Antioxidant Effects of Black Rice Extract through the Induction of Superoxide Dismutase and Catalase Activities

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ABSTRACT: Our *ex vivo* study revealed that BRE had significantly stronger ability to inhibit LDL oxidation than white rice extract (WRE). The purpose of this study was to investigate whether black rice extract (BRE) supplementation might ameliorate oxidative stress and enhance antioxidant enzyme activities in HepG2 cells and in C57BL/6 mice. In the cellular study, superoxide anions ($O2^{\bullet-}$) and reactive oxygen species (ROS) in the BRE group were significantly suppressed. The BRE group also showed significant increases in superoxide dismutase (SOD) and catalase (CAT) activities by 161.6% and 73.4%, respectively. The major components responsible for the free–radical-scavenging and antioxidative properties might be cyanidin-3-O-glucoside chloride and peonidin-3-O-glucuside chloride. In the animal study, male C57BL/6 mice were divided into three groups (control, BRE, and WRE). Plasma HDL-cholesterol was significantly higher, and thiobarbituric, acid-reactive substances were significantly lower in the BRE group, whereas plasma levels of total cholesterol and triglyceride were not affected by BRE supplementation. Increased hepatic SOD and CAT activities were observed in BRE-treated mice as compared to the control mice. However, no changes were detected for the protein expression of antioxidant enzymes by Western blot analysis. Our data suggest that antioxidative effects exerted by BRE are mediated through decreases in free-radical generation as well as increases in SOD and CAT activities both *in vitro* and *in vivo*.

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Rice is a widely consumed food source for over half of the world's population (1). There are many different kinds of rice, including white rice and a variety of colored rice. Rice with colored hulls has long been considered to be a health food. Black and red rice are planted mainly in South Asian countries and other places such as the United States, Italy, and Greece. Europeans eat more black rice than South Asians (2). Recent reports have shown that supplementation of diets with black rice pigments markedly reduced atherosclerotic lesions in hypercholesterolemic rabbits and apolipoprotein–E-deficient mice (3,4). Black rice pigments are mainly located in the aleurone layer, which is characterized as dark purple to black in color and probably represents a mixture of anthocyanins. Most dietary intake of anthocyanins comes from fruits and vegetables (5). There are about 17 anthocyanins found in nature (6), whereas only six of them (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin) are ubiquitously distributed (7). The water-soluble fraction of black rice might serve as an extra source of dietary anthocyanins (8). Most people consumed a diet of rice by boiling the natural grain in water, but there are no studies concerning the effects of water extract of rice on oxidative stress. This raises the question of whether supplementation with water extract of black rice may be of importance in controlling oxidative stress.

Oxidative damage usually accompanies the development of many age-related diseases (9). Accumulating evidence suggests that oxidative modification of low-density lipoprotein (ox-LDL) may play a critical role in the development of atherosclerosis (10,11). Oxidative stress is one of the main risk factors for LDL oxidation. Reduction of LDL oxidation is an essential target that is used to evaluate dietary factors with antioxidant activity. Conjugated diene (CD) is one of the intermediate products formed during the peroxidation of polyunsaturated fatty acids in LDL lipids (12). Furthermore, thiobarbituric acid-reactive substances (TBARS) are measured as a means of indicating aldehydic breakdown products of oxidized LDL (13). In this study, we assess the effect of BRE on LDL oxidation using assays that measure CD formation *ex vivo* and TBARS levels *in vivo*.

