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Effects of Thai black sticky rice extract on oxidative stress and lipid metabolism gene expression in HepG2 cells

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ABSTRACT. Anthocyanins, which are found in some food, including Thai black sticky rice, are reported to have health-promoting properties. Oxidative stress plays a major role in the pathogenesis of many degenerative diseases induced by free radicals, such as cardiovascular disease, stroke and cancer. We evaluated the anthocyanin-rich extract (ARE) from Thai black sticky rice for antioxidative and antihyperlipidemic effects on HepG2 cells. Cell viability was investigated with the neutral red assay and the MTT assay, and oxidative stress was determined by the DCFH-DA assay. RT-PCR was used to evaluate the effect of ARE on LDLR, HMG-CoAR, PPAR (α1,γ) and LXRα gene expression. We found that ARE at high doses ($\geq$800 mg/L) induces cytotoxicity. However, at 600-1000 mg/L it reduced intracellular oxidative stress ($P < 0.05$) in a dose-dependent manner, and at 200 mg/L

it significantly enhanced the expression of the LDLR gene in HepG2 cells. We concluded that ARE can be beneficial for health promotion by reducing oxidative stress and enhancing LDL clearance, regulating LDLR production on the cell surface membrane, thereby maintaining lipid homeostasis.

Key words: Thai black sticky rice; Antioxidant; Oxidative stress; Lipid metabolism genes

INTRODUCTION

Natural products have been used for therapeutic purposes in traditional and folk medicine. Black rice (*Oryza sativa* L. *indica*), an enriched source of flavonoids, is widely used for therapeutic purposes and is gradually becoming popular in Thailand. Anthocyanins are found more in black sticky rice than in red rice or black rice (Sangkitikomol et al., 2008). Oxidative stress plays a major role in the pathogenesis of many degenerative diseases induced by free radicals such as cardiovascular disease, stroke and cancer, and in aging as well (Halliwell and Gutteridge, 2001; Galati and O'Brien, 2004). Experimental and clinical studies have reported a positive correlation between cardiovascular health status and the consumption of black rice and/or black rice pigments. Supplementation of black rice pigments has been shown to improve the antioxidant and anti-inflammatory status in patients with coronary heart disease (Wang et al., 2007), reduced oxidative stress and inflammation, improved plasma lipid levels and alleviated atherosclerotic lesions in animal models (Auger et al., 2002). Black rice pigments attenuate atherosclerotic plaque formation in rabbits (Ling et al., 2002) and in apolipoprotein E-deficient mice (Xia M et al., 2003; Xia X et al., 2006). Some flavonoids have been found to act differently depending on their doses, particularly a cytoprotective effect at low doses, while a cytotoxic effect and induction of DNA strand breaks at high doses (Wätjen et al., 2005). The regulation of cholesterol homeostasis is associated with low-density lipoprotein receptor (LDLR), a membrane receptor, for removal of LDL from the blood circulation, which contributes to lower plasma cholesterol levels. The activity of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoAR), the rate controlling enzyme, is expressed to synthesize cholesterol for cholesterol homeostasis inside the cells. The peroxisome proliferator-activated receptors [PPAR (α1,γ)] and the liver X receptor α (LXRα), nuclear membrane receptors, are important regulators of cholesterol, fatty acid, and glucose homeostasis (Powell and Kroon, 1994; Bays and Stein, 2003; Kaul et al., 2005).

The present investigation aimed to determine the dose-response of anthocyanin-rich extract (ARE) from Thai black sticky rice against oxidative stress and the mRNA expression of lipid metabolism genes including LDLR, HMG-CoAR, PPAR (α1,γ), and LXRα in HepG2 cells.

