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Anthocyanins Induce the Activation of Phase II Enzymes through the Antioxidant Response Element Pathway against Oxidative Stress-Induced Apoptosis

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Reactive oxygen species (ROS)-induced cell damage is inevitable and severe and is involved in numerous diseases, including cancer. Reducing oxidative stress is one of the strategies of chemoprevention. Anthocyanins are naturally occurring flavonoids that show multiple benefits. We first pointed out the effects of anthocyanins in the contributions to activation of phase II antioxidant and detoxifying enzymes, chemopreventive potency, and involved transcriptional regulation. Our results obtained in rat liver Clone 9 cells showed that treatment of anthocyanins leads to positive effects on elevating the antioxidant capacity, including activated expression of glutathione-related enzymes (glutathione reductase, glutathione peroxidase, and glutathione *S*-transferase) and recruited GSH content. In addition, the activity of NAD(P)H: quinone oxidoreductase (NQO1) was also promoted under the treatment of anthocyanin. This influential functions as the defense system against programmed cell death induced by H_2O_2. The capacity for induction of luciferase expression by anthocyanins in cells transfected with rat *nqo1*-promoter constructed plasmid was further investigated; we found that the molecular mechanism is related to the activation of antioxidant response element (ARE) upstream of genes that are involved in antioxidation and detoxification. Our data suggest that natural anthocyanins are recommended as chemopreventive phytochemicals and could stimulate the antioxidant system to resist oxidant-induced injury. And, more important, the promoting effect of anthocyanins on ARE-regulated phase II enzyme expression seems to be a critical point in modulating the defense system against oxidative stress.

KEYWORDS: Anthocyanins; reactive oxygen species; chemoprevention; antioxidant response element (ARE); apoptosis

INTRODUCTION

Anthocyanins are glycosides of anthocyanidins universally associated with attractive, colorful, and flavorful fruits. Recently, anthocyanins have attracted lots of attention because of their potential biological and pharmacology benefits, such as antioxidant (*1*), anti-inflammatory (*2*), and antitumor properties (*3*), and its ability to reduce the risk of cardiovascular diseases (*4*). Animal studies have also demonstrated that feeding with anthocyanin-rich extract protected against *tert*-butyl hydroperoxide-induced hepatic toxicity (*5*) and decreased lipid peroxidation and DNA damage in vitamin E-depleted rats (*6*). More recently, anthocyanins have been shown to be an effective chemopreventive agent against 1,2-dimethyhydrazine- and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced mammary carcinogenesis in rats (*7*). In addition, anthocyanins can be directly absorbed and distributed to the blood (*8*) and can be incorporated into cellular components, both in the plasma membrane and in the cytosol (*9, 10*). Extensive studies indicate that anthocyanins have strong free radical scavenging and antioxidant activity (*11*), suggesting that they play an important role in preventing mutagenesis and carcinogenesis (*12*).

Cells are constantly exposed to toxic, mutagenic, and carcinogenic agents that make up reactive intermediates, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and other reactive electrophilic metabolites, that result in the destruction of physiological function. A major protective strategy against oxidizing substances that are able to damage cells and initiate carcinogenesis is through the induction of phase II detoxifying and antioxidant enzymes by substances that show chemopreventive effects (*13*). Recently, the redox-sensitive transcription factor NF-E2-related factor-2 (Nrf2) has been demonstrated to be a critical transcription factor that binds to

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the antioxidant response element (ARE) or stress response element (StRE) in the promoter region of a number of genes that code for antioxidative and detoxifying enzymes in several types of cells and tissues (*14*). Activation of phase II detoxifying enzymes, such as UDP-glucuronyl transferase (UGT), glutathione S-transferase (GST), and NAD(P)H:quinone oxidoreductase (QR), by phytochemicals and nutraceuticals results in the detoxification of carcinogens and represents one mechanism of their anticarcinogenic effects (*15, 16*).

Although the multifunctions of anthocyanins have been widely discussed, their influences on phase II and antioxidant enzymes as well as involved mechanisms have never been revealed. In the present study, we evaluated the relationship between antioxidant capacity of anthocyanins and their efficacy on the activation of phase II antioxidant (glutathione-dependent) and detoxifying (quinon-oxido metabolizing) enzymes in hepatocytes (Clone 9 cells). The chemopreventive effects of anthocyanins on oxidative stress-induced cell death and the ARE-regulated signal transduction pathway were also investigated.

MATERIALS AND METHODS

Reagents. Anthocyanidins (cyanidin chloride, delphinidin chloride, malvidin chloride, pelargonidin chloride, peonidin chloride, and petunidin chloride), anthocyanins (cyanidin-3-O-glucoside chloride, malvidin-3-O-glucoside chloride (oenin), pelargonidin-3-O-glucoside chloride, and peonidine-3-O-glucoside chloride) were obtained from Extrasynthese Corporation (Genay Cedex, France). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT), a cell viability detection chemical, was obtained from Sigma (St. Louis, MO) and Takara Bio Corporation (Shiga, Japan), respectively. Annexin V:FITC assay kit was obtained from Serotec Corporation (Raleigh, NC). A CaspaTag Caspase-3 (DEVD) activity kit was purchased from Serologicals Corporation (Norcross, GA). Tri_{Zol} RNA extraction reagent and GenoMaker genomic DNA isolation reagent were purchased from MDBio Corporation (Frederick, MD) and Blossom Corporation (Taipei, Taiwan). All analytical reagents were of the highest grade.