Reactive oxygen species (ROS) are free radicals produced during metabolism and the aging process. ROS include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH). ROS can cause DNA strand breaks, base modification, lipid peroxidation, and protein modification, resulting in oxidative stress. Emerging evidence indicates that ROS are important risk factors in the pathogenesis of many diseases if the antioxidant system is impaired. The measurement of changes in endogenous antioxidant enzyme activity is considered a fairly sensitive biomarker of the response to oxidative stress. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are primary antioxidant enzymes that protect cells from damage caused by ROS. The protective mechanisms against oxidative stress by black rice extract in he-

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Abbreviations: BRE, black rice extract; C3G, cyanidin-3-O-glucoside chloride ; CAT, catalase; CD, conjugated diene; GPx, glutathione peroxidase; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; MDA, malondialdehyde; P3G, peonidin-3-O-glucuside chloride; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TG, triglycerides; TC, total cholesterol; WRE, white rice extract.

patic cells and in the animal model remain largely unknown. In this study, we investigate effects of water extracts from black and white rice on free radical generation and antioxidant enzyme activities in HepG2 cells, as well as in C57BL/6 mice. The results cast light on the physiological significance and the mechanism by which black rice suppress oxidative stress and decrease lipid peroxidation.

MATERIALS AND METHODS

Materials. The raw materials of white and black rice came from an Agricultural Research Center for the Taiwan Region, and the whole rice was washed, dried, and ground to powder. One hundred grams of rice powder was refluxed with 700 mL of double distilled water for 5 h at 80°C, cooled, and vacuum filtered through Whatman filter paper. This was repeated three times, and all extracts were pooled after lyophilization. The residue was re-dissolved in water and diluted with the reaction mixture for the subsequent assay.

Anthocyanins (cyanidin-3-O-glucoside chloride, delphinidin chloride, malvidin chloride, pelargonide chloride, peonidin-3-O-glucoside chloride, and petunidin chloride) were purchased from Extrasynthese (Genay, France) and were HPLC grade. Anti-Mn-SOD and anti-Cu,Zn-SOD antibodies were obtained from Upstate (Charlottesville, VA). Anti-catalase and anti-β-actin antibodies were obtained from Calbiochem (San Diego, CA) and Sigma Chemical Co. (St. Louis, MO), respectively.

Human LDL oxidation. Determination of CD formation was conducted in accordance with our previously reported method (14). Briefly, blood from healthy subjects was collected in the presence of 1 mM EDTA in order to prevent oxidation during lipoprotein separation. Plasma was isolated by centrifugation at 1500 × g for 15 min, and LDL was isolated by sequential density gradient ultracentrifugation (15). The isolated LDL was dialyzed against PBS (10 mM, pH 7.4) overnight and stored at 4°C before being used. Oxidation of LDL (50 μg of protein/mL) was initiated at 37°C by 5 μM $CuSO_4$ for at least 6 h. The extent of CD formation in the absence or presence of 10 μg/mL or 50 μg/mL of BRE or WRE was measured by monitoring the increase in absorbance at 234 nm as previously described (16). The protein content of the LDL fraction was determined using the Bradford assay (Bio-Rad, Hercules, CA) with BSA as the standard.

Identification of anthocyanin content in black and white rice extracts. To obtain a more comprehensive knowledge of the composition of BRE and WRE, we determined the content of anthocyanin components in BRE and WRE by HPLC and LC-MS (8). The anthocyanins were extracted using methanol/water/acetic acid (0.5:15:0.5, vol/vol/vol) according to the method described by Wu et al. (17). The extracted solutions were then diluted with acidic methanol and filtered using a 0.22-μm filter before HPLC analysis equipped with an autosampler/injector and diode array detector. An analytical SB-C_{18} column (4.6 × 250 mm, 5 μm, Agilent Technologies, Rising Sun, MD) was used for separation. The mobile phase was prepared from water/formic acid/acetonitrile (87:10:3, vol/vol/vol), eluent A, and water/formic acid/acetonitrile (40:10:50, vol/vol/vol), eluent B. The gradient program was: 5% B (5 min), 5% B to 20% B (15 min), 20% B to 40% B (15 min), 40% B to 100% B (7 min), 100% B to 5% B (3 min), 5% B (5 min). The flow-rate was 0.4 mL min^{-1}. Monitoring was performed at 520 nm, and the diode-array detector was set at an acquisition range from 200 nm to 600 nm at a spectral acquisition rate of 1.25 scan s^{-1}. To construct calibration plots, volumes of standard stock solution (1000 μM) of cyanidin-3-O-glucoside chloride, delphinidin chloride, malvidin chloride, pelargonide chloride, peonidin-3-O-glucoside chloride, and petunidin chloride were diluted with eluent A, and six concentration levels (1, 5, 20, 100, 200, 500 μM) were analyzed. For quantification, peak areas were correlated with concentrations in accordance with the calibration plot. Final concentrations were expressed as mg per 100 g of dry weight of BRE or WRE. The LC-MS was controlled by Masslynx 3.5 software (Micromass). Positive ion mode was performed with capillary voltage, 3.0 kV; cone voltage, 40V; source temperature, 120°C; desolvation temperature, 250°C; cone gas flow, 50 L/h; and desolvation gas flow, 250 L/h. Anthocyanin components were identified by both retention time and mass profile in comparison to authentic standards.

