Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 20 (2009) 443–452
Journal of Nutritional Biochemistry

Resveratrol, a unique phytoalexin present in red wine, delivers either survival signal or death signal to the ischemic myocardium depending on dose

Jocelyn Dudley, Samarjit Das, Subhendu Mukherjee, Dipak K. Das*
Cardiovascular Research Center, University of Connecticut School of Medicine, Farmington, 06030-1, USA
Received 19 September 2007; received in revised form 20 March 2008; accepted May 2008

Abstract

Recent studies have demonstrated the cardioprotective abilities of resveratrol, a polyphenolic antioxidant present in red wine. Resveratrol can also kill cancer cells at relatively higher doses by exerting a death signal. We reasoned that resveratrol might possess the ability to protect the cells at lower doses as observed during pharmacological preconditioning of the heart, while at higher doses cause cell death as found for cancer cells. To test this hypothesis, rats were randomly fed for 14 days by gavaging any of the four doses of resveratrol — 2.5, 5.0, 25 or 50 mg/kg — while vehicle-fed animals served as placebo control. After 14 days, isolated working hearts were prepared from both experimental and control animals, and the hearts were subjected to 30-min global ischemia followed by 2 of reperfusion. The rats fed either 2.5 or 5 mg/kg dose of resveratrol for 14 days provided cardioprotection as evidenced by improved post-ischemic ventricular recovery and reduction of myocardial infarct size and cardiomyocyte apoptosis compared to control. In contrast, the hearts fed either 25 or 50 mg/kg dose of resveratrol depressed cardiac function and increased myocardial infarct size and number of apoptotic cells. The results for Western blots and RT-PCR demonstrated an increase of protein and RNA transcripts of redox proteins including thioredoxin (Trx)-1, Trx-2, glutaredoxin (Grx)-1, Grx-2, redox factor Ref-1 as well as redox-sensitive transcription factor NF-κB and survival factors such as phosphorylated-Akt (p-Akt), and Bcl-2 in the animals fed lower doses (2.5 and 5 mg/kg) of resveratrol, while the reverse was true for the animals fed higher doses (25 and 50 mg/kg) of resveratrol. The results thus indicate that at low doses (2.5 or 5 mg/kg), resveratrol exerts survival signal by up-regulating anti-apoptotic and redox proteins Akt and Bcl-2, while at high doses (>25 mg/kg), it potentiates a death signal by down-regulating redox proteins and up-regulating pro-apoptotic proteins.

Keywords: Resveratrol; Death signal; Survival signal; Heart; Redox; Thioredoxin; Glutaredoxin

1. Introduction

Eating a well-balanced diet that includes fruits and vegetables is the key component for maintaining a healthy heart. Certain foods are known for their cardioprotective properties such as red wine, grape skins, peanuts and blueberries [1]. All of them have one thing in common, i.e., the presence of resveratrol (Table 1). Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a naturally occurring phytoalexin that does provide cardioprotection through the generation of a survival signal [22–27]. It provides cardioprotection in red wine by multiple routes such as through antioxidant action [23,26–28], inhibiting low-density lipoprotein [29], activating NO production [25–27,30], hindering platelet aggregation [32] and promoting anti-inflammatory effects [24,33]. Resveratrol protects the heart at relatively low concentration, at about 2.5 to 10 mg/kg doses [34].

In contrast, resveratrol destroys cancer cells at relatively higher doses [34–37]. For example, it causes death to cancer cells by apoptosis at 100–1000 mg/kg doses. [34–39]. Whether such high does of resveratrol are also destructive to normal cells is not known. Several existing studies indicate that high doses of resveratrol could produce adverse effects to the biological system. For example, high doses of *trans*-resveratrol given for 60 days to hypercholesterolemic rabbits increased atherosclerotic lesions while lower doses of resveratrol were protective [40]. We hypothesized that, at lower doses, resveratrol functions as an antioxidant, while at higher doses it may function as a pro-oxidant. The present

* Corresponding author. Tel.: +1 860 679 3687; fax: +1 860 679 4606.
E-mail address: ddas@neuron.uchc.edu (D.K. Das).

0955-2863/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.05.003

Table 1
The amount of resveratrol found in natural foods

Source	Resveratrol concentration	Reference
100% Natural peanut butter	~0.65 μg/g	[2,3]
Bilberries	~16 ng/g	[4]
Blueberries	~32 ng/g	[4]
Boiled peanuts	~5.1 μg/g	[5,6]
Cranberry raw juice	~0.2 mg/L	[7]
Dry grape skin	~24.06 μg/g	[8]
Grapes	0.16–3.54 μg/g	[6,9–11]
Peanut butter	0.3–1.4 μg/g	[3,5,6]
Peanuts	0.02–1.92 μg/g	[12,13]
Pistachios	0.09–1.67 μg/g	[13]
Ports and sherries	<0.1 mg/L	[14]
Ref grape juice	~0.50 mg/L	[14,15]
Red wines	0.1–14.3 mg/L	[6,15–21]
Roasted peanuts	~0.055 μg/g	[5]
White grape juice	~0.05 mg/L	[15]
White wines	<0.1–2.1 mg/L	[16–21]

study was designed to compare the effects of resveratrol on cardioprotection at low doses with high doses (based on the results of previous studies) simultaneously comparing the redox cycling of resveratrol by examining its effects on several redox-sensitive transcription factors and proteins.

2. Materials and methods

2.1. Animals

All animals in this study received humane care in agreement with "The Principles of Laboratory Animal Care" established by the National Society for Medical Research and *The Guide for the Care and Use of Laboratory Animals* devised by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 85-23, revised 1985). Male Sprague–Dawley rats between 250 and 300 g were fed ad libitum regular rat chow (purchased from Harlan TEKLAD Company, Madison, WI, USA) that contains 18% crude protein, 5% crude fat and 5% crude fiber. Ingredients are as follows: ground wheat, ground corn, dehulled soybean meal, soybean oil, calcium carbonate, dicalcium phosphate, iodized salt, brewers dried yeast, choline chloride, niacin, vitamin A, biotin, thiamine mononitrate, vitamin D_3 supplement, vitamin E supplement, vitamin B12 supplement, riboflavin, magnesium oxide, folic acid, copper sulfate, calcium lodate, cobalt carbonate, etc., with free access to water until the start of the experiment. The rats were assigned to five groups with seven rats per group and gavaged for 14 days under the following conditions: the control group (gavaged with 0.25 ml of 21% ethanol in H_2O), the low-dose group [gavaged with 2.5 and 5.0 mg/kg doses of resveratrol (Sigma-Aldrich, St. Louis, MO, USA) in 0.25 ml of 21% ethanol in H_2O] and the high-dose group (gavaged with 25 and 50.0 mg/kg doses of resveratrol in 0.25 ml of 21% ethanol in H_2O). The different oral treatment of resveratrol after 14 days had no effect on body weight, food or water consumption.

