JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Ameliorative Effects of Proanthocyanidin on Oxidative Stress and Inflammation in Streptozotocin-Induced Diabetic Rats

YOUNG A. LEE,[†] YOU JUNG KIM,[‡] EUN JU CHO,[§] AND TAKAKO YOKOZAWA*,[†]

Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama, 930-0194, Japan, Department of Cosmetology, Pusan Women's College, Pusanjin-Gu, Busan, Korea, and Department of Food Science and Nutrition, Research Institute of Ecology for the Elderly, Pusan National University, 30 Jangjeon-dong, Geumjeong-gu, Busan 609-735, Korea

Recent evidence strongly suggests that oxidative stress due to redox imbalance is causally associated with inflammatory processes and various diseases including diabetes. We examined the effects of proanthocyanidin from persimmon peel, using both oligomers and polymers, against oxidative stress with elucidation of the underlying mechanisms in streptozotocin-induced diabetic rats. The elevation of lipid peroxidation in the kidney and serum under the diabetic condition was decreased by the administration of proanthocyanidin. The suppression of reactive oxygen species generation and elevation of the reduced glutathione/oxidized glutathione ratio were observed in the groups administered proanthocyanidin. These results support the protective role of proanthocyanidin from oxidative stress induced by diabetes. Moreover, proanthocyanidin, especially its oligomeric form, affected the inflammatory process with regulation of related protein expression, inducible nitric oxide synthase, cyclooxygenase-2, and upstream regulators, nuclear factor κB, and inhibitor-binding protein κB-α. Proanthocyanidin ameliorated the diabetic condition by decreases of serum glucose, glycosylated protein, serum urea nitrogen, urinary protein, and renal advanced glycation endproducts. In particular, oligomeric proanthocyanidin exerted a stronger protective activity than the polymeric form. This suggests that the polymerization of proanthocyanidin has an effect on its protective effect against diabetes. The present study supports the beneficial effect of proanthocyanidin against diabetes and oxidative stress-related inflammatory processes.

KEYWORDS: Proanthocyanidin; oxidative stress; inflammation; diabetes

INTRODUCTION

Oxidative stress occurs when the production of reactive oxygen species (ROS) overwhelms antioxidant defenses via antioxidants and antioxidative enzymes. This oxidative stress leads to cellular damage and is a causative factor in chronic degenerative diseases. In biological systems, antioxidants such as reduced glutathione (GSH), a major nonenzymatic antioxidant involved in the maintenance of redox balance, ameliorate cellular oxidative damage. At cellular and molecular levels, redox imbalance causes the activation of redox-sensitive transcription factors that lead to inflammation (*1*). Therefore, enhanced oxidative stress due to uncontrolled ROS is a major factor in both acute and chronic inflammation and inflammatory-related diseases including diabetes (*2*).

Diabetes mellitus is characterized by hyperglycemia and an abnormal elevation in the blood glucose level, which has been associated with oxidative stress (*3*). In diabetes, products of lipid peroxidation, advanced glycation end products (AGEs), and damaged DNA accumulate and eventually result in pathological diabetic complications (*4–6*). Furthermore, the development of diabetes is closely related with inflammatory processes regulated by cyclooxygenase (COX)-2 and nuclear factor-κB (NF-κB) (*7, 8*).

To elucidate the protective role of proanthocyanidin against diabetes in relation to its polymerization, we extracted proanthocyanidin from persimmon peel and then disintegrated polymers and oligomers from it. Proanthocyanidin polymers have antioxidative, antifungal, and anticancer properties (*9–11*). They are widely distributed in plants, fruits, legume seeds, wine, and tea, and persimmon is a proanthocyanidin-rich food with higher contents in the peel than in the pulp (*9, 12*). Although other reports have shown the effect of proanthocyanidin from other plants on diabetes (*13, 14*), the protective potential of proanthocyanidin from persimmon peel against oxidative stress and inflammation in streptozotocin (STZ)-induced diabetic rats has not been reported to date. We investigated the effect of proanthocyanidin on the regulation of blood glucose, and the protective effect against oxidative stress was also observed

* To whom correspondence should be addressed. Fax: +81-76-434-5068. E-mail: yokozawa@inm.u-toyama.ac.jp.
[†] University of Toyama.
[‡] Pusan Women's College.
[§] Pusan National University.

10.1021/jf071523u CCC: $37.00
Published on Web 10/17/2007

through the measurement of lipid peroxidation, ROS generation, and the GSH/oxidized glutathione (GSSG) ratio. We studied the expression of proteins such as inducible nitric oxide synthase (iNOS), COX-2, NF-κB p65, and inhibitor-binding protein κB-α (IκB-α). Furthermore, the protective potential of proanthocyanidin against the diabetic condition was observed by employing several diabetes-related parameters.

MATERIALS AND METHODS

Materials. The following reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan): 4,6-dihydroxy-2-mercaptopyrimidine [2-thiobarbituric acid (TBA)], 5-hydroxymethylfurfural (5-HMF), oxalic acid, bovine serum albumin (BSA), 2-amino-2-hydroxylmethyl-1,3-propanediol [Tris(hydroxymethyl)aminomethane], NP-40, Tween 20, phenylmethyl sulfonyl fluoride, protease inhibitor cocktail, and skim milk powder. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). Dithiothreitol (DTT) was purchased from BioVision, Inc. (Mountain View, CA). The Bio-Rad protein assay kit and pure nitrocellulose membrane were purchased from Bio-Rad Laboratories (Tokyo, Japan). β-Actin, STZ, *o*-phthalaldehyde, and *N*-ethylmaleimide were purchased from Sigma Chemical Co. (St. Louis, MO). NF-κB p65, IκB-α, iNOS, COX-2, goat antirabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody, and goat antimouse IgG HRP-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ECL Western Blotting Detection Reagents were purchased from Amersham Bioscience (Piscataway, NJ).