Cell culture and ROS assays. HepG2 cells were cultured in DMEM containing 100 units of penicillin/streptomycin and 10% fetal bovine serum and seeded at a density of 2×10^5 cells/well into 24-well plates. After 24-h growth at 37°C under 5% CO_2, cells were treated with BRE or WRE at a final concentration of 5 mg/mL for another 24 h. To avoid interferences due to cytotoxicity, we used a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the cytotoxic effect of BRE or WRE in HepG2 cells (18). Briefly, cultured cells were treated with BRE or WRE at a final concentration of 5 mg/mL for 24 h, and MTT solution (0.5 mg/mL) was added into the cell medium. Cells were incubated at 37°C and 5% CO_2 for an additional 4 h. DMSO and a glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added to each well, and the dissolved solutions were detected at 570 nm by a microplate ELISA reader (Dynex technologies, Chantilly, VA). The absorbance of untreated cells was considered as 100%. Treatment of cells with 5 μg/mL of cyanidin-3-O-glucoside chloride or peonidin-3-O-glucoside chloride was performed as the positive control group. Cultured cells were harvested in triplicate to assess the generation of $O_2^{\bullet-}$ and ROS. The ROS levels in HepG2 cells were measured using the dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (19). This reduced dye was added to cells (1×10^6 cells/mL) at a final concentration of 10 μM. The fluorescence of the oxidized dichlorofluorescein was monitored by Flow Cytometry with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Similar measurements were made with 10-μM hydroethidine (HE), which has been reported to be more specifically oxidized by $O_2^{\bullet-}$ (20). In this case, the fluorescence was monitored by Flow Cytometry with excitation at 495 nm and emission at 637 nm. The results were expressed as the relative fluorescence intensity. $O_2^{\bullet-}$ and ROS levels in HepG2 cells

without rice extract treatment were used as the negative control.

Assessment of antioxidant enzyme activities. Assays of antioxidant enzyme activities were carried out as previously described (21). Briefly, SOD activity in the extract of HepG2 or the liver homogenates of C57BL/6 mice was assayed using the hydroxylamine-reduction method (22). In this method, the reduction of hydroxylamine by $O_2^{\bullet -}$ is monitored at 550 nm, utilizing the hypoxanthine/xanthine oxidase system as the source for $O_2^{\bullet -}$. One unit (U) of SOD activity is defined as the amount of enzyme necessary to decrease the reduction of hydroxylamine by 50%. CAT activity in the extract of HepG2 or the liver homogenates of C57BL/6 mice was assayed using the method of Aebi (23). In this method, the decomposition of H_2O_2 due to CAT activity was assayed by monitoring the decrease in the absorbance of H_2O_2 at 240 nm. One U of CAT activity is defined as the amount of enzyme required to catalyze 1 μmol of H_2O_2 per min at 25°C. GPx activity in the extract of HepG2 or the liver homogenates of C57BL/6 mice was quantified by a coupled-enzyme (GPx and glutathione reductase) procedure (24). This method measures the decrease in absorbance at 340 nm as NADPH is converted to NADP. One U of GPx activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min. Specific activities of SOD and CAT were expressed in U/mg of protein, but GPx activity was expressed in mU/mg of protein with the protein content determined as stated above.

