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Resveratrol Supplementation Gender Independently Improves Endothelial Reactivity and Suppresses Superoxide Production in Healthy Rats

Selen Soylemez · Aylin Sepici · Fatma Akar

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Abstract
Purpose Resveratrol, a polyphenolic compound mainly abundant in red wines, has beneficial cardiovascular effects on various pathological conditions. However, at present, the effect of resveratrol on health promotion remains unclear. Therefore, in this study, we assessed whether long-term resveratrol supplementation changes endothelial function, vascular contractility, nitric oxide and superoxide production in healthy male and female rats.
Methods Wistar rats were treated with resveratrol (50 mg/l) in their drinking water for 3 weeks. We investigated relaxation to acetylcholine (10^{-9}–10^{-4} M) and contractions to phenylephrine (10^{-9}–3×10^{-4} M) and angiotensin II (10^{-10}–10^{-5} M) in either endothelium-intact or denuded aortae from control and resveratrol-treated male and female rats. Aortic superoxide production capacity was measured in response to provocation by angiotensin II and NAD(P)H. Plasma nitrite/nitrate levels and superoxide dismutase (SOD) activity were also evaluated.
Results Resveratrol supplementation gender independently increased relaxation to acetylcholine and decreased contractions to phenylephrine and angiotensin II in endothelium-intact aortic rings, but not in endothelium-denuded arteries, from healthy male and female rats. This was associated with increased plasma nitrite/nitrate levels. Furthermore, resveratrol caused a refractoriness to angiotensin II and NAD(P)H-induced provocation in superoxide production.
Conclusion Our results suggest that resveratrol supplementation gender independently could improve the capacity of endothelial function and suppression of oxidative stress under physiological conditions. Resveratrol ingestion indicates a potential for cardiovascular health promotion.

Key words Resveratrol · Endothelial reactivity · Superoxide · Angiotensin II · Health promotion · Male and female rats

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Introduction

Epidemiological studies have proposed that moderate consumption of red wine is associated with a reduction in the incidence of cardiovascular diseases [1] however, the underlying mechanism is not completely understood. A number of studies on red wine have shown that resveratrol could be the main component responsible for the observed beneficial cardiovascular effects [2]. Resveratrol has been shown to possess both endothelium-dependent and independent vasodilatory effects in animal and human vessels [3–5]. Resveratrol was also found to enhance the expression of eNOS mRNA and eNOS protein as well as the activity of eNOS in cultured endothelial cells [6]. Furthermore, resveratrol was shown to activate genomic and nongenomic estrogen receptors, thereby stimulating nitric oxide synthesis in cell lines [7, 8]. On the other hand, the activity of NAD(P)H oxidase, which is major potential source of superoxide in the vasculature, has been shown to decrease following resveratrol treatment in endothelial cells and the rat aorta [4, 9, 10]. Long-term treatment with resveratrol

was reported to increase flow-mediated vasodilation in hypercholesterolemic rabbits [11], and endothelium-dependent relaxations to acetylcholine in spontaneously hypertensive rats and obese and elderly mice [12–14]. However, very little is known about the effect of resveratrol supplementation under physiological conditions which could reflect its potential effect on health promotion and disease prevention. In a very recent study, we showed that dietary resveratrol administration caused functional activation of estrogen receptors in arteries from healthy male and female rats suggesting a novel mechanism for beneficial cardiovascular effects of resveratrol [10]. In the present study, we assessed whether relaxation to acetylcholine and vasoconstrictions to phenylephrine and angiotensin II are changed in aortae from resveratrol-treated healthy rats, whether aortic superoxide production capacity in response to provocation by angiotensin II and NAD(P)H is reduced, and whether the effect of resveratrol is modified by gender and by the presence or absence of endothelium. It is well known that endothelium modulates vascular contractility, and its function may change between males and females [15–17]. Increasing evidence demonstrated that endothelial factor nitric oxide promotes vascular health against superoxide, angiotensin II and catecholamine-induced insults [18]. Hence, the potential enhancer effect of resveratrol on endothelial reactivity could contribute to health promotion.

Methods

Animal care and diet

The study protocol was performed in accordance with institutional guidelines and approved by the Ethical Animal Research Committee of Gazi University. Wistar rats (14–16 weeks old) of male and female genders were housed in temperature-controlled rooms (21–24°C) under a 12 h light/dark cycle. Rats were fed with standard diet (Korkuteli Yem Sanayii, Turkey) and allowed free access to food and water. The analysis of the food in TUBITAK showed no traces of resveratrol. The rats were separated into four groups: male control, male resveratrol, female control and female resveratrol. The female rats were randomly selected on different days of the estrous period. Resveratrol (50 mg/l) or its vehicle (0.05% ethanol) was administrated to rats in drinking water by means of a bottle wrapped with light protective material for 21 days. The former was described as the resveratrol (long-term resveratrol treatment) group, while the latter was the control (vehicle) group. The concentration of resveratrol was selected based on previously reported in vivo observations in which the biological effects of resveratrol were determined at concentrations ranging from 3–204 mg/kg administered orally [10–12, 14]. The water intake and body weights of the rats were recorded as reported previously [10]. The initial and terminal body weights of rats of both genders were between 210–230 g. Consumption of water was 12–14 ml/100 g bw/day in all experimental groups. The amount of resveratrol ingested in drinking water was calculated as approximately 6.6 mg/kg bw/day in both genders. Experiments were carried out the day after the last drinking of resveratrol or its vehicle to study its long-term effects with no implication of acute influences. Age-matched male and female rats were euthanized using a CO_2 gas chamber.

