd are controversial because of its proinflammatory effects in cardiac fibroblasts, but it appears that globular adiponectin is significantly more potent in reversing insulin resistance and exerts vascular protective effects by enhancing NO availability in endothelial cells. gAd incubation, 2 mg/mL, for 2 hours improved endothelium-dependent relaxation and total production of NO as a result of enhanced eNOS activity. In Leprdb mice, serum adiponectin levels are significantly reduced compared with those in m Leprdb (supplemental Table I). By treating the mice with recombinant globular adiponectin, we found that long-term adiponectin administration rescues both coronary microvascular and aortic macrovascular dysfunction in type 2 diabetic mice (Figures 1 and 2). This vasoprotection by adiponectin may be partly through the direct vascular effects of stimulating endothelial NO production and ameliorating oxidative stress based on previous studies using APN mice. In APN mice, ACh-induced vasodilation in aortas was impaired, accompanied by increased superoxide and peroxynitrite production. eNOS expression was conserved in APN mice, but NO production and eNOS phosphorylation were significantly reduced. Adiponectin also causes endothelium-independent vasodilation by opening voltage-gated K channels. However, the endothelium-independent vasodilatory effects of adiponectin do not represent a common pathway for the regulation of vascular dysfunction in type 2 diabetic mice because SNP-induced vasodilation is similar among m Leprdb, untreated diabetic mice, diabetic mice treated with adiponectin, or the neutralizing antibody to TNF (anti–TNF-α) (Figure 1C and Figure 2B and D).

Reciprocal Regulation Between Adiponectin and TNF-α
The reciprocal regulation between adiponectin and TNF-α has been studied in various tissues and cells. Adiponectin

Figure 4. Colocalization and regulation of adiponectin expression in coronary microvessels. Dual fluorescence combining adiponectin with markers for endothelial cells (von Willebrand factor [vWF], vascular smooth muscle [α-actin], and fibroblast marker) with the use of specific primary antibodies followed by fluorescence-labeled secondary antibodies. A through C, Dual labeling of adiponectin (red) and vWF (green) in control mouse heart tissue. D through F, Dual labeling of adiponectin (red) and vWF (green) in Leprdb mouse heart tissue. G through I, Dual labeling of adiponectin (red) and vWF (green) in db/db mouse heart tissue. White arrows in A, D, G, J, and M indicate the staining of adiponectin. Grey arrows in B, E, and H indicate the staining of endothelial cells. Blue arrows in C, F, and I indicate the colocalization of adiponectin and endothelial cells (yellow). J through L, Dual labeling of adiponectin and endothelial cells (yellow). The pink arrows in K and L show the specific α-actin staining with absence of adiponectin staining. M through O, Dual labeling of adiponectin (red) and marker of fibroblast in Leprdb mice heart tissue. The brown arrows in N and O indicate the specific fibroblast staining with absence of adiponectin staining. P and Q, Negative control: the purple arrows show an absence of staining in vessels with isotype control IgG and without primary antibodies. R, Nuclear staining with 4',6-diamidino-2-phenylindole (blue) in m Leprdb heart tissue. Magnification ×40. Data are representative of 4 separate experiments.
suppresses lipopolysaccharide-stimulated TNF-α production in cultured cardiac myocytes and macrophages,\textsuperscript{7,27,28} whereas adiponectin deficiency leads to an increase in circulating TNF-α in mouse models.\textsuperscript{29} TNF-α also has a regulatory effect on adiponectin. By incubating human visceral adipose tissue with TNF-α in vitro, the mRNA and protein expressions of adiponectin were significantly reduced.\textsuperscript{30} However, there are no in vivo studies examining the reciprocal association between adiponectin and TNF-α in the vasculature. Also, the role of this reciprocal regulation in the pathogenesis of diabetes-induced microvascular and macrovascular dysfunction has not been investigated. Our results suggest that anti–TNF-α treatment upregulates adiponectin expression; however, adiponectin treatment inhibits TNF-α expression in coronary arterioles and aortas of diabetic mice (Figure 3). Thus, adiponectin and TNF-α not only exert vascular effects independently by affecting vascular NO bioavailability and oxidative stress\textsuperscript{13,14,25} but also act interactively to participate in the complex regulation of their combined vascular expression in type 2 diabetes.