Animals. At eight weeks of age, 33 male C57BL/6J mice were randomly assigned to one of the three groups. Three or four animals were housed in each cage and maintained at 25°C in an animal room with a 12-h light/dark cycle. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Mice were allowed free access to food and tap water for 10 weeks during the experiment. The composition of the experimental diet is shown in Table 1. The mineral and vitamin mixtures were obtained from ICN Biochemicals (Costa Mesa, CA). To exclude that ROS generation and lipid peroxidation were affected by differences in energy utilization or hyperlipidemia, we compared body weight and plasma lipid levels of animals of the control, BRE, and WRE groups. After a 12-h overnight fast, the mice were anesthetized with CO_2, and blood samples were taken for biochemical analyses. Plasma was isolated by centrifugation at 1500 × g for 15 min and was stored at –35°C for subsequent lipid and TBARS analyses. The liver tissues were rapidly removed, blotted dry, weighed, frozen in liquid nitrogen, and then stored at –80°C for antioxidant enzyme activity analyses. Before analyses, the liver tissue was homogenized in 10 volumes of a 50-mM phosphate buffer (pH 7.4) on ice for 30 s using a polytron homogenizer. The homogenate was transferred into centrifuge tubes and centrifuged at 9000 × g at 4°C for 20 min. The supernatant was used for the measurement of protein concentration and antioxidant enzyme activities. Twenty micrograms protein was separated on SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with rabbit anti-human Mn-SOD, Cu,Zn-SOD, and catalase antibodies (dilution as recommended) using the standard Western-blot analysis. Immune complexes were visualized with horseradish peroxidase-conjugated immunoglobins and detected using enhanced chemiluminescence (Amersham Biosciences).

TABLE 1
Compositions of Normal (Control) and Experimental Diets (%)

Ingredient	Control	BRE[a]	WRE[a]
Casein	20	20	20
Corn starch	40	40	40
Sucrose	15	10	10
Rice extract	0	5	5
Soybean oil	13.5	13.5	13.5
Cellulose	5	5	5
Mineral mix	3.5	3.5	3.5
Vitamin mix	1	1	1
Choline bitartrate	0.25	0.25	0.25
tert-Butylhydroquinone	1.45	1.45	1.45
L-Cystine	0.3	0.3	0.3

[a]BRE, black rice extract; WRE, white rice extract.

Plasma lipids and TBARS analyses. The concentrations of total cholesterol (TC) and triglyceride (TG) were assayed enzymatically using commercial reagents (Merck). The levels of HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) were determined by precipitation with phosphotungstic acid/magnesium chloride and heparin/sodium citrate, respectively, using reagents supplied by Merck. The products of lipid peroxidation were measured as TBARS at 532 nm (25) using a standard curve of thiobarbituric acid (TBA) adduct formation with freshly diluted 1,1,3,3-tetraethoxypropane as the standard.

Statistical analyses. Data are given as the mean ± SD. Results were analyzed by one-way analysis of variance (ANOVA) using the SAS program (version 6.12, SAS Institute, Cary, NC). Differences between mean values were evaluated by the post-hoc test and were considered significant if $P < 0.05$.

RESULTS

Inhibition of LDL oxidation by BRE. Conjugated diene formation on exposure of copper-catalyzed LDL to 10 or 50 μg/mL rice extract is shown in Fig. 1. The initial amount of CD formed did not differ among groups. Addition of 10 and 50 μg/mL BRE significantly decreased the oxidation rates and maximal amounts of diene formation, whereas WRE had little or no effect. The lag-phase duration, which represents the resistance of LDL to lipid peroxidation, was prolonged from 72 min to 198 min when the BRE treatment was increased from 10 to 50 μg/mL. The presence of WRE at increased concentrations had no significant effect on the lag time, LDL oxidation rate, or maximal amounts of diene formation.