Fractionation of Polymers and Oligomers from Proanthocyanidin of Persimmon Peel. As described previously (*15*), a mixture of freshly crushed persimmon peel (green peel 5–7 cm in diameter, 3 kg) and dried green tea leaves (450 g) in water containing citric acid (240 g) was boiled for 3 h. At this stage, nucleophilic substitution at the C-4 positions of polymeric proanthocyanidin with monomeric tea catechins occurred, and consequently, the polymeric molecules were converted to oligomers. After cooling, the insoluble materials were removed by filtration, and the filtrate was directly applied to a Sepabeads SP 825 column (10 cm i.d. $\times$ 45 cm, Mitsubishi Chemical Co.). Elution with water (4 L) washed out nonphenolic compounds consisting of citric acid, sugar, minerals, amino acids, etc. Further elution with water containing increasing amounts of ethanol (20–80% ethanol, 20% stepwise elution, each 2 L) yielded a mixture of oligomeric proanthocyanidin and tea catechins (72.2 g). The mixture was subsequently subjected to Sephadex LH-20 column chromatography with ethanol. The monomeric tea catechins were eluted out with ethanol, and further elution with 50% aqueous acetone yielded oligomers (51.5 g). The degree of oligomeric polymerization was estimated as 3.3 by quantitative high-performance liquid chromatography (HPLC) analysis of thiol degradation products (*16*), while the unit ratios of epigallocatechin, epicatechin, epigallocatechin 3-*O*-gallate, and epicatechin 3-*O*-gallate in oligomers were determined as 47, 15, 31, and 6%, respectively. The preparation of persimmon polymeric proanthocyanidin was as follows: An aqueous acetone extract of fresh persimmon peel (500 g) was concentrated, and the resulting insoluble precipitates were removed by filtration. The filtrate was subjected to MCI-gel CHP 20P (Mitsubishi Chemicals Co.) column chromatography with water containing methanol (0–80%, 20% stepwise elution) to give polymers (6.93 g).

Experimental Animals and Treatment. The "Guidelines for Animal Experimentation" approved by the University of Toyama were followed for the current study. Male Wistar rats (120–130 g) from Japan SLC Inc. (Hamamatsu, Japan) were used. They were kept in a plastic-bottom cage and exposed to a 12 h light/dark cycle. The room temperature (about 25 °C) and humidity (about 60%) were controlled automatically. The rats were allowed free access to laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan, comprising 24.0% protein, 3.5% lipids, and 60.5% carbohydrate), and water was given ad libitum. After several days of adaptation, STZ dissolved in citrate buffer (pH 4.5) was injected intraperitoneally at a dose of 50 mg/kg of body weight following overnight fasting. Ten days after injection, the glucose level of blood taken from the tail vein was measured. Animals were divided into three groups (seven rats/group) as follows: DC, diabetic control; DP, polymeric proanthocyanidin-fed; and DO, oligomeric proanthocyanidin-fed. The nondiabetic normal (ND) group was comprised of five rats. The diabetic control group was given water (vehicle), while the other groups were administered polymers or oligomers orally at a dose of 10 mg/kg body weight/day using a stomach tube for 20 consecutive days. At the end of this experiment, 24 h urine samples were collected from rats housed in metabolic cages. After the rats were anesthetized with pentobarbital injection, blood samples were collected from the abdominal aorta. Serum was separated immediately from blood samples by centrifugation. Subsequently, the renal artery of each rat was perfused with ice-cold physiological saline (0.9% NaCl, pH 7.4), and the kidneys were collected, snap frozen in liquid nitrogen, and stored at -80 °C until analysis.

Assessment of ROS Generation. ROS generation was measured by the method of Ali et al. (*17*). Kidney tissue was homogenized on ice with 1 mM EDTA–50 mM potassium phosphate buffer (pH 7.4). In brief, 25 mM DCFH-DA was added to homogenates, and after 30 min under dark conditions, changes in fluorescence were determined at an excitation wavelength of 486 nm and an emission wavelength of 530 nm.

Determination of GSH and GSSG Levels. GSH and GSSG assays were carried out by the method of Pandey and Katiyar (*18*). Kidney tissue was homogenized on ice with 1 mM EDTA–50 mM potassium phosphate buffer (pH 7.4). Then, 25% *meta*-phosphoric acid was added to precipitate protein. The total homogenate was centrifuged at 4 °C at 100000*g* for 30 min to obtain a supernatant for the assay of GSH and GSSG. To assay for GSH, 1 mM EDTA–50 mM potassium phosphate buffer (pH 7.4) was added to the supernatant followed by *o*-phthalaldehyde. After 20 min at room temperature, fluorescence was estimated at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. GSSG was assayed after preincubation with *N*-ethylmaleimide for 20 min, and 0.1 M NaOH was substituted for 1 mM phosphate buffer.

