Chronic Resveratrol Enhances Endothelium-Dependent Relaxation but Does Not Alter eNOS Levels in Aorta of Spontaneously Hypertensive Rats

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Spontaneously hypertensive rats (SHRs) were administered the red wine polyphenol resveratrol in drinking water at 0, 0.448, or 4.48 mg/l (control, low, or high, respectively) for 28 days. The low dosage was chosen to mimic moderate red wine consumption. After the treatment period, thoracic aorta rings were excised for *in vitro* assessment of vasomotor function. Chronic resveratrol significantly improved endothelium-dependent relaxation to acetylcholine (Ach), increasing maximal values to 80.8% ± 5.2% and 80.8% ± 5.0% in low and high groups, respectively, compared with 60.7% ± 1.4% in controls ($P < 0.01$). This treatment effect was eliminated in the presence of the endothelial nitric oxide synthase (eNOS) blocker Nω-nitro-L-arginine methyl ester. Resveratrol did not affect relaxation to sodium nitroprusside or systolic blood pressure in SHRs. In contrast to the SHR results, chronic resveratrol in Sprague Dawley rats did not affect vasomotor function in aorta rings in response to Ach. Hydrogen peroxide was reduced in the SHR thoracic aorta by a high dosage of resveratrol ($P < 0.05$), but it was not significantly altered in other tissues tested. Thoracic aorta immunoblots revealed no significant treatment effects in SHRs on eNOS, superoxide dismutases 1 and 2, gp91phox, or Hsp90. Thus, these data provide novel evidence of improved endothelium-dependent vasorelaxation in hypertensive, but not normotensive, animals as a result of chronic resveratrol consumption mimicking dosages resulting from moderate red wine consumption. This response was not dependent on increases in eNOS expression but was dependent on improved NO bioavailability. Exp Biol Med 232:814–822, 2007

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Introduction

Resveratrol (3,4′,5-trihydroxystilbene) is a naturally occurring phenolic compound found in grape skins (1–3). It has been suggested that transresveratrol is one of the components responsible for the potential benefits of moderate red wine consumption in reducing cardiovascular disease risk (4, 5). *In vitro* studies have demonstrated that resveratrol is associated with a variety of specific benefits, including reduced platelet aggregation, protection against low-density lipoprotein oxidation and inflammation, and improved plasma lipid profiles, that could collectively contribute to the putative cardioprotective action of this compound (4, 5). In addition, resveratrol and other red wine polyphenols have vasodilatory effects when applied to isolated artery segments *in vitro* at pharmacologic concentrations (5–10).

The mechanisms accounting for resveratrol's vasodilatory action are not known but could include stimulation of nitric oxide (NO) synthesis based on physiologic studies that indicate enhanced NO production *in vitro* and *in vivo* in response to acute resveratrol treatment (6, 11). In addition, endothelial cell culture studies have revealed that resveratrol increases both the expression and activity of endothelial NO synthase (eNOS; Refs. 12–14), which raises the possibility that in addition to the acute vasodilatory effects, chronic exposure to resveratrol could affect vasomotor function. An isolated report found that prolonged resveratrol administration improved endothelium-dependent vasodilation in ovariectomized, stroke-prone, spontaneously hypertensive rats (15). However, the dosage of resveratrol used, 5 mg/kg/day, was very high (weight adjusted for a human this would be equivalent of the resveratrol content in approximately 70 liters of red wine; Ref. 3), and the effect was limited to the ovariectomized group, suggesting some interaction with this

treatment. Thus, the influence of chronic moderate resveratrol consumption on vascular function and phenotype has not been investigated thoroughly in hypertensive animals.

Blunted endothelium-dependent vasodilation is a common finding associated with cardiovascular disease risk factors, including hypertension (16–20). The underlying causes of this impaired dilation often include impaired NO bioavailability resulting from either decreased synthesis or sensitivity to NO, or increased oxidative stress–mediated destruction of NO (16–20). For instance, in the spontaneously hypertensive rat (SHR) model, elevated NAD(P)H oxidase-dependent oxidative stress impairs NO bioavailability and contributes to the endothelial dysfunction observed in these animals compared with normotensive Wistar Kyoto (WKY) control rats (16, 17, 21). Physiologic stimuli, such as exercise, and drug and gene manipulation interventions that increase eNOS and/or antioxidant enzyme content of the vascular wall can reverse the endothelial dysfunction in hypertensive animals (reviewed in Ref. 16). Since resveratrol is an antioxidant scavenger of reactive oxygen species (ROS; Ref. 22), a potential NAD(P)H oxidase inhibitor (23), and an inducer of eNOS expression in cultured endothelial cells (12–14), it is reasonable to predict that chronic dietary administration of this compound to hypertensive animals may improve endothelium-dependent vasorelaxation by increasing NO bioavailability. Thus, the purpose of this study was to test the hypothesis that chronic dietary resveratrol administration to SHR animals would improve endothelium-dependent, NO-mediated vasorelaxation of the aorta as a result of resveratrol's reported effect of increasing eNOS expression.

