Anthocyanins from purple sweet potato attenuate dimethylnitrosamine-induced liver injury in rats by inducing Nrf2-mediated antioxidant enzymes and reducing COX-2 and iNOS expression

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Abstract
Anthocyanins of the purple sweet potato exhibit antioxidant and hepatoprotective activities via a multitude of biochemical mechanisms. However, the signaling pathways involved in the actions of anthocyanin-induced antioxidant enzymes against chronic liver injury are not fully understood. We examined whether an anthocyanin fraction (AF) from purple sweet potato may prevent dimethylnitrosamine (DMN)-induced liver injury by inducing antioxidants via nuclear erythroid 2-related factor 2 (Nrf2) pathways and by reducing inflammation. Treatment with AF attenuated the DMN-induced increased serum alanine aminotransferase and aspartate aminotransferase activities. It also prevented the formation of hepatic malondialdehyde and the depletion of glutathione and maintained normal glutathione-S-transferase (GST) activity in the livers of DMN-intoxicated rats. Furthermore, AF increased the expression of Nrf2, NADPH:quinone oxidoreductase-1, heme oxygenase-1, and GSTa, which were reduced by DMN, and decreased the expression of cyclooxygenase-2 and inducible nitric oxide synthase. An increase in the nuclear translocation of nuclear factor kappa B (NF-κB) was observed in the DMN-induced liver injury group, but AF inhibited this translocation. Taken together, these results demonstrate that AF increases the expression of antioxidant enzymes and Nrf2 and at the same time decreases the expression of inflammatory mediators in DMN-induced liver injury. These data imply that AF induces antioxidant defense via the Nrf2 pathway and reduces inflammation via NF-κB inhibition.

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1. Introduction

Dimethylnitrosamine (DMN) is an N-nitroso compound found in processed meats and industrial products. It is a potent hepatotoxin, carcinogen, and mutagen (George et al., 2001) whose damaging effects are induced by cytochrome P450 2E1 (CYP2E1) (Yamazaki et al., 1992). The activation of DMN by CYP2E1 in the mouse liver stimulates Kupffer cells, leading to the generation of superoxide and other reactive oxygen species (ROS) that can damage liver cells (Teufelhoefer et al., 2005). ROS cause inflammation and contribute to the pathogenesis of various acute and chronic liver ailments such as acetaminophen (APAP) overdose, alcohol-induced liver injury, toxin exposure, and viral hepatitis (Chen et al., 1993). Antioxidant enzymes including heme oxygenase 1 (HO-1), NADPH: quinone oxidoreductase-1 (NQO1), and glutathione-S-transferase (GST) provide protection against the deleterious effects of ROS (Banerjee et al., 1999). The induction of antioxidant enzymes is one of the most important determinants of cancer susceptibility and is related to both cancer chemopreventive and cytoprotective effects.

The induction of antioxidant proteins is mediated by nuclear erythroid 2-related factor 2 (Nrf2), which is sequestered in the cytoplasm by the actin-binding protein Keap1 (Zhang and Gordon, 2004). Upon stimulation, Nrf2 is translocated from the cytosol to the nucleus, where it sequentially binds to the antioxidant response element (ARE), resulting in a cytoprotective response characterized by an upregulation of antioxidant enzymes and decreased sensitivity to oxidative stress damage (Dhakshinamoorthy and Jaiswal, 2001; Jaiswal, 2004). In addition, Nrf2 plays a broader role in the modulation of inflammatory responses (Guo and Ward, 2007).

Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are enzymes involved in both inflammatory processes and...
tumor development (Mohan and Epstein, 2003; Chung et al., 2007). Recent studies have demonstrated the carcinogen-induced expression of COX-2 and iNOS through the activation of nuclear factor kappa B (NF-κB) (Suru et al., 2001; Senthil Kumar and Wang, 2009), and as such, the targeted inhibition of COX-2 or iNOS and the modulation of NF-κB upregulation have been identified as the molecular basis of cancer chemoprevention by structurally diverse dietary phytochemicals in several organs (Chung et al., 2007).

