JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. XXXX, XXX, 000–000 A
DOI:10.1021/jf102224b

Protective Effects of Black Rice Bran against Chemically-Induced Inflammation of Mouse Skin

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We investigated the inhibitory effects of black rice (cv. LK1−3−6−12−1−1) bran against 12-*O*-tetradecanolylphorbol-13-acetate (TPA)-induced skin edema and 2,4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD) in inflammatory mouse models. We also determined the effects of the bran extract on the following biomarkers: pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), eicosanoids leukotriene B_4 (LTB_4), and prostaglandin E_2 (PGE_2). Topical application of TPA to ears of CD-1 mice induced inflammation accompanied with substantial increase in TNF-α, IL-1β, IL-6, LTB_4, and PGE_2 levels and an elevation in intercellular adhesion molecule-1 (ICAM-1) gene expressions in ear skin tissues. Intraperitoneal injection of black rice bran extract prior to TPA application in mice significantly suppressed TPA-induced inflammation (edema) and induced a marked decrease in the production of TNF-α, IL-1β, IL-6, and LTB_4. Feeding mice a standard diet with added 10% black rice bran also significantly suppressed DNFB-induced allergic contact dermatitis on the skin of the mice. By contrast, a nonpigmented brown rice bran extract did not inhibit the TPA-induced edema and failed to significantly suppress production of pro-inflammatory biomarkers (mediators). These in vivo findings further demonstrate the potential value of black rice bran as an anti-inflammatory and antiallergic food ingredient and possibly also as a therapeutic agent for the treatment and prevention of diseases associated with chronic inflammation.

KEYWORDS: Black rice bran; brown rice bran; health food; biomarkers; cytokine; eicosanoid; mice; skin inflammation

INTRODUCTION

Rice (*Oryza sativa* L.) is a basic food for a large part of the world's population, especially in Asia. Rice bran is a rich source of a large number of bioactive secondary metabolites including phytosterols, γ-oryzanol, tocopherol, tocotrienols, simple phenolic compounds, and anthocyanins (cyanidin-3-glucoside, cyanidin-3,5-diglucoside, malvidin, peonidin-3-glucoside, and pelargonidin-3,5-diglucoside) (*1*−*7*) and volatile aroma compounds (*8*). The bioactive components of black rice are anthocyanin pigments, whereas those in brown rice are simple phenolic compounds such as caffeic, ferulic, and gallic acids (*7*, *9*, *10*).

These rice bran ingredients and dark rice brans have been shown to possess a number of health-promoting functions. These include cholesterol reduction in plasma and liver, inhibition of platelet aggregation, prevention of ulcer formation, inhibition of cancer cell invasion, and immunomodulating effects in cell assays (*11*−*15*). A Chinese study showed that dietary black rice fractions improved cardiovascular risk factors in humans (*16*).

In previous studies we found that 70% ethanolic bran extracts from several black rice cultivars exhibited potent antioxidative, antimutagenic, and anticarcinogenic effects in chemical and cell assays (*17*−*19*), and that brans from the black rice cultivars, especially cv. LK1-3-6-12-1-1, suppressed the release of histamine from mast and basophilic cells (*20*) and inhibited ovalbumin-induced airway inflammation in a mouse asthma model (*21*). To further demonstrate the potential therapeutic value of dark rice bran, the major objective of this study was to compare anti-inflammatory effects of intraperitoneally and orally administered black and brown bran extracts in the ear skin of mice and to define several molecular biomarkers that govern anti-inflammatory effects.

MATERIALS AND METHODS

Animals and Reagents. Female CD-1 mice, aged 4−5 weeks, were obtained from Orient Inc. (Seoul, Korea). The average body wt of the mice injected with TPA was 23.9 ± 1.99 g and of the mice in the control group, 24.7 ± 1.78 g. After acclimation for 1 week, the mice were housed under a 12-h light/dark cycle with a temperature range of 20−22 °C and relative humidity of 50 ± 10%. The mice were fed for six weeks with pelletized commercial chow diet and sterile tap water ad libitum during the entire experimental period. DNFB (> 99% pure), TPA (molecular biology grade), and other reagents were purchased from Sigma (St. Louis, MO). The AMV reverse transcriptase and dNTP mix were products of Takara Bio Inc. (Kyoto, Japan). PCR primers were custom-synthesized and purified by Bioneer Co. (Daejon, Korea).

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Table 1. Primer Sets Representing Target Genes

primer	sequence
tumor necrosis factor-α (TNF-α) sense	5′-TACTGAACTTCGGGGTGATCGGTCC-3′
TNF-α antisense	5′-CAGCCTTGTCCCTTGAAGAGAACC-3′
interleukin-1β (1 L-1β) sense	5′-GTAGCCCACGTCGTAGCAAA-3′
IL-1β antisense	5′-CCCTTCTCCAGCTGGGAGAC-3′
interleukin-6 (IL-6) sense	5′-GAAATGATGGATGCTTCCAAACTGG-3′
IL-6 antisense	5′-GGATATATTTTCTGACCACAGTGAGG-3′
intercellular adhesion molecule-1 (ICAM-1) sense	5′-TTCCTCTCTGCAAGAGACT-3′
ICAM-1 antisense	5′-TGTATCTCTCTGAAGGACT-3′
cyclooxygenase-2 (COX-2) sense	5′-TCTCAACCTCTCCTACTAC-3′
COX-2 antisense	5′-GCACGTAGTCTTCGATCACT-3′
5-lipoxygenase (5-LOX) sense	5′-ATGAGCTGTTTCTAGGCATGTACC-3′
5-LOX antisense	5′-GAATAAAGTACCCCTGACCCAGCC
β-actin sense	5′-GTGGGGCGCCCCAGGCACCA-3′
β-actin antisense	5′-GTCCTTAATGTCACGCACGATTTC-3′.