Cell Culture. The rat hepatocyte Clone 9 cell line (ATCC number: CRL-1439) was purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan), and cells were maintained in minimum essential Ham's F-12K medium with 2% fetal bovine serum and 100 units/mL penicillin/streptomycin antibiotics and incubated at 37 °C with 5% CO_2.

Enzymatic Activity Assays. The glutathione peroxidase (GPx) activity was determined spectrophotometrically according to the method described previously (*17*). The following solutions were pipetted into a cuvette: 0.1 mL of homogenate and 0.8 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GSH reductase, and 1 mM GSH. This mixture was preincubated for 5 min at 37 °C. Thereafter, the overall reaction was initiated by adding 0.1 mL of 2.5 mM H_2O_2. Enzyme activity was calculated by the change of the absorbance value at 340 nm for 5 min. The nonenzymatic reaction rate was correspondingly assayed by replacing the homogenate sample with potassium phosphate buffer. GPx activity could be expressed as nanomoles of NADPH per minute per milligram of protein.

The glutathione reductase (GR) assay monitored the oxidation of NADPH consumed in the reduction of glutathione disulfide (GSSG) by the change in absorbance at 340 nm as described previously (*18*). The following solutions were pipetted into a 1 cm spectrophotometric cuvette: 0.1 mL of homogenate and 0.9 mL of 0.10 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH. This mixture was preincubated for 5 min at 37 °C. GR activity was calculated by the change in the absorbance value at 340 nm for 5 min; GR activity was expressed as nanomoles of NADPH per minute per milligram of protein.

Glutathione S-transferase (GST) was measured according the method previously described (*19*) and with a slight modification. One hundred microliters of homogenate was reacted with 880 μL of potassium phosphate buffer (100 mM, pH 6.5, containing 1 mM GSH) and well-mixed with 20 μL of 1-chloro-2,4-dinitrobenzene (CDNB). GST activity was calculated by the change in the absorbance value at 340 nm for 3 min and expressed as nanomoles of CDNB-GSH conjugated formation per minute per milligram of protein.

NAD(P)H:quinone oxidoreductase 1 (NQO1) was measured in 1 mL of reagent as reported previously (*20*) with slight modifications. Reduction of cytochrome *c* (50 μM) in the presence of liver homogenate, 10 μM menadione, and 1 mM NADPH was monitored for 2 min. The reactions were carried out in 100 mM potassium phosphate buffer, pH 7.7, containing 0.04% Triton X-100 at 25 °C. Activity of dicumarol-inhibitable menadione reductase was determined spectrophotometrically at 550 nm.

GSH/GSSG was evaluated by the method from previous studies (*21, 22*) with a slight modification. One-tenth of one milliliter of the homogenate was mixed well with 0.9 mL of Tris/EDTA solution (0.4 M, pH 8.9), and the reaction was started after 20 μL of 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) was added, followed by co-incubation for 5 min at room temperature. The concentration of reduced GSH was calculated by monitoring photometrically at 412 nm. For the GSSG assay, 100 μL of homogenate was incubated at room temperature with 200 μL of *N*-ethylmaleimide solution (40 mM) for 30 min. Seven hundred microliters of 0.1 M NaOH was then added to the mixture. Finally, 100 μL of this mixture was taken for the measurement of GSSG using the procedure described above for GSH assay.

Cell Viability and Apoptosis Assay. Cells that seeded in Petri dish were preincubated with anthocyanins for 24 h following exposure to H_2O_2 for indicated time. Cytotoxicity was evaluated by analysis of lactate dehydrogenase (LDH) activity using the LDH detection kit. To examine early programmed cell death, cells were stained with an Annexin V:FITC Assay kit (Serotec, Oxford, U.K.) using FITC-conjugated Annexin V and propidium iodide (PI) reagents according to the manufacturer's protocol. The stained cells were analyzed by a laser flow cytometer (FACS Calibur). The fluorescence intensity is expressed in a log scale. To determine the expression of apoptosis execution, we investigated the downstream caspase cascade. Caspase-3 activity was measured with the Caspase-3 assay kit (Intergen Co., Purchase, NY) according to the manufacturer's instruction. Cells (1×10^6 cells/mL) from different treatments were collected and incubated with the Working Dilution reagent at 37 °C for 1 h, and after being washed with the wash buffer, the cells were resuspended with PBS and the fluorescence intensity was detected by the fluorescence spectrophotometer with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Plasmid Construction. Briefly, the pGL3-Basic luciferase reporter vector (Promega Corporation, Madison, WI) was digested with *Sac*I and *Hind*III and harvested by 0.8% agarose gel electrophoresis. The ~1000 bp of the rat *nqo1* 5′-upstream region containing the antioxidant response elementlike (AREL) segment is shown in **Figure 5** (GeneID: 24314, and the full sequence was further extended to created 5′-terminal *Sac*I and 3′-terminal *Hind*III restriction sites). The genomic DNA of rat liver Clone 9 cells was purified with commercial kit (Bio Basic Inc., Markham Ontario, Canada) and used as template. The amplified double-stranded DNA was digested with the restriction enzymes *Sac*I/ *Hind*III (Promega, Madison, WI) and inserted into the prepared pGL3 basic vector. The resulting construct containing the ARE from the rat *nqo1* gene was designated pGL3 (rNQO1-ARE) vector. Plasmid DNA was purified using the Endotoxin-free Ultrapure Plasmid DNA purification kit (Bay Gene, Inc., San Francisco, CA). Prior to use, the orientation, integrity and sequence of the cloned construct was confirmed by DNA sequencing.

