JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Synergistic Effect of Cyanidin and PPAR Agonist against Nonalcoholic Steatohepatitis-Mediated Oxidative Stress-Induced Cytotoxicity through MAPK and Nrf2 Transduction Pathways

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ABSTRACT: Nonalcoholic steatohepatitis (NASH) is caused by an elevation in oxidative stress, which might further lead to hepatic fibrogenesis. Importantly, both peroxisome proliferator-activated receptor (PPAR) and nuclear factor erythroid 2-related factor 2 (Nrf2) play roles in modulating oxidative stress-mediated hepatic dysfunction. The objective of this study was to investigate the mechanisms of the multifunctional effects of cyanidin on regulating antioxidant enzymes and oxidative stress-induced hepatotoxicity. The data indicated that cyanidin-mediated antioxidant enzyme expression involved the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways and Nrf2 activation. Furthermore, the synergistic effect of cyanidin and the PPAR agonist, troglitazone, on Nrf2-PPAR activation, was also observed. Besides, treatment of cyanidin and troglitazone abolished H_2O_2-induced downregulation of genes involved in lipid metabolism. In addition, H_2O_2-mediated cytotoxicity, which was caused by inducing ROS formation and apoptotic cell death, was also ameliorated upon cyanidin and troglitazone stimulation. In conclusion, mitogen-activated protein kinases (MAPKs) and the transcription factor Nrf2 played regulatory roles in cyanidin-mediated antioxidant enzyme activation. Furthermore, the combination of cyanidin and troglitazone activated PPARγ-Nrf2 and improved H_2O_2-mediated perturbation of genes involved in lipid metabolism. These data suggested that cyanidin and PPAR agonists might have synergistic benefits against metabolic dysfunction-related oxidative damage.

KEYWORDS: *anthocyanidin, oxidative stress, chronic nonalcoholic fatty liver disease, synergistic effect, thiazolidinedione*

INTRODUCTION

High fat and high carbohydrate diet habits result in glucotoxic and lipotoxic phenomena, which might lead to physical degeneration.[1] It is postulated that oxidative stress plays an important role in the pathogenesis of human diseases.[2] Hyperglycemia induces reactive oxygen species (ROS) formation through different pathways, including activation of NAD(P)H:oxidase by the protein kinase C (PKC) pathway and accumulation of advanced glycation end products (AGEs). Because the complexity of chronic oxidative stress conditions, such as the metabolic syndrome, is strongly associated with insulin resistance and cardiovascular diseases, it is imperative to rectify dietary behavior and prevent deterioration.

Chronic nonalcoholic fatty liver disease (NAFLD), including nonalcoholic steatohepatitis (NASH), is an indicator of metabolic syndrome.[3] Oxidative stress is one of the crucial factors induced by excess fatty acid accumulation that triggers cellular oxidative damage.[4] Free radicals are generated through different pathways including the cytochrome P450 family, mitochondrial abnormality, and the accumulation of AGEs.[5,6] Since ROS plays an important role in the modulation of macromolecules involved in cellular function and metabolism, it is a practical strategy to prevent further atherosclerosis and cardiovascular diseases by blocking ROS formation.

Peroxisome proliferator-activated receptors (PPARs) are members of the crucial nuclear hormone receptor superfamily that contribute to nutrient−gene interactions, which are regulatory genes involved in lipid and carbohydrate metabolism, inflammatory modulation, and antineoplasia.[7,8] PPARs heterodimerize with the retinoid X receptor (RXR) and bind to the PPAR response element (PPRE) in enhancer sites of responsive genes to activate gene transcription.[9] PPARs are suggested to play a critical role in the prevention of dyslipidemia and the maintenance of metabolic homeostasis to regulate storage, oxidation, and synthesis of lipids and fatty acids.[10] PPAR-regulated genes are involved in β-oxidation of fatty acids and are predominantly expressed in the liver. PPAR upregulates fatty acid transport proteins and facilitates the uptake of long chain fatty acids. The fatty acids are further esterified by acyl-CoA synthetase to acyl-CoA derivatives. Sequentially, medium chain acyl-CoA dehydrogenase, acyl-CoA oxidase, and cytochrome P450 fatty acid hydroxylase, which are the key enzymes involved in β-oxidation of the activated acyl-CoA esters, are also activated by PPAR.[11] PPAR functions as a

Received: January 2, 2012
Revised: February 22, 2012
Accepted: February 26, 2012
Published: February 26, 2012

dx.doi.org/10.1021/jf300005v | *J. Agric. Food Chem.* 2012, 60, 2924−2933

fatty acid regulator and, by activating its target genes, maintains fatty acid metabolism homeostasis. Therefore, PPAR agonists are attractive pharmacological targets for the treatment of hyperlipidemia.

Thiazolidinedione (TZD) is one of the medication classes that act as an adjuvant therapy for diabetes mellitus and related diseases since the 1990s. The antidiabetic and antihyperglycemic effects of TZD are mediated by increasing the insulin sensitivity of peripheral tissues, such as skeletal muscles and adipose.[12] TZD is widely used to treat the later period diabetic patients with severe glucose abnormality. Furthermore, TZD also functions as a ligand of PPAR and shows modulatory effects on PPAR-regulated genes relevant to lipid metabolism.[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 the antioxidant response element (ARE) in the promoter region of a number of genes that code for antioxidative and detoxifying enzymes in several types of cells and tissues.[14] 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, hemeoxygenase, and UDP-glucuronosyltransferase that are involved in metabolism. Furthermore, the upstream signals are tightly regulated through phosphorylation by several protein kinase signaling pathways, including mitogen-activation protein kinases (MAPKs), PKC, and phosphatidylinositol-3-kinase (PI3K). Activation of phase II detoxifying enzymes, such as superoxide dismutase (SOD), catalase, 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]

Anthocyanins are colorful pigments that exist in naturally occurring fruits and vegetables. It is postulated that anthocyanins show multifunctional benefits including antioxidant, anti-inflammatory, chemopreventive, and cardioprotective effects.[17,18] Besides, anthocyanin was a potent antagonist in various cancer types. Therefore, it is critical to clarify the molecular type and composition of the anthocyanins that confer specific health benefits and to conduct further investigation into the underlying molecular mechanisms. Recently, it has been suggested that cherries and berries, which are both rich in phenolics and anthocyanins, reduced hyperlipidemia and inhibited hepatic steatosis.[19] Previously, we have shown that cyanidin upregulates antioxidant and detoxifying enzymes through the antioxidant response element pathway.[18] In the present study, we further investigated the molecular role of upstream signal regulators in cyanidin-mediated chemopreventive effects. The effects of cyanidin and TZD on oxidative stress-induced liver dysfunction have never been reported. Thus, we also evaluated the combinatorial effects of cyanidin and troglitazone on H_2O_2-mediated hepatotoxicity.