Preparation of thoracic aortic rings

The thoracic aorta was removed and immediately placed in cold Krebs solution of the following composition (mM): NaCl 118, KCl 4.73, KH_2PO_4 1.2, $MgSO_4$.7H2O 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11 and EDTA 0.026. The aorta was cleaned of fat and connective tissues and cut into rings 3 to 4 mm in length. The rings were mounted in a 10 ml organ bath containing Krebs solution at 37°C and aerated with 95% O_2 and 5% CO_2. Care was taken to preserve the endothelial layer during preparation of the aortic rings. To study the endothelium-denuded rings, the endothelium was removed by gently rubbing the intimal surface of the ring with a roughed polyethylene tube. Changes in isometric force were measured using force displacement transducers (PowerLab ML750). The optimal point of length-tension relation had been ascertained previously by repeated application of phenylephrine (3×10^{-6} M) at different resting tensions. An initial tension of 1 g was determined to be optimal for maximal phenylephrine responsiveness in the aortae of both genders. The rings were allowed to stabilize for approximately 1 h with renewal of the bathing solution every 15 min. Each ring was subjected to only one assay.

Experimental protocol for vascular contraction and relaxations

Two reproducible contractions in response to KCl (40 mM) and phenylephrine (2×10^{-6} M or 3×10^{-6} M) were obtained in the aortic rings of both males and females. The intactness of the endothelium was verified functionally by applying acetylcholine (10^{-6} M) to phenylephrine (2×10^{-6} M or 3×10^{-6} M)-precontracted aortic rings. The rings exhibiting <70% relaxation in response to acetylcholine were excluded from the experiments. Acetylcholine-induced relaxations were completely abolished after the removal of the endothelium.

The cumulative concentration-response curves to phenylephrine (10^{-9}–3×10^{-4} M) and angiotensin II (10^{-10}–10^{-5} M) were obtained in aortic rings with and

without endothelium from both genders in the control and long-term resveratrol treatment groups. To study the relaxant effects of acetylcholine and sodium nitroprusside, arterial rings with endothelium were precontracted with 2×10^{-6} M (female) and 3×10^{-6} M (male) phenylephrine, which corresponds to 80–90% of maximal contractions in aortic rings from both groups. These doses of phenylephrine produced almost the same contraction levels in the control and resveratrol groups. KCl (40 mM) caused similar absolute tensions in the all groups. Acetylcholine (10^{-9}–10^{-4} M) and sodium nitroprusside (10^{-10}–10^{-5} M), at cumulative concentrations, were applied in phenylephrine-contracted rings. In pilot time-match experiments, we determined that the plateau of precontraction elicited by phenylephrine was stable enough for the period required to perform the cumulative-relaxation curves of acetylcholine and sodium nitroprusside.

Measurement of nitric oxide production in plasma

Basal levels of plasma nitric oxide in control and long-term resveratrol-treated male and female rats were evaluated by the measurement of nitrite/nitrate concentrations using a commercially available colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Blood samples taken from the heart were centrifuged at 4°C and 10,000 g for 10 min. Plasma samples were immediately stored at −20°C until the analysis. Potassium nitrate was used as a standard.

Measurement of superoxide production in the aorta

The generation of superoxide in the aortic segments with and without endothelium from control and resveratrol-treated male and female rats was measured using lucigenin-enhanced chemiluminescence at a low concentration (5 μM) of lucigenin, which is described as a sensitive and reliable sensor for monitoring of superoxide production in intact vessels [19]. Aortic segments (3–4 mm) were placed in Krebs-HEPES buffer and incubated in the dark for 10 min at 37°C. HEPES buffer (200 μl) with lucigenin (5 μmol/L) was added in the wells of scintillation plates and equilibrated in the dark for 10 min at 37°C. The chemiluminescence signals were recorded every minute for 10 min in a scintillation counter (1450 Microbeta Wallac TRILUX liquid scintillation counter). The amount of superoxide was calculated by comparison with a standard curve using xanthine/xanthine oxidase [19, 20]. Superoxide generation in aortic segments was expressed as counts/min/mg dry tissue weight.

The superoxide production was stimulated with angiotensin II (10^{-7} M) and its combination with NADH or NADPH (both 100 μM) for 10 min at 37°C in the aortae. Additionally, angiotensin II and NAD(P)H-induced signals were determined in the arteries preincubated with resveratrol (1 μM, acute application of resveratrol), SOD (200 U/ml) or the flavin-containing enzyme inhibitor DPI (10 μM) for 10 min at 37°C. The chemiluminescence signals were also recorded in the wells containing buffer, lucigenin and the chemicals in vascular segments to determine the possible interaction between lucigenin and the chemicals.

Measurement of superoxide dismutase (SOD) in plasma

The total SOD activity in the plasma was measured using a spectrophotometer. The principle of the method is based on the inhibition of superoxide dismutase activity by reduction of nitroblue-tetrazolium with xanthine–xanthine oxidase used as a superoxide generator. One IU was defined as the quantity of SOD required to produce 50% inhibition. The results are expressed as units/ml.

Measurement of copper and zinc in plasma

Plasma samples were treated with trichloroacetic acid and separated from the proteins. The resulting supernatant was used as the source of copper and zinc. The plasma levels of the metals were determined with an atomic absorption spectrophotometer at wavelength 324.7 nm and 213.7 nm, respectively.

Chemicals

Chemicals for bioassay and superoxide measurement were purchased from Sigma Chemical Co (St Louis, MO). Chemicals and kits for PCR measurement were obtained from Fermentas and Qiagen. *Trans-resveratrol* was purchased from Sigma or Herb-Tech (China). The purity of resveratrol from two sources was compared by HPLC analysis and there were no differences between them. The drinking water stocks with resveratrol or vehicle were prepared weekly in 0.05% vol/vol ethanol and stored in dark bottles at 2–4°C. Acetylcholine was dissolved in 0.001 N HCl. Stock solutions of phenylephrine and angiotensin II were prepared in distilled water. All subsequent dilutions were made in Krebs solution and kept cold in the dark.