Assays of Serum and Urine Samples. Serum glucose and serum urea nitrogen were measured using commercial kits (Glucose CII-Test Wako was obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan; BUN Kainos was obtained from Kainos Laboratories Inc., Tokyo, Japan). The serum glycosylated protein level was determined by a modified TBA assay of Fluckiger and Winterhalter (*19*), in which nonenzymatically bound glucose was released as 5-HMF and quantified colorimetrically. In brief, serum (100 μL) was diluted to 1.0 mL, mixed with 0.5 mL of oxalic acid (0.1 M), and hydrolyzed for 4.5 h at 100 °C, and then, the glycosylated protein level was quantified by measuring the absorbance at 443 nm after reacting with TBA. Levels of TBA reactive substances (TBARS) were measured using the method of Naito and Yamanaka (*20*). The urinary protein level was measured by the sulfosalicylic acid method (*21*).

Measurements of AGEs and Lipid Peroxidation in the Kidney. Renal AGEs were determined by the method of Mitsuhashi et al. (*22*). Minced kidney tissue was washed three times in cold PBS and was dilipidated with chloroform and methanol (2:1, vol/vol) overnight. The solvent was removed by suction, and the pellet was washed sequentially three times with methanol and three times with deionized water. The tissue was then homogenized with 0.1 M NaOH and stirred for 12 h at 4 °C, followed by centrifugation at 8000*g* for 15 min at 4 °C. The amounts of AGEs in these alkali-soluble samples were determined by measuring the fluorescence at an emission wavelength of 465 nm and an excitation wavelength of 369 nm using a Microplate reader (GENios, TECAN Austria GmbH, Salzburg, Austria). TBARS levels were determined according to the method of Mihara and Uchiyama (*23*).

Western Blotting. The kidney was homogenized with ice-cold lysis buffer containing 250 mM NaCl, 25 mM Tris-HCl (pH 7.5), 1% v/v NP-40, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (10 μg/mL aprotinin and 1 μg/mL leupeptin). Samples were then centrifuged at 20000*g* for 15 min at 4 °C. To ensure equal loading of lanes, the protein concentration of each tissue was determined using a Bio-Rad protein kit with BSA as a standard, and then, immunoblotting was carried out. To determine the protein levels of NF-κB, IκB, iNOS, and COX-2, 30 μg of protein from each sample was electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Sepa-

Table 1. Effects of Proanthocyanidin on Renal and Serum TBARS Levels[a]

group	renal TBARS (nmol/mg protein)	serum TBARS (nmol/mL)
ND	$0.72 \pm 0.10^{*,a}$	$2.31 \pm 0.16^{*}$
DC	1.17 ± 0.04	3.98 ± 0.44
DP	$0.86 \pm 0.07^{*,b}$	3.09 ± 0.21
DO	$0.79 \pm 0.07^{*,c}$	3.09 ± 0.13

[a] Results are expressed as the means ± SEM. Significance: $^{*}p < 0.05$ vs DC; $^{a}p = 0.0009$ vs DC; $^{b}p = 0.0026$ vs DC; and $^{c}p = 0.0006$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

rated proteins were transferred electrophoretically to a pure nitrocellulose membrane, blocked with 2% skim milk solution for 1 h, subsequently incubated with 2% skim milk solution for 3 h, and then incubated with primary antibodies overnight at 4 °C. After the blots were washed, they were incubated with goat antirabbit and/or goat antimouse IgG HRP-conjugated secondary antibody for 40 min at room temperature. Each antigen–antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (Fujifilm, Tokyo, Japan). Band density was determined by Image J software (Wayne Rasband, National Institute of Health) and normalized to β-actin.

Protein Assay. Protein assays were carried out according to the method of Itzhaki and Gill (*24*) using BSA as a standard. All samples were assayed in duplicate.

Statistical Analysis. All results are expressed as the mean ± standard error of the mean (SEM) and were analyzed using Kruskal–Wallis analysis of variance on ranks followed post hoc by Bonferroni's or Dunn's test for comparisons among all groups. When normality and equal variance assumptions were met, we used the *t* test for comparisons between diabetic control and nondiabetic, normal, or proanthocyanidin-administered rats. In this case, a *p* value of less than 0.05 was considered to indicate significance.

RESULTS

Lipid Peroxidation. As shown in **Table 1**, TBARS levels in the kidney and serum increased in the DC group as compared with the ND group. However, the administration of proanthocyanidin inhibited this increase. In renal TBARS, the DP and DO groups showed significant decreases from 1.17 nmol/mg protein to 0.86 and 0.79 nmol/mg protein, respectively, indicating that the increase in lipid peroxidation under diabetes was effectively suppressed by proanthocyanidin intake. No significant difference was observed between DP and DO groups.

ROS Generation. The effect of proanthocyanidin administration on ROS generation in the STZ diabetic rat model is shown in **Figure 1**. The generation of ROS was elevated in the DC group as compared with the ND group, while the administration of proanthocyanidin led to a significant decrease in ROS generation.