MATERIALS AND METHODS

Reagents. Cyanidin chloride was purchased from Extrasynthese Corporation (Genay Cedex, France). Troglitazone, 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and propidium iodide (PI) were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). PD98059, SP600125 and SB203580 were purchased from BioSource International Co. (Camarillo, CA, USA). siNrf2 transfection kit was obtained from Santa Cruz Co. (Santa Cruz, CA, USA). TRIzol RNA extraction reagent was obtained from MDBio Co. (Frederick, MD, USA). Other high grade reagents were purchased from commercial companies. Cyanidin (Cy) and troglitazone (Tz) were dissolved in DMSO, and the final working concentration was adjusted at 0.05% DMSO as the control.

Cell Culture. Human hepatoblastoma HepG2 (BCRC number: 60177) and rat normal hepatocyte Clone 9 (BCRC number: 60201) were obtained 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 7% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin antibiotics and then incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Antioxidant Enzyme Activity. HepG2 cells were treated with 50 μM cyanidin for 24 h. Various pharmacological kinase inhibitors, PD98059, SP600125 and SB203580, were added into the culture medium for 1 h before cyanidin treatment. The cell lysate used for analyzing antioxidant enzyme activity was prepared in PBS containing protease inhibitor cocktail (Sigma). Total lysates were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was then transferred to a clean tube and was preserved at −20 °C for further study.

The protein determination was performed spectrophotometrically by Bio-Rad Protein Assay kit following the magnifications. The solubilized protein was detected with the acidic solution of Coomassie Brilliant Blue G-250 dye and subsequent measurement at 595 nm with a spectrophotometer. In brief, prepare dye reagent by diluting 1 part dye reagent concentrate with 4 parts distilled, deionized (DDI) water. 100 μL of cell diluted lysate or standard (bovine serum albumin) was well mixed with 100 μL of dye reagent and incubated for 10 min at room temperature. At the end of incubation, measure the absorbance at 595 nm.

Catalase activity was evaluated spectrophotometrically as previously described.[20] The assay mixture consisted of 975 μL H_2O_2 (25 mM) and 25 μL of the cell lysate. Changes in absorbance were recorded at 240 nm for 2 min. Catalase activity was expressed as nanomoles of H_2O_2 per minute per microgram of protein.

Superoxide dismutase (SOD) activity was evaluated using a commercial kit (Dojindo, Tokyo, Japan) and with the manufacturer's protocol. Absorbance was determined at 450 nm, and SOD activity was defined as 50% of inhibition on the formation of formazan. Catalase and SOD activities were normalized to the control group.

RNA Interference. Silencing of Nrf2 was achieved by transfection with siRNA duplexes targeting Nrf2. HepG2 cells, which were incubated in antibiotic-free normal growth medium supplemented with FBS, were transfected with 10 μM control siRNA (used as nonspecific siRNA control) or Nrf2 siRNA using the siRNA Transfection Reagent (Santa Cruz). After transfection, the cells were maintained in 2× normal growth medium without removing the transfection mixture and incubated for 6 h in the conditioned incubator. Then, the medium was changed to 1× normal growth medium and incubated for an additional 24 h. The cells were then incubated in medium with 50 μM cyanidin or 6.25 μM troglitazone for 24 h. The cells were harvested for RT-PCR and immunoblot analyses.

Western Blot Assay. The cytosolic/nuclear fractionation kit (TransFactor Extraction Kit, Clontech, Mountain View, CA, USA) was used to generate cytoplasmic and nuclear extracts. After treatment, HepG2 cells were trypsinized and washed with PBS twice and were then centrifuged to discard the supernatant. A volume of lysis buffer equal to five times the cell pellet volume was added to the cell pellet. The cell pellet was gently resuspended and incubated on ice for

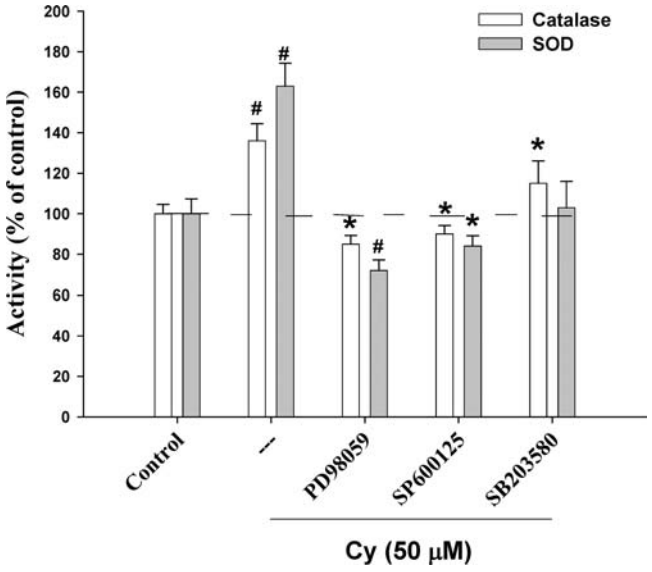


Figure 1. Signaling pathways involved in cyanidin-mediated induction of antioxidant enzymes. HepG2 cells were pretreated with MAPK inhibitors (20 μM) for 1 h and then treated with cyanidin for 24 h. The activity of antioxidant enzymes was examined. Cy, cyanidin. * and # mean significantly different from control group at $P < 0.05$ and $P < 0.01$, respectively. Data are from triplicate and independent experiments.