Statistical analysis

The results are given as mean ± standard error of the mean. Contractile responses to phenylephrine and angiotensin II were expressed as a percentage of the 40 mM KCl-induced contraction. The relaxations to

acetylcholine were expressed as percent decreases of the precontraction to phenylephrine. The maximal response (E_{max}) and potency (EC_{50}) of the agents were determined by non-linear curve fitting using the Prism 4.03 GraphPad program. EC_{50} values are given as –log M. Statistical analyses were performed using the Student's *t*-test or ANOVA followed by the Bonferroni post-hoc analysis to compare selected groups. Values were considered to be significantly different when the *p* value was less than 0.05.

Results

The endothelium-dependent relaxation to acetylcholine in aortic rings from male and female rats and the influence of long-term resveratrol treatment

The relative contractions in response to $2–3\times10^{-6}$ M phenylephrine were not different in aortic rings of control and resveratrol-treated female and male rats (80–85% vs. 86–89%, respectively, $n=10$, $p>0.05$). Acetylcholine (10^{-9}–10^{-4} M) produced endothelium-dependent relaxations, in a concentration-dependent manner, in the aortic rings of control male and female rats (Fig. 1). The maximal relaxation in response to acetylcholine was more pronounced in aortic rings from females than in those of males (E_{max} values in males and females: 76% vs. 90%, $n=10$, $p<0.05$), but the potencies were similar between genders (Table 1). Resveratrol treatment significantly increased the maximal relaxation, but not the potency of acetylcholine in the aortic rings of males (E_{max}: 96% vs. control, $n=10$, $p<0.05$). However in the female gender, this treatment increased the potency, but not the maximal response to acetylcholine (Fig. 1, Table 1). The relaxations in response to acetylcholine disappeared in endothelium-denuded aortic rings or after pretreatment with L-NOARG (10^{-4} M) from control and resveratrol-treated male and female rats. The endothelium-independent relaxations to sodium nitroprusside (10^{-10}–10^{-5} M) were similar in all of the groups, in terms of potency (EC_{50}) or maximal response (E_{max}). In control male and female rats: EC_{50}: 8.69±0.07 vs. 8.75±0.12, $n=8$, $p>0.05$ and E_{max}: 98±3% vs. 100±3%, $n=8$, $p>0.05$, respectively; in resveratrol-treated male and female rats: EC_{50}: 8.71±0.1 vs. 8.81±0.09, $n=8$, $p>0.05$ and E_{max}: 98±2% vs. 101±2%, $n=8$, $p>0.05$, respectively.

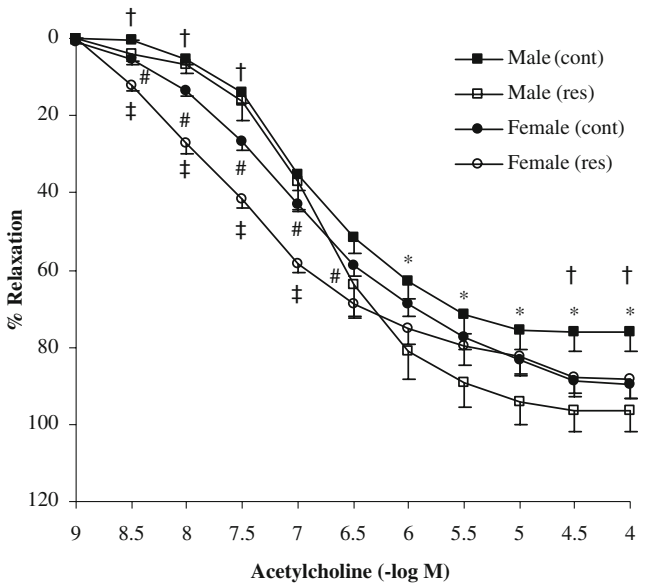

Fig. 1 The influence of resveratrol treatment (res) on concentration-response curves for acetylcholine in endothelium-intact aortic rings from male and female rats. Values are expressed as means±SEM, $n=10$. $^{\dagger}p<0.05$ between control genders; $^{*}p<0.05$ between control and resveratrol-treated males; $^{\#}p<0.05$ between control and resveratrol-treated females; $^{\ddagger}p<0.05$ between resveratrol-treated genders

Table 1 The potency (EC_{50}; –log M) and maximum relaxation (E_{max}) values to acetylcholine in endothelium-intact (+E) aortic rings from control (cont) or resveratrol-treated (res) male and female rats

	Protocols	EC_{50}	E_{max}	n
Male				
Acetylcholine	+E (cont)	6.73±0.07	76.3±4.7	10
	+E (res)	6.62±0.07	96.5±5.6[a]	10
Female				
Acetylcholine	+E (cont)	6.83±0.06	89.9±3.3[b]	10
	+E (res)	7.35±0.08[a,b]	88.5±4.8	10

Values are expressed as mean±SEM

[a] $p<0.05$ between control and resveratrol-treated groups

[b] $p<0.05$ between genders

Contractile response to phenylephrine in aortic rings from male and female rats and the influence of long-term resveratrol treatment

Phenylephrine-induced maximal contraction was greater in aortic rings from males than in those from females. Endothelium removal increased the contractions and abolished the gender difference. Resveratrol treatment significantly decreased maximal contractions to phenylephrine in endothelium-intact aortae obtained from male and female rats as compared to corresponding controls, but did not abrogate the gender difference (Fig. 2, Table 2). Additionally, this treatment reduced the sensitivity to phenylephrine in endothelium-intact aortae from females. In endothelium-denuded aortae from male and female rats, resveratrol treatment did not affect either the