Transfection and Luciferase Reporter Gene Assays. Cells were transiently transfected with constructed plasmids by TransFast Transfection reagent (Promega) according to the manufacturer's guidelines. 5×10^4 cells/well were seeded into 24-well microplates 24 h before transfection. Cells were transfected with the *nqo1*-ARE promoter reporter construct (1 μg) with or without cotransfection of 0.1 μg of

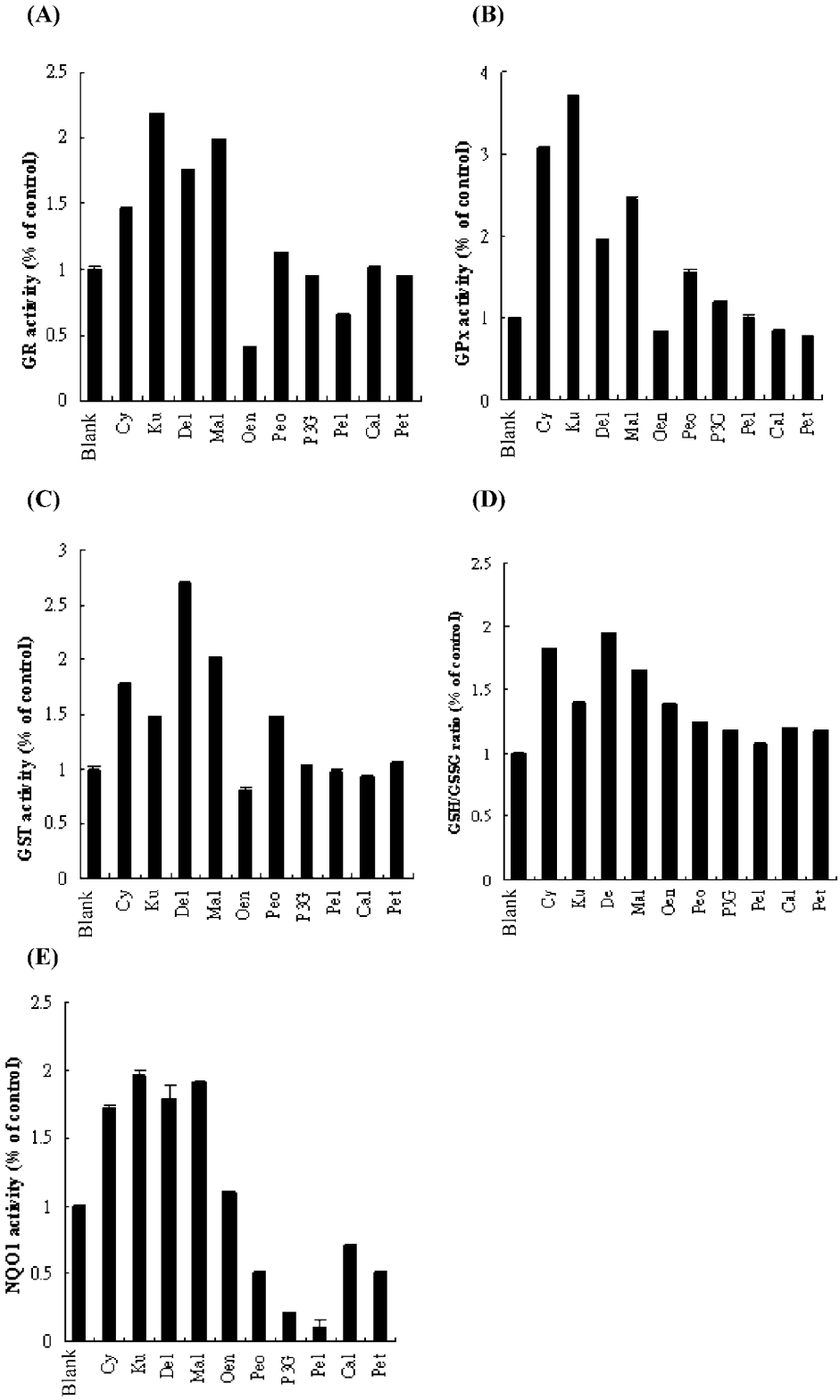
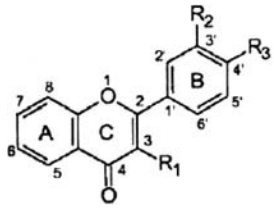


Figure 1. Effects of anthocyanins on the activity of antioxidant enzymes (**A**) glutathione reductase (GR), (**B**) glutathione peroxidase (GPx), (**C**) glutathione S-transferase (GST), (**D**) GSH/GSSG ratio, and (**E**) NAD(P)H: quinone oxidoreductase 1 (NQO1) in rat Clone 9 cells. Cells were treated with 10 different types of anthocyanins (50 μM) independently for 24 h. Abbreviation: Cy, cyanidin; Ku, kuromanin; Del, delphinidin; Mal, malvidin; Oen, oenin; Peo, peonidin); P3G, peonidin-3-O-glucoside); Pel, pelargonidin; Cal, callistephin; Pet, petunidin.

pRL-TK plasmid that was used as an internal control. After being washed, cells were treated with H_2O_2 for 3 h and washed with cold phosphate buffer solution (PBS) twice. Cells were treated immediately with or without anthocyanins for 24 h. Finally, cells were washed twice with PBS and then lysed in 100 μL of Passive Lysis Buffer (Promega); Firefly and Renilla luciferase activity was excited by using the Dual-Luciferase Reporter Assay System (Promega) and examined with FLUO star galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg, Germany). All results were normalized to Renilla luciferase internal control luminescence.

