Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 489 (2004) 39–48
www.elsevier.com/locate/ejphar

Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury

Zhuoxiao Cao, Yunbo Li*

Department of Pharmaceutical Sciences, St. John's University College of Pharmacy and Allied Health Professions, 8000 Utopia Parkway, Jamaica, NY 11439, USA

Received 13 October 2003; received in revised form 10 February 2004; accepted 20 February 2004

Abstract

Resveratrol is known to be protective against oxidative cardiovascular disorders. However, the underlying mechanisms remain unclear. This study was undertaken to determine if resveratrol could increase endogenous antioxidants and phase 2 enzymes in cardiomyocytes, and if such increased cellular defenses could provide protection against oxidative and electrophilic cell injury. Incubation of cardiac H9C2 cells with low micromolar resveratrol resulted in a significant induction of a scope of cellular antioxidants and phase 2 enzymes in a concentration- and/or time-dependent fashion. To investigate the protective effects of the resveratrol-induced cellular defenses on oxidative and electrophilic cell injury, H9C2 cells were first incubated with resveratrol, and then exposed to xanthine oxidase (XO)/xanthine, 4-hydroxy-2-nonenal or doxorubicin. We observed that resveratrol pretreatment afforded a marked protection against the above agent-mediated cytotoxicity in H9C2 cells. Moreover, the resveratrol pretreatment led to a great reduction in XO/xanthine-induced intracellular accumulation of ROS. Taken together, this study demonstrates that resveratrol induces antioxidants and phase 2 enzymes in cardiomyocytes, which is accompanied by increased resistance to oxidative and electrophilic cell injury.

Keywords: Resveratrol; Antioxidant; Cardiomyocyte; Reactive oxygen species; 4-Hydroxy-2-nonenal; Doxorubicin

1. Introduction

Cardiovascular diseases remain a leading cause of death worldwide. It is estimated that approximately 6.4 million US citizens have cardiovascular diseases, which accounts for ~40% of all deaths in the US (American Heart Association, 2004). Considerable evidence has suggested that oxidative and/or electrophilic stress are intimately involved in the pathogenesis of various forms of cardiovascular diseases, including myocardial ischemia–reperfusion injury, congestive heart failure, atherosclerosis, and drug-induced cardiomyopathy (Lefer and Granger, 2000; Uchida, 2000; Wattanapitayakul and Bauer, 2000; Steinberg, 2002; Kang, 2003). In support of the above notion, administration of exogenous antioxidative agents, including resveratrol has been shown to exert protective effects on oxidative cardiovascular injury (Marchioli, 1999; Lefer and Granger, 2000; Cuzzocrea et al., 2001; Wu et al., 2001; Hattori et al., 2002).

Resveratrol (structure in Fig. 1) is a polyphenol phytoalexin synthesized by a variety of plant species, including grapes, peanuts and mulberries in response to pathogenic attacks. Resveratrol has been reported to exhibit a wide range of biological actions that include cardiovascular protection, anticancer, and anti-inflammatory effects (Fremont, 2000; Gusman et al., 2001; Wallerath et al., 2002; Bwradamante et al., 2003; Dong, 2003). Resveratrol along with other phenolic compounds present in red wine has been considered to largely contribute to the "French paradox" (i.e., low incidence of cardiovascular events in spite of diet relatively high in saturated fat in people in Southern France) (Fremont, 2000; Gusman et al., 2001; Wu et al., 2001). The cardiovascular protective effects rendered by resveratrol are thought to be ascribed to its antioxidant properties, and its ability to modulate lipid metabolism, increase cellular nitric oxide levels, as well as inhibit platelet aggregation (Fre-

* Corresponding author. Tel.: +1-718-990-5253; fax: +1-718-990-1877.
E-mail address: liy@stjohns.edu (Y. Li).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.02.031

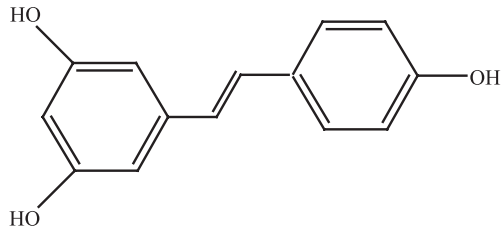

Fig. 1. Structure of resveratrol.

mont, 2000; Gusman et al., 2001; Wallerath et al., 2002; Wu et al., 2001). However, it remains unknown whether the cardiovascular protective effects of resveratrol may also occur through other mechanisms, such as the induction of endogenous antioxidants and phase 2 enzymes in cardiovascular tissues/cells. Accordingly, in this study, using rat cardiac H9C2 cells as a model system, we have investigated the induction of cellular antioxidants and phase 2 enzymes by resveratrol, and the protective effects of the resveratrol-upregulated cellular defenses on reactive oxygen species (ROS)- and electrophile-mediated cardiac cell injury. Our results demonstrate for the first time that a battery of cellular antioxidants and phase 2 enzymes can be potently induced by resveratrol in cultured cardiomyocytes, which is accompanied by a dramatically increased resistance to oxidative and electrophilic cardiac cell injury.

2. Materials and methods

2.1. Chemicals and materials

Resveratrol, xathine oxidase (XO), xanthine, doxorubicin, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), 1-chloro-2,4-dinitrobenzene (CDNB), hydrogen peroxide (H_2O_2), *o*-phthalaldehyde, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate, and bovine serum albumin were from Sigma (St. Louis, MO). 4-Hydroxy-2-nonenal (HNE) was from Cayman Chemical (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum, and Dulbecco's phosphate-buffered saline (PBS) were from Gibco-Invitrogen (Carlsbad, CA). Tissue culture flasks and 24-well tissue culture plates were from Corning (Corning, NY).

2.2. Cell culture

Rat cardiac H9C2 cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in 150 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. The cells were fed every 2–3 days, and subcultured once they reached 70–80% confluence.

2.3. Preparation of cell extract for measurement of antioxidants and phase 2 enzymes

Cardiac H9C2 cells were collected by centrifugation and then resuspended in ice-cold 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA. The cells were sonicated, followed by centrifugation at 13,000 × *g* for 10 min at 4 °C. The resulting supernatants were collected and kept on ice for immediate measurement of the antioxidants and phase 2 enzymes, as described below.