2.2. Isolated working rat heart preparation

Twenty-four hours after the last dose of resveratrol administered by gavaging, the rats were anesthetized with sodium pentobarbital (80 mg/kg body weight ip injection) (Abbott Laboratories, North Chicago, IL, USA) and anticoagulated with heparin sodium (500 IU/kg body weight ip injection) (Elkin-Sinn, Inc., Cherry Hill, NJ, USA). After an adequate period of anesthesia, thoracotomy was conducted, and the hearts were perfused with KHB buffer (118 mM sodium chloride, 4.7 mM potassium chloride, 1.7 mM calcium chloride, 25 mM sodium bicarbonate, 0.36 mM potassium biphosphate, 1.2 mM magnesium sulfate and 10 mM glucose) in retrograde Langendorff mode at 37°C at a constant perfusion pressure of 100 cm of water (10 kPa) in the aorta for a 15-min washout and equilibration period [30]. After the Langendorff mode, the heart was switched to the working mode (antegrade perfusion) at a constant perfusion pressure of 17 cm of water (1.7 kPa) in the left atrium for 5 min. The baseline functional parameters were collected after steady-state cardiac function was established. For the control group, the hearts were perfused continuously in antegrade mode and cardiac function measurements for heart rate, coronary flow, aortic flow, left ventricular end-diastolic pressure, left ventricular developed pressure and its first derivative were done at 0 (baseline), 10, 30, 60, 90 and 120 min during the reperfusion. After the stabilization period in the antegrade mode, the ischemia reperfusion effects consisted of closing the antegrade perfusion line and subjecting the heart to 30 min of ischemia. The heart was then switched to the retrograde mode for 5 min to avoid the development of high incidence ventricular fibrillation and switched to the working heart antegrade mode where the cardiac function parameters were measured as described previously [30]. All hearts were subjected to 30 min of global ischemia followed by 2 h of reperfusion. Any heart that showed any cardiac disturbance (ventricle arrhythmia and fibrillation) during the entire experiment was excluded from this study.

2.3. Measurements of cardiac function

Cardiac function was measured at baseline and during reperfusion. The aortic flow was measured with a flowmeter (Gilmont Instrument, Inc., Barrington, IL, USA), and the coronary flow was measured by timed collection of the coronary effluent dripping from the heart [30]. A Gould p23XL transducer (Gould Instruments System, Inc., Valley View, OH, USA) was used to measure the heart rate, left ventricular developed pressure (LVDP) (the variation between the maximum systolic and diastolic pressure), the left ventricular end-diastolic pressure and the first derivative of developed pressure (dP/dT). A Gould 6600 series signal conditioner (Gould Instruments System) was used to amplify the signal, and a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA, USA) was used to monitor the signal. The pressure transducer was

attached to the aortic cannula side arm. All data collection was in working mode with the buffer being perfused through the pulmonary vein to the left atrium and from there to the left ventricle and then to the aortic valve into the aorta leading to the system.

2.4. Measurements of the infarct size

At the end of each experiment, the heart was sliced into 1 mm thickness of cross-sectional pieces and placed into a 2% solution of triphenyl tetrazolium (TTC) in phosphate buffer for 10 min at 37°C, then fixed with 2% paraformaldehyde. Each slice was scanned by a computer-assisted scanner (HP Scanjet 5370C). The NIH Image 5.1 software (a public domain software package) was used to quantify the infarct size in pixels. The living myocardium tissue was stained a dark deep red, and the infarcted tissue was left unstained pale tan color.

2.5. Cardiomyocyte apoptosis

Immunohistochemical detection of apoptotic cells was carried out using TUNEL [30]. The sections were incubated again with mouse monoclonal antibody recognizing cardiac myosin heavy chain to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser microscope. The number of apoptotic cells was counted and expressed as a percentage total myocyte population.

2.6. Western blot analysis

One hundred milligrams of the heart's left ventricle was homogenized in a buffer consisting of 25 mM Tris, 25 mM NaCl, 1 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA and 1 mM phenylmethylsulphonyl fluoride. Fifty micrograms of protein for each heart sample was boiled in loading buffer for 10 min, loaded and separated by 12% SDS-polyacrylamide gel electrophoresis in running buffer [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3] at 180 V. Broad range molecular weight standards (5 μl) (Bio-Rad Laboratories, California, USA) were used. The gel was transferred onto a nitrocellulose membrane (Bio-Rad Laboratories) for 1 h at 100 V in transfer buffer [25 mM Tris base, 19.2 mM glycine, 20% (v/v) methanol, pH 8.3]. The membrane was blocked for 1 h in Tris-buffered saline (TBS-T) [50 mM Tris, pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween-20] and 5% (w/v) nonfat dry milk overnight at 4°C with the primary antibody. All antibodies were acquired from Cell Signaling Technology and were used according to manufacturer's suggested dilutions. The membrane was washed three times with TBS-T, followed by a 1-h incubation of horseradish peroxide-conjugated secondary antibody in 5% (w/v) nonfat dry milk and TBS-T. The Western blots were developed using ECL Detection Reagents A and B (Santa Cruz Biotechnology, California, USA) and exposed on Kodak XOMAT film.

2.7. Total RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was extracted from left ventricular tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and dissolved in 50 μl of DEPC-treated water. Reverse transcription–polymerase chain reaction (RT-PCR) was performed with RETROscript (Ambion, Austin, TX, USA), according to the manufacturer's instructions. The following primers were used in our study.

Thioredoxin-1:
Forward — 5′-GCCAAAA GGTGAAGC GA-3′
Reverse — 5′-CTGG AGTC CCACG CT-3′
Thioredoxin-2:
Forward — 5′- CATCC GAGCAC TCCTAC-3′
Reverse — 5-′C GC CATGT GTGTGTG-3′
Glutaredoxin-1:
Forward — 5′-ACGT CT TCCTGGAATTTG-3′
Reverse — 5′-GCAGAC TCCAATCTGCTTC-3′
Glutaredoxin-2:
Forward — 5′-T GCCAAGAAGATTT TCCAT-3′
Reverse — 5′-AGGCAGCAATTTCCCTTCTT-3′
GAPDH:
Forward — 5′-AGACAGCCGCATCTTCTTGT-3′
Reverse — 5′-CTTGCCGTGGGTAGAGTCAT-3′

The PCR products were visualized on a UV-transillumination and digitized after electrophoresis on 2% agarose gel containing ethidium bromide.

2.8. Statistical analysis

The values for myocardial functional parameters, total and infarct volumes and infarct sizes, and cardiomyocyte apoptosis are all expressed as the mean±S.E.M. Analysis of variance test followed by Bonferroni's correction was first carried out to test for any differences between the mean values of all groups. If differences between groups were established, the values of the treated groups were compared with those of the control group by a modified *t*-test. The results were considered significant if $P<05$.