GSH and GSSG Levels, and the GSH/GSSG Ratio. Figure 2 illustrates the levels of GSH and GSSG and their ratio. The GSH level significantly decreased in the DC group; however, it was significantly increased in the DP and DO groups as compared to the DC group (**Figure 2A**). The GSSG level was elevated in the DC group, but it was reduced in the DP and DO groups (**Figure 2B**). In addition, as shown in **Figure 2C**, the GSH/GSSG ratio showed a marked decrease in the DC group, while it increased in the DP and DO groups, as compared to the DC group. The oligomer-treated rats had a higher ratio of GSH/GSSG than the polymer-treated rats through an elevation in GSH and a decline in GSSG.

iNOS, COX-2, NF-κB p65, and IκB-α Protein Levels. Figures 3–5 show the effect of proanthocyanidin from persimmon peel on protein expression related to inflammation in the diabetic

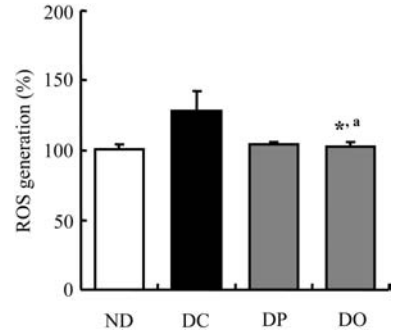

Figure 1. Changes in ROS generation by proanthocyanidin. Results are expressed as the mean ± SEM. Significance: $^{*}p < 0.05$ vs DC; $^{a}p = 0.0467$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

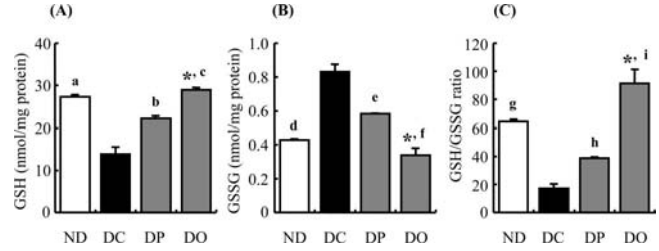

Figure 2. Effects of proanthocyanidin on GSH and GSSG levels and their ratios. (**A**) Effects of proanthocyanidin on GSH levels. Results are expressed as the mean ± SEM. Significance: $^{*}p < 0.05$ vs DC; $^{a}p = 0.0034$ vs DC; $^{b}p = 0.0173$ vs DC; and $^{c}p = 0.0003$ vs DC. (**B**) Effects of proanthocyanidin on GSSG levels. Results are expressed as the mean ± SEM. Significance: $^{*}p < 0.05$ vs DC; $^{d}p = 0.002$ vs DC; $^{e}p = 0.0092$ vs DC; and $^{f}p = 0.0006$ vs DC. (**C**) Effects of proanthocyanidin on the GSH/GSSG ratio. Results are expressed as the mean ± SEM. Significance: $^{*}p < 0.05$ vs DC; $^{g}p \leq 0.0001$ vs DC; $^{h}p = 0.0034$ vs DC; and $^{i}p = 0.0014$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

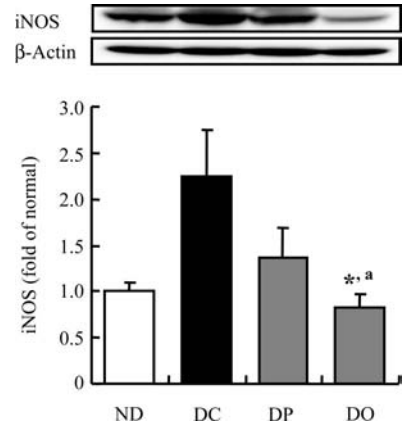

Figure 3. Modulation of iNOS protein levels by proanthocyanidin. Results are expressed as the mean ± SEM. Significance: $^{*}p < 0.05$ vs DC; $^{a}p = 0.0162$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

rat model. As observed in **Figure 3**, the iNOS protein level in diabetic rats was increased, but it was decreased by proanthocyanidin administration; inhibition of iNOS protein expression was greater in the DO than the DP group. The expression of COX-2 protein (**Figure 4**) was up-regulated in the DC group as compared with the ND group, while the DO group showed that the expression

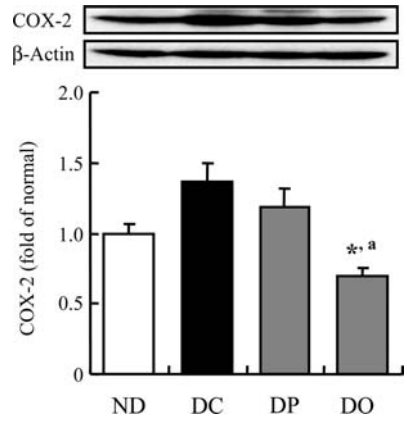

Figure 4. Effects of proanthocyanidin on COX-2 protein levels. Results are expressed as the mean ± SEM. Significance: $*p < 0.05$ vs DC; $^{a}p = 0.0079$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