Preparation of Rice Bran Extracts. The pigmented black rice (*Oryza sativa* cv. LK1-3-6-12-1-1) and nonpigmented brown rice (*Oryza sativa* cv. Chuchung) were harvested at the experimental rice field of the College of Agriculture and Life Science, Seoul National University (Suwon, Korea). The rice seeds were dehulled, degermed, polished in a laboratory mill, and passed through a 60-mesh sieve, resulting in a uniform fraction of rice bran. The rice bran fractions were then extracted by shaking overnight at room temperature with 10× the sample weight of 70:30 ethanol:water. We optimized the solvent to bran ratio used to extract the rice bran in several related studies (*17−19*). The 70% ethanol extraction retained the highest activities. The extracts were filtered through Whatman no. 2 filter paper (Maidstone, U.K.). The filtrates were concentrated to dry solids by sequential use of rotary evaporator and lyophilizer. The dried extracts were stored at −20 °C until use.

TPA-Induced Ear Inflammation in Mouse Skin. Persistent inflammation in ear skin was induced by multiple topical application of TPA based on the method described by Nakamura and others (*22*) and Zhaorigetu and others (*23*). Prior to application of TPA, bran extracts were intraperitoneally administered to CD-1 mice (10 mg/kg body weight) for 14 d. Both ears were then treated topically once a day for 4 d with 160 μM (10 μL) TPA in acetone. Changes in the ear thickening/swelling were measured at 24 h intervals using a thickness gauge (Mitsutoyo, Tokyo, Japan). After the last TPA treatment and subsequent measurement of ear thickening/swelling, the mice were sacrificed to obtain the ear skin using an 8 mm-diameter punch. The ear skins were used for immunohistochemical examination, ELISA analysis, and histology.

To demonstrate that long-term daily ingestion of a black rice bran-containing diet can prevent the incidence of contact dermatitis, we compared the effects of the intraperitoneal injection 14-day dosage of the black rice bran extract in the TPA-model to the feeding of 10% black rice bran powder added to a normal mouse diet fed for 6 weeks. The 10 mg/kg intraperitoneal injection dose is based on related studies with other natural products (*24*, *25*).

DNFB-Induced Allergic Contact Dermatitis (ACD) in Mouse Skin. Allergic contact dermatitis was induced in ear skin according to the method of Flint and Tinkle (*26*) with slight modifications. The mice were fed with a commercial chow diet supplemented with 10% rice bran for 6 weeks. On days 0 and 1, the back of each mouse was shaved using surgical clippers. DNFB (0.5%, 20 μL) diluted in a vehicle of acetone:olive oil (4:1) was then applied to the back skin. On day 5, the right ears were topically challenged with DNFB (0.2%, 20 μL). The left ears were treated only with the vehicle. The resultant change of ear thickening/swelling was measured at 24 h intervals using the same approach described above for the TPA-induced ear edema.

Histology and Immunochemistry. For histological analysis, the ear tissue of the mice was fixed overnight with 4% paraformaldehyde in 0.5 M phosphate buffer (PBS, pH 7.4). The tissues were rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sectioned into 4 μm and mounted on glass slides. The sections were then dewaxed using xylene and ethanol, and stained with hematoxylin and eosin (H&E). The number of dermal infiltrating inflammatory cells was counted under a microscope. An average number of stained cells were counted for each section in six fields.

For immunohistochemical analysis, the deparaffinized skin sections were treated with 3% H_2O_2 to block undesirable effects of endogenous peroxidase. Following incubation with 10% fetal calf serum in PBS to reduce background staining and nonspecific antibody binding, samples were incubated with primary antibodies and then labeled with HRP-conjugated anti-IgG antibody. After washing, the tissue sections were incubated in diaminobenzidine (DAB) solution, followed by counterstaining with 0.2% modified Harris hematoxylin solution (Sigma, St. Louis, MO). Rabbit polyclonal antimouse myeloperoxidase antibody and antimouse tryptase monoclonal antibody (Abcam, Cambridge, MA), were used as the primary antibodies. For histological and immunohistochemical examination, histological samples were selected as representative samples ($n = 3$) from the tissue samples ($n = 10$).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Cellular RNA. Total cellular RNA was prepared from tissue samples following acid phenol guanidium thiocyanate-chloroform extraction (*27*). For reverse transcription, 1 μg of the total RNA was incubated with 5 units of AMV reverse transcriptase and 100 ng of oligo (dT_{18}) as primer. DNA amplification was then primed in a reaction mixture containing 400 μM of dNTP mix, 2.5 U of *Taq* polymerase, and 20 μM each of the primer sets representing the target genes as represented in **Table 1**. PCR was conducted using a thermocycler (PTC-200, MJ Research Inc., Reno, NV) with one cycle for 5 min at 94 °C, followed by 30 cycles for 30 s at 94 °C, 45 s at 58 °C, and 45 s at 72 °C, and finally one cycle for 5 min at 72 °C. All amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with UV illuminator. The intensity of separated bands under DNA was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan).

Western Blot Analysis of Cell Proteins. The tissues were extracted in a homogenizer with RIPA buffer (50 mM Tris Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, pH 7.4). Protein concentrations were determined according to the Bradford method using a Bio-Rad Protein kit (Hercules, CA). Bovine serum albumin (BSA) was used as standard. The protein extracts were separated on 10% polyacrylamide gels and electrophoretically transferred onto nitrocellulose membrane (Millipore, Billerica, MA).