MATERIAL AND METHODS

Chemicals

Neutral red dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethidium bromide and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were pur-

chased from Sigma-Aldrich, St. Louis, MO, USA. Dulbecco's modified Eagle's medium (DMEM, with 4 mM glutamine, 4500 mg/L glucose without sodium pyruvate), fetal bovine serum (FBS), 1X EDTA-trypsin 0.25% were obtained from Thermo Scientific HyClone, Logan, UT, USA. TRI reagent was purchased from Molecular Research Center Inc., Cincinnati, OH, USA. RNase inhibitor, 100-bp DNA ladder, deoxyribonuclease I and primers were purchased from Bio Basic Inc., Ontario, Canada. M-MuLV Reverse transcriptase was purchased from Finnzymes Inc., Espoo, Finland. Amphotericin B (Fungizone) and penicillin-streptomycin solutions came from Biochrom AG, Berlin, Germany. All other basic reagents were of analytical grade.

Preparation of ARE from Thai black sticky rice

ARE fraction was prepared from Thai black sticky rice that was previously shown by our group to possess certain characteristics: including total antioxidant properties, 1368.34 ± 41.27 mM Trolox equivalents/kg dry mass (ORAC - fluorescein assay); total phenolic compounds, 922.03 ± 9.42 mM gallic acid equivalents/kg dry mass (Folin - Ciocalteu assay), and anthocyanins, 218.97 ± 1.82 mM catechin equivalents/kg dry mass (vanillin assay) (Sangkitikomol et al., 2008). In brief, 100 g black sticky rice was milled and extracted in 2000 mL 80% methanol in water. This crude extract was refluxed at 75-78°C for 3 h, shaken in an ultrasonic bath for 30 min, cooled, and stored in the dark at 4-8°C for 7 days, after which the sediment was removed by centrifugation at 3000 rpm for 15 min. The extract was concentrated using a vacuum rotary evaporator at 50°C and freeze-dried giving a yield of dried extract of 0.5 g. This dried extract was kept in a deep freezer at -80°C until used.

Preparation of HepG2 cells and biological analysis

The HepG2 cell line was a generous gift from Assoc. Prof. Dr. Parvapan Bhattarakosol, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. HepG2 cells were grown in DMEM supplemented with 4 mM glutamine, 4.5 g/L glucose, 10% heat-inactivated FBS, 0.1% amphotericin B (Fungizone) and 1% penicillin-streptomycin, and they were maintained at 37°C in a humidified atmosphere at 5% CO_2. The culture medium was changed twice a week, and the cells were subcultured once a week. The cells were seeded at a density of 1 x 10^6 cells/well in 6-well plates with a final volume of 5 mL/well for reverse transcriptase-polymerase chain reaction (RT-PCR) assay and 1 x 10^4 cells/well in 96-well plates with a final volume of 0.2 mL/well for oxidative stress (DCFH-DA assay) and cell viability test using the neutral red (NR) assay and MTT assay, respectively.

Neutral red assay

HepG2 cell viability was assessed by using the NR assay as previously described (Zhang et al., 1990) with some modifications. Briefly, following the cell treatments, HepG2 cells were exposed to 100 μL NR solution (4 mg/mL in phosphate-buffered saline (PBS), pH 7.5) for 3 h at 37°C to allow the lysosomes of viable cells to take up the vital stain (NR). This process requires metabolically active cells. Failure to take up NR, therefore, indicates that those cells have suffered damage. The cultures were rapidly washed with 1% formaldehyde-

l% calcium chloride to remove the excess NR. A mixture of 1% acetic acid-50% ethanol was added to the HepG2 cells to extract the NR from these cells at room temperature for 30 min. The supernatants were transferred to 96-well plates, and the absorbance at 550 nm was then measured. The % cell survival was calculated according to the following formula: % cell survival = [(absorbance of treatment group - blank) / (absorbance of control group - blank)] x 100. This method was carried out using VICTOR^2 Multilabel Counter (Perkin Elmer Life and Analytical Sciences, Finland).

