Procyanidins from grape seeds protect against phorbol ester-induced oxidative cellular and genotoxic damage[1]

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ABSTRACT

AIM: To evaluate the inhibitory effects of *Vitis vinifera* procyanidins (PAs) on carcinogen-induced oxidative stress. **METHODS:** The single cell gel electrophoresis technique (comet assay) was employed to detect DNA damage induced by the carcinogen phorbol-12-myristate-13-acetate (PMA). The release of hydrogen peroxidase from polymorphonuclear leukocytes (PMNs) was assayed by the horseradish peroxidase-mediated oxidation of phenol red. The microplate assay was used to detect the presence of oxidative products by means of 2',7'-dichlorofluorescin-diacetate (DCFH-DA). The superoxide dismutase (SOD) activity of liver mitochondria was assayed, based on the ability of SOD to inhibit the generation of superoxidate anions by the xanthine-xanthine oxidase system. The malondialdehyde (MDA) level was determined by the thiobarbituric acid (TBA) assay. **RESULTS:** DNA of NIH3T3 cells was significantly damaged after addition of PMA. The length of the comet tail was observed ,while in normal cells the comet tail could not be observed. PAs showed significant protective effects on carcinogen PMA-induced DNA damage. Through assessment of DCFH-DA oxidation, PAs were shown to inhibit the PMA-induced release of hydrogen peroxide by PMNs, and to inhibit respiratory burst activity in NIH3T3 mouse fibroblasts. *Ex vivo* study showed that serum from rats administered with PAs displayed similar effects in a dose-dependent manner. In addition, PAs suppressed liver mitochondrial lipid peroxidation induced by PMA. PAs protected the activity of SOD and decreased the level of MDA in liver mitochondria damaged by PMA. **CONCLUSION:** Dietary PAs from grape seeds protect against carcinogen-induced oxidative cellular and genotoxic damage.

INTRODUCTION

Cancer, currently the second leading cause of death in the western world, may in several decades outrank cardiovascular disease as the highest ranking cause of death. The activated oxygen theory is one of the major theories of carcinogenesis. The human diet contains a great variety of natural mutagens and carcinogens, as well as many natural antimutagens and anticarcinogens. Many of these mutagens and carcinogens may act through the generation of oxygen radicals. Oxygen radicals may also play a major role as endogenous initiators of degenerative processes, such as DNA damage, mutation and tumor promotion. Tumor pro-

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moters stimulate the induction of oxygen radicals by direct chemical generation and through the indirect activation or alteration of cellular sources (including membrane oxidases, peroxisomes, and electron transport chains in mitochondria and endoplasmic reticulum). Oxygen radicals cause damage to cells and could be possible contributing factors in the development of cancer[1].

Dietary intake of natural antioxidants could be an important aspect of the body's defense mechanism against these factors. Many antioxidants have been identified as anticarcinogens for protection against oxidative stress induced by free radicals and active oxygen species. Antioxidants exert their protective action either by suppressing the formation of free radicals or by scavenging free radicals. Antioxidants have a wide range of biological effects, including effects on tumor initiation, promotion and progression, cell proliferation and differentiation, as well as DNA repair, cell membrane stability and immune function, and may inhibit carcinogenesis[2].

Grapes are one of the most widely consumed fruits in the world. Moderate consumption of wine is associated with a reduced risk of cancer. Grape seeds are rich in dimers, trimers and other oligomers of flavan-3-ols, named procyanidins (PAs)[3,4]. There is a growing interest in the utilization of PAs for their dietary and pharmacological properties. Red grape seed extract containing PAs and other antioxidants is being used as a nutritional supplement by many health individuals[5,6]. It was suggested by some reports that PAs could be a potential cancer chemopreventive agent against various epithelial cancers in animal studies[7-9]. In addition to its chemopreventive benefits, PAs has been shown to exert anticancer effects against various human carcinoma cells in culture[10-13] including breast and prostate cancers, *etc*. However, the precise mechanism by which PAs mediates chemoprevention is not yet fully understood.

