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Grape Seed Polyphenols Protect Cardiac Cells from Apoptosis via Induction of Endogenous Antioxidant Enzymes

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Grape seed extract (GSE) has been reported to exert protective effects on various forms of cardiac disorders. The cardiovascular protective effects of GSE are believed to be ascribed to its antioxidative properties. A series of studies have demonstrated that polyphenols are instrumental for the antioxidative properties of GSE. This study was undertaken to investigate whether two major polyphenols isolated from GSE (catechin and proanthocyanidin B_4) could increase the endogenous antioxidant enzymes in cardiomyocytes, and whether such increased cellular defenses could provide protection against oxidative cardiac cell injury. Incubation of cardiac H9C2 cells with micromolar concentrations of catechin or proanthocyanidin B_4 resulted in a significant induction of cellular antioxidant enzymes in a concentration-dependent fashion. Furthermore, catechine or proanthocyanidin B_4 pretreatment led to a marked reduction in xanthine oxidase(XO)/xanthine-induced intracellular reactive oxygen species (ROS) accumulation and cardiac cell apoptosis. These results indicated that grape seed polyphenols (GSP) could protect against cardiac cell apoptosis via the induction of endogenous antioxidant enzymes. This may be an important mechanism underlying the protective effects of GSE observed with various forms of cardiovascular disorders.

KEYWORDS: Catechin; proanthocyanidins; antioxidant; apoptosis; reactive oxygen species; cardiomyocytes.

INTRODUCTION

Cardiovascular disease remains a leading cause of death worldwide. It comprises its own set of pathologies, chief among which are atherosclerosis, hypertension, congestive heart failure, cardiomyopathy, coronary heart disease, hypertrophy, arrhythmias, ventricular fibrillation (VF), ventricular tachycardia (VT), myocardial infarction, and stroke (*1*−*6*). Strong evidence for the role of oxidative stress in the pathogenesis of heart failure has been provided by studies on experimental animals as well as humans (*7*). When the equilibrium between free-radical production and cellular antioxidant defenses is disturbed in favor of more free radicals, it causes oxidative stress which can promote cellular injury (*8*). Free radicals and oxidative stress also appear to be a common mediator of apoptosis, either directly or via the formation of lipid peroxidation and lipid hydroperoxides (*3*, *6*). Free-radical scavengers and antioxidants have been found to be cardioprotective against various ischemic heart diseases (*9*, *10*).

Grape seeds are waste products of the winery and grape juice industry. These seeds contain lipid, protein, carbohydrates, and 5−8% polyphenols, depending on the variety. Polyphenols in grape seeds are mainly flavonoids, including gallic acid, the monomeric flavan-3-ols catechin, epicatechin, gallocatechin, epigallocatechin, and epicatechin 3-*O*-gallate, and dimeric-, trimeric-, and even more-polymeric proanthocyanidins (*11*). A large number of studies have been conducted on GSE and have demonstrated excellent free-radical scavenging and cardioprotective properties (*12*). GSE provided unique protection against myocardial ischemia−reperfusion injury, myocardial infarction in rats (*13*), and doxorubicin-induced cardiotoxicity in mice (*14*). In a recent study, it was concluded that GSE prevents oxidative injury of HepG2 cells (a hepatocarcinoma cell line) by modulating the expression of antioxidant enzyme systems (*15*). In most cases, activities of GSE are related to its antioxidative properties and are mainly attributed to the phenolic compounds. Catechin and proanthocyanidin B_4 (pc B_4) (**Figure 1**) are two major phenolics extracted from grape seeds. One of the dimeric proanthocyanidins is pc B_4. Our previous studies have demonstrated that catechin and pc B_4 exhibit better antioxidative activities on oxidative damage to DNA in mice spleen cells than other compounds we have obtained from GSE (*16*). In the present study, using rat cardiac H9C2 cells, we examined the regulation of cellular antioxidant enzymes induced by grape seed catechin and pc B_4 as well as the effects of these two grape seed polyphenols-regulated cellular defenses on xanthine oxidase (XO)/xanthine-induced intracellular ROS accumulation and cardiac cell apoptosis.

