Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2009 August ; 29(8): 1164–1171. doi:10.1161/ATVBAHA.109.187146.

Resveratrol Improves Endothelial Function: Role of TNFα and Vascular Oxidative Stress

Hanrui Zhang[*], **Jing Zhang**[*], **Zoltan Ungvari**[#,%], and **Cuihua Zhang**[*]

[*]Department of Internal Medicine, Medical Pharmacology & Physiology and Nutritional Sciences, Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO 65211

[#]Department of Physiology, New York Medical College, Valhalla, NY 10595

[%]Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine, University of Oklahoma Health Science Center, Oklahoma City, OK 73104

Abstract

Objective—Oxidative stress plays an important role in type 2 diabetes-related endothelial dysfunction. We hypothesized that resveratrol protects against oxidative stress-induced endothelial dysfunction in aortas of diabetic mice by inhibiting tumor necrosis factor-alpha (TNFα)-induced activation of NAD(P)H oxidase and preserving phosphorylation of endothelial nitric oxide synthase (eNOS).

Methods and Results—We examined endothelial-dependent vasorelaxation to acetylcholine (ACh) in diabetic mice ($Lepr^{db}$) and normal controls (m $Lepr^{db}$). Relaxation to ACh was blunted in $Lepr^{db}$ compared with m $Lepr^{db}$ while endothelial-independent vasorelaxation to sodium nitroprusside (SNP) was comparable. Resveratrol improved ACh-induced vasorelaxation in $Lepr^{db}$ without affecting dilator response to SNP. Impaired relaxation to ACh in $Lepr^{db}$ was partially reversed by incubating the vessels with NAD(P)H oxidase inhibitor apocynin and a membrane-permeable superoxide dismutase mimetic TEMPOL. Dihydroethidium (DHE) staining showed an elevated superoxide ($O_2^{\cdot -}$) production in $Lepr^{db}$ while both resveratrol and apocynin significantly reduced $O_2^{\cdot -}$ signal. Resveratrol increased nitrite/nitrate levels and eNOS (Ser1177) phosphorylation, and attenuated H_2O_2 production and nitrotyrosine (N-Tyr) content in $Lepr^{db}$ aortas. Furthermore, resveratrol attenuated the mRNA and protein expression of TNFα. Genetic deletion of TNFα in diabetic mice (db^{TNF-}/db^{TNF-}) was associated with a reduced NAD(P)H oxidase activity and vascular $O_2^{\cdot -}$ production and an increased eNOS (Ser1177) phosphorylation, suggesting that TNFα plays a pivotal role in aortic dysfunction in diabetes by inducing oxidative stress and reducing NO bioavailability.

Conclusions—Resveratrol restored endothelial function in type 2 diabetes by inhibiting TNFα-induced activation of NAD(P)H oxidase and preserving eNOS phosphorylation, suggesting the potential for new treatment approaches to promote vascular health in metabolic diseases.

Correspondence: Cuihua Zhang, MD, PhD, Departments of Internal Medicine, Medical Pharmacology & Physiology and Nutritional Sciences, Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO 65211, Phone: 573-882-2427 (O); 573-884-6208 (Lab), Fax: 573-884-4232, ZhangCu@missouri.edu.

Subject Codes:
(91) Oxidant stress
(95) Endothelium/vascular type/nitric oxide
(190) Type 2 diabetes

Conflicts of Interest:
None.

Keywords

Antioxidants; Nitric Oxide; Nitric Oxide Synthase; Superoxide; Vasodilation

Introduction

Epidemiological studies indicate that resveratrol, a plant polyphenol widely consumed in the Mediterranean diet, is associated with reduced risk of cardiovascular diseases,[1] which account for the majority of the mortality in type 2 diabetes.[2] Vascular dysfunction is the initial step in the occurrence of many disease states in the cardiovascular system concurrent with diabetes.[3] Obesity is an established risk factor for type 2 diabetes. Recent studies suggest that resveratrol, by activating silent mating type information regulation 2 homolog 1 (SIRT1), safely mimics the effects of dietary restriction in laboratory animals.[4] In diet-induced obese mice, resveratrol improves insulin sensitivity, lowers plasma glucose, and increases mitochondrial capacity.[5] Furthermore, resveratrol attenuates aortic dysfunction in aging and high fat-induced obesity animal models.[6] However, there is no study regarding the effects of resveratrol on insulin sensitivity and vascular dysfunction in type 2 diabetic mice.

Oxidative stress is known to be the key mechanism in the pathogenesis of diabetes-related vascular dysfunction. Oxidative stress is due to excessive production of reactive oxygen species (ROS). The inactivation of nitric oxide (NO, a potent vasodilator synthesized by endothelial NO synthase, eNOS) by ROS is recognized to be a crucial factor in reducing NO bioavailability and the development of endothelial dysfunction.[7] NAD(P)H oxidase is a key source of superoxide ($O_2^{\cdot -}$) in the vasculature.[8] Previous work shows that anti-tumor necrosis factor–alpha (TNFα)-treatment inhibits NAD(P)H oxidase activation in coronary arterioles from type 2 diabetic mice.[9] TNFα is reported to down-regulate eNOS expression in fat and muscle of obese rodents.[10] Several studies also reported that resveratrol attenuates TNFα expression activated by lipopolysaccharides (LPS),[11, 12] leading us to hypothesize that resveratrol protects against vascular dysfunction occurring during diabetes by attenuating TNFα-induced vascular oxidative stress, therefore improving NO bioavailability. To test this, we examined the effects of resveratrol on hyperglycemic status and vascular function using a type 2 diabetic mouse model.

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. At the age of 10 weeks, m $Lepr^{db}$ and $Lepr^{db}$ mice were either treated with resveratrol (20 mg/kg/day, Cayman Chemical) or vehicle (0.5% methylcellulose) orally for 4 weeks.[13]

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.[14] Quantification was performed using the efficiency-corrected ΔΔCT method and β-actin was used for internal normalization.[14]

Functional Assessment of Murine Aortas

2 mm of aortic rings were isometrically mounted in a myograph (model 610M, DMT, Denmark) and 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, 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),[15] 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.[16]

Protein Expression of TNFα, gp91^{phox}, eNOS, phospho-eNOS, and Nitrotyrosine (N-Tyr) by Western Blot Analyses

TNFα, gp91^{phox}, eNOS, phospho-eNOS, and N-Tyr protein expressions were detected in aortas by Western Blot. The relative amounts of protein expression were quantified to those of the corresponding m Lepr^{db} control.