In this study, we investigated the mechanism of action of PAs which effectively protected against carcinogen-induced toxic effects in normal fibroblasts.

MATERIALS AND METHODS

Materials NIH3T3 mouse fibroblasts were grown in DMEM with 10 % FCS at 37 ºC, in 95 % air and 5 % CO_2 atmosphere in culture flasks. Sprague-Dawley (SD) rats and BALB/c mice were bred under specific pathogen-free (SPF) conditions, kept under standard laboratory conditions (temperature, 20-24 ºC; humidity, 50 %-60 %; 12 h light/12 h dark), and given a laboratory diet and water. Phorbol-12-myristate-13-acetate (PMA, Calbiochem, La Jolla, CA, USA) was dissolved in dimethylsulfoxide (Me_2SO) at a concentration of 10 μmol/L. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-aldrich, St Louis, MO, USA) was dissolved in ethanol at a concentration of 2.5 g/L and stored in the dark at -20 ºC. PAs (consisting primarily of oligomeric and polymeric PAs, Traco Labs, USA) was dissolved in PBS at a concentration of 12.5 g/L and stored at 4 ºC.

Single cell gel electrophoresis and scoring (Comet assay) The comet assay was carried out using the method described previously[14]. Briefly, cells were treated with PAs (final concentration 12.5 mg/L, 6.25 mg/L, 3.12 mg/L, 1.56 mg/L, respectively) and PMA (final concentration 1 μmol/L) for 40 min at 37 ºC, 5 % CO_2 atmosphere. Cell suspension (1×10^8/L) 10 μL of the were mixed with 65 μL of low melting point (LMP) agarose at 37 ºC. This mixture was placed on top of the previous layer of 0.5 % normal melting point (NMP) agarose on the slide, then covered with a coverslip and returned to 4 ºC until solid. The coverslip was gently removed and 75 μL NMP agarose was added onto the slide. The slide was then covered again with a coverslip and placed at 4 °C until the mixture was solid. The coverslip was then removed and the slide was placed in chilled lysis solution (Tris 10 mmol/L, pH 10.0, NaCl 2.5 mol/L, edetic acid 100 mmol/L, 1 % Triton X-100) at 4 ºC for 1 h. Electrophoresis was performed at 1 V/cm for 10 min in TBE buffer (1 % Tris base, 0.5 % boric acid, 0.093 % edetic acid), with a maximum current of 300 mA. The slide was neutralized by slowly washing it with neutralization buffer (0.4 mol/L Tris, pH 7.5). Cellular DNA was stained with ethidium bromide (20 g/L) and visualized under a fluorescence microscope (200× or 400× magnification) with an excitation filter of 515-560 nm and barrier filter of 590 nm. The damage profile of DNA was assessed by Leica Qwin software visual scoring of the tail length (from the center of the comet head to the end of the tail) of the comets randomly observed for at least 100 cells on each slide with.

Measurements of carcinogen-induced active oxygen species[15] Cells were suspended in Hanks' balanced salt solution (HBSS) to give a concentration of 10^8/L. Cell suspension 150 μL was added to 96-well flat bottom microtiter plates. Cells were treated with

PAs (final concentration 12.5, 25, 50, 100 μmol/L, respectively) and PMA (final concentration 1 μmol/L) for 40 min at 37 ºC, 5 % CO_2 atmosphere. Each sample was loaded with DCFH-DA. Fluorescence intensity was determined by the methods described previously[15].