The primary antibodies used for Western blot analysis were as follows: goat antimouse COX-2 antibody (Santa Cruz, Delaware, CA), goat antimouse 5-LOX antibody (Abcam, Cambridge, MA), and mouse β-actin monoclonal antibody (Millipore, Billerica, MA). After blocking with 5% skim milk, membranes were incubated with each primary antibody, followed by peroxidase-conjugated IgG (Millipore, Billerica, MA). Blots were developed using the ECL detection kit (Pierce, Rockford, IL). The intensity of separated protein bands was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan). At least three separate replicates were determined for each experiment.

ELISA of Cytokines and Eicosanoids. Extraction of cytokines and eicosanoids from ear tissue was conducted by the method described by Huang and others (*28*). Briefly, ear tissues were homogenized in a phosphate (PBS) pH 7 buffer containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1 mM PMSF, and 10 mM EDTA. The homogenates were microcentrifuged at 14,000*g* for 15 min at 4 °C to recover the supernatant.

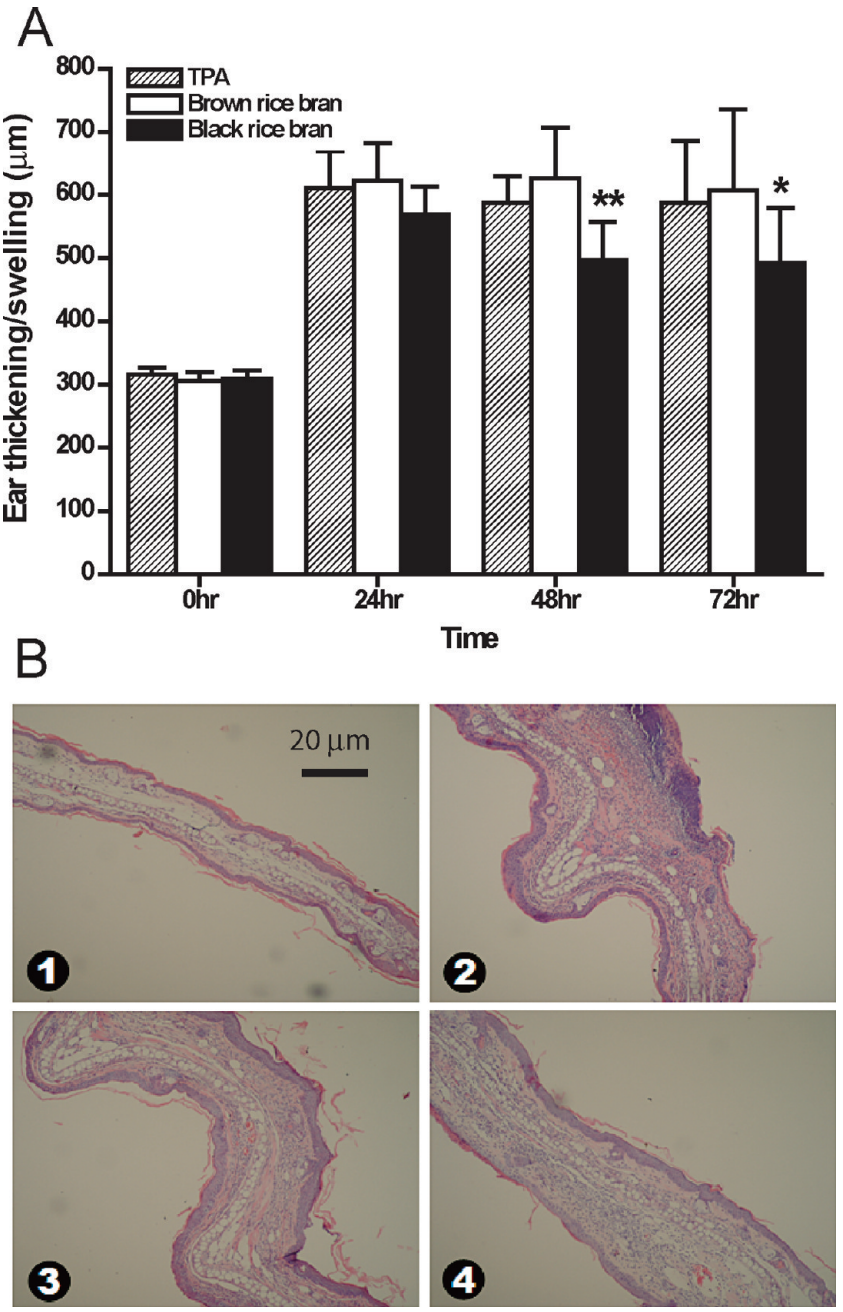

Figure 1. Changes in thickening/swelling of ear skins of mice. (A) Effect of treatment of rice bran extracts by intraperitoneal injection on TPA-induced ear thickening/swelling at various time points. The intraperitoneal treatment of vehicle (DMSO) alone (stripped bar), brown rice bran extract (open bar), and black rice bran extract (closed bar) was performed for 14 days followed by topical application of TPA. Data are expressed as mean ± SD ($n = 10$). (B) Effect of the treatment of rice bran extracts by intraperitoneal injection on histological changes in TPA-induced ear tissues stained with hematoxylin and eosin. Panel 1, normal skin; panel 2, intraperitoneal injection with vehicle alone prior to topical application of TPA; panel 3, intraperitoneal injection with brown rice bran extract prior to topical application of TPA; panel 4, intraperitoneal injection with black rice bran extract prior to topical application of TPA. * and ** statistically different from the group subjected to intraperitoneal injection with vehicle alone at $p < 0.05$ and $p < 0.01$, respectively.