Statistical Analysis. Results are presented as mean ± SD. The statistical analyses were performed by two-way analysis of variance

Table 1. Chemical Characteristics and Antioxidant Capacity of Anthocyanins



compd	free OH-substituents	glycosylated position	TEAC (mM)
cyanidin	3, 5, 7, 3′, 4′		3.8 ± 0.3
kuromanin	5, 7, 3′, 4′	3-glu	1.9 ± 0.1
delphinidin	3, 5, 7, 3′, 4′, 5′		3.6 ± 0.9
malvidin	3, 5, 7, 4′		2.9 ± 0.7
oenin	5, 7, 4′	3′, 5′-diOMe, 3-glu	1.7 ± 0.2
peonidin	3, 5, 7, 4′	3′-OMe	1.3 ± 0.01
peonidin-3-glucoside	5, 7, 4′	3-glu	1.2 ± 0.1
pelargonidin	3, 5, 7, 4′		1.3 ± 0.03
callistephin	5, 7, 4′	3-glu	1.3 ± 0.1
petunidin	3, 5, 7, 3′, 4′	5-OMe	1.4 ± 0.2

(ANOVA) for multiple comparisons. $p < 0.05$ was taken as an indication of significant differences between means.

RESULTS

Correlation between Antioxidant Capacity of Anthocyanins and Their Induction Effects on Phase II Enzymes. To evaluate the activating effects of anthocyanins on antioxidant and detoxifying enzymes, we treated rat liver cells (Clone 9) with 10 kinds of naturally occurring anthocyanins for 24 h. As shown in **Figure 1**, the expression of glutathione-related enzymes, including GR, GPx, and GST, was activated by treating cells with the anthocyanins, and the ratio of GSH/GSSG was also elevated. Furthermore, the activity of the detoxifying enzyme NQO1 was changed, as well. Before the antioxidant activity assay, the cell viability has already been investigated, and there was no significant ($p < 0.05$) cytotoxicity of every anthocyanin that treated cells for 24 h at the same concentration (50 μM, data not shown). And the data shown in **Table 1** suggested that all anthocyanins exhibited potent antioxidant capacity. In our experimental conditions, not all anthocyanins showed positive effects on the activation of antioxidant and detoxifying enzymes. Overall, cyanidin, kuromanin, delphinidin, and malvidin showed much positive representation and were used for further examination.

Chemopreventive Effect of Anthocyanins Against Oxidative Stress-Induced Cell Death. Because oxidative stress results in inevitable damage during metabolism, we investigated whether pretreatment with anthocyanins represses cell death induced by H_2O_2. Cells were pretreated with four kinds of anthocyanins for 24 h, respectively, and were further treated with of H_2O_2 for 12 h. Cell viability was evaluated by LDH assay. As shown in **Figure 2**, the cytotoxicity-induced by $H_2O_2$was significantly increased compared to the control group. Pretreatment of anthocyanins suppressed the damaged triggered by H_2O_2. Among four anthocyanins, cyanidin, and delphinidin showed more inhibitory effects on oxidative stress-induced cytotoxicity.

Anthocyanins Repress H_2O_2-Induced Early Apoptosis. The evidence that anthocyanins suppress H_2O_2-induced cytotoxicity prompted us to further examine whether anthocyanins prevent the appearance of apoptosis induced by H_2O_2. Cells were pretreated with four kinds of anthocyanins for 24 h, which was followed by treatment of cells with H_2O_2 for another 6 h. The cell population was then evaluated by flow cytometry. As shown in **Figure 3**, cells treated with H_2O_2 significantly ($p < 0.05$) increased the level of apoptosis as compared with the blank group. Cells that were pretreated with anthocyanins suppressed the phenomenon of early apoptosis, and maximal inhibition in the level of early apoptosis was observed in cyanidin-treated cells (about 60%), followed by treatment of delphinidin (about 50%).

