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Black Rice (*Oryza sativa* L. *indica*) Pigmented Fraction Suppresses both Reactive Oxygen Species and Nitric Oxide in Chemical and Biological Model Systems

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Anthocyanins, present in fruits and vegetables as natural colorants, have been well characterized to possess bioactive properties. Anthocyanin components extracted from black rice (*Oryza sativa* L. *indica*) separated by gel filtration and identified using LC-MS were cyanidin 3-glucoside and peonidin 3-glucoside. A standardized extract of black rice pigmented fraction (BRE) containing known proportions of cyanidin 3-glucoside and peonidin 3-glucoside exhibited marked antioxidant activities and free radical scavenging capacities in a battery of in vitro model systems. Significant ($p < 0.05$) prevention of supercoiled DNA strand scission induced by reactive oxygen species (specifically, peroxyl radical and hydroxyl radicals) and suppression of the oxidative modification of human low-density lipoprotein was obtained with BRE. In addition, BRE reduced ($p < 0.05$) the formation of nitric oxide by suppressing inducible nitric oxide synthase expression in murine macrophage RAW264.7 cells, without introducing cell toxicity. The results of this study show that black rice contains anthocyanin pigments with notable antioxidant and anti-inflammatory properties for potential use in nutraceutical or functional food formulations.

KEYWORDS: Black rice; anthocyanin; antioxidant; nitric oxide

INTRODUCTION

Rice is a major cereal crop in the developing world and an important staple food source for over half of the world's population (*1*). Although widely consumed as white rice, there are many special cultivars of rice that contain color pigments, such as black rice and red rice. Southeastern Asia (20−28° latitude) is the major production area for this agricultural crop, and inhabitants of this area have a long history of black rice consumption.

It is well documented that consumption of rice bran can produce a hypocholesterolemic effect as well as antioxidant activity, attributed in major part to the presence γ-oryzanol and tocotrienols (*2*, *3*). Feeding black rice to rodents in place of white rice has resulted in increased high-density lipoprotein concentrations in hypercholesterolemic rabbits, which corresponded to a reduction in the size of atherosclerotic lesions in these same animals (*4*). A recent report showed that the supplementation of atherogenic diets with black rice pigment markedly reduced oxidative stress and inflammation in addition to modulating atherosclerotic lesions in the apolipoprotein E deficient mice (*5*). These findings indicate that compounds present in pigmented black rice, and absent in white rice, provide cardiovascular protection in addition to the lipid-soluble components present in rice bran (*3*).

Endogenous antioxidant activity has recently been attributed to a variety of specific phenolic acid derivatives in white rice hull (*6*). In addition, pigmented rice, such as red rice, contains an acetylated procyanidin, a common anthocyanin with free radical scavenging activity (*7*). Anthocyanins are a group of natural colorants that belong to the family of flavonoids. The most commonly occurring anthocyanin aglycons are cyanidin, peonidin, malvidin, and pelargonidin (*8*). In black rice, pigments are located in the aleurone layer, which is characterized as dark purple to black in color and probably represents a mixture of anthocyanins. Little is known about the black rice pigment profile and its potential ability to scavenge free radicals, including both oxygen- and nitrogen-derived radicals. The purpose of the present study therefore was to extract and quantitatively characterize the anthocyanin profile present in black rice, specifically a black rice pigmented fraction extract (BRE). In addition, we investigated the efficacy of an anthocyanin standardized extract from black rice to neutralize both reactive oxygen and nitrogen reactive species in chemical and cell culture models.

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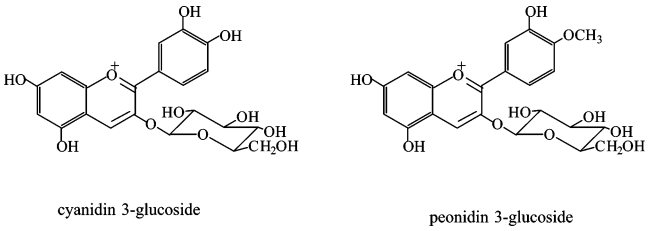

Figure 1. Structures of cyanidin 3-glucoside and peonidin 3-glucoside.