### Adiponectin and AdipoR Expression in Coronary Microvessels and Aortas

Two receptor forms have been cloned for adiponectin, and the receptors have unique distributions and affinities for the molecular forms of adiponectin. AdipoR1 is a high-affinity receptor for gAd with low affinity for full-length protein, and AdipoR2 has intermediate affinity for both forms of adiponectin.\textsuperscript{3} Interestingly, AdipoR1 is abundantly expressed in skeletal muscle and moderately expressed in other tissues, whereas AdipoR2 is predominantly expressed in the liver.\textsuperscript{20,31,32} Aortic endothelial cells express both adiponectin isoforms but appear to preferentially express mRNA of AdipoR1. In human umbilical vein endothelial cells, globular adiponectin-induced phosphorylation of eNOS at serine 1177 and NO production\textsuperscript{22} were abrogated when expressions of AdipoR1 and AdipoR2 were simultaneously suppressed.\textsuperscript{34} Overexpression of AdipoR1 and AdipoR2 in human umbilical vein endothelial cells significantly enhanced the suppressive effect of an otherwise symptomless dose of globular adiponectin on TNF-α–induced intercellular adhesion molecule 1 expression and NFκB activation, suggesting the involvement of AdipoRs in adiponectin-induced vasoprotection against the proinflammatory effects of TNF-α.\textsuperscript{35} To our knowledge, this study provides the first in vivo documentation that AdipoR1 expression is similar between control and diabetic mice but AdipoR2 expression is significantly reduced in both coronary arterioles and aortas in diabetic mice (Figure 5). Furthermore, AdipoR2 expression in aortas of diabetic mice is only 25% of that in aortas of m Lepr\textsuperscript{db}, but AdipoR2 expression in coronary arterioles of diabetic mice is approximately 47% of that in coronary vessels of m Lepr\textsuperscript{db}. The mechanisms accounting for the reduced AdipoR2, but not AdipoR1, expression in diabetic mice vasculature, and the possible differences in AdipoR2 expression between vascular beds, remain unknown. However, our results suggest that, in addition to the reduced circulating level of adiponectin in diabetic mice (supplemental Table I), suppressed AdipoR2 expression in skeletal muscle and moderately expressed in other tissues, whereas AdipoR2 is predominantly expressed in the liver.\textsuperscript{20,31,32} Aortic endothelial cells express both adiponectin isoforms but appear to preferentially express mRNA of AdipoR1.\textsuperscript{3} In human umbilical vein endothelial cells, globular adiponectin-induced phosphorylation of eNOS at serine 1177 and NO production\textsuperscript{22} were abrogated when expressions of AdipoR1 and AdipoR2 were simultaneously suppressed.\textsuperscript{34} Overexpression of AdipoR1 and AdipoR2 in human umbilical vein endothelial cells significantly enhanced the suppressive effect of an otherwise symptomless dose of globular adiponectin on TNF-α–induced intercellular adhesion molecule 1 expression and NFκB activation, suggesting the involvement of AdipoRs in adiponectin-induced vasoprotection against the proinflammatory effects of TNF-α.\textsuperscript{35} To our knowledge, this study provides the first in vivo documentation that AdipoR1 expression is similar between control and diabetic mice but AdipoR2 expression is significantly reduced in both coronary arterioles and aortas in diabetic mice (Figure 5). Furthermore, AdipoR2 expression in aortas of diabetic mice is only 25% of that in aortas of m Lepr\textsuperscript{db}, but AdipoR2 expression in coronary arterioles of diabetic mice is approximately 47% of that in coronary vessels of m Lepr\textsuperscript{db}. The mechanisms accounting for the reduced AdipoR2, but not AdipoR1, expression in diabetic mice vasculature, and the possible differences in AdipoR2 expression between vascular beds, remain unknown. However, our results suggest that, in addition to the reduced circulating level of adiponectin in diabetic mice (supplemental Table I), suppressed AdipoR2
expression and the receptor-mediated response may also contribute to the impaired adiponectin-mediated vascular protective effects. Moreover, an increased serum TNF-α level (supplemental Table 1) and TNF-α receptor 1 expression in diabetic mice synergistically exacerbated the detrimental effects of TNF-α on vascular function.36 Thus, in addition to modulating adiponectin and TNF-α production and circulatory levels, treatments mediating AdipoR2 and TNF-α receptor 1 expression may have potential therapeutic applications for vascular complications associated with the metabolic syndrome and diabetes.