Anthocyanins are a class of natural polyphenol compounds present in a wide variety of fruits, beans, cereals, and vegetables. In animal models, anthocyanins have powerful antioxidant (Shih et al., 2007), anti-inflammatory (Karlsen et al., 2007), and anti-tumor effects (e.g., delaying the growth of pre-malignant cells; Shih et al., 2005). In addition, anthocyanins could prevent obesity, hyperglycemia (Tsuda et al., 2003), and asthma (Park et al., 2007). Recently, the purple sweet potato Ipomoea batatas has received much attention for its role in health care (Lila, 2004). It contains a high level of anthocyanin pigments, and they are more stable than those found in strawberry, red cabbage, perilla, and other plants; thus, the potato has been suggested as a good source of stable anthocyanins. In addition, these anthocyanins possess numerous biological functions including ROS-scavenging, anti-mutagenic, anti-carcinogenic, and anti-hypertensive effects (Ahmed et al., 2010).

We previously reported that the anthocyanin fraction (AF) obtained from the purple sweet potato has a potent hepatoprotective effect in an APAP-induced hepatic damage mouse model. AF upregulated the activity of antioxidants such as glutathione (GSH) and GST, scavenged ROS, and inhibited APAP-induced hepatotoxicity by attenuating CYP2E1-mediated APAP bioactivation (Choi et al., 2009).

Anthocyanins from black raspberries, blackberries, and strawberries protect against a number of hepatotoxic agents including nitrosamines (Reen et al., 2006). However, the mechanism by which AF elicits hepatoprotective and antioxidant effects in association with Nrf2 is unclear. Here, we report that AF attenuates DMN-induced liver injury in rats by inducing Nrf2-mediated antioxidant enzymes and attenuating the inflammatory mediators COX-2 and iNOS through the inhibition of NF-κB.

2. Materials and methods

2.1. Preparation of AF

AF was purified from aqueous extracts of whole purple sweet potato I. batatas supplied by the Ji San Food Co. (Hamyang, Korea) and prepared as previously described (Lee et al., 2000). The anthocyanins in this potato include cyanidin-3-O-glucoside chloride, malvidin-3-O-glucoside chloride, pelargonidin-3-O-glucoside chloride, and peonidin-3-O-glucoside chloride (Goda et al., 1997). Briefly, uniformly sized tubers without defects were washed, peeled, diced into 0.5-cm cubes, and freeze-dried. Then samples (0.5 g) were homogenized in 15 mL ethanol/water (85:15 vol/vol) using an Ultra Turrax Tissumizer (30,000 rpm; Divtech Equipment Co., Cincinnati, OH) and stored for 12 h at −20 °C. Supernatants obtained by centrifugation were diluted to 5 mL using 0.01% aqueous HCl (whole extract) and passed through C-18 Sep-Pak cartridges (Waters, Milford, MA) preconditioned with 0.1% acetic acid methanol to absorb anthocyanins (Lee et al., 1997). Anthocyanins were obtained by eluting the columns with 0.01% methanolic HCI, concentrating eluates under a nitrogen flow, and reconstituting with either alcohol or dimethyl sulfoxide. The yield of dried residue corresponded to 2.5% of the original whole dry weight. This powder was stored in a freezer, passed through a 40-mesh sieve, and stored at −20 °C until use.

2.2. Animals and DMN-induced liver injury

Five-week-old male Sprague–Dawley rats were obtained from Daehan Biolink (Chungbuk, Korea). The animals were allowed free access to Purina rodent chow (Seoul, Korea) and tap water and were maintained under specific pathogen-free conditions. They were acclimatized to the temperature (22 ± 2 °C) and humidity (55 ± 5%) of controlled rooms with a 12-h light/dark cycle for at least 1 week prior to experimentation. All experiments were performed according to the rules and regulations of the Animal Ethics Committee, Chosun University.