MTT assay

HepG2 cell viability was assessed using the MTT assay as previously described (Twentyman and Luscombe, 1987) with some modifications. The assay is dependent on the ability of viable cells to metabolize MTT, a water-soluble tetrazolium salt (yellow color), by mitochondrial succinate dehydrogenase into a water-insoluble formazan product (dark purple color). Briefly, following the cell treatments, HepG2 cells in each well of the 96-well plate were subjected to MTT assay by mixing with 20 µL MTT solution [5 mg/mL in PBS], and incubation at 37°C in 5% CO_2 incubator for 4 h. Subsequently, the medium was removed, 150 µL 50% ethanol in DMSO was added to each well to dissolve the formazan product, and the absorbance was measured at 550 nm. Since the reduction of MTT can occur in metabolically active cells, the level of activity is a measure of the viability of the cells. The % cellular activity was calculated according to the following formula: % cellular activity = [(absorbance of treatment group - blank) / (absorbance of control group - blank)] x 100. This method was carried out using VICTOR^2 Multilabel Counter (Perkin Elmer Life and Analytical Sciences).

DCFH-DA assay

DCFH-DA has been used as a substrate for measuring intracellular oxidant production in HepG2 cells (Wang and Joseph, 1999). Briefly, following the cell treatments, HepG2 cells were centrifuged for 10 min at 2500 rpm and then washed three times with PBS, pH 7.4. The cells were mixed with 100 µL of 100 µM DCFH-DA in PBS, and incubated for 90 min at 37ºC in a humidified 5% CO_2 atmosphere. The non-ionic, non-polar DCFH-DA crosses cell membranes and is hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescin (DCFH). In the presence of reactive oxygen species (ROS) inside the cells, DCFH is oxidized to highly fluorescent, dichlorofluorescein (DCF). The cells were centrifuged for 10 min at 2500 rpm and then washed three times with PBS, pH 7.4. The fluorescent measurement was monitored using VICTOR^2 Multilabel Counter (Perkin Elmer Life and Analytical Sciences) at 485 and 535 nm for excitation and emission, respectively. Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in the cells. The % cellular oxidative stress was calculated according to the following formula: % cellular oxidative stress = [(fluorescence of treatment group - blank) / (fluorescence of control group - blank)] x 100.

Detection of lipid metabolism gene expression using RT-PCR method

In this investigation, mRNA expression of lipid metabolism genes including LDLR, HMG-

CoAR, PPAR (α1,α2,γ), and LXRα in HepG2 cells was detected using the RT-PCR method according to previous reports with minor modification (Powell and Kroon, 1994; Kaul et al., 2005). Briefly, 1 x 10^6 cells HepG2 suspended in DMEM in 6-well plates were mixed with ARE (0 to 600 mg/L with 0 = control). After incubation for 29 h at 37°C in a humidified 5% CO_2 atmosphere, total RNA was isolated from HepG2 cells using the TRI reagent following the manufacturer protocol. Purity and quantity of total RNA were determined using agarose gel electrophoresis and a spectrophotometer. The synthesis of cDNA was performed using 2 µg total RNA, random primers and M-MuLV-reverse transcriptase at 42ºC for 1 h. Subsequently, PCR was carried out using specific primer pairs in order to generate PCR products, which were 258-bp LDLR (sense 5'-CAATGTCTCACCAAGCTCTG-3' and antisense 5'-TCTGTCTCGAGGGGTAGCTG-3'), 247-bp HMG-CoAR (sense 5'-CTTGTGTG TCCTTGGTATTAGAGCTT-3' and antisense 5'-TTATCATCTTGACCCTCTGAGTTACAG-3'), 728-bp PPARα1 and 525-bp PPARα2 (sense 5'-AGTCTCCCAGTGGAGCATTGAACA-3' and antisense5'-ATACGCTACCAGCATCCCGTCTTT-3'),434-bpPPARγ(sense5'-AGCCTCATGA AGAGCCTTCCAACT-3' and antisense 5'-TGTCTTTCCTGTCAAGATCGCCCT-3'), 818-bp LXRα (sense 5'-AACCCACAGAGATCCGTCCACAAA-3' and antisense 5'-ATTCATGGCC CTGGAGAACTCGAA-3') and 656-bp β-actin (sense 5'-ACGGGTCACCCACACTGTGC-3' and antisense 5'-CTAGAAGCATTTGCGGTGGACGATG-3'). RT-PCR products along with a DNA ladder were electrophoresed on an agarose gel and visualized by ethidium bromide staining using a gel documentation system. For data analysis, the GeneTools software 3.08 (SynGene, Cambridge, UK) was utilized. The expression of genes was normalized to that of β-actin, and data for treatment with each ARE concentration were presented as fold change in normalized mRNA expression relative to that of no ARE treatment (control).