**Adiponectin and TNF-α Converge on NFκB to Regulate Their Reciprocal Suppression**

Adiponectin-mediated suppression of TNF-α expression and inflammatory responses by the inhibition of NFκB signaling functionally contribute to the beneficial actions of adiponectin.37,38 Full-length protein suppresses TNF-α–induced inflammatory changes in human aortic endothelial cells by blocking IkBα phosphorylation and NFκB activation without affecting TNF-α–mediated activation of c-JUN N-terminal kinase, p38, and protein kinase B (Akt).38 GAd has attenuated lipopolysaccharide-stimulated NFκB production in macrophages by suppression of NFκB activation.37 TNF-α per se suppresses adiponectin secretion in 3T3-L1 adipocytes; this suppression was reversed by an IkB kinase-β inhibitor, IMD-0354.39 These data suggest that NFκB signaling may act as a pivot for a reciprocal association between adiponectin and TNF-α.

Our studies demonstrate this pivotal role of NFκB signaling in the reciprocal association between adiponectin and TNF-α in both coronary microcirculation and aortas in the type 2 diabetic murine model. The results reveal that adiponectin and anti–TNF-α treatment remarkably inhibit IkBα phosphorylation and NFκB expression in coronary arterioles and aortas of Leprdb without affecting total IkBα expression (Figure 6). Therefore, adiponectin and TNF-α may converge on NFκB signaling to reciprocally regulate their expression and function in coronary microvessels and aortas in type 2 diabetic mice. In conclusion, although TNF-α is a key adipokine promoting endothelial dysfunction, adiponectin may prevent vascular injury. This paradigm for adipokine regulation of endothelial function may have important therapeutic implications in diabetes-associated vascular complications.

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**Disclosures**

None.

**References**


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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\textsuperscript{db}) (Background Strain: C57BLKS/J), homozygote type 2 diabetic mice (Lepr\textsuperscript{db}) (Background Strain: C57BLKS/J) and Lepr\textsuperscript{db} null for TNF\textalpha (dbTNF\textsuperscript{-}/dbTNF\textsuperscript{-}) (Background Strain: C57BL/6J) were purchased from Jackson Laboratory and Adiponectin knockout mice (APN\textsuperscript{+/-}) (Background Strain: C57BL/6J) were from Dr. William P. Fay’s laboratory. All of these mice were maintained on a normal rodent chow diet. Male, 20-35g m Lepr\textsuperscript{db} and APN\textsuperscript{+/-}, 40-60 g Lepr\textsuperscript{db}, and dbTNF\textsuperscript{-}/dbTNF\textsuperscript{-} mice of either sex were used in this study. The cross (dbTNF\textsuperscript{-}/dbTNF\textsuperscript{-}) of Lepr\textsuperscript{db} with TNF\textalpha knockout mice is heterozygous for Lepr\textsuperscript{db} and homozygous for TNF knockout mice (TNF\textsuperscript{+/-}). These dbTNF\textsuperscript{-}/dbTNF\textsuperscript{-} 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\textsuperscript{db} and TNF\textsuperscript{+/-} were used in experimentation.\textsuperscript{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:\textsuperscript{2}

\[ \text{HOMA-IR} = \left( \frac{\text{non-fasting glucose} \times \text{non-fasting insulin}}{22.5} \right) \]
Treatment with Adiponectin, TNFα Neutralization, or Recombinant TNFα