Materials and Methods

Experimental Procedures. These experiments were performed using 20-week-old male SHR rats ($n = 24$) obtained from Harlan (Indianapolis, IN) and group housed at a constant air temperature (20°–21°C) and humidity (~50%) in a 12:12-hr reverse light:dark cycle facility. Sprague Dawley rats (300–350 g; $n = 8$) were obtained and housed identically for use as normotensive control animals in select experiments. The University of Waterloo Animal Care Committee approved all animal-related procedures in this study. Rats were randomly assigned to one of three groups: control (no resveratrol), low resveratrol, and high resveratrol. Target doses of transresveratrol were 0.01568 mg (low) and 0.1568 mg (high) per animal per day. Under dim light conditions, solutions were prepared in water containing 10 g/l carboxymethylcellulose at either 0 mg/ml (control, just water and carboxymethylcellulose), 0.000448 mg/ml, or 0.00448 mg/ml of resveratrol which, assuming 35 ml of fluid intake per day per rat, would result in the administration of the target resveratrol doses. The low and high resveratrol doses were chosen based on scaling down to an approximately 0.333-kg rat from a 70-kg human consuming moderate daily amounts of red wine (low: human dose equivalent to 3.3 mg resveratrol), and 10 times this dose (high: human dose equivalent to 33 mg resveratrol; Refs. 3, 24). Rats had free access to standard 22/5 Rodent Diet (W) lab chow (Harlan) and the assigned drinking fluid, which was replaced daily after recording the volume consumed. For Sprague Dawley rat experiments only the control and high resveratrol conditions were studied.

On the day of experimentation and tissue sampling, body mass was recorded, and rats were anesthetized with sodium pentobarbital injection (50 mg/kg intraperitoneally; MTC Pharmaceuticals, Cambridge, ON, Canada). Systolic blood pressure (SBP) was recorded via a heparinized, saline-filled PE-50 cannula inserted into the left common carotid artery and attached to a transducer (Harvard, South Natick, MA). Continuous blood pressure data were acquired using a PowerLab system (ADInstruments, Colorado Springs, CO). A 10-min equilibration period was observed after cannula insertion prior to collecting 10 mins of data for analyses. After obtaining blood pressure measurements, a midline thoracotomy was performed, and the heart was cut free and removed, as was the thoracic aorta from the distal end of its arch to the point at which it entered the diaphragm. The heart and aorta were cleaned by dissection, and the aorta was divided into two segments: the proximal portion (~1 cm) was prepared for vascular myography as described below, and the distal portion (~1 cm) was frozen in liquid nitrogen and stored at –80°C until use in immunoblot or biochemical analyses. Similarly, liver, kidney, plantaris muscle, and plasma samples were obtained and stored for biochemical analyses.

Assessment of Vasomotor Function. Four rings, each 2 mm in axial length, were cut from the proximal segment of the thoracic aortas and prepared for myography. The optimal resting length of the vessels (L_o) was determined through KCl length–tension curve experiments as previously described (25). Rings were allowed to equilibrate for 30 mins at L_o, and all further testing was done at the L_o. During the equilibration period some baths were supplemented with Nω-nitro-L-arginine methyl ester (L-NAME) at 10^{-4} *M*. After preconstriction with submaximal phenylephrine (10^{-7} *M*), a cumulative acetylcholine (Ach), dose–response protocol was performed (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} *M*), followed by washes every 5 mins (re-adjusting L-NAME concentrations in appropriate baths) until tension returned to resting levels. After preconstriction with submaximal phenylephrine (10^{-7} *M*), a cumulative sodium nitroprusside (SNP), dose–response protocol was performed (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} *M*). In all vasomotor function experiments, two rings from each animal were exposed to the same pharmacologic treatment *in vitro* so that the data from these two rings could be averaged to represent each animal's response in the analyses.

Immunoblot Analyses. Aortas were prepared for immunoblotting as previously described (17, 25). Briefly, samples containing 30 μg protein were electrophoresed and

Table 1. SHR Treatment Characteristics[a]

	Control	Resveratrol	
		Low	High
Average daily H_2O (ml)	41.7 ± 1.9	44.5 ± 1.2	39.1 ± 0.9
Average daily resveratrol (mg)	—	0.020 ± 0.001*	0.175 ± 0.004*,**
Initial body weight (g)	335.0 ± 4.3	333.0 ± 8.7	332.8 ± 8.7
Final body weight (g)	374.2 ± 5.9	360.8 ± 11.1	361.8 ± 7.1
Systolic blood pressure (mm Hg)	198.0 ± 4.2	194.7 ± 0.7	193.7 ± 2.0

[a] Values are means ± SE. Initial indicates measurement made on treatment Day 1, and final indicates measurement made on Day 28. Blood pressure measurement was made on Day 28.
* $P < 0.001$ versus control group; ** $P < 0.001$ versus low-resveratrol group.

electroblotted onto microporous polyvinylidene difluoride membranes (Roche Diagnostics, Mannheim, Germany) which were stained with ponceau red dye, and the predominant 43-kDa actin band was quantified using the ChemiGenius 2 Bio-Imaging system (Syngene, Cambridge, UK) to verify equal protein loading prior to immunoblotting for target proteins using the same membranes. In addition, vascular smooth muscle actin was assessed by immunoblotting (1:1000; Sigma, St. Louis, MO) to further confirm that there was no systematic treatment effect on actin protein level. Membranes were probed with primary antibodies specific for the following target proteins: eNOS (1:750; BD Biosciences, Franklin Lakes, NJ); gp91phox subunit of NADPH oxidase (1:250; BD Biosciences); SOD-1 and SOD-2 (1:1000 and 1:2500, respectively; Stressgen, Victoria, BC, Canada); and Hsp90 (1:1000; Stressgen). Target protein detection was performed *via* horseradish peroxidase–conjugated secondary antibody in conjunction with enhanced chemiluminescence reagents (Amersham, Little Chalfont, UK) and a Syngene gel detection system.