3. Results

3.1. The effects of high and low doses of resveratrol on ventricular recovery

The baseline values of left ventricular function did not vary appreciably between the groups. During the post-ischemic reperfusion, all the parameters were depressed appreciably as expected except for the coronary flow, which did not change significantly. At 2.5 and 5 mg/kg doses, aortic flow, LVDP and LVmaxdp/dt were higher during the reperfusion period. As shown in Fig. 1, at 30 and 120 min after reperfusion, all the functional parameters, except coronary flow, were higher for resveratrol doses of 2.5 and 5 mg/kg compared to control. In contrast, these functional

parameters were reduced significantly compared to control for resveratrol doses of 25 and 50 mg/kg.

3.2. The effects of high and low doses of resveratrol on myocardial infarct size

As shown in Fig. 2, infarct size expressed as the percent area of risk was lower compared to control at resveratrol doses of 2.5 and 5 mg/kg. Resveratrol at a dose of 25 mg/kg did not have any significant effect on the infarct size, while at 50 mg/kg it increased the amount of infarct size significantly.

3.3. The effects of high and low doses of resveratrol on cardiomyocyte apoptosis

Similar to the results of myocardial infarct size, resveratrol at 2.5 and 5 mg/kg doses reduced the number of apoptotic cardiomyocytes significantly compared to control (Fig. 3). In contrast, there was a significant increase in cardiomyocyte apoptosis with 50 mg/kg dose of resveratrol, while the number of apoptotic cells remained unaltered with 25 mg/kg dose of resveratrol.

3.4. The effects of high and low dose of resveratrol on the RNA transcripts of redox proteins

Since our previous studies demonstrated enhancement of cardioprotective redox proteins like Trx and Grx with resveratrol, and since Trx/Grx are directly related to cardioprotection, we determined the effects of low and high doses of resveratrol on the RNA transcripts of Trx and Grx. As shown in Fig. 4, RT-PCR results showed reduced transcripts of Trx-1, Trx-2, Grx-1 and Grx-2 in the hearts

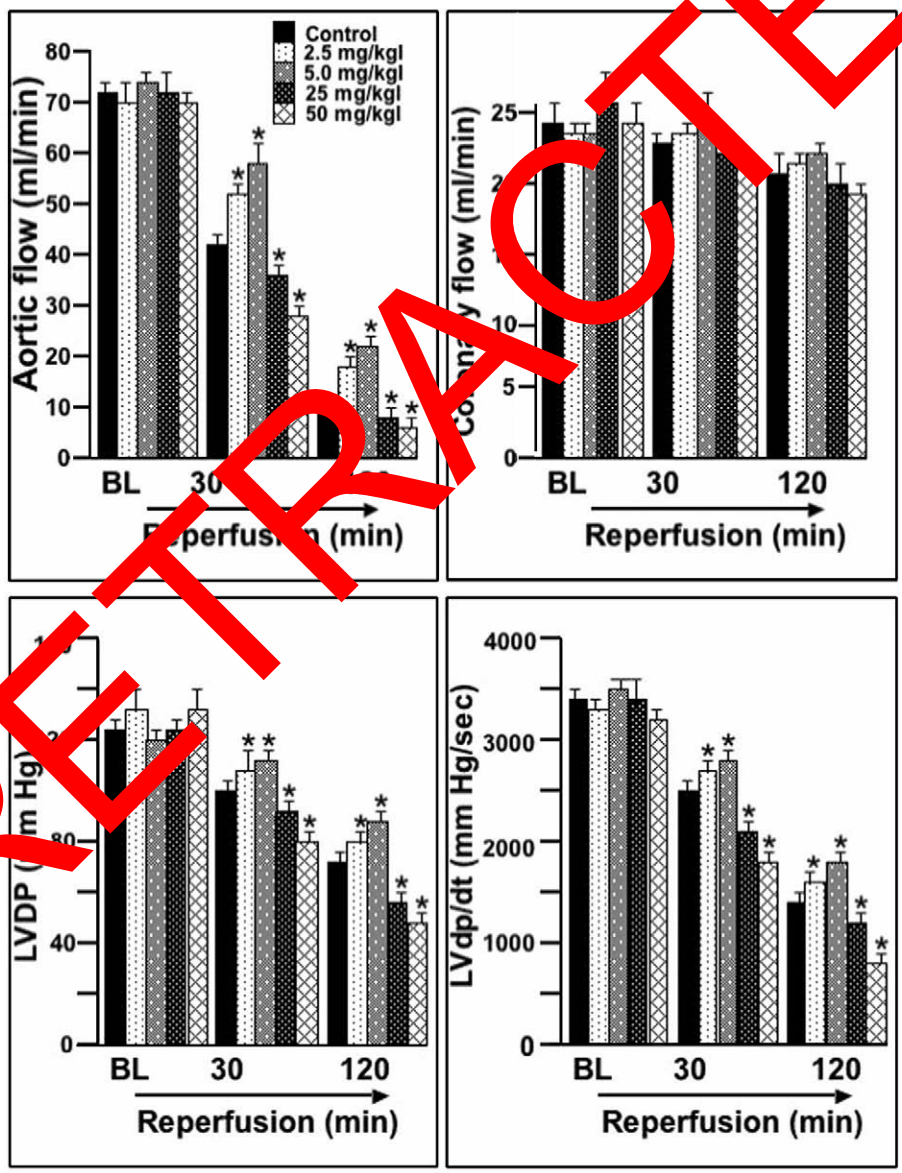

Fig. 1. Effects of high and low doses of resveratrol on the aortic flow, coronary flow, LVDP and maximum first derivative of LVDP (LVdp/dt). Results are expressed as means±S.E.M. of six animals per group. *P<.05 vs. control.

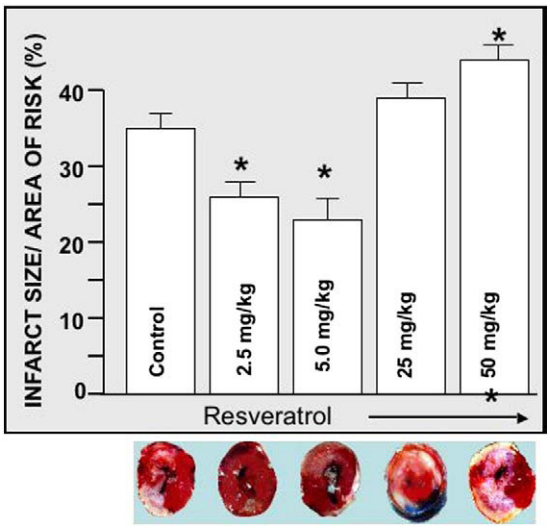

Fig. 2. Effects of high and low doses of resveratrol on the myocardial infarct size determined by TTC staining. Representative infarcts are shown at the bottom. Results are expressed as means±S.E.M. of six animals per group. *P<.05 vs. control.

subjected to ischemia/reperfusion. At low doses of 2.5 and 5 mg/kg of resveratrol, there was an increase in Trx/Grx transcripts, while at higher doses of 25 and 50 mg/kg, resveratrol reduced the transcript of these redox proteins.