At 12-16 weeks of age, Lepr\textsuperscript{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\textsuperscript{db} mice received the neutralizing anti-TNFα (anti-TNF, 0.625 mg/ml/kg/day, i.p. for 10 days).\textsuperscript{3} m Lepr\textsuperscript{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.\textsuperscript{3} Coronary arterioles (40 to 100 µm in diameter) from mouse heart were carefully dissected for \textit{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\textsubscript{2}O) were assessed in isolated coronary arterioles in m Lepr\textsuperscript{db}, Lepr\textsuperscript{db} and Lepr\textsuperscript{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\textsubscript{2}O 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 KH₂PO₄, 1.17 mM MgSO₄•7H₂O, 2.50 mM CaCl₂•2H₂O, 14.9 mM NaHCO₃, 5.5 mM D-Glucose, and 0.03 mM EDTA. Aortas were maintained in PSS in 95% O₂-5% CO₂ 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. The contribution of NO in vasorelaxation was assessed by incubating the vessels with NOS (eNOS and neuronal NOS) inhibitor N°-nitro-L-arginine methyl ester (L-NAME; 100 µmol/L for 20min).

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.\textsuperscript{3-4} The relative amounts of protein expression in various groups were quantified and normalized to those of the corresponding \textit{mLepr}\textsuperscript{db} control, which were set to a value of 1.0.\textsuperscript{3}

\textbf{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).\textsuperscript{6}

\textbf{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 Fibrous 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).\textsuperscript{7} Efficiency of the PCR reaction was determined using dilution series of a standard vascular sample. Quantification was performed using the 2\textsuperscript{-ΔΔCT} method as reported.\textsuperscript{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<sup>db</sup> mice and Lepr<sup>db</sup> mice treated with adiponectin or anti-TNFα, compared with the mean transcript abundance in aortas of mice Lepr<sup>db</sup> 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. 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

<table>
<thead>
<tr>
<th>Groups</th>
<th>m Lepr&lt;sup&gt;db&lt;/sup&gt;</th>
<th>Lepr&lt;sup&gt;db&lt;/sup&gt;</th>
<th>Lepr&lt;sup&gt;db&lt;/sup&gt;+adiponectin</th>
<th>Lepr&lt;sup&gt;db&lt;/sup&gt;+anti-TNF</th>
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</thead>
<tbody>
<tr>
<td>Body Weight, g</td>
<td>25.54±0.62</td>
<td>45.18±0.47*</td>
<td>45.11±0.89*</td>
<td>45.03±1.08*</td>
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<tr>
<td>Abdominal Girth, cm</td>
<td>8.56±0.12</td>
<td>12.56±0.30*</td>
<td>12.75±0.33*</td>
<td>12.64±0.27*</td>
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<tr>
<td>Blood Glucose, mg/dl (non-Fasting)</td>
<td>156.25±8.06</td>
<td>529.63±11.58*</td>
<td>515.75±18.15*</td>
<td>523.38±20.83*</td>
</tr>
<tr>
<td>Insulin, ng/ml (non-Fasting)</td>
<td>2.83±0.77</td>
<td>6.46±0.70*</td>
<td>6.93±0.24*</td>
<td>7.03±0.11*</td>
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<tr>
<td>HOMA</td>
<td>29.78±7.66</td>
<td>234.31±22.39*</td>
<td>250.47±18.34*</td>
<td>244.59±9.32*</td>
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<tr>
<td>Adiponectin (μg/ml)</td>
<td>9.54±0.89</td>
<td>5.43±0.37*</td>
<td>8.88±0.85#</td>
<td>5.24±0.29*</td>
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<tr>
<td>TNFα (pg/ml)</td>
<td>9.01±1.43</td>
<td>33.51±2.92*</td>
<td>28.83±2.82*</td>
<td>10.49±1.56#</td>
</tr>
</tbody>
</table>

Table II. – Log EC<sub>50</sub> and E<sub>max</sub> Values for ACh Concentration-Response Curves