NADP(H) Oxidase Activity

NAD(P)H oxidase activity was assayed in homogenized aorta samples using lucigenin-derived chemiluminescence assay as previously reported.[9, 17] $O_2^{\cdot -}$ 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).

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.[18] 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 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 TEMPOL (1 mmol/L, 60 min). Quantification of fluorescence intensity was determined by using Image-J (NIH, Bethesda, MD) and normalized to m Lepr^{db}.

Measurement of Serum Hydrogen Peroxide (H_2O_2)

Serum production of H_2O_2 was determined by using QuantiChrom™ peroxide assay kit (BioAssay Systems).

Measurement of Nitrite/Nitrate

Aortas were homogenized and supernatants were collected for the quantification of nitrite/nitrate level using amperometric sensors (WPI, World Precision Instruments) according to previous publication.[19] Briefly, nitrate was converted to nitrite using Nitralyzer Nitrate to Nitrite Reduction Kit (WPI). 2 mm sensor (ISO-NOP) was calibrated by chemical generation of NO. 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. Significance was accepted at $P < 0.05$.

Results

Resveratrol Attenuated mRNA and Protein Expression of TNFα in Type 2 Diabetes

mRNA and protein expression of TNFα were examined in isolated aortas of m $Lepr^{db}$ and $Lepr^{db}$ mice either treated with resveratrol (m $Lepr^{db}$+RSV and $Lepr^{db}$+RSV) or vehicle (m $Lepr^{db}$ and $Lepr^{db}$). mRNA and protein expression of TNFα were significantly elevated in $Lepr^{db}$ compared with m $Lepr^{db}$. Resveratrol treatment reduced TNFα mRNA and protein expression in $Lepr^{db}$ without affecting that in m $Lepr^{db}$ (Figure 1).

Resveratrol Improved Aortic Endothelial Dysfunction in Type 2 Diabetes

Isolated aortic rings from m $Lepr^{db}$ and $Lepr^{db}$ either treated with resveratrol or vehicle dilated dose-dependently to endothelium-dependent and endothelium–independent agonists (ACh and SNP respectively). Relaxation to ACh was blunted in $Lepr^{db}$ compared with m $Lepr^{db}$ while endothelial-independent vasorelaxation to SNP was comparable. Resveratrol improved ACh-induced vasorelaxation in $Lepr^{db}$ without affecting dilator response to SNP (Figure 2).

Role of TNFα and NAD(P)H Oxidase in Diabetes-induced Aortic Vascular Dysfunction

In db^{TNF-}/db^{TNF-}, ACh-induced vasorelaxation was greater than that in $Lepr^{db}$ while recombinant TNFα incubation impaired endothelial-dependent vasorelaxation in m $Lepr^{db}$ (Figure 3A). Although resveratrol restored endothelial function in diabetic mice, the improvement was abolished by incubating the vessel with recombinant TNFα (Figure 3B). Furthermore, administration of apocynin and TEMPOL rescued impaired vasorelaxation to ACh in $Lepr^{db}$ (Figure 3C).

Resveratrol Inhibited NAD(P)H Activation and Subsequent $O_2^{\cdot -}$ Production

$O_2^{\cdot -}$ production was significantly elevated in diabetic mice compared with control mice and resveratrol decreased vascular $O_2^{\cdot -}$ production (Figure 4A). Although db^{TNF-}/db^{TNF-} showed decreased $O_2^{\cdot -}$ production compared with $Lepr^{db}$, the $O_2^{\cdot -}$ production in m $Lepr^{db}$ was enhanced after incubating the vessels with recombinant TNFα. In $Lepr^{db}$, vascular $O_2^{\cdot -}$ production was reduced to the level comparable to m $Lepr^{db}$ by incubating the vessel with apocynin (Figure 4A). Quantification of fluorescence intensity is available in online supplemental materials (Figure II. Please see www.ahajournals.org). Figure 4B and 4C show that NAD(P)H oxidase activity and its subunit, $gp91^{phox}$ protein expression were significantly elevated in diabetic mice. Resveratrol inhibited NAD(P)H oxidase activation and $gp91^{phox}$ expression. db^{TNF-}/db^{TNF-} mice also showed reduced NAD(P)H oxidase activity compared with that of $Lepr^{db}$ (Figure 4B).

Resveratrol Increased NO Bioavailability and eNOS Phosphorylation

Aortic nitrite/nitrate levels were reduced in diabetic mice and resveratrol enhanced aortic NO release (Figure 5A). Although total eNOS protein expression was comparable among groups, eNOS phosphorylation was decreased in diabetic mice. Resveratrol increased eNOS phosphorylation at Ser1177 in the aorta of diabetic mice (Figure 5B). Protein expression of phospho-eNOS was greater in db^{TNF-}/db^{TNF-} compared with that in $Lepr^{db}$ (Figure 5B).

Resveratrol Decreased N-Tyr Expression and Serum H_2O_2 Production

Western blot analysis (Figure 6A) of N-Tyr protein expression in aortas from m $Lepr^{db}$ and $Lepr^{db}$ revealed a significantly higher level of N-Tyr in $Lepr^{db}$. Figure 6B shows elevated serum H_2O_2 production in $Lepr^{db}$ compared with control mice. Resveratrol significantly diminished the aortic N-Tyr protein expression and serum H_2O_2 level in diabetic mice without affecting those in control mice (Figure 6A and 6B).

Discussion

The pathogenic relationships among obesity, type 2 diabetes, and its vascular complications, remain poorly understood. Intensification of oxidative stress and inflammation has been implicated to accelerate vascular dysfunction in type 2 diabetes despite anti-hyperglycemic treatment. In past decades, numerous interventions have been put forward to counteract the vulnerability of the vasculature to oxidative challenges and inflammation in diabetes. Our major findings are: 1) Chronic resveratrol administration rescues aortic dysfunction in type 2 diabetes. 2) Resveratrol has potent effects that inhibit TNFα expression in aortas of diabetic mice. 3) Resveratrol attenuates macrovascular oxidative stress partially by inhibiting TNFα-induced NAD(P)H oxidase activation. 4) Resveratrol greatly improves aortic NO bioavailability through enhancing eNOS phosphorylation in type 2 diabetes. 5) Resveratrol does not induce weight loss, nor improve hyperglycemic status and insulin sensitivity in type 2 diabetic $Lepr^{db}$ mice.