3.5. The effects of high and low doses of resveratrol on the expression of redox-sensitive transcription factor NFκB

Since proteasomal degradation of phosphorylated IκB is the hallmark of NFκB activation, we examined the effect of different doses of resveratrol for the relative abundance of

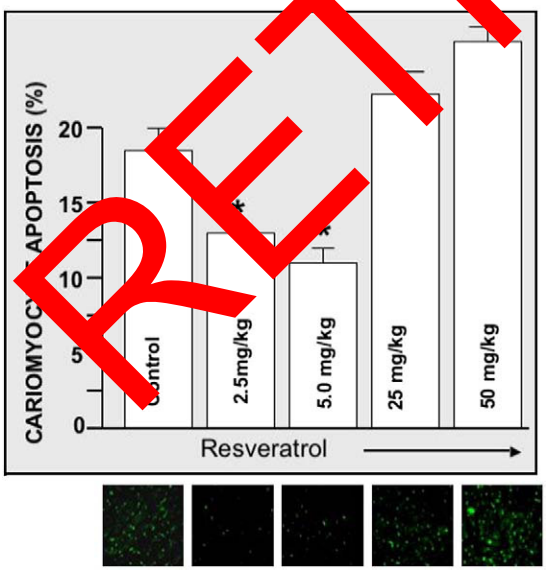

Fig. 3. Effects of high and low doses of resveratrol on the cardiomyocyte apoptosis determined by TUNEL staining. Representative apoptotic cells are shown at the bottom. Results are expressed as means±S.E.M. of six animals per group. *P<.05 vs. control.

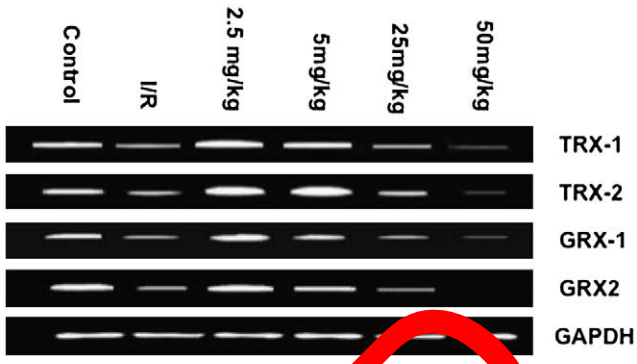

Fig. 4. Effects of low and high doses of resveratrol on the RNA transcripts performed by RT-PCR. Results shown are representative of three experiments per group.

IκB and phosphorylated IκB. As shown in Fig. 5, after ischemia/reperfusion, there was no detectable change in IkB, but p-IκB level was reduced. Again, none of the doses of resveratrol caused any change in IkB, but at lower doses of 2.5 and 5 mg/kg resveratrol increased the abundance of phospho IκB, while at higher doses of 25 and 50 mg/kg, it resulted in a reduction of the amount of phospho IκB.

3.6. The effects of high and low doses of resveratrol on the expression of redox factor Ref-1

We examined the effects of low and high doses of resveratrol on Ref-1, since we recently observed an interaction of Ref-1 with NFκB and Trx-1 [41]. Induction of Ref-1 was significantly reduced in the hearts subjected to I/R compared to control (Fig. 6). Significant increase of Ref-1 proteins was observed in the hearts treated with both 2.5 and 5 mg/kg doses of resveratrol. The expression of Ref-1 proteins was significantly lowered in the hearts treated with resveratrol at higher doses.

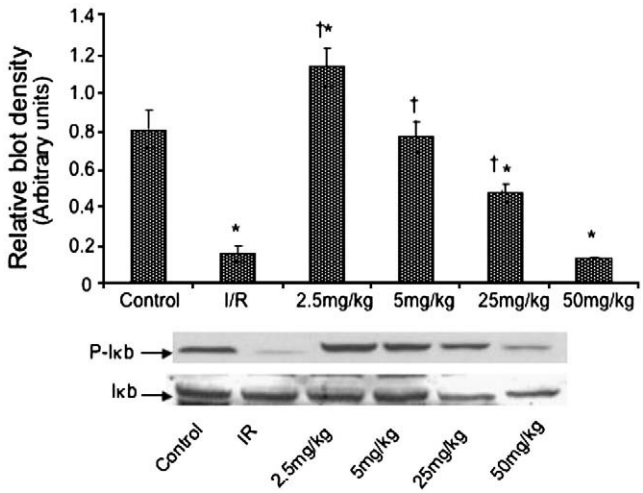

Fig. 5. Effects of low and high doses of resveratrol on the Western blot analysis of the expression of IkB and phospho-IkB. Results shown are representative of three experiments per group. *P<.05 vs. control; †P<.05 vs. I/R.

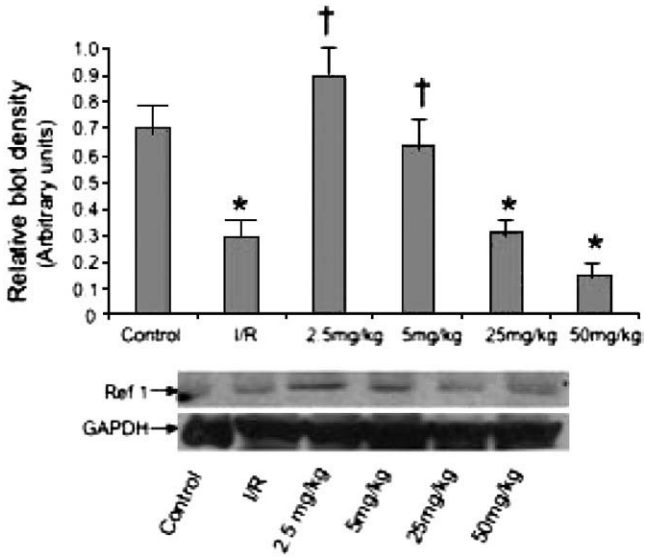

Fig. 6. Effects of low and high doses of resveratrol on the Western blot analysis of the expression of Ref-1. Results shown are representative of three experiments per group. *P<05 vs. control; †P<05 vs. I/R.

3.7. The effects of high and low doses of resveratrol on the generation of survival signal

We examined the expression of several members of survival proteins in the hearts treated with both high and low doses of resveratrol. Fig. 7 shows the results. Consistent with our previous observation, the phosphorylation of Akt was reduced significantly after ischemia and reperfusion. The expression of Bcl-2 protein followed a similar pattern. Low doses (2.5 and 5 mg/kg) of resveratrol enhanced the induction of Akt phosphorylation and expression of Bcl-2 protein, while at higher doses (25 and 50 mg/kg) both Akt phosphorylation and Bcl-2 protein expression were reduced.

4. Discussion

The most significant finding of the present study is that at lower doses (2.5 and 5 mg/kg per day, resveratrol protects the ischemic heart by inducing a survival signal. Cardioprotection was confirmed from the improved post-ischemic ventricular function and reduced myocardial infarct size and apoptotic cardiomyocytes by resveratrol. Cardioprotection was further supported by the generation of survival signal as evidenced by increased phosphorylation of Akt and activation of Bcl-2. In contrast, there was increased activation of Ref-1 and the transcription factor IκB. At a higher dose of 25 mg/kg per day, resveratrol failed to achieve any cardioprotective effects, and at 50 mg/kg per day, resveratrol deteriorated the recovery of cardiac function and increased myocardial infarct size and apoptotic cardiomyocytes. At both doses of 25 and 50 mg/kg per day, resveratrol induced a death signal as indicated by the reduced phosphorylation of Akt and IκB, and depression of Ref-1 and Bcl-2.

