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Effects of Proanthocyanidin Preparations on Hyperlipidemia and Other Biomarkers in Mouse Model of Type 2 Diabetes

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The protective effect of proanthocyanidins from persimmon peel, using both oligomers and polymers, was investigated in a *db*/*db* type 2 diabetes model. Male *db*/*db* mice were divided into three groups: control (vehicle), polymer-, or oligomer- (10 mg/(kg body weight·day·p.o.)) administered mice. Age-matched nondiabetic *m*/*m* mice were used as a normal group. The administration of proanthocyanidins reduced hyperglycemia in *db*/*db* mice through a decline in the serum level of glucose and glycosylated protein. In addition, it had a strong effect on hyperlipidemia through lowering levels of triglyceride, total cholesterol, and nonesterified fatty acids. The protective effect against hyperglycemia and hyperlipidemia was greater in the groups administered the oligomeric rather than polymeric form. The increased oxidative stress in *db*/*db* mice was attenuated by the administration of oligomers through inhibiting the generation of reactive oxygen species and lipid peroxidation and elevating the reduced glutathione/oxidized glutathione ratio. On the other hand, polymers did not show such an effect. Moreover, expressions in the liver of sterol regulatory element binding protein (SREBP)-1 and SREBP-2 were downregulated by the administration of proanthocyanidins, especially the oligomeric form. Oligomers caused a slight elevation in the expression of peroxisome proliferator-activated receptors α. Furthermore, oligomeric proanthocyanidin regulated the expression of nuclear factor κB in *db*/*db* type 2 diabetes via the activation of inhibitor protein κB-α. It also attenuated the protein expressions of cyclooxygenase-2 and inducible nitric oxide synthase. This suggests that oligomers would act as a regulator in inflammatory reactions associated with oxidative stress in type 2 diabetes. The present study results suggest that proanthocyanidin administration, especially the oligomeric form, may improve oxidative stress via the regulation of hyperlipidemia than hyperglycemia in type 2 diabetes.

KEYWORDS: Type 2 diabetes; *db*/*db* mice; proanthocyanidin; oxidative stress; hyperlipidemia

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder that continues to present a major worldwide health problem. The predominant type of diabetes mellitus, type 2 diabetes, is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action that lead to chronic hyperglycemia, with deleterious effects on β-cell function. In addition to hyperglycemia, type 2 diabetic individuals almost invariably show a marked disruption of lipid dynamics, often reflected by elevated levels of circulating free fatty acids (FFA) and triglycerides (TG), together with excess fat deposition in various tissues including the liver. An abnormal accumulation of fat in the liver and muscle plays an important role in the etiology of insulin resistance and possibly also in β-cell reduction in type 2 diabetes (*1, 2*). Moreover, hyperglycemia causes oxidative stress due to increased mitochondrial production of O_2^-, nonenzymatic glycation of proteins, and glucose autoxidation. In the absence of an appropriate compensatory response from the endogenous antioxidant network against glucotoxicity and lipotoxicity caused by hyperglycemia and hyperlipidemia under diabetes, oxidative stress becomes marked, leading to activation of the stress-sensitive intracellular signaling pathway (*3, 4*). Accordingly, the attenuation of oxidative stress and regulation of stress-sensitive signaling pathways have been considered as ways to alleviate diabetes and diabetic complications.

Persimmon is a proanthocyanidin-rich food, with higher contents in peel than in pulp (*5, 6*). Proanthocyanidin is known as condensed tannin, a member of a specific group of polyphenolic compounds, and it has been reported to exhibit powerful antioxidant activity (*7, 8*). In our previous studies, we demon-

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strated that proanthocyanidins from persimmon peel exhibited protective activity against diabetes in vitro and in a streptozotocin-induced diabetic rat model (*9, 10*). However, the protective potential of proanthocyanidin and its oligomers on the serum glucose level and lipid metabolism, important goals for the treatment of type 2 diabetes, has yet to be investigated. As an experimental model of obesity-associated type 2 diabetes mellitus, *db*/*db* mice are widely used and well-established (*11, 12*). C57BL/Ksj-*db*/*db* mice develop diabetes due to mutation of the mouse diabetes (*db*) gene that encodes a receptor for leptin. The lack of leptin-receptor signaling results in increased food intake in combination with a phenotype of reduced energy expenditure, reminiscent of the neuroendocrine starvation response (*13*). Consequently, the homozygotes (*db*/*db*) after birth show unrepressed eating behavior, become obese, and by 3−6 months after birth, develop severe insulin resistance associated with hyperinsulinemia, hyperglycemia, and hypertriglycemia. Therefore, we investigated the antidiabetic potential of proanthocyanidin and its oligomers/polymers in *db*/*db* mice, as type 2 diabetic mice, and focused on the regulation of hyperglycemia and/or hyperlipidemia together with protective mechanisms.