Quantitative analyses of anthocyanin components in rice extract. HPLC analysis was performed to estimate the content of anthocyanin components in brown and white rice extract. As shown in Table 2, cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride were identified as the two major

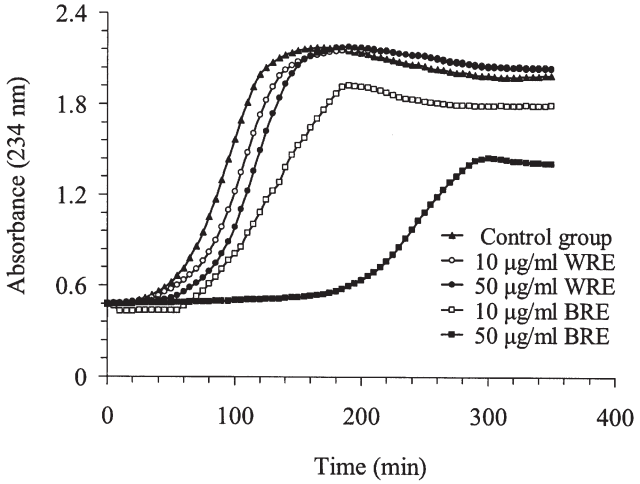

FIG. 1. Effects of white (WRE) and black (BRE) rice extracts on the generation of conjugated dienes (CD) in LDL. The oxidation of LDL (50 μg protein/mL) was initiated by the addition of 5 μM copper. White and black rice extracts were added to LDL at final concentrations as shown. CD generation was monitored by measuring the increase in absorbance at 234 nm. Values shown are from a representative experiment.

TABLE 2
Anthocyanin Content (mg/100 g dry weight) of Black Rice Extract (BRE) and White Rice Extract (WRE)

Compound	BRE	WRE
Cyanidin-3-O-glucoside chloride	26.8	14.2
Delphinidin chloride	4.3	n.d.a
Malvidin chloride	2.4	n.d.
Pelargonidin chloride	n.d.	n.d.
Peonidin-3-O-glucoside chloride	11.2	6.8
Petunidin chloiride	n.d.	n.d.

[a]n.d., not detected.

contents of BRE by comparison of the retention time and the photodiode array spectrum with those of authentic anthocyanins. We thus used cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride as the positive control to compare their effects on free-radical scavenging capacities and antioxidant enzyme activities in HepG2 cells.

Effects of rice extracts and anthocyanins on ROS levels and antioxidant enzyme activities in HepG2 cells. Cell toxicity was evaluated by MTT assay. The result from the cell viability assay showed that BRE and WRE had no obvious toxicity (94.7 ± 10.3% and 92.3 ± 6.5% of control, respectively) to HepG2 cells at the concentration of 5 mg/mL. The oxidative status was reflected from the levels of $O_2^{\bullet -}$ and ROS in HepG2 cells (Fig. 2). BRE (5mg/mL) supplementation significantly suppressed $O_2^{\bullet -}$ and ROS levels by 34.0% and 39.3%, respectively, as compared to the control group. Significant suppression of ROS generation was also obtained by cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride by 63.0% and 44.3%, respectively, at a final concentration of 5 μg/mL. However, HepG2 cells treated with a WRE (5 mg/mL) diet showed no significant difference in levels of $O_2^{\bullet -}$ and ROS compared to the control cells.

Effects of BRE, WRE, and anthocyanins on activities of antioxidant enzymes in HepG2 cells are shown in Fig. 3. SOD and CAT activities were significantly higher in the BRE group than in the control group, whereas GPx activity was not altered. However, cells treated with WRE and anthocyanins did not cause significant changes in antioxidant enzyme activities compared to the control group.