The findings that, at a low dose of 2.5 mg/kg per day, resveratrol renders the heart resistant to ischemic reperfusion injury by generating a survival signal support previous reports [34] and further demonstrate that even at 5 mg/kg per day, the cardioprotection persists. In this study [34], 2.5 mg/kg per day of resveratrol provided significant cardioprotection as evidenced by superior post- ischemic ventricular recovery, reduced myocardial infarct size and decreased number of apoptotic cardiomyocytes. SnPP abolished the cardioprotective effect of resveratrol, suggesting a direct role of HO-1 in cardioprotection. Resveratrol generated a survival signal by inducing the activation of p38 MAP kinase β and Akt, and inhibition of p38 MAP kinase α, which were completely reversed by treating the hearts with SnPP. Resveratrol also increased the DNA binding of NFκB, but SnPP had no effect on resveratrol-mediated activation of NFκB. Consistent with these results, the present study also demonstrated increased DNA binding of NFκB and increased Akt phosphorylation with low doses of resveratrol.

Striking similarities of the cardioprotective properties between resveratrol and NO prompted researchers to determine the role of NO in resveratrol-mediated cardioprotection. A direct role of NO was shown from a study which found resveratrol-mediated increase in NOS activity in cultured pulmonary artery endothelial cells, suggesting that resveratrol could afford cardioprotection by affecting the expression of NOS [42]. This result was further supported by a finding that resveratrol protected isolated working rat hearts through the up-regulation of iNOS [25,30]. Resveratrol failed to provide cardioprotection in iNOS knockout mice devoid of any copy of iNOS gene, further supporting the role of NO [26]. In a more recent study, resveratrol

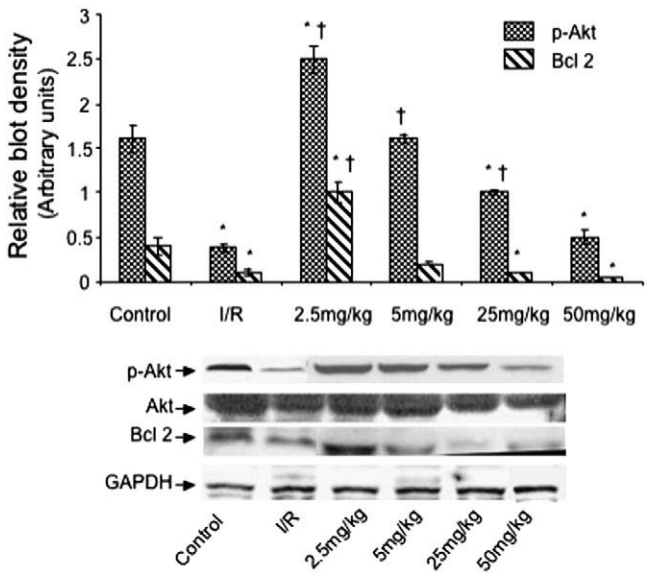

Fig. 7. Effects of low and high doses of resveratrol on the Western blot analysis of the expression of Akt and phospho-Akt and Bcl2 proteins. Results shown are representative of three experiments per group. *P<05 vs. control; †P<05 vs. I/R.

reduced myocardial ischemia/reperfusion injury through both an iNOS-dependent and an iNOS-independent manner [23]. Similar to NO, resveratrol significantly reduced the amount of proadhesive molecules including sICAM-1, sVCAM-1 and E-selectin in the ischemic reperfused myocardium [33]. All these results demonstrating resveratrol-mediated cardioprotection were achieved at low resveratrol dose.

In the present study, we sought to compare the effects of low and high doses of resveratrol on redox cycling of several redox proteins, redox-sensitive transcription factors and death/survival proteins. Since previous studies indicated modification of several redox-regulated proteins by resveratrol, we examined the effects of resveratrol on thioredoxins (Trx) and glutaredoxins (Grx). Both Trx and Grx can exist in the nucleus and cytosol (Trx1 and Grx1) as well as in the mitochondria (Trx-2 and Grx-2). Because Trx proteins exist in extremely low quantities which allows easy detection by Western blot analysis, we performed RT-PCR to detect the RNA transcripts of Trx and Grx proteins. Several recent studies from our laboratory have implicated the cardioprotective role of thioredoxins [41,43,44]. The cellular changes associated with ischemic heart diseases are redox regulated. Ischemia and reperfusion render the heart in the oxidized environment maintained by the stabilizing disulfides present in the extracellular surface, while the intracellular environment is maintained in the reduced state with the help of free sulfhydryl groups. The principal disulfide reductase responsible for maintaining the inside of the cell in the reduced state is thioredoxin [44]. Thioredoxins and other members of the thioredoxin superfamily such as glutaredoxins and peroxiredoxins are ubiquitously present in mammalian cells including the heart [44] and play an important role in maintaining the redox environment of the cell. Glutaredoxins (Grx), also known as thiol transferase, is also a ubiquitous protein found in most of the organs including the heart. Like Trx, two Grx genes have been found in mammals. Grx1 is initially considered to be a cytosolic enzyme. However, recent results of immunolabeling experiments have shown that it is also present in the nucleus [45]. The second Grx (*Grx2*) gene, which was reported recently, encodes two proteins as a result of alternative RNA splicing. One of the Grx2 isoforms is located in the mitochondria and the other in the nucleus [46]. Similar to Trx, Grx also functions in catalyzing thiol/disulfide exchange [47]. Our recent studies demonstrated that transgenic mice overexpressing Grx1 reduced the number of apoptotic cardiomyocytes in the ischemic reperfused heart [44]. The results of the present study indicated down-regulation of the transcripts of Grx1 and Grx2 as well as of Trx1 and Trx2 in the ischemic reperfused hearts and with high doses of resveratrol. Lower doses of resveratrol increased the transcripts of Trx1 and Trx2 as well as of Grx1 and Grx2 compared to control.

Since our recent studies showed potentiation of a survival signal through the redox activation of Ref-1 following myocardial ischemia reperfusion injury [41] and since Ref-1 is known to be modulated by Trx, we examined the effects of low and high doses of resveratrol on Ref-1 expression. Ref-1, a redox effector factor-1/apurinic/apyrimidinic endonuclease, is a bifunctional protein that is ubiquitously present in the mammalian system including the heart. While it is a major protein of the DNA base excision repair pathway [48], it also serves as a transcriptional coactivator by stimulating the DNA binding activity of the transcription factors [49]. A recent study showed that ischemia/reperfusion could potentiate a rapid translocation of TRx-1 into the nucleus, which then interacts with Ref-1, leading to the generation of a survival signal [49]. Consistent with these previous reports, low doses of resveratrol generated a survival signal by activating both Trx and Ref-1, while high doses of resveratrol produced in a death signal by reducing both Ref-1 and Trx expression.