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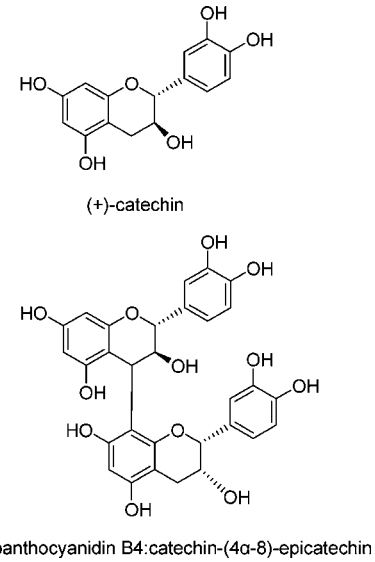

Figure 1. Structures of catechin and pc B_4.

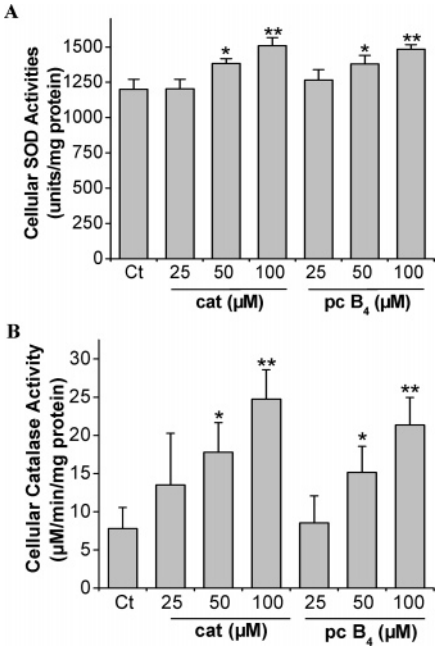

Figure 2. Effects of catechin and pc B_4 treatment on SOD (**A**) and CAT (**B**) activities in cardiac H9C2 cells. H9C2 cells were incubated with the indicated concentrations of chemicals for 24 h. Cellular SOD and CAT activities were measured as described in Materials and Methods. Values represent means ± SD from three independent experiments. *Significantly different from control, $P < 0.05$; **significantly different from control, $P < 0.01$.

MATERIALS AND METHODS

Cell Culture Materials and Chemicals. Catechin and pc B_4 were extracted from grape seeds in our laboratory (*16*); xanthine oxidase (XO), xanthine, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH), and phenylmethyl sulfonyl fluoride (PMSF) were from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was from Gibco-Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Tianjin TBD biotechnology development center (Tianjin, China). All other reagents were of analytical purity.

Cell Culture. Rat heart cell line H9C2(2-1) (ATCC, Rockville, MD) were cultured in DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 80 μg/mL of streptomycin in tissue culture flasks at

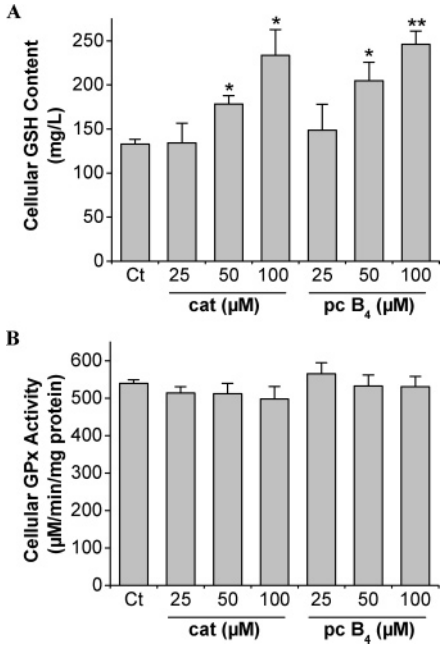

Figure 3. Effects of catechin and pc B_4 treatment on GSH content (**A**) and GPx (**B**) activity in cardiac H9C2 cells. Cells were incubated with the indicated concentrations of chemicals for 24 h. Cellular GSH content and GPx activities were measured as described in Materials and Methods. Values represent mean ± SD from three independent experiments. *Significantly different from control, $P < 0.01$; **significantly different from control, $P < 0.001$.