Release of hydrogen peroxide (H_2O_2) from polymorphonuclear leukocytes (PMNs) stimulated by PMA Rat PMNs were isolated by the method of Boyum[16] with minor modifications. Briefly, fresh EDTA-anticoagulated blood was mixed at 1:1 with 0.9 % sodium chloride containing 3.0 % Dextran T-500 (Amersham Biosciences, Piscataway, NJ, USA), then incubated for 20 min at room temperature to sediment the erythrocytes. The resulting leukocyte-rich supernatant was centrifuged at 550×*g* for 10 min, and cells were resuspended in 35 mL of 0.9 % sodium chloride. The leukocyte suspension was underlayed with 10 mL of Ficoll-Paque PLUS (1.077 g/L, Amersham Biosciences, Piscataway, NJ, USA) and centrifuged for 25-30 min to separate the PMNs from peripheral blood mononuclear cells (PBMCs). After standard hypotonic lysis of erythrocytes (cells suspended in sterile water for 20 s and then mixed immediately with an equal volume of 1.7 % sodium chloride), purified PMNs were suspended in RPMI-1640 medium (GIBCO, Grand Island, NY, USA), buffered with 10 mmol/L Hepes, and incubated on ice until used. The entire procedure for the purification of PMNs was performed at room temperature, and all reagents used contained <10.0 ng/L endotoxin. PMNs in each preparation were enumerated visually on a hemocytometer in 2 % acetic acid, and slides were routinely prepared and stained with a modified Wright-Giemsa (Sigma-aldrich, St Louis, MO, USA). Each PMN preparation contained 95 %-98 % neutrophils, with the remaining cells being predominantly eosinophils.

Isolated PMNs were stimulated to produce superoxide anions by adding the PMA. Super oxide anion generation was measured by the horseradish peroxidase-mediated oxidation of phenol red[17]. Triplicate reaction mixtures containing PMNs (1×10^9/L) and PMA were incubated at 37 ºC with phenol red buffer containing phenol red 100 μg/L and horseradish peroxidase 50 mg/L (268 U/mg, Sigma-aldrich, St Louis, MO, USA) for 30 min in the presence or absence of PAs (final concentration 12.5, 25, 50, 100 μmol/L) (Tab 1). Retinoic acid (RA, final concentration 83.3 μmol/L) was used as a positive control, and Me_2SO as a negative control.

Tab 1. Reaction system for H_2O_2 generation.

Reagent	Blank tube	Control tube	Sample tube
PMNs/μL	50	50	50
Phenol red buffer/μL	900	800	800
PAs/μL	-	-	50
Solvent/μL	50	50	-
1 % PMA-Me_2SO/μL	-	100	100

Superoxide anion generation by PMNs was expressed as nanomoles of H_2O_2 per 5×10^6 PMNs.

***In vitro* study of the serum from rats administrated with PAs on H_2O_2 release from PMNs stimulated by PMA** Blood was collected from rats before or 40 min, 60 min, 120 min after oral administration of PA or retinoic acid. Retinoic acid was given at the dose of 1 g/kg and PA was given at doses of 0.54 g/kg, 1.08 g/kg, and 2.16 g/kg. Six rats were used in each group. Blood was centrifuged at 700×*g* for 15 min. The serum was inactivated at 56 ºC for 30 min, and stored at -20 ºC. While the release of H_2O_2 was being monitored, PA was replaced by the serum from rats administrated with PA or retinoic acid in the reaction system as described above.

Hepatic mitochondrial lipid peroxidation induced by PMA Mice liver mitochondria were isolated by modification of the method described previously[18]. Protein concentration was estimated by the Bradford assay as described previously[19]. The mitochondrial suspension (900 μL) was added to a test tube containing 50 μL PA or control solutions, vortexed gently, then incubated at 37 °C for 20 min. After incubation with 50 μL 1 % PMA at 37 ºC for 30 min, malondialdehyde (MDA) level was determined by thiobarbituric acid (TBA) assay as previous described[20]. The SOD activity was assayed on the basis of superoxide anions generated by the xanthine-xanthine oxidase system[21].