Body weight and plasma lipid levels in the C57BL/6J mice. The body weight of mice in each group before study entry (eight weeks of age) was 21.2 ± 2.6 g, and at the end of the study (18 weeks of age) was 27.5 ± 4.4 g, 25.6 ± 2.7 g, and 26.8 ± 3.3 g in the control, BRE, and WRE groups, respectively. The differences in body weight across the three groups were not significant.

No significant differences were found for the plasma levels of triglyceride, total cholesterol, and LDL-cholesterol among the BRE, WRE, and control groups. However, plasma HDL-cholesterol was significantly higher, and TBARS showed a significant decrease, in mice fed with the BRE diet, whereas no change was found in the WRE group (Table 3).

Effects of rice extracts on antioxidant enzyme activities and protein expression in livers of C57BL/6J mice. The levels of the hepatic antioxidant enzyme activities are shown in Table 4. The BRE-treated animals exhibited markedly increased hepatic SOD and CAT activities. In contrast, there was no increase in GPx activity in livers of BRE-treated animals. Hepatic SOD activity of the BRE group also displayed a greater increase than the WRE group, whereas CAT and GPx activities did not differ between the BRE and WRE groups. By using Western-blot analysis, the relatively small differences in the expression of Mn-SOD, Cu,Zn-SOD, and catalase were statistically non-significant among the BRE, WRE, and control groups (Fig. 4).

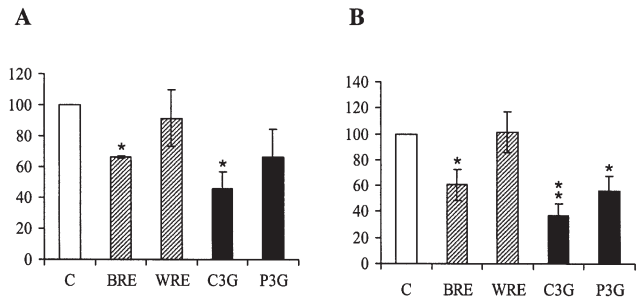

FIG. 2. Effects of rice extracts and anthocyanin components on the levels of $O_2^{\bullet -}$ (A) and ROS (B) in HepG2 cells. Cells were incubated with 5 mg/mL of rice extracts (black rice extract, BRE, and white rice extract, WRE) or 5 μg/mL of anthocyanin components (cyanidin-3-O-glucoside chloride, C3G, and peonidin-3-O-glucoside chloride, P3G) for 24 h at 37∞C. $O_2^{\bullet -}$ and ROS were determined as described in Methods. The results are representative of three different assays. All of the corresponding control values (arbitrary unit) were converted as 100%. The data represent the mean ± SD. Statistical analyses: $^*P < 0.05$ and $^{**}P < 0.01$ versus the control.