2.4. Measurement of cellular superoxide dismutase activity

Total cellular superoxide dismutase (SOD) activity was determined by the method of Spitz and Oberley (1989) with slight modifications. Briefly, the reaction mix (to be prepared freshly) contained in 50 mM potassium phosphate buffer, pH 7.8, 1.33 mM diethylenetriaminepentaacetic acid, 1.0 U/ml catalase, 70 μM nitroblue tetrazolium, 0.2 mM xanthine, 50 μM mM bathocuproinedisulfonic acid, and 0.13 mg/ml BSA. 0.8 ml of the reaction mix was added to each cuvette, followed by addition of 100 μl of sample. The cuvettes were pre-warmed at 37 °C for 3 min. The reaction was then started by adding 100 μl of XO (0.1 U/ml). The formation of formazan blue was monitored at 560 nm, 37 °C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma) standard curve, and expressed as units per mg of cellular protein. Cellular protein content was quantified with Bio-Rad protein assay dye (Hercules, CA) based on the method of Bradford (1976) using bovine serum albumin as the standard.

2.5. Measurement of cellular catalase activity

The method of Aebi (1984) was used to measure the catalase activity. In brief, to a quartz cuvette, 0.65 ml of 50 mM potassium phosphate buffer (pH 7.0) and 50 μl of sample were added. The reaction was started by adding 0.3 ml of 30 mM H_2O_2. The decomposition of H_2O_2 was monitored at 240 nm, 25 °C for 2 min. The catalase activity was expressed as μmol of H_2O_2 consumed per min per mg of cellular protein.

2.6. Measurement of cellular GSH content

The cellular GSH content was measured according to the procedures described previously by Cao and Li (2002) with slight modifications. Briefly, 10 μl of the supernatant as described above was incubated with 12.5 μl of 25% HPO_3, and 37 μl of 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 8.0 at 4 °C for 10 min. The samples were centrifuged at 13,000 × *g* for 5 min at 4 °C. The resulting supernatant (10 μl) was incubated with 0.1 ml of *o*-phthalaldehyde solution (0.1% in methanol) and 1.89 ml of the above phosphate buffer for 15 min at room temperature. Fluorescence was then read using a Perkin-Elmer lumines-

cence spectrometer (LS50B) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Cellular GSH content was calculated using a concurrently run standard curve and expressed as nmol of GSH per mg of cellular protein.

2.7. Measurement of cellular glutathione reductase activity

Cellular glutathione reductase (GR) activity was measured by the method of Wheeler et al. (1990) according to the procedures previously described (Cao and Li, 2002). GR activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nmol of NADPH consumed per min per mg of cellular protein.

2.8. Measurement of cellular GSH peroxidase activity

Cellular glutathione peroxidase (GPx) activity was measured by the method of Flohe and Gunzler (1984). Briefly, to an assay cuvette containing 0.5 ml of 50 mM potassium phosphate (pH 7.0), 1 mM EDTA and 2 mM sodium azide, 100 μl of sample, 100 μl of 10 mM GSH, 100 μl of glutathione reductase (2.4 U/ml) and 100 μl of 1.5 mM NADPH were added. The cuvette was incubated at 37 °C for 3 min. After addition of 100 μl of 2 mM H_2O_2, the rate of NADPH consumption was monitored at 340 nm, 37 °C for 5 min. This was designated as the total rate of NADPH consumption. The non-enzyme-dependent consumption of NADPH was also measured as above except that the 100 μl of sample was replaced by 100 μl of sample buffer. The rate of enzyme-dependent NADPH consumption was obtained by subtracting the non-enzyme-dependent NADPH consumption rate from the total NADPH consumption rate. GPx activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nmol of NADPH consumed per min per mg of cellular protein.

2.9. Measurement of cellular glutathione S-transferase activity

Cellular glutathione *S*-transferase (GST) activity was measured by the method of Habig et al. (1974) according to the procedures described previously (Cao and Li, 2002). 1-Chloro-2,3-dinitrobenzene (CDNB) was used as the substrate for GST. GST activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1}, and expressed as nmol of CDNB–GSH conjugate formed per min per mg of cellular protein.

2.10. Measurement of cellular NAD(P)H:quinone oxidoreductase 1 activity

Cellular NAD(P)H:quinone oxidoreductase 1 (NQO1) activity was determined according to the procedures described previously (Benson et al., 1980). Briefly, the reaction mix (to be prepared freshly) contained 50 mM Tris–HCl, pH 7.5, 0.08% Triton X-100, 0.25 mM NADPH, 80 μM 2,6-dichloroindophenol (DCIP) in the presence or absence of 60 μM dicumarol. To an assay cuvette, 0.695 ml of reaction mix was added. The reaction was started by adding 5 μl of sample, and the 2-electron reduction of DCIP was monitored at 600 nm, 25 °C for 3 min. The dicumarol-inhibitable NQO1 activity was calculated using the extinction coefficient of 21.0 mM^{-1} cm^{-1}, and expressed as nmol of DCIP reduced per min per mg of cellular protein.

2.11. Detection of cell viability with MTT reduction assay

Cell viability was determined by a slightly modified MTT cytotoxicity assay as described previously (Cao and Li, 2002). In brief, cells were plated into 24-well tissue culture plates. After incubation of the cells with chemicals in DMEM supplemented with 0.5% fetal bovine serum at 37 °C for 24 h, 50 μl of MTT (2 mg/ml PBS) was added to each well. The plates were incubated at 37 °C for another 2 h. Media were removed and wells were rinsed with PBS. To each well, 60 μl of dimethyl sulfoxide, 240 μl of isopropanol, and 300 μl of deionized water were added at room temperature to solubilize the formazan crystals. The dissolved formazan was then transferred into semi-micro-cuvettes, and the absorbance was measured at 570 nm.