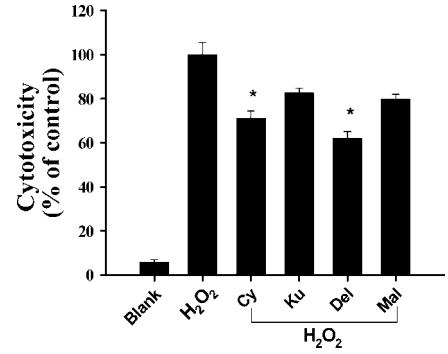
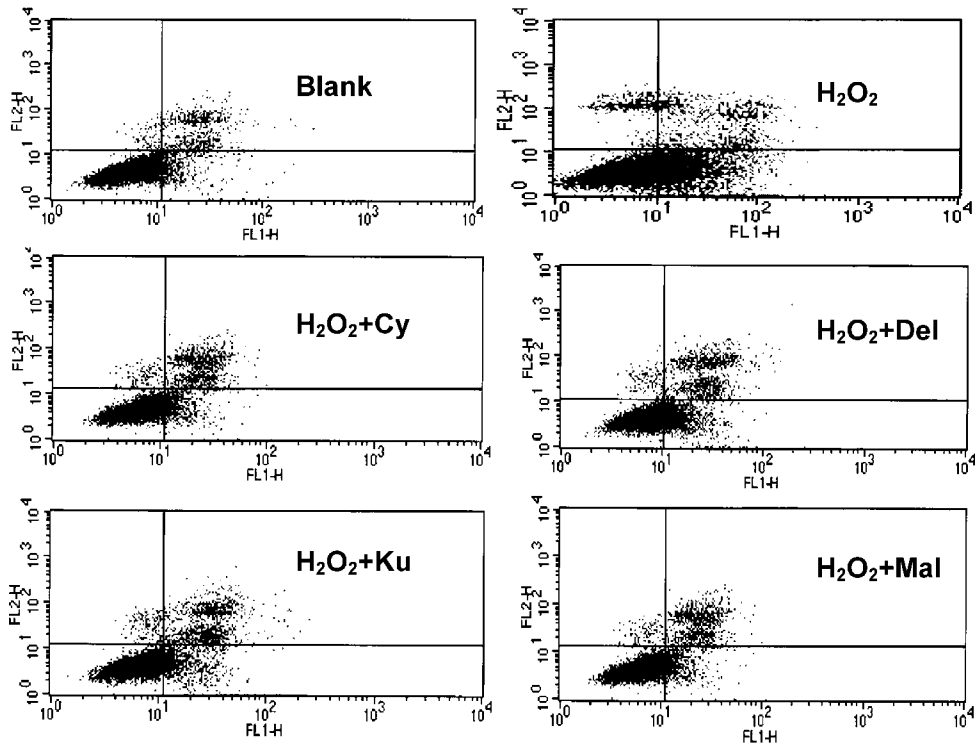


Figure 2. Inhibitory effect of anthocyanins on H_2O_2-induced cytotoxicity. Cells were treated with four kinds of anthocyanins (50 μM) independently for 24 h, followed by treatment with H_2O_2 (70 μM) for 12 h. Cell viability was evaluated by LDH detection kit. Data are means ± SD from three independent experiments. Statistical analysis was performed using ANOVA ($p < 0.05$) and comparing values from H_2O_2-treated cells with or without the pretreatment of anthocyanins.

Anthocyanins Mediate Inhibition of Expression of Caspase-3. To further examination whether anthocyanins suppress the activation of caspase and then retard programmed cell death,we investigated the downstream executor of apoptosis, caspase-3. As shown in **Figure 4**, caspase-3 activity in cells treated with H_2O_2 for 3 h was significantly ($p < 0.05$) higher than that of the blank group. Pretreatment with anthocyanins repressed the activity of caspase-3 triggered by excessively oxidative stress; interestingly, among four anthocyanins, cyanidin and delphinidin showed more inhibitory effects on the activation of caspase-3 induced by H_2O_2.

Anthocyanins Induce ARE-Regulated Activation of Phase II Enzymes. The results obtained previously suggested that anthocyanins activated the expression of antioxidant and detoxifying enzymes and suppressed H_2O_2-induced apoptosis and cytotoxicity. However, the relationship between the antioxidant-activity-inducing phenomenon of anthocyanin and its inhibitory effect on oxidative-stress-triggered apoptosis is not clear. Cells were transiently transfected with the *nqo1* plasmid containing the 5′ promoter segment linked to the luciferase gene. Cells were then treated with quercetin (Quer) or four anthocyanins for another 24 h, respectively, which was followed by incubating with H_2O_2 (70 μM) for 3 h. Luminescence activity was then examined. As shown in **Figure 6**, the cells that transfected with the *nqo1*-promoter containing plasmid displayed constitutive promoter activity under basal maintenance (blank group). Interestingly, we found that the level of luminescence was significantly ($p < 0.05$) increased following treatment with flavonoids, and more importantly, cell viability was also improved compared with the group of H_2O_2-treated alone (data not shown). The overall results indicated that anthocyanins not only play the role of free radical scavengers but are also the phase II enzyme inducer through the ARE signal transduction pathway and protect cells against oxidative-stress-induced apoptosis.

(A)



(B)

Treatment	Early apoptosis (% of total cells)
Blank/H_2O_2	6±2.4*/30±3.3
H_2O_2+Cy	10±2.9*
H_2O_2+Ku	21±3.1
H_2O_2+Del	15±4.2*
H_2O_2+Mal	19±1.9*

Figure 3. Inhibitory effect of anthocyanins on H_2O_2-induced apoptosis. Cells were treated with four kinds of anthocyanins (50 μM) independently for 24 h, followed by treatment with H_2O_2 (70 μM) for 6 h. Apoptotic cells were analyzed using an Annexin V/PI staining assay. (**A**) Flow cytometric illustration and (**B**) statistics by CELL QuestTM software. Statistical analysis was performed using ANOVA ($p < 0.05$) and comparing values from H_2O_2-treated cells with or without pretreatment by anthocyanins. Results are representative of three separate experiments. *, $p < 0.05$ is significantly different from group of H_2O_2-treated alone.