MATERIALS AND METHODS

Black rice (*Oryza sativa* L. *indica*) was obtained from Guangdong province, China. HPLC grade cyanidin 3-*O*-β-glucoside and peonidin 3-*O*-β-glucoside (**Figure 1**) standards were purchased from Polyphenol AS (Sandnes, Norway). Bio-Gel P-2 resin and electrophoresis grade agarose were obtained from Bio-Rad Laboratories (Hercules, CA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA Inc. (Richmond, VA). Murine macrophage RAW264.7 cells (TIB-71) were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics were purchased from Gibco Invitrogen Corp. (Burlington, ON, Canada). Other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

Sample Preparation. Black rice aleurone layer was removed by an abrasive dehuller (Satake) at 10% yield. Both aleurone layer and whole rice were used for the extraction purpose and quantitation of anthocyanin. Both whole rice and black rice pigmented fraction were extracted overnight at room temperature with methanol containing 1% HCl, followed by filtration through Whatman filter paper no 4. Methanol was removed by rotary evaporation at <40 °C. Whole black rice (WBR) and black rice pigmented fraction extract (BRE) were obtained for further study.

Separation of Anthocyanins by Bio-Gel P-2 Column Chromatograph. A preconditioned 40 cm × 2.5 cm Bio-Gel P-2 column (Bio-Rad Laboratories) was used for anthocyanin separation with aqueous acetic acid, pH 2.5. Elution sample volumes (5 mL/tube) were collected using a fraction collector and were monitored by spectrophotometer at 520 nm (Shimadzu 160 spectrophotometer). Anthocyanin-rich fractions were identified and concentrated under vacuum prior to further identification.

Quantitation and Identification of Anthocyanin by LC-MS. Anthocyanin quantitation was conducted by HPLC using a Waters Alliance 2690 separation module equipped with a model 996 photodiode array detector (Waters Corp., Franklin, MA). A 50 × 2.1 mm i.d., 2.5 μm Xterra MS C18 column (Waters Corp.), used at 40 °C was employed with a linear gradient mobile phase containing solvent A (water), solvent B (methanol), and solvent C (5% formic acid in water, v/v) at a flow rate of 0.3 mL/min. Mobile phase composition increased from A:B:C 70/10/20 to 40/40/20 within 5 min, before returning to initial conditions for 3 min for the next sample. Injection volume was 5 μL. Micromass ZQ2000 mass spectrometry (Micromass, Manchester, U,K,) with electrospray ionization interface (ESI) was also employed to identify the anthocyanins. The LC-MS system was controlled by Masslynx 3.5 software (Micromass). Positive ion mode was applied with the following conditions: capillary voltage, 3.0 kV; cone voltage, 40 V; source temperature, 120 °C; desolvation temperature, 250 °C; cone gas flow, 50 L/h; desolvation gas flow, 250 L/h. Anthocyanins were identified by both retention time and mass profile in comparison to authentic standards.

DNA Nicking Prevention. Both AAPH-induced peroxyl and Fenton reaction-induced hydroxyl radicals were generated in the presence of DNA. Effects of black rice extracts on preventing DNA from free radical induced scission in vitro were evaluated (*9*). Supercoiled pBR322 DNA strands were separated on 0.7% agarose gel electrophoresis with 0.5 μg/mL ethidium bromide using a TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5). DNA bands were visualized by an ultraviolet benchtop transilluminator (UVP Inc., Upland, CA), and individual band densities were analyzed by LabWork software (UVP Inc.). The inhibition of DNA nicking was calculated according to the procedure described previously (*9*).

Effect of BRE on Preventing Human LDL (hLDL) Oxidation in Vitro. The evaluation of BRE on the prevention of hLDL oxidation in vitro was conducted in accordance with our previously reported method (*10*). Briefly, hLDL was dialyzed against PBS (10 mM, pH 7.4) under continuous nitrogen treatment in the dark at 4 °C for 24 h to remove transition metal ions and EDTA. Oxidation of LDL (0.5 mg of protein/mL) was initiated at 37 °C by 10 μM $CuSO_4$ for 16 h. The oxidation was stopped by adding 100 μM EDTA. The extent of LDL oxidation was further measured in multiple assays including conjugated dienes measured at 234 nm, thiobarbituric acid reactive substances (TBARs) measured at 532 nm, and electrophoretic migration using 0.6% (w/v) agarose gel electrophoresis with 50 mM barbital buffer (pH 8.6).