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; reduced glutathione (GSH); oxidized glutathione (GSSG); metaphosphoric acid; bovine serum albumin (BSA); 2-amino-2-hydroxylmethyl-1,3-propanediol (Tris (hydroxymethyl) aminomethane); NP-40; tween 20; phenylmethyl sulfonyl fluoride (PMSF); 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, *o*-phthalaldehyde, and *N*-ethylmaleimide were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies against nuclear factor κB (NF-κB) p65, inhibitor protein κB (IκB)-α, sterol regulatory element binding protein (SREBP)-1, SREBP-2, and peroxisome proliferator-activated receptors (PPAR) α, and mouse monoclonal antibody against cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat antirabbit and goat antimouse IgG horseradish peroxidase (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 (*10*), 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 into oligomers. After cooling, the insoluble materials were removed by filtration, and the filtrate was directly applied to a Sephabeads SP 825 column (10 cm i.d. × 45 cm, Mitsubishi Chemical Co.). Elution with water (4 L) washed out nonphenolic compounds consisting of citric acid, sugars, minerals, amino acids, and so forth. 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 HPLC analysis of thiol degradation products (*14*), 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 was followed in the present study. Male, 5-wk-old, C57BL/Ksj-*db*/*db* mice and their age-matched nondiabetic *m*/*m* littermates were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were kept in a plastic-bottomed cage and exposed to a 12-h light/dark cycle. The room temperature (about 25 °C) and humidity (about 60%) were controlled automatically. The mice 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 10 days of adaptation, glucose and total cholesterol (TC) levels of blood taken from the tail vein were measured. Animals were divided into three groups (13∼14 mice/group, 3∼4 mice/cage). The *db*/*db* control group was given water (vehicle), while the other two groups were administered polymers or oligomers orally at a dose of 10 mg/(kg body weight·day) using a stomach tube. The nondiabetic *m*/*m* group comprised eight mice. After administration for 6 weeks, blood samples were collected from the inferior vena cava of anesthetized mice. The serum was separated immediately from blood samples by centrifugation. Subsequently, each mouse was perfused with ice-cold physiological saline (0.9% NaCl, pH 7.4) after cardiac puncture, and the livers were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis. Food and water intakes were determined by estimating the amount consumed once in two days during the treatment period.

Measurement of Serum Parameters. Serum glucose, TG, TC, and nonesterified fatty acids (NEFA) were measured using a commercial kit (Glucose CII-Test Wako, Triglyceride E-Test Wako, Cholesterol E-Test Wako and NEFA C-Test Wako, Tokyo, Japan). The serum glycosylated protein level was determined by a modified TBA assay of Fluckiger and Winterhalter (*15*), in which nonenzymatically bound glucose is 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; then, the glycosylated protein level was quantified by measuring the absorbance at 443 nm after reaction with TBA.

Measurement of Hepatic TG and TC Contents. The liver of each mouse was homogenized, total lipids of the liver homogenates were extracted with a mixture of chloroform and methanol (2:2, v/v) according to the method of Folch et al. (*16*), and the amounts of TG and TC were determined using the Wako kit, as described previously.

Assessment of Reactive Oxygen Species (ROS) Generation and TBA-Reactive Substance (TBARS) Levels. ROS generation was measured by the method of Ali et al. (*17*). Liver tissue was homogenized on ice with 1 mM EDTA−50 mM sodium phosphate buffer (pH 7.4). In brief, 25 mM DCFH-DA was added to homogenates, and after 30 min, changes in fluorescence were determined at an excitation wavelength of 486 nm and emission wavelength of 530 nm. TBARS levels were determined according to the method of Mihara and Uchiyama (*18*).

Determination of GSH and GSSG Levels. GSH and GSSG assays were carried out by the method of Hissin and Hilf (*19*). Liver tissue was homogenized on ice with 1 mM EDTA−50 mM sodium phosphate buffer (pH 7.4). Then, 25% metaphosphoric acid was added to the protein precipitation. The total homogenate was centrifuged at 4 °C at 100 000*g* for 30 min to obtain the supernatant for the assays of GSH and GSSG. To assay for GSH, 1 mM EDTA−50 mM sodium 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 emission wavelength of 460 nm. GSSG was assayed after preincubation with *N*-ethylmaleimide for 20 min, and 0.1 M NaOH was substituted for

phosphate buffer. After 20 min at room temperature, fluorescence was estimated at an excitation wavelength of 360 nm and emission wavelength of 460 nm.

Determination of Protein. Protein assays were carried out according to the method of Itzhaki and Gill using BSA as a standard (*20*).

Preparation of Total and Nuclear Protein Extracts. The liver 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, 1 μg/mL leupeptin). Samples were then centrifuged at 20 000*g* for 15 min at 4 °C. The supernatants were used as total protein extracts. For nuclear protein extracts, liver was homogenized with ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 15 mM $CaCl_2$, 1.5 M sucrose, 1 mM DTT, and protease inhibitor cocktail (10 μg/mL aprotinin, 1 μg/mL leupeptin). Then, samples were centrifuged at 11 000*g* for 20 min at 4 °C. The supernatants were resuspended with extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 10 mM DTT, and protease inhibitor cocktail (10 μg/mL aprotinin, 1 μg/mL leupeptin). The samples were shaken gently for 30 min and centrifuged at 21 000*g* for 5 min at 4 °C (*21*). The pellets were used as nuclear protein extracts. The total and nuclear protein contents were determined by the Bio-Rad protein kit with BSA as the standard.

Western Blotting. Total protein (25 μg) for IκB, iNOS, and COX-2 protein levels and nuclear protein (25 μg) for NF-κB, PPARα, SREBP-1, and SREBP-2 were electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gel. Separated proteins were transferred electrophoretically to a pure nitrocellulose membrane, blocked with 5% skim milk solution for 2 h, and then incubated with primary antibodies overnight at 4 °C. After washing of the blots, they were incubated with goat antirabbit or goat antimouse IgG HRP-conjugated secondary antibody for 2 h 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 densities were determined by an image analyzer ATTO densitograph (ATTO, Tokyo, Japan) and normalized to β-actin for total protein and histone H1 for nuclear protein.