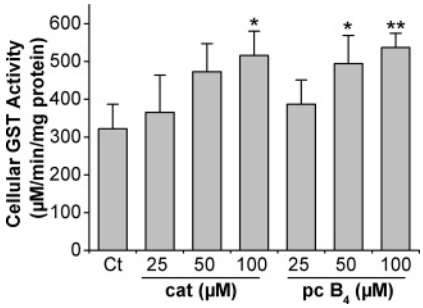

Figure 4. Effects of catechin and pc B_4 treatment on GST activity in cardiac H9C2 cells. H9C2 cells were incubated with the indicated concentrations of chemicals for 24 h. Cellular GST activities was measured as described in Materials and Methods. Values represent mean ± SD from three independent experiments. *Significantly different from control, $P < 0.05$; **significantly different from control, $P < 0.01$.

37 °C in a humidified atmosphere of 5% CO_2. The cells were fed every 2−3 days, and they were subcultured once they reached 70−80% confluence.

Assays for Activities of Cellular Antioxidant Enzymes. Catechin and pc B_4 were dissolved in dimethyl sulfoxide and diluted in the culture medium to the required concentration; the final dimethyl sulfoxide content should never exceed 0.05%. Cells were seeded at 10^5 cells/well and cultured overnight, then the cells were cultured with or without catechin and pc B_4 in 24-well culture plates for 24 h. After this incubation, cells were harvested and disrupted in 100 μL of lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.6, 50 mM NaCl, 0.1% bovine serum albumin (BSA), 1 mM PMSF, 1% aprotinin, 5 mM EDTA, 50 mM NaF, 0.1% 2-mercaptoethanol, 5 mM phenylarsine oxide, and 100 mM sodium orthovanadate by three cycles of freezing/thawing. After centrifugation at 13000*g* for 10 min at 4 °C, the supernatants were used for cellular superoxide dismutase(SOD), catalase, reduced glutathione (GSH), GSH peroxidase (GPx), and glu-