Effect of BRE on Preventing Nitric Oxide Production in Lipopolysaccharide (LPS)-Stimulated Macrophage Cells. Murine macrophage RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C under humidified air with 5% CO_2. Cells (2×10^5 cells/well) were seeded in a 96-well plate. After cells had been cultured overnight, test samples were added 30 min prior to exposure of the cells to 1 μg/mL bacterial LPS (*Escherichia coli*, serotype 0111:B4). Cells were then cultured for another 24 h, and culture medium (100 μL) was mixed with 100 μL of Greiss reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water, 1:1 v/v) in a 96-well microplate (*11*). The absorbance at 540 nm was measured within 30 min at room temperature (Multiskan Ascent, ThermoLabsystems). The concentration of nitrite was calculated according to a standard curve obtained from sodium nitrite. The inhibition on nitric oxide production was calculated according to the equation

$$\text{inhibtion \%} = \frac{[NO_2^-]_{LPS} - [NO_2^-]_{sample}}{[NO_2^-]_{LPS} - [NO_2^-]_{blank}} \times 100$$

of which $[NO_2^-]_{LPS}$, $[NO_2^-]_{blank}$, and $[NO_2^-]_{sample}$ represent nitrite concentration of cultural media with LPS, without LPS, and sample with LPS, respectively.

Evaluation of Cell Viability in RAW264.7 Cells. RAW264.7 cells were seeded at a density of 2×10^5 cells/well into 96-well plates. After overnight growth, cells were treated with BRE for another 24 h, followed by adding 0.5 mg/mL methylthiazoletetrazolium (MTT). After incubation for 4 h at 37 °C under 5% CO_2, DMSO was used to dissolve the crystal and absorbance readings were taken at 570 nm (Multiskan Ascent, ThermoLabsystems).

Western Blotting for the Inducible Nitric Oxide Synthase (iNOS). Cells were cultured in 100-mm dishes with cultural conditions the same as described above. Cells were collected into 2× sample reducing buffer and incubated in a boiling water bath for 5 min. Samples (e.g., 20 μL) were loaded to 8% SDS-PAGE, followed by electrophoretic transfer to a nitrocellulose membrane (Bio-Rad Laboratory). The membrane was then blocked with 3% skim milk powder in 50 mM Tris buffer, pH 7.5, containing 150 mM NaCl and 0.05% Tween-20, at room temperature for 1 h. The membrane was then incubated with mouse anti-iNOS antibody (Pharmingen Transduction Laboratories) and mouse anti-α-tubulin antibody (Sigma-Aldrich) in 50 mM Tris buffer with 150 mM NaCl, pH 7.5, at 4 °C overnight, followed by incubation with goat anti-mouse IgG conjugated with horseradish peroxidase (Pharmingen Transduction Laboratories) for 1 h at room temperature. Protein was visualized with 4-chloro-1-naphthol and hydrogen peroxide (*12*).

Statistics. Tests were run in triplicate, and data were expressed as means ± SD. Differences in treatment means were analyzed using Student's *t* test (SPSS Inc., Chicago, IL) with the significance difference level set at $p < 0.05$.

RESULTS AND DISCUSSION

Total anthocyanin contents of whole black rice and alueurone layer of black rice were 0.16 and 1.36%, respectively, measured by LC-MS using cyanidin 3-glucoside and peonidin 3-glucoside