Statistical Analysis. All results are expressed as the mean ± SEM and analyzed statistically using one-way ANOVA followed by posthoc Bonferroni's or Dunn's test for comparison among all groups. When the normality and equal variance assumptions were met, we used the *t*-test to compare *db*/*db* control and proanthocyanidin-administered mice. In this case, a *p* -value of less than 0.05 was considered to indicate significance.

RESULTS

Body Weight and Food and Water Intakes. Figure 1 shows the changes in body weight and food and water intakes during the experimental period. In the *db*/*db* control group, these parameters were increased markedly compared with the *m*/*m* group. However, 6-wk administration of proanthocyanidin did not lead to any significant changes in body weight, food, or water intake.

Hematological Change. As shown in **Table 1**, the levels of glucose and glycosylated protein in the *db*/*db* control group were increased significantly compared with the *m*/*m* group. Although the glucose level was not significantly decreased by the administration of proanthocyanidins, a tendency to reduce the glucose level was exhibited by oligomers. Regarding glycosylated protein, oligomer administration led to a significant decrease in the level. Moreover, the serum concentrations of TG, TC, and NEFA were significantly elevated in the *db*/*db* control group compared with the *m*/*m* group; these concentrations were significantly reduced in the proanthocyanidin-administered groups. However, no significant difference between polymer- and oligomer-administered groups was observed.

Hepatic Lipid Contents. As shown in **Table 2**, the liver weight of *db*/*db* control mice was increased 3.5 times compared with that of *m*/*m* mice. However, the administration of proanthocyanidins led to a slight decrease in its weight, although no significant difference among *db*/*db* groups was observed. Concerning the hepatic TG level, the *db*/*db* control group showed a marked increase compared with the *m*/*m* group, from 23.27 to 78.24 mg/(liver·10 g B.W.), showing an increase of 3.3 times. In addition, the hepatic TC level was elevated 1.7 times. These hepatic concentrations of TG and TC were decreased significantly by proanthocyanidin administration. In particular, the decrease in these levels was more significant in the group administered oligomers than polymers.

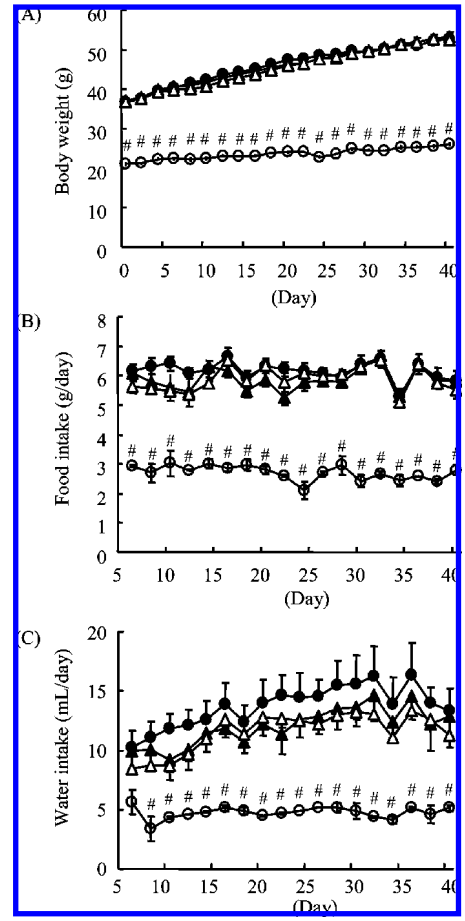

Figure 1. Metabolic analyses in *db*/*db* mice. (**A**) During the intervention period, body weight was measured once every two days: (○) *m*/*m* mice; (●) *db*/*db* mice treated with vehicle; (△) *db*/*db* mice treated with oligomers; (▲) *db*/*db* mice treated with polymers; (#) significance, $p < 0.05$ vs *db*/*db* mice treated with vehicle. (**B**) During the intervention period, food intake was measured once every two days: (○) *m*/*m* mice; (●) *db*/*db* mice treated with vehicle; (△) *db*/*db* mice treated with oligomers; (▲) *db*/*db* mice treated with polymers; (#) significance, $p < 0.05$ vs *db*/*db* mice treated with vehicle. (**C**) During the intervention period, water intake was measured once every two days: (○) *m*/*m* mice; (●) *db*/*db* mice treated with vehicle; (△) *db*/*db* mice treated with oligomers; (▲) *db*/*db* mice treated with polymers; (#) significance, $p < 0.05$ vs *db*/*db* mice treated with vehicle.

Biomarkers Associated with Antioxidative Effect in Hepatic Tissues. Table 3 shows the effect of polymers and oligomers against oxidative stress. TBARS levels were increased from 0.63 nmol/mg protein to 1.18 nmol/mg protein (1.9 times) in the *db*/*db* control group compared with the *m*/*m* group. However, the groups administered polymers and oligomers showed a decrease of the TBARS level from 1.18 to 1.11 and 0.71 nmol/mg protein, respectively. In addition, an elevation in ROS generation was also observed in the *db*/*db* control group compared with the *m*/*m* group, while it was decreased by

Table 1. Hematological Analyses of *db*/*db* Mice Treated with Polymers and Oligomers[a]

group	dose (mg/(kg B.W. · day))	glucose (mg/dL)	glycosylated protein (nmol/mg protein)	TG (mg/dL)	TC (mg/dL)	NEFA (mequiv/L)
+/+		127.82 ± 13.38[b,c]	57.28 ± 7.49[d]	68.41 ± 5.83[b,f]	68.80 ± 2.15[b,i]	0.72 ± 0.07[b,l]
db/*db*						
vehicle		663.43 ± 58.42	112.13 ± 12.59	152.20 ± 20.21	167.54 ± 5.93	1.47 ± 0.07
polymers	10	607.76 ± 69.71	98.63 ± 18.27	76.29 ± 9.46[b,g]	150.34 ± 3.87[j]	1.06 ± 0.07[b,m]
oligomers	10	572.72 ± 70.46	75.47 ± 8.93[e]	75.24 ± 3.30[b,h]	149.79 ± 4.92[k]	1.06 ± 0.07[b,n]

