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Antiglycative Effects of Protocatechuic Acid in the Kidneys of Diabetic Mice

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ABSTRACT: Protocatechuic acid (PCA) at 2 or 4% was supplied to diabetic mice for 12 weeks. PCA treatments increased its deposit in organs and significantly reduced the plasma HbA1c level, the urinary glycative albumin level, and renal production of carboxymethyllysine (CML), pentosidine, sorbitol, and fructose ($p < 0.05$). However, PCA treatments only at 4% significantly decreased brain content of CML, pentosidine, fructose, and sorbitol ($p < 0.05$). PCA treatments at 2 and 4% significantly lowered renal activity and mRNA expression of aldose reductase and sorbitol dehydrogenase ($p < 0.05$), and PCA treatments only at 4% significantly enhanced renal glyoxalase I mRNA expression ($p < 0.05$). PCA treatments also dose-dependently decreased the renal level of type-IV collagen, fibronectin, and transforming growth factor-β1 ($p < 0.05$), as well as dose-dependently diminished renal protein kinase C (PKC) activity ($p < 0.05$); however, PCA treatments only at 4% suppressed renal mRNA expression of PKC-α and PKC-beta ($p < 0.05$). PCA treatments at 4% significantly restored renal mRNA expression of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ, as well as suppressed expression of the advanced glycation end-product receptor ($p < 0.05$). These findings suggest that the supplement of PCA might be helpful for the prevention or alleviation of glycation-associated diabetic complications.

KEYWORDS: Protocatechuic acid, diabetes, glycation, aldose reductase, protein kinase C

INTRODUCTION

Advanced glycation end-products (AGEs), such as glycated hemoglobin, carboxymethyllysine (CML), pentosidine, and glycated albumin, have been implicated in the pathogenesis of diabetic nephropathy and other complications.[1,2] Hyperglycemia that occurred in diabetic conditions favors glucose metabolism through the polyol pathway and promotes AGE formation.[3] Aldose reductase (AR), the first and rate-limiting enzyme in this pathway, reduces glucose to sorbitol, which is further metabolized to fructose by sorbitol dehydrogenase (SDH), the other key enzyme in this pathway.[4,5] Consequently, the massive fructose facilitates AGE production and causes vascular abnormalities.[5] Glyoxalase I (GLI), part of the glyoxalase system presented in the cytosol of cells, could metabolize physiologically reactive α-carbonyl compounds, such as glyoxal and methylglyoxal, and, subsequently, decrease the available precursors for AGE generation.[6] AR, SDH, and GLI are involved in diabetes-associated glycation reactions; thus, any agent with the ability to affect the activity and/or mRNA expression of these enzymes might potentially mediate AGE production.

It is documented that glycated albumin enhances protein kinase C (PKC) activity and stimulates transforming growth factor (TGF)-β1 expression in kidneys, which in turn leads to massive production of the extracellular matrix (ECM), such as fibronectin and type-IV collagen, and causes glomerular trophy and/or renal fibrosis.[7,8] Peroxisome proliferator-activated receptors (PPARs) play important roles in the transcriptional control of glucose homeostasis, inflammation, and ECM remodeling, and PPAR-γ activation by its agonists could attenuate AGE-induced renal ECM accumulation and fibrosis.[9,10] Thus, any agent with a capability to lower the renal level of glycated albumin and TGF-β1 and/or promote PPAR-γ activation may be more efficacious in attenuating diabetes-associated renal injury.

Protocatechuic acid (PCA; 3,4-dihydroxybenzoic acid) is a phenolic compound found in many plant foods, such as olives, *Hibiscus sabdariffa* (roselle), *Eucommia ulmoides* (du-zhong), calamondin (*Citrus microcarpa* Bonge), and white grape wine.[11−13] Kwon et al.[14] observed that PCA provided *in vitro* inhibitory activity for α-glucosidase and suggested that this compound might be able to manage diabetes and hypertension. The study by Harini and Pugalendi[15] indicated that PCA could alleviate hyperlipidemia in diabetic rats. Our previous animal study reported that this compound could provide antioxidative, anti-inflammatory, and anticoagulatory protection against diabetic deterioration.[13] Although these previous studies support that PCA is a potent antidiabetic agent, it remains known that this compound possesses antiglycative and renal protective activities.

The major purpose of this study was to examine the antiglycative effects and possible actions of PCA in the kidneys of diabetic mice. The impact of this compound on AGE production and renal activity and/or mRNA expression of AR, SDH, GLI, PKC, and PPARs was determined. Furthermore, the content of PCA in organs of mice that consumed PCA was measured.

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Table 1. Forward and Reverse Primers for Real-Time PCR Analysis

target	forward	reverse
AR	5′-CCC AGG TGT ACC AGA ATG AGA-3′	5′-TGG CTG CAA TTG CTT TGA TCC-3′
SDH	5′-TGG GAG CTG CTC AAG TTG TG-3′	5′-GGT CTC TTT GCC AAC CTG GAT-3′
GLI	5′-CGT GAG ACA GCA AGC AGC TAG A-3′	5′-ACC ATG AGG CAT AGG CAT ACC C-3′
RAGE	5′-CCA TCC TAC CTT CTC CTG-3′	5′-AGC GAC TAT TCC ACC TTC-3′
PPAR-α	5′-CTG CAG AGC AAC CAT CCA GAT-3′	5′-GCC GAA GGT CCA CCA TTT T-3
PPAR-γ	5′-TCC GTG ATG GAA GAC CAC TC-3′	5′-CCC TTG CAT CCT TCA CAA GC-3′
PKC-α	5′-GAA CCA TGG CTG ACG TTT AC-3′	5′-GCA AGA TTG GGT GCA CAA AC-3′
PKC-β	5′-TTC AAG CAG CCC ACC TTC TG-3′	5′-AAG GTG GCT GAA TCT CCT TG-3′
PKC-γ	5′-GAC CCC TGT TTT GCA GAA AG-3′	5′-GTA AAG CCC TGG AAA TCA GC-3′
GAPDH	5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′	5′-CCT TGG AGG CCA TGT AGG CCA T-3′

MATERIALS AND METHODS

Materials. PCA (99.5%) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used in these measurements were of the highest purity commercially available.

Animals and Diet. Male Balb/cA mice, 3−4 weeks old, were obtained from the National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). Mice were housed on a 12 h light/dark schedule; water and mouse standard diet were consumed *ad libitum*. The use of mice was reviewed and approved by the China Medical University Animal Care Committee (CMU-97-22-N). To induce diabetes, mice with a body weight of 23.0 ± 1.3 g were treated with a single intravenous (iv) dose (50 mg/kg) of streptozotocin dissolved in citrate buffer (pH 4.5) into the tail vein. The blood glucose level was monitored on days 5 and 10 from the tail vein using a one-touch blood glucose meter (Lifescan, Inc., Milpitas, CA). Mice with fasting blood glucose levels ≥ 14.0 mmol/L were used for this study. PCA at 2 or 4 g was mixed with 98 or 96 g of powder diet (PMI Nutrition International, LLC, Brentwood, MO) to prepare a 2 or 4% PCA diet and supplied to mice for 12 weeks. All mice had free access to food and water at all times.

Experimental