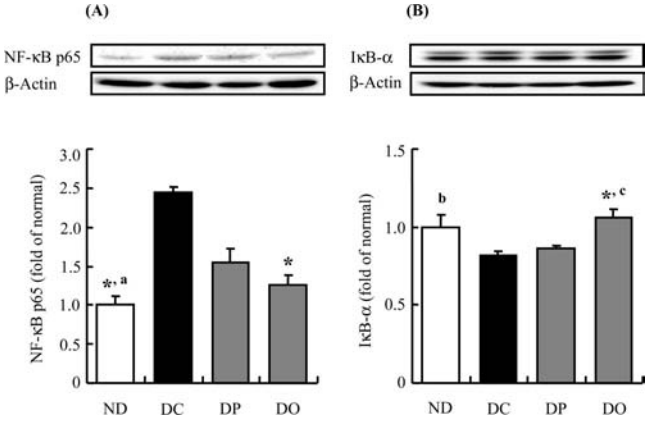

Figure 5. Modulation of NF-κB p65 and IκB-α protein levels by proanthocyanidin. (**A**) Effects of proanthocyanidin on NF-κB p65 protein levels. Results are expressed as the mean ± SEM. Significance: $*p < 0.05$ vs DC; $^{a}p \leq 0.0001$ vs DC. (**B**) Effects of proanthocyanidin on IκB-α protein levels. Results are expressed as the mean ± SEM. Significance: $*p < 0.05$ vs DC; $^{b}p \leq 0.033$ vs DC; and $^{c}p \leq 0.0068$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

was significantly suppressed. Moreover, the DC group showed a marked increase in the NF-κB p65 protein level (**Figure 5A**), but the administration of polymeric and oligomeric proanthocyanidin markedly reduced the expression. The IκB-α protein level, which decreased in the DC group, was elevated in the DO group (**Figure 5B**). The modulating effect on the expression of inflammatory proteins, iNOS, COX-2, NF-κB p65, and IκB-α, under diabetes was greater in the oligomer than the polymer group.

Glucose and Glycosylated Protein Levels in Serum. The effect of proanthocyanidin on changes in the serum glucose and glycosylated protein level is shown in **Table 2**. Marked increases in the levels of serum glucose and glycosylated protein were observed in the DC group (591.3 mg/dL and 46.84 nmol/mg protein, respectively) as compared with the ND group (116.5 mg/dL and 28.30 nmol/mg protein, respectively). However, the administration of proanthocyanidin led to a decrease in the levels. In particular, the DO group showed a significant decline in these values to 532.8 mg/dL of glucose and 40.60 nmol/mg protein of glycosylated protein.

Table 2. Effects of Proanthocyanidin on Glucose and Glycosylated Protein Levels in Serum[a]

group	glucose (mg/dL)	glycosylated protein (nmol/mg protein)
ND	$116.5 \pm 4.2^{*,a}$	$28.30 \pm 1.20^{*}$
DC	591.3 ± 18.4	46.84 ± 1.42
DP	583.8 ± 18.0	44.11 ± 1.13
DO	532.8 ± 9.0^{b}	40.60 ± 1.90^{c}

[a] Results are expressed as the mean ± SEM. Significance: $*p < 0.05$ vs DC; $^{a}p \leq 0.0001$ vs DC; $^{b}p = 0.0491$ vs DC; and $^{c}p = 0.032$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

Table 3. Modulation of Renal Functional Parameters by Proanthocyanidin[a]

group	serum urea nitrogen (mg/dL)	urinary protein (mg/day)
ND	$19.3 \pm 0.8^{*,a}$	13.10 ± 0.76
DC	33.1 ± 1.5	23.02 ± 4.78
DP	30.3 ± 0.6	16.64 ± 2.19
DO	$24.0 \pm 1.3^{*,b}$	13.06 ± 0.22

[a] Results are expressed as the mean ± SEM. Significance: $*p < 0.05$ vs DC; $^{a}p = 0.0002$ vs DC; and $^{b}p = 0.0029$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

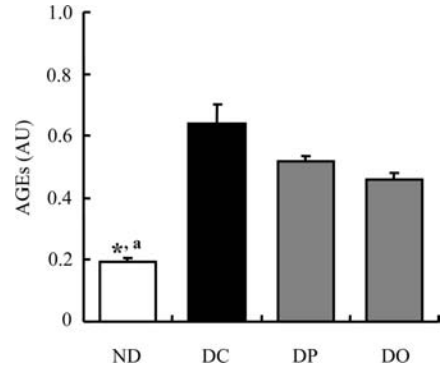

Figure 6. Changes in renal AGEs level by proanthocyanidin. Results are expressed as the mean ± SEM. Significance: $*p < 0.05$ vs DC; $^{a}p = 0.0023$ vs DC. ND, nondiabetic normal rats; DC, diabetic control rats; DP, polymeric proanthocyanidin-administered rats; and DO, oligomeric proanthocyanidin-administered rats.

Serum Urea Nitrogen and Urinary Protein. Table 3 shows the effect of proanthocyanidin on renal function in the STZ diabetic rat model. A marked increase of serum urea nitrogen in the DC group was observed as compared with the normal group, from 19.3 to 33.1 mg/dL. However, the administration of oligomers led to a significant decrease to 24.0 mg/dL. In addition, urinary protein was also elevated in the DC group, while in the DO group, it was decreased to 13.06 mg/day as compared with the control level of 23.02 mg/day. The DP group did not show any significant effect regarding serum nitrogen and urinary protein.

Renal AGEs. The changes in renal AGEs are represented in **Figure 6**. The levels showed a significant increase in the DC group. However, the decrease in AGEs was shown in both DO and DP groups, although no significant effect among all diabetic groups was noted.