Statistical analysis Results were expressed as mean±SD. Statistical analysis was performed by the least-significant difference test, consisting of two steps. Firstly, an analysis of variance was performed. The null hypothesis was accepted for all numbers in which F was not significant at the level of $P<0.05$. Secondly, the sets of data in which F was significant were examined by the modified t-test, using $P<0.05$ as the critical limit.

RESULTS

Protection by grape seed PAs on PMA-induced DNA damage The results revealed that the DNA of NIH3T3 cells was significantly damaged after addition of PMA. The length of the comet tail after PMA treatment reached 34.24±7.95 μm, while in normal cells the comet tail could not be observed. PA showed significantly protective effects on carcinogen PMA-induced DNA damage and this effect was dose-dependent (Fig 1). At the concentration of 12.5 mg/L the comet tail length was 5.22±1.83 μm (P<0.01).

Inhibition by grape seed PAs on PMA-generated activated oxygen species (ROS) Results presented in Fig 2 showed that the basal DCF fluorescence was strongly increased in cells pretreated with PMA. Incubation of DCFH-loaded NIH3T3 with increasing concentrations of PA led to a concentration-dependent decrease in DCF fluorescence.

Inhibition of PAs on H_2O_2 release from PMNs induced by PMA The H_2O_2 concentration was measured by the horseradish peroxidase-mediated oxidation of phenol. The standard curve of H_2O_2 oxidation phenol red was OD=17.8C-0.159 (r=0.9942). The results showed that release of H_2O_2 from PMNs was increased after stimulation by PMA. Different concentrations of PAs could inhibit PMNs releasing H_2O_2 induced by PMA (Fig 3A). To test if PAs could still have the inhibitory effect on H_2O_2 release stimulated by PMA after metabolism by the body, we used the serum from rats administered with PAs to replace the original form of PAs. As shown in Fig 3B, the procyanidin metabolites could also inhibit PMNs releasing H_2O_2 induced by PMA. This inhibitory effect was dose- and time-dependent and persisted for at least 60 min after oral administration. These tests further confirmed that the inhibitory effect of PAs was effective *in vitro*.

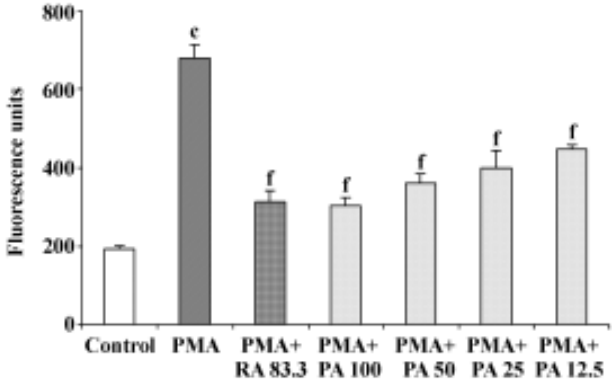

Fig 2. The basal DCF fluorescence was strongly increased in cells pretreated with PMA. Stimulation of DCFH-loaded NIH3T3 with increasing concentrations (mol/L) of PAs led to a concentration-dependent decrease in DCF fluorescence. Mean±SD. ^cP<0.01 *vs* control. ^fP<0.01 *vs* PMA group.

Inhibition of PAs on hepatic mitochondrial lipid peroxidation Normal hepatic mitochondria have high SOD activity and low level of MDA. Data presented in Fig 4 showed that PMA decreased the SOD activity and increased the MDA production of hepatic mitochondria. Preincubation of hepatic mitochondrial with PA markedly increased the SOD activity and decreased the MDA level. These results suggested that PA had protective effects against PMA-induced hepatic mitochondrial lipid peroxidation.

DISCUSSION

Carcinogenesis is a multistage process and often has a latency of many years or decades. Potential targets for chemoprevention have recently been identified.