Cytokines TNF-α, IL-β, and IL-6 and eicosanoids leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) in the supernatants were measured by an ELISA (Biosource International (Camarillo, CA) according to the manufacturer's instructions. The absorbance of final reaction mixture at 420 nm was measured in a microplate reader (model 550, Bio-Rad, Hercules, CA).

Statistical Analysis. Significant difference among the groups was obtained using SAS software with Duncan's multiple range test at $p < 0.05$. The data are expressed as mean ± SD.

We evaluated the difference among groups at $p < 0.05$ level for all experiments. Differences from controls in **Figures 1** and **2** were determined at $p < 0.01$ and $p < 0.001$, respectively.

RESULTS

Effect of Black Rice Bran on TPA-Induced Skin Inflammation (Edema). The inhibitory effect of the black rice bran extract on skin inflammation of mice was assessed using the persistent skin inflammation model induced by topical application with TPA. As shown in **Figure 1A**, the increase in skin thickening/swelling reached a plateau after 24 h and persisted for 72 h. This TPA-induced skin edema was significantly inhibited by intraperitoneal injection of the black rice bran extract prior to TPA application ($p < 0.01$ and $p < 0.05$ at 48 and 72 h, respectively). In contrast, the nonpigmented brown rice bran extract had no inhibitory

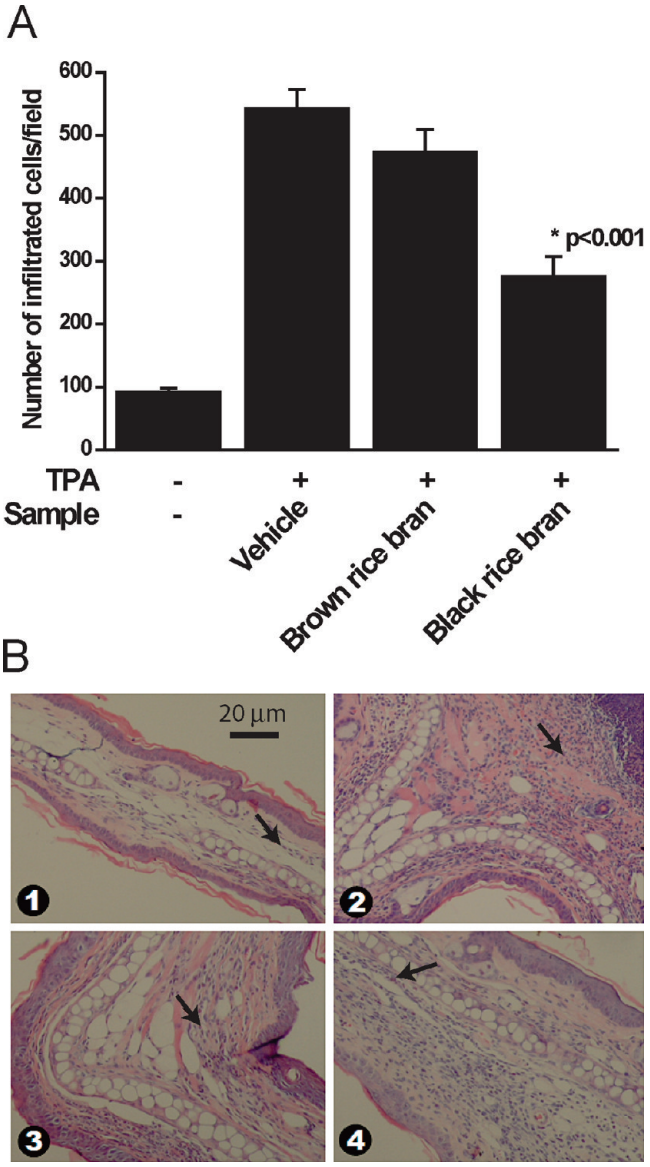

Figure 2. Infiltration of cells into ear skin tissues of mice. (A) Effect of the treatment of rice bran extracts by intraperitoneal injection on inflammatory cell infiltration in ear tissues examined by histological analysis. Data are expressed as mean ± SD (n = 3). * Statistically different from the group subjected to the intraperitoneal injection with vehicle alone at $p < 0.001$. (B) Infiltrated cells under high power-field microscopy (×400). Panel 1, normal skin; panel 2, intraperitoneal injection with vehicle alone (DMSO) prior to topical application of TPA; panel 3, intraperitoneal injection with brown rice bran extract prior to topical application of TPA; panel 4, intraperitoneal injection with black rice bran extract prior to topical application of TPA. Arrows indicate representative infiltrated inflammatory cells.

effect. Instead, the mice exhibited a slight increase in ear thickening/swelling after TPA application. Histological analysis revealed that at 72 h swelling of dermis applied with TPA alone was evident as compared with the section of a normal skin and that treatment with pigmented black rice bran markedly reduced the chemical-induced thickening of dermis (**Figure 1B**).

Effect of Black Rice Bran on Infiltration of Cells and Expression of Intercellular Adhesion Molecule-1 (ICAM-1). TPA-induced increase in ear thickening/swelling indicates massive leukocyte infiltration into ear tissues. As expected, a large number of leukocytes were found to have infiltrated the dermis in response to multiple TPA applications relative to that of the normal skin (**Figure 2**). The black rice bran extract treatment by intraperitoneal injection significantly suppressed leukocyte infiltration in the dermis as compared with that observed with the nonpigmented brown rice.

Calculation of % decrease in ear thickness/swelling or % increase in leukocyte infiltration are based on the initial ear thickness at 0 h or infiltrated leukocyte number of unstimulated mice. The calculated net changes in ear thickness or leukocyte infiltration are ~32% or a ~59% decrease. Therefore, there were no major discrepancies between the rate of decrease in ear thickness and the rate of increase in leukocyte infiltration.