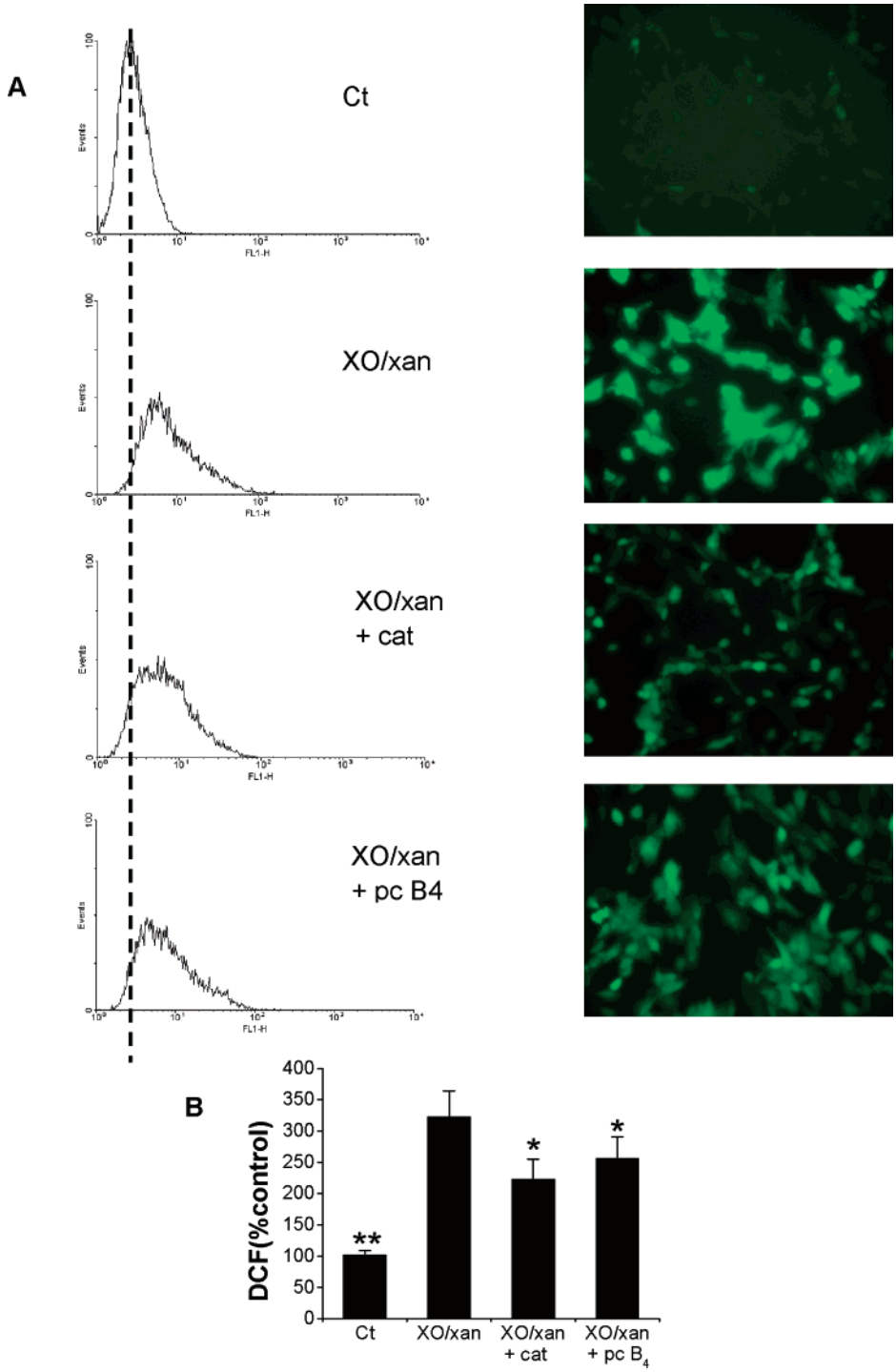

Figure 5. Effects of catechin and pc B_4 treatment on intracellular ROS accumulation induced by XO/xan. Following pretreatment with catechin, or pc B_4 for 24 h, the medium was changed, followed by incubation with 20 μM DCF-DA for 30 min. The intracellular ROS accumulation was determined by measuring the DCF-derived fluorescence after incubation of cells with XO (100 mU/mL) and xanthine(0.5 mM) for another 30 min. (**A**) Flow cytometric histogram of DCF in H9C2 cells. Events: cell number, FL1-H: relative DCF fluorescence intensity. Data are the results of a single experiment. (**B**) Column bar graph of mean cell flurescence for DCF. The fluorescence intensities in untreated control cells are expressed as 100%. Results are means ± SD of three independent experiments. *P < 0.05 vs XO/xan; **P < 0.001 vs XO/xan.

tathione S-transferase (GST) assays using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively (*17*−*21*).

Flow-Cytometric Assay of 2′,7′-Dichlorodihydrofluorescein (DCFH). Intracellular ROS levels were determined by flow cytometry using the peroxide-sensitive fluorescent probe DCFH, which in the presence of intracellular ROS is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) (*22*).To detect XO/xanthine-induced intracellular ROS accumulation, H9C2 cells grown on 6-well tissue culture plates were rinsed once with PBS, and then DCFH was added at a final concentration of 20 μM and incubated for 30 min at 37 °C. After this incubation, the cells were washed once with PBS followed by incubation with 100 mU/mL XO in the presence of 0.5 mM xanthine at 37 °C for another 30 min. Then, the cells were washed three times with PBS and maintained in 500 μL of PBS. Cellular fluorescence was determined by a flow cytometry apparatus (FACS-SCAN Becton-Dickinson.