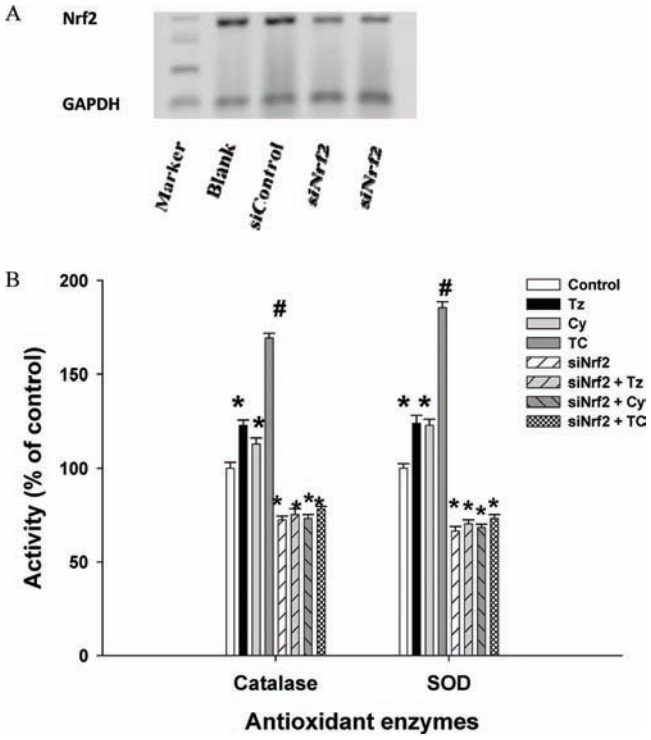


Figure 2. Nrf2 is a target involved in the induction of antioxidant enzymes upon cyanidin stimulation. HepG2 cells were transfected with Nrf2 siRNA for 48 h and were then treated with 25 μM cyanidin for another 24 h. (A) Nrf2 gene expression. Lane 1, marker; lane 2, blank; lane 3, siControl (control siRNA); lanes 4 and 5, siNrf2 (Nrf2 siRNA). (B) HepG2 cells were transfected with Nrf2 siRNA for 48 h and then were lysed, and the activities of catalase and superoxide dismutase (SOD) were examined. Data are from triplicate and independent experiments and expressed as mean ± SD. Tz, troglitazone (3.125 μM); Cy, cyanidin (25 μM); TC (3.125 μM troglitazone + 25 μM cyanidin). * and # mean significantly different from control group at $P < 0.05$ and $P < 0.01$, respectively.

15 min, and then centrifuged at 3,000 rpm for 5 min. The cell pellet was resuspended in a volume of lysis buffer equal to twice the cell pellet volume, and then the cells were disrupted by passing through a syringe. Following centrifugation at 12,000 rpm for 20 min, the supernatant, which is the cytosolic fraction, was transferred to a fresh tube. The pellet, which is the nuclear fraction, was resuspended in a volume of extraction buffer equal to two-thirds the pellet volume and disrupted using a fresh syringe. After shaking the nuclear suspension gently for 30 min at 4 °C, the mixture was centrifuged at 14,000 rpm for 5 min. The supernatant was the nuclear extract. Specimens were preserved at −80 °C for further study.

Protein concentrations were quantified using a Bio-Rad Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions as mentioned above. Protein extracts were diluted in 5 × SDS sample buffer (8% SDS; 0.04% Coomassie Blue R-250; 40% glycerol; 200 mM Tris, pH 6.8 and 10% 2-mercaptoethanol) and boiled for 10 min. Samples were electrophoresed in a 10% SDS−PAGE mini-gel and then transferred onto polyvinylidene difluoride membranes (PVDF; Millipore Corp., Bedford, MA, USA) with transfer buffer (48 mM Tris; 39 mM glycine; 0.0037% SDS and 20% methanol) at 350 mA for 60 min. The membranes were blocked with 5% nonfat milk in a PBS solution containing 0.1% Tween-20 (PBST) for 1 h. The membrane was immunoblotted with primary antibodies of rabbit anti-rat Nrf2 (Santa Cruz, CA, USA) and rabbit anti-rat PPARγ (Upstate, Lake Placid, NC, USA) in PBST solution containing 5% bovine serum albumin (BSA) at 4 °C overnight. After consecutive 30 min PBST washes, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 60 min at room temperature and then washed with PBST for 30 min. The final detection was performed using enhanced chemiluminescence (ECL kit) Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Real-Time PCR Analysis. Clone 9 cells were harvested at 18 h after incubation with 50 μM cyanidin or 6.25 μM troglitazone. In addition, Clone 9 cells were treated with 50 μM H_2O_2 for 3 h and then were incubated with cyanidin or troglitazone for a total period of 24 h. Total cellular RNA was isolated using TRIzol (MDBio) reagent according to the manufacturer's protocols and was quantified by measuring the absorbance at 260 nm. RNA purity was determined using A260/A280 ratio (average ≥1.8). Total RNA of each specimen was first reverse-transcribed into cDNA using SYBR GreenER Two-Step qRT-PCR kit (Invitrogen), and then PCR amplification was performed using an ABI7300 Real Time PCR System as follows: 1 cycle of 50 °C for 2 min and denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A linear regression was performed and the slope, relating Ct to the log of ng RNA, was calculated, and the Ct value of the target gene was normalized to the housekeeping gene GAPDH. Gene *Nrf2* was amplified using SuperScript One-Step RT-PCR with Platinum *Taq* System (Invitrogen). Specimens were subjected to 30 cycles of amplification (cDNA synthesis at 50 °C for 30 min followed by predenaturation at 94 °C for 2 min, and PCR amplification was performed with denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min). PCR products were then electrophoresed on 1.8% agarose and visualized by ethidium bromide staining and then were digitized using UVP BioDoc-It System. The following primers were used: GAPDH (NM002046) cga cca ctt tgt caa gct ca (sense) and agg ggt cta cat ggc aac tg (antisense); PPARα (M88592) act atg gag tcc acg cat gtg a (sense) and ttg tcg tac gcc agc ttt agc (antisense); RXR (L06482) gga tgg cct gtg tgg atc tt (sense) and atg atg atg gcg agg atg gt (antisense); HMG-CoA Rd (X55286) aac ctg ctg cca taa act gga t (sense) and acc acc ttg gct gga atg ac (antisense); Acyl-CoA Od (J02752) act atg gag tcc acg cat gtg a (sense) and ttg tcg tac gcc agc ttt agc (antisense); PMP70 (D90038) aac ctg ctg cca taa act gga t (sense) and acc acc ttg gct gga atg ac (antisense). Normalization of the expression levels allowed data to be expressed relative to GAPDH mRNA, and therefore, any difference in reverse-transcriptase efficiency