[a] Values are the mean ± S.E. [b] Significance: $p < 0.05$ vs *db*/*db* mice treated with vehicle. [c] Significance: $p = 0.00$ vs *db*/*db* mice treated with vehicle. [d] Significance: $p = 0.0044$ vs *db*/*db* mice treated with vehicle. [e] Significance: $p = 0.0245$ vs *db*/*db* mice treated with vehicle. [f] Significance: $p = 0.00$ vs *db*/*db* mice treated with vehicle. [g] Significance: $p = 0.0066$ vs *db*/*db* mice treated with vehicle. [h] Significance: $p = 0.00$ vs *db*/*db* mice treated with vehicle. [i] Significance: $p = 0.00$ vs *db*/*db* mice treated with vehicle. [j] Significance: $p = 0.028$ vs *db*/*db* mice treated with vehicle. [k] Significance: $p = 0.0331$ vs *db*/*db* mice treated with vehicle. [l] Significance: $p \leq 0.0001$ vs *db*/*db* mice treated with vehicle. [m] Significance: $p = 0.0005$ vs *db*/*db* mice treated with vehicle. [n] Significance: $p = 0.0004$ vs *db*/*db* mice treated with vehicle.

Table 2. Changes in the Weight, TG, and TC Contents in Livers of *db*/*db* Mice Treated with Polymers and Oligomers[a]

group	dose (mg/(kg B.W. · day))	weight (g)	TG (mg/(liver · 10 g B.W.))	TC (mg/(liver · 10 g B.W.))
+/+		1.21 ± 0.02[b]	23.47 ± 4.55[b,c]	32.23 ± 1.71[b,d]
db/*db*				
vehicle		4.25 ± 0.15	78.24 ± 9.22	53.97 ± 2.53
polymers	10	4.02 ± 0.10	59.12 ± 3.36	51.70 ± 1.69
oligomers	10	3.98 ± 0.20	56.94 ± 2.10[b]	36.79 ± 3.56[b,e]

[a] Values are the mean ± S.E. [b] Significance: $p < 0.05$ vs *db*/*db* mice treated with vehicle. [c] Significance: $p = 0.0015$ vs *db*/*db* mice treated with vehicle. [d] Significance: $p \leq 0.0001$ vs *db*/*db* mice treated with vehicle. [e] Significance: $p = 0.0006$ vs *db*/*db* mice treated with vehicle.

Table 3. Effects of Polymers and Oligomers on Biomarkers Associated with Oxidative Stress[a]

group	dose (mg/(kg B.W. · day))	TBARS (nmol/mg protein)	ROS generation (%)	GSH (μmol/mg protein)	GSSG (μmol/mg protein)	GSH/GSSG
+/+		0.63 ± 0.07[c]	100.3 ± 9.3[b,e]	6.63 ± 0.36[b,f]	4.65 ± 0.52	1.49 ± 0.12[b,h]
db/*db*						
vehicle		1.18 ± 0.20	248.2 ± 30.3	4.91 ± 0.56	5.67 ± 0.57	0.86 ± 0.03
polymers	10	1.11 ± 0.12	206.5 ± 15.7	4.63 ± 0.22	5.45 ± 0.24	0.85 ± 0.02
oligomers	10	0.71 ± 0.07[d]	185.1 ± 12.3	4.07 ± 0.36	3.76 ± 0.31[g]	0.98 ± 0.05[i]

[a] Values are the mean ± S.E. [b] Significance: $p < 0.05$ vs *db*/*db* mice treated with vehicle. [c] Significance: $p = 0.0236$ vs *db*/*db* mice treated with vehicle. [d] Significance: $p = 0.0348$ vs *db*/*db* mice treated with vehicle. [e] Significance: $p = 0.0003$ vs *db*/*db* mice treated with vehicle. [f] Significance: $p = 0.0241$ vs *db*/*db* mice treated with vehicle. [g] Significance: $p = 0.0283$ vs *db*/*db* mice treated with vehicle. [h] Significance: $p = 0.000583$ vs *db*/*db* mice treated with vehicle. [i] Significance: $p = 0.0044$ vs *db*/*db* mice treated with vehicle.

proanthocyanidin administration. The changes in TBARS and ROS generation were more pronounced by the administration of oligomers rather than polymers. Moreover, the *db*/*db* control group showed declining GSH/GSSG ratios through the decrease in the GSH level and increase in the GSSG level compared with the *m*/*m* group. The oligomer-treated group showed elevated GSH/GSSG ratios due to the significant decrease in GSSG levels and slight increase in GSH levels, whereas the polymer-treated group did not show a significant effect.

Alterations of Nucleic SREBP-1, SREBP-2, and PPARα Protein Levels in Hepatic Tissues. As revealed in **Figure 2**, protein expressions of hepatic SREBP-1 and SREBP-2 were increased markedly in the *db*/*db* control group compared with the *m*/*m* group. However, the administration of oligomers led to a significant downregulation of these protein expressions (**Figure 2A**, **B**). On the other hand, polymers did not show a significant effect. In addition, the *db*/*db* control group exhibited a marked decrease in hepatic PPARα expressions. Although no significant difference among *db*/*db* groups was observed, administrations of both polymers and oligomers slightly increased PPARα expressions (**Figure 2C**).