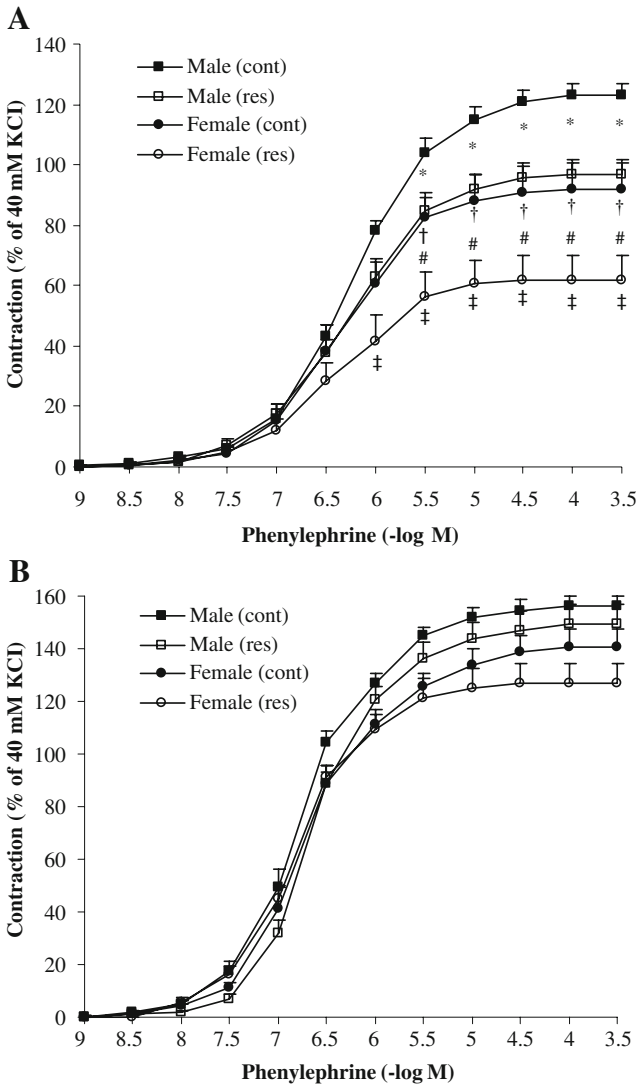

Fig. 2 The influence of resveratrol treatment (res) on concentration-response curves for phenylephrine in endothelium-intact (**a**) and endothelium-denuded (**b**) aortic rings from male and female rats. Values are expressed as means±SEM, n=7–13. $^{\dagger}p<0.05$ between control genders; $^{*}p<0.05$ between control and resveratrol-treated males; $^{\#}p<0.05$ between control and resveratrol-treated females; $^{\ddagger}p<0.05$ between resveratrol-treated genders

maximal response or the potency of phenylephrine (Fig. 2, Table 2).

Contractile response to angiotensin II in aortic rings from male and female rats and the influence of long-term resveratrol treatment

Angiotensin II caused concentration-dependent contractions in aortic rings of rats, with a higher potency in males than females. Interestingly, endothelium removal markedly reduced the sensitivity to angiotensin II in females, preserving the gender difference, whereas did not increase

Table 2 The potency (EC_{50}; –log M) and maximum relaxation (E_{max}) values to phenylephrine in endothelium-intact (+E) and denuded (−E) aortic rings from control (cont) or resveratrol-treated (res) male and female rats

	Protocols	EC_{50}	E_{max}	n
Male				
Phenylephrine	+E (cont)	6.22±0.07	123±4.1	12
	+E (res)	6.21±0.07	96.6±5.2[a]	13
	−E (cont)	6.72±0.05[b]	156±3.8[b]	12
	−E (res)	6.64±0.07[b]	149.5±7.3[b]	9
Female				
Phenylephrine	+E (cont)	6.36±0.07	91.6±9[c]	8
	+E (res)	6.06±0.07[a]	61.7±8.2[a,c]	9
	−E (cont)	6.70±0.07[b]	140.5±6.9[b]	9
	−E (res)	6.79±0.04[b]	127.1±6.9[b]	7

Values are expressed as mean±SEM

[a] $p<0.05$ between control and resveratrol-treated groups

[b] $p<0.05$ between endothelium-intact and denuded rings in the same gender

[c] $p<0.05$ between genders

the maximal contractions. Resveratrol treatment significantly diminished both sensitivity and maximal contractions to angiotensin II in endothelium-intact aortae from male and female rats as compared to corresponding controls, and did not change the gender difference. We did not observe any effect of resveratrol treatment in endothelium-denuded aortic rings from both genders (Fig. 3, Table 3).

Nitric oxide levels in plasma from male and female rats and the influence of long-term resveratrol treatment

The nitrite/nitrate levels were similar in the samples of plasma from control male and female rats. The nitric oxide production increased in the plasma from resveratrol-treated rats compared to controls in a gender-independent manner (Fig. 4).