DISCUSSION

Proanthocyanidin, naturally and widely distributed as an antioxidant, is reported to exhibit various biological activities

against free radicals and oxidative stress (*25*). Ariga (*26*) suggested the utilization of proanthocyanidin related to its antioxidative function and preventive action on diseases. Proanthocyanidin is absorbed through the gut barrier, and its absorption depends on its degree of polymerization. It is considered that this degree of polymerization dictates the functional roles of proanthocyanidin in a biological system. However, the functional properties brought about by their polymerization are little understood. In the current study, we provided the first evidence that dietary proanthocyanidin from persimmon peel, especially oligomers, potently protects STZ diabetic rats against oxidative stress-related inflammation. We examined the effects of proanthocyanidin against oxidative stress with the elucidation of the underlying mechanisms behind the possible beneficial effect against diabetes.

It has been well-established that lipid peroxidation increases under the diabetic pathological condition, since diabetes is related to oxidative stress. The present results also showed the elevation of lipid peroxidation under the diabetic condition. However, the administration of proanthocyanidin suppressed lipid peroxidation in both the kidney and the serum. The increase in ROS generation under diabetes was attenuated by proanthocyanidin. This suggests that proanthocyanidin would probably ameliorate diabetic oxidative stress. Its actions are possibly related to upregulation of the GSH/GSSG ratio through an increase in GSH and reduction in GSSG. Elevation of the GSH/GSSG ratio and suppression of ROS generation may be the primary role of proanthocyanidin in ameliorating oxidative stress and maintaining the redox balance.

In diabetes mellitus, oxidative stress may be attributed to a combination of hyperglycemia-induced glycoxidation, sorbitol system activation, and reduced GSH synthesis due to limited hexose monophosphate shunt. It has been reported that under hyperglycemic conditions, as much as 30% of glucose is shunted to the polyol pathway (*27*), causing a substantial depletion of NADPH and, consequently, a significant decrease in the GSH level. Our current data on the GSH/GSSG ratio are in line with another report (*28*), which demonstrated that an elevation of the GSH/GSSG ratio is effective in ameliorating oxidative stress under diabetes.

Because a redox imbalance is causally linked to inflammatory processes (*29*), we evaluated expression levels of some major players implicated in inflammation in the diabetic rat model. The inflammatory process is regulated by COX-2 and NF-κB. ROS induces the activation of NF-κB activity, and NF-κB, in turn, up-regulates the transcription of genes that encode enzymes such as iNOS and COX-2. The present results showed that the protein levels of COX-2 and iNOS were increased in the diabetic oxidative rat model. Feng et al. (*30*) reported that ROS induces the expression of COX-2 protein, the key enzyme in proinflammatory prostanoid synthesis, and COX-2 is induced readily by cytokines, hormones, growth factors, and tumor promoters in selected tissues (*31, 32*). In addition, iNOS is also readily inducible by proinflammatory cytokines and has a close relationship with ROS generation. The administration of oligomeric proanthocyanidin suppressed the high-level expression of these proteins under diabetes. This suggests that the modulation of COX-2 and iNOS expressions may contribute an important protective role of proanthocyanidin against diabetes.

A molecular explanation supporting the aforementioned findings comes from the present results on NF-κB expression. The NF-κB complex is a heterodimer of two subunits, p50 and p65, which exist in the cytoplasm in an inactive form, and it is related to the inhibitory subunit, IκB-α. Molecular investigations revealed that inflammation and ROS stimulate NF-κB activation by enhancing the dissociation of cytoplasmic NF-κB from IκB-α, thereby allowing NF-κB to migrate to the nucleus (*33*), where it binds to promoters of NF-κB-regulated genes to initiate gene transcription (*34*). In the present study, an increase in the NF-κB p65 protein level and reduction of the IκB-α protein level were observed in the diabetic rat. However, the administration of proanthocyanidin led to a decrease in NF-κB p65 and elevation of IκB-α protein, indicating that proanthocyanidin suppressed the translocation of NF-κB to the nucleus, where it binds to the promoters of NF-κB-regulated genes and initiates gene transcription. In particular, among the proanthocyanidins from persimmon peel, the oligomeric form exerted stronger effects on inflammatory protein regulation than the polymeric form, suggesting the crucial role of polymerization of proanthocyanidins in inflammation-related conditions. Our findings on NF-κB p65, COX-2, and iNOS protein levels indicate the crucial protective role of proanthocyanidins through its antidiabetic action by modulating key proinflammatory genes.

Under the diabetic condition, glucose itself and an increase in protein glycosylation induced by hyperglycemia are significant sources of free radicals and inducers of oxidative stress (*35*). The data on the decrease in glucose and glycosylated protein in serum by proanthocyanidin administration indicated that proanthocyanidin attenuated the pathological condition of diabetes by the control of blood glucose and protein glycosylation. Although the blood glucose level was decreased by proanthocyanidin as compared to control diabetic rats, it was still high, and a clinical trial also has to be conducted to clarify its effect. In addition, the inhibition of renal AGE formation by proanthocyanidin would be associated with attenuation of the pathogenesis of diabetic complications, since evidence that the renal accumulation of AGEs is linked to the pathogenesis of various diabetic complications has been well-established (*36*). Our study also showed that renal functional markers such as serum urea nitrogen and urinary protein levels were downregulated by proanthocyanidin. Oligomeric proanthocyanidin exerted a stronger protective activity than the polymeric form, suggesting that polymerization affects the functional properties of proanthocyanidin. These results are in agreement with our recent reports showing that maintenance of the antioxidative status plays a crucial role in protecting against renal insufficiency (*37, 38*).