Generation of survival signal was demonstrated by confirming the ability of low doses of resveratrol to induce Akt phosphorylation and Bcl-2 expression as well as to increase DNA binding of NFκB. High doses of resveratrol completely reversed the survival signal into a death signal by reducing Bcl-2 expression, Akt phosphorylation and NFkB activation. Akt is a critical regulator of PI-3-kinase–mediated cell survival, and constitutive activation of Akt is sufficient to block cell death by a variety of apoptotic stimuli [50]. Once activated, Akt can phosphorylate and inactivate proapoptotic proteins such as Bad and pro-caspase 9, and activate anti-apoptotic redox-sensitive transcription factor NFκB, a finding consistent with our results that indicated increase in Bcl-2 and activation of NFκB as evidenced by increased phosphorylation of IκB by low doses of resveratrol.

To the best of our knowledge, there has been no report showing the cardiovascular effects at higher resveratrol doses nor was there any report available on the comparison of low and high doses of resveratrol on cardioprotection. However, indirect results exist to show detrimental cellular effects of resveratrol at relatively higher doses. For example, a study by Bråkenhielm et al. [51] showed that resveratrol not only hindered tumor growth but also inhibited wound healing, endothelial cell growth by fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor, and angiogenesis in healthy tissue cells in a dose-dependent manner. Resveratrol inhibited FGF-2-induced phosphorylation of MAPKp44 and MAPKp42 in bovine capillary endothelial cells at a relatively high dose. A study conducted by Dubash et al. [52] showed that resveratrol prevented the incorporation of [^{3}H]thymidine into DNA and [^{3}H]uridine into RNA with increasing resveratrol concentration. This study indicates that at high dose, resveratrol inhibits synthesis of RNA, DNA and protein, and blocks cell proliferation, leading to cell death. Another related study conducted by Matsuoka et al. [53] found that resveratrol grossly affected Chinese hamster lung with increased concentration. At higher doses, resveratrol caused structural chromosome aberrations, chromatin breaks, chromatin exchanges, weak aneuploidy, higher S-phase arrest, lower

cell proliferation and apoptosis. A 28-day high-dose (300, 1000 and 3000 mg/kg) rat study conducted by Crowell et al [54] showed that the rats experienced signs of nephrotoxicity, dehydration, decreased activity, rough coat, diarrhea, soft stool and red material around the nose. The male rats had leukocytosis, and it is believed that both sexes may have had anemia. The rats also showed increased amounts of BUN, creatinine, alkaline phosphatase, alanine aminotransferase, total bilirubin, white cell counts and albumin, with reduced levels of hemoglobin, hematocrit and red cell counts. A study by Vieira et al. [55] in 2007 showed a dose-dependent manner of glutamate uptake on primary cortical astrocytes of the cerebral cortex from neonate Wistar rats. As the resveratrol concentration increased from 25 to 250 mM, there was a decrease in glutamate uptake and glutamate content, and an increase in glutamine synthetase activity. A study by Wilson et al. [40] on the effects of *trans*-resveratrol on hypercholesterolemic rabbits for 60 days at 0.6 to 1.0 g/kg found an increase in atherosclerotic lesions in the higher resveratrol dose. Resveratrol promotes death signal and apoptosis at higher concentrations (100–1000 mg/kg) in cancer cells [34–39,56].

The precise reason for generation of death signal at higher resveratrol doses is not clear. However, it is speculated that differential redox cycling of resveratrol between low and high dose might be responsible for survival vs. death signal generated by resveratrol. Resveratrol has two phenol groups. A study by Boyer et al. [57] showed that phenol reduces Fe^{3+} to Fe^{2+}. At higher concentration, resveratrol may produce a higher accumulation of reduced iron. Iron plays a crucial part in free radical reactions such as the Fenton reaction and peroxidation mechanisms leading to iron–oxygen complexes that remove hydrogen atoms from the polyunsaturated fatty acid membrane [58–60]. Miura et al. [61] discovered that resveratrol has both an antioxidant effect and a prooxidant effect. Resveratrol promoted the reduction of Fe^{3+} by increasing the formation of hydroxyl radicals through the Fenton reaction producing hydroxyl radicals and iron species [62]. At higher dose, resveratrol is likely to cause DNA strand breakage by the accumulation of reduced ADP-Fe^{3+} in the presence of hydrogen peroxide. Fukuhara and Miyata [63] found that resveratrol could bind to DNA. In the presence of Cu^{2+} under aerobic conditions and neutral pH, resveratrol promoted DNA plasmid cleavage. Resveratrol also reduces Cu^{2+} to Cu in the presence of the reactive oxygen species.

Inverse relationship between the consumption of red wine and incidence of cardiovascular disease has been popularly known as the "French paradox" [64]. The cardioprotective abilities of red wine have been attributed to resveratrol [23,64]. Resveratrol, a polyphenol phytoalexin (*trans*-3,5,4′-trihydroxystilbene) abundantly found in grape skins and in wines, possesses diverse biochemical and physiological actions, which include estrogenic, antiplatelet and anti-inflammatory properties [32,33]. *trans*-Resveratrol was originally identified as the active ingredient of an Oriental herb (*Kojo-kon*) used for the treatment of a wide variety of diseases including dermatitis, gonorrhea, fever, hyperlipidemia, atherosclerosis and inflammation. There are many foods as well as vitamins and minerals whose daily allowance requirement is safe and promotes good health when taken at a low dose. When the same substances are taken in large quantities, they become toxic and produce adverse effects to the cells at the subcellular levels. The recommended dosage for resveratrol in humans is 5–10 mg/day [65]. The most notable natural source of resveratrol is grapes, especially muscadine grapes which are used to make red wine, which remains the most important dietary source of resveratrol; a fluid ounce of red wine averages 0 µg of resveratrol. In a recent study, after 28 days of dietary supplementation with white or red wine (200 ml/day), urinary *trans*-resveratrol and *cis*-resveratrol-3-*O*-glucuronide concentrations increased by 211.5 nmol/g after white wine consumption and by 560.5 nmol/g after red wine consumption [66]. In an experimental study, levels of [^{3}H]resveratrol after 2 h, as % of that gavaged, were 03% in the heart and the brain and 0.02% in the spleen and the lung, whereas they were about 0.6% in the kidneys and 0.9% in the liver [67]. Thus, it appears that the amount of resveratrol after the consumption of moderate amount of wine is very low. In the present study, the blood concentrations of resveratrol were 0.1, 0.12, 1.1 and 5.7 µM after feeding resveratrol at doses of 2.5, 5, 25 and 50 mg/kg, respectively. These results are comparable with the data available in the literature on the blood concentrations of resveratrol achieved after moderate [one 5-oz (150 ml) serving] consumption of red wine (containing 14.3 mg/L of resveratrol that equates to 2.145 mg resveratrol/5 oz) [49,60–66]. In order to obtain a 2.5 mg/kg dose of resveratrol, one must consume at least 0.17 L of red wine, while to achieve higher concentrations of resveratrol, e.g., 5, 25 and 50 mg/kg, one needs to consume at least 0.35, 1.75 and 3.5 L of red wine, respectively. It is tempting to speculate that the cardioprotective ability at low doses of resveratrol is related to the current popular proposition about the health benefits of "moderate" wine drinking.