DISCUSSION

In the present study, we first pointed out that naturally occurring anthocyanins protect hepatocytes against H_2O_2-induced apoptosis and the involved molecular mechanism. The Clone 9 normal liver cell line has been extensively used for studies of carcinogenesis in vitro and clonal assays for screening sera and other nutritional supplements (*23, 24*) and was used as cell model to investigate the up-regulatory effects of anthocyanins on the activity of detoxifying and antioxidant enzymes in this study.

It has been extensively discussed that supplementation of functional food improves health and activity. Dietary antioxidant phytochemicals, most notably phenolic acids and flavonoids that are abundant in fruits and vegetables, show multifunctional benefits to human beings. The molecular relationship between consumption of antioxidants and elevation of antioxidant ability was not completely clear until the identification of the antioxidant response element (ARE), which is controlled by nuclear factor erythroid 2-related factor 2 (Nrf2) (*25*). It has been confirmed that this key factor plays a critical role in the regulation of antioxidant products, including antioxidant and detoxifying enzymes and endogenous antioxidants, such as glutathione, one of the abundant antioxidants that participate in protecting cells against oxidative stress.

In this study, we first evaluated the induction effect of naturally occurring antioxidants, the anthocyanins, on the activity of phase II enzymes. We focused on the glutathione (GSH)-related enzymes, including glutathione *S*-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GRd). GST is involved in the detoxification of xenobiotics through catalyzing the conjugation of GSH and considered as one of the key enzymes associated with chemoprevention. GPx, a

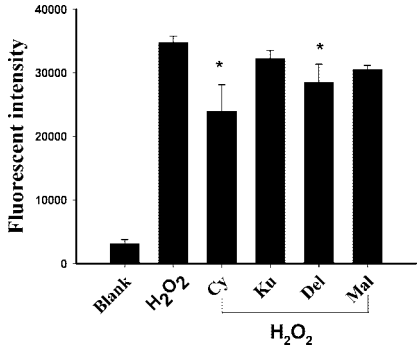


Figure 4. Inhibitory effect of anthocyanins on H_2O_2-induced caspase-3 activation in Clone 9 cells. Cells were treated with four kinds of anthocyanins (50 μM) independently for 24 h, followed by treatment with H_2O_2 (70 μM) for 6 h. The caspase-3 activity was analyzed with a caspase activity detection kit. Data are the means ± SD from three independent experiments. Bars with an asterisk are significantly different ($p < 0.05$) from group of H_2O_2-teated alone.

selenium-dependent enzyme, catalyzes the reduction of peroxides and then formation of selenoles and is suggested to act as barrier against hydroperoxide attack (*26*). Both GST and GPx are involved in eliminating peroxides that are formed during metabolism. GRd regulates the equivalent of reduced GSH and oxidized GSH (GSSG), and the ratio of GSH/GSSG is a well-known index of oxidative stress. The activation of GRd plays an important role in elevating the concentration of GSH, which maintains the oxido-redox status in the organism. Moreover, NAD(P)H:quinone oxidoreductase 1 (NQO1), a well-defined Nrf2-ARE regulated enzyme also known as DT-diaphorase or quinone reductase, is another cellular enzyme that protects cells against deleterious reactive semiquinones by converting exogenous quinones into hydroquinones through a two-electron reduction pathway. As shown in **Figure 1** and **Table 1**, they seem to reveal a positive correlation between antioxidant capacity and promoting an effect on enzymatic activity. This finding might also speak response to the results that showed that phytochemicals exhibit higher activity and have greater influence (*27*).

During metabolism, reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion, and hydroxyl radical, etc, are produced irreversibly (*28*). These active molecules will attack functional components like protein, lipid, and genetic material. The rise in such metabolic intermediates will result in the elevation of a hazardous condition called oxidative stress that might lead to dysfunction, mutation, and disease. Oxidative stress is precipitated in lots of metabolic syndrome, such as cardiovascular disease, neuron degeneration, and diabetes mellitus (DM), to name a few (*29*). The report showed that the strawberry and its anthocyanins reduce the oxidative-stress-induced apoptosis in neuronlike cells (*30*). Thus, how to reduce the damage induced by oxidative stress is extensively investigated and attracts much attention. As shown in our data, among 10 anthocyanins tested, cyanidin, kuromanin, cyanidin, and malvidin showed higher efficacy in their antioxidant capacity and induction of phase II enzymes. We further evaluated the protective effect of these active compounds against oxidative-stress-induced cell damage. H_2O_2 is one of oxidative toxicants that with longer life-span than other free radicals in organism. H_2O_2 is always generated in peroxisomes that function to rid the cell of toxic substances. However, it is also an active carcinogen that will induce serious damage to the cellular membrane and nucleus. Our result indicates that cyanidin shows greater inhibitory effect on the activity of Caspase-3 and is similar to the data for PI/Annexin IV double staining. The above mentioned evidence could reveal the biological potency and chemopreventive benefit of anthocyanins that are abundant in berries and red wine. Apoptosis is one mode of cell death that is triggered by several factors. Evidence reveals that antioxidant capacity is also a critical element that plays an important role against oxidative stress-induced apoptosis. Supplementation of phytochemicals and nutraceuticals not only increases antioxidant ability through contribution of their own natural antioxidant endowment but also stimulates the expression of antioxidant and detoxifying genes.