Tissue and Plasma Hydrogen Peroxide Analyses. Hydrogen peroxide (H_2O_2) levels were determined in tissue and plasma using the Amplex Red Hydrogen Peroxide kit (Molecular Probes, Eugene, OR) as previously described (25). Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in a 1:1 stoichiometry in the presence of peroxidase to produce the oxidation product resorufin, which can be measured spectrophotometrically (26). Briefly, aorta, liver, kidney, and plantaris muscle tissues were homogenized in phosphate-buffered saline (PBS; pH 7.4) on ice. A total of 50 µl of diluted tissue homogenate or plasma was added to 50 µl Amplex Red reagent (100 µ*M* Amplex Red stock and 0.2 U/ml horseradish peroxidase in 50 m*M* sodium phosphate buffer, pH 7.4) and incubated in the dark at room temperature for 60 mins. Absorbance was measured at 560 nm using a SPECTRAmax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). H_2O_2 concentrations were calculated using a H_2O_2 standard curve ($R^2 = 0.999$). Tissue concentrations are expressed relative to milligrams of protein.

Tissue ROS Generation. ROS generation was measured in tissue homogenates using a 2′-7′-dichlorofluorescin-diacetate (DCFH-DA) as previously described (27, 28). Briefly DCFH-DA, a nonfluorescent dye, is cleaved by esterase activity to yield DCFH, which is subsequently oxidized by a variety of ROS to form dichlorofluorescein (DCF), which is fluorescent. Aorta, liver, kidney, and plantaris muscle were homogenized in PBS using a glass homogenizer. Samples containing 25 µg tissue protein diluted in PBS were incubated with 5.0 µ*M* DCFH-DA (Molecular Probes) in the dark for 15 mins at 37°C. Fluorescence was measured every 15 mins for 1 hr with excitation and emission wavelengths of 488 nm and 525 nm, respectively, using a SPECTRAmax GEMINI-XS (Molecular Devices). Values are expressed as relative fluorescence units (RFU) normalized per milligram of protein.

Data Analyses. Values are reported as mean ± standard error, with *n* referring to the number of animals per group. ANOVA was used to test for treatment group differences, and sigmiodal curve fitting was employed in the analysis of dose–response curves resulting from myography experiments. Tukey HSD tests were performed as *post hoc* tests for individual differences after significant F ratios were detected using ANOVA. Group mean differences were considered significant if $P < 0.05$. All statistical analyses were performed using GraphPad Prism 4 and SAS software (GraphPad Software Inc., San Diego, CA; SAS Institute, Cary, NC).

Results

Animal Characteristics and Efficacy of Treatment. As expected, average daily resveratrol consumption was significantly different between all SHR groups (Table 1), and the desired 10-fold difference in resveratrol consumption between low and high resveratrol groups was achieved ($P < 0.001$). Average daily fluid consumption over the 28-day experimental period did not differ among treatment groups (Table 1) but was approximately 25% greater in all groups than was projected; thus, total estimated resveratrol consumption was correspondingly greater in

Table 2. Tension Characteristics of SHR Aortic Rings and Kinetic Constants for Dilatory Curves[a]

	Control	Resveratrol	
		Low	High
Resting tension at L_o (g)	6.1 ± 0.5	6.1 ± 0.5	6.5 ± 0.6
Developed tension 60 m*M* KCl (g)	1.5 ± 0.1	1.8 ± 0.1	1.7 ± 0.2
Developed tension 10^{-7} *M* PE (g)	1.2 ± 0.1	1.2 ± 0.2	1.4 ± 0.2
Developed tension 10^{-4} *M* PE (g)	2.2 ± 0.1	2.3 ± 0.2	2.4 ± 0.2
Ach dilation			
Log EC_{50} (log *M*)	−7.5 ± 0.1	−7.4 ± 0.1	−7.5 ± 0.1
Maximal dilation (%)	60.7 ± 1.4	80.8 ± 5.2*	80.8 ± 5.0*
SNP dilation			
Log EC_{50} (log *M*)	−8.5 ± 0.1	−8.6 ± 0.5	−8.7 ± 0.1
Maximal dilation (%)	109 ± 2	103 ± 6	97 ± 6

[a] Values are means ± SE. All developed tension measurements made at L_o. PE, phenylephrine.
*$P < 0.01$ versus control group.