Role of Resveratrol in Weight Loss and Hyperglycemic Status

The $Lepr^{db}$ mouse is a genetic model of non-insulin-dependent diabetes (type 2 diabetes), which has defects in receptors for the obese gene product, leptin.[20] The defects of leptin receptors, especially its long form Ob-Rb in $Lepr^{db}$ lead to impairments of leptin regulation on food intake and body weight mainly via activation of the hypothalamic leptin receptors.[21] and to a lesser extent via its peripheral and varied targets,[22] and result in the expression of diabetes, preceded by hyperinsulinemia, hyperglycemia, and extreme obesity.[23]

Resveratrol reverses hyperglycemic status and improves insulin sensitivity in high fat-induced obese rodents and in the type 1 diabetic animal model.[5, 24, 25] Oral administration of resveratrol at a dose of 22.4 mg/kg/day was shown to improve insulin sensitivity and to slightly reduce the body weight of 1-year-old mice fed a high fat diet.[24] At a dose of 400 mg/kg/day, resveratrol prevented diet-induced obesity and alleviated obesity-related insulin resistance.[5] In streptozotocin-induced type 1 diabetic rats, resveratrol dose-dependently decreased plasma glucose and lipid levels.[25] In contrast, our results show that resveratrol does not alter body weight nor reduce hyperinsulinemic hyperglycemia in $Lepr^{db}$ mice (Table I and Figure I. Please see www.ahajournals.org). Body weight is considered important in the control of type 2 diabetes[26] and the calorie restriction mimetic effects of resveratrol have been proposed to largely explain its anti-aging benefits.[4] Our work suggests there are therapeutic effects of resveratrol, especially on vascular complications, that can also occur independently of weight loss and hyperglycemic status.

Role of Resveratrol in TNFα Expression and anti-TNFα Treatment in Vascular Dysfunction

Our previous study demonstrated that TNFα contributes to microvascular dysfunction in type 2 diabetes evidenced by increased TNFα mRNA and protein expression in coronary arterioles of diabetic mice and improved coronary arteriolar endothelial function in db^{TNF-}/db^{TNF-}.[9] This study further supports the central role of TNFα in aortic dysfunction in type 2 diabetes and the protective effects of resveratrol in TNFα-induced vascular dysfunction. Resveratrol potently decreases TNFα mRNA and protein expression in diabetic mice aorta, suggesting profound anti-inflammatory effects of resveratrol in the macrovasculature in type 2 diabetes (Figure 1). As a potent inflammatory trigger, the central role of TNFα in vascular dysfunction has been demonstrated by the ability of agents that block the action of TNFα to treat a range of cardiovascular disorders and inflammatory conditions. Although anti-TNFα treatment has been extensively studied in vascular dysfunction accompanied by various inflammatory diseases such as rheumatoid arthritis and Crohn's disease, the beneficial effects of TNFα inhibition have not been fully clarified in cardiovascular diseases and in vascular complications associated with type 2 diabetes.

Our results demonstrated for the first time that resveratrol administration improves aortic endothelial function in type 2 diabetic mice without affecting the dilator response in control mice. Although the E_{max} is only slightly decreased in diabetic mice, the EC_{50} is significantly higher in diabetic mice compared with control mice (Table II. Please see www.ahajournals.org), suggesting the aortas of $Lepr^{db}$ exhibit attenuated endothelium-dependent relaxation in response to ACh. ACh interacts with endothelial muscarinic M3 receptors and causes NO release and vasodilation.[27] To rule out the possibility that the vascular dysfunction in $Lepr^{db}$ may be due to impaired muscarinic receptor activity, we examine adenosine-induced vasorelaxation, which is fully endothelial-dependent in murine aorta (data not shown). Our results reveal that diabetic mice show impaired adenosine-induced vasorelaxation and resveratrol restores vascular function (Figure IV. Please see www.ahajournals.org), which is consistent with the results of ACh-induced vasorelaxation. Furthermore, in db^{TNF-}/db^{TNF-}, ACh-induced vasorelaxation is greater than that of $Lepr^{db}$ and TNFα impairs endothelial function in m $Lepr^{db}$ control (Figure 3A). Although resveratrol restores endothelial function in diabetic mice, incubating the vessel with recombinant TNFα abolishes the improvement (Figure 3B). Therefore, TNFα appears to play a pivotal role in macrovascular dysfunction in type 2 diabetes and the vascular protective effects of resveratrol may be related to its TNFα inhibitory properties. Recently, long-term resveratrol treatment was also shown to improve aortic endothelial function in aged and high fat diet-induced obese mice.[4] Furthermore, chronic resveratrol consumption improved endothelium-dependent vasorelaxation in hypertensive (but not normotensive) rats.[28] These results suggest that resveratrol treatment has the potential to preserve vascular health when multiple cardiovascular risk factors are present.

Interestingly, in vitro administration of supra-physiological concentrations of resveratrol causes both endothelium-dependent and endothelium-independent relaxation of rat aortic ring preparations.[29, 30] In endothelium-intact rings, resveratrol can dose-dependently inhibit the contractile response of the vasculature to noradrenaline. This inhibitory effect can be blocked by NO synthase inhibitor N^G-nitro-L-arginine (L-NNA), suggesting an acute activation of NO formation/release induced by resveratrol. In addition, at higher concentrations resveratrol may also act directly on the vascular smooth muscle cells,[29] likely by modulating the function of voltage-gated K^+ (Kv) channels.[30] Although direct vasoactive effects of resveratrol are likely not responsible for the improvement of endothelial function in the present study, they may contribute to the in vivo effects of chronic resveratrol treatment.

Role of Resveratrol in TNFα-induced NAD(P)H Oxidase Activation and Subsequent Oxidative/Nitrative Stress

There are multiple cellular sources of superoxide anion, including NAD(P)H oxidase, xanthine oxidase, the mitochondrial respiratory chain, and the arachidonic acid cascade (including lipoxygenase and cycloxygenase).[31] Strong evidence supports the idea that activation of NAD(P)H oxidase contributes to vascular oxidative stress in virtually every forms of experimental diabetes.[32, 33] Furthermore, inhibition of ROS generation by genetic depletion of $gp91^{phox}$, by administration of dominant-negative Rac1, or by structurally different pharmacological inhibitors of NAD(P)H oxidase also significantly attenuate vascular $O_2^{\cdot -}$ production in diabetes.[32, 33] Activation of NAD(P)H oxidase by TNFα is a main contributor to the generation of oxidative stress in coronary microcirculation in type 2 diabetes.[9] In aortas from $Lepr^{db}$, NAD(P)H oxidase activity is greatly elevated (Figure 4B). db^{TNF-}/db^{TNF-} shows decreased NAD(P)H oxidase activity (Figure 4B), and inhibiting NAD(P)H oxidase activation by apocynin (100 μmol/L) potently reduces vascular $O_2^{\cdot -}$ production (Figure 4A) as well as improving endothelial function in diabetic mice (Figure 3C), while a xanthine oxidase inhibitor allopurinol (10 μmol/L), or the mitochondrial respiratory chain inhibitor rotenone (1 μmol/L), did not improve aortic function (data not shown). Although a recent study posit that apocynin

at high concentrations may exert direct antioxidant effects, at concentrations less than 1,000 μmol/L, apocynin did not scavenge $O_2^{\cdot -}$ produced by cell-free systems, as detected by either dihydroethidium (DHE) or lucigenin assays.[34] In the present study we used apocynin at a concentration of 100 μM, which in the study by Heumüller et al did not scavenge $O_2^{\cdot -}$ in DHE assay. Thus, we attribute the finding that low concentrations of apocynin decreased $O_2^{\cdot -}$ production in the aorta of $Lepr^{db}$ mice to the inhibition of NADPH oxidases by apocynin.