It is worth pointing out that the duel effects of proanthocyanidin derived from its antioxidative and antiinflammatory properties are useful in the amelioration of diabetic conditions. In particular, oligomeric proanthocyanidin showed a stronger effect than polymeric proanthocyanidin, indicating that absorption through the gut barrier is likely to be limited to molecules with a low degree of polymerization (*39*).

In summary, our present study demonstrated that the administration of proanthocyanidin attenuated oxidative stress induced by diabetes through the inhibition of lipid peroxidation, ROS generation, and elevation of the GSH/GSSG ratio. In addition, proanthocyanidin regulated the expression of proteins related to inflammation such as iNOS, COX-2, NF-κB p65, and IκB-α protein. Proanthocyanidin exerted a protective effect against hyperglycemia and hyperglycemia-related changes through modulating glucose, glycosylated protein, serum urea nitrogen, urinary protein, and renal AGEs in STZ diabetic rats. On the basis of the results, we propose that the suppression of oxidative stress-related inflammation is a plausible mechanism underlying the protective effect of proanthocyanidin in diabetic rats. Further studies should focus on the absorption of proanthocyanidin in biological systems based on its degree of polymerization.

LITERATURE CITED

(1) Chung, H. Y.; Kim, H. J.; Kim, J. W.; Yu, B. P. The inflammation hypothesis of aging: Molecular modulation by calorie restriction. *Ann. N. Y. Acad. Sci.* **2001**, *928*, 327–335.

(2) Lin, Y.; Berg, A. H.; Iyengar, P.; Lam, T. K.; Giacca, A.; Combs, T. P.; Rajala, M. W.; Du, X.; Rollman, B.; Li, W.; Hawkins, M.; Barzilai, N.; Rhodes, C. J.; Fantus, I. G.; Brownlee, M.; Scherer, P. E. The hyperglycemia-induced inflammatory response in adipocytes: The role of reactive oxygen species. *J. Biol. Chem.* **2005**, *280*, 4617–4626.

(3) Bonnefont-Rousselot, D.; Beaudeux, J. L.; Therond, P.; Peynet, J.; Legrand, A.; Delattre, J. Diabetes mellitus, oxidative stress and advanced glycation end products. *Ann. Pharm. Fr.* **2004**, *62*, 147–157.

(4) Anderson, D.; Yu, T. W.; Wright, J.; Ioannides, C. An examination of DNA strand breakage in the comet assay and antioxidant capacity in diabetic patients. *Mutat. Res.* **1998**, *398*, 151–161.

(5) Kilhovd, B. K.; Berg, T. J.; Birkeland, K. I.; Thorsby, P.; Hanssen, K. F. Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. *Diabetes Care* **1999**, *22*, 1543–1548.

(6) Ugochukwu, N. H.; Bagayoko, N. D.; Antwi, M. E. The effects of dietary caloric restriction on antioxidant status and lipid peroxidation in mild and severe streptozotocin-induced diabetic rats. *Clin. Chim. Acta* **2004**, *348*, 121–129.

(7) Kim, H. J.; Kim, K. W.; Yu, B. P.; Chung, H. Y. The effect of age on cyclooxygenase-2 gene expression: NF-κB activation and IκB-α degradation. *Free Radical Biol. Med.* **2000**, *28*, 683–692.

(8) Chung, H. Y.; Sung, B.; Jung, K. J.; Zou, Y.; Yu, B. P. The molecular inflammatory process in aging. *Antioxid. Redox Signal.* **2006**, *8*, 572–581.

(9) Gorinstein, S.; Zemser, M.; Weisz, M.; Halevy, S.; Deutsch, J.; Tilis, K.; Feintuch, D.; Guerra, N.; Fishman, M.; Bartnikowska, E. Fluorometric analysis of phenolics in persimmons. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1087–1092.

(10) de Pascual-Teresa, S.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *J. Agric. Food Chem.* **2000**, *48*, 5331–5337.

(11) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds—Nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094–1117.

(12) Gorinstein, S.; Zachwieja, Z.; Folta, M.; Barton, H.; Piotrowicz, J.; Zemser, M.; Weisz, M.; Trakhtenberg, S.; Martin-Belloso, O. Comparative contents of dietary fiber, total phenolics, and minerals in persimmons and apples. *J. Agric. Food Chem.* **2001**, *49*, 952–957.

(13) Landrault, N.; Poucheret, P.; Azay, J.; Krosniak, M.; Gasc, F.; Jenin, C.; Cros, G.; Teissedre, P. L. Effect of a polyphenols-enriched chardonnay white wine in diabetic rats. *J. Agric. Food Chem.* **2003**, *51*, 311–318.

(14) El-Alfy, A. T.; Ahmed, A. A.; Fatani, A. J. Protective effect of red grape seeds proanthocyanidins against induction of diabetes by alloxan in rats. *Pharmacol. Res.* **2005**, *52*, 264–270.

(15) Yokozawa, T.; Kim, Y. A.; Kim, H. Y.; Lee, Y. A.; Nonaka, G. Protective effect of persimmon peel polyphenol against high glucose-induced oxidative stress in LLC-PK_1 cells. *Food Chem. Toxicol.* **2007**, *45*, 1979–1987.

(16) Tanaka, T.; Takahashi, R.; Kouno, I.; Nonaka, G. Chemical evidence for the de-astringency (insolubilization of tannins) of persimmon fruit. *J. Chem. Soc. Perkin Trans.* **1994**, *I*, 3013–3022.