Modulations of NF-κB, IκB, COX-2, and iNOS Protein Levels in Hepatic Tissues. NF-κB p65 expression in the *db*/*db* control group was upregulated, while it was suppressed significantly by the administration of oligomers (**Figure 3A**). IκB-α expression was downregulated in the *db*/*db* control group, but its expression intensity was increased by oligomer administration. On the other hand, the polymer-treated group showed no significant change in NF-κB and IκB expressions (**Figure 3A**, **B**). In addition, upregulations of COX-2 and iNOS expressions were observed compared with the *m*/*m* group by 1.6 and 1.9 times, respectively. However, proanthocyanidin administration, especially oligomers rather than polymers, suppressed protein expressions of COX-2 and iNOS (**Figure 3C**, **D**).

DISCUSSION

Type 2 diabetes mellitus leads to the abnormal metabolism of glucose, FFA, and other reactive metabolites caused by insulin-deficient and -resistant states (*22*). Especially in the liver, insulin resistance elevates the hepatic output of TG-rich particles. When the FFA supply exceeds utilization, nonadipose tissues start accumulating TG, which is aggravated by the simultaneous presence of hyperglycemia. Subsequently, the formation of reactive, long-chain fatty acyl-CoAs and toxic metabolites such as ceramide, the activation of protein kinase C-δ, and increase of oxidative stress may all contribute to apoptosis and the decline of β-cells (*3, 23*). Thus, the regulation of hyperlipidemia would play an important role in the etiology of diabetes and the complication of hyperglycemia. In this study, we investigated whether proanthocyanidin, especially its oligomeric form,