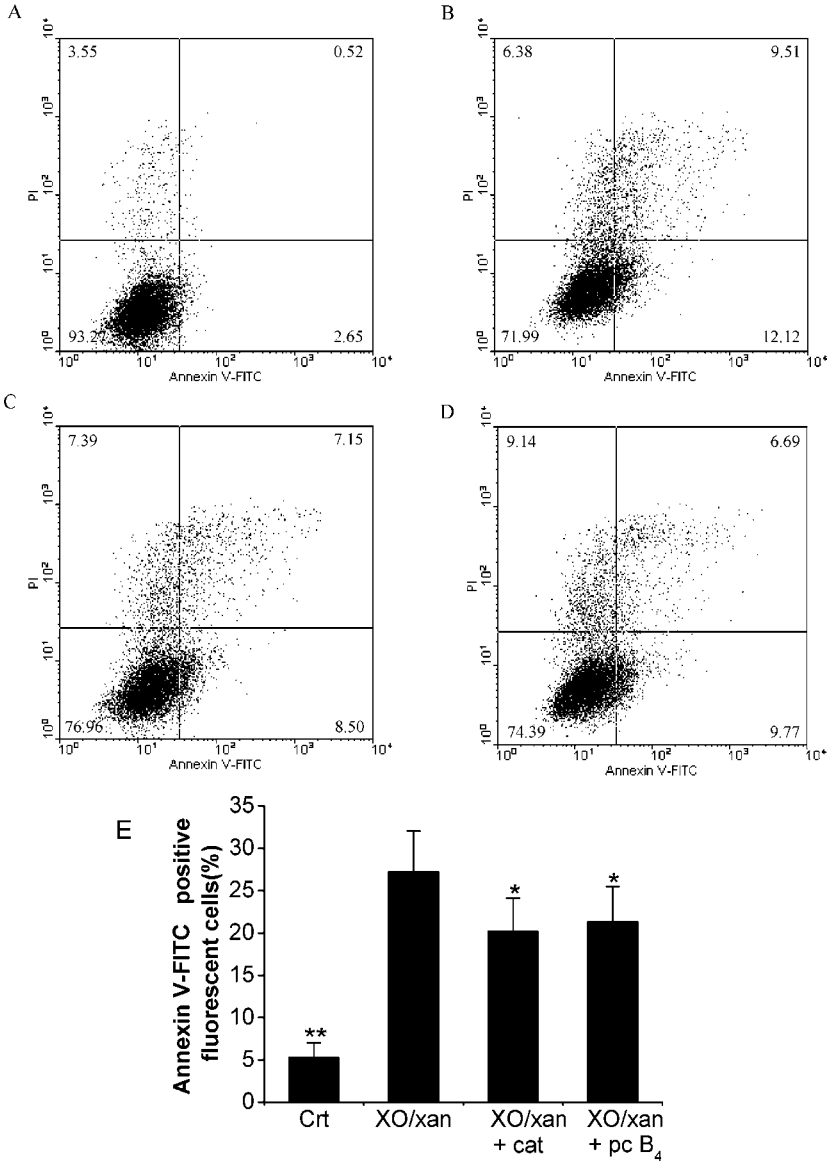

Figure 6. Flow cytometric analysis of apoptosis in H9C2 cells that were pretreated with catechin and pc B_4. The upper panels are the results of a single experiment. (**A**) Untreated control cells; (**B**) cells that were treated with 100 mU/mL XO in the presence of 0.5 mM xanthine for 30 min; (**C**) cells that were pretreated with 100 μM catechin for 24 h and then incubated with XO/xan for 30 min; (**D**) cells that were pretreated with 100 μM pc B_4 for 24 h and then incubated with XO/xan for 30 min. The lower panel (E) shows a column bar graph analysis. Results are means ± SD of three independent experiments. **P* < 0.05 vs XO/xan; ***P* < 0.01 vs XO/xan.