2.12. Measurement of intracellular ROS accumulation

2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA) was used to detect intracellular ROS levels in H9C2 cells. DCF-DA is cell membrane permeable. Once inside the cells, DCF-DA is hydrolyzed by cellular esterases to form DCF, which is trapped intracellularly due to its membrane impermeability. DCF then reacts with intracellular ROS to form the fluorescent product, 2′,7′-dichlorofluroscein. To detect XO/xanthine-induced intracellular ROS accumulation, H9C2 cells grown on 25-cm^2 tissue culture flasks were rinsed once with PBS and then incubated with 10 μM DCF-DA in DMEM at 37 °C for 30 min. After this incubation, the cells were washed once with PBS followed by incubation with XO/xanthine in DMEM supplemented with 0.5% fetal bovine serum at 37 °C for another 30 min. Then, the cells were washed once with PBS and lysed in 3 ml ice-cold 10 mM Tris–HCl buffer, pH 7.4, containing 0.2% sodium dodecyl sulfate. The cell lysates were collected and centrifuged at $2000 \times g$ for 5 min at 4 °C. The fluorescence of the supernatants was measured using a Perkin-Elmer luminescence spectrometer (LS50B) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

2.13. Statistical analyses

All data are expressed as mean ± S.E.M. from at least three independent experiments. Differences between mean

values of multiple groups were analyzed by one-way analysis of variance (ANOVA) or Student's *t*-test. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Effects of resveratrol treatment on SOD and catalase activities in cardiac H9C2 cells

SOD and catalase are two key enzymes in detoxifying intracellular $O_2^{\cdot -}$ and H_2O_2 (Kehrer, 1993). As shown Fig. 2A, incubation of H9C2 cells with 25–100 μM resveratrol for 24 h did not result in any significant increase in SOD activity, however, treatment with 100 μM resveratrol for 48 h or with 50 and 100 μM resveratrol for 72 h led to a significant ~ 50% increase in total cellular SOD activity. In contrast to the relatively weak induction of SOD by resveratrol, incubation of H9C2 cells with 25–100 μM resveratrol resulted in a marked increase in cellular catalase activity in a concentration- and time-dependent manner (Fig. 2B). At 24 h, a significant increase in cellular catalase activity was observed with 50 and 100 μM resveratrol, but not with 25 μM resveratrol. Incubation of cells with 25, 50, and 100 μM resveratrol for 48 h caused a significant 25%, 90%, and 200% increase in catalase activity, respectively. A ~ 50% increase in catalase activity was observed in cells treated with 25 μM resveratrol for 72 h; treatment of cells with 50 and 100 μM resveratrol for 72 h led to a remarkable 2.7- and 5.7-fold induction of cellular catalase, respectively (Fig. 2B).

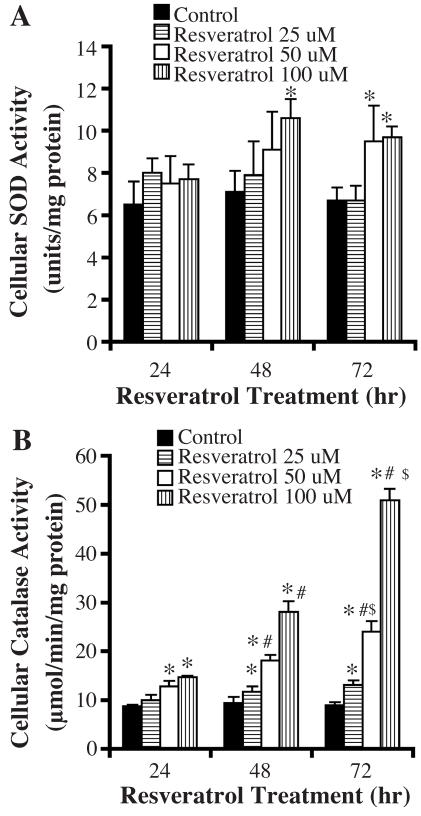

Fig. 2. Effects of resveratrol treatment on SOD (A) and catalase (B) activities in H9C2 cells. H9C2 cells were incubated with the indicated concentrations of resveratrol for 24–72 h. Cellular SOD and catalase activities were measured as described in Materials and methods. Values represent mean ± S.E.M. from three independent experiments. *Significantly different from control; #Significantly different from 24 h; $Significantly different from 48 h.

3.2. Effects of resveratrol treatment on GSH content, and GR and GPx activities in cardiac H9C2 cells

GSH and GSH-linked antioxidants, including GR and GPx are critically involved in the detoxification of ROS and electrophiles, and have been suggested to be protective against various forms of oxidative cardiovascular injury (Lapenna et al., 1998; Lefer and Granger, 2000; Forgione et al., 2002). We therefore investigated the inducibility of the above antioxidants by resveratrol in cardiac cells. As shown in Fig. 3A, incubation of H9C2 cells with 50 and 100 μM resveratrol resulted in a significant elevation of cellular GSH content at all of the three time points (24, 48, and 72 h). Moreover, at 48 h, a significant induction of cellular GSH was also observed with 25 μM resveratrol. Fig. 3B shows that incubation of H9C2 cells with 25–100 μM resveratrol for 72 h led to a concentration-dependent increase in GR activity. At 24 and 48 h, 100 μM, but not 25 and 50 μM resveratrol caused a significant increase in the GR activity. In contrast to the induction of GSH and GR, incubation of H9C2 cells with 25–100 μM resveratrol for 24–72 h did not result in any significant increases in cellular GPx activity (Fig. 3C).

3.3. Effects of resveratrol treatment on GST and NQO1 activities in cardiac H9C2 cells

GST and NQO1 are phase 2 enzymes that are critically involved in the detoxification of xenobiotics as well as ROS (Hayes and Pulford, 1995; Ross et al., 2000; Xie et al., 2001; Yang et al., 2001). Incubation of H9C2 cells with resveratrol also led to marked increases in cellular GST and NQO1 activities (Fig. 4). The induction of GST and NQO1 by resveratrol exhibited a concentration- and/or time-dependent manner. At 48 and 72 h, significant increases in both GST and NQO1 activities were observed with all of the three concentrations of resveratrol. Notably, incubation of H9C2 cells with 100 μM resveratrol for 72 h resulted in a remarkable 3.4- and 5.2-fold induction of cellular GST and NQO1, respectively (Fig. 4).