(17) Ali, S. F.; LeBel, C. P.; Bondy, S. C. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. *Neurotoxicology* **1992**, *13*, 637–648.

(18) Pandey, A.; Katiyar, S. S. Inactivation of yeast glutathione reductase by o-phthalaldehyde. *J. Enzyme Inhib.* **1996**, *11*, 141–149.

(19) Fluckiger, R.; Winterhalter, K. H. In vitro synthesis of hemoglobin AIc. *FEBS Lett.* **1976**, *71*, 356–360.

(20) Naito, C.; Yamanaka, T. Lipid peroxides in atherosclerotic diseases. *Nippon Ronen Igakkai Zasshi* **1978**, *15*, 187–191.

(21) Sakagishi, Y. Total protein. In *Rinsho Kagaku Bunseki II*; Saito, M., Kitamura, M., Niwa, M., Eds.; Tokyo Kagaku Dojin: Tokyo, Japan, 1968; pp 115–142.

(22) Mitsuhashi, T.; Nakayama, H.; Itoh, T.; Kuwajima, S.; Aoki, S.; Atsumi, T.; Koike, T. Immunochemical detection of advanced glycation end products in renal cortex from STZ-induced diabetic rat. *Diabetes* **1993**, *42*, 826–832.

(23) Mihara, M.; Uchiyama, M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* **1978**, *86*, 271–278.

(24) Itzhaki, R. F.; Gill, D. M. A micro-biuret method for estimating proteins. *Anal. Biochem.* **1964**, *9*, 401–410.

(25) Bagchi, D.; Bagchi, M.; Stohs, S. J.; Das, D. K.; Ray, S. D.; Kuszynski, C. A.; Joshi, S. S.; Pruess, H. G. Free radicals and grape seed proanthocyanidin extract: Importance in human health and disease prevention. *Toxicology* **2000**, *148*, 187–197.

(26) Ariga, T. The antioxidative function, preventive action on disease and utilization of proanthocyanidins. *Biofactors* **2004**, *21*, 197–201.

(27) Chung, S. S.; Ho, E. C.; Lam, K. S.; Chung, S. K. Contribution of polyol pathway to diabetes-induced oxidative stress. *J. Am. Soc. Nephrol.* **2003**, *14*, S233–S236.

(28) Ugochukwu, N. H.; Cobourne, M. K. Modification of renal oxidative stress and lipid peroxidation in streptozotocin-induced diabetic rats treated with extracts from Gongronema latifolium leaves. *Clin. Chim. Acta* **2003**, *336*, 73–81.

(29) Touyz, R. M.; Schiffrin, E. L. Reactive oxygen species in vascular biology: implications in hypertension. *Histochem. Cell. Biol.* **2004**, *122*, 339–352.

(30) Feng, L.; Xia, Y.; Garcia, G. E.; Hwang, D.; Wilson, C. B. Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide. *J. Clin. Invest.* **1995**, *95*, 1669–1675.

(31) Smith, W. L.; Meade, E. A.; DeWitt, D. L. Pharmacology of prostaglandin endoperoxide synthase isozymes-1 and -2. *Ann. N. Y. Acad. Sci.* **1994**, *714*, 136–142.

(32) Goppelt-Struebe, M. Regulation of prostaglandin endoperoxide synthase (cyclooxygenase) isozyme expression. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **1995**, *52*, 213–222.

(33) Noh, E. J.; Ahn, K. S.; Shin, E. M.; Jung, S. H.; Kim, Y. S. Inhibition of lipopolysaccharide-induced iNOS and COX-2 expression by dehydroevodiamine through suppression of NF-kappaB activation in RAW 264.7 macrophages. *Life Sci.* **2006**, *79*, 695–701.

(34) Li, Q.; Verma, I. M. NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.* **2002**, *2*, 725–734.

(35) Shimoi, K.; Okitsu, A.; Green, M. H.; Lowe, J. E.; Ohta, T.; Kaji, K.; Terato, H.; Ide, H.; Kinae, N. Oxidative DNA damage induced by high glucose and its suppression in human umbilical vein endothelial cells. *Mutat. Res.* **2001**, *480*−*481*, 371–378.

(36) Figarola, J. L.; Scott, S.; Loera, S.; Xi, B.; Synold, T.; Rahbar, S. Renoprotective and lipid-lowering effects of LR compounds, novel advanced glycation end product inhibitors, in streptozotocin-induced diabetic rats. *Ann. N. Y. Acad. Sci.* **2005**, *1043*, 767–776.

(37) Kim, Y. J.; Yokozawa, T.; Chung, H. Y. Effects of energy restriction and fish oil supplementation on renal guanidino levels and antioxidant defences in aged lupus-prone B/W mice. *Br. J. Nutr.* **2005**, *93*, 835–844.

(38) Kim, Y. J.; Yokozawa, T.; Chung, H. Y. Suppression of oxidative stress in aging NZB/NZW mice: effect of fish oil feeding on hepatic antioxidant status and guanidino compounds. *Free Radical Res.* **2005**, *39*, 1101–1110.

(39) Scalbert, A.; Deprez, S.; Mila, I.; Albrecht, A. M.; Huneau, J. F.; Rabot, S. Proanthocyanidins and human health: Systemic effects and local effects in the gut. *Biofactors* **2000**, *13*, 115–120.

Received for review May 24, 2007. Revised manuscript received September 13, 2007. Accepted September 17, 2007.

JF071523U