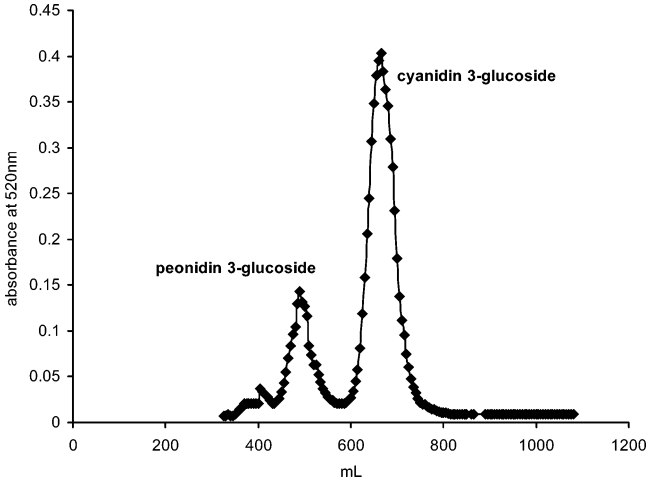

Figure 2. Chromatogram of black rice pigment on Bio-Gel P-2 column eluted with acidified aqueous solution; two major peaks were further identified as peonidin 3-glucoside and cyanindin 3-glucoside.

as standards. Therefore, anthocyanins are concentrated in the pigmented layer of black rice granule, and the study focused on this layer to generate a standardized extract. The anthocyanin present in the pigmented layer (aleurone) accounted for 85% of the total anthocyanin content in whole black rice. The physical removal of pigmented layer provided a unique method for obtaining anthocyanin from black rice.

Figure 2 shows a typical chromatogram of BRE derived from the Bio-Gel P-2 column and containing two absorbance peaks at 520 nm under low pH. Employing Bio-Gel P-2 resin was effective for separating anthocyanins present in the black rice. Further LC-MS analysis revealed the content and composition of anthocyanins present in whole rice and the outer layer of black rice. The positive charge of anthocyanins favors fast and effective electrospray mass spectroscopy detection of intact molecule ions (*13*). The LC-MS profile indicated that peak I from the Bio-Gel P-2 column was peonidin 3-glucoside ($[M + H]^+$, *m/z* 463.1; $[M -$ glucoside$]^+$, *m/z* 301.1), and peak II from the Bio-Gel P-2 column was cyanidin 3-glucoside ($[M + H]^+$, *m/z* 449.1; $[M -$ glucoside$]^+$, *m/z* 287.1). This was also confirmed by the spiking with authentic cyanidin 3-glucoside and peonidin 3-glucoside standards. The use of LC-MS provided a fast and reliable method for the measurement of anthocyanin contents in food materials, based on not only the retention time on the chromatographic method but also the compound fragment information obtained from mass spectrum. **Figure 1** illustrates the chemical structures of cyanidin 3-glucoside and peonidin 3-glucoside, which were found to exist in the black rice used in this study.

Numerous studies have shown the usefulness of using DNA base scission as an effective in vitro method for evaluating antioxidant activity against active oxygen species, including both hydroxyl radicals and peroxyl radicals (*9*, *14*−*16*). The transition metal−ascorbic acid−hydrogen peroxide system produces marked sequence-dependent damage of DNA with preferential oxidation of guanine bases, which indicates a localized production of reactive oxygen species as a result of metal binding to specific regions on the DNA molecule (*17*−*19*). Halliwell et al. (*20*) developed a simple method to generate hydroxyl radicals through the action of Fenton reactants, of which both site-specific and non-site-specific sources of hydroxyl radicals were defined. In our current study, both non-site-specific and site-specific hydroxyl radicals were applied to the DNA scission assays for evaluating the antioxidant activity of the anthocyanin

Table 1. Inhibition Percentage of Black Rice and Black Rice Pigmented Fraction on the Prevention of DNA Nicking Induced by Hydroxyl Radical

	inhibition % on non-site-specific hydroxyl radical[a]	inhibition % on site-specific hydroxyl radical[a]
whole rice		
100 μg/mL	65.7 ± 5.1*	51.4 ± 6.4*
1000 μg/mL	83.9 ± 7.5**	74.1 ± 3.2**
black rice pigmented fraction		
10 μg/mL	54.4 ± 5.6*	49.0 ± 4.5*
100 μg/mL	82.8 ± 6.5**	69.5 ± 5.9*
Trolox		
0.17 μg/mL	44.0 ± 6.0*	25.7 ± 1.9*
1.7 μg/mL	80.8 ± 3.2**	61.0 ± 5.4**

[a] *, $p < 0.05$, and **, $p < 0.01$, versus control.