Angiotensin II and NAD(P)H-induced superoxide productions in aortic segments from male and female rats and the influence of long-term resveratrol treatment

Angiotensin II (10^{-7} M), alone or in combination with NADH or NADPH (100 μM), increased the superoxide production in endothelium-intact and denuded aortae of rats with minor differences between males and females. Considering amount of generated superoxide, angiotensin II was more productive in endothelium-intact aortae, whereas its combination with NAD(P)H was more effective in endothelium-denuded aortae from both genders. The aortae obtained from resveratrol-treated rats displayed refractoriness to superoxide stimulation in response to angiotensin

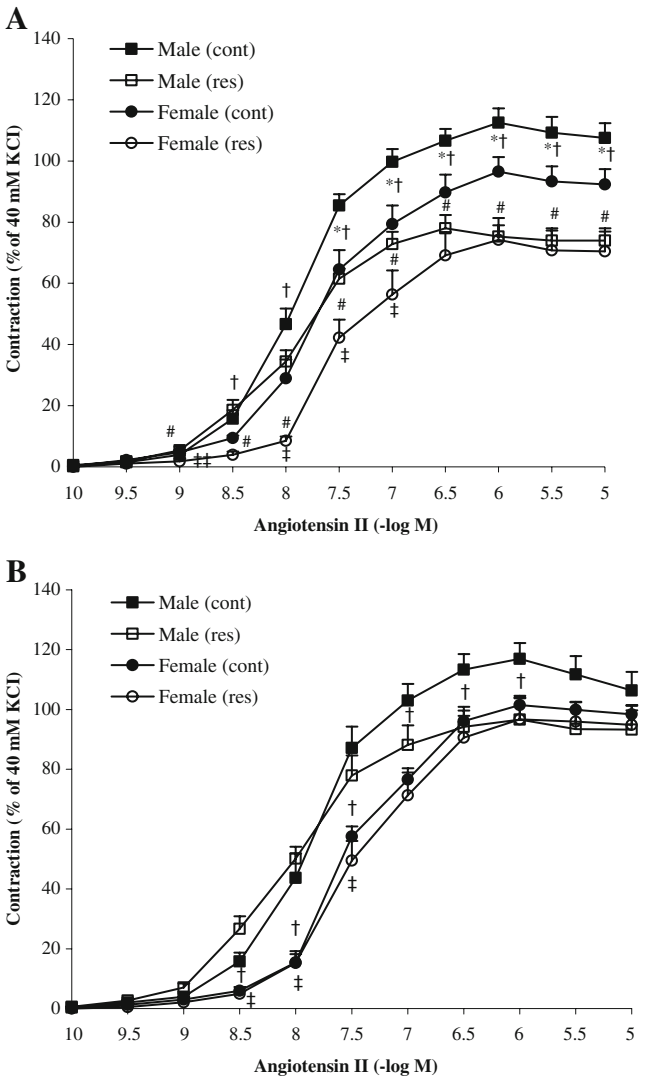

Fig. 3 The influence of resveratrol treatment (res) on concentration-response curves for angiotensin II in endothelium-intact (**a**) and endothelium-denuded (**b**) aortic rings from male and female rats. Values are expressed as means±SEM, n=9–13. $^{\dagger}p<0.05$ between control genders; $^{*}p<0.05$ between control and resveratrol-treated males; $^{\#}p<0.05$ between control and resveratrol-treated females; $^{\ddagger}p<0.05$ between resveratrol-treated genders

II and NAD(P)H. In the similar experimental setting, we have recently demonstrated that NADPH or NADH (100 μM) stimulation generated less superoxide in the aortae isolated from resveratrol-treated male and female rats compared to controls [10]. Gender and the presence or absence of endothelium did not modify the influence of resveratrol (Figs. 5, 6). Furthermore, preincubation with resveratrol (10 μM) or SOD (200 U/ml) significantly reduced angiotensin II and NAD(P)H-induced superoxide production in the aortae of male and female rats. DPI (10 μM) almost completely abolished superoxide generation (Fig. 7).

Table 3 The potency (EC_{50}; –log M) and maximum relaxation (E_{max}) values to angiotensin II in endothelium-intact (+E) and denuded (−E) aortic rings from control (cont) or resveratrol-treated (res) male and female rats

	Protocols	EC_{50}	E_{max}	n
Male				
Angiotensin II	+E (cont)	7.95±0.08	112.5±4.7	10
	+E (res)	7.80±0.01[a]	77.9±4.4[a]	13
	−E (cont)	7.81±0.07	116.9±5.2	10
	−E (res)	7.96±0.08	96.6±7.4	9
Female				
Angiotensin II	+E (cont)	7.73±0.05[b]	96.5±4.8	10
	+E (res)	7.53±0.08[a,b]	74.2±7.1[a]	9
	−E (cont)	7.49±0.05[b,c]	101.5±3.1	13
	−E (res)	7.42±0.09[b]	96.7±7.1	11

Values are expressed as mean±SEM

[a] $p<0.05$ between control and resveratrol-treated groups

[b] $p<0.05$ between genders

[c] $p<0.05$ between endothelium-intact and denuded rings in the same gender

SOD, copper and zinc levels in plasma samples from male and female rats and the influence of long-term resveratrol treatment

Plasma SOD activity was measured to assess its possible modulation by resveratrol treatment. There were no differences in plasma SOD activities and zinc levels among four groups. However, plasma copper levels were slightly higher in females than in males and further increased after resveratrol treatment (Table 4).