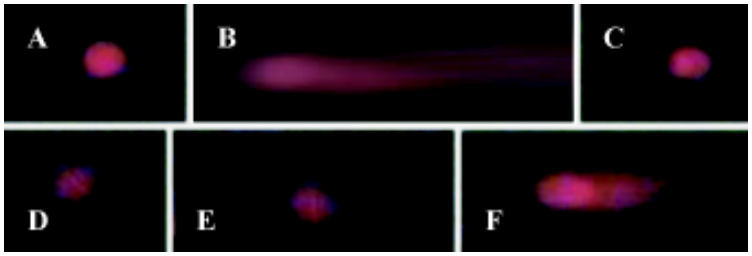

Fig 1. Significant differences of comet tail of NIH3T3 cells treated with or without PMA. Untreated cells were round, the DNA remains intact without any DNA migrated out of the cells (A). Cells were treated with PMA. DNA tail was much longer than the diameter of the nucleus (B). Cells treated with 12.5, 6.25, 3.12, and 1.56 mg/L PAs with a little DNA migrated out of the cell but the tail was shorter than the diameter of the cell, or the DNA tail length was similar to the diameter of the cell (C, D, E, F). Each figure represented the typical comet tail of the 100 observed cells.

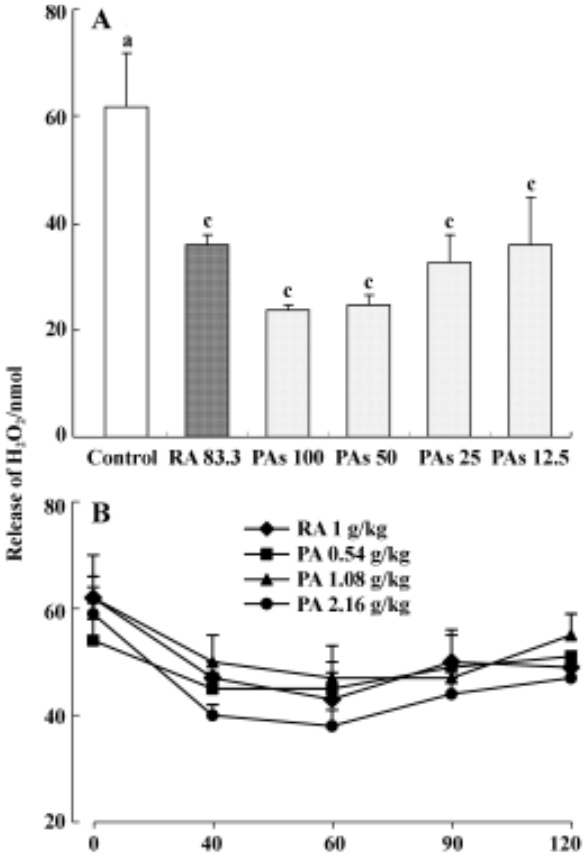

Fig 3. Inhibition of PAs on H_2O_2 release from PMNs induced by PMA. (A) PMA stimulated PMNs to release H_2O_2 and PAs at different concentrations (mol/L) inhibited the H_2O_2 release induced by PMA ($^cP<0.01$ *vs* control). (B) The serum from rats administered with PAs markedly decreased generation of H_2O_2 at the dose of 1.08 and 2.16 g/kg at the time point 40, 60, 90, and 120 min ($P<0.01$ *vs* the serum before treatment). At the dose of 0.54 g/kg this inhibitory effect also was observed but to a shortened period, at the time point 40 and 60 min ($P<0.01$ *vs* the serum before treatment).