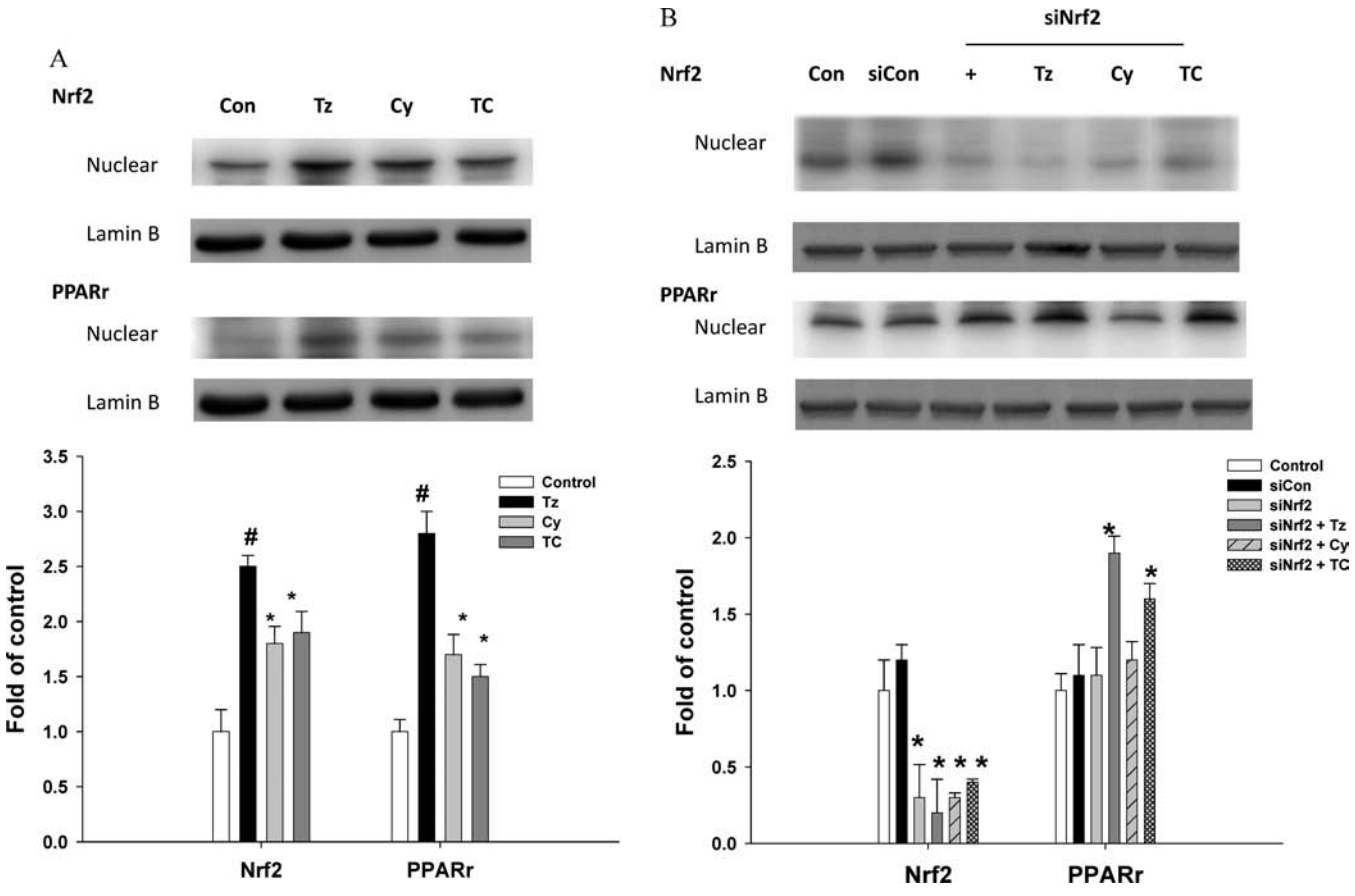


Figure 3. Effect of Nrf2 inhibition on PPARγ activation upon troglitazone and cyanidin stimulation. HepG2 cells were treated with or without Nrf2 siRNA for 48 h, and then treated with cyanidin (Cy) or troglitazone (Tz) for another 6 h. Nuclear and cytosolic fractions were separated and prepared for a Western blot assay; (A) Nrf2 normal expression and (B) Nrf2 siRNA-treated groups. Con, control; siCon; control siRNA; Tz, siNrf2 + troglitazone (6.25 μM); Cy, siNrf2 + cyanidin (50 μM); TC, siNrf2 + 3.125 μM troglitazone and 25 μM cyanidin combination. Data are from triplicate and independent experiments with similar results. * and # mean significantly different from control group at $P < 0.05$ and $P < 0.01$, respectively.

was compensated. All standards and samples were analyzed as duplicates. Data were obtained as Ct values (the cycle where the emitted fluorescence signal is significantly above background levels and is inversely proportional to the initial template copy number) according to manufacturer's guidelines and used to determine ΔCt values (ΔCt = Ct of the target gene − Ct of the housekeeping gene 18S) of each sample.

ROS Formation. Clone 9 cells were preincubated with 50 μM H_2O_2 for 3 h and then incubated with 50 μM cyanidin or 6.25 μM troglitazone for a total period of 24 h. Cells were harvested and incubated with DCFH-DA/PBS solution at the reaction concentration of 20 μM for 30 min. After washing, ROS levels were analyzed by flow cytometry (FACS Calibur, Becton-Dickinson Immunocytometry System, Franklin Lakes, NJ, USA). Data were collected from 10,000 cells and were evaluated by CELL Quest software.

Cell Cycle Evaluation. Clone 9 cells were preincubated with 50 μM H_2O_2 for 3 h and then incubated with 50 μM cyanidin or 6.25 μM troglitazone for a total period of 24 h. Cells were harvested and incubated with 40 μg/mL propidium iodide (PI) solution for 15 min. After washing, the cells were resuspended in PBS and analyzed by flow cytometry. Data were collected from 10,000 cells and were evaluated by CELL Quest software.

Cell Viability Assay. Clone 9 cells were preincubated with 50 μM H_2O_2 for 3 h and then incubated with cyanidin (0−50 μM) or troglitazone (0−6.25 μM) for a total period of 24 h. The cells were then washed with PBS and incubated with MTT (0.5 mg/mL) for 3 h in a conditioned incubator. The blue formazan crystals formed were dissolved using DMSO and then were quantified spectrophotometrically by measuring the absorbance at 570 nm by spectrophotometer (Hitachi, Tokyo, Japan).

Median Effect and Combination Index (CI). The results were analyzed using the median effect/combination index (CI) by Chou and Talalay,[21] which is based on the median-effect principle (mass-action law). The analysis used in the present study was under the assumption of mutual nonexclusiveness of the mechanism of compound action.

Statistical Analysis. All data are presented as mean ± SD. The statistically significant differences were compared with the untreated groups, and were calculated by one- or two-way analysis of variance (ANOVA). Statistically significant individual group means were then compared using a Student's t test.