3.4. Inhibitory effects of resveratrol pretreatment on XO/xanthine-mediated cytotoxicity and intracellular ROS accumulation in cardiac H9C2 cells

To examine if the resveratrol-elevated cellular defenses could lead to cytoprotection against oxidative injury, H9C2

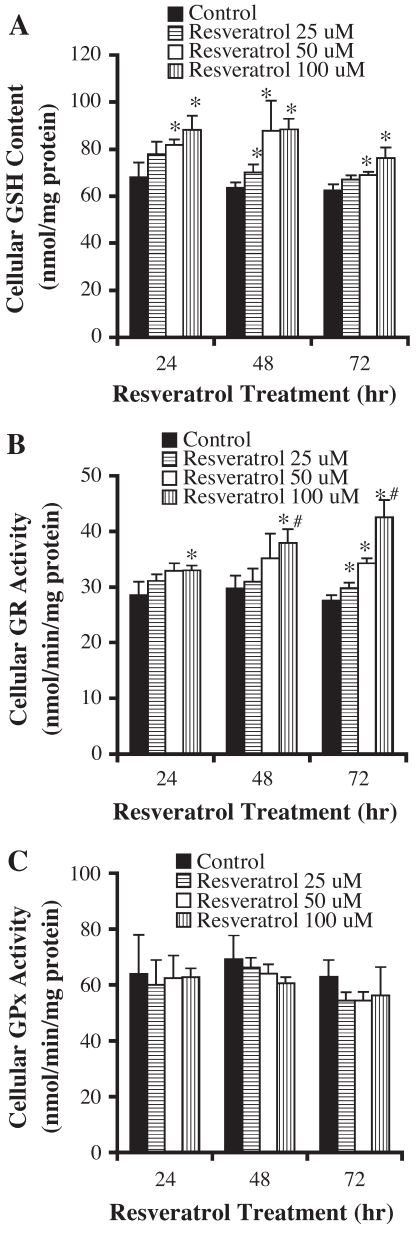

Fig. 3. Effects of resveratrol treatment on GSH content (A), and GR (B) and GPx (C) activities in H9C2 cells. Cells were incubated with the indicated concentrations of resveratrol in for 24–72 h. Cellular GSH content, and GR and GPx activities were measured as described in Materials and methods. Values represent mean ± S.E.M. from three independent experiments. *Significantly different from control; #Significantly different from 24 h.

cells were pretreated with resveratrol and then exposed to XO/xanthine, a system that generates both superoxide ($O_2^{\bullet -}$) and H_2O_2. As shown in Fig. 5A, incubation of H9C2 cells with various concentrations of XO in the presence of 0.5 mM xanthine for 24 h led to significant decreases in cell viability, as detected by MTT reduction assay. The decrease of cell viability was dependent on the concentrations of XO. Pretreatment of H9C2 cells with 25–100 μM resveratrol for 72 h afforded a marked protection against XO/xanthine-induced decreases in cell viability. The cytoprotection resulting from resveratrol pretreatment exhibited a resveratrol concentration-dependent manner; the protection was most dramatic with 100 μM resveratrol pretreatment, followed by 50 μM resveratrol pretreatment. Notably, pretreatment of H9C2 cells with 25 μM resveratrol for 72 h also resulted in a significant protection against XO/xanthine-induced cytotoxicity (Fig. 5A). The effects of resveratrol pretreatment on XO/xanthine-mediated intracellular ROS accumulation were also determined. As shown in Fig. 5B, incubation of control H9C2 cells with 15 mU/ml XO and 0.5 mM xanthine for 30 min resulted in a dramatic intracellular accumulation of ROS as indicated by a ~10-fold increase in DCF-derived fluorescence (Fig. 5B). As compared with the control cells, the XO/xanthine-induced intracellular accumulation of ROS was reduced by 70% in the resveratrol-pretreated H9C2 cells (Fig. 5B).

As aforementioned, resveratrol may direct scavenge ROS due to its antioxidant properties (Fremont, 2000). To further investigate if the cytoprotective effects of resveratrol pretreatment on XO/xanthine-induced cell injury (Fig. 5) might be at least partially ascribed to its direct antioxidant properties, H9C2 cells were pretreated with 100 μM resveratrol for 2 h, and then exposed to the same concentrations of XO/xanthine as used in Fig. 5. The results shown in Fig. 6

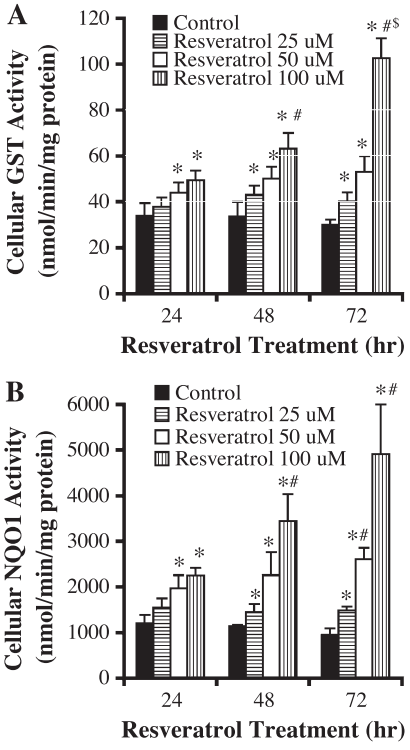

Fig. 4. Effects of resveratrol treatment on GST (A) and NQO1 (B) activities in H9C2 cells. H9C2 cells were incubated with the indicated concentrations of resveratrol for 24–72 h. Cellular GST and NQO1 activities were measured as described in Materials and methods. Values represent mean ± S.E.M. from three independent experiments. *Significantly different from control; #Significantly different from 24 h; $Significantly different from 48 h.

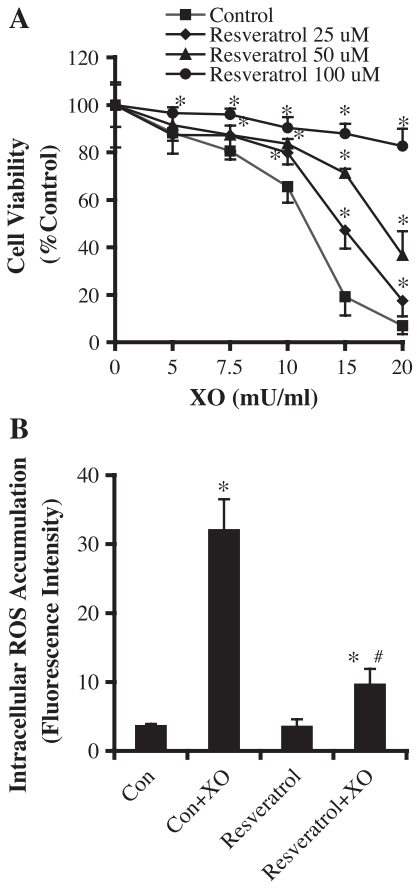

Fig. 5. Inhibitory effects of resveratrol pretreatment on XO/xanthine-mediated cytotoxicity (A) and intracellular ROS accumulation (B) in H9C2 cells. In A, cells were incubated with or without resveratrol for 72 h, followed by incubation with XO in the presence of 0.5 mM xanthine for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. In B, cells were incubated with or without 100 μM resveratrol for 72 h, followed by incubation with 10 μM DCF-DA for 30 min. The intracellular ROS accumulation was determined by measuring the DCF-derived fluorescence after incubation of the cells with XO (15 mU/ml) and xanthine (0.5 mM) for another 30 min. Values in both A and B represent mean ± S.E.M. from three (A) or four (B) independent experiments. *Significantly different from control; #Significantly different from control + XO.

indicated that pretreatment with resveratrol for 2 h failed to protect H9C2 cells against XO/xanthine-elicited cytotoxicity.