The rats were divided into six groups of five animals. To induce hepatic fibrosis, DMN (200 μL/Sigma Chemical Co., St. Louis, MO) dissolved in sterile saline (10 mg/kg body weight, 200) was administered as an intraperitoneal injection three times per week for 4 weeks. AF (100 μL) was dissolved in saline and rats were intragastrically administered 50, 100, or 200 mg/kg of AF each day, six times per week for 4 weeks. The control and DMN-treated groups were administered saline (intragastrically) without the drug. The animals were sacrificed on day 29.

2.3. Serum biochemistry

To assess hepatotoxicity, we measured the serum activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using spectrophotometric diagnostic kits according to the manufacturer’s recommendations (Sigma Chemical Co., St. Louis, MO) (Lee et al., 2004).

2.4. Determination of lipid peroxidation

Hepatic lipid peroxidation levels were determined by measuring thiobarbituric acid reactive substances (TBARS) (Lee et al., 2004). Briefly, samples were mixed with TBA reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25 M HCl. The reaction mixture was boiled in a water bath for 30 min and centrifuged at 2000 rpm for 10 min at 4 °C. Then the TBARS concentration was determined based on the absorbance at 532 nm measured with a spectrophotometer (Varioskan, Thermo Electron Co., Berthold, Germany). Control tests were performed to ensure that AF did not interfere with the lipid peroxidation assays. Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

2.5. Hepatic GSH and GST activity

Livers were removed, weighed, perfused with ice-cold 0.15 M KCl, and homogenized with 4 μL (w/v) 10 mM Tris·HCl (pH 7.4) containing 0.15 M KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol, and 0.01 mM phenylmethoxy sulfonyle fluoride in a Potter–Elvehjem homogenizer. Hepatic microsomal fractions were obtained by differential centrifugation as described previously (Lee et al., 2004), and hepatic GSH levels were estimated colorimetrically using Ellman’s reagent as in Lee et al. (2004). The activity of GST was determined by using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The reaction mixture contained 1 mL of CDNB and 1 mM reduced glutathione in 0.1 M phosphate buffer (pH 6.5). The formation of the reduced GSH–CDNB conjugate was measured spectrophotometrically at 340 nm with CDNB according to Habig et al. (1974).

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from liver tissue using Trizol reagent (Gibco-BRL, Grand Island, NY). Total RNA (0.5–1 μg) was subjected to RT-PCR using a one-step RT-PCR premix kit (iNTRON Biotechnology, Seoul, Korea) containing specific primers for GSTα, HE-1, Nrf2, NQO1, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. Amplified products were resolved by 1.5% agarose gel electrophoresis and imaged under ultraviolet light.

2.7. Western blot analysis

To analyze protein expression, liver homogenates (20 μg) were normalized by Bradford method (Bradford, 1976) and resolved on 10.0% polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the indicated antibodies against mouse HO-1, Nrf2, GSTα, and NQO1 and monoclonal antibodies against mouse β-actin with the appropriate horse-radish peroxidase-conjugated secondary antibodies. Blots were visualized using an enhanced chemiluminescence Western blotting detection kit (iNTRON Biotechnology Co., Ltd., Korea). The intensity of the immunoreactive bands was determined by densitometric analysis (LAS-4000 mini, Fujifilm Co., Ltd., Tokyo).

2.8. Statistical analysis

All experiments were repeated a minimum of three times. Results were presented as means ± SD. Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test. A value with P > 0.01 was considered as statistically significant.