RESULTS AND DISCUSSION

ERK and JNK but Not p38 Are Required for Cyanidin To Promote Antioxidant Enzyme Activation. Previously, we cloned the rat ARE, which is the well-known targeting element of transcription factor Nrf2, and the activation of Nrf2-drived luciferase expression was induced upon anthocyanidin treatment.[18] Herein, we wanted to clarify the upstream signaling pathway involved in Nrf2-ARE. Several distinct and unique MAPK pathways have been identified and are activated by sequential phosphorylation cascades. ERKs, JNKs, and p38 pathways are involved in cell proliferation, stress stimuli, and inflammatory regulation, respectively.[22] Recently, delphenidin has been proved that inhibits EGF-induced autophosphorylation of

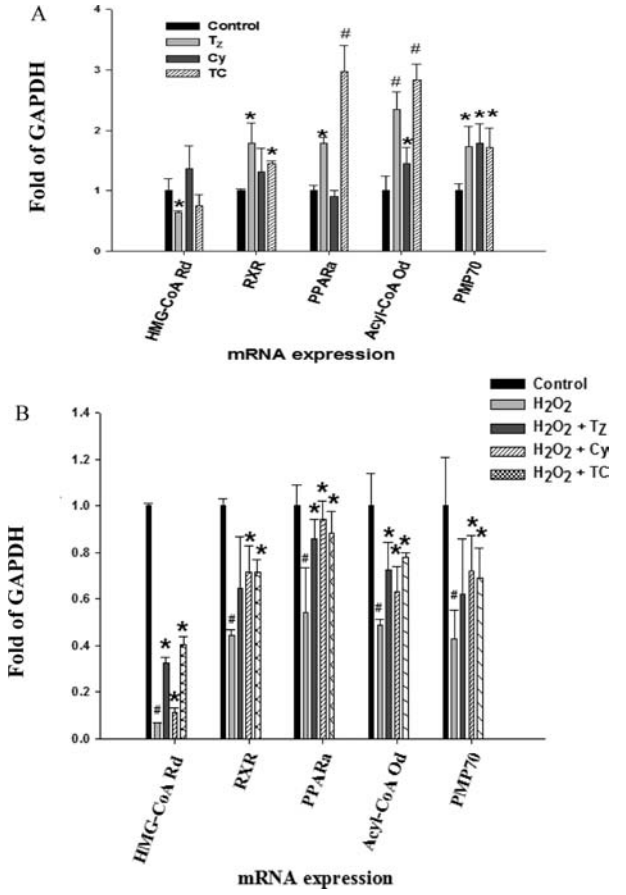


Figure 4. Effects of cyanidin and troglitazone on PPAR-regulated gene expression in Clone 9 cells. (A) Cells were treated with cyanidin (50 μM) and troglitazone (6.25 μM), and the combination of Cy (25 μM) and Tz (3.125 μM) for 18 h, respectively. (B) effects of cyanidin and troglitazone on PPAR-regulated gene expression after stimulation with H_2O_2. Data are from three independent experiments and expressed as mean ± SD. * and #, significantly different as compared with control group at $P < 0.05$ and $P < 0.01$, respectively. Tz, troglitazone (6.25 μM); Cy, cyanidin (50 μM); TC (3.125 μM troglitazone and 25 μM cyanidin combination). HMG-CoA Rd, HMG-CoA reductase; RXR, retinoid X receptor; PPARa, PPARα; Acyl-CoA Od, Acyl-CoA oxidase; PMP70, peroxisomal membrane protein.

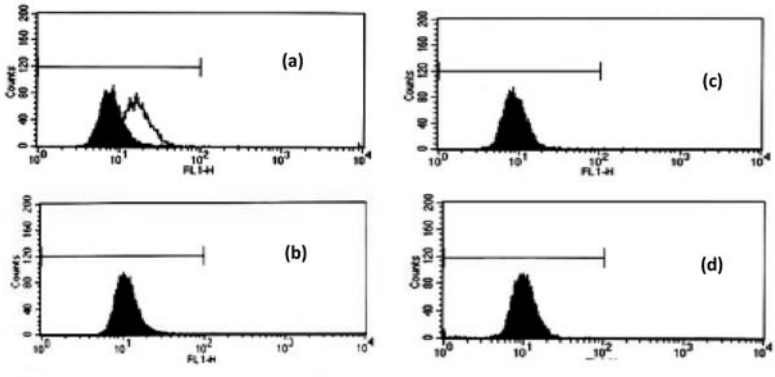
EGFR, activation of PI3K, phosphorylation of AKT and MAPK.[23] Besides, we had proven that anthocyanin triggered MAPK family members in HepG2 cells.[24] To confirm the signaling transduction pathways involved in cyanidin-induced antioxidant enzyme expression, we used specific pharmacological inhibitors to inhibit MAP kinases, which are involved in the upstream signal transduction. Figure 1 shows the catalase and SOD activity in HepG2 cells upon the treatment of cyanidin (50 μM) for 24 h. Before the cyanidin treatment, cells were incubated with various MAPK inhibitors for 3 h. The activity of catalase and SOD increased upon cyanidin stimulation; however, the activation of catalase and SOD by cyanidin was significantly blocked by preincubation with the MEK inhibitor (PD98059) and JNK inhibitor (SP600125), but not by the p38 inhibitor (SB203580). Interestingly, we found that treatment with the MAPK inhibitors PD98059 and SP600125 alone reduced the activity of catalase and SOD, but no significant difference was observed in SB203580-treated group (data not shown). It is suggested that MAPKs play crucial roles in the upregulation of phase II detoxifying enzymes by dietary chemopreventive compounds.[25] Unlike the p38 pathway, MEK and JNK pathways act as positive regulators in the induction of NAD(P)H:quinone oxidoreductase (NQO1) by *tert*-butylhydroquinone (tBHQ) in HepG2 cells.[26]

Nrf2 Is Necessary for Cyanidin-Induced Antioxidant Enzyme Activation. Nrf2 is the crucial transcription factor that regulates phase II detoxifying enzymes. Nrf2 binds to antioxidant response elements located in the promoter of responsive genes. To prove whether Nrf2 and subsequent antioxidant enzyme activation are involved in cyanidin stimulation, we next determined the effect of cyanidin-mediated catalase and SOD activation in Nrf2 siRNA-treated HepG2 cells. After transfection of HepG2 cells with Nrf2 siRNA for 48 h, the basal expression levels of Nrf2 genes were decreased as compared to control Nrf2 siRNA-treated or blank cells (Figure 2A). The basal activities of catalase and SOD were significantly decreased in Nrf2 siRNA-treated cells than in cyanidin-treated cells (Figure 2B). With the administration of cyanidin or troglitazone, the activities of antioxidant enzymes were still significantly lower than those of the cyanidin-treated group. It has also been observed that Nrf2 knockout ($Nrf2^{-/-}$) mice had relatively lower mRNA and protein levels of phase II enzymes.[27]