Table 2. Effects of Black Rice Pigmented Fraction and Anthocyanin in Protecting Supercoiled DNA Strand from Peroxyl Radical Induced Scission

sample	inhibition %[a]
black rice pigmented fraction	
1 μg/mL	1.0 ± 0.7
10 μg/mL	7.4 ± 0.3*
25 μg/mL	25.8 ± 8.7*
100 μg/mL	82.2 ± 7.8**
cyanidin 3-glucoside/peonidin 3-glucoside[b]	
9:1	63.2 ± 1.6**
4:1	50.4 ± 2.1**
1:1	38.7 ± 0.3**
1:4	50.2 ± 0.6**
1:9	40.7 ± 0.0***
Trolox	
10 μg/mL	100

[a] *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus control. [b] Concentration of cyanidin 3-glucoside + peonidin 3-glucoside was 10 μg/mL.

standardized BRE (**Table 1**). The presence of hydroxyl radicals, regardless of whether from site-specific or from non-site-specific induction procedures, resulted in supercoiled DNA strand scission (**Table 1**). The standardized BRE exhibited greater ($p < 0.05$) prevention of DNA nicking in the non-site-specific generation of hydroxyl radicals, compared to the test that produced site-specific hydroxyl radical-induced DNA nicking (**Table 1**). The positive control in this test was the antioxidant Trolox, which produced a similar result with higher ($p < 0.05$) inhibition of DNA damage induced by non-site-specific hydroxyl radical generation (**Table 1**). In the non-site-specific model, the presence of EDTA interfered with sequence-specific interaction between the iron and DNA, similar to that reported by others (*18*, *21*). Moreover, the observation that the extent of prevention against hydroxyl radical-induced supercoiled DNA nicking was also associated with the distribution of anthocyanins in the outer layer reflects simply the greater concentration of anthocyanins in this part of the rice granule (**Table 1**). The findings herein with anthocyanin, which are protective against hydroxyl radical-induced DNA scission, have also been reported with specific flavonoids (*9*, *22*) and lignans (*14*). In our study, we also found that the BRE suppressed the supercoiled DNA scission, induced by peroxyl radical generated from AAPH, in a concentration-dependent manner at a minimal threshold concentration of 10 μg/mL (**Table 2**). LC-MS data confirmed the presence of cyanidin 3-glucoside and peonidin 3-glucoside as the major anthocyanin pigments in the BRE. In both cases, these specific anthocyanins have potential antiradical activity (*23*).