3.5. Protective effects of resveratrol pretreatment on HNE-mediated cytotoxicity in cardiac H9C2 cells

Since both GSH and GST have been shown to be major cellular defenses against reactive aldehyde-induced cell injury (Cao et al., 2003a,b), we determined if resveratrol-pretreated cardiac cells also acquired increased resistance to toxicity induced by 4-hydroxy-2-nonenal (HNE), a major aldehydic product of lipid peroxidation (Uchida, 2000, 2003). To determine the protective effects of resveratrol-induced cellular defenses on HNE-elicited cytotoxicity, H9C2 cells were pretreated with 25–100 μM resveratrol for 72 h, and then exposed to various concentrations of HNE

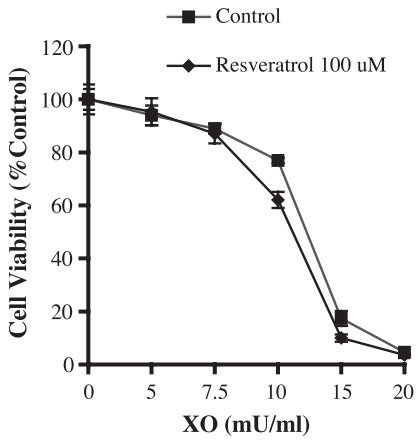

Fig. 6. Effects of resveratrol pretreatment for 2 h on XO/xanthine-mediated cytotoxicity in H9C2 cells. Cells were incubated with or without resveratrol for 2 h, followed by incubation with XO in the presence of 0.5 mM xanthine for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent mean ± S.E.M. from at four independent experiments.

for another 24 h. As shown in Fig. 7, resveratrol pretreatment afforded a marked cytoprotection against HNE-mediated toxicity in a concentration-dependent fashion. The cytoprotection was most remarkable with 100 μM resveratrol pretreatment, followed by pretreatment with 50 μM resveratrol. Pretreatment of H9C2 cells with 25 μM resveratrol also resulted in a significant protection against cytotoxicity induced by 10 μM HNE (Fig. 7).

3.6. Protective effects of resveratrol pretreatment on doxorubicin-mediated cytotoxicity in cardiac H9C2 cells

Doxorubicin is an effective anticancer agent, which can also elicit severe cardiotoxicity largely through an oxidative mechanism (Gewirtz, 1999). To investigate if resveratrol

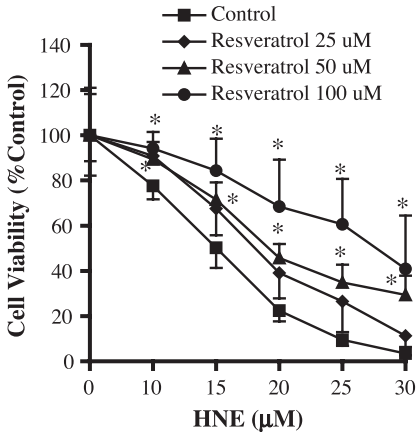

Fig. 7. Protective effects of resveratrol pretreatment on HNE-mediated cytotoxicity in H9C2 cells. Cells were incubated with or without resveratrol for 72 h, followed by incubation with HNE for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent mean ± S.E.M. from three independent experiments. *Significantly different from control.

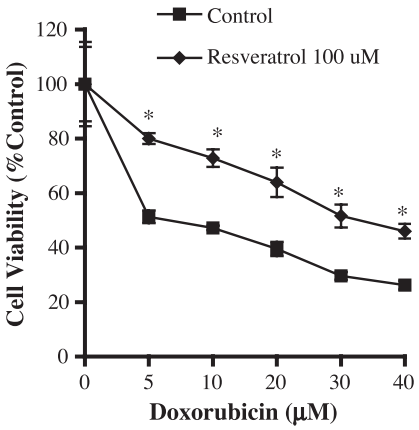

Fig. 8. Protective effects of resveratrol pretreatment on doxorubicin-mediated cytotoxicity in H9C2 cells. Cells were incubated with or without resveratrol for 72 h, followed by incubation with doxorubicin for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent mean ± S.E.M. from four independent experiments. *Significantly different from control.

pretreatment could also result in cytoprotection against doxorubicin-induced cardiac cell injury, H9C2 cells were pretreated with 100 μM resveratrol for 72 h, and then expose to various concentrations of doxorubicin for another 24 h. As shown in Fig. 8, resveratrol pretreatment provided significant protective effects on doxorubicin-mediated cytotoxicity at all of the concentrations of doxorubicin used.

4. Discussion

ROS and electrophilic species, including $O_2^{\cdot -}$, H_2O_2, lipid hydroperoxides, and lipid peroxidation-derived aldehydes are constantly formed during the normal cellular metabolism and/or under certain pathophysiological conditions, including tissue ischemia–reperfusion, inflammatory responses as well as exposure to toxic substances (Kehrer, 1993). Due largely to the detrimental nature of ROS and electrophilic species, mammalian cells have evolved a number of antioxidative and phase 2 enzymes to protect against oxidative and electrophilic cell damage. For example, SOD catalyzes the dismutation of $O_2^{\cdot -}$ to form H_2O_2. The toxic H_2O_2 can further be decomposed by catalase or GPx to produce water. During GPx-catalyzed decomposition of H_2O_2, the enzyme cofactor GSH is oxidized to the oxidized form of glutathione (GSSG). The GSSG can then be reduced to GSH via the action of GR. On the other hand, GST utilizing GSH as a cofactor is crucially involved in the detoxification of organic hydroperoxides as well as electrophiles, such as reactive aldehydes derived from lipid peroxidation (Xie et al., 2001; Yang et al., 2001; Cao et al., 2003a,b). NQO1 catalyzes the two electron reduction of quinone chemicals, thus limiting the formation of semiquinone radicals through one electron reduction, and the subsequent generation of ROS (Ross et al., 2000). Moreover, NQO1 has been suggested to be able to maintain the cellular levels of ubiquinol and vitamin E, two important biological antioxidants involved in the detoxification of ROS (Ross et al., 2000). Therefore, as depicted in Fig. 9, the coordinate actions of various cellular antioxidants and phase 2 enzymes ensure the effective detoxification of ROS and electrophilic species. In this context, the induction of a scope of endogenous antioxidants and phase 2 enzymes via the use of pharmacological agents appears to be a promising strategy for protecting against oxidative cardiovascular disorders.