Acknowledgments

This study was supported in part by NIH HL 22559, HL 33889, HL 56803 and HL 56322. JD was supported by a NIH Minority grant.

References

[1] Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 2006;5:493–506.
[2] Ibern-Gomez M, Roig-Perez S, Lamuela-Raventos RM, de la Torre-Boronat MC. Resveratrol and piceid levels in natural and blended peanut butters. J Agric Food Chem 2000;48:6352–4.
[3] Lyons MM, Yu C, Toma RB, Cho SY, Reiboldt W, Lee J, et al. Resveratrol in raw and baked blueberries and bilberries. J Agric Food Chem 2003;51:5867–70.
[4] Sobolev VS, Cole RJ. *trans*-Resveratrol content in commercial peanuts and peanut products. J Agric Food Chem 1999;47:1435–9.

[5] Burns J, Yokota T, Ashihara H, Lean ME, Crozier A. Plant foods and herbal sources of resveratrol. J Agric Food Chem 2002;50:3337–40.
[6] Vitrac X, Bornet A, Vanderlinde R, Valls J, Richard T, Delaunay JC, et al. Determination of stilbenes (δ-viniferin, *trans*-astringin, *trans*-piceid, *cis*- and *trans*-resveratrol, e-viniferin) in Brazilian wines. J Agric Food Chem 2005;53:5664–9.
[7] Wang Y, Catana F, Yang Y, Roderick R, van Breemen RB. An LC-MS method for analyzing total resveratrol in grape juice, cranberry juice, and in wine. J Agric Food Chem 2002;50:431–5.
[8] Romero-Perez AI, Lamuela-Raventos RM, Andres-Lacueva C, de La Torre Boronat MC. Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. J Agric Food Chem 2001;49:210–5.
[9] Soleas GJ. A derivatized gas chromatographic mass spectrometric method for the analysis of both isomers of resveratrol in juice and wine. Am J Enol Vitic 1995;46:346–52.
[10] Creasy LL, Coffee M. Phytoalexin production potential of grape berries. J Am Soc Hortic Sci 1988;11:3230–4.
[11] Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR. Resveratrol, pterostilbene, and piceatannol in vaccinium berries. J Agric Food Chem 2004;52:4713–9.
[12] Sanders TH, McMichael RW, Hendrix KW. Occurrence of resveratrol in edible peanuts. J Agric Food Chem 2000;48:1243–6.
[13] Tokusoglu O, Unal MK, Yemis F. Determination of the phytoalexin resveratrol (3,5,4′-trihydroxystilbene) in peanuts and pistachios by high performance liquid chromatographic diode array (HPLC-DAD) and gas chromatography-mass spectrometry (GC MS). J Agric Food Chem 2005;53:5003–9.
[14] Romero-Perez AI, Ibern-Gomez M, Lamuela-Raventos RM, de La Torre Boronat MC. Piceid, the major resveratrol derivative in grape juices. J Agric Food Chem 1999;47:1533–6.
[15] Soleas GJ, Diamandis EP, Goldberg DM. Resveratrol: a mo... whose time has come? And gone? Clin Biochem 1997;30:91–11...
[16] Careri M, Corradini C, Elviri L, Nicoletti I, Zagnoni I. Liq... chromatography-electrospray tandem mass spectr...try of *cis*-resveratrol and *trans*-resveratrol: development, va...ation, and appli-cation of the method to red wine, grape, and wi...making b...roducts. J Agric Food Chem 2004;52:6868–74.
[17] Goldberg DM. A global survey of trans-sveri... co...ntrations in commercial wines. Am J Enol Vitic 19...;46:159–...
[18] Mark L, Nikfardjam MS, Avar P...macht R. A ...dated HPLC method for the quantitative an...sis ...*trans*-resveratr...nd *trans*-piceid in Hungarian wines. J Chromatogr ... 2005;43:445–9.
[19] Rattan SI. Aging, anti-ag..., and hormesis. ...ch Ageing Dev 2004; 125:285–9.
[20] Kiraly-Veghely Z...yihak E...lbert L, Nemeth ZI, Katay G. Identification and ...asurem... of resveratrol and formaldehyde in parts of white and ...ape berries. Acta Biol Hung 1998;49: 281–9.
[21] Ribeiro ...e Lima ...T. Deter...nati... of stilbenes (*trans*-astringin, *cis*- and *...ans*-piceid, and *cis*- and *...ns*-resveratrol) in Portuguese wines. J ...ic Food ... 2...;47:2666–70.
[22] Brada...t..., Piccinini F, Barenghi L, Bertelli AA, De Jonge R, Beemster ...et al. Does resveratrol induce pharmacological preconditioning? Int ...issue React 2000;22:1–4.
[23] Hung LM, Su ... Chen JK. Resveratrol protects myocardial ischemia reperfusion injury through both NO-dependent and NO-independent mechanisms. Free Radic Biol Med 2004;36:774–81.
[24] Sato M, Maulik G, Bagchi D, Das DK. Myocardial protection by protykin, a novel extract of *trans*-resveratrol and emodin. Free Radic Res 2000;32:135–44.
[25] Hattori R, Otani H, Maulik N, Das DK. Pharmacological preconditioning with resveratrol: role of nitric oxide. Am J Physiol 2002;282: H1988–95.
[26] Imamura G, Bertelli AA, Bertelli A, Otani H, Maulik N, Das DK. Pharmacological preconditioning with resveratrol: an insight with iNOS knockout mice. Am J Physiol 2002;282:H1996–2003.
[27] Das S, Cordis GA, Maulik N, Das DK. Pharmacological preconditioning with resveratrol: a role of CREB-dependent Bcl-2 signaling via adenosine A3 receptor activation. Am J Physiol 2005;288: H328–35.
[28] Ray PS, Maulik G, Cordis GA, Bertelli AA, Bertelli A, Das DK. The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury. Free Radic Biol Med 1999;27: 160–9.
[29] Chen CK, Pace-Asciak CR. Vasorelaxing activity of resveratrol and quercetin in isolated rat aorta. Gen Pharmacol 1996;27:363–6.
[30] Das S, Alagappan VK, Bagchi D, Sharma HS, Maulik N, Das DK. Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: a potential mechanism for ...era... preconditioning of the heart. Vascul Pharmacol 2005;42...1–9.
[31] Ma XI, Weyrich AS, Lefer DJ, ...er AM. Diminish... basal nitric oxide release after myocardial ische...a and reperfu...n promotes neutrophil adherence to c...nary endo...lium. Circ...es 1993;72: 403–12.
[32] Bertelli AAE, Giova...ni DE, C...na RL, L...ni W, Migliori M, Fregoni M, et al. An...platelet ...ivity of *cis*-resveratrol. Drugs Exp Clin Res 1996;...:61–3.
[33] Das S, Fal...M, Bertelli ... Maulik ..., Das DK. Attenuation of ischemia ...p...sion injury in ... ...he anti-inflammatory action of resver...ol. Arz...mittelforschung...2006;56:700–6.
[34] Das S, Fraga CG, ...as DK. Cardioprotective effect of resveratrol ...-1 expressio...volves p38 map kinase and PI-3-kinase signaling, but does not involve NFkappaB. Free Radic Res 2006; 40:1066–75.
[35] Fremont L. ...ological effects of resveratrol. Life Sci 2000;66: 663–73.
[36] ...hat KPL, ...zzuto JM. Resveratrol exhibits cytostatic and antiestrogenic properties with human endometrial adenocarcinoma (Ishikawa) cells. Cancer Res 2001;61:6137–44.
[37] ...g HH, Ren HL. New progression in the study of protective properties of resveratrol in anticardiovascular disease. Bratisl Lek Listy 2004;105:225–9.
[38] Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res 2004;24:2783–840.
[39] Lee KW, Lee HJ. The roles of polyphenols in cancer chemoprevention. Biofactors 2006;26:105–21.
[40] Wilson T, Knight TJ, Beitz DC, Lewis DS, Engen RL. Resveratrol promotes atherosclerosis in hypercholesterolemic rabbits. Life Sci 1996;59:15–21.
[41] Malik G, Gorbounov N, Das S, Gurusamy N, Otani H, Maulik N, et al. Ischemic preconditioning triggers nuclear translocation of thioredoxin and its interaction with Ref-1 potentiating a survival signal through the PI-3-kinase-Akt pathway. Antioxid Redox Signal 2006;8:2101–9.
[42] Hsieh TC, Juan G, Darzynkiewicz Z, Wu JM. Resveratrol increases nitric oxide synthase, induces accumulation of p53 and p21(WAF1/CIP1), and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G2. Cancer Res 1999;59:2596–601.
[43] Turoczi T, Chang VW, Engelman RM, Maulik N, Ho YS, Das DK. Thioredoxin redox signaling in the ischemic heart: an insight with transgenic mice overexpressing Trx1. J Mol Cell Cardiol 2003;35: 695–704.
[44] Das DK. Thioredoxin regulation of ischemic preconditioning. Antioxid Redox Signal 2004;6:405–12.
[45] Reimer KA. The slowing of ischemic energy demand in preconditioned myocardium. Ann NY Acad Sci 1996;793:13–26.
[46] Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 1998;17:2596–606.
[47] Shioji K, Kishimoto C, Nakamura H, Masutani H, Yuan Z, Oka S, et al. Overexpression of thioredoxin-1 in transgenic mice attenuates adriamycin-induced cardiotoxicity. Circulation 2002;106:1403–9.