Immunohistochemical analysis using antibody raised against myeloperoxidase identified the infiltrated cells as neutrophils. Treatment with pigmented black rice bran extract induced significant reduction in neutrophil levels (**Figure 3A**). The expression of cell adhesion molecules in TPA-induced neutrophil infiltration was elucidated by RT-PCR to semiquantify the mRNA expression levels for ICAM-1. The attenuation of neutrophil influx into ear tissue resulting from exposure to rice bran extract was inversely related to the transcription level of ICAM-1 gene (**Figure 3B**).

Effect of Black Rice Bran on Production of Pro-Inflammatory Cytokines and Eicosanoids. TPA application increased levels of the representative pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and eicosanoids (LTB_4 and PGE_2) in ear skin tissues (**Tables 2** and **3**). The intraperitoneal treatment with the pigmented black rice bran extract significantly suppressed TNF-α, IL-1β, and IL-6 production in ear tissue by 62.3%, 10.8%, and 7.8%, respectively (**Table 2**). The treatment also significantly suppressed LTB_4 formation by 55.7% but did not significantly suppress PGE_2 production (**Table 3**).

By contrast, the nonpigmented brown rice treatment failed to elicit significant suppression of pro-inflammatory mediators TNF-α, IL-1β, IL-6, PGE_2, and LTB_4 in the local inflamed skin tissue. Examination by RT-PCR of inhibition of expression of genes for pro-inflammatory cytokines and eicosanoid synthesis showed marked reductions in TNF-α, IL-1β, IL-6, and 5-LOX gene expressions in mice treated with black rice bran. The treatment also significantly reduced expression of the 5-LOX gene to a much greater extent than that of COX-2 gene (**Figure 4**). Data from Western blot analysis show similar trends of the bran treatments on modulation of 5-LOX and COX-2 gene expressions (**Figure 5**).

Effect of Black Rice Bran Extract on DNFB-Induced Allergic Contact Dermatitis in Mouse Skin. We then addressed the question as to whether oral feeding of the black rice bran will also ameliorate DNFB-induced allergic contact dermatitis (ACD) in skin. **Figure 6** shows that ear swelling from chemical-induced contact hypersensitivity peaked at 48 h. The duration of inhibitory effect lasted for 48 h after topical application of DNFB.

The results show that oral feeding of a standard mice diet supplemented with 10% black rice bran significantly suppressed ear swelling in mice at 24 h ($p < 0.01$) as compared to that observed with the diet without black rice bran. By contrast, the diet with 10% brown rice bran did not suppress contact hypersensitivity. Instead, it induced a slight increase in ear thickening/swelling.

DISCUSSION

The skin represents a unique immunological interface for interaction with environmental agents, including sunlight and chemicals. It also provides a path for interactions between the peripheral immune and the central nerve systems for maintenance of homeostasis of tissues (*29*). Stress that imparts injury on this interface may cause inflammation of the skin and other tissues (*28*, *30*).