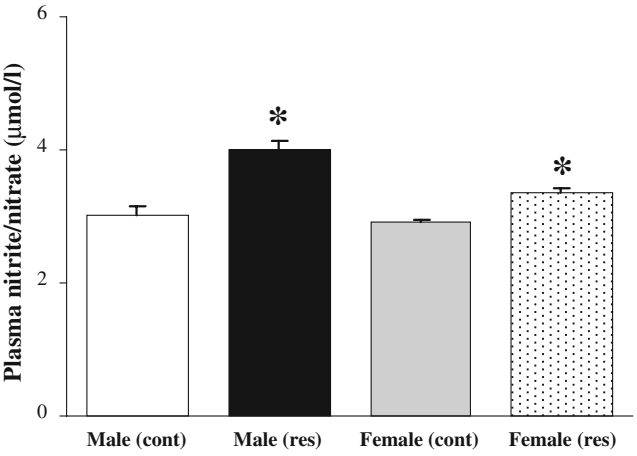

Fig. 4 Nitric oxide (NO) production was detected as nitrite/nitrate in plasma obtained from control (cont) and resveratrol-treated (res) male and female rats. The nitrite/nitrate levels were significantly increased in the plasma of male and female resveratrol-treated rats. Results are means±SEM (n=6–22). $^{*}p<0.05$ between control and resveratrol-treated groups

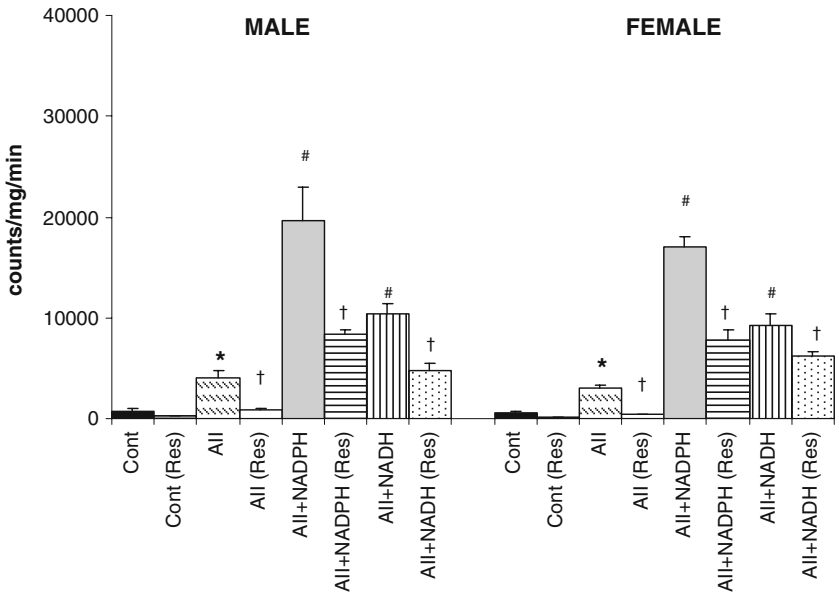

Fig. 5 Angiotensin II alone or in combination with NAD(P)H-stimulated superoxide production in aortae with endothelium from control (Cont) and long-term resveratrol-treated (Res) male and female rats. Values are expressed as means±SEM. n= 6–12. *p<0.01 control vs. AII; $^{\#}p$<0.05 AII vs. AII+ NAD(P)H; $^{\dagger}p$<0.05 shows the inhibitory effect of resveratrol treatment

Discussion

Resveratrol, a polyphenolic compound present in red wine, produces mainly endothelium-dependent relaxation in vascular preparations [3–5]. We have reported that resveratrol enhances relaxations to estrogen via functional activation of estrogen receptors [10]. Here, we showed that resveratrol supplementation increases relaxation to acetylcholine and decreases contractile responses to angiotensin II and phenylephrine in endothelium-intact aortic rings from healthy male and female rats. Furthermore, this treatment causes a refractoriness to angiotensin II and NAD(P)H-induced provocation in superoxide generation. Our data demonstrated that resveratrol administration in drinking water to healthy rats improves endothelial reactivity in a gender-independent fashion. Resveratrol intake as a nutritional supplement indicates a potential for cardiovascular health.

Angiotensin II type 1 (AT_1) and alpha-adrenergic receptor-mediated vasoconstriction may balance with endothelial vasodilatory factors such as nitric oxide and prostacyclin. The imbalance among their functions could play an important role in cardiovascular diseases [18]. Moreover, the female sex hormone estrogen seems to be involved in the regulation of vascular tension because estrogen, at physiological concentrations, causes relaxation by activating nitric oxide synthesis, which may lead to gender-related differences [21]. Consistent with results reported by others in rat aorta [15–17], we demonstrated a

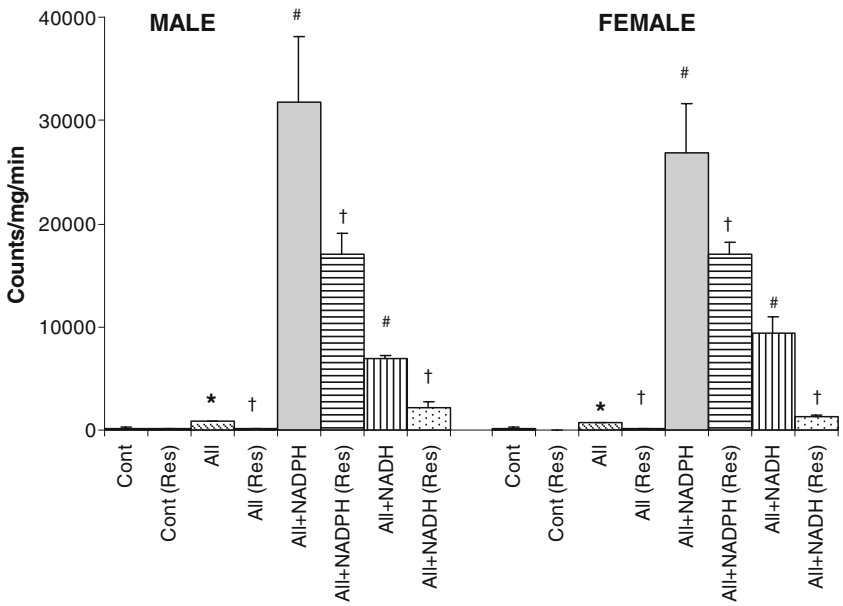

Fig. 6 Angiotensin II alone or in combination with NAD(P)H-stimulated superoxide production in aortae without endothelium from control (Cont) and long-term resveratrol-treated (Res) male and female rats. Values are expressed as means ±SEM. n=6–12. *p<0.01 control vs. AII; $^{\#}p$<0.05 AII vs. AII+ NAD(P)H; $^{\dagger}p$<0.05 shows the inhibitory effect of resveratrol treatment