In situ detection of $O_2^{\cdot -}$ in aortas by DHE staining shows that the increased $O_2^{\cdot -}$ production mainly accumulates in the adventitia layers (Figure 4A), which is consistent with previous work using different staining methods.[35] Furthermore, immunohistochemical staining revealed specific labeling of the adventitia with NAD(P)H oxidase subunits $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$, whereas no substantial staining was observed in other areas of the aorta.[35] Our results reveal that $gp91^{phox}$ protein expression is elevated in $Lepr^{db}$ mice, suggesting the up-regulation of $gp91^{phox}$ in diabetic mice is associated with apocynin-inhibitable, NAD(P)H oxidase-derived $O_2^{\cdot -}$ production. Furthermore, resveratrol reduces $gp91^{phox}$ expression in $Lepr^{db}$ mice (Figure 4C). In light of previous results from studies using genetically altered mice and structurally different inhibitors of NADPH oxidase, our data support the conclusion that resveratrol-induced down-regulation of NAD(P)H oxidase expression contributes to the attenuation of NADPH oxidase-derived $O_2^{\cdot -}$ production and consequent improvement of endothelial function in the aorta of $Lepr^{db}$ mice.

The increase in vascular NAD(P)H oxidase-induced $O_2^{\cdot -}$ production may initiate profound oxidative/nitrative stress. On the one hand, inactivation of NO by $O_2^{\cdot -}$ anion is recognized to be a key mechanism underlying reduced NO bioavailability and the development of endothelial dysfunction.[7] The interaction of $O_2^{\cdot -}$ and NO produces peroxynitrite ($ONOO^-$), which leads to protein tyrosine nitration to generate nitrotyrosine.[36] Nitrotyrosine was initially considered as a specific marker of peroxynitrite generation, but now is generally regarded as an index of reactive nitrogen species, rather than a specific indicator of peroxynitrite formation. Accumulating evidence suggests that diabetes is associated with increased nitrosative stress and peroxynitrite formation.[37] Toxic actions of peroxynitrite and/or nitrotyrosine in the cardiovascular system are also supported by evidence showing that the degree of cell death and/or dysfunction correlates with levels of nitrotyrosine in endothelial cells, myocytes and fibroblasts from heart biopsies of diabetic patients.[38,39] Nitrotyrosine content is higher in endothelial cells of microvasculature in diabetic patients, which correlates with endothelial dysfunction,[40] suggesting that nitrotyrosine level is associated with diabetes-related endothelial dysfunction. On the other hand, $O_2^{\cdot -}$ can be dismutased spontaneously or by the catalytic effects of superoxide dismutase (SOD) to produce H_2O_2.[41] We show that aortic N-Tyr protein content is elevated in diabetic mice and is significantly reduced by resveratrol treatment (Figure 6A). Furthermore, resveratrol also attenuated H_2O_2 levels measured in the serum of diabetic mice (Figure 6B), suggesting that resveratrol has potent anti-oxidative activities. Resveratrol also up-regulates natural antioxidant enzymes. Superoxide dismutase (SOD)-1 and glutathione peroxidase (GPx) protein expression are decreased in $Lepr^{db}$ and resveratrol up-regulated aortic SOD-1 and GPx protein expression, whereas SOD-3 and catalase protein expression were similar among groups (Figure III. Please see www.ahajournals.org). More interestingly, in db^{TNF-}/db^{TNF-}, SOD-1 and GPx protein expression were similar to $Lepr^{db}$, indicating that the genetic deletion of TNFα in $Lepr^{db}$ did not affect the expression of antioxidant enzyme while resveratrol elevated antioxidant enzyme expression in $Lepr^{db}$. These results may explain why aortic function in db^{TNF-}/db^{TNF-} fails to return to the levels of the control and resveratrol treated $Lepr^{db}$.

Whether these antioxidant effects result primarily from a direct scavenging effect or are the result of the activation of pathways that up-regulate natural antioxidant defenses in the cells, or via the inhibition of oxidase enzymes, etc. is not yet clear. Because the resveratrol

concentration in the plasma of treated mice was reported to be much lower[42] than the resveratrol concentration needed in vitro to effectively scavenge free radicals (1 μmol/L to 100 μmol/L),[6, 14, 43] we posit that direct anti-oxidant effects of resveratrol are likely play only a secondary role in vasoprotection.

Therefore, our results provide direct evidence that chronic resveratrol administration inhibits TNFα-induced NAD(P)H oxidase activation and subsequent vascular oxidative stress *in vivo*, as well as up-regulating antioxidant enzymes, which is postulated to be the beneficial effects of dietary supplement of resveratrol on vascular dysfunction in type 2 diabetes.

Role of Resveratrol in the Prevention of TNFα-induced Imapirment of NO Bioavailability

In rat aortic endothelial cells, resveratrol treatment (100 μmol/L, 16 hours) increased nitrite/nitrate level 2-fold. This effect was completely abolished by knockdown of SIRT1 with siRNA.[6] Resveratrol inhibits the acetylation of endogenous eNOS on lysine residues *in vitro*, suggesting that SIRT1 activation may play a fundamental role in regulating endothelial NO and endothelium-dependent vascular tone by deacetylating eNOS.[6] Our study examined the effects of resveratrol on the (Ser1177) phosphorylation of eNOS. Although total eNOS protein expression is not statistically different among groups, phospho-eNOS protein content is reduced in the aorta of diabetic mice (Figure 5B). Resveratrol treated $Lepr^{db}$ mice exhibit increased (Ser1177) phosphorylation of eNOS (Figure 5B), which is associated with an increased NO bioavailability, as evidenced by increased aortic nitrite/nitrate level (Figure 5A). TNFα plays an important role in regulating eNOS expression and activity, as evidenced by down-regulation of mRNA and protein expression of eNOS following TNFα stimulation in endothelial cells.[44] TNFα also inhibits endothelial NO synthase gene promoter activity in bovine aortic endothelial cells.[45] Therefore, TNFα exerts transcriptional, as well as post-transcriptional, effects on eNOS gene expression. Since db^{TNF-}/db^{TNF-} also shows elevated phospho-eNOS expression in aortas (Figure 5B), we postulate that resveratrol's effects on enhancing eNOS phosphorylation and improving NO bioavailability may be related to its role in inhibiting TNFα expression.