The potential cardiovascular protective effects of resveratrol have been documented in various animal models (Wu et al., 2001). However, the exact mechanisms underlying its cardiovascular protective effects remain to be elucidated. The results of this studies demonstrated for the first time that incubation of rat cardiomyocytes with low micromolar concentrations of resveratrol resulted in a significant induction of a scope of cellular antioxidants and phase 2 enzymes, including SOD, catalase, GSH, GR, GST, and NQO1, in a concentration- and/or time-dependent fashion (Figs. 2–4). Induction of the above antioxidants and phase 2 enzymes by resveratrol in cardiac cells has not previously been demonstrated in the literature. The low micromolar concentrations of resveratrol employed in the present studies are similar to those used by others in the investigation of the biological effects of resveratrol in cultured cells (Fremont, 2000; Gusman et al., 2001; Wallerath et al., 2002; Dong, 2003). One critical question was that could such concentrations of resveratrol be achieved in the target organs of whole animals upon oral administration? While there is no direct answer to the above question, recent studies did indicate that because of its high lipophilicity, resveratrol could accumulate in tissues, such as heart, liver, and kidney in rats after oral administration in wine (Gusman et al., 2001). In this context, our preliminary in vivo studies demonstrated that resveratrol was also capable of inducing cardiac antioxidants and phase 2 enzymes in mice after oral administration (Li and Cao, 2004). These studies suggested that a significant amount of resveratrol could be achieved in cardiac tissue in whole animals upon oral administration, leading to increased expression of endogenous antioxidants and phase 2 enzymes in myocardium (Li and Cao, 2004).

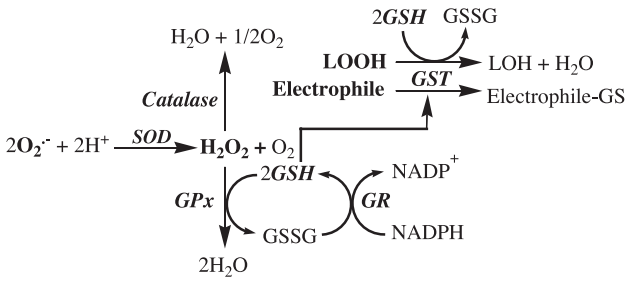

Fig. 9. A schematic illustration of the coordinate actions of various cellular antioxidants and phase 2 enzymes in detoxification of ROS and electrophiles. LOOH, lipid hydroperoxide; LOH, lipid alcohol.

The molecular mechanisms underlying resveratrol-mediated elevation of the above antioxidants and phase 2 enzymes in cardiac cells remain to be investigated. Recently, studies by Kwak et al. (2001) have demonstrated that induction of antioxidative and phase 2 enzymes by chemoprotective agents, including 1,2-dithiole-3-thiones in rodent hepatic tissues occurs via a nuclear factor E2-related factor 2 (Nrf2)-mediated antioxidant response element-driven transcriptional mechanism. Studies are currently underway in our laboratory to investigate if the above Nrf-2-mediated transcriptional mechanism is also involved in the resveratrol-mediated upregulation of cellular antioxidative and phase 2 defenses in cardiomyocytes.

Because of the ability of resveratrol treatment to increase SOD, catalase, GSH, GR, GST and NQO1, which are important cellular defenses against oxidative and electrophilic stress (Fig. 9), we investigated if the induction of the above antioxidants and phase 2 enzymes by resveratrol led to protection against ROS-mediated cytotoxicity. The results clearly showed that pretreatment of H9C2 cells with resveratrol resulted in a marked protection against XO/xanthine-mediated cytotoxicity and intracellular accumulation of ROS (Fig. 5). XO has been extensively implicated in the pathogenesis of various cardiovascular diseases, including ischemia–reperfusion injury (Thompson-Gorman and Zweier, 1990; Wattanapitayakul and Bauer, 2000). As aforementioned, SOD, catalase and GPx/GSH are critical cellular defenses involved in the detoxification of $O_2^{\cdot -}$ and H_2O_2, and have been demonstrated to protect against myocardial ischemia–reperfusion injury as well as drug-induced oxidative cardiotoxicity in animals (Yen et al., 1996; Lefer and Granger, 2000; Kang et al., 2002). The simultaneous induction of SOD, catalase and GSH by resveratrol may thus largely contribute to the increased resistance of the resveratrol-pretreated H9C2 cells to XO/xanthine-mediated cytotoxicity as well as intracellular ROS accumulation (Fig. 5). Furthermore, the induction of GR by resveratrol may lead to increased regeneration of GSH from GSSG produced during GPx-catalyzed decomposition of H_2O_2 in H9C2 cells. GSH is also a cofactor for GST, an abundant cellular enzyme in mammalian tissues. GST is generally viewed as a phase 2 enzyme, primarily involved in the detoxification of electrophilic xenobiotics via catalyzing the formation of GSH–electrophile conjugate (Hayes and Pulford, 1995; Strange et al., 2001). Several recent studies have also reported that GST plays an important role in protecting cells against ROS-mediated injury through catalyzing the decomposition of lipid hydroperoxides generated from oxidative damage of cellular lipid molecules (Xie et al., 2001; Yang et al., 2001; also see Fig. 9). Accordingly, the induction of GST by resveratrol in H9C2 cells may contribute partially to the increased resistance of the resveratrol-pretreated cells to XO/xanthine-elicited toxicity. The potent induction of NQO1 by resveratrol may also be involved in the cytoprotective effects of resveratrol on XO/xanthine-induced cytotoxicity. In this context, as mentioned above NQO1 may act as an antioxidative enzyme via its ability to maintain the cellular levels of ubiquinol and vitamin E, two important non-protein antioxidants (Ross et al., 2000). To further determine the causal involvement of the elevated endogenous antioxidants and phase 2 enzymes in cytoprotective effects of resveratrol pretreatment on ROS-induced cardiac cell injury, H9C2 cells were pretreated with resveratrol for 2 h, and then exposed to XO/xanthine. The failure of the 2-h resveratrol pretreatment to protect against XO/xanthine-induced cytotoxicity (Fig. 6) strongly suggested that the resveratrol associated with cells was unlikely to directly react with the ROS, leading to cytotprotection. On the other hand, this observation indicated that the cytoprotective effects of resveratrol pretreatment (Fig. 5) were most likely due to the elevation of the endogenous cellular antioxidants and phase 2 enzymes. Studies are currently under way in our laboratory to investigate the discrete role on the individual resveratrol-inducible antioxidants and phase 2 enzymes in protecting cardiac cells against oxidative injury.