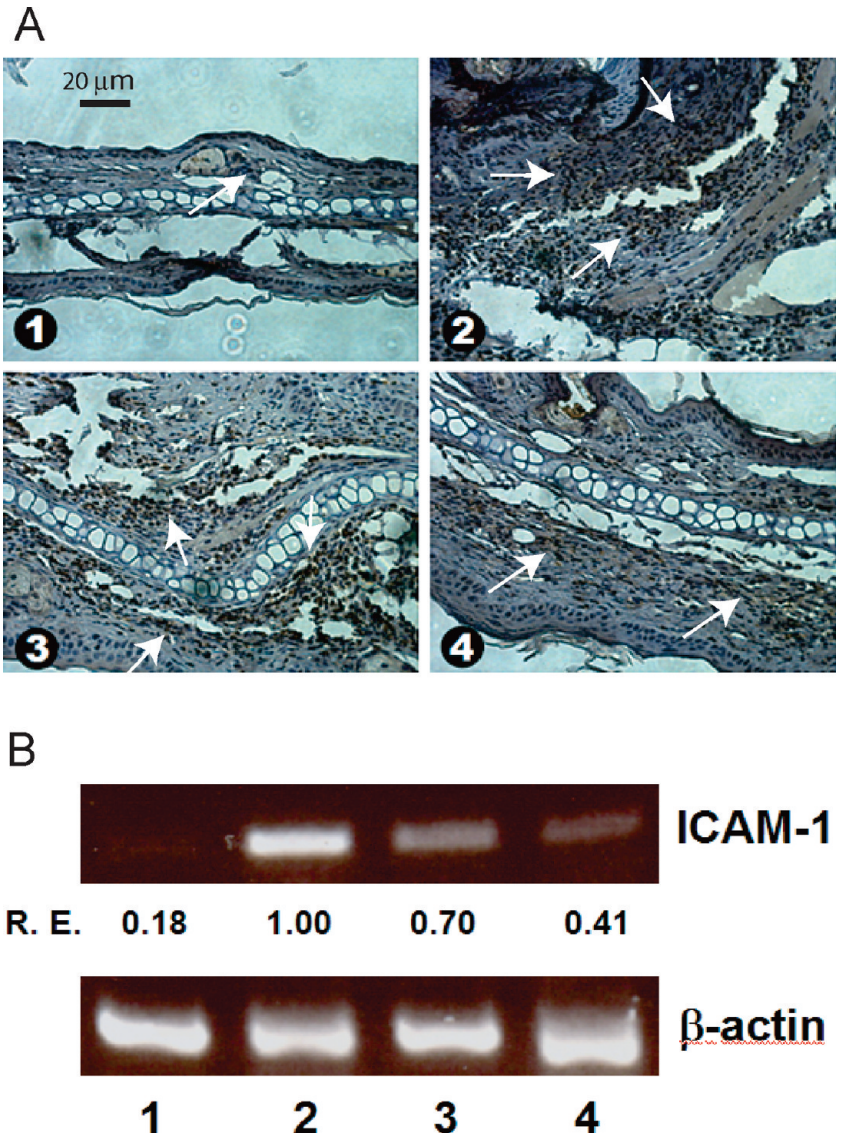

Figure 3. Immunostaining of infiltrated leukocytes with antimyeloperoxidase antibody and semiquantitative RT-PCR analysis for expression of ICAM-1 in ear skin tissues. (A) Effect of the intraperitoneal treatment with rice bran extracts on staining patterns observed under high power-field microscopy (×400). Arrows indicate representative positive immunostained cells (brown). (B) Effect of the treatment with rice bran extracts by intraperitoneal injection on transcription of ICAM-1 gene in ear tissues. Panel 1 and Lane 1, normal skin; panel 2 and lane 2, intraperitoneal injection with vehicle alone (DMSO) prior to topical application of TPA; panel 3 and lane 3, intraperitoneal injection with brown rice bran extract prior to topical application of TPA; panel 4 and lane 4, intraperitoneal injection with black rice bran extract prior to topical application of TPA. R.E. means relative expression of ICAM-1/β-actin gene. Figures represent at least three individual experiments.

Table 2. Inhibitory Effect of Brown and Black Rice Bran Extracts on TNF-α, IL-1β, and IL-6 Production in TPA-Induced Mouse Ear Tissue Inflammation (Edema)[a]

	pg/mL (% inhibition)		
experiments	TNF-α	IL-1β	IL-6
control (-TPA)	15.2 ± 2.60[†] (−)	241.8 ± 28.0[†] (−)	270.6 ± 4.8[†] (−)
control (+TPA)[b]	68.3 ± 3.6[‡] (0.00)	1064.1 ± 16.3[‡] (0.00)	800.9 ± 9.8[‡] (0.00)
brown rice bran[c]	74.5 ± 2.1[‡] (−9.05)	1037.6 ± 19.7[‡] (2.49)	785.7 ± 15.5[‡] (1.90)
black rice bran[d]	25.8 ± 5.9[§] (62.28)	949.7 ± 29.4[§] (10.75)	738.7 ± 9.3[§] (7.77)

[a] The data are expressed as mean ± S.D. (n = 3). Values in the same column not sharing a common superscript are significantly different at $p < 0.05$. [b] Intraperitoneal injection of mice with vehicle only (DMSO) for 14 days prior to TPA application. [c] Intraperitoneal injection of mice with brown rice bran extract (10 mg/kg body weight) for 14 days prior to TPA application. [d] Intraperitoneal injection of mice with black rice bran extract (10 mg/kg body weight) for 14 days prior to TPA application.

During inflammation, cells associated with the immune response migrate to the site of injury and release pro-inflammatory mediators, cytokines, and eicosanoids derived primarily from arachidonic acid to counter events induced by the injury (*31*). Topical application of TPA or DNFB to the skin induces inflammatory responses by attracting inflammatory cells, increasing level of pro-inflammatory mediators, and inducing hyperplasia (*32*). Such chemical-induced ear edema or allergic contact dermatitis has been employed as a useful mouse model of testing anti-inflammatory activity.

Table 3. Inhibitory Effect of Brown and Black Rice Bran Extracts on LTB_4 and PGE_2 Production in TPA-Induced Mouse Ear Tissue Inflammation (Edema)[a]

experiments	ng/mL (% inhibition)	
	LTB_4	PGE_2
control (−TPA)	1.45 ± 0.05[†] (−)	8.44 ± 1.29[§] (−)
control (+TPA)[b]	8.31 ± 1.82[‡] (0.00)	14.7 ± 1.01[‡] (0.00)
brown rice bran[c]	9.23 ± 0.65[‡] (−11.07)	15.9 ± 0.09[‡] (−7.90)
black rice bran[d]	3.68 ± 0.38[§] (55.72)	14.1 ± 0.79[‡] (4.18)

[a] The data are expressed as mean ± S.D. ($n = 3$). Values in the same column not sharing a common superscript are significantly different at $p < 0.05$. [b] Intraperitoneal injection of mice with vehicle only (DMSO) for 14 days prior to TPA application. [c] Intraperitoneal injection of mice with brown rice bran extract (10 mg/kg body weight) for 14 days prior to TPA application. [d] Intraperitoneal injection of mice with black rice bran extract (10 mg/kg body weight) for 14 days prior to TPA application.

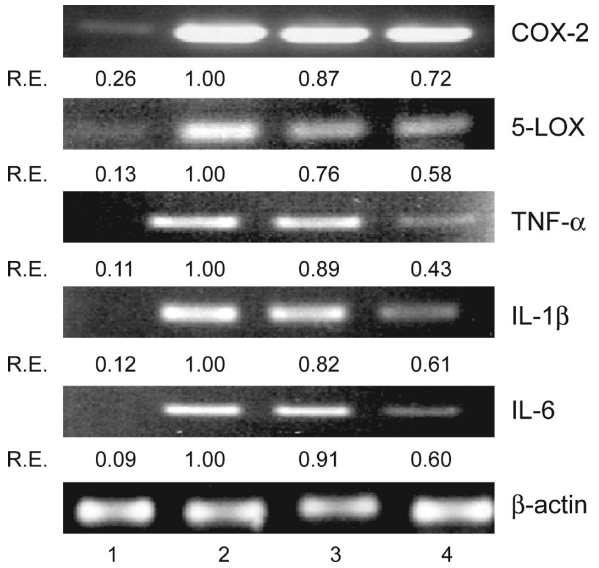

Figure 4. Semiquantitative analysis of gene expressions for synthesis of pro-inflammatory cytokines and eicosanoids. The relative ratio of expression of each gene is expressed as R.E. value calculated from target gene/β-actin gene expression. Lane 1, normal skin; lane 2, intraperitoneal injection with vehicle alone (DMSO) prior to topical application of TPA; lane 3, intraperitoneal injection with brown rice bran extract prior to topical application of TPA; lane 4, intraperitoneal injection with black rice bran extract prior to topical application of TPA. Figures represent at least three individual experiments.