Collectively, the vasoprotective activity of resveratrol against vascular oxidative stress and decreased NO bioavailability has been explained by the capacity of this polyphenol to inhibit the activation of vascular NAD(P)H oxidase and down-regulation of eNOS phosphorylation induced by TNFα. The detailed characterization of resveratrol's therapeutic effects in vascular tissue may identify novel therapeutic approaches that modulate this system to ameliorate the heightened cardiovascular risk associated with obesity and type 2 diabetes. We believe that a better understanding of the mechanisms by which resveratrol attenuates vascular oxidative stress as well as improves NO bioavailability in diabetes will lead to novel pharmacological interventions using resveratrol to prevent or delay the development of vascular complications in type 2 diabetic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

None.

Funding Sources

This study was supported by grants from the American Diabetes Association (to ZU) and the American Heart Association (110350047A, to CZ) and NIH (RO1-HL077566 and RO1-HL085119, to CZ).

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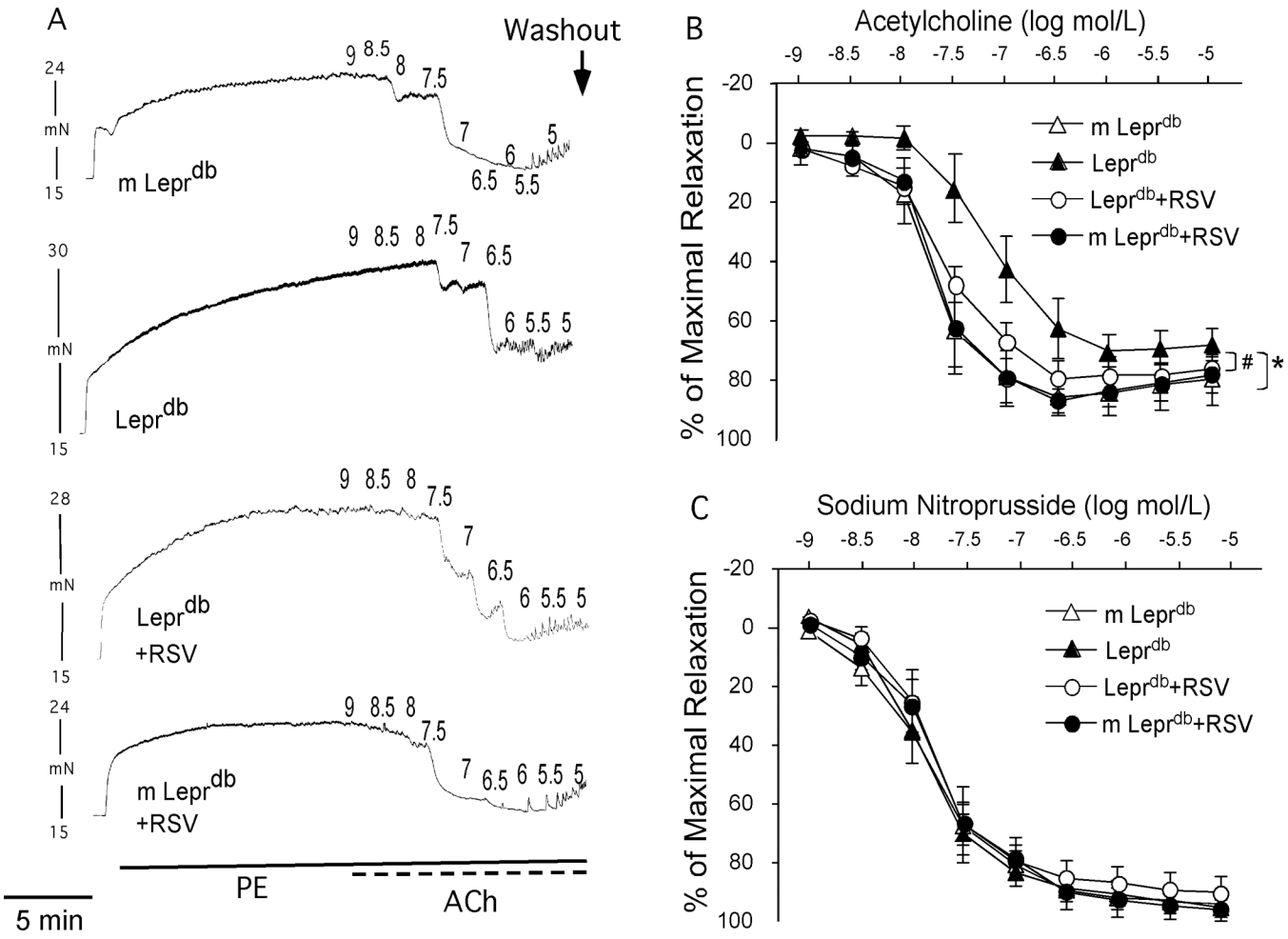

Figure 1.

mRNA (A) and protein expression (B) of TNFα (fold change) were significantly higher in Lepr^{db} than in m Lepr^{db}. Resveratrol (RSV) reduced TNFα expression (both mRNA and protein) in Lepr^{db}. n=5 separate experiments. *p<0.05 vs. m Lepr^{db}; #p<0.05 vs. Lepr^{db}.