Franklin Lakes, NJ). Measurements were taken at 510−540 nm after excitation of cells at 488 nm with an argon ion laser.

Flow Cytometric Detection of Apoptosis; FITC-Annexin V/PI Double Staining. Double staining for FITC-annexin V binding and for cellular DNA using propidium iodide (PI) was performed according to the kit's instructions (Jinmei Biotechnology Co. Ltd, Beijing, China).

Fluorescent Staining of Nuclei. The nuclei of H9C2 cells were stained with chromatin dye (Hoechst 33258). Briefly, cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and incubated with 10 μM Hoechst 33258 in PBS at room temperature for 30 min. After three washes, the cells were observed under a fluorescence microscope (IX-71, Olympus, Japan).

Statistical Analysis. All data are expressed as means ± SD from at least three independent experiments. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA). Statistical significance was considered at $P < 0.05$.

RESULTS

Effects of Catechin and pc B_4 Treatment on SOD and Catalase Activities. SOD and catalase are two key enzymes in detoxifying intracellular $O_2^{\cdot -}$ and H_2O_2. As shown in **Figure 2A**, significant increases in cellular SOD activity were observed both with 50 and 100 μM catechin or pc B_4, but not with 25 μM concentration. Incubation of cells with catechin (50 and 100 μM) caused a significant 128 and 218% increase in catalase activity, respectively. Treatment of cells with pc B_4 (50 and 100 μM) also led to a remarkable 94 and 174% induction of cellular catalase, respectively (**Figure 2B**).

Effects of Catechin and pc B_4 Treatment on GSH Content and GPx Activity. GSH and GSH-linked antioxidants GPx are critically involved in the detoxification of ROS and have been suggested to be protective against various forms of oxidative cardiovascular injuries (*23*, *24*). We therefore investigated the inducibility of the antioxidant enzymes above by catechin and pc B_4 in cardiac cells. As shown in **Figure 3A**, incubation of H9C2 cells with 50 and 100 μM catechin or pc B_4 for 24 h resulted in a significant elevation of cellular GSH content. Celluar GPx activity was not significantly increased by treatment

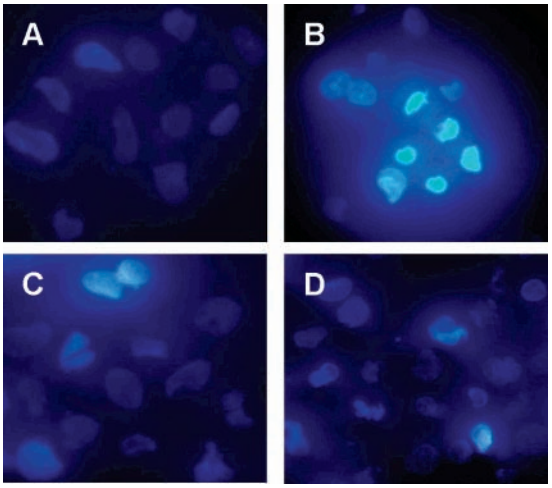

Figure 7. Fluorescent staining of nuclei. Cells were treated as described in Materials and Methods. Then, cells were visualized under a fluorescent microscope. (**A**) Untreated control cells; (**B**) cells that were treated with 100 mU/mL XO in the presence of 0.5 mM xanthine for 30 min; (**C**) cells that were pretreated with 100 μM catechin for 24 h and then incubated with XO/xan for 30 min; (**D**) cells that were pretreated with 100 μM pc B_4 for 24 h and then incubated with XO/xan for 30 min.

with catechin or pc B_4 at the indicated concentrations (25−100 μM) (**Figure 3B**).