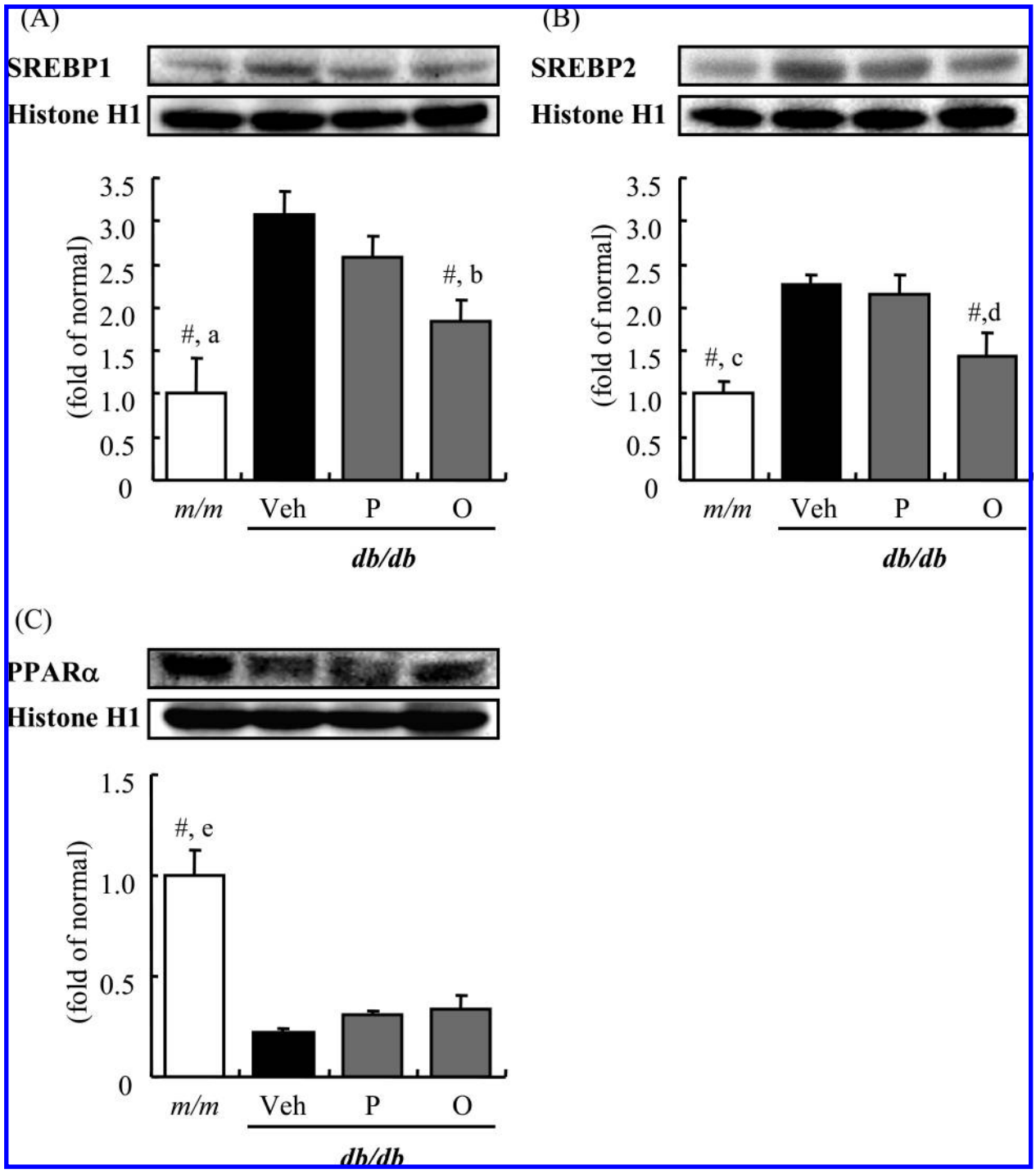

Figure 2. Analyses of SREBP-1, SREBP-2, and PPARα expressions in liver. (**A**) Western blot analysis of SREBP1. Histone H1 was utilized as an internal control. Significance: $^{\#}p < 0.05$ vs *db*/*db* mice treated with vehicle; $^{a}p = 0.00622$ vs *db*/*db* mice treated with vehicle; $^{b}p = 0.0079$ vs *db*/*db* mice treated with vehicle. (**B**) Western blot analysis of SREBP2. Histone H1 was utilized as an internal control. Significance: $^{\#}p < 0.05$ vs *db*/*db* mice treated with vehicle; $^{c}p \leq 0.0001$ vs *db*/*db* mice treated with vehicle; $^{d}p = 0.0333$ vs *db*/*db* mice treated with vehicle. (**C**) Western blot analysis of PPARα. Histone H1 was utilized as an internal control. Significance: $^{\#}p < 0.05$ vs *db*/*db* mice treated with vehicle; $^{e}p = 0.0121$ vs *db*/*db* mice treated with vehicle.

ameliorates metabolic disorders including hyperlipidemia as well as hyperglycemia to reduce the risk of type 2 diabetes, using well-established *db*/*db* type 2 diabetic mice. *db*/*db* mice develop metabolic disorders caused by severe insulin resistance; therefore, they are considered as a model of type 2 diabetes. Consistent with other reports (*11*), *db*/*db* mice showed a marked increase in body weight due to increases in food and water intake. The genotypes of *db*/*db* mice lead to a lack of signaling by leptin that regulates food intake and systemic fuel metabolism (*24*). Proanthocyanidin administration had no effect on food and water intakes; consequently, body weights among *db*/*db* groups showed no significant change.

Diabetes represents a progressive and cumulative load caused by cellular glucose and lipid metabolites. Therefore, the regulation of circulating metabolites including glucose, FFA, and so on can be considered as a part of metabolic modulation