Effect of PPARγ-Nrf2 Expression on the Stimulation of the PPAR Agonist and Cyanidin. The relationship between dietary uptake and health is a widely discussed topic. Westernized lifestyle typically includes an excess intake of fatty acids and carbohydrates. Body mass index (BMI) and waist circumference are the major external parameters to evaluate metabolic risk factors, and serum cholesterol and triglyceride levels are very important internal indexes that represent direct metabolic conditions.[28] NASH is one of the nonalcoholic fatty liver diseases that are caused by the imbalance of lipid uptake and energy depletion. The alteration of lipogenesis and β-oxidation further contributes to impaired lipid turnover and leads to lipid accumulation in the liver.[29] Lipid retention within hepatocytes results in oxidative stress and is defined as a secondary level of gluco- and lipotoxicity, which triggers cellular oxidative damage and might participate in the early stage of insulin resistance and other cardiovascular diseases.[30] Troglitazone, which is a TZD class of antidiabetic drugs, elevates insulin sensitivity of peripheral tissues, acts as a ligand to activate PPARs, and regulates genes involved in lipid metabolism.[31] However, the side effects and unexpected contraindications of TZDs have been reported and discussed worldwide. Troglitazone has been removed from worldwide markets due to severe drug-induced liver injury.[32] Thus, the use of TZD to treat patients with complicated diabetes or related cardiovascular diseases shall require more careful monitoring.

This study examined the effects of the troglitazone and cyanidin on PPARγ and Nrf2 expression. HepG2 cells were transfected with or without Nrf2 siRNA for 48 h and were then treated with troglitazone or cyanidin for a further 6 h. As shown in Figure 3A, the expression of nuclear Nrf2 and PPARγ was significantly elevated upon the treatment of troglitazone (6.25 μM) and cyanidin (50 μM), and the combination (3.125 μM troglitazone and 25 μM cyanidin) still induced PPARγ and Nrf2 translocation from the cytosol to the nucleus. However, after treatment with Nrf2 siRNA, the activation of Nrf2 by troglitazone and cyanidin was significantly inhibited, and this inhibitory effect was also accompanied by a decrease in PPARγ expression (Figure 3B). These data revealed that Nrf2 is a major determinant of catalase and SOD activation, and both troglitazone and cyanidin activated Nrf2 and PPARγ expression. Furthermore, Nrf2 is also

A



B

Treatment	ROS formation (% of control)
(a) Control/H_2O_2 (50 μM)	100/195#
(b) H_2O_2 + Tz (6.25 μM)	135*
(c) H_2O_2 + Cy (50 μM)	120*
(d) H_2O_2 + Tz (3.125 μM) + Cy (25 μM)	107*

Figure 5. Effects of cyanidin and troglitazone on H_2O_2-induced ROS production. Clone 9 cells were treated with H_2O_2 (50 μM) for 3 h, and then incubated for a total period of 24 h in the absence or presence of cyanidins and troglitazone. Cells were collected and incubated with DCFH-DA/PBS solution (20 μg/mL) for 15 min. After washing, ROS levels were analyzed by flow cytometry (A) and the data were calculated (B). Tz, troglitazone; Cy, cyanidin. * and # mean significantly different from control group at $P < 0.05$ and $P < 0.01$, respectively.

involved in PPARγ activation. Recently, Kronke and colleagues reported that a PPAR agonist (rosiglitazone) activates heme oxygenase-1 (a phase II detoxifying enzyme) through a PPRE pathway.[33] The major cause is that upstream promoters of detoxifying enzymes might contain both ARE and PPRE, and PPAR activation results in the recruitment of Nrf2 and other coregulators upon PPAR agonist stimulation.[34] PPAR promotes detoxifying enzyme upon the treatment of PPAR agonist as well as cyanidin. On the other hand, Nrf2 inducer might activate PPAR expression through a similar signaling pathway.

Effects of PPAR Agonist and Cyanidin on PPAR-Related Gene Expression. PPARs are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors that regulate genes relevant to lipid metabolism and storage.[9] PPARα-regulated genes involved in hepatic lipid metabolism have been revealed and classified by the various functions, including mitochondrial fatty acid oxidation, fatty acid activation, peroxisomal fatty acid oxidation, lipogenesis, lipase, glycerol metabolism, and lipid transport.[13] To evaluate PPAR-related gene expression upon the treatment of troglitazone and cyanidin, real-time PCR analysis was performed with the total RNA extracts from the Clone 9 cells treated with troglitazone, cyanidin or their combination. Troglitazone (6.25 μM) significantly increased the peroxisomal membrane (PMP70), peroxisomal β-oxidation (Acyl-CoA Od), and RXR, which are all involved in the metabolism of fatty acids (Figure 4A). However, the levels of HMG-CoA Rd, which is the rate-limiting enzyme in cholesterol synthesis, were significantly inhibited. Interestingly, we found that cells treated with cyanidin (50 μM) significantly increased the acyl-CoA Od and PMP70 levels, and troglitazone (3.125 μM) + cyanidin (25 μM) still showed enhancing effects on the expression of these genes, especially for PPARα and Acyl-CoA Od. Next, we treated cells with H_2O_2 (50 μM) for 3 h and then the medium was changed, followed by incubation for a total period of 24 h in the absence or presence of troglitazone and cyanidin. The expression of the genes mentioned above was significantly decreased upon H_2O_2 stimulation, but was restored by troglitazone and cyanidin treatment (Figure 4B). Inflammatory stress is also one of the major mediators that exacerbate lipid accumulation in the liver of ApoE knockout mice, and downregulated expression of PPARα and ATP-binding cassette transporter A1 (ABCA1) were noted in TNFα and IL-1β damaged hepatocytes.[35] Our data suggested that oxidative stress is another determinant that induces dysfunction in PPAR-regulated genes that are involved in lipid metabolism.