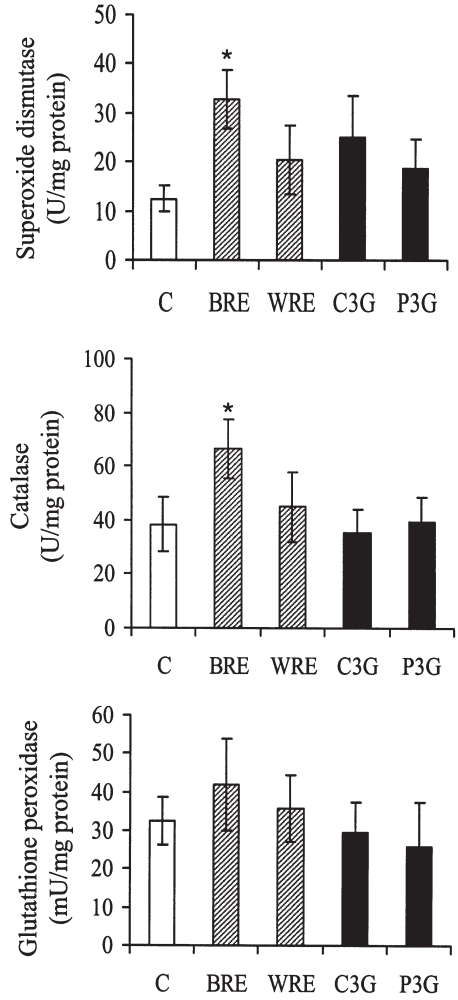

FIG. 3. Effects of rice extracts and anthocyanin components on the levels of antioxidant enzyme activities in HepG2 cells. Antioxidant enzyme activities were analyzed as described in Methods. The results are representative of three different assays. The data represent the mean ± SD. Statistical analyses: *$P < 0.05$ versus the control.

DISCUSSION

The results of this study show that black rice contains anthocyanin components with notable antioxidative properties for potential use in nutraceutical benefit in preventing human diseases pathologically related to oxidative stress (9). Cupric ion-induced LDL oxidation is a common marker for evaluating antioxidant activity relative to the prevention of lipid and protein (apolipoprotein B) oxidation (14). We have clarified for the first time that BRE evoked a remarkably greater inhibition of CD formation than that in WRE. This prompted us to investigate the antioxidant effects of BRE in cellular and animal models.

HepG2, a well–differentiated, transformed cell line, is a reliable model and widely used for biochemical and nutritional studies. In this study, we investigated the ROS scavenger effect by rice extract and anthocyanins (cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride) in HepG2 cells to assist in elucidating the mechanism of reduction in oxidative

TABLE 3
Plasma Lipid Profiles and TBARS Levels[a] in C57BL/6J Mice Fed a Control Diet with and without Black Rice Extract (BRE) or White Rice Extract (WRE

Variable	Control	BRE	WRE
n	10	12	11
TGb, mg/dL	86.7 ± 20.9	76.1 ± 22.4	94.3 ± 23.3
TCb, mg/dL	97.6 ± 15.2	102.2 ± 19.3	105.8 ± 24.2
HDL-Cb, mg/dL	58.7 ± 11.4	69.7 ± 12.0[c]	66.9 ± 14.4
LDL-Cb, mg/dL	37.5 ± 14.8	32.3 ± 08.9	35.3 ± 14.0
TBARSb, nmol MDAa/L	257.4 ± 31.5	226.7 ± 23.5[c]	243.5 ± 27.9

[a]Values are expressed as the mean ± SD.
[b]Abbreviations used: TG, triglyceride; TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde.
[c]Significant difference from the control ($P < 0.05$).

stress by BRE. Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They are widely distributed in the human diet through crops, beans, fruits, and vegetables and seem to possess strong antioxidant, anti-inflammatory, and anticarcinogenic properties (26). However, little is known about the anthocyanin profile of whole black rice. We thus quantitatively characterized the anthocyanin contents in black rice—specifically, a black rice pigmented fraction extract (BRE)—in the present study. The molecular mechanisms of the ROS-scavenging effect by BRE and anthocyanins were also determined.

The mechanism for the antioxidative effect of BRE was unclear at the start of this study. We hypothesized that BRE might exert its antioxidant effects through suppression of ROS and induction of antioxidant enzyme activities. To test this hypothesis, we determined free-radical generation and activities of SOD, CAT, and GPx in HepG2 cells treated with BRE or WRE, as well as cyanidin-3-O-glucoside chloride or peonidin-3-O-glucoside chloride. It is of note that BRE exerted its antioxidative effect via the induction of SOD and CAT activities in hepatoma cells. By contrast, enzyme activities of SOD and CAT were not affected by the addition of cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride to the culture medium. Recent studies have suggested that cyanidin-3-O-glucoside chloride is thought to play an antioxidant role through the suppression of peroxynitrite-induced oxidation (8,27). Our results indicate that cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside

TABLE 4
Hepatic Antioxidant Enzyme Activities[a] in C57BL/6J Mice Fed the Control or Experimental Diets