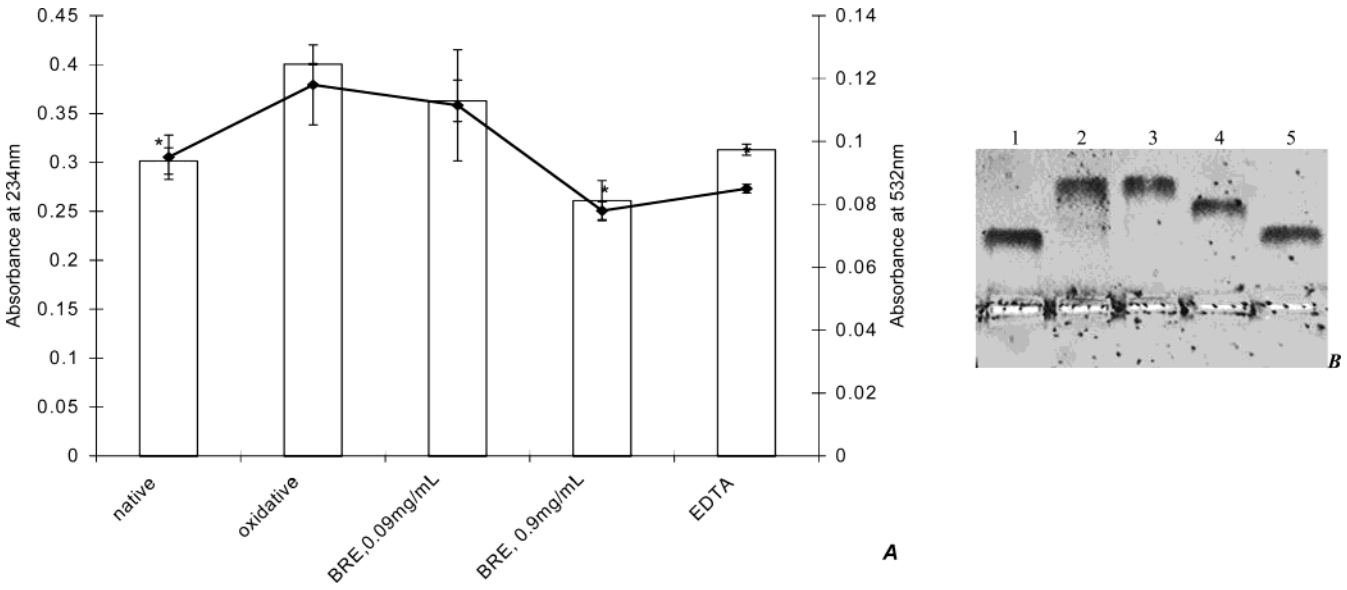

Figure 3. (A) Black rice pigmented fraction (BRE) suppresses oxidative modification of human LDL in vitro (bars = conjugated diene, line = TBARs); *, $p < 0.05$ versus oxidatively modified LDL. (B) Effect of black rice pigmented fraction (BRE) in suppressing the change of negative charge of oxidative modified LDL: (lane 1) native LDL; (lane 2) LDL + Cu^{2+}; (lane 3) LDL + Cu^{2+} + 0.09 mg/mL BRE; (lane 4) LDL + Cu^{2+} + 0.9 mg/mL BRE; (lane 5) LDL + Cu^{2+} + EDTA.

The significance of the antioxidant activity attributed to BRE is related to the fact that peroxyl radicals are generated freely from the reaction between carbon-centered radicals and O_2 at a diffusion-controlled rate, which thus represents a common source of reactive oxygen species (*24*). Although peroxyl radicals have a longer half-life than the nanosecond half-life of hydroxyl radicals (*25*), this particular source of free radical damage exhibits potential toxicity toward supercoiled DNA scission as well as inducing lipid peroxidation (*16*). In our study, we found that cyanidin 3-glucoside and peonidin 3-glucoside exhibited 11.5 and 11.4% protection on the DNA strand scission at 1 μg/mL, respectively, whereas protection increased to 45.3 and 44.1% at the 5 μg/mL level, respectively. The combined effect of cyanidin 3-glucoside and peonidin 3-glucoside was also measured in this peroxyl radical induced DNA scission model. Our results showed that the protection against DNA nicking decreased ($p < 0.05$) with the reduced presence of cyanidin 3-glucoside in the mixture (**Table 2**). A concentration-dependent suppression of supercoiled DNA scission was also observed with BRE (**Table 2**).

Cupric ion-induced LDL oxidation is also a common in vitro model for evaluating antioxidant activity relative to the prevention of protein−lipid oxidation (*9*, *10*, *26*, *27*). The implication that oxidized LDL in vivo will promote the formation of macrophage foam cells, which in turn triggers the damage of endothelial cells and the proliferation of smooth muscle cells that precedes endothelial dysfunction and fatty streak formation (*28*), makes this assay relevant to understanding how phytochemicals may act to reduce LDL oxidation and its related incidence of coronary heart disease. For example, vitamin E concentration in plasma has been associated with the resistance of human LDL against oxidation ex vivo (*29*), and vitamin E intake has been inversely correlated with the incidence of myocardial infarction (*30*). Moreover, epidemiological surveys have demonstrated that higher dietary consumption of flavonoids reduces the incidence of coronary disease (*31*, *32*). The prevention of cardiovascular events by the increased intake of flavonoids was suggested to be attributed to the prevention of LDL oxidation (*33*).