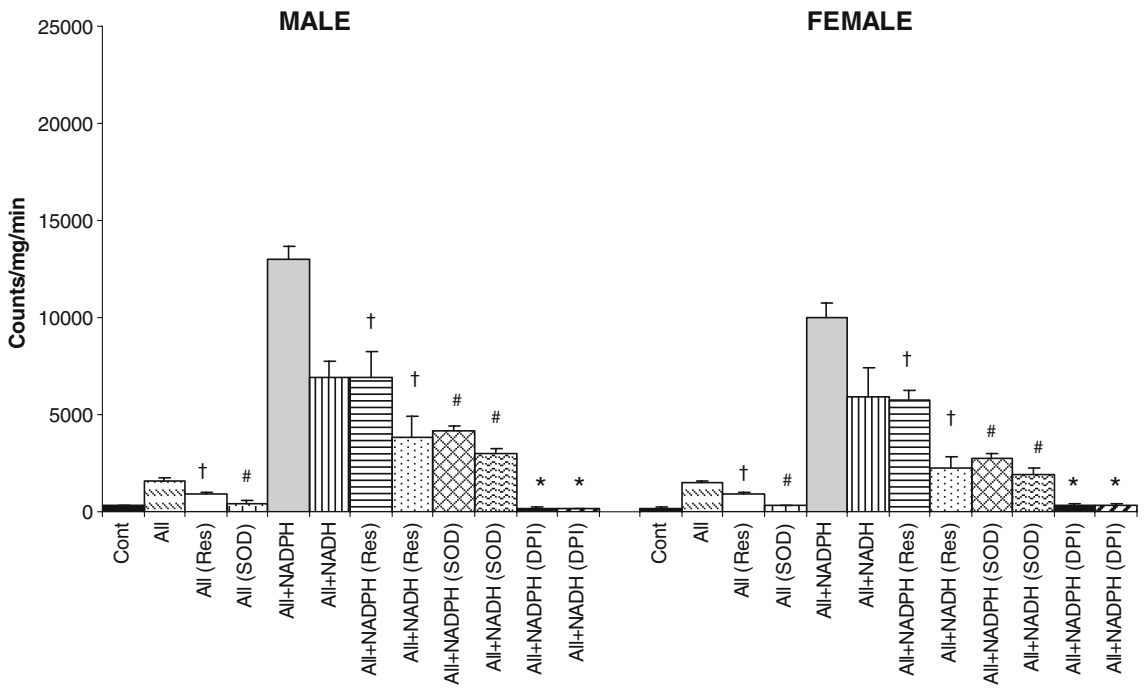

Fig. 7 The inhibitory effects of acute resveratrol (Res; 1 μM) or SOD (200 U/ml) or DPI (10 μM) application on angiotensin II alone or in combination with NAD(P)H-stimulated superoxide production in aortae with endothelium from male and female rats. Values are expressed as means±SEM. n=7–11. $^{\dagger}p<0.05$ for resveratrol, $^{\#}p<0.05$ for SOD and $^{*}p<0.05$ for DPI, as compared to corresponding controls

higher maximal relaxation to acetylcholine, and a lower contractile response to phenylephrine and less sensitivity to angiotensin II in aortae from females compared to males. Estrogen deficiency was shown to augment contractions to angiotensin II, with AT_1 receptor overexpression, and phenylephrine, which is reversed by estrogen substitution therapy, in isolated aortic rings from female rats [16, 17]. Administration of estrogen for 7 days to male and female rats was demonstrated to increase endothelial relaxation or suppress the contractions most likely by increasing production of endothelial nitric oxide [22–24].

We recently reported that resveratrol promoted the response to estrogen by activating classical estrogen receptors, which can be inhibited by pretreatment with ICI 182,780, an estrogen receptor antagonist [10]. Previously, it was shown that resveratrol has agonistic or antagonistic properties on estrogen receptors that vary across cell types [25, 26]. In cell-based studies, resveratrol was reported to activate genomic or rapid membrane-associated estrogen receptors, and, in turn, lead to stimulation of nitric oxide synthesis [7, 8]. In cultured endothelial cells, resveratrol enhanced the expression of eNOS mRNA and eNOS protein, as well as the activity of eNOS [6]. In an in vivo study, it may, therefore, be helpful to investigate the endothelial function of arteries obtained from long-term resveratrol-treated animals under physiologic conditions in association with nitric oxide production.

It has been demonstrated that resveratrol, like estrogen, decreases the expression of AT_1 receptor mRNA in mouse aorta and blunts angiotensin II-induced hypertension [27]. To our knowledge, this is first report showing that resveratrol supplementation (50 mg/l, approximately 6.6 mg/kg bw/day for 3 weeks) increased the endothelial relaxation to acetylcholine and reduced contractility to angiotensin II and phenylephrine in endothelium-intact aortae from healthy rats. This treatment appears to have only minor or no effects on endothelium-denuded arteries, thus revealing its effect on endothelial function. However, we have previously demonstrated that resveratrol treatment increased both endothelium-dependent and independent relaxations to estrogen, without affecting the response to sodium nitroprusside, suggesting that resveratrol could

Table 4 Plasma superoxide dismutase (SOD), copper and zinc levels in control (cont) or resveratrol-treated (res) male and female rats
Values are expressed as mean ±SEM (n=9–22)

	Male (cont)	Male (res)	Female (cont)	Female (res)
SOD activity, U/ml	18.7±1	17.8±1	20.6±1	20.9±1
Copper, μg/dl	182±11	214±30	251±17	295±18
Zinc, μg/dl	207±25	210±21	207±12	221±27

have a direct effect on endothelial and vascular estrogen receptors [10]. Prolonged resveratrol administration did not importantly influence gender-related differences established for the agonists in divergence with our above study in which resveratrol has differentially modified the response to estrogen between males and females. Our data from healthy rats now provide some evidence for improving effect of resveratrol on the endothelial function through activation of the nitric oxide because resveratrol treatment increased blood nitrite/nitrate levels in both genders. Moreover, we have very recently determined increased nitrite/nitrate levels and basal nitric oxide production in aortae from rats applied with the same treatment protocol of resveratrol [10].