each group than anticipated *a priori*. Within a treatment group neither the daily water nor the resveratrol consumption significantly differed as a function of day during the 28-day treatment period (data not shown). Likewise, neither initial nor final body weights differed between groups, and all groups had a similar, significant weight gain over the experimental period (Table 1). Systolic blood pressure was not significantly different between treatment groups ($P = 0.599$; Table 1). In the normotensive Sprague Dawley animals used as controls, resveratrol average daily dose in the high-resveratrol group (0.165 ± 0.008 mg, $n = 4$) was not significantly different from in the high-dose SHR group (Table 1), and the Sprague Dawley animals were of similar size (final body weights 382 ± 8 g and 395.9 ± 10.2 g in the control and high-resveratrol Sprague Dawley groups, respectively, $n = 4$ per group) compared with the SHR groups (Table 1). Resveratrol had no significant effect on systolic blood pressure in the Sprague Dawley rats (139 ± 5 vs. 135 ± 4 mm Hg in control and high-resveratrol groups, respectively, $n = 4$ per group).

Vasomotor Function. There were no differences in resting tension at L_o among treatment groups (Table 2). Likewise, there was no significant difference between treatment groups in developed tension elicited by maximal (60 m*M*) KCl, or by submaximal (10^{-7} *M*) or maximal (10^{-4} *M*) phenylephrine (Table 2).

Ach-elicited, dose-dependent relaxation from 10^{-7} *M* phenylephrine preconstriction in all treatment groups ($P < 0.0001$) and a significant treatment group effect in the Ach dose–response data were identified ($P < 0.0001$). Significant differences between groups at specific Ach concentrations are indicated in Figure 1A. Curve fitting using a sigmoidal dose–response model revealed significant increases in maximal vasorelaxation in both low- and high-resveratrol groups compared with the control group (Table 2), whereas maximal relaxation was not significantly different between the low- and high-resveratrol groups. No significant difference in EC_{50} (effective concentration resulting in 50% relaxation from preconstruction) among

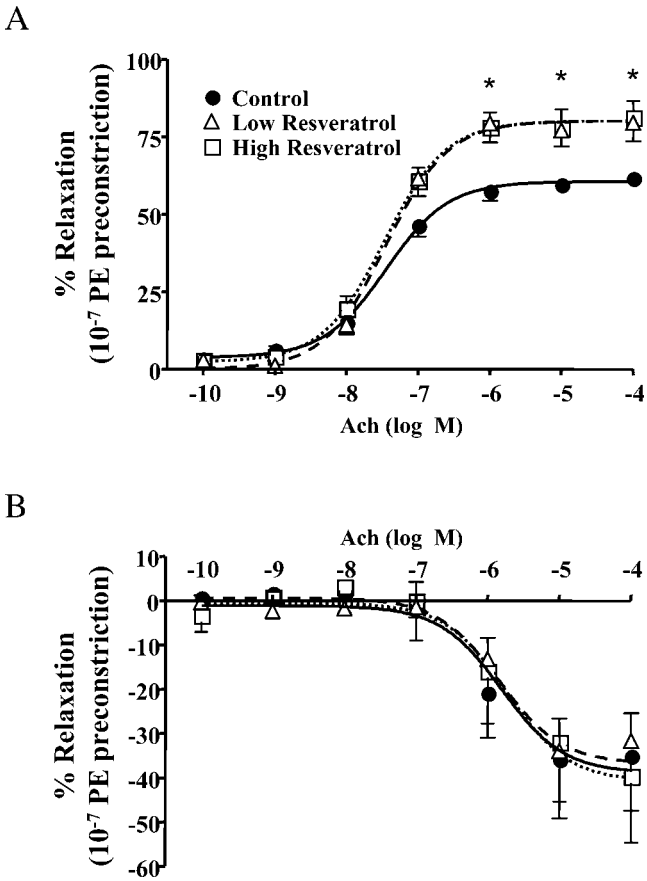

Figure 1. Ach-induced relaxation of thoracic aorta rings from control (filled circles, solid lines), low-resveratrol (open triangles, dashed lines), and high-resveratrol (open squares, dotted lines) SHRs after phenylephrine (PE) preconstriction (10^{-7} *M*). (A) Relative relaxation to Ach in the absence of L-NAME ($n = 8$ rats per group). Ach-induced relaxation was significantly improved in both resveratrol groups compared with controls ($P < 0.0001$). *$P < 0.01$ for low- and high-resveratrol versus control at specific Ach concentrations. (B) Relative constriction to Ach in the presence of L-NAME ($n = 8$ rats per group). No significant treatment group differences existed under these conditions ($P = 0.991$).