<table>
<thead>
<tr>
<th></th>
<th>-Log EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>E&lt;sub&gt;max&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>m Lepr&lt;sup&gt;db&lt;/sup&gt;</td>
<td>7.71±0.06</td>
<td>87.12±2.42</td>
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<tr>
<td>Lepr&lt;sup&gt;db&lt;/sup&gt;</td>
<td>7.10±0.10*</td>
<td>73.31±1.74*</td>
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<td>Lepr&lt;sup&gt;db&lt;/sup&gt; + adiponectin</td>
<td>7.32±0.05*</td>
<td>83.13±1.62#</td>
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<tr>
<td>Lepr&lt;sup&gt;db&lt;/sup&gt; + anti-TNF</td>
<td>7.21±0.04*</td>
<td>83.04±1.79#</td>
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<tr>
<td>m Lepr&lt;sup&gt;db&lt;/sup&gt; + TNF</td>
<td>7.70±0.09#</td>
<td>78.12±1.24*</td>
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</table>

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession #</th>
<th>Sequence</th>
<th>Target Length</th>
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<tr>
<td>TNFα s</td>
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<td>GTCCCCAAGGGATGAGAAG</td>
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<td>TNFα as</td>
<td>NM_013693</td>
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<td>134</td>
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<tr>
<td>Adiponectin s</td>
<td>NM_009605</td>
<td>AGGTTGGATGGCGAGGC</td>
<td>129</td>
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<tr>
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<td>GTTCACCCCTTAGGACCAAGAA</td>
<td>129</td>
</tr>
<tr>
<td>β-actin s</td>
<td>NM_007393</td>
<td>GCTTTTTTGACCTTCTCTTT</td>
<td>168</td>
</tr>
<tr>
<td>β-actin as</td>
<td>NM_007393</td>
<td>CTTCTGCGATCCTGTCAGCAA</td>
<td>168</td>
</tr>
</tbody>
</table>
Figure I.

A

mRNA Expression of TNFα in Aorta (Fold of Control)

B

mRNA Expression of Adiponectin in Aorta (Fold of Control)

Figure II.

Adiponectin  vWF  Merge

A  B  C

E  F  G

I  J  K

M  N  O

Q  R  S

m Lepr<sup>db</sup>  Lepr<sup>db</sup>  Lepr<sup>db</sup>+ Adiponectin  Lepr<sup>db</sup>+ anti-TNF

db<sup>vWF-/-</sup>/db<sup>−/−</sup>

Adiponectin  α-actin  Merge

m Lepr<sup>db</sup>

Negative Ctl  Negative Ctl  DAPI

m Lepr<sup>db</sup>
Table I. Baseline Plasma Parameters

Body weight and abdominal girth were higher in Lepr<sup>db</sup> vs. m Lepr<sup>db</sup>. Non-fasting blood glucose level, plasma insulin level and HOMA-IR were elevated in Lepr<sup>db</sup>. Adiponectin or anti-TNFα treatment did not affect the above parameters in Lepr<sup>db</sup>. Serum adiponectin level was reduced in Lepr<sup>db</sup>, while TNFα level was increased in Lepr<sup>db</sup>. 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<sup>db</sup>, #p<0.05 vs. Lepr<sup>db</sup>. 
Table II. –Log EC50 and E\text{max} Values for ACh Concentration-Response Curves

The –log EC50 was significantly lower in Lepr\text{db}. Lepr\text{db} treated with adiponectin or anti-TNF\text{\alpha} (anti-TNF) and m Lepr\text{db} treated with recombinant TNF\text{\alpha} (TNF) showed no changes in –log EC50. The E\text{max} for ACh-induced vasorelaxation was decreased in Lepr\text{db}. Adiponectin and anti-TNF\text{\alpha} treatment increased E\text{max} in Lepr\text{db}. m Lepr\text{db} treated with recombinant TNF\text{\alpha} showed lower E\text{max} compared with that of m Lepr\text{db}. Data were shown as mean±SEM. n=4-10 mice. *P<0.05 vs. m Lepr\text{db}, #p<0.05 vs. Lepr\text{db}.

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

Figure Legends

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

Figure II. Dual fluorescence combining adiponectin with markers for endothelial cells [von Willebrand factor (vWF)] and vascular smooth muscle (\text{\alpha}-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\text{db} mouse aorta. I, J and K dual labeling of adiponectin (red) and vWF (green) in db\text{TNF–}/db\text{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 adiponectin (red) and \( \alpha \)-actin (green) in m Lepr\(^{db} \) mouse aorta. The pink arrow in O shows the specific \( \alpha \)-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 \( \mu \)M 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


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-