Variable	Control	BRE[b]	WRE[b]
n	10	11	11
SOD[b] (U/mg protein)	15.6 ± 4.1	24.1 ± 06.3[c]	19.1 ± 4.8[d]
CAT[b] (U/mg protein)	40.3 ± 8.2	53.7 ± 11.4[c]	45.9 ± 9.0
GPx[b] (mU/mg protein)	32.1 ± 7.0	39.5 ± 10.2	37.8 ± 9.8

[a]Values are expressed as the mean ± SD.
[b]Abbreviations used: BRE, black rice extract; WRE, white rice extract; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.
[c]and [d]denote a significant difference from the control ($P < 0.01$) and BRE ($P < 0.05$) groups, respectively.

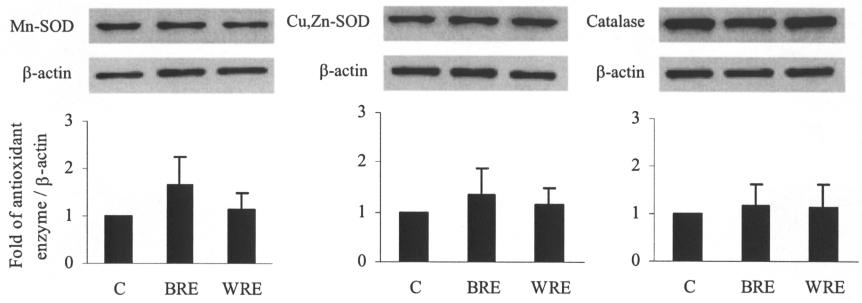

FIG. 4. Effects of black (BRE) and white (WRE) rice extracts on protein expression of Mn-superoxide dismutase (Mn-SOD), Cu,Zn-SOD, and catalase in livers of C57BL/6 mice (n = 6). Representative blots of protein expression are depicted and antioxidant enzyme expression was normalized to β-actin level as the internal control. Histograms show densitometric analyses of protein expression levels. The results are shown as fold increases above the untreated controls. Data are the mean ± SD from at least six independent experiments.

chloride may scavenge ROS through other biological pathways independent of the induction of antioxidant enzyme activities.

Antioxidant enzymes, such as SOD, CAT, and GPx, are endogenous factors that protect cells from oxidative damage caused by ROS. SOD catalyzes the dismutation of the $O2^{\bullet-}$ to molecular oxygen and H_2O_2, which in turn is metabolized to harmless water and oxygen by CAT and GPx. The activities of SOD and CAT were higher in livers of C57BL/6 mice treated with the BRE diet. These results suggest that BRE supplementation may attenuate oxidative stress by reducing ROS and increasing antioxidant enzyme activities both in vitro and in vivo. However, the increased enzyme activities observed in the BRE group may not be the result of increased gene expression via the evaluation of the protein expression of Mn-SOD, Cu,Zn-SOD, and CAT by Western-blot analysis. The lack of an exact relationship between the changes in the antioxidant enzyme activities and those in the protein levels indicates that antioxidant enzyme activities are possibly post-translationally regulated.

Previous studies have revealed that lipids are susceptible to oxidative damage. Lipid peroxidation is usually quantified by using a TBARS assay (28). Our data from plasma TBARS analyses are in accordance with the results obtained from the *ex vivo* study. Among all the major lipoproteins, HDL is considered to be an endogenous protective factor against atherosclerosis (29). Our previous studies have suggested that HDL has the potential to limit the oxidative modification of LDL (14). The results from this study suggest that BRE may also exert its antioxidative effects through the induction of plasma HDL in C57BL/6 mice.

In summary, our data shed new light on the molecular basis that BRE contributes to marked increases in SOD and CAT activities that may cause a decrease in free-radical generation and oxidative stress. This observation points to a new direction when trying to understand the physiological function of black rice as a benefit to human health.

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