A first study with prolonged resveratrol treatment (5 mg/kg/day for 3 weeks) found significantly increased endothelium-dependent vascular relaxation to acetylcholine in ovariectomized stroke-prone spontaneously hypertensive rats [12]. Additionally, in hypercholesterolemic rabbits, the administration of resveratrol (3 mg/kg/day for 12 weeks) improved flow-mediated vasodilation and increased plasma nitrite level [11]. Chronic treatment with high dose of resveratrol (204 mg/kg/day for 10 months) prevented both age-related and obesity-related declines in endothelial function in response to acetylcholine in mice [14]. A recent study also demonstrated that resveratrol increases endothelial relaxation to acetylcholine in the absence of significant changes in aorta eNOS protein levels in hypertensive rats, but not in healthy animals [13]. In that study, to mimic moderate red wine consumption, resveratrol was employed at lower doses (0.44–4.48 mg/l, for 4 weeks). Contrary to the above study, but in accord with our results, resveratrol treatment (20 mg/kg/day for 8 weeks) was shown to improve the relaxations to acetylcholine in female rats fed a standard or high fat diet [28]. Collectively, these data suggested that high doses of resveratrol may have been required to detect its effect on endothelial function and nitric oxide under physiological conditions. The doses of resveratrol used in the current and above studies are not readily achievable during the moderate consumption of red wine. However, in our recent study with the same protocol, the recovery of resveratrol was detectable only in the liver, at levels in the range of 40–50 ng/g, but not in plasma and aorta indicating its rapid metabolism [10], which is in accord with previous studies [29]. Finally, it is also important to note that harmful doses of resveratrol are reported to be as high as 1,500 mg/kg in mice [14].

Angiotensin II increases NAD(P)H oxidase activity, which is a major source of superoxide in endothelium and vascular smooth muscle cells, and thus causes an enhancement in oxidative stress [30]. Exogenous addition of NADH and NADPH as a substrate are commonly used to stimulate NAD(P)H oxidase activity in vascular preparations [31, 32]. We showed that angiotensin II, alone or in combination with NAD(P)H, stimulated superoxide production in aortic segments, with minor differences between males and females. This appears to be related to the activation of NAD(P)H oxidase because SOD was significantly reduced, whereas DPI, a selective inhibitor of NAD(P)H oxidase, almost completely abolished the superoxide production. Interestingly, angiotensin II–induced stimulation was pronounced in endothelium-intact aorta, whereas its combination with NAD(P)H was more effective in endothelium-denuded aorta, indicating potency differences in the action side. The decreased capacity of superoxide production at basal and upon stimulation with angiotensin II and NAD(P)H suggests that resveratrol may suppress its generation by inhibiting NAD(P)H oxidase in accordance with previous studies [4, 9, 10, 20]. Showing similarity in the suppressive effects of resveratrol in either endothelium-intact or denuded aortae, and between males and females, revealed that its effect was endothelium and gender-independent upon stimulation with angiotensin II. The inhibitory effect of resveratrol on oxidative stress may contribute to its enhancer effect on endothelial reactivity. With the presented data, we cannot conclude that the suppressive effect of resveratrol treatment on the superoxide production does not contribute to the aortic tone, although it seems unlikely, since this effect of resveratrol in endothelium-denuded aortic rings was not associated with a change of vascular reactivity to the contractile agonists.

In aortic segments, preincubation with resveratrol at micromolar concentrations for 24 h was found to upregulate the expression of enzymes involved in the scavenging of reactive oxygen species such as glutathione peroxidase and catalase, but not Cu, Zn-SOD and Mn-SOD [33]. Our results showed that plasma total SOD activity, as well as copper and zinc levels, were not changed after resveratrol treatment indicating lack of interaction with SOD enzyme, which is one of the extracellular antioxidative defense markers, in physiological states without provocation of oxidative stress. Free oxygen radical scavenging with simple antioxidants as a therapeutic approachment in cardiovascular diseases was not considered as a successful strategy in clinical trials, partially due to their failure in restoring endothelial nitric oxide production [34]. Therefore, a compound like resveratrol, which directly stimulates nitric oxide production, has the potential to be more useful for therapeutic intervention. Moreover, estrogen substitution therapy for cardiovascular diseases is not encouraging due the possible induction of cancer, whereas resveratrol has not only estrogen mimetic cardiovascular properties, but also exerts anticancer and antiaging effects [35].

Resveratrol supplementation, gender independently, improved endothelial relaxations to acetylcholine and suppressed contractions to angiotensin II and phenylephrine, in association with increased nitrite/nitrate levels under physiological

conditions. It also produced a refractoriness to superoxide provocation. Our results suggest that capacity of endothelial function and suppression of oxidative stress could be improved by a diet containing resveratrol. The presented beneficial effects of resveratrol may have important implications in the prevention of cardiovascular disease.

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Conflict of interest The authors state no conflict of interest.

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