Resveratrol improves left ventricular diastolic relaxation in type 2 diabetes by inhibiting oxidative/nitrative stress: in vivo demonstration with magnetic resonance imaging

Hanrui Zhang,1 Brandon Morgan,2,3 Barry J. Potter,4 Lixin Ma,2,3 Kevin C. Dellsperger,1 Zoltan Ungvari,5 and Cuihua Zhang1

1Department of Internal Medicine, Medical Pharmacology and Physiology, and Nutrition and Exercise Physiology, Dalton Cardiovascular Research Center, and 2Department of Radiology, and Nuclear Science and Engineering Institute, University of Missouri, Columbia, Missouri; 3Biomolecular Imaging Center, Harry S. Truman Memorial Veterans Hospital, Columbia, Missouri; 4Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, Louisiana; and 5Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma

TRANSLATIONAL PHYSIOLOGY

Resveratrol improves left ventricular diastolic relaxation in type 2 diabetes by inhibiting oxidative/nitrative stress: in vivo demonstration with magnetic resonance imaging

Submitted 20 May 2010; accepted in final form 26 July 2010

Hanrui Zhang,1 Brandon Morgan,2,3 Barry J. Potter,4 Lixin Ma,2,3 Kevin C. Dellsperger,1 Zoltan Ungvari,5 and Cuihua Zhang1

1Department of Internal Medicine, Medical Pharmacology and Physiology, and Nutrition and Exercise Physiology, Dalton Cardiovascular Research Center, and 2Department of Radiology, and Nuclear Science and Engineering Institute, University of Missouri, Columbia, Missouri; 3Biomolecular Imaging Center, Harry S. Truman Memorial Veterans Hospital, Columbia, Missouri; 4Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, Louisiana; and 5Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma

Diabetes is associated with an excessive cardiovascular morbidity and mortality. Diabetes is an independent risk factor for left ventricular (LV) dysfunction (17). Although one frequently associates cardiac dysfunction with enhanced coronary atherosclerosis in diabetic patients, evidence has accumulated for the existence of a specific “diabetic” cardiomyopathy, which has been shown even in the absence of hypertension and atherosclerosis in diabetic patients (47). The relative pathogenic significance of multiple factors that may alter myocardial performance in diabetic patients awaits further delineation (22). Furthermore, such delineation will aid in the discovery of effective therapies for diabetes-induced myocardial dysfunction.

Epidemiological studies (8, 18) have indicated that the Mediterranean diet is associated with a reduced risk of cardiovascular disease. Resveratrol is an important dietary constituent in the Mediterranean diet (37). Previous studies have revealed that resveratrol exerts cardioprotective effects in fructose-fed rat (21) and rats with streptozotocin-induced type 1 diabetes (41). However, the effects and mechanisms by which resveratrol protects against cardiac dysfunction in type 2 diabetes await elucidation.

MRI is a noninvasive technique that can assess both the systolic and diastolic function of the heart in vivo. Using this method, we aimed to assess cardiac function in control m-Leprdb mice and diabetic Leprdb mice treated with either resveratrol or vehicle. We hypothesized that resveratrol rescues cardiac dysfunction in type 2 diabetes by attenuating oxidative/nitrative stress as well as improving nitric oxide (NO) availability. To test this, we examined the effects of resveratrol on TNF-α-mediated NF-κB activation and the downstream signaling that contributes to the oxidative/nitrative stress and impaired NO availability to elucidate the mechanisms of resveratrol’s cardioprotective effects in type 2 diabetes.

METHODS

Animals and Treatments

Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Missouri-
Columbia and conformed to the current guidelines of the National Institutes of Health and the American Physiological Society for the care and use of laboratory animals. Heterozygote control (m-Lepr<sup>db</sup>) mice (background strain: C57BLKS/J), homozygous type 2 diabetic (Lepr<sup>db</sup>) mice (background strain: C57BLKS/J), and Lepr<sup>db</sup> mice null for TNF-α (dbTNF<sup>-/-</sup>/dbTNF<sup>-/-</sup>; background strain: C57BL/6J) were purchased from Jackson Laboratory and maintained on a normal rodent chow diet. Male m-Lepr<sup>db</sup> mice (weight: 20–35 g) as well as Lepr<sup>db</sup> (weight: 40–60 g) and dbTNF<sup>-/-</sup>/dbTNF<sup>-/-</sup> (weight: 40–60 g) mice of either sex were used in this study. These dbTNF<sup>-/-</sup>/dbTNF<sup>-/-</sup> mice show the phenotypes of hyperglycemia and obesity, the diabetic phenotype that is consistent with the penetrance of the leptin receptor (Lepr) mutation. Resveratrol (Cayman Chemical) was dispersed in 0.5% methylcellulose (Sigma) (9). At the age of 12 wk, male m-Lepr<sup>db</sup> and Lepr<sup>db</sup> mice were treated with either resveratrol (20 mg·kg<sup>-1</sup>·day<sup>-1</sup>) or methylcellulose vehicle for 4 wk by gastric gavage (34, 49).

**In Vivo Cine MRI**

Noninvasive MRI scans were performed on age-matched m-Lepr<sup>db</sup> and Lepr<sup>db</sup> mice with or without 4 wk of resveratrol treatment using a Varian 7-T horizontal-bore MRI (Varian, Palo Alto, CA) instrument equipped with a 38- and 60-mm quadrature-driven birdcage radiofrequency coil. ECG and respiratory monitoring and gating were done with a small animal monitoring system (Alto, CA) instrument equipped with a 38- and 60-mm quadrature-driven birdcage radiofrequency coil. ECG and respiratory signals were referenced to the MRI bore to maintain the animal’s body temperature. In addition, respiration monitoring and gating were done with a small animal monitoring system (Alto, CA) instrument equipped with a 38- and 60-mm quadrature-driven birdcage radiofrequency coil. ECG and respiratory signals were referenced to the MRI bore to maintain the animal’s body temperature.

**Protein Expression of TNF-α, IκB-α, Phospho-IκB-α, NF-κB, gp91<sup>phox</sup>, endothelial NO synthase, inducible NO synthase, and Nitrotyrosine by Western Blot Analyses**

Protein expressions were detected using TNF-α, IκB-α, and phospho-IκB-α primary antibodies (Santa Cruz Biotechnology), NF-κB, inducible NO synthase (iNOS), and nitrotyrosine (N-Tyr) primary antibodies (Abcam), and endothelial NO synthase (eNOS) and gp91<sup>phox</sup> primary antibodies (BD Science Pharmingen). Signals were scanned densitometrically using Fuji LAS3000 and quantified with Multi Gauge software (Fujifilm). The relative amounts of protein expression were quantified to those of the corresponding m-Lepr<sup>db</sup> control, which were set to a value of 1.0.

**NAD(P)H Oxidase Activity**

NAD(P)H oxidase activity was assayed in homogenized heart tissue extracts using a lucigenin (Sigma)-derived chemiluminescence assay as previously described (11, 49). Samples were read by Fluoroscan Ascent FL (Thermal Scientific), and NAD(P)H oxidase activity was normalized to the m-Lepr<sup>db</sup> control.

**Immunofluorescence Staining**

Immunohistochemistry was used to identify and localize gp91<sup>phox</sup> proteins in sections of vessels or myocardial tissue. Formalin-fixed hearts were sectioned at 5 μm. Primary antibodies to gp91<sup>phox</sup> (BD Biosciences Pharmingen), the endothelial cell marker von Willebrand factor (Abcam), the smooth muscle marker α-actin (Abcam), or the macrophage marker F4/80 (Serotec) were used for sequential double-immunofluorescence staining. Secondary fluorescent antibodies were either FITC or Texas Red conjugated. Sections were mounted in an anti-fading agent (Slowfade gold with 4',6-diamidino-2-phenylindole, Invitrogen). Slides were observed and analyzed using a fluorescence microscope with a ×40 objective (Zeiss Axiosplan Microscope).

**Measurement of O<sub>2</sub>− Using Electron Paramagnetic Resonance Spectroscopy**

O<sub>2</sub>− quantification from the electron paramagnetic resonance (EPR) spectra was determined in the heart tissue homogenate as previously described (48). In brief, a 10% tissue homogenate was prepared in 50 mmol/l phosphate buffer containing 0.01 mmol/l EDTA. The supernatants containing 2 mmol/l 1-hydroxy-3-carboxy-pyrrolidine were incubated for 30 min at 37°C and quickly frozen in liquid nitrogen. Superoxide quantification from the EPR spectra was determined by double integration of the peaks, with reference to a standard curve generated from horseradish peroxidase generation of the anion from standard solutions of hydrogen peroxide, using p-acetamidophenol as the cosubstrate, and then normalized by protein concentration.

**Measurement of Nitrite/Nitrate**

Nitrite/nitrate levels in cardiac tissue were assessed using amperometric sensors (World Precision Instruments) as reported by Zhang et al. (49). Briefly, nitrate was converted to nitrite using Nitralyzer metric sensors (World Precision Instruments) as reported by Zhang et al. (49). Results are reported as means ± SD except as specifically stated. Data were analyzed by one-way ANOVA followed by a
Dysfunction in diabetic mice. Resveratrol did not affect HR or respiratory rate. Lepr db mice had lower HRs versus m-Lepr db mice. CO was significantly increased in Lepr db mice compared with m-Lepr db mice. However, there were no statistical differences (Table 1). EF was comparable among all groups (Table 1).

LV volume-time curves and diastolic relaxation. LV volume-time curves indicated that PER and PFR were reduced in Lepr db mice. Resveratrol greatly increased PFR without significantly affecting PER (Table 1 and Fig. 1).

Resveratrol Attenuated Protein and mRNA Expression of TNF-α and Protein Expression of Phospho-IκB-α and NF-κB p65 in Type 2 Diabetes

Protein and mRNA expression of TNF-α (Fig. 2) in Lepr db mice were significantly elevated compared with m-Lepr db mice. Resveratrol attenuated TNF-α expression in Lepr db mice, IκB-α expression was decreased in Lepr db mice, whereas IκB-α phosphorylation and NF-κB p65 expression were increased in Lepr db mice. Resveratrol attenuated IκB-α phosphorylation and NF-κB p65 protein expression without affecting total IκB-α expression (Fig. 3).

Table 1. Cardiac function parameters and morphological parameters

<table>
<thead>
<tr>
<th></th>
<th>m-Lepr db Mice</th>
<th>Lepr db Mice</th>
<th>Lepr db Mice + RSV</th>
<th>m-Lepr db Mice + RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV mass, mg</td>
<td>89.91 ± 16.76</td>
<td>77.49 ± 9.25</td>
<td>88.92 ± 22.68</td>
<td>91.09 ± 15.85</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>28.94 ± 1.55</td>
<td>49.79 ± 4.39*</td>
<td>48.74 ± 4.50*</td>
<td>33.93 ± 3.71†</td>
</tr>
<tr>
<td>Respiratory rate, breaths/min</td>
<td>3.11 ± 0.63</td>
<td>1.56 ± 0.11*</td>
<td>1.81 ± 0.28*</td>
<td>2.70 ± 0.52†</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>31.50 ± 4.43</td>
<td>40.20 ± 8.49</td>
<td>38.75 ± 1.26</td>
<td>38.00 ± 4.97</td>
</tr>
<tr>
<td>LV mass-to-body mass ratio, mg/g</td>
<td>318.89 ± 47.31</td>
<td>315.66 ± 14.27*</td>
<td>322.48 ± 26.30*</td>
<td>370.37 ± 25.49†</td>
</tr>
<tr>
<td>LV end-diastolic volume, μl</td>
<td>49.10 ± 7.78</td>
<td>42.68 ± 7.75</td>
<td>49.26 ± 12.02</td>
<td>51.15 ± 8.38</td>
</tr>
<tr>
<td>LV end-systolic volume, μl</td>
<td>16.10 ± 5.15</td>
<td>11.90 ± 5.03</td>
<td>15.18 ± 7.68</td>
<td>15.28 ± 4.67</td>
</tr>
<tr>
<td>Stroke volume, μl</td>
<td>33.00 ± 2.98</td>
<td>30.77 ± 3.91</td>
<td>34.08 ± 5.23</td>
<td>35.88 ± 3.80</td>
</tr>
<tr>
<td>Cardiac output, m/min</td>
<td>12.69 ± 2.55</td>
<td>9.76 ± 1.59*</td>
<td>11.01 ± 1.95</td>
<td>13.30 ± 1.77†</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>0.71 ± 0.14</td>
<td>0.49 ± 0.14*</td>
<td>0.56 ± 0.09</td>
<td>0.67 ± 0.07†</td>
</tr>
<tr>
<td>Peak ejection rate, μl/ms</td>
<td>0.75 ± 0.06</td>
<td>0.50 ± 0.09*</td>
<td>0.71 ± 0.18†</td>
<td>0.77 ± 0.13†</td>
</tr>
<tr>
<td>Peak filling rate, μl/ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD; n = 4–7 mice/group. RSV, resveratrol; LV, left ventricular. *P < 0.05 vs. m-Lepr db mice; †P < 0.05 vs. Lepr db mice.

least-significant-difference post hoc test. Significance was accepted at P < 0.05.

RESULTS

Effects of Resveratrol on Diabetes-Induced Cardiac Dysfunction

Morphometric parameters. LVM was lower in Lepr db mice, although there was no statistical difference. However, the LVM-to-body mass ratio was significantly lower in m-Lepr db mice. Resveratrol had no effect on LVM and the LVM-to-body mass ratio (Table 1).

HR and respiratory rate. Lepr db mice had lower HRs compared with m-Lepr db mice, although the respiratory rate was similar among groups. Resveratrol did not affect HR or respiratory rate in both Lepr db and m-Lepr db mice (Table 1).

Systolic function parameters. LVEDV, LVESV, and SV were marginally decreased in Lepr db mice. CO was significantly diminished in Lepr db versus m-Lepr db mice. Resveratrol slightly increased LVEDV, LVESV, SV, and CO, although
Resveratrol Increased eNOS Expression and Nitrite/Nitrate Levels in Type 2 Diabetic Mice

Cardiac nitrite/nitrate levels were reduced in diabetic mice, and resveratrol enhanced NO production (Fig. 4A). eNOS protein expression was downregulated in diabetic mice. Resveratrol increased eNOS expression (Fig. 4B). Protein expression of eNOS was greater in db/dbTNF−/H11002/dbTNF−/H11002 and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Decreased Protein and mRNA Expression of gp91phox in Type 2 Diabetes and Cellular Sources of gp91phox

We studied the expression of NAD(P)H oxidase subunits p22phox, gp91phox, and p67phox. Figure 6, A and B, show that protein and mRNA expression of gp91phox were significantly higher in Leprdb mice. Resveratrol decreased gp91phox protein activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Inhibited Increased NAD(P)H Oxidase Activity and Subsequent O2− Production in Type 2 Diabetes

Figure 5A shows that O2− production was significantly increased in Leprdb mice. Resveratrol reduced O2− production in Leprdb mice but not in m-Leprdb mice. The NAD(P)H oxidase inhibitor apocynin (20 mg·kg−1·day−1 ip, 5 days) attenuated O2− production in Leprdb mice to levels not significantly different from normal control mice. NAD(P)H oxidase activity was higher in Leprdb mice than in m-Leprdb mice (Fig. 5B). Resveratrol significantly decreased NAD(P)H oxidase activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Inhibited Increased NAD(P)H Oxidase Activity and Subsequent O2− Production in Type 2 Diabetes

Figure 5A shows that O2− production was significantly increased in Leprdb mice. Resveratrol reduced O2− production in Leprdb mice but not in m-Leprdb mice. The NAD(P)H oxidase inhibitor apocynin (20 mg·kg−1·day−1 ip, 5 days) attenuated O2− production in Leprdb mice to levels not significantly different from normal control mice. NAD(P)H oxidase activity was higher in Leprdb mice than in m-Leprdb mice (Fig. 5B). Resveratrol significantly decreased NAD(P)H oxidase activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Decreased Protein and mRNA Expression of gp91phox in Type 2 Diabetes and Cellular Sources of gp91phox

We studied the expression of NAD(P)H oxidase subunits p22phox, gp91phox, and p67phox. Figure 6, A and B, show that protein and mRNA expression of gp91phox were significantly higher in Leprdb mice. Resveratrol decreased gp91phox protein activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Inhibited Increased NAD(P)H Oxidase Activity and Subsequent O2− Production in Type 2 Diabetes

Figure 5A shows that O2− production was significantly increased in Leprdb mice. Resveratrol reduced O2− production in Leprdb mice but not in m-Leprdb mice. The NAD(P)H oxidase inhibitor apocynin (20 mg·kg−1·day−1 ip, 5 days) attenuated O2− production in Leprdb mice to levels not significantly different from normal control mice. NAD(P)H oxidase activity was higher in Leprdb mice than in m-Leprdb mice (Fig. 5B). Resveratrol significantly decreased NAD(P)H oxidase activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Decreased Protein and mRNA Expression of gp91phox in Type 2 Diabetes and Cellular Sources of gp91phox

We studied the expression of NAD(P)H oxidase subunits p22phox, gp91phox, and p67phox. Figure 6, A and B, show that protein and mRNA expression of gp91phox were significantly higher in Leprdb mice. Resveratrol decreased gp91phox protein activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Inhibited Increased NAD(P)H Oxidase Activity and Subsequent O2− Production in Type 2 Diabetes

Figure 5A shows that O2− production was significantly increased in Leprdb mice. Resveratrol reduced O2− production in Leprdb mice but not in m-Leprdb mice. The NAD(P)H oxidase inhibitor apocynin (20 mg·kg−1·day−1 ip, 5 days) attenuated O2− production in Leprdb mice to levels not significantly different from normal control mice. NAD(P)H oxidase activity was higher in Leprdb mice than in m-Leprdb mice (Fig. 5B). Resveratrol significantly decreased NAD(P)H oxidase activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Decreased Protein and mRNA Expression of gp91phox in Type 2 Diabetes and Cellular Sources of gp91phox

We studied the expression of NAD(P)H oxidase subunits p22phox, gp91phox, and p67phox. Figure 6, A and B, show that protein and mRNA expression of gp91phox were significantly higher in Leprdb mice. Resveratrol decreased gp91phox protein activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Inhibited Increased NAD(P)H Oxidase Activity and Subsequent O2− Production in Type 2 Diabetes

Figure 5A shows that O2− production was significantly increased in Leprdb mice. Resveratrol reduced O2− production in Leprdb mice but not in m-Leprdb mice. The NAD(P)H oxidase inhibitor apocynin (20 mg·kg−1·day−1 ip, 5 days) attenuated O2− production in Leprdb mice to levels not significantly different from normal control mice. NAD(P)H oxidase activity was higher in Leprdb mice than in m-Leprdb mice (Fig. 5B). Resveratrol significantly decreased NAD(P)H oxidase activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Decreased Protein and mRNA Expression of gp91phox in Type 2 Diabetes and Cellular Sources of gp91phox

We studied the expression of NAD(P)H oxidase subunits p22phox, gp91phox, and p67phox. Figure 6, A and B, show that protein and mRNA expression of gp91phox were significantly higher in Leprdb mice. Resveratrol decreased gp91phox protein activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.

Resveratrol Inhibited Increased NAD(P)H Oxidase Activity and Subsequent O2− Production in Type 2 Diabetes

Figure 5A shows that O2− production was significantly increased in Leprdb mice. Resveratrol reduced O2− production in Leprdb mice but not in m-Leprdb mice. The NAD(P)H oxidase inhibitor apocynin (20 mg·kg−1·day−1 ip, 5 days) attenuated O2− production in Leprdb mice to levels not significantly different from normal control mice. NAD(P)H oxidase activity was higher in Leprdb mice than in m-Leprdb mice (Fig. 5B). Resveratrol significantly decreased NAD(P)H oxidase activity in Leprdb mice. Furthermore, db/dbTNF−/H11002/dbTNF− and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced NAD(P)H oxidase activity, whereas m-Leprdb mice treated with recombinant TNF-α exhibited enhanced NAD(P)H oxidase activation.
Resveratrol Downregulated Protein Expression of iNOS and N-Tyr in Type 2 Diabetes

Western Blot analysis (Fig. 7A) for N-Tyr revealed significantly higher levels of N-Tyr in Lepr<sup>db</sup> mice, which were reduced by resveratrol and treatment with the iNOS inhibitor 1400W (10 mg·kg<sup>−1</sup>·day<sup>−1</sup> ip, 4 days). Protein expression of iNOS was higher in Lepr<sup>db</sup> mice compared with m-Lepr<sup>db</sup> mice. The administration of resveratrol reduced iNOS expression in Lepr<sup>db</sup> mice. Moreover, db<sup>Tnf<sup>−/−</sup>·Tnfα−/−</sup> and Lepr<sup>db</sup> mice treated with the NF-κB inhibitor MG-132 showed decreased iNOS protein expression, whereas m-Lepr<sup>db</sup> mice treated with recombinant TNF-α exhibited elevated iNOS expression (Fig. 7B).

DISCUSSION

The major finding of this study is that resveratrol is cardioprotective in mice with diabetic cardiomyopathy. Cardiomyopathy in this model is manifested as impaired diastolic relaxation measured by a reduction in the early filling phase of diastole. In addition, during systole, we measured reduced peak ejection. Despite reduced PER, LV EF was not impaired. We found that the resveratrol-treated diabetic mice showed lower levels of oxidative/nitrative stress with an improvement of NO availability. This suggests that the mechanisms responsible for the improvement in LV diastolic relaxation with resveratrol were due to the inhibition of oxidative/nitrative stress and improvement of NO availability in this model (Fig. 8).

Cardioprotective Effects of Resveratrol in Type 2 Diabetes

The cardioprotective effects of resveratrol have been studied in various disease models. Echocardiographic analyses showed that resveratrol reduced isovolumic relaxation time, a parameter of diastolic function, in aging (3) and pressure overload-induced cardiac hypertrophy (aortic...
banded rat model) (16). In a rat model of myocardial ischemia-reperfusion injury, in vivo LV catheterization showed that resveratrol pretreatment increased LV maximum systolic pressures and dP/dt_{max} and decreased myocardial infarct size (35). Using in vivo MRI, we demonstrated that resveratrol improved cardiac function in type 2 diabetic mice. The results suggested that at 16 wk old, although SV was only marginally decreased in male Leprdb mice, CO was significantly diminished (Table 1 and Fig. 1). Both PER and PFR were significantly lower in Leprdb mice, suggesting the early impairment of systolic and diastolic function. Systolic dysfunction is more likely dependent on the degree of myocyte loss and myocyte injury, which account for reduced contractility, decreased pump function, and EF (10). Resveratrol did not exhibit significant benefits in improving SV, CO, or PER, but greatly increased PFR (Table 1 and Fig. 1). Therefore, we posit that resveratrol exerts a beneficial role in diabetic cardiomyopathy mainly by improving diastolic function. Although resveratrol prevented diet-induced obesity and alleviated insulin resistance in high-fat diet-fed mice and streptozotocin-induced type 1 diabetic rats, in Leprdb mice, resveratrol did not alter body weight or reduce hyperinsulinemic hyperglycemia (49). Thus, the therapeutic effects of resveratrol, especially on cardiac dysfunction, can also occur independently of weight loss and hyperglycemic status.

Furthermore, Leprdb mice had a lower LVM-to-body mass ratio compared with m-Leprdb mice (Table 1). These results are consistent with a previous investigation of hearts using MRI in type 2 diabetic mice by Panagia et al. (28). Although resveratrol improved diastolic function, it showed no marked effect via a morphological change of the heart in both m-Leprdb and Leprdb mice. Therefore, early intervention by resveratrol supplementation might be a promising approach to prevent or delay the onset of diabetic cardiomyopathy.

Fig. 6. RSV diminished protein and mRNA expression of NAD(P)H oxidase subunit gp91^{phox}. A and B: gp91^{phox} protein and mRNA expression were enhanced in Leprdb mice. RSV decreased gp91^{phox} expression in Leprdb mice. Data are means ± SD; n = 4 separate experiments. *P < 0.05 vs. m-Leprdb mice; #P < 0.05 vs. Leprdb mice. C: dual fluorescence combining gp91^{phox} with markers for endothelial cells (von Willebrand Factor (vWF)), vascular smooth muscle (α-actin), and macrophages (F4/80) with the use of specific antibodies followed by fluorescent-labeled secondary antibodies. a–c, Dual labeling of gp91^{phox} (red) and vWF (green) in m-Leprdb heart tissue. d, Higher magnification view of a, e, g, and i. Dual labeling of gp91^{phox} (red) and vWF (green) in Leprdb heart tissue. j, h, and j. Higher magnification views of e, g, and i, respectively. The gray arrow in j shows the staining of gp91^{phox} (red), and the pink arrows in d and j show the colocalization of gp91^{phox} and endothelial cells (yellow). k–m, Dual labeling of gp91^{phox} (red) and α-actin (green) in Leprdb heart tissue. The white arrow in m shows specific α-actin staining with an absence of gp91^{phox} staining. n, Higher magnification view of m. o–q, Dual labeling of gp91^{phox} (red) and F4/80 (green) in Leprdb heart tissue. The purple arrow in q shows colocalization of gp91^{phox} and F4/80 (yellow). r, Higher magnification view of q, s, and t, negative controls (Ctl). The blue arrow shows an absence of staining in vessels with the secondary antibodies. u, Nuclear staining with 4',6-diamidino-2-phenylindole (blue) in Leprdb heart tissue. Magnification: ×40. Data shown are representative of 4 separate experiments.
Resveratrol may contribute to its cardioprotective benefits since in type 2 diabetes (Fig. 2). The TNF-α protein expression were elevated in type 2 diabetic mouse expression. Protein expression of iNOS was higher in rotid arteries (7). We demonstrated that TNF-α Activation

\[
\text{Leprdb} \quad \text{db-mice. The administration of resveratrol reduced iNOS expression in m-Leprdb mice. Data are means } \pm \text{ SE; } n = 4 \text{ separate experiments. } ^* P < 0.05 \text{ vs. m-Leprdb mice; } \# P < 0.05 \text{ vs. Leprdb mice. B: effects of RSV on iNOS protein expression. Protein expression of iNOS was higher in Leprdb versus m-Leprdb mouse. The administration of resveratrol reduced iNOS expression in Leprdb mice. } db^{TNF-} / db^{TNF-} \text{ and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced iNOS expression, whereas iNOS expression was markedly elevated in m-Leprdb mice treated with recombinant TNF-α. Data are means } \pm \text{ SE; } n = 4 \text{ separate experiments. } ^* P < 0.05 \text{ vs. m-Leprdb mice; } \# P < 0.05 \text{ vs. Leprdb mice.}
\]

**Resveratrol Reduced Cardiac TNF-α Expression and NF-κB Activation**

Resveratrol inhibited TNF-α production by the LPS-activated brain macrophage, the microglia (4). It also abrogated cigarette smoking-induced upregulation of TNF-α in rat carotid arteries (7). We demonstrated that TNF-α mRNA and protein expression were elevated in type 2 diabetic mouse cardiac tissue and that resveratrol inhibited TNF-α expression in type 2 diabetes (Fig. 2). The TNF-α inhibitory effects of resveratrol may contribute to its cardioprotective benefits since TNF-α has been shown to be a prognostic marker in patients with heart failure. Etanercept, a soluble TNF-α receptor antag-

\[
\text{Fig. 7. RSV reduced nitrotyrosine (N-Tyr) and inducible NO synthase (iNOS) protein expression. A: both 50- and 30-kDa bands of N-Tyr expression were higher in } Leprdb \text{ mice than in m-Leprdb mice. Treatment with RSV and the iNOS inhibitor (10 mg·kg$^{-1}$·day$^{-1}$ ip, 4 days) decreased N-Tyr expression in Leprdb mice. Data are means } \pm \text{ SD; } n = 4 \text{ separate experiments. } ^* P < 0.05 \text{ vs. m-Leprdb mice; } \# P < 0.05 \text{ vs. Leprdb mice. B: effects of RSV on iNOS protein expression. Protein expression of iNOS was higher in Leprdb versus m-Leprdb mouse. The administration of resveratrol reduced iNOS expression in Leprdb mice. } db^{TNF-} / db^{TNF-} \text{ and Leprdb mice treated with the NF-κB inhibitor MG-132 showed reduced iNOS expression, whereas iNOS expression was markedly elevated in m-Leprdb mice treated with recombinant TNF-α. Data are means } \pm \text{ SE; } n = 4 \text{ separate experiments. } ^* P < 0.05 \text{ vs. m-Leprdb mice; } \# P < 0.05 \text{ vs. Leprdb mice.}
\]

\[
\text{Effects of Resveratrol on NO Production and eNOS Expression}
\]

Agnostist-stimulated release of NO from eNOS in the coronary endothelium exerts paracrine effects on cardiomyocytes, predominantly affecting the timing of relaxation as well as myocardial O$_2$ consumption (32). The autocrine role of eNOS within cardiomyocytes may include the modulation of basal inotropy and relaxation, β-adrenergic responsiveness, and the force-frequency relationship (32). eNOS$^{-/-}$ mice are not only hypertensive but also characterized by enhanced systolic function and depressed diastolic function without LV hypertrophy (30). Moreover, inhibition of basal NO production by the NOS inhibitor N$^\diamond$-monomethyl-L-arginine significantly impaired LV diastolic filling (6). This suggests that NO derived from eNOS contributes to myocardial relaxation (29).

In a pressure overload-induced cardiac hypertrophy rat model, eNOS and redox factor-I expression were significantly reduced. Resveratrol increased eNOS and redox factor-I expression and improved diastolic relaxation (16). Our results suggest that resveratrol at a dose of 20 mg·kg$^{-1}$·day$^{-1}$ increased eNOS protein expression in Leprdb mice (Fig. 4B), which was associated with enhanced myocardial nitrite/nitrate levels, a parameter of NO production (Fig. 4A). Furthermore, $db^{TNF-}$ / $db^{TNF-}$ and Leprdb mice treated with MG-132 exhibited elevated eNOS protein expression, whereas eNOS expression was reduced in m-Leprdb mice with recombinant TNF-α treatment (Fig. 4B), suggesting that resveratrol may affect eNOS expression by inhibiting TNF-α-NF-κB signaling. Thus, resveratrol’s benefits in improving cardiac filling of diabetic mice may ameliorate cardiomyopathy by enhancing eNOS-derived NO production.

**Effects of Resveratrol on NAD(P)H Oxidase Subunit Expression, NAD(P)H Oxidase Activation, and Subsequent O$_2^-$ Production**

NO production by eNOS does not accurately reflect NO availability since inactivation of NO by ROS is recognized to be a key mechanism underlying reduced NO bioavailability...
A previous study (36) suggested that LV diastolic function is correlated with oxidative stress. Therefore, we evaluated oxidative stress in cardiac tissue of diabetic mice. Despite the presence of multiple ROS sources, studies (13, 14) in the last decade have indicated that the major cardiovascular source of ROS, particularly in the vasculature, originates from a family of NADPH oxidases.

Our previous studies (11, 12) have indicated that dbTNF/Leprdb and MG-132-treated Leprdb mice exhibit reduced NAD(P)H oxidase activity in coronary arterioles. The present study showed that NAD(P)H oxidase activity was elevated in the cardiac tissue of Leprdb mice, whereas dbTNF/Leprdb and MG132-treated Leprdb mice revealed ameliorated NAD(P)H oxidase activation (Fig. 5B). O2·− production was elevated in Leprdb mice, and the NAD(P)H oxidase inhibitor apocynin reduced O2·− production back to the level of the m-Leprdb control, suggesting that NAD(P)H oxidase may be one of the main sources of O2·− production in diabetic mouse cardiac tissue (Fig. 5A). Resveratrol inhibited NAD(P)H oxidase activity and subsequent O2·− production, possibly by inhibiting TNF-α-induced NF-κB activation.

The classical NADPH oxidase complex comprises membrane-bound cytochrome b558 (composed of one gp91phox subunit and one p22phox subunit), which forms the catalytic core of the enzyme, and four cytosolic regulatory subunits (p47phox, p67phox, p40phox, and Rac) (19). gp91phox is one of the most important subunits in NAD(P)H oxidase-mediated oxidative stress since deletion of gp91phox severely inhibits activated O2·− production (1). We determined that gp91phox protein and mRNA expression were elevated in diabetic mice (Fig. 6, A and B), whereas p22phox and p67phox protein expression showed no significant differences between m-Leprdb and Leprdb mice (data not shown). Resveratrol greatly reduced gp91phox mRNA and protein expression, suggesting that resveratrol may abrogate NAD(P)H oxidase activation by interrupting NAD(P)H oxidase subunit gp91phox expression. Immunofluorescence staining suggested that gp91phox is mainly colocalized with endothelial cells and macrophages in cardiac tissue (Fig. 6C). Thus, the antioxidative effects may contribute to the cardio-protective effects by resveratrol via LV relaxation.

Effects of Resveratrol on iNOS Expression and Subsequent Nitrative Stress

iNOS is induced by inflammatory cytokines and produces a much higher level of NO compared with constitutive NOS (43). Cardiac myocytes, as well as a number of other parenchymal cells within the myocardium, including the endothelium of the coronary microvasculature, endocardium, and infiltrating inflammatory cells, are all able to express iNOS in response to soluble inflammatory mediators (2). A recent study (43) demonstrated that pathological concentrations of NO produced by iNOS may result in nitrative stress and tissue injury by generating the powerful nitrative molecule peroxynitrite (ONOO−). A transgenic mouse model conditionally targeting the expression of human iNOS cDNA to the myocardium displayed upregulation of iNOS, which led to an increased production of ONOO−. Those mice infrequently developed overt heart failure but displayed a high incidence of sudden cardiac death due to bradyarrhythmia (25). Myocardial reperfusion injury increased iNOS expression and subsequent generation of RNS (N-Tyr), which was prevented by pretreatment with 1400W, a selective iNOS inhibitor, or M-40401, an ONOO− scavenger (39). Thus, nitrative stress and peroxynitrite play a crucial role in the pathogenesis of diabetic cardiomyopathy (26, 27). Our study reveals that iNOS expression was enhanced in the cardiac tissue of Leprdb mice, concurrent with elevated N-Tyr expression in the presence of excessive O2·−. The iNOS...
inhibitor 1400W reversed the increased N-Tyr expression, suggesting a role for iNOS in the increased RNS production in the diabetic cardiomyocyte (Fig. 7A). Therefore, we postulate that the effects of resveratrol on reducing cardiac N-Tyr expression might be partially explained by its role in inhibiting iNOS expression (Fig. 7B). Although iNOS expression was increased in the cardiac tissue of diabetic mice versus control mice, iNOS protein expression in aortas was not different between control and diabetic mice (data not shown). Moreover, 50-kDa N-Tyr protein expression was over 10 times higher in the heart of diabetic mice compared with control mice, whereas it was only 3 times higher in the aorta of diabetic versus control mice (49), supporting the view of more marked nitrative stress in the cardiac tissue of type 2 diabetic mice.

NF-κB activation is required for TNF-α-induced iNOS gene expression in both human liver (AKN-1) and human lung (A549) epithelial cell lines (40). We found that dbTNF-/dbTNF- and Leprdb mice treated with MG-132 exhibited decreased iNOS expression, whereas iNOS expression was elevated in m-Leprdb mice treated with anti-TNF-α versus control m-Leprdb mice (Fig. 7B). These findings suggest that TNF-α-induced NF-κB activation may upregulate iNOS expression in diabetic mouse cardiac tissue.

**Resveratrol Regulates the Imbalance of NO and O2⁻ in Type 2 Diabetes**

A complex paracrine interaction exists between endothelial cells and cardiac myocytes in the heart. Cardiac endothelial cells release (or metabolize) several diffusible agents that exert direct effects on myocyte function, independent of changes in coronary flow (33). The role of NO in this paracrine/autocrine pathway is active due to the short diffusion distance between cardiac microvessel endothelial cells and ventricular cardiomyocyte (<1 μm) (38). In pathological situations, the beneficial effects of NO resulting from eNOS-derived NO were diminished by either reduced production or enhanced inactivation by ROS. There can also be deleterious effects of NO that result from excessive NO production by iNOS, usually with concurrent ROS production and ONOO⁻ formation (33). Thus, the balance between NO and ROS production is a major factor in the pathophysiological state of the cardiovasculature.

Resveratrol inhibits NAD(P)H oxidase-derived O₂⁻ as well as iNOS-derived NO but stimulates eNOS-derived NO production. This prevents cardiac oxidative/nitrative stress and improves NO availability. Therefore, by regulating the imbalance between NO and ROS, resveratrol is postulated to be efficient in ameliorating oxidative/nitrative stress and subsequent tissue damage in the diabetic heart.

**Conclusions**

In conclusion, MRI assessment of cardiac function in diabetic mice supports the existence of diabetic cardiomyopathy in this animal model of type 2 diabetes. Reduced systolic function and abnormal diastolic function were evident in 16-week-old diabetic mice. Importantly, systolic and diastolic dysfunction in the diabetic hearts were not associated with cardiac hypertrophy because the measured wall thickness (data not shown) and calculated LVM were not increased (Table 1). This alteration coexisted with enhanced TNF-α expression, NF-κB activation, and NF-κB downstream signaling, which contributed to the perpetuation of oxidative/nitrative stress as well as decreased NO availability. Our results in resveratrol-treated Leprdb mice showed normalized diastolic function, suggesting that pharmacological interventions to promote NO availability and suppress oxidative/nitrative stress may reduce diabetes-induced cardiac dysfunction. We suggest that resveratrol has a potential role as a novel pharmacological agent in restoring cardiac function during the early stages of diabetic cardiomyopathy.

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge Ashley Brown for technical assistance as well as the MRI facility support provided by the Veterans Affairs Biomedical Imaging Center at the Harry S. Truman Memorial Veterans Hospital and the University of Missouri (Columbia, MO).

**GRANTS**

This work was supported by Pfizer Atorvastatin Research Award 2004–37, American Heart Association Scientific Development Grant 11035004TA, and National Institutes of Health (NIH) Grants R01-HL-077566 and R01-HL-085119 (to C. Zhang). L. Ma was supported by NIH Grant P50-CA-10313 and United States Department of Defense Prostate Cancer Research Program Grant PC081264. H. Zhang was supported by NIH Clinical Biodetecives Training Grant R90-DK-70105. B. Morgan was supported by NIH Clinical Biodetecives Training Grant T90-DK-071510.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**

ROLE OF RESVERATROL IN TYPE 2 DIABETES