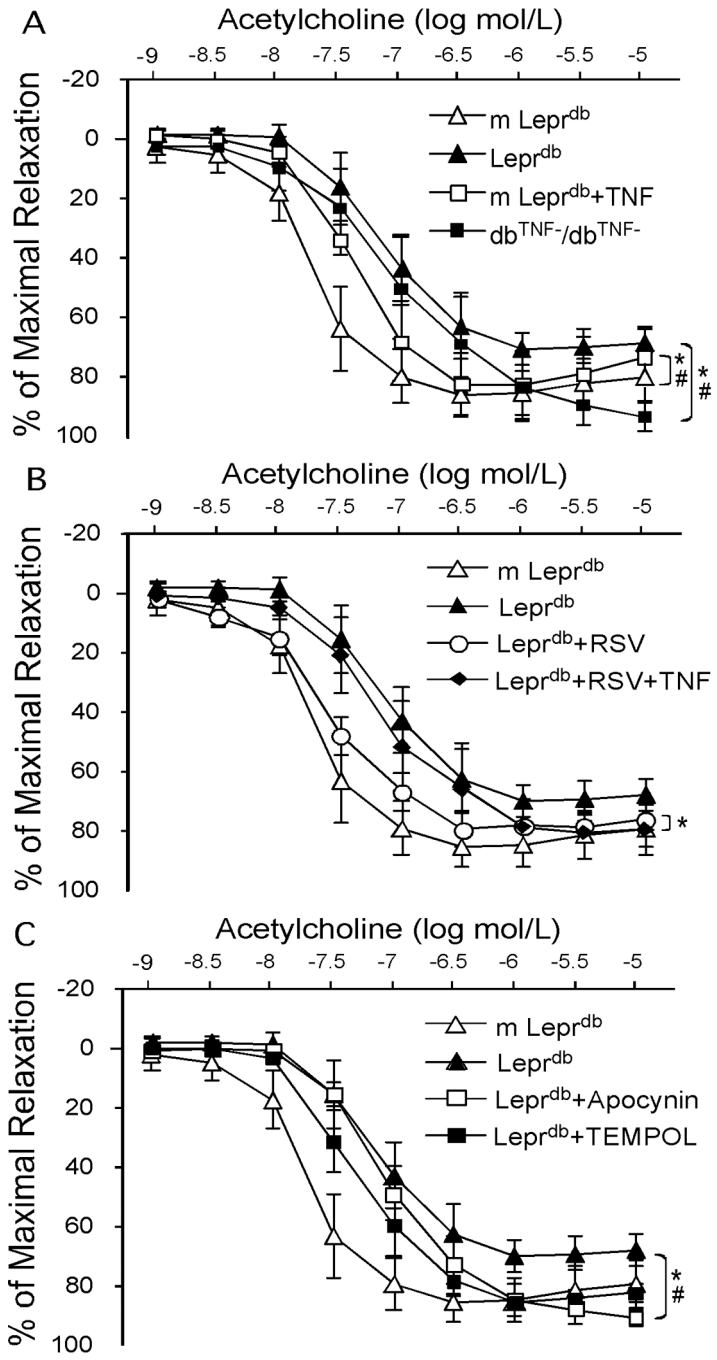

Figure 2.

Representative traces (A) and concentration-response curve (B) showed that endothelial-dependent vasorelaxation to ACh was significantly impaired in aortas of Lepr^{db}. Resveratrol treatment restored impaired vasorelaxation. (C) Endothelial-independent vasorelaxation to SNP was not statistically different among groups. n=7–12 mice. *p<0.05 vs. m Lepr^{db}; #p<0.05 vs. Lepr^{db}.

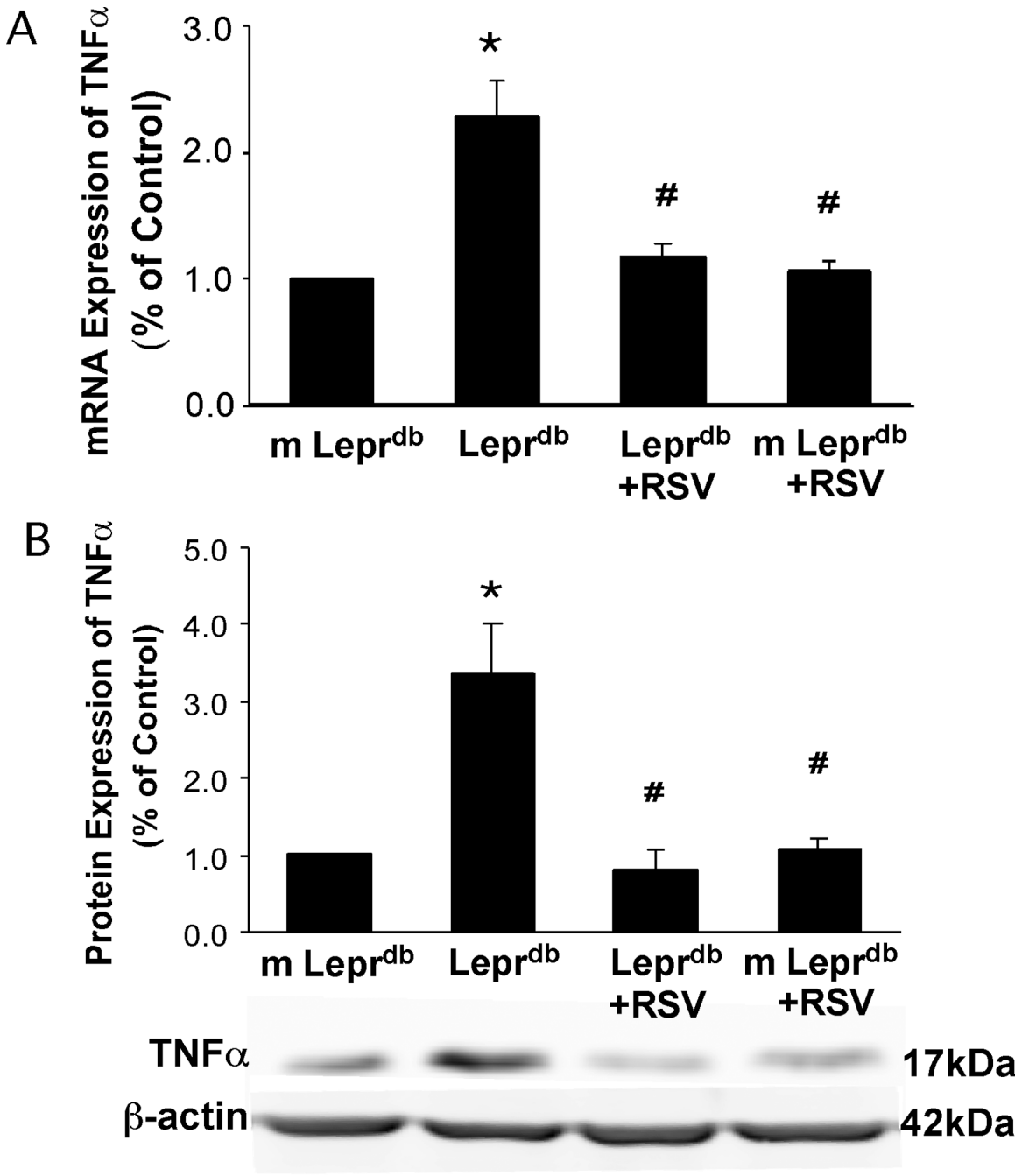

Figure 3.