To place the results of the present study in proper perspective, we will briefly describe reported observations that are relevant to the theme of this study. The TPA and DNFB inflammatory animal models are widely used to evaluate anti-inflammatory activity (*26*, *28*, *33*−*39*). The studies with the TPA-model showed that the antiallergic and inflammatory activities of black rice bran were largely due to suppression of mast cell activation, whereas the DNFB-model revealed suppressive activity of black rice bran against contact dermatitis. DNFB-induced contact dermatitis or hypersensitivity results from dendritic cell and CD8 cytotoxic T cell activation, and down-regulation of CD4 T cells of the immune system.

The onset of local inflammation following TPA application was previously reported to be associated mainly with ROS generation and oxidative tissue injury, followed by sequential production of pro-inflammatory mediators (cytokines and eicosanoids), and subsequent massive infiltration of leukocytes, leading to local inflammatory reactions (*40*−*42*). Previously, it was also shown that inhibition of TNF-α and IL-1β formation

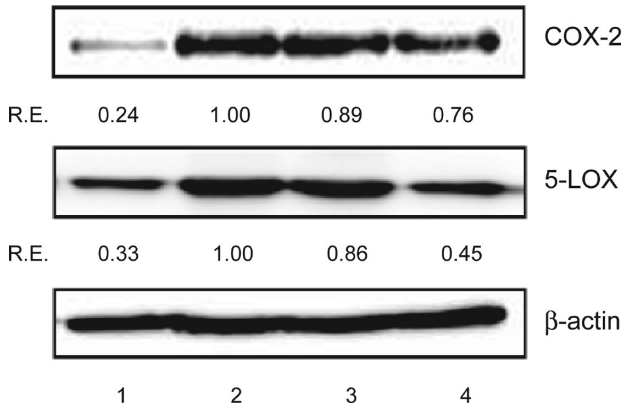

Figure 5. Western blot analysis of COX-2 and 5-LOX protein expressions in ear skin tissues. Each protein expression level was expressed as R.E. value calculated from target protein/β-actin protein expression. Lane 1, normal skin; lane 2, intraperitoneal injection with vehicle alone (DMSO) prior to topical application of TPA; lane 3, intraperitoneal injection with brown rice bran extract prior to topical application of TPA, lane 4, intraperitoneal injection with black rice bran extract prior to topical application of TPA. Figures represent at least three individual experiments.

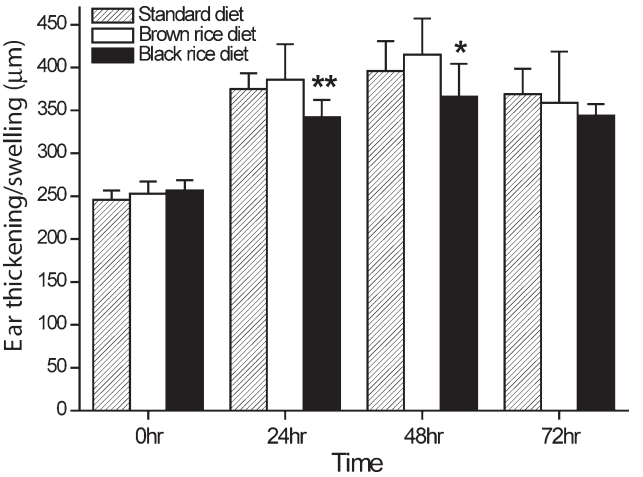

Figure 6. Effect of dietary administration of rice bran on ear swelling due to DNFB-induced allergic contact dermatitis in mice at various time points. Three groups of animals were orally fed standard mouse diet (stripped bar), diet supplemented with 10% brown rice bran (open bar), and diet supplemented with 10% black rice bran (closed box), respectively, for six weeks prior to topical application with DNFB. Data expressed as mean ± SD ($n = 10$). * and ** statistically different from the group subjected to oral administration of standard diet at $p < 0.05$ and $p < 0.01$, respectively.

significantly prevented development of the inflammatory process (*43*) and that a stabilized rice bran extract inhibited pro-inflammatory cyclooxygenase and lipooxoxygenase enzymes (*6*).

We assessed the anti-inflammatory activity of the black rice bran extract by the two in vivo models. The mechanisms that govern these two mice models are known to differ; that is, the TPA model mimics an early phase skin inflammation, mainly representing the innate immune system, and the DNFB model represents a chronic skin inflammation where the adaptive immune system operates to expand a specific lymphocyte clone recognizing allergens. We used the DNFB-model to assess the inhibitory activity of a mouse diet supplemented with 10% black rice bran powder. Results from previous studies and the present study imply that blockade of cytokine production is an important anti-inflammatory action exerted by black rice bran. Among the

cytokines, TPA-induced TNF-α production was the most prominently suppressed by the bran. This finding is noteworthy because TNF-α has been reported to be a potent mediator for increased vascular permeability (*44*).