It has been well-defined that chemopreventive blocking reagents, such as phenolic antioxidants, dithiolethiones, isothiocyanates, etc., selectively induce the activation of phase II detoxifying and antioxidant enzymes through the Keap1–Nrf2 pathway. Nrf2, which is a nuclear factor with a basic leucine zipper DNA binding domain homologous to *Drosophila* cap 'n' collar proteins, is constrained by a cytoplasmic protein homologous to *Drosophila* actin binding protein Kelch-like erythroid cell-derived protein (ECH)-associated protein 1 (Keap1). Dissociation of Nrf2 from Keap1 allows it to translocate to the nucleus for further heterodimerization with small muscle aponeurotic fibrosarcoma (Maf) protein and then specifically bind to the ARE, leading to transcriptional activation of genes such as epoxide hydrolase, glutamate cysteine ligase, heme oxygenase, and UDP-glucuronosyltransferase that are involved in metabolism. Furthermore, upstream signals are tightly regulated through phosphorylation by several protein kinase signaling pathways, including mitogen-activation protein kinases (MAPKs), protein kinase *c* (PKC), and phosphatidylinositol 3-kinase (PI3K) (*13*). It is a practicable strategy to modulate the oxido–redox condition through supplementation of naturally occurring antioxidants. Antioxidants terminate the chain reaction of oxidation induced by free radicals through scavenging radical intermediates directly and are oxidized themselves. Furthermore, the potential effects of many phytochemicals and nutraceuticals are involved in the regulation of genes that contribute to defense against oxidative stress. These active compounds, such as dithioethiones, isothiocyanates, carotenoids, and triterpenoids, etc., are considered to be the inducers that stimulate phase II and antioxidant enzymes perform conjugation and antioxidation via the ARE signaling transduction pathway (*13*). It has been revealed that resveratrol, a phytochemical found in red wine and grape seed, shows an attenuating effect on H_2O_2-induced PC12 cell death through induction of glutamate-cysteine ligase (GCLC) at nontoxic concentration (15 μM) (*31*). Moreover, anthocyanin-rich Pomegranate fruit extract (2 mg/mouse) can inhibit skin tumorigenesis in CD-1 mice through MAPK and NF-κB pathways (*32*). Although the bioactivity of phytochemicals has always been queried because the dosage tested in vitro could not be reflected to that in vivo, the physiological and chemopreventive effect of these phytochemicals should not be ignored.

In the present study, we supposed that the ARE is an authentic and reliable selection marker for evaluation of phytochemicals. We first constructed an ARE-containing reporter vector that expresses luciferase activity. The rat *nqo1*-ARE used to construct the reporter plasmid conserved the ARE full consensus sequence. NQO1 is widely known to play an important role in defenses against carcinogens and xenobiotics and is regulated through the Nrf2–ARE pathway (*33*). As shown in **Figure 5**, the transcription start site is indicated by +1, and the previously characterized AREs (nucleotides −468 to −455 and −904 to −895) are underlined and labeled as ARE-like-1 and -2 (named