Statistical analysis

Statistically significant differences between control and exposed groups were evaluated by one-way ANOVA (SPSS version 17.0 for Windows) and the Student *t*-test. P value was two-tailed, and $P < 0.05$ was considered to be statistically significant when comparing the data sets.

RESULTS

Effect of ARE on cell survival and oxidative stress in HepG2 cells

According to the HepG2 viability tests, ARE in the 800-1000 mg/L concentration range significantly reduced the number of living cells (neutral red assay, Figure 1A), while the 100-400 mg/L concentration range significantly increased the activity of cellular metabolizing enzymes (MTT assay, Figure 1B), upon incubation for 58 h ($P < 0.05$). In the DCFH-DA assay, HepG2 cells treated with ≥600 mg/L ARE significantly reduced oxidative stress ($P < 0.05$) in a dose-dependent manner (Figure 1C).

The effect of ARE on lipid metabolism gene expression in HepG2 cells

With regard to the mRNA expression of lipid metabolism genes (Figures 2 and 3), 200 mg/L ARE significantly enhanced the expression of LDLR gene in HepG2 cells ($P < 0.05$),

whereas ARE at higher concentrations (400-600 mg/L) resulted in lower levels of LDLR mRNA as compared to those for 200 mg/L ARE. As far as the mRNA expression of PPARγ gene in response to ARE is concerned, its expression was similar to that of LDLR gene. The difference in mRNA expression in response to various concentrations of ARE was not observed for the HMG-CoAR, PPARα1 and LXRα genes. The expression of PPARα2 mRNA detected simultaneously when using the PCR primer pair for PPARα1 mRNA was not of interest in our present study since it is known to be an inactive isoform.

DISCUSSION

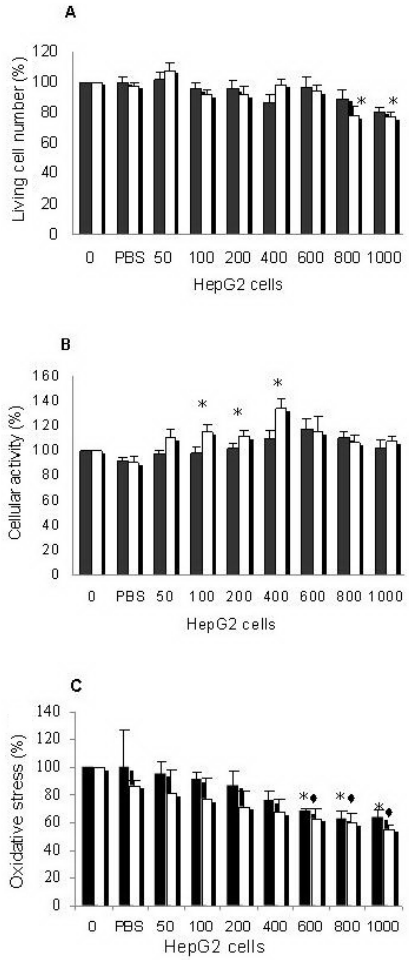

Figure 1. The effect of anthocyanin-rich extract (ARE) on cytotoxicity and oxidative stress of HepG2 cells. HepG2 cells were incubated with different concentrations of ARE (0-1000 mg/L) at the end of experimental periods of 29 h (filled columns) and 58 h (open columns). **A.** The number of living HepG2 cells was determined using neutral red assay **B.** The cellular activity of living HepG2 cells was assessed using the MTT assay. **C.** The oxidative stress in HepG2 cells was evaluated using the DCFH-DA assay. Values are reported as means with their standard error of the mean (SEM) depicted by vertical bars. All experiments were performed in triplicate (N = 3). PBS = phosphate-buffered saline. *,♦$P < 0.05$ for significant change as compared to control (no treatment).