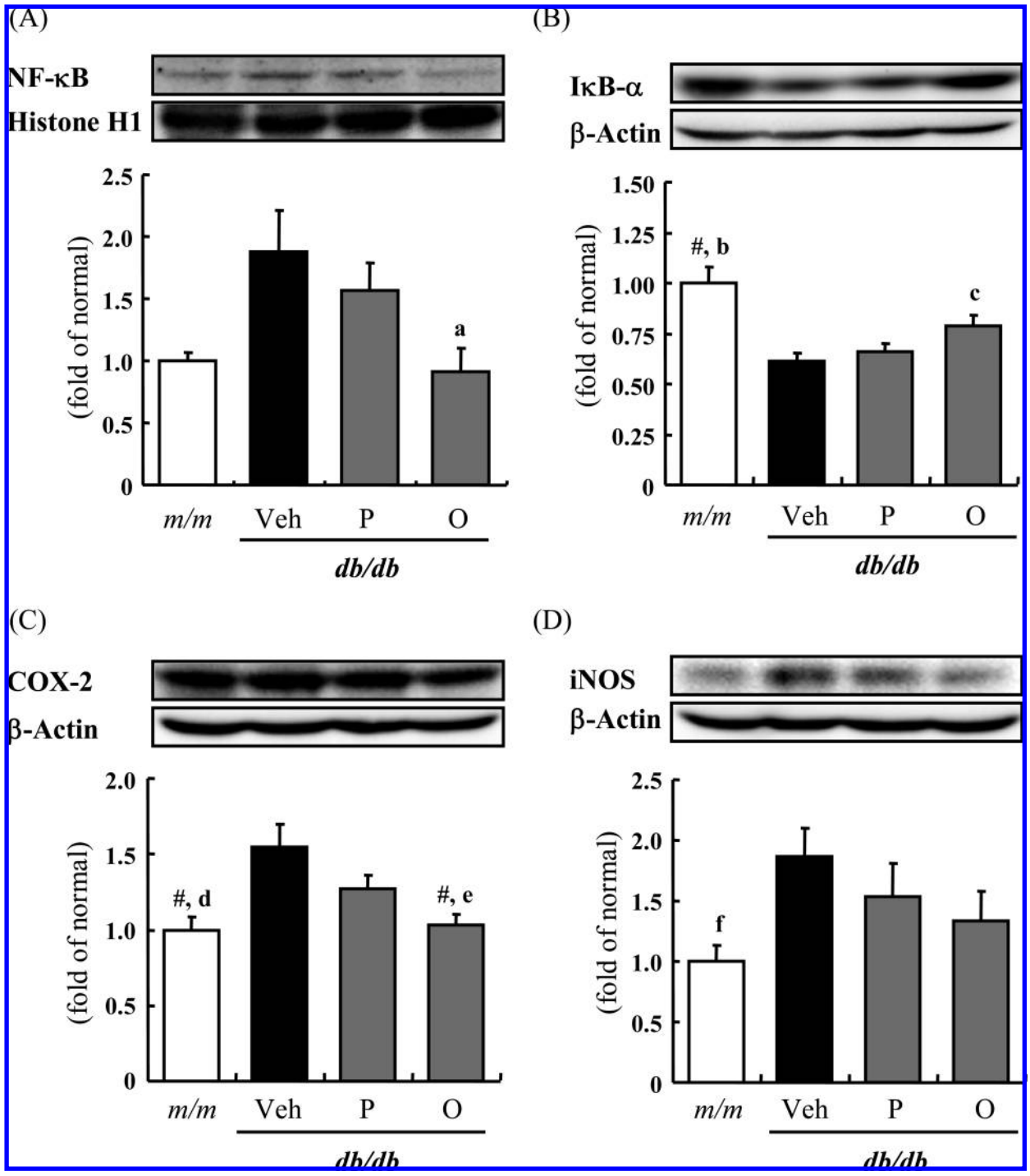

Figure 3. Change in NF-κB, IκB-α, COX-2, and iNOS expressions in liver. (**A**) Western blot analysis of NF-κB. Histone H1 was utilized as an internal control. Significance: $^{a}p = 0.0349$ vs *db*/*db* mice treated with vehicle. (**B**) Western blot analysis of IκB-α. β-Actin was utilized as an internal control. Significance: $^{\#}p < 0.05$ vs *db*/*db* mice treated with vehicle; $^{b}p = 0.0003$ vs *db*/*db* mice treated with vehicle; $^{c}p = 0.0213$ vs *db*/*db* mice treated with vehicle. (**C**) Western blot analysis of COX-2. β-Actin was utilized as an internal control. Significance: $^{\#}p < 0.05$ vs *db*/*db* mice treated with vehicle; $^{d}p = 0.0162$ vs *db*/*db* mice treated with vehicle; $^{e}p = 0.0048$ vs *db*/*db* mice treated with vehicle. (**D**) Western blot analysis of iNOS. β-Actin was utilized as an internal control. Significance: $^{f}p = 0.0325$ vs *db*/*db* mice treated with vehicle.

in various tissues, especially the liver. However, the pathological changes in the kidney of *db*/*db* mice have not been observed until 12 weeks-old (*25*). In this study, we investigated glucose and glycosylated protein as hematologic factors of hyperglycemia and TG, TC, and NEFA as indicators of hyperlipidemia. We confirmed that *db*/*db* mice showed hyperglycemia as well as hyperlipidemia. However, the administration of proanthocyanidin reduced hyperglycemia through a decline in the serum level of glucose and glycosylated protein. In addition, it had a strong effect on hyperlipidemia through lowering TG, TC, and NEFA. The protective effect against hyperglycemia and hyperlipidemia was greater in the group administered the oligomeric rather than polymeric form. An increase in the level of polymerization leads to the elevation of lipase activity leading to dietary fat digestion and absorption (*26, 27*). Therefore, polymeric proanthocyanidin with high-level polymerization may suppress fat absorption in the gastrointestinal tract; consequently, the inflow of lipidemic metabolites into the blood may be

inhibited. Even if lipase activity is decreased by oligomerization, oligomeric proanthocyanidin improves not only hyperlipidemia but also hepatic lipid accumulation in *db*/*db* mice through mechanisms distinct from those of the polymeric form.

Hyperglycemia and elevated FFA levels result in the generation of ROS and consequently increase oxidative stress. ROS not only directly damage cells by oxidizing DNA, proteins, and lipids, but they also indirectly damage them by activating a variety of stress-sensitive intracellular signaling pathways such as NF-κB, p38 mitogen-activated protein kinase (MAPK), NH_2-terminal Jun kinase/stress-activated protein kinase, hexosamines, protein kinase C, advanced glycation end-products (AGE)/receptor for AGE (RAGE), and others. Activation of these pathways results in the increased expression of numerous gene products that cause cellular damage and play a major role in the etiology of the later-stage complications of diabetes (*28*). Thus, the upregulation of endogenous antioxidative systems and suppression of oxidative stress are important factors to ameliorate diabetes and its complications. In this study, we investigated ROS generation and lipid peroxidation, as biomarkers associated with oxidative stress, and also measured GSH and GSSG as indicators of an endogenous antioxidative system. Our results showed that the levels of ROS generation including H_2O_2, O_2^-, $\cdot$OH, ROO$\cdot$, and so on and that of lipid peroxidation were increased in *db*/*db* mice. ROS are mainly generated from mitochondrial electron transport, and the liver is the most important tissue rich in mitochondria. Therefore, in the present study, ROS were mainly generated in the mitochondria of the liver as a result of oxidative stress in *db*/*db* mice. ROS generation induces the oxidation of membrane lipids as one of the primary events in oxidative cellular damage. Lipid peroxidation also leads to oxidant production from many molecules and thus amplifies oxidative damage (*29*). Therefore, our results suggest that *db*/*db* mice may show increased oxidative damage due to an elevation of ROS generation induced by hyperglycemia and hyperlipidemia. During ROS overproduction, intracellular antioxidant GSH is oxidized to GSSG, which is then reconverted to GSH by GSH reductase. The GSH/GSSG ratio defines the so-called GSH redox state, which plays an important role in cellular activation, gene expression, mRNA stability, protein folding, metabolic regulation, and cell protection against oxidative damage (*30*). Decreased GSH and increased GSSG, and consequently the downregulation of the GSH/GSSG ratio in *db*/*db* mice, were implicated in the disruption of the intracellular antioxidative system. In our study, the administration of oligomeric proanthocyanidin attenuated the increase in ROS generation and lipid peroxidation and elevated the GSH/GSSG ratio, whereas polymeric proanthocyanidin did not show any effect. However, it did not show a significant effect against ROS generation. Another report also demonstrated that the compounds that improve cellular function can also reduce oxidative stress, even though they do not act as direct free radical scavengers (*31*). This is related to the recent evidence that oxidative stress is primarily a biomarker for inflammation and other cellular dysfunctions rather than the cause of them. The antioxidative effect of oligomeric proanthocyanidin is probably associated with the inhibition of glycosylated protein levels, because it is generated as glucose reacts with an amino group to form a labile Schiff base that is highly prone to oxidation and free radical generation (*32*). From our data, oligomeric proanthocyanidin exhibited more effective antioxidative and antihyperlipidemic activities than polymeric proanthocyanidins. This suggests that the oligomerization of proanthocyanidin plays an important role in type 2 diabetes, although further studies on pathological changes in the liver are needed. The beneficial effect of proanthocyanidin in a biological system depends on the intake of foods rich in proanthocyanidin and also on the absorption related to the degree of polymerization. On the basis of the results, the antioxidative effect of proanthocyanidin from persimmon can be expected by an intake of about 50 g/day in the case of adults with a body weight of 60 kg.