PPAR Agonist and Cyanidin Protect Cells from H_2O_2-Mediated Cytotoxicity through Blocking ROS Formation. Apoptosis, also named programmed cell death, is postulated to play an important role in liver pathology and NASH.[36] It is suggested that reactive oxygen species are closely associated with liver fibrogenesis and cirrhosis.[37] Elevating oxidative stress is an acute damage to cellular homeostasis. Thus, inhibition of ROS formation and increasing antioxidant capacity is one of the strategies to prevent oxidative stress-triggered hepatotoxicity. To investigate whether treating cells with H_2O_2 results in the production of free radicals and the protective effects of PPAR agonist and cyanidin, we evaluated the cellular ROS content. The results indicated that basal ROS levels increased by approximately 95% in H_2O_2-treated Clone 9 cells, and cotreatment with troglitazone (6.25 μM) or cyanidin (50 μM) significantly inhibited ROS formation (Figure 5). Furthermore, cotreatment of troglitazone (3.125 μM) and cyanidin (25 μM) also showed potent ROS scavenging effects.

dx.doi.org/10.1021/jf300005v | J. Agric. Food Chem. 2012, 60, 2924−2933

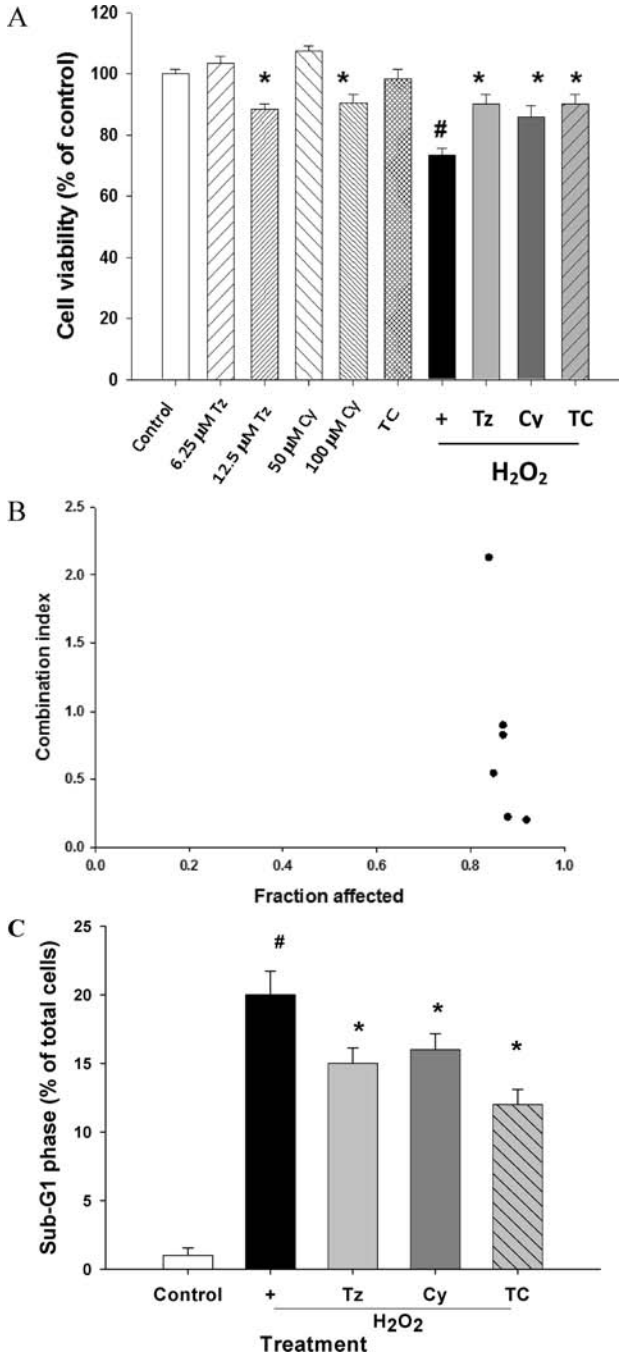


Figure 6. Effects of cyanidin and troglitazone on H_2O_2-induced cytotoxicity of Clone 9 cells. (A) Cells were treated with or without H_2O_2 (50 μM) for 3 h, and then incubated for a total period of 24 h in the absence or presence of cyanidin and troglitazone. Tz, troglitazone; Cy, cyanidin; TC (3.125 μM troglitazone and 25 μM cyanidin). Cell viability was evaluated by MTT assay. Data are from three independent experiments and expressed as mean ± SD. * and # mean significantly different from control group at $P < 0.05$ and $P < 0.01$, respectively. (B) Combination index (CI) of cyanidin–troglitazone combination vs the protection ability (fraction affected) of cell viability. (C) Cells were treated with H_2O_2 (50 μM) for 3 h prior to the treatment with anthocyanins for 21 h. Cells were harvested and stained with propidium iodide (PI) and analyzed by flow cytometry. Data are evaluated by ModFit LT software. For each treatment, 10000 cells were collected and all events were defined as 100%. Tz, troglitazone; Cy, cyanidin. #, significantly different from control group. *, significantly different compared to H_2O_2-treated alone. Data are from triplicate and independent experiments.

To investigate the protective effects of PPAR agonist and cyanidin against H_2O_2-induced cytotoxicity, the MTT assay, which is widely used as a method to evaluate cell proliferation, was performed to evaluate the cell viability at the end of incubation. As shown in Figure 6A, cell viability was significantly inhibited by treatment with H_2O_2 as compared with control cells. The cytotoxic effect of H_2O_2 was suppressed by the treatment of troglitazone or cyanidin. We performed a dose-dependent assay to investigate the synergistic characteristics of cyanidin and troglitazone. Using a mutually nonexclusive model, the combination index (CI) is shown to represent the combination effect, and the values below 1 indicate synergistic effects of the combination, whereas those equal or close to 1 are additive and those above 1 are antagonistic.[21] We found that the CI values were 0.22 and 0.198 upon cyanidin (12.5 μM) and troglitazone (1.5625 μM) and cyanidin (25 μM) and troglitazone (3.125 μM) treatments, respectively (Figure 6B). sub-G1 cell accumulation is a well-known hallmark of apoptotic cell death. Withdrawal of growth signals from proliferating cells sends them into a stage of self-destruction. Patients with NASH also have oxidative DNA damage biomarkers such as 8-hydroxydeoxyguanosine (8-OHdG).[38] Recently, cytoprotective/genoprotective effects of flavonoids in nonstressed and hydrogen peroxide stressed human peripheral lymphocytes has been proved.[39] Furthermore, they also found that the investigated flavonoids also induced DNA damage, indicating their prooxidative capacity. The balance between the protection of DNA from oxidative damage and pro-oxidative effects was strongly dependent on flavonoid concentration and the incubation period. To investigate whether the inhibitory effect of H_2O_2 on cell proliferation is mediated through influencing cell cycle progression or inducing apoptotic cell death, we further determined the cell cycle profile using flow cytometry. After H_2O_2 stimulation (50 μM, 3 h), cells were incubated with troglitazone or cyanidin, respectively, for a total period of 24 h, and then the cell cycle evaluation was performed. The proportion of cells in the sub-G1 phase significantly increased with H_2O_2 treatment, compared with control cells (Figure 6C). H_2O_2-treated cells had 20 ± 1.2% of cells distributed in the sub-G1 phase as compared with 1 ± 0.5% of control cells. However, cells treated with cyanidin or troglitazone significantly decreased H_2O_2-mediated apoptotic cell death. Furthermore, the synergistic effect of cyanidin and troglitazone was also observed and led to the decrease in apoptotic cells. Over the past decade, we and many colleagues have focused on the multifunctional benefits of anthocyanins. However, the bioavailability of anthocyanins is still a complex issue. Since 1999, Kault and colleagues have shown that foods such as blueberries are often promoted as dietary sources of anthocyanins (2.67 ± 0.097 μmol/g).[40] Recently, a review paper summarized much evidence about bioavailability and protective effects of anthocyanins.[41] In an earlier feeding study with strawberries, Carkeet et al.[42] reported a urinary excretion equivalent to 1.8% of the 179 mmol of ingested pelargonidin-3-*O*-glucoside, and this is similar to values obtained in a 15–60 μmol dose study with strawberries.[42] Furthermore, in a separate human feeding study with 200 g of blackberries containing 960 μmol of cyanidin-3-*O*-glucoside, twelve anthocyanins were excreted including unmetabolized cyanidin-3-*O*-glucoside, a cyanidin-*O*-glucuronide and a peonidin-*O*-glucuronide in quantities equivalent to 0.16% of intake.[43] Data presented here indicated that anthocyanidins, in concentrations lower