Pretreatment of cardiac H9C2 cells with resveratrol also resulted in increased resistance to HNE-induced cytotoxicity (Fig. 7). HNE is formed during oxidation of low-density lipoprotein as well as lipid peroxidation of cell membranes (Uchida, 2000, 2003). HNE is known to cause cell dysfunction at lower concentrations and elicit cytotoxicity at higher levels (Cao et al., 2003a; Uchida, 2003). As a major electrophilic aldehydic product of lipid peroxidation, HNE has been extensively implicated in the pathogenesis of various cardiovascular diseases, including atherosclerosis, myocardial ischemia–reperfusion injury, and drug-induced cardiomyopathy (Blasig et al., 1995; Eaton et al., 1999; Uchida, 2000). Previous studies have demonstrated that both GSH and GST are principal defenses against HNE-induced cell injury (Hubatsch et al., 1998; Cao et al., 2003a). Thus, in this study, the induction of GSH and GST by resveratrol may largely account for the increased resistance of resveratrol-pretreated cardiac H9C2 cells to HNE-induced cytotoxicity (Fig. 7).

Doxorubicin is a commonly used anticancer drug. It is effective in the treatment of various forms of malignancies. However, the clinical use of doxorubicin is associated with the development of life-threatening cardiomyopathy (Gewirtz, 1999). Accumulating evidence over the last decade has suggested that ROS are intimately involved in the doxorubicin-induced cardiotoxicity (Gewirtz, 1999; Horenstein et al., 2000). As such, extensive efforts have focused on the identification of exogenous antioxidative compounds which can be administrated to protect against doxorubicin-induced cardiotoxicity (Horenstein et al., 2000; Quiles et al., 2002). However, whether induction of endogenous antioxidants in cardiac cells by pharmacological agents also affords protection against doxorubicin cardiotoxicity has not been carefully investigated. The results of this study demonstrated for the first time that pretreatment of cardiac H9C2 cells with resveratrol led to a significant protection against doxorubicin-induced decrease in cell viability (Fig.

8). Previous studies using transgenic animal models have shown that overexpression of catalase or SOD results in great protection against doxorubicin-induced cardiotoxicity (Yen et al., 1996; Kang et al., 2002). Accordingly, the increased resistance of resveratrol-pretreated H9C2 cells to doxorubicin toxicity, as shown in Fig. 8, most likely resulted from the elevated levels of SOD and catalase in the resveratrol-pretreated cardiac cells (Fig. 2).

In conclusion, this study demonstrates for the first time that a number of endogenous antioxidants and phase 2 enzymes in cultured cardiomyocytes can be induced by low micromolar concentrations of resveratrol, and that this chemically mediated upregulation of cellular defenses is accompanied by a markedly increased resistance to cardiac cell injury elicited by ROS, HNE, and doxorubicin. As mentioned above, efficient detoxification of ROS and electrophilic species requires the coordinate actions of various cellular antioxidants and phase 2 enzymes (Fig. 9). Accordingly, simultaneous induction of a scope of key cellular antioxidants and phase 2 enzymes by resveratrol in cardiovascular cells may be an important mechanism underlying the protective effects of resveratrol observed with various forms of cardiovascular disorders (Fremont, 2000; Wu et al., 2001; Hattori et al., 2002; Bradamante et al., 2003).

Acknowledgements

This work was supported in part by the National Institutes of Health grant CA91895 (Y.L.), St. John's University Professor Research Fund, and the Doctoral Fellowship from St. John's University (Z.C.).

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–127.

American Heart Association, 2003. Heart Disease and Stroke Statistics—2004 Update. http://www.americanheart.com.

Benson, A.M., Hunkeler, M.J., Talalay, P., 1980. Increase of NAD(P)H: quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. Proc. Natl. Acad. Sci. U. S. A. 77, 5216–5220.

Blasig, I.E., Grune, T., Schonheit, K., Rohde, E., Jakstadt, M., Haseloff, R.F., Siems, W.G., 1995. 4-Hydroxynonenal, a novel indicator of lipid peroxidation for reperfusion injury of the myocardium. Am. J. Physiol. 269, H14–H22.

Bradamante, S., Barenghi, L., Piccinini, F., Bertelli, A.A., De Jonge, R., Beemster, P., De Jong, J.W., 2003. Resveratrol provides late-phase cardioprotection by means of a nitric oxide- and adenosine-mediated mechanism. Eur. J. Pharmacol. 465, 115–123.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation ofmicrogram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cao, Z., Li, Y., 2002. Chemical induction of cellular antioxidants affords marked protection against oxidative injury in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 292, 50–57.

Cao, Z., Hardej, D., Trombetta, L.D., Li, Y., 2003a. The role of chemically-induced glutathione and glutathione *S*-transferase in protecting against 4-hydroxy-2-nonenal-mediated cytotoxicity in vascular smooth muscle cells. Cardiovasc. Toxicol. 3, 165–178.

Cao, Z., Hardej, D., Trombetta, L.D., Trush, M.A., Li, Y., 2003b. Induction of cellular glutathione and glutathione *S*-transferase by 3*H*-1,2-dithiole-3-thione in rat aortic smooth muscle A10 cells: protection against acrolein-induced toxicity. Atherosclerosis 166, 291–302.

Cuzzocrea, S., Riley, D.P., Caputi, A.P., Salvemini, D., 2001. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol. Rev. 53, 135–159.