The formation of malonedialdehyde (MDA), as measured by TBARS, was found to increase in the oxidatively modified LDL and could be reduced when hLDL was exposed to the BRE (**Figure 3A**). Confirmation of the TBARs result was made with simultaneous measurements of conjugated diene (**Figure 3A**) and relative electrophoretic migration of treated hLDL on agarose gel (**Figure 3B**). The fact that the BRE suppressed the change of electrophoretic migration characteristic to the ox-LDL (**Figure 3B**) is evidence that the chemical change of lysine residues present in apoB-100 by reactive aldehydes formed during lipid oxidation was reduced as a consequence of antioxidant activity (*34*). Finally, the generation of conjugated dienes, a primary lipid oxidation product measured at 234 nm, was also found to be lower ($p < 0.05$) in hLDL when in the presence of BRE.

In addition to ROS, nitric oxide and its metabolite peroxynitrite are considered to have mutagenic activity (*35*, *36*). Of the two sources of cellular nitric oxide synthase (NOS) that include constituted NOS (cNOS) and inducible NOS (iNOS), the latter is activated by LPS or cytokines to result in the production of nitric oxide. Leeuwenburgh et al. (*37*) implicated reactive nitric species with aortic LDL oxidation and atherosclerosis with the finding that LDL from aortic atherosclerotic intima contained significantly higher 3-nitrotryosine than plasma LDL. Further evidence has shown that iNOS inhibitor *N*-iminoethyl-L-lysine limited the progression of pre-existing atherosclerosis in hypercholesterolemic rabbits (*38*), thus suggesting that inhibition of iNOS is beneficial for the progression of atherosclerosis.

Nitric oxide expression was obtained in a mouse macrophage cell line RAW264.7 co-incubated with bacterial LPS. The measurement of nitric oxide was quantified indirectly from the metabolite nitrite in the supernatant of cell cultural media using the Greiss reagent. The concentration of nitrite in the cultural media from macrophage at 25 μM following exposure to LPS was higher ($p < 0.05$) than that obtained from cells cultured without LPS treatment. The addition of the standardized BRE significantly ($p < 0.05$) suppressed nitric oxide output in activated macrophage cells (**Figure 4**). The inhibition of nitric

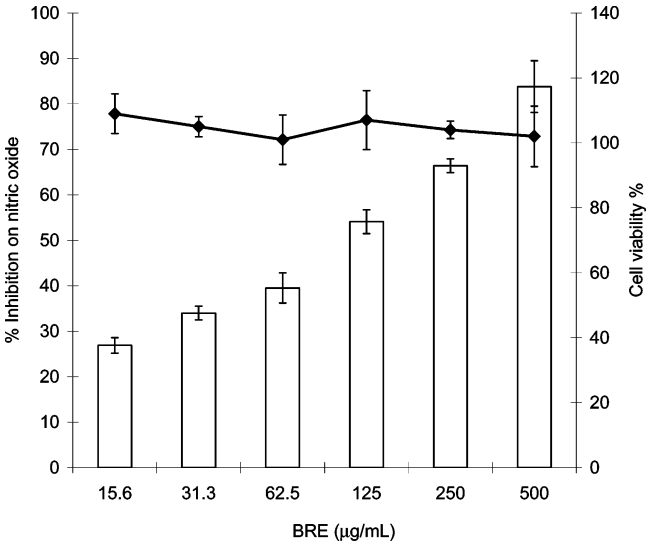

Figure 4. Effect of black rice pigmented fraction (BRE) on the inhibition of nitric oxide stimulated by bacterial lipopolysacharide in mouse macrophage cell RAW264.7 (bars = % inhibition of nitric oxide, solid line = cell viability).