Effects of Catechin and pc B_4 Treatment on GST Activity. GST is a phase 2 enzyme that is also critically involved in the detoxification of ROS (*25*−*27*). Treatment with 100 μM catechin for 24 h led to a significant 60% increase in cellular GST activity; 53 and 67% increases were observed, respectively, in cells treated with 50 and 100 μM pc B_4 (**Figure 4**).

Flow Cytometric Detection of Free-Radical Production. Adding 100 mU/mL XO in the presence of 0.5 mM xanthine to H9C2 cells at 37 °C for 30 min caused a 2.2-fold increase in DCF fluorescence, thus suggesting intracellular production of ROS. Pretreatment with 100 μM catechin or 100 μM pc B_4 for 24 h both dramatically lowered XO/xanthine-induced free-radical release (**Figure 5**).

Protective Effects of Catechin and pc B_4 on Oxidative-Stress-Induced Cardiac Cell Apoptosis. Exposure of cardiac H9C2 cells to 100 mU/mL XO in the presence of 0.5 mM xanthine for 30 min induced apoptosis, as demonstrated by the increase from 5 (control cells) to 27% (XO/xan-treated cells) in the number of Annexin V-FITC positive fluorescent cells. Pretreatment with 100 μM catechin or 100 μM pc B_4 for 24 h both led to significant decreases in the number of Annexin V-FITC positive fluorescent cells (**Figure 6E**). XO/xanthine induced the rapid changes in the nuclear morphology of H9C2 cells with heterogeneous intensity and chromatin condensation under a fluorescent microscope. Control cells were shown as round-shaped nuclei with homogeneous fluorescence intensity. Pretreatment of 100 μM catechin or 100 μM pc B_4 significantly protected cells from morphological changes by XO/xanthine (**Figure 7**).

DISCUSSION

Grape seed polyphenols mainly included proanthocyanidins (*28*). Generally the dimer- and trimer-proanthocyanidins, referred to as extractable proanthocyanidins or bioflavonoids, have been shown to be highly bioavailable chemicals and to provide excellent health benefits (*12*). These low-molecular proanthocyanidins are also known as sustained-release antioxidants and can remain in the plasma and tissues for up to 7−10 days and exert antioxidant properties, which is mechanistically different from other soluble antioxidants (*29*). Using gel chromatography and semipreparative HPLC, 11 pure individual phenolic compounds were separated from GSE. Epicatechin lactone A and B and catechin lactone are beyond the usual varieties of compounds from GSE, which are presumed oxidative derivatives of epicatechin and catechin after analyzing the homology of their structures. In previous studies, we observed that catechin and pc B_4 had substantial antioxidant activities, which prevent the DNA damage induced by ROS in mice spleen cells (*16*).

ROS, including $O_2^{\cdot -}$ and H_2O_2, are common products formed during the normal cellular aerobic metabolism and/or under certain pathophysiological conditions (*30*−*33*). Due largely to the detrimental nature of ROS, mammalian cells have evolved a number of antioxidant enzymes to protect against oxidative cell damage. Among the cellular antioxidant enzymes, SOD, catalase, GPx, and GR have been studied extensively. SOD catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2. Because H_2O_2 is still harmful to cells, catalase and GPx further catalyze the decomposition of H_2O_2 to H_2O. In the reaction catalayzed by GPx, GSH is oxidized to GSSG, which can then be reduced back to GSH by GR. Thus, the coordinate actions of various cellular antioxidant enzymes ensure the effective detoxification of ROS.

A series of studies were conducted using GSE to demonstrate its cardioprotective ability in animals and humans. GSE supplementation improved cardiac functional assessment including post-ischemic left ventricular function, reduced myocardial infarct size, reduced VF and VT, decreased the amount of ROS, and reduced malondialdehyde (MDA) formation in the heart perfusate (*34*). Interaction with cellular antioxidants/antioxidant enzymes is considered to evaluate the therapeutic potential of a given antioxidant (*12*). In this report, we demonstrated that incubation of cardiomyocytes with low micromolar concentrations of two major phenolics in GSE (catechin and pc B_4) resulted in significant induction of a scope of cellular antioxidant enzymes, including SOD, catalase, GSH, and GST, in a concentration-dependent fashion. Induction of these antioxidant enzymes by catechin or pc B_4 in cardiac cells has not been previously reported in the literature.