[48] Demple B, Harrison L. Repair of oxidative damage to DNA: enzymology and biology. Annu Rev Biochem 1994;63:915–48.
[49] Edwards M, Rassin DK, Izumi T, Mitra S, Perez-Polo JR. APE/Ref-1 responses to oxidative stress in aged rats. J Neurosci Res 1998;54: 635–8.
[50] Das S, Tosaki A, Bagchi D, Maulik N, Das DK. Resveratrol-mediated activation of cAMP response element-binding protein through adenosine A3 receptor by Akt-dependent and -independent pathways. J Pharmacol Exp Ther 2005;314:762–9.
[51] Bråkenhielm E, Cao R, Cao Y. Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. FASEB J 2001;15:1798–800.
[52] Dubash BD, Zheng BL, Kim CH, He K, Shao Y, Zheng QY, et al. Inhibitory effect of resveratrol and related compounds on the macromolecular synthesis in HL-60 cells and the metabolism of 7,12-dimethylbenz[*a*]anthracene by mouse liver microsomes. In: Shahidi F, Ho C-T, editors. Phytochemicals and phytopharmaceuticals. AOCS Press; 1999.
[53] Matsuoka A, Furuta A, Masayasu O, Fukuhara K, Miyata N. Resveratrol, a naturally occurring polyphenol, induces sister chromatid exchanges in a Chinese hamster lung (CHL) cell line. Mutat Res 2001; 494:107–13.
[54] Crowell JA, Korytko PJ, Morrissey RL, Booth TD, Levine BS. Resveratrol-associated renal toxicity. Toxicol Sci 2004;82:614–9.
[55] Vieira de Almeida LM, Pineiro CC, Leite MC, Brolese G, Tramontina F, Feoli AM, et al. Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures. Cell Mol Neurobiol 2007;27:661–8.
[56] Whitsett T, Carpenter M, Lamartiniere CA. Resveratrol, but not EGCG, in the diet suppresses DMBA-induced mammary cancer in rats. J Carcinog 2006;5:15.
[57] Boyer RF, Clark HM, LaRoche AP. Reduction and release of ferritin iron by plant phenolics. J Inorg Biochem 1988;32:171–81.
[58] Minotti G, Aust SD. The role of iron in oxygen radical mediated lipid peroxidation. Chem-Biol Interactions 1989;71:1–19.
[59] Sotomatsu A, Nakano M, Hirai S. Phospholipid peroxidation induced by the catechol-$Fe3^{1}(Cu2^{1})$ complex: a possible mechanism of nigrostriatal cell damage. Arch Biochem Biophys 1990;283: 334–41.
[60] Rice-Evans C, Burdon R. Free radical–lipid interactions and their pathological consequences. Prog Lipid Res 1993;32:71–110.
[61] Miura T, Muraoka S, Ikeda N, Watanabe M, Fujimoto Y. Antioxidative and prooxidative action of stilbene derivatives. Pharmacol Toxicol 2000;86:203–8.
[62] Yamazaki L, Piette H. ESR spin-trapping studies on the reaction of $Fe2^{1}$ ions with H_2O_2-reactive species in oxygen toxicity in biology. J Biol Chem 1990;265:13594.
[63] Fukuhara K, Miyata N. Resveratrol as a new type of DNA-cleaving agent. Bioorg Med Chem Lett 1998;8:3187–
[64] Kopp P. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the "French paradox"? Eur J Endocrinol 1998;138:619–
[65] Turner RT, Evans GL, Zhang M, Maran A, Sibonga JD. Is resveratrol a phytoestrogen agonist in growing rats? Endocrinology 1999; 40:50–
[66] Zamora-Ros R, Urpi-Sarda M, Lamulela-Raventos RM, Estruch R, Vazquez-Agell M, Serra-Martinez M, et al. Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. Clin Chem 2006;52:7.
[ ] El-Mohsen M, Bayele H, Kuhnle G, Gibson G, Debnam E, Srai SK, et al. Distribution of [^{3}H] trans-resveratrol in rat tissues following oral administration. Brit J Nutr 2006;96:62–70.