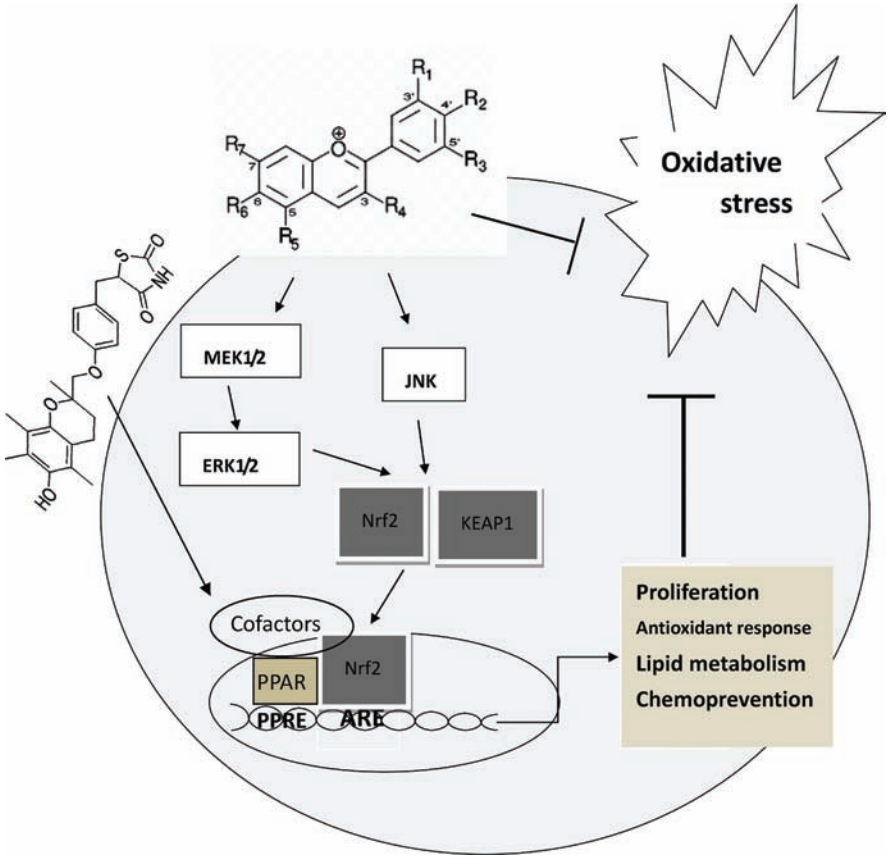


Figure 7. Schematic representation of the proposed chemopreventive mechanism by which anthocyanidin and PPAR agonist induced activation of antioxidant and detoxifying enzymes in Clone 9 liver cells against oxidative stress and modulated lipid metabolism.

than 50 μM, had protective activity against H_2O_2-induced cytotoxicity of human hepatocytes. The concentration of anthocyanidins used in the present study is consistent with other study on the chemopreventive effect of anthocyanins in in vitro research.[44] It has been first revealed that PPAR activation results in the recruitment of Nrf2 and other coregulators upon PPAR agonist stimulation by Park et al.[34] Recently, researchers showed that Nrf2-regulated PPARγ expression is critical to protection against acute lung injury in mice, and then played a cooperative role in chemoprevention against oxidant-induced lung damage.[45,46] They also suggested that silenced Nrf2 critically resulted in a decrease in PPARγ activation. Thus, in the present study, we wondered whether anthocyanidin, which acts as Nrf2-ARE signaling transmitter, shows a synergistic effect on PPAR-Nrf2 activation accompanied with PPAR-agonist treatment, and the effect of Nrf2 deletion on PPAR activation. These data suggested that the cytoprotective effects of troglitazone and cyanidins might act through blocking ROS-induced oxidative damage by upregulating the antioxidant and detoxifying capacity.

The effect of cyanidin on oxidative stress-induced hepatotoxicity could be illustrated as shown in Figure 7. We concluded that MAPKs played a regulatory role in cyanidin-mediated antioxidant enzyme activation, and the transcription factor Nrf2 was a crucial determinant that induced downstream antioxidant enzyme gene activation. Furthermore, the PPAR agonist plus cyanidin activated and induced Nrf2 and PPAR. The combined treatment of cyanidin and the PPAR agonist ameliorated the oxidative stress-related perturbation of genes involved in lipid metabolism and decreased the dosage of the drug. These data suggested that cyanidin and the PPAR agonist might have synergistic benefits against metabolic dysfunction-related oxidative damage.

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Funding

This research work was supported by the National Science Council, NSC99-2628-B005-003-MY3, Taiwan, Republic of China.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

Acyl-CoA Od, acyl-CoA oxidase; AGEs, glycation end products; ARE, antioxidant response element; Cy, cyanidin; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; HMG-CoA Rd, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; QR, NAD(P)H:quinone oxidoreductase; RXR, retinoid X receptor; PMP70, peroxisome membrane

protein 70 kDa; SOD, superoxide dismutase; TC, troglitazone plus cyanidin; Tz, troglitazone; TZD, thiazolidinedione

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