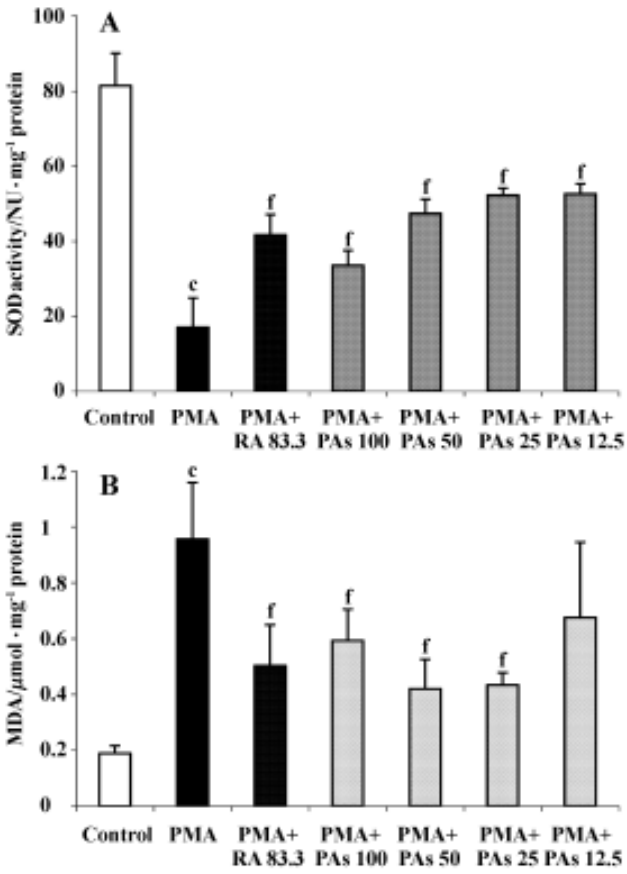

Fig 4. The protective effect of PAs on liver mitochondrial lipid peroxidation induced by PMA. PAs could protect PMA induced SOD activity decrease in mitochondria while inhibit the generation of MDA. Mean±SD. $^cP<0.01$ *vs* control, $^fP<0.01$ *vs* PMA group.

Many classes of agents including anti-estrogens, anti-inflammatories, antioxidants and other diet-derived agents have shown a great deal of promise. Agents derived from dietary source have an inherent appeal for chemoprevention aimed at healthy populations, as they can be taken for long periods of time with simple dietary modification and no apparent health risk. Many diets derived agents are currently being evaluated for potential chemopreventive activity.

PAs are compounds from grape seeds used for protection against oxidative stress induced by free radicals and active oxygen species. The phorbol ester PMA has been studied extensively in this regard and has been shown to cause oxidative stress as one of its mechanisms of tumor promotion. Polymorphonuclear leukocytes provide a useful system for exploring the roles of reactive oxygen species in mechanism of action. Through the assessment of oxidation of 2',7'-dichlorofluorescin-diacetate, it was determined that PAs inhibited the release of H_2O_2 from PMA-stimulated PMNs, and inhibited respiratory burst activity in NIH3T3 cells. PAs also protect hepatic mitochondrial lipid peroxidation damaged by PMA. The H_2O_2 releasing assay showed that PAs could inhibit H_2O_2 release from PMA-stimulated PMNs. *In vitro* study using the serum from rats administered with PAs also had the same effect. Donovan *et al* have recently reported that after a single meal containing a mixture of PAs, these compounds could not be detected in the plasma of rats. In contrast, these compounds are extensively metabolized to yield catechin and epicatechin[22]. It suggests that the *in vitro* antioxidation effect of PAs may be different from its *in*

vivo effect. It would be our interest to compare the effects of catechin and epicatechin to those observed with PA-treated rat serum.

Antioxidants act as free radical scavengers, binding with the free radicals to make them stable and benign, and thereby helping to limit damage. The cancer chemopreventive effects of PAs is specifically important since environmental pollutants, radiation, pesticides, and UV radiation exhibit the ability to produce enormous amount of free radicals which cause many diseases, including tumor promotion and cancer. Since PAs are already in human use as a dietary supplement for their multiple health benefits[23,24], our study suggests that PAs from grape seeds could protect against the carcinogen-induced oxidative cellular and genotoxic damage, and could be beneficial for cancer chemoprevention.

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