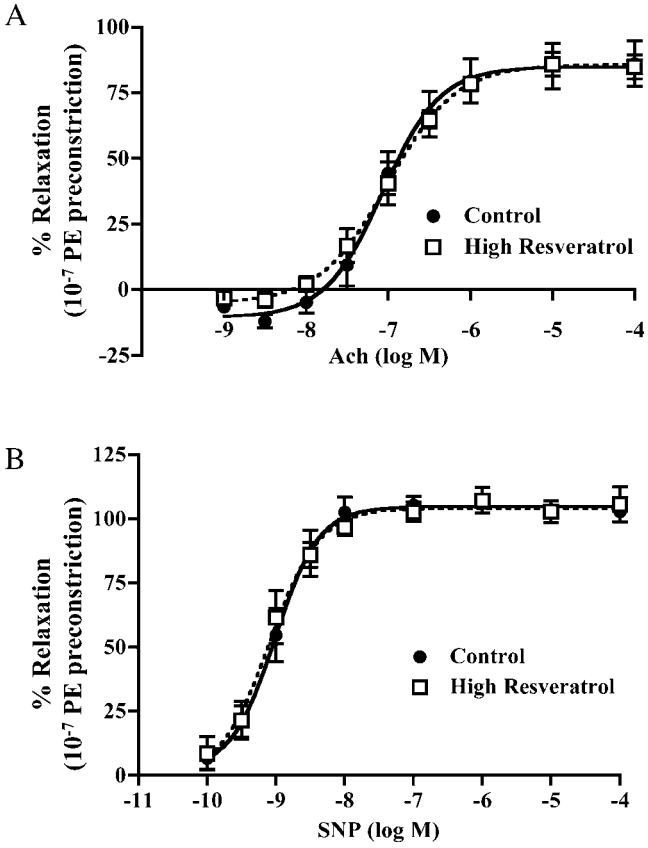

Figure 2. Ach-induced relaxation of thoracic aorta rings from control (filled circles, solid lines) and high-resveratrol (open squares, dotted lines) Sprague Dawley rats after phenylephrine (PE) preconstriction (10^{-7} *M*). (A) Relative relaxation to Ach in the absence of L-NAME ($n = 4$ rats per group). Ach-induced relaxation was not affected by resveratrol treatment in Sprague Dawley rats. (B) Relative relaxation of thoracic aorta rings from control and high-resveratrol Sprague Dawley rats to SNP in the absence of L-NAME ($n = 4$ rats per group) after phenylephrine (PE) preconstriction (10^{-7} *M*). No treatment group differences were evident in SNP responses.

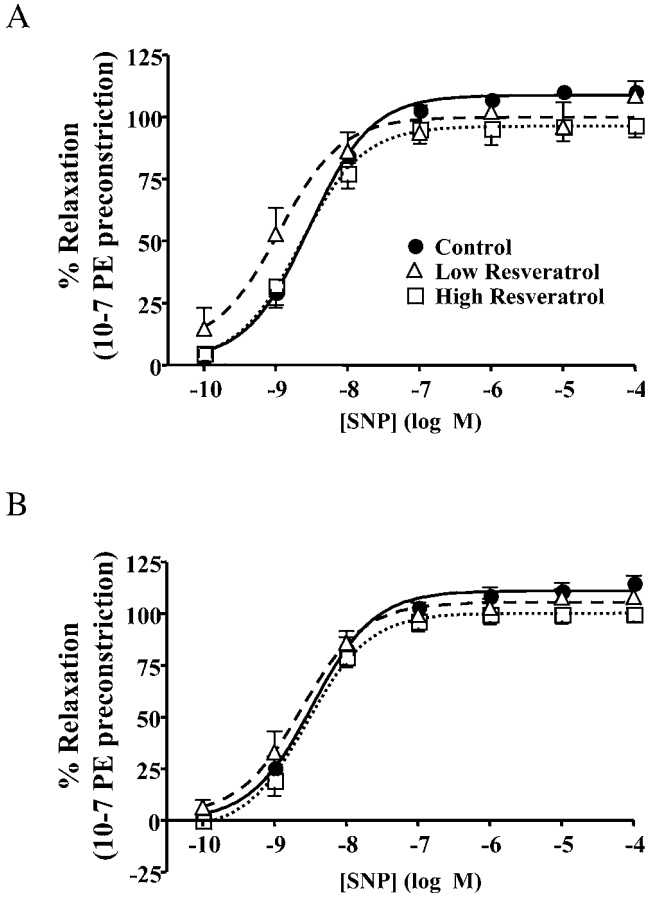

Figure 3. SNP-induced relaxation of thoracic aorta rings from control (filled circles, solid lines), low-resveratrol (open triangles, dashed lines), and high-resveratrol (open squares, dotted lines) SHR after phenylephrine (PE) preconstriction (10^{-7} *M*). (A) Relative relaxation to SNP in the absence of L-NAME ($n = 8$ rats per group). Relaxation was similar in control and resveratrol groups across SNP concentrations. (B) Relative relaxation to SNP in the presence of L-NAME ($n = 8$ rats per group). No treatment group differences were evident in SNP responses in the presence of L-NAME.

treatment groups was detected (Table 2). Inhibition of eNOS with L-NAME eliminated treatment group differences in response to Ach ($P = 0.991$; Fig. 1B). In the presence of L-NAME, Ach failed to induce vasorelaxation, and it elicited contraction at higher concentrations (10^{-6} *M* and greater, as has been previously observed in SHR aorta; Fig. 1B; Ref. 17).

As illustrated in Figure 2A, Ach-induced dilation of aortic rings from normotensive Sprague Dawley rats was not significantly affected by resveratrol with respect to maximal dilation (84.8% ± 9.1% and 85.5% ± 4.0% in control and high-resveratrol groups, respectively; $P = 0.956$) and EC_{50} (–7.08 ± 0.08 and –7.06 ± 0.15 in control and high-resveratrol groups, respectively; $P = 0.912$).