Several studies have indicated that apoptosis occurs in the human heart during the end stage of cardiac failure or acute myocardial infarction, which suggests that apoptosis is involved in cardiovascular disease (*35*). Multiple lines of evidence support the role of ROS in apoptotic cell death. A direct role of oxygen free radicals has also been implicated in the pathogenesis of apoptosis (*36*). It has been demonstrated that SOD or an expression vector containing SOD cDNA can delay apoptotic cell death. Injection of antisense SOD expression vector into the neurons decreased the amount of SOD simultaneously delaying apoptosis (*37*). In view of the ability of catechin and pc B_4 treatment to increase SOD, catalase, GSH, and GST, which are important cellular defenses against oxidative injury, we investigated whether the induction of the above antioxidant enzymes led to protection against ROS-mediated cardiac cell apoptosis. The results clearly showed that pretreatment of H9C2 cells with catechin or pc B_4 resulted in a marked protection against XO/xanthine-mediated intracellular accumulation of ROS and cell apoptosis. XO has been extensively implicated in the pathogenesis of various cardiovascular diseases, including ischemia−reperfusion injury (*38*).

As mentioned earlier, SOD, CAT, and GPx/GSH are critical cellular defenses involved in the detoxification of $O_2^{\cdot -}$ and H_2O_2,

and they have been demonstrated to protect against myocardial ischemia−reperfusion injury as well as drug-induced oxidative cardiotoxicity in animals (*39*, *40*). The simultaneous induction of SOD, catalase, and GSH by catechin or pc B_4 may thus largely contribute to the increased resistance of the catechin- or pc B_4-pretreated H9C2 cells to XO/xanthine-mediated cell apoptosis as well as intracellular ROS accumulation. 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 (*41*). A large number of studies show 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 (*26*, *27*). Accordingly, the induction of GST by catechin or pc B_4 in H9C2 cells may contribute partially to the increased resistance of the catechin- or pc B_4-pretreated cells to XO/xanthine-elicited injury. At 100 μM dose, catechin or pc B_4 blocked the free-radical production and simultaneously reduced the appearance of the apoptotic cardiomyocytes. But results were a little beyond anticipation, since pc B_4 did not show a significantly better effect than catechin; this was not consistent with its double number of phenolic hydroxyl groups. The protecting effects in cells should relate positively to antioxidant activities in a non-cellular environment in theory, but it is not plausible to take it for granted. After all, the absorbability and actual concentrations of the compounds in cells also can influence the effect.

In conclusion, our experiments confirm that a number of antioxidant enzymes in cultured cardiomyocytes can be induced by low micromolar concentrations of two major polyphenols isolated from GSE (catechin and pc B_4) and that this chemically mediated up-regulation of cellular defenses is accompanied by a markedly increased resistance to cardiac cell apoptosis elicited by ROS. As mentioned above, efficient detoxification of ROS requires the coordinate actions of various cellular antioxidant enzymes. Accordingly, simultaneous induction of a scope of key cellular antioxidant enzymes by grape seed polyphenols in cardiovascular cells may be an important mechanism underlying the protective effects of GSE observed with various forms of cardiovascular disorders.

ABBREVIATION USED

BSA, bovine serum albumin; DCFH, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GPx, glutathione peroxidase; GSE, grape seed extract; GSH, reduced glutathione; GSP, grape seed polyphenols; GST, glutathione S-transferase; MDA, malondialdehyde; pc B_4, proanthocyanidin B_4; PI, propidium iodide; PMSF, phenylmethyl sulfonyl fluoride; ROS, reactive oxygen species; SOD, superoxide dismutase; VF, ventricular fibrillation; VT, ventricular tachycardia; XO, xanthine oxidase.

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