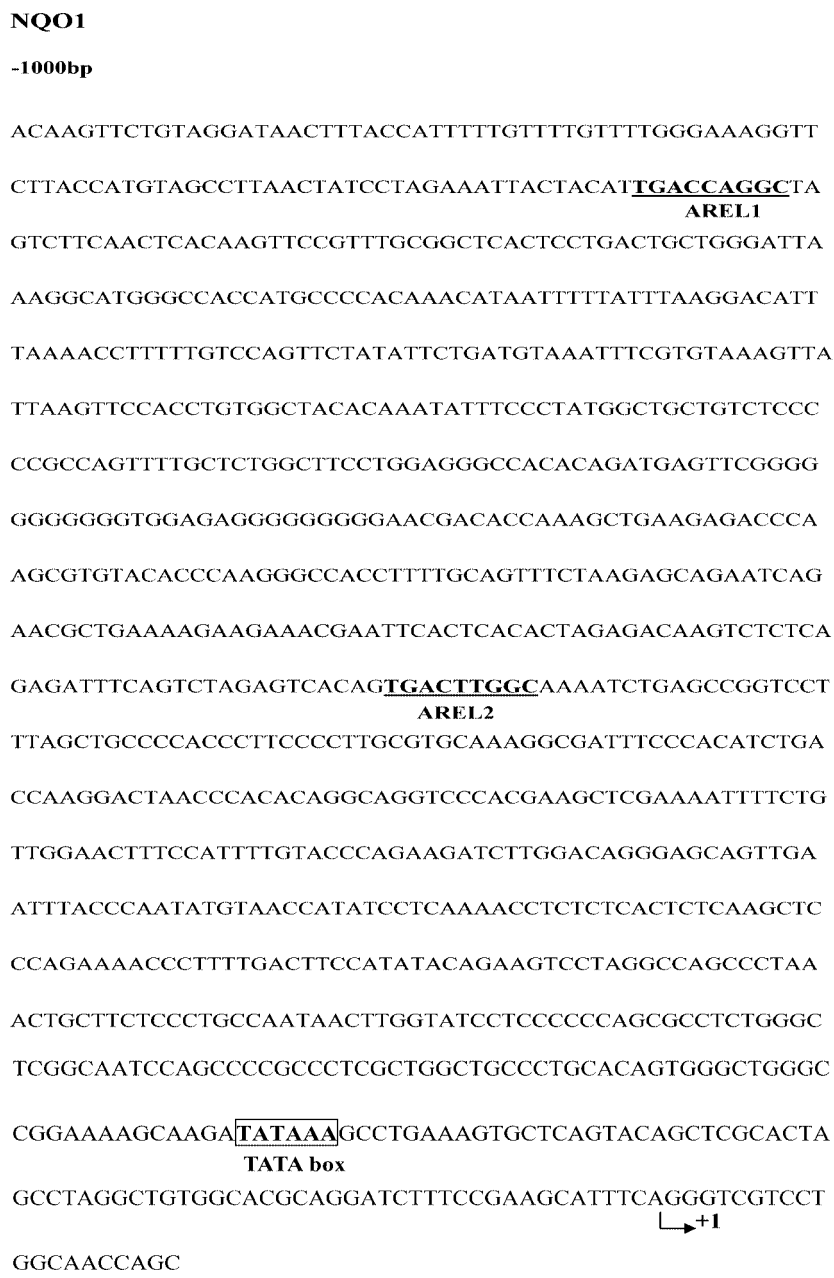


Figure 5. Antioxidant response elements within the 5′-upstream region of rat *nqo1* (GeneID: 24314). The transcriptional start site is indicated by +1 and a probable TATA box is indicated. The position of the antioxidant response elementlike (AREL) sequence is underlined.

AREL1 and AREL2, respectively). And this is the first time that the rat *nqo1* gene has been investigated, to the best of our knowledge. We cloned two predicted ARE regions, which seem to be quite different from their expression in mouse. We observed a luciferase induction effect of quercetin on Clone 9 cells that had been transiently transfected with a rat *nqo1* promoter-containing vector when compared with the blank group transfected with an empty vector. Quercetin, which is also a flavonoid that shows multifunctional benefits for health and can induce the activation of the Nrf2-ARE pathway, was taken as a positive control here to set-up the experimental model. All other anthocyanins possessed ARE-activating effects. However, there was no significant difference between treatments. To check which ARE found in the rat *nqo1* promoter shows mort efficacy, oligocucleotides containing AREL1, AREL2, or the full promoter were amplified, respectively. The results suggested that Nrf2 significantly binds to the rat *nqo1* promoter at the AREL2-site and triggers the expression of luminescence (data not shown). The relationship between antioxidant capacity and ARE-inducing effect of flavonoids was first discussed (*27*). Their findings suggest that there is no significantly consistent relationship between luciferase induction activity and antioxidant potential. For this reason, it may not be appropriate to evaluate the phase II and antioxidant enzyme-inducing effects of phytochemicals regarding only their antioxidant capacity. On the basis of this screening model, we proved that anthocyanins activated the ARE signaling transduction pathway and stimulated the expression of Nrf2–ARE-related phase II and antioxidant genes.

In conclusion, we find that naturally occurring anthocyanins that are abundant in vegetables and fruits are able to elevate the expression of phase II and antioxidant enzymes. Among 10 kinds of anthocyanins, cyanidin, kuromanin, delphinidin, and malvidin showed the best efficacy. Furthermore, pretreatment of Clone 9 liver cells with anthocyanins could elevate the activity of glutathione-related enzymes and NQO1 and protect

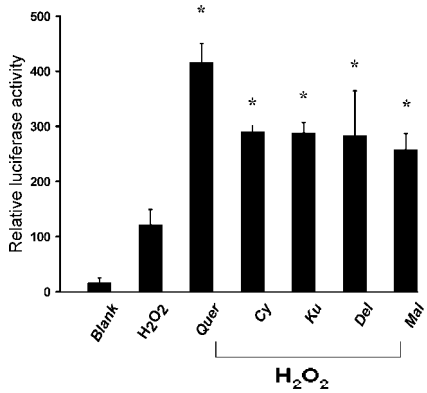


Figure 6. Effects of anthocyanins on luciferase activity mediated by the rat *nqo1* promoter. Construct consisting of the firefly luciferase gene driven by the 5′-flanking region of *the rat nqo1* gene was transfected into Clone 9 cells. The transfected cells were treated with 10 μM of quercetin (Quer) or 50 μM of four anthocyanins for further 24 h, respectively, which was followed by treatment with H_2O_2 (70 μM) for 3 h. Luminescence was analyzed and the representative sets of experiments were performed in triplicate. Bars with an asterisk are significantly different ($p < 0.05$) from the group of H_2O_2-treated alone.

cells against oxidative-stress-induced apoptosis by directly scavenging free radicals and activating the defense system. Finally, we showed that the molecular mechanism was closely involved in the ARE signaling transduction. Synthetic medicines available for treating cancers and other diseases are much expensive and toxic. Thus, the development of substances derived from natural sources, described traditionally and showing cancer preventive effects, is indispensable. Our data suggested that anthocyanin is a kind of potential phytochemical that shows chemopreventive effects against oxidative-stress-induced cell damage.

ABBREVIATIONS USED

AOEs, antioxidant enzymes; ARE, antioxidant response element; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; MAPK, Mitogen-activated protein kinase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; NQO1, NAD(P)H:quinone oxidoreductase 1; PI3K, phosphatidylinositol-3 kinase.

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