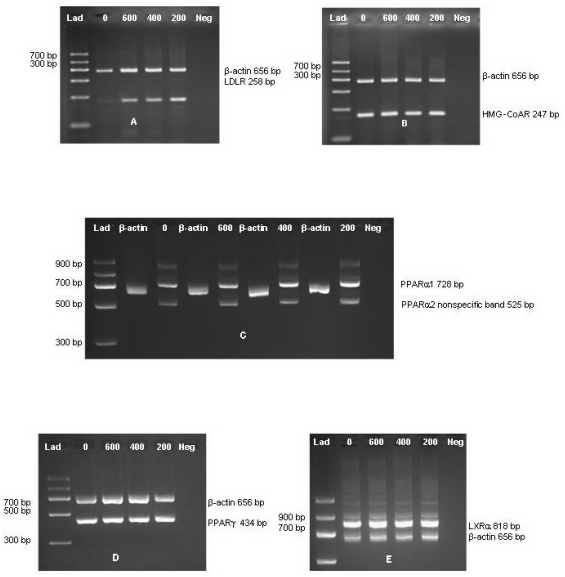

Figure 2. Effect of anthocyanin-rich extract (ARE) on mRNA expression of certain lipid metabolism genes in HepG2 cells in a dose-dependent manner as determined using the RT-PCR approach. Representative agarose gel photographs showed RT-PCR products corresponding to expected sizes of the lipid metabolism genes studied. **A.** LDLR. **B.** HMG-CoAR. **C.** PPARα1 and PPARα2 nonspecific band (isoform). **D.** PPARγ. **E.** LXRα. The mRNA expression of β-actin gene was used for normalization. HepG2 cells were treated with ARE (0 to 600 mg/L; 0 = control). LDLR = low-density lipoprotein receptor; HMG-CoAR = 3-hydroxy-3-methyl-glutaryl-CoA reductase; PPAR = proliferator-activated receptors; LXRα = liver X receptor α.

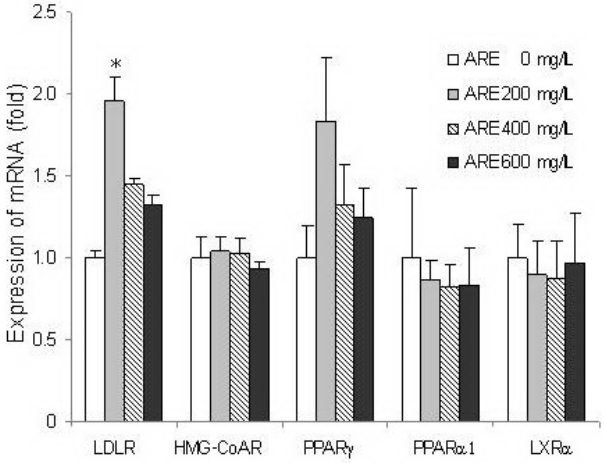

Figure 3. The mRNA expression of lipid metabolism genes (LDLR, HMG-CoAR, PPARα1, PPARγ, and LXRα) in HepG2 cells treated with varying concentrations of anthocyanin-rich extract (ARE, 0-600 mg/L; 0 = control) was evaluated using RT-PCR. The mRNA expression of the genes studied was normalized to that of the β-actin gene. Values derived from normalized band intensities are means with their standard error of the mean (SEM) depicted by vertical bars. All experiments were performed in triplicate (N = 3). *P < 0.05 for significant change in normalized gene expression as compared to control. For abbreviations, see legend to Figure 2.