3. Results

3.1. Effects of AF in rats with DMN-induced liver injury

The liver uses a variety of transaminases to synthesize and break down amino acids allowing for the inter-conversion of energy storage molecules. When the liver becomes damaged, the
3.2. Effects of AF on hepatic GSH levels and GST activity

Increased oxidative stress has been reported in DMN-induced fibrosis and hepatocarcinogenesis in rats (Vendemiale et al., 2001). Because the oxidative stress pathway generally involves the GSH system, we measured the level of GSH in each liver group. The administration of DMN significantly depleted GSH, whereas treatment with AF significantly and dose-dependently protected the liver against this effect (Fig. 1A). In addition, there was lower GST activity in the liver homogenates of DMN-treated rats than in those of normal controls. The AF-treated group had significantly higher GST activity (Fig. 1B). Also, the hepatic GSH levels and GST activity were increased by treatment with AF alone (200 mg/kg). These results demonstrate that the protection afforded by AF against DMN-induced liver injury may be related to its ability to increase cellular GSH content and GST activity.

3.3. Involvement of Nrf2 by AF in rats with DMN-induced liver injury

Nrf2 is sequestered in the cytoplasm by Keap1 under normal conditions and its translocation into the nucleus is essential for the transactivation of various target genes. To identify whether AF exerts its effects by regulating Nrf2 activity, we investigated the transactivation of various target genes. To determine whether AF exerts its effects by regulating Nrf2 activity, we investigated the mRNA and protein expression of Nrf2, HO-1, and GST by semi-quantitative RT-PCR and Western blot analysis, respectively. As shown in Fig. 2A, the mRNA levels of Nrf2 were lower following DMN administration. However, oral administration of AF (100 and 200 mg/kg) reverses the suppression of Nrf2 mRNA levels by DMN treatment (Fig. 2A). In addition, the AF-treated group exhibited increased nuclear translocation of Nrf2 than the DMN-induced liver injury group (Fig. 2B).

3.4. The expression levels of NQO1, HO-1, and GSTα following AF treatment in rats with DMN-induced liver injury

To determine whether Nrf2-regulated genes are induced by AF in DMN-induced liver injury, we examined the mRNA levels of HO-1, NQO1, and GSTα by semi-quantitative RT-PCR. The mRNA levels of HO-1 were lower in the DMN-induced liver injury group than the control group (Fig. 3A), but significantly higher in the AF-treated group than the DMN-induced liver injury group (Fig. 3A). In addition, significantly lower in the DMN-induced liver injury group than the control group.

3.5. Analysis of COX-2/iNOS expression and NF-κB activity following AF treatment in rats with DMN-induced liver injury

To identify the anti-inflammatory effects of AF, we investigated the expression of COX-2 and iNOS in rats with DMN-induced liver injury. As shown in Fig. 4A, COX-2 and iNOS mRNA levels were significantly higher than those of the DMN-induced liver injury group. To confirm these results, we examined the protein levels of HO-1, NQO1, and GSTα following AF treatment in the injury group. As shown in Fig. 3B, AF induced the expression of various Nrf2-stimulated proteins and significantly augmented the expression of HO-1, NQO1, and GSTα compared to the injury group.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum ALT (U/l)</th>
<th>Serum AST (U/l)</th>
<th>Malondialdehyde (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42 ± 4.1</td>
<td>73 ± 5.4</td>
<td>0.191 ± 0.0021</td>
</tr>
<tr>
<td>AF 200</td>
<td>39 ± 4.1</td>
<td>68 ± 5.9</td>
<td>0.188 ± 0.0023</td>
</tr>
<tr>
<td>DMN</td>
<td>118 ± 9.1</td>
<td>160 ± 13.8</td>
<td>0.235 ± 0.0025</td>
</tr>
<tr>
<td>DMN + AF 50</td>
<td>110 ± 8.9</td>
<td>148 ± 10.4</td>
<td>0.231 ± 0.0021</td>
</tr>
<tr>
<td>DMN + AF 100</td>
<td>97 ± 9.4</td>
<td>136 ± 9.9</td>
<td>0.228 ± 0.0025</td>
</tr>
<tr>
<td>DMN + AF 200</td>
<td>91 ± 7.4</td>
<td>117 ± 8.9</td>
<td>0.215 ± 0.0024</td>
</tr>
</tbody>
</table>

To induce hepatic fibrosis, DMN dissolved in sterile saline (10 mg/kg body weight) was administered by intraperitoneal (i.p.) injection three times per week for 4 weeks. Rats were intragastrically (i.g.) administered 50, 100, or 200 mg/kg/day of AF (dissolved in saline) six times per week for 4 weeks. The control and DMN-treated groups were administered saline alone (i.g.) without AF. Hepatotoxicity was determined by quantifying the serum activity of ALT and AST, and malondialdehyde formation of fibrotic rats. Values are expressed as means ± SD for five rats.