Black rice bran markedly inhibited LTB_4 generation in tissue exudates from TPA-induced local edema lesion, while no significant difference was observed for PGE_2 production under the same experimental condition (**Table 3**). This observation indicates that the black rice bran extract has a selective inhibitory potency toward the 5-LOX pathway. Results of RT-PCR and Western blot analyses also showed reduced level of 5-LOX mRNA and protein expressions induced by the black rice bran treatment (**Figures 4** and **5**).

LTB_4 has been reported to be a potent chemotactic and chemokinetic factor that recruits neutrophils (*45*). Naturally occurring dihydroflavonols inhibited LTB_4 more effectively than they did PGE_2 generation in activated rat leukocytes (*34*). Flavonoids may also participate in the inhibition of ICAM-1 expression via a modulated action of 5-LOX. Suppression of 5-LOX expression by black rice bran extract appears to be linked to the down-regulation of ICAM-1 expression that contributes to the blockade of migration of inflammatory neutrophils into the tissue (**Figure 3A**) and to the subsequent aggravation of inflammation induced by the infiltrated cells. These suggestions are supported by reports that 5-LOX regulates the expression of adhesion molecules (*46*, *47*).

We do not know the specific reason for the selective inhibitory effect on LTB_4 compared to PGE2. A literature search showed that the black tea compound theaflavin had a stronger inhibitory effect on the 5-LOX than on the COX pathway in TPA-induced ear edema (*28*) and that cyanidine-3-glucoside showed a weak inhibitory effect and cyanidine-3-rutinoside had no effect on PGE_2 formation in macrophage cells (*48*).

The immunohistochemical analysis revealed that the infiltrated leukocytes were mainly neutrophils positively stained with an antibody against myeloperoxidase, a critical neutrophil marker. RT-PCR also showed that treatment with black rice bran extract down-regulated ICAM-1 expression at the transcription level. These findings suggest that the reversal of TPA-induced neutrophil influx in ear tissue of mice injected with the black rice bran might be due to interrupted interaction between neutrophils and endothelial cells (*49*).

It was also of interest to find out whether dietary administration of black rice bran would suppress chemical-induced allergic contact dermatitis. This turned out to be the case. **Figure 6** shows significant suppression of ear edema after 24 h that lasted for 48 h. Oral administration of black rice bran also suppressed DNFB-induced skin swelling. The inhibitory effect of black rice bran extract on ear edema in the DNFB model was observed to start earlier than was the case with the TPA model.

Collectively, the results the present study show that (a) levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were markedly elevated in TPA-induced edema tissue exudates of mouse skin as compared to levels of normal skin tissue; (b) intraperitoneal administration of black rice bran extract significantly suppressed development of skin inflammation (**Table 2**, **Figure 4**); and (c) active constituents of pigmented black rice bran powder delivered via the diet through the gastrointestinal barrier also suppressed local allergic contact dermatitis induced by topical application of DNFB (**Figure 6**).

Summary and Research Needs. The described findings indicate that bran from the black rice cultivar LK1-3-6-12-1-1 exhibited anti-inflammatory activities in vivo. The data also suggest that the suppression of the intercellular adhesion molecule ICAM-1 expression and neutrophil infiltration and the inhibition of pro-inflammatory mediators TNF-α and LTB_4 are critical parts of the mechanism of protection against skin injury, contact hypersensitivity, and inflammation. The observed changes in these and other biomarkers associated with the described events (IL-1β, IL-6, LTB_4, PGE_2, TNF-α, ICAM-1, COX-2, 5-LOX, and cellular mRNA expression levels) could serve as a guide for future studies on the protective effects of other food ingredients against inflammation. The present study also contributes to the current debate about the role of disease-related biomarkers as indicators of therapeutic benefits of functional foods (*50*).

As mentioned in the Introduction, because the composition of black rice bran differs significantly from that of brown rice, knowledge of the composition of the extracts may not permit conclusions as to which compound or which combination of compounds induces the anti-inflammatory effect. One would have to isolate and test each compound individually in the in vivo experiments. This aspect awaits further study.

The present study showed that the administration of black rice bran extract elicited marked changes in the expression of 6 genes, well-known to be associated with inflammation (**Table 1**). However, it is difficult to offer a detailed molecular action mechanism of the black rice bran for attenuating the inflammatory progress triggered by TPA and DNFB because of the reported complexity of the signal pathway in the inflammatory response (*51*, *52*) and because of the chemical heterogeneity of the black rice bran extract. This aspect merits further study.

Finally, because prolonged inflammation is associated with the development of infections (*49*), allergies, atherosclerosis, heart diseases, and cancer (*53*−*56*), it would also be of interest to evaluate the potential of black rice bran-containing foods to prevent or treat these disorders.

ABBREVIATIONS USED

ACD, allergic contact dermatitis; COX-2, cyclooxygenase 2; DMSO, dimethyl sulfoxide; DNFB, 2,4-dinitrofluorobenzene; ELISA, enzyme-linked immunosorbent assay; ICAM-1, intercellular adhesion molecule-1; IL-1β, interleukin-1β; IL-6, interleukin-6; 5-LOX, 5-lipooxygenase; LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2; RT-PCR, reverse transcription-polymerase Chain reaction; R.E., relative expression of a gene; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanolylphorbol-13-acetate.

ACKNOWLEDGMENT

We thank Carol E. Levin for assistance with the preparation of the manuscript.

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Received for review June 10, 2010. Revised manuscript received August 4, 2010. Accepted August 09, 2010. This work received grant support from the Agenda Program (No. 200901OFT113068122), Rural Development Administration, Republic of Korea.