Dong, Z., 2003. Molecular mechanism of the chemopreventive effect of resveratrol. Mutat. Res. 523–524, 145–150.

Eaton, P., Li, J.M., Hearse, D.J., Shattock, M.J., 1999. Formation of 4-hydroxy-2-nonenal-modified proteins in ischemic rat heart. Am. J. Physiol. 276, H935–H943.

Flohe, L., Gunzler, W.A., 1984. Assays of glutathione peroxidase. Methods Enzymol. 105, 114–119.

Forgione, M.A., Cap, A., Liao, R., Moldovan, N.I., Eberhardt, R.T., Lim, C.C., Jones, J., Goldschmidt-Clermont, P.J., Loscalzo, J., 2002. Heterozygous cellular glutathione peroxidase deficiency in the mouse: abnormalities in vascular and cardiac function and structure. Circulation 106, 1154–1158.

Fremont, L., 2000. Biological effects of resveratrol. Life Sci. 66, 663–673.

Gewirtz, D.A., 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem. Pharmacol. 57, 727–741.

Gusman, J., Malonne, H., Atassi, G.A., 2001. Reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. Carcinogenesis 22, 1111–1117.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hattori, R., Otani, H., Maulik, N., Das, D.K., 2002. Pharmacological preconditioning with resveratrol: role of nitric oxide. Am. J. Physiol. 282, H1988–H1995.

Hayes, J.D., Pulford, D.J., 1995. The glutathione *S*-transferase supergene family: regulation of GST and the contribution of the isozymes to cancer chemoprotection and drug resistance. Crit. Rev. Toxicol. 30, 445–600.

Horenstein, M.S., Heide, R.S.V., L'Ecuyer, T.J., 2000. Molecular basis of anthracycline-induced cardiotoxicity and its prevention. Mol. Genet. Metab. 71, 436–444.

Hubatsch, I., Ridderstrom, M., Mannervik, B., 1998. Human glutathione transferase A4-4: an alpha class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxidation. Biochem. J. 330, 175–179.

Kang, Y.J., 2003. New understanding in cardiotoxicity. Curr. Opin. Drug. Discov. Dev. 6, 110–116.

Kang, Y.J., Sun, X., Chen, Y., Zhou, Z., 2002. Inhibition of doxorubicin chronic toxicity in catalase-overexpressing transgenic mouse hearts. Chem. Res. Toxicol. 15, 1–6.

Kehrer, J.P., 1993. Free radicals as mediators of tissue injury and disease. Crit. Rev. Toxicol. 23, 21–48.

Kwak, M.-K., Itoh, K., Yamamoto, M., Sutter, T.R., Kensler, T.W., 2001. Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemprotective agent, 3*H*-1,2-dithiole-3-thione. Mol. Med. 7, 135–145.

Lapenna, D., de Gioia, S., Ciofani, G., Mezzetti, A., Ucchino, S., Calafiore, A.M., Napolitano, A.M., Ilio, C.D., Cuccurullo, F., 1998. Glutathione-related antioxidant defenses in human atherosclerotic plaques. Circulation 97, 1930–1934.

Lefer, D.J., Granger, N., 2000. Oxidative stress and cardiac disease. Am. J. Med. 109, 315–323.

Li, Y., Cao, Z., 2004. Chemical induction of endogenous antioxidants in mouse cardiac tissue: implications for cardioprotection. Toxicol. Sci. 78 (S-1), 168.

Marchioli, R., 1999. Antioxidant vitamins and prevention of cardiovascular

disease: laboratory, epidemiological and clinical trial data. Pharmacol. Res. 40, 227–238.

Quiles, J.L., Huertas, J.R., Battino, M., Mataix, J., Ramirez-Tortosa, M.C., 2002. Antioxidant nutrients and adriamycin toxicity. Toxicology 180, 79–95.

Ross, D., Kepa, J.K., Winski, S.L., Beall, H.D., Anwar, A., Siegel, D., 2000. NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. Chem.-Biol. Interact. 129, 77–97.

Spitz, D.R., Oberley, L.W., 1989. An assay for superoxide dismutase activity in mammalian tissue homogenates. Anal. Biochem. 179, 8–18.

Steinberg, D., 2002. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. Nat. Med. 8, 1211–1217.

Strange, R.C., Spiteri, M.A., Ramachandran, S., Fryer, A.A., 2001. Glutathione-*S*-transferase family of enzymes. Mutat. Res. 482, 21–26.

Thompson-Gorman, S.L., Zweier, J.L., 1990. Evaluation of the role of xanthine oxidase in myocardial reperfusion injury. J. Biol. Chem. 265, 6656–6663.

Uchida, K., 2000. Role of reactive aldehyde in cardiovascular diseases. Free Radic. Biol. Med. 28, 1685–1696.

Uchida, K., 2003. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog. Lipid Res. 42, 318–343.

Wallerath, T., Deckert, G., Ternes, T., Anderson, H., Li, H., Witte, K., Forstermann, U., 2002. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. Circulation 106, 1652–1658.

Wattanapitayakul, S.K., Bauer, J.A., 2000. Oxidative pathways in cardiovascular diseases: roles, mechanisms, and therapeutic implications. Pharmacol. Ther. 89, 187–206.

Wheeler, C.R., Salzman, J.A., Elsayed, N.M., Omaye, S.T., Korte, D.W., 1990. Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. Anal. Biochem. 184, 193–199.

Wu, J.M., Wang, Z.R., Hsieh, T.C., Bruder, J.L., Zou, J.G., Huang, Y.Z., 2001. Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine. Int. J. Mol. Med. 8, 3–17.

Xie, C., Lovell, M.A., Xiong, S., Kindy, M.S., Guo, J.-T., Xie, J., Amaranth, V., Montine, T.J., Markesbery, W.R., 2001. Expression of glutathione-*S*-transferase isozyme in the SY5Y neuroblastoma cell line increases resistance to oxidative stress. Free Radic. Biol. Med. 31, 73–81.

Yang, Y., Cheng, J.-Z., Singhal, S.S., Saini, M., Pandya, U., Awasthi, S., Awasthi, Y.C., 2001. Role of glutathione *S*-transferases in protection against lipid peroxidation. J. Biol. Chem. 276, 19220–19230.

Yen, H.-C., Oberley, T.D., Vichitbandha, S., Ho, Y.-S., St. Clair, D.K., 1996. The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. J. Clin. Invest. 98, 1253–1260.