Genetic deletion of TNFα (db^{TNF-}/db^{TNF-}) (A, n=8 mice), apocynin and TEMPOL (C, n=4 mice) partially restored impaired vasorelaxation in $Lepr^{db}$. TNFα incubation impaired endothelial function in m $Lepr^{db}$ (A, n=6 mice) and abolished the improvement in endothelial function by resveratrol treatment in $Lepr^{db}$ (B, n=6 mice). *p<0.05 vs. m $Lepr^{db}$; #p<0.05 vs. $Lepr^{db}$.

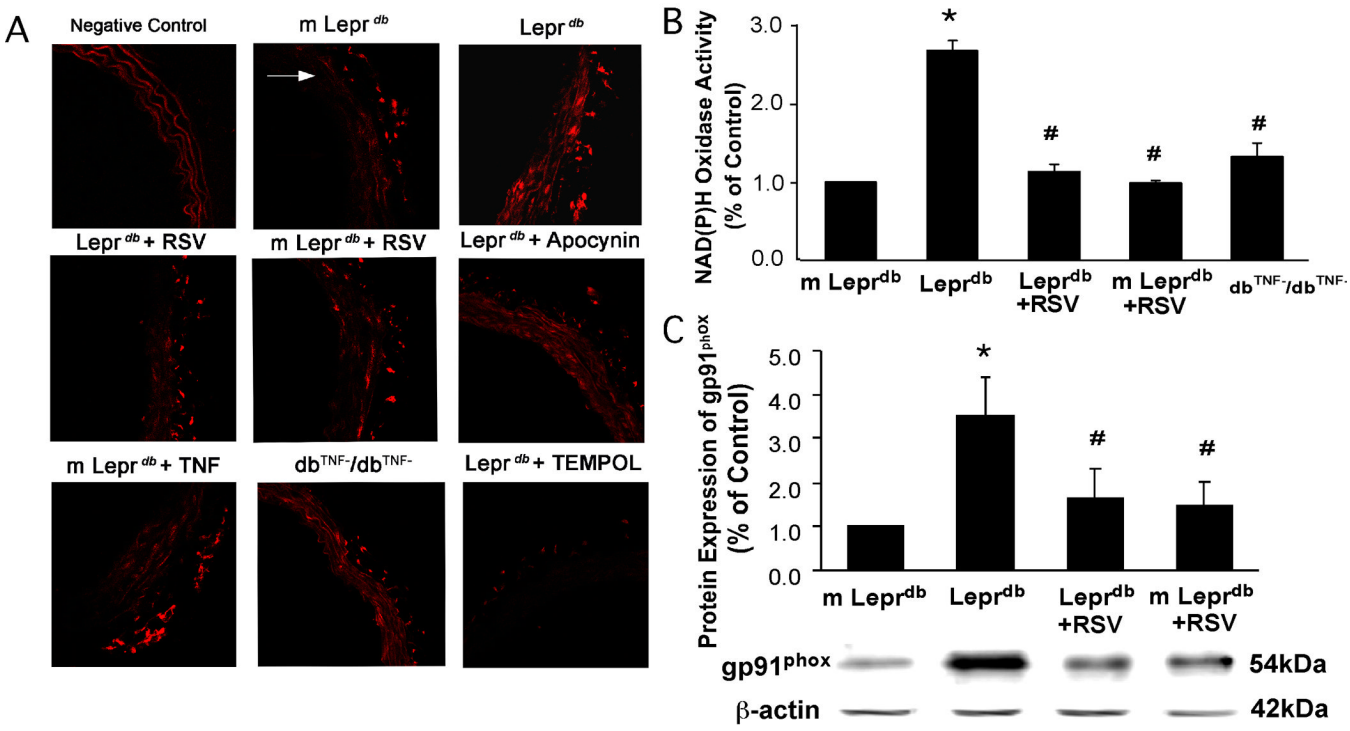

Figure 4.

(A) Resveratrol, apocynin and genetic deletion of TNFα significantly reduced aortic $O_2^{\cdot -}$ production in Lepr^{db}. Incubating aortas of m Lepr^{db} with TNFα increased $O_2^{\cdot -}$ production. White arrow indicates the lumen of aorta. (B) and (C), Resveratrol significantly decreased NAD(P)H oxidase activity and gp91^{phox} protein expression in Lepr^{db}. NAD(P)H oxidase activity was lower in db^{TNF-}/db^{TNF-} than in Lepr^{db} (B). n=4 separate experiments. *P < 0.05 vs. m Lepr^{db}; #P<0.05 vs. Lepr^{db}.