* Significantly different from control.
† Significantly different from AF.
‡ Significantly different from DMN.
In addition, the AF-treated group exhibited significantly lower COX-2 and iNOS protein levels than the injury group (Fig. 4B). In response to oxidative stress and carcinogenic insult, the upregulation of COX-2 requires the activation of several transcription factors, including Nrf2, which is known to bind to antioxidant response elements in the promoter regions of target genes. This binding activates the expression of genes involved in the detoxification and antioxidant defense systems, such as HO-1, NQO1, and GSTα.

**Fig. 2.** Effects of AF on Nrf2 expression and nuclear translocation in rats with DMN-induced liver injury. (A) The mRNA levels of Nrf2 after AF treatment of rats with DMN-induced liver injury, as determined by semi-quantitative RT-PCR. (B) Nuclear Nrf2 expression after AF treatment of injured rats, assessed via Western blot analysis. Each value represents the means ± SD of at least three independent experiments. *P < 0.01 denotes a significant difference from the DMN-treated group.

**Fig. 3.** Effects of AF on the expression of antioxidant enzymes in rats with DMN-induced liver injury. (A) mRNA expression of HO-1, NQO1, GSTα, and GAPDH following AF treatment of rats with DMN-induced liver injury was measured by semi-quantitative RT-PCR. (B) Expression of HO-1, NQO1, GSTα, and β-actin after AF treatment of injured rats, measured by Western blot analysis. Blots were quantified via densitometry analysis. Each value represents the means ± SD of at least three separate experiments. *P < 0.01 denotes a significant difference from the DMN-treated group.
transcription factors including NF-κB and AP-1 (Lee et al., 2007), particularly NF-κB (Surh et al., 2001). To evaluate whether the preventive effects of AF on DMN-induced liver injury are related to its ability to regulate the activity of NF-κB, we assessed the nuclear translocation of NF-κB following AF treatment. As shown in Fig. 5A, AF treatment attenuated NF-κB nuclear translocation in the injury group and inhibited the degradation of IκBα in a dose-dependent manner (Fig. 5B).
4. Discussion

Anthocyanins possess powerful antioxidant (Shih et al., 2007), anti-inflammatory (Karlsen et al., 2007) and anti-tumor properties (Shih et al., 2005). We previously reported that AF possesses potent hepatoprotective effects in the APAP-induced hepatic damage mouse model. In addition, AF upregulates the activity of antioxidants such as GSH and GST and acts as an ROS scavenger (Choi et al., 2009). However, the mechanisms of antioxidant induction by AF have remained unclear. In the present study, we report that AF attenuates DMN-induced liver injury in rats by inducing Nrf2-mediated antioxidant enzymes and attenuating the inflammatory mediators COX-2 and iNOS through NF-κB inhibition. The apparent reduction of DMN-stimulated activity of serum enzymes and lipid peroxidation associated with AF treatment is in agreement with previous observations reporting the hepatoprotective ability of several hepatotoxicants (Table 1) (Reen et al., 2006; Choi et al., 2009). In addition, our findings show that AF treatment significantly attenuates DMN-induced hepatic GSH depletion and DMN-reduced GST activity (Fig. 1). Also, the hepatic GSH levels and GST activity were increased by treatment with AF alone (Fig. 1).