Table 3. Inhibition Percentage of Nitric Oxide Generated in the LPS-Activated Mouse Macrophage Cell RAW264.7

	cyanidin 3-glucoside	peonidin 3-glucoside
1 μM	12.7 ± 2.7*	14.4 ± 0.6*
10 μM	23.1 ± 0.9*	22.3 ± 3.7*
100 μM	72.9 ± 4.8***	35.0 ± 2.5**

[a] *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus control.

oxide production occurred without noticeable cytotoxicity, with normal macrophage cell viability present with the addition of the BRE (**Figure 4**). Inhibition ($p < 0.05$) of nitric oxide was also apparent when LPS-activated RAW264.7 cells were incubated with cyanidin 3-glucoside and peonidin 3-glucoside under identical culture conditions (**Table 3**). It was noteworthy that cyanidin 3-glucoside evoked a relatively greater inhibition of nitric oxide formation than that of peonidin 3-glucoside, at an equivalent concentration of 100 μM. Kim et al. (*39*) demonstrated that flavonoids possessing a C-2,3 double bond and 5,7-dihydroxyl groups in the A-ring were strongly active at suppressing NO production; however, the 4′-substitution negatively affected such activity. This is of interest to our results, because the 4′-position of peonidin 3-glucoside is substituted by a methyl group and it exhibits reduced activity against nitric oxide in comparison to cyanidin 3-glucoside.

There is controversy as to whether flavonoids inhibit nitric oxide production by suppressing iNOS enzymatic activity (*39*, *40*). In our study, the addition of LPS to the RAW264.7 cells clearly induced the expression of iNOS protein as demonstrated by western blotting (**Figure 5**). The addition of the BRE to culture media containing macrophage cells further reduced the expression of iNOS protein (MW 130 kDa) in a concentration-dependent manner. Cell lysate blotted with anti-α-tubulin antibody showed that this housekeeping protein remained unchanged with or without LPS and BRE sample treatments. These results show that the BRE constituents specifically suppressed iNOS of cultured macrophage, thus reducing nitric oxide production under this condition. Similar findings of a flavonoid-rich extract suppressing nitric oxide by reducing the expression of iNOS protein and corresponding to the reduction of iNOS mRNA have been reported with the

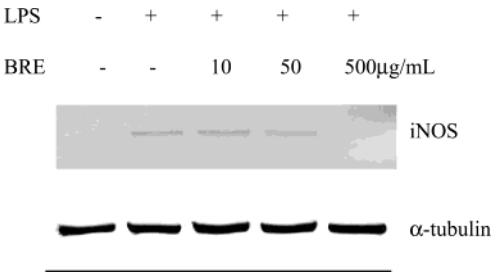

Figure 5. Effect of black rice pigmented fraction (BRE) on the expression of iNOS in LPS-stimulated macrophage cells.

Ginkgo biloba extract EGb761 (*11*). More recently, Xia et al. (*5*) reported a reduction in iNOS protein expression in aortic tissue collected from atherosclerotic susceptible C57BL/6J knock-out mice fed with a black rice pigment fraction. The suppression of iNOS in apo-E knock-out mice fed black rice pigment corresponded to lower oxidized LDL and reduced atherosclerotic lesions in arotic sinus compared to counterparts fed white rice.

A typical American diet contributes 180−215 mg of daily intake of anthocyanins, which varies with the season, mostly from the intake level of fruits and vegetables (*8*). In the current study, we demonstrated the existence of cyanidin 3-glucoside and peonidin 3-glucoside as primary anthocyanin components present in black rice, suggesting that black rice could serve as an extra source of dietary anthocyanins. Bioavailability and metabolism studies of cyanidin 3-glucoside and peonidin 3-glucoside have been attempted in human subjects (*41*, *42*), and anthocyanins have been shown to undergo both methylation and glucuronide conjugation (*43*). Intact cyanidin glycoside was also found in human plasma within 30 min after oral administration of blueberry extract (*44*). Although not yet confirmed, it is possible that anthocyanins from the black rice fraction are bioavailable following human oral administration in a form that maintains the function of suppressing both reactive oxygen and nitrogen species.

In conclusion, our results demonstrated the presence of anthocyanins in the aleurone layer of black rice, in particular, cyanidin 3-glucoside and peonidin 3-glucoside. The anthocyanins contributed to marked antioxidant activities in preventing DNA damage and LDL deterioration in vitro. Black rice and specific anthocyanin components present in black rice also suppressed the production of nitric oxide in the activated macrophage without introducing cytotoxicity. These data suggest that black rice may have some health benefits associated with the relief of oxidative stress.

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