SNP elicited dose-dependent relaxation from 10^{-7} *M* phenylephrine precontractions in all SHR treatment groups, but there was no significant effect of treatment on EC_{50} or maximal dilation to SNP (Fig. 3A; Table 2). There was no effect of L-NAME on the response of aortas to SNP from any SHR treatment group, and there also were no treatment group differences in SNP responses in the presence of L-NAME (Fig. 3B).

As illustrated in Figure 2B, SNP-induced dilation of aortic rings from normotensive Sprague Dawley rats was likewise not significantly affected by resveratrol with respect to maximal dilation (105.1% ± 2.6% and 104.7% ± 5.0% in control and high-resveratrol groups, respectively; $P = 0.949$) and EC_{50} (–9.00 ± 0.11 and –9.18 ± 0.16 in control and high-resveratrol groups, respectively; $P = 0.381$).

Immunoblots. There were no significant treatment group differences in the SHR aorta protein expression levels of eNOS, the antioxidant enzymes SOD-1 and SOD-2, or the gp91phox subunit of the pro-oxidant enzyme NAD(P)H oxidase (Fig. 3). Relative to the control group, Hsp90 increased by 23% and 40% in the low- and high-resveratrol groups, respectively, although this did not reach statistical significance ($P < 0.07$; Fig. 4). Actin protein levels were

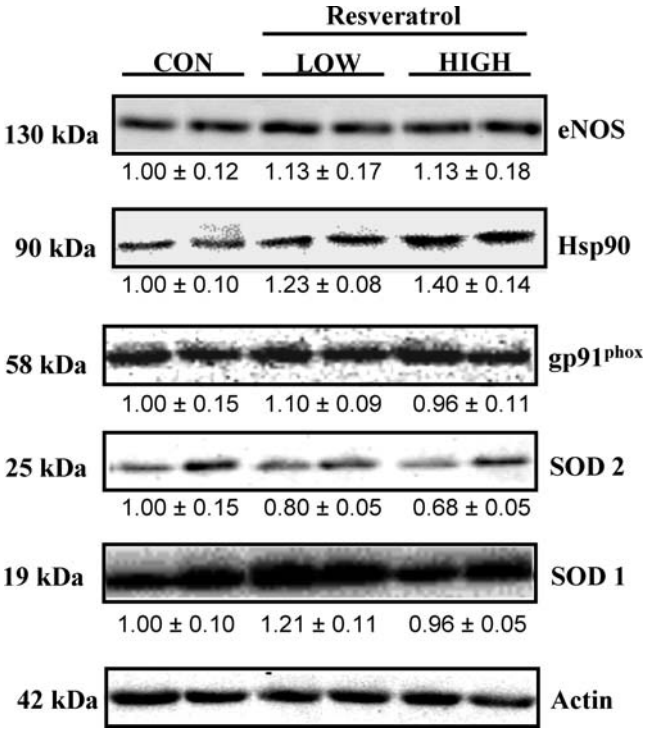

Figure 4. Immunoblot analysis of thoracic aorta protein expression in SHRs. Representative immunoblots for the proteins of interest in two samples from each of the control, low- and high-resveratrol SHR groups are shown, and mean data for relative levels of the proteins, normalized to sample actin content and adjusting control group mean to 1.00 for each protein, are indicated in the line below each luminogram ($n = 6$ rats per group, each sample run in triplicate). Proteins analyzed are eNOS, heat shock protein 90 (Hsp90), the gp91phox subunit of NAD(P)H oxidase (gp91phox), and SOD-1 and SOD-2.

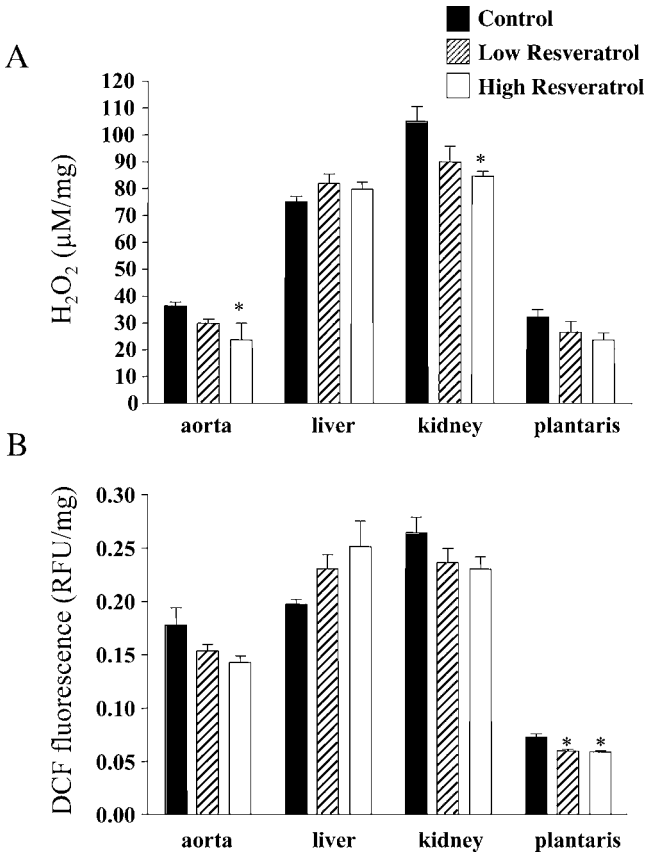

Figure 5. Amplex Red and DCF fluorescence measurements of H_2O_2 and ROS in SHR tissue homogenates. (A) Amplex Red fluorescence measurement of H_2O_2 in tissue homogenates from SHR treatment groups; treatment key is inset. *$P < 0.05$ versus control group within a tissue type. (B) DCF fluorescence measurement of ROS in tissue homogenates from SHR treatment groups. *$P < 0.05$ versus control group within a tissue type.

not systematically different across treatment groups (1.00 ± 0.11, 0.89 ± 0.13, and 0.92 ± 0.10 in control, low, and high groups, respectively; $P = 0.77$).

Tissue Hydrogen Peroxide Levels and ROS Generation. The concentration of H_2O_2, which was assessed by Amplex Red in tissue homogenates, was significantly lower in aorta and kidney of high-resveratrol compared with control SHRs ($P < 0.05$) but was not significantly different between treatment groups in liver or plantaris (Fig. 5). ROS generation, which was assessed in tissue homogenates by DCF fluorescence, was significantly lower in plantaris muscle of low- and high-resveratrol compared with control SHRs ($P < 0.001$) but was not significantly different between treatment groups in the other tissues assayed (Fig. 5).

Discussion

Previous reports have demonstrated that endothelium-dependent dilation is impaired in SHR compared with normotensive WKY animals and that elevated oxidative stress–mediated destruction of NO contributes to this impairment (17–21). Thus, it was reasoned that the SHR was an appropriate model in which to test the current hypothesis that chronic dietary resveratrol administration could improve endothelium-dependent, NO-mediated vasorelaxation and increase eNOS expression. The main finding of this study is that 28 days of resveratrol ingestion at a weight-adjusted daily ingestion similar to that resulting from moderate daily red wine consumption in humans improved the maximal Ach-induced, endothelium-dependent, NO-mediated relaxation of aorta in SHRs. However, contrary to our hypothesis, this occurred in the absence of significant changes in aorta eNOS protein levels. Thus, the hypothesis was only partially supported by the current results.

A crucial distinction that bolsters the novelty and relevance of the current study is that in contrast to previous studies that examined acute vasoactive responses to pharmacologic resveratrol treatment *in vitro* (6–10), arterial tissue adaptations to moderate resveratrol ingestion, which was designed to mimic chronic daily resveratrol consumption that would occur as a result of moderate red wine ingestion, were examined. An obvious limitation in this comparison of models, however, is in the pattern of

resveratrol ingestion, which is phasic during the day in the red wine–drinking humans and more spread out during the day in the animal models as a result of its presence in the drinking water.

Ach-induced relaxation was improved in aorta of resveratrol-treated SHRs in both the low- and high-dose groups, but in the presence of L-NAME this treatment group difference was abolished, suggesting that enhanced NO bioavailability is the cause of the improved relaxation response. This effect is similar to those of chronic pharmacologic antioxidant treatments used in other studies to recover impaired vascular function in SHRs (29, 30). It also builds on the previous observation that resveratrol at 100 times the daily dose of the low-resveratrol group in the current study improves endothelium-dependent dilation in ovariectomized, stroke-prone SHRs (15) by demonstrating the effect at much lower doses and in the absence of other confounding treatments. Resveratrol did not affect Ach-induced relaxation in normotensive Sprague Dawley aortas, suggesting that the preexisting impairment in the SHR aortas was required to expose the beneficial effect of resveratrol. On the basis of the observation that aortic rings from all three SHR treatment groups responded similarly to the NO donor SNP in either the presence or absence of L-NAME, it can be inferred that the sensitivity of the vascular smooth muscle to NO is similar among treatment groups. Thus, it is likely that either Ach-induced NO production must be enhanced or destruction of NO must be tempered by the chronic resveratrol treatment to account for the functional effects observed. However, the experimental design limitation that only one endothelium-dependent agonist, Ach, was used in this study to test endothelium-dependent dilation should be recognized. The possibility that resveratrol effects are parochial to Ach thus cannot be eliminated.

Endothelial cell culture studies demonstrated an upregulation of eNOS expression and activity as a result of acute and short-term resveratrol exposure (12–14), and this formed the basis for the current hypothesis that improved Ach-induced relaxation with resveratrol would result from upregulated eNOS expression. However, no significant differences in aorta eNOS protein levels were found between control and resveratrol-treated SHRs in the current study. At present it is not known why the *in vivo* results in SHRs differ from the *in vitro* results in cultured endothelial cells. It is known from previous studies that the protein expression levels of aortic eNOS are already elevated in SHRs compared with control normotensive WKYs (17, 31), possibly preventing further resveratrol-induced increases. However, the eNOS protein expression was likewise not elevated in the high-resveratrol Sprague Dawley rat group with respect to controls (0.92 ± 0.08 vs. 1.00 ± 0.16 units, respectively; $P = 0.647$). Alternatively, it may be that the eNOS-inducing effect of resveratrol is either not as robust *in vivo*, regardless of rat strain, as it is in cultured endothelial cells, or that higher doses of resveratrol may have been required to elicit such a response. The eNOS protein expression data do not eliminate the possibility that resveratrol administration may affect eNOS activity, although the latter was not assessed in the current study. Many regulatory factors (170 factors) affect eNOS activity, and it is possible that one or several of these contributes to the resveratrol effect by enhancing NO synthesis but, again, no specific studies were performed to evaluate specific mechansims. The expression level of Hsp90 was modestly increased in aortas of resveratrol-treated animals, and as Hsp90 binds directly to eNOS in a protein–protein interaction that increases eNOS activity (32–34), this is one example of the types of eNOS regulatory mechanisms that could be operative, although the data in this study are not substantive enough to implicate the responsible mechanisms.

There was no treatment effect on the protein levels of either gp91phox, a subunit of the pro-oxidant enzyme NAD(P)H oxidase, or the antioxidant enzymes SOD-1 or SOD-2 in the aorta. Thus, if improved handling of ROS contributes to the improved NO bioavailability in chronic resveratrol animals, it does not appear to be due to alterations in the protein expression levels of these pro-oxidant and antioxidant enzymes. As with the eNOS results, however, these findings do not eliminate the possibility that resveratrol treatment might change the activities of pro-oxidant or antioxidant enzymes by regulatory mechanisms independent of changes in expression. A possible ROS-managing effect of resveratrol is consistent with previous results showing partial protection by this compound against oxidative damage in stroke-prone SHRs (35), and indeed with the data in this study indicating lower ROS in some, but not all, tissues of resveratrol-treated SHRs.

Resveratrol did not affect blood pressure in the SHR animals used in this study. This finding is consistent with the previously reported lack of effect of 1 mg/kg per day of resveratrol (approximately 6 and 60 times the high and low doses used in the current study, respectively) on blood pressure in stroke-prone SHRs throughout an 8-week feeding period (35). Thus, the resveratrol-induced improvements in vasodilatory function noted in the aorta occurred in the absence of an improvement in blood pressure. As the aorta is not a resistance artery, it is perhaps not surprising that improvements in its vasodilatory capacity do not necessarily result in lowered blood pressure *in vivo*. From the present data we cannot conclude whether there was an effect of resveratrol ingestion on the resistance vasculature, although it seems unlikely, since there was no reduction in blood pressure. However, the improvement in conduit artery endothelial function as a result of resveratrol ingestion in hypertensive rats may be consistent with enhancement of the capacity of this segment of the vasculature to defend against dysfunction and disease. This is interesting, since the improved vasomotor function occurs independently of changes in blood pressure, and because large, conduit arteries are the types of vessels most prone to atherosclerosis

in humans. Further studies of resistance artery function and regional blood flow distribution would allow for comparison of the resveratrol effects on resistance vessels to contrast or complement the initial studies, reported herein, performed in a conduit artery.

The resveratrol doses used in this study were within the range previously reported to be nonharmful to rats with respect to histopathologic, biochemical, and hematologic parameters (36). The doses were designed to mimic chronic resveratrol intake from moderate red wine consumption (low) and 10 times this level (high), weight adjusted to the rat from the human. The target low dose was ~47 μg/kg body weight per day for 28 days, which is roughly equivalent to the dose of 43 μg/kg per day for 15 days used by Bertelli and colleagues (24), in which it was also demonstrated that this modest dose mimicking moderate red wine consumption resulted in absorption of a sufficient quantity of resveratrol to inhibit platelet aggregation, one clinical index of the beneficial effects of red wine. Actual estimated resveratrol administration exceeded target doses by approximately 25% as a result of apparent fluid consumption in excess of that expected *a priori*. Still, the low dose administered falls within the qualitative description of that equivalent to weight-adjusted moderate red wine consumption. Bertelli *et al*. (24) argued that, based on kinetics of the absorption of resveratrol administered over a prolonged period of time, after a sufficiently high tissue concentration of resveratrol has been attained, equilibrium between absorbed and eliminated resveratrol can prevent further increases. This argument could contribute to the observation of no additional effect of higher-dose over lower dose resveratrol on the improvement in NO-mediated relaxation of SHR aortas in the current study. One limitation to this argument, however, is that for some of the biochemical parameters (e.g., Amplex Red measurements and Hsp90 immunoblots) there appeared to be a dose-dependent effect.

In summary, chronic resveratrol ingestion at a dose mimicking that obtained from moderate red wine consumption improves maximal Ach-induced, endothelium-dependent, NO-mediated relaxation of arteries in SHRs, but it does not affect blood pressure. At present, the particular determinant of NO bioavailability that is targeted by resveratrol *in vivo* in this model is not known, although it is known that the functional effect did not result from increased expression of eNOS, as had been hypothesized based on cell culture results. The implications of these findings must be further examined by determining the effects of this treatment on hemodynamics and perfusion *in vivo*, including assessment of atherosclerotic phenotype in large conduit vessels and functional adaptations in resistance arteries that might impact resistance and blood flow distribution.

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