The induction of the chemopreventive and antioxidant enzyme system is an important event in the cellular stress response during which a diverse array of electrophilic and oxidative toxicants can be eliminated or inactivated before they damage cellular macromolecules (Rushmore and Kong, 2002). Antioxidant agents can either scavenge ROS or stimulate detoxification mechanisms within cells, resulting in ROS removal. Several dietary phytochemicals such as triterpenoid compounds, isothiocyanates, flavonoids, and curcuminoids induce ARE-mediated gene expression through either direct increasing the expression of Nrf2 or suppressing its turnover by ubiquitination (Jeong et al., 2005; Dobrovolskaia and Kozlov, 2005). Nrf2-null mice are particularly susceptible to chemical oxidative and electrophilic stress (Ma et al., 2006; Reisman et al., 2009), contributing to an increased hepatotoxicity by APAP (Reisman et al., 2009), ethanol (Lamle et al., 2008), and high-fat diet (Tanaka et al., 2008). In the present study, Nrf2 expression in the DMN-induced liver injury group was lower; however, AF treatment both induced Nrf2 expression and enhanced its nuclear translocation (Fig. 2). This observation implicates AF in the stabilization and activation of Nrf2 in the nucleus.

The majority of genes that encode antioxidant enzymes possess an ARE sequence in their promoter region. Because Nrf2 regulates ARE-driven HO-1, NQO1, and GST gene expression, we investigated whether AF induces the expression of these antioxidants, using semi-quantitative RT-PCR and Western blot analysis. AF-induced increases in Nrf2 levels increased the levels of HO-1, NQO1, and GSTα mRNA and protein expression (Fig. 3), suggesting that AF indeed promotes the transcription of key antioxidant genes by triggering the translocation of Nrf2 into the nucleus.

The activation of the NF-κB signaling pathway is central to the pathophysiology of the inflammatory response and can be activated by oxidative stress, bacterial endotoxins, and cytokines (Chen et al., 1999; Dobrovolskaia and Kozlov, 2005). The functional importance of NF-κB in inflammation is based on its ability to regulate the promoters of multiple inflammatory genes including tumor necrosis factor-alpha (TNF-α), COX-2, and iNOS. Although diverse mechanisms have been reported to account for the chemopreventive effects of dietary phytochemicals (Surh, 2003; Lee et al., 2007), recent studies have focused on the signaling molecules that mediate inflammation and cancer. Because of a causal link between inflammation and cancer (Clevers, 2004), intracellular signaling pathways that mediate inflammatory responses are now considered a viable target for the development of chemopreventive agents (Surh et al., 2005). The modulation of the cellular signaling network involved in the induction and activity of COX-2 and/or iNOS has been considered a new paradigm for the prevention of carcinogenesis (Chung et al., 2007).

Anthocyanins have strong anti-inflammatory effects by inhibiting NF-κB and pro-inflammatory chemokine expression in monocytes (Karlsen et al., 2007). Anthocyanins also inhibit the expression of matrix metalloproteinase (MMP)-2 and MMP-9 and the activation of NF-κB stimulated by TNF-α and attenuate inflammatory COX-2 and iNOS gene expression by regulating NF-κB (Pergola et al., 2006; Wang et al., 2008). NF-κB and Nrf2 are major transcription factors involved in the regulation of pro-inflammatory and antioxidant genes, respectively. Recent studies have suggested the activation of Nrf2-mediated antioxidant enzymes and attenuating the inflammatory COX-2 and iNOS expression by inactivating NF-κB (Figs. 4 and 5). In addition, AF increases the expression of the antioxidant enzymes HO-1, NQO1, and GSTα by activating Nrf2 and decreases inflammatory mediators by inhibiting NF-κB. These data imply that the ability of AF to mediate NF-κB inhibition may be achieved through the activation of Nrf2, as well as by having a direct anti-inflammatory effect.

In conclusion, we demonstrate a novel mechanism for AF-mediated protection following DMN-induced liver damage. We demonstrate that AF achieves hepatoprotective effects and antioxidant defense through the Nrf2 pathway and an anti-inflammation effect by inhibiting NF-κB.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Y. P.Hwang et al. / Food and Chemical Toxicology 49 (2011) 93–99