ARE enhanced cellular enzymatic activities, as shown in the MTT assay. Oxidative stress-induced ROS production detected with DCFH-DA was significantly decreased by ARE in a dose-dependent manner. Based on this particular experiment, we found that ARE in the 600-1000 mg/L concentration range reduced oxidative stress in HepG2 cells in a dose-dependent manner. Therefore, the toxic effect of ARE (≥800 mg/L) on HepG2 cells may be due to its inhibitory or stimulatory actions via kinase signaling pathways, which are likely to affect cellular function by altering the phosphorylation state of target molecules and by modulating gene expression (Williams et al., 2004). These findings suggest that ARE at high concentrations may not be appropriate for health-promoting purposes. Other studies indicated that lower concentrations of flavonoids (nM to low μM) could lead to antioxidant response element-mediated gene expression, including that of phase II detoxifying enzymes. In contrast, higher concentrations of flavonoids may sustain the activation of mitogen-activated protein kinases or stress-activated protein kinases, which could induce apoptosis (Chen et al, 2000; Kong et al, 2000). Many reports have described the adverse actions of flavonoids at the cellular level. For example, due to the toxic effects of flavonoids at high concentrations, several lines of evidence have reported induction of DNA strand breaks when using such flavonoids as epigallocatechin-3-gallate (Tian et al., 2007), quercetin (Beatty et al., 2000), and kaempferol (Niering et al., 2005). Other natural products used for lowering lipids, such as phytoestrogens, estrogens and soy proteins have been shown to have the ability to increase hepatic LDLR activity (Owen et al., 2004).

Analyzing the effect of ARE on lipid metabolism gene expression using the RT-PCR method, ARE significantly induced the expression of LDLR gene in HepG2 cells in a unique dose-dependent manner. ARE at 600, 400 and 200 mg/L progressively increased LDLR gene expression, respectively corresponding to 1.3-, 1.5- and 2-fold higher levels relative to control (untreated HepG2 cells). Also, a similar trend was true for PPARγ gene, since its expression was respectively 1.2-, 1.3- and 1.8-fold higher than that of control. ARE exhibits this particular biological activity similar to that of α-tocopherol, which has been demonstrated to function in a concentration-dependent fashion with a biphasic 'up then down' effect on LDLR in HepG2 cells (Pal et al., 2003). In particular, at low levels of α-tocopherol (up to 50 μM), LDLR binding activity, protein and mRNA were progressively induced to maximum levels, while they gradually decreased to baseline levels at concentrations higher than 50 μM. PPARγ is a ligand-activated nuclear receptor with essential roles in adipogenesis, glucose and lipid homeostasis, and inflammatory responses. These roles may be considered possible mechanisms for reducing the risk of cardiovascular diseases (Xu and Li, 2008). Since the present study showed that ARE induces the expression of LDLR and PPARγ genes, these particular genes may regulate energy metabolism in the HepG2 cells. According to microarray analysis, gene expression patterns of liver and skeletal muscles were significantly affected by treatment with anthocyanins (Lefevre et al., 2008). In particular, the first set discovered included down-regulated pathways in both muscle and liver involved in cellular defense (inflammatory response genes and oxidative stress genes), whereas another set included hepatic genes involved in energy metabolism in the liver, with major metabolic pathways down-regulated such as the TCA cycle, fatty acid β-oxidation, and cholesterol biosynthesis.

In conclusion, ARE showed protective effects at low concentrations (≤600 mg/L), while it exhibited a toxic effect at high concentrations (≥800 mg/L). At the molecular level, ARE at a low concentration (200 mg/L) regulated the production of LDLR on cell surface membranes, thereby maintaining lipid homeostasis. Taken together, these results support the

notion that ARE is biologically active in a dose-dependent manner.

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