Lipid homeostasis is regulated by a family of membrane-bound transcription factors called SREBPs. SREBP-1 is a key transcription factor that nutritionally regulates the hepatic gene expression of lipogenic enzymes and TG deposition in the liver (*33*). On the other hand, SREBP-2 regulates genes involved in cholesterol synthesis through the cleavage of its precursor form to an active nuclear form via interaction with SREBP cleavage activating protein and protease in a sterol-dependent manner (*34*). Upregulations of SREBP-1 and SREBP-2 were reported in leptin-resistant mice such as *ob*/*ob* mice and $FAB^{db/db}$ mice (*35, 36*). In this study, the increase in hepatic SREBP-1 and SREBP-2 in *db*/*db* mice was downregulated by the administration of proanthocyanidin, especially in the oligomeric form. This was probably related to the inhibition of hepatic TG and TC accumulations. Furthermore, PPARs, with three isoforms (α, δ, and γ), are also involved in the long term regulation of lipid metabolism, and their activity is modulated by endogenous lipid-derived ligands. When PPARα is activated, it promotes fatty acid oxidation, ketone body synthesis, and glucose sparing (*37*). In the current study, hepatic PPARα was decreased in *db*/*db* mice; however, it was increased slightly by proanthocyanidin administration. In our study, we clarified that proanthocyanidin, especially in its oligomeric form, exhibits an effect on regulations of both PPARα and SREBP.

Under type 2 diabetes, the stress-sensitive intracellular signaling pathway is altered. In particular, one major intracellular target of hyperglycemia and oxidative stress is the transcription factor NF-κB. NF-κB can be activated by a wide array of exogenous and endogenous stimuli including hyperglycemia, elevated FFA, ROS, tumor necrosis factor-α, interleukin-1β, other proinflammatory cytokines, AGE-binding RAGE, and p38 MAPK. The aberrant regulation of NF-κB is associated with a number of chronic diseases including diabetes and atherosclerosis (*38*). NF-κB is present in the cytoplasm as an inactive heterodimer, consisting of p50 and p65 subunits complexed with an inhibitor protein subunit, IκB. After stimulation, IκB was phosphorylated by the activation of a serine kinase cascade. This event primes IκB as a substrate for ubiquitination and subsequent degradation, facilitating NF-κB heterodimer translocation to the nucleus. NF-κB regulates the expression of a large number of genes, including growth factors, proinflammatory cytokines, and others (*38, 39*). NF-κB is involved in regulations of COX-2 and iNOS expressions that mediate the inflammatory process (*40*). In *db*/*db* mice, hepatic NF-κB was upregulated with the downregulation of IκB compared with *m*/*m* mice, while the administration of proanthocyanidin, especially in its oligomeric form, led to the downregulation of NF-κB with the upregulation of IκB. NF-κB activation induces insulin resistance by lipid/fatty acid infusion and the inhibition of insulin signaling by lipid metabolites such as diacylglycerol and ceramide (*41*). Accordingly, our results suggest that the regulation of NF-κB and IκB by oligomers may be associated with the amelioration of hyperlipidemia and hyperglycemia. Moreover, COX-2 and iNOS mediate inflammation-mediated/induced insulin resistance. The expressions were increased in *db*/*db* compared with *m*/*m* mice. COX-2 and iNOS expressions were upregulated under insulin resistance, obesity, hyperglycemia, and oxidative stress

(*42*). Their downregulations by oligomers shown by the present results indicated the regulation of hyperlipidemia with the inhibition of TG and TC deposition. The present results suggest the crucial role of oligomers in stress-activated signaling pathways such as NF-κB, COX-2, and iNOS.

In summary, the present results show that proanthocyanidins ameliorated hyperlipidemia as well as hyperglycemia. In particular, the oligomeric form of proanthocyanidins ameliorated oxidative damage and lipid deposition in the liver more effectively than the polymeric form. Oligomerization may be associated with ameliorations of oxidative stress and abnormal lipid metabolism in type 2 diabetes. The oligomeric form of proanthocyanidins reversed hyperlipidemia in parallel with regulations of hepatic SREBP-1 and SREBP-2 expressions. Furthermore, oligomeric proanthocyanidin ameliorated the up-regulation of NF-κB via the activation of IκB. In addition, oligomeric proanthocyanidin also attenuated COX-2 and iNOS expressions. This suggests that oligomers would act as regulators in inflammatory reactions caused by oxidative stress under type 2 diabetes. Oxidative stress in type 2 diabetes was also attenuated by proanthocyanidin through the reduction of ROS generation and lipid peroxidation, and elevation of the GSH/GSSG ratio. The present study suggests that proanthocyanidins, especially oligomers, would improve oxidative stress under type 2 diabetes via the regulation of hyperlipidemia rather than hyperglycemia.

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