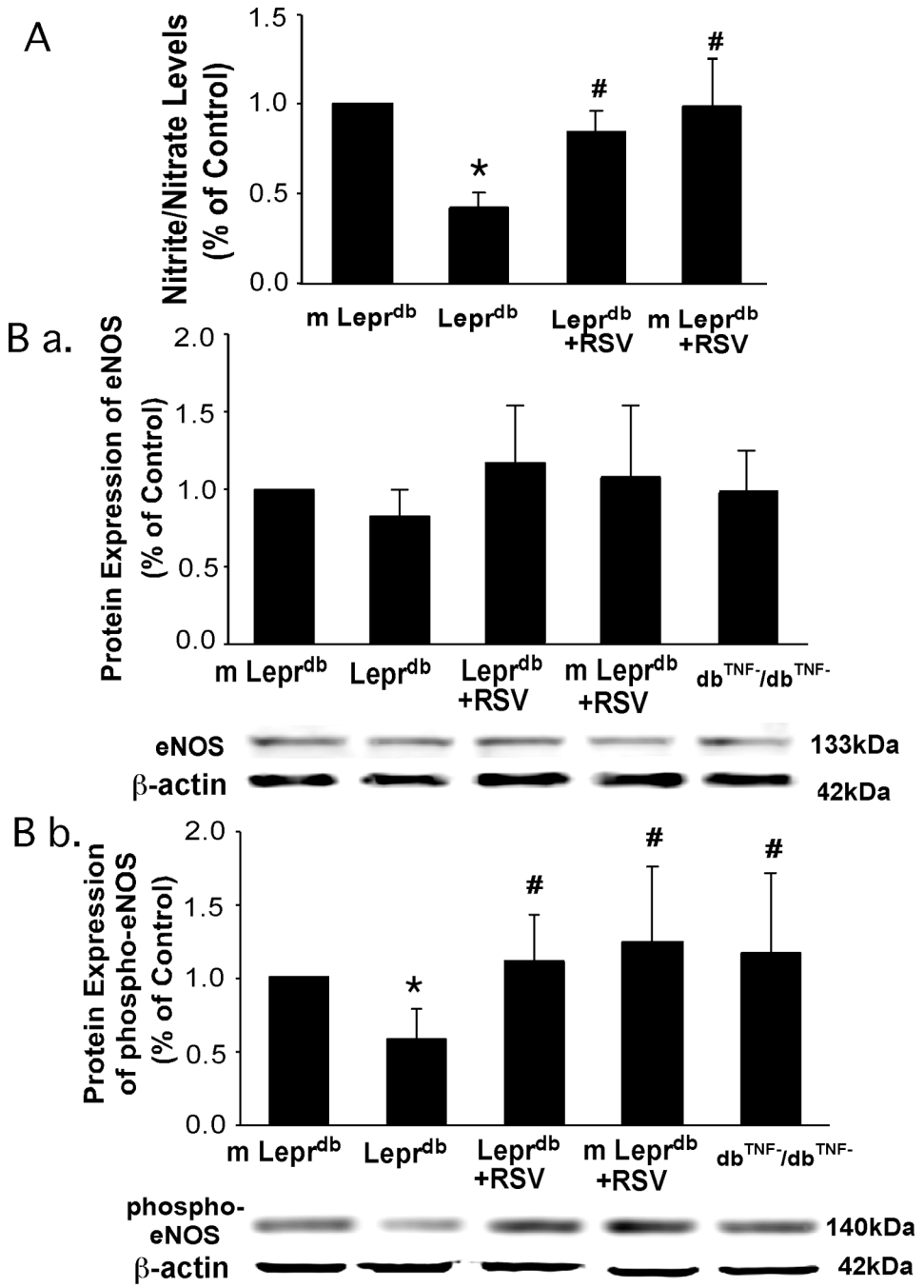

Figure 5.

Aortic nitrite/nitrate level (A) and phosphorylation of eNOS at Ser1177 (B b.) were lower in $Lepr^{db}$, while total eNOS expression (B a.) was similar among groups. Resveratrol enhanced nitrite/nitrate production and eNOS phosphorylation. eNOS phosphorylation was higher in db^{TNF-}/db^{TNF-} than in $Lepr^{db}$ (B b.). n=5 separate experiments. *P<0.05 vs. m $Lepr^{db}$; #P<0.05 vs. $Lepr^{db}$.

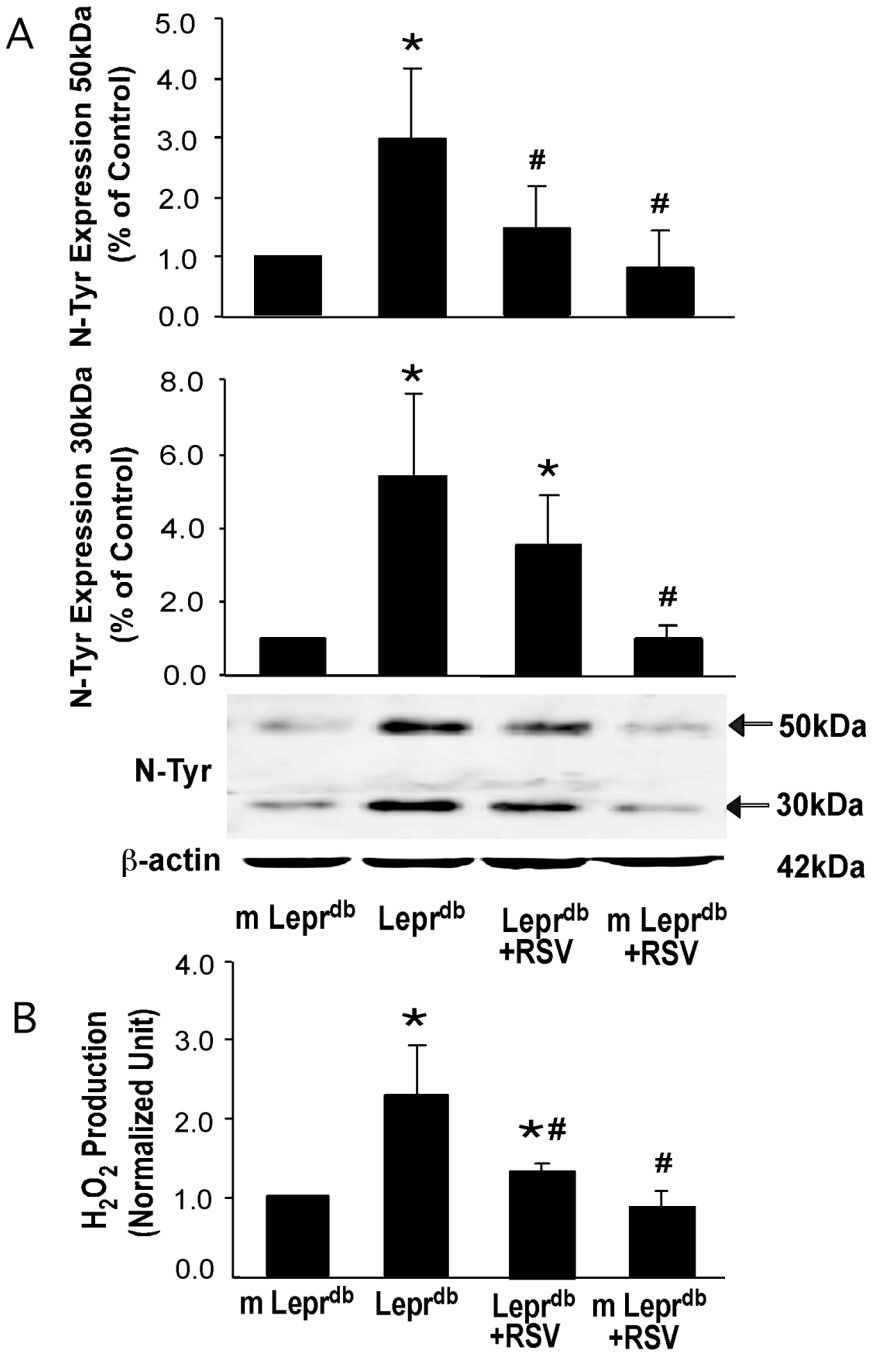

Figure 6.

Aortic N-Tyr expression (A) and serum H_2O_2 production (B) were higher in $Lepr^{db}$ than in m $Lepr^{db}$. Resveratrol decreased N-Tyr content and H_2O_2 levels in $Lepr^{db}$. n=4 separate experiments. *P<0.05 vs. m $Lepr^{db}$; #P<0.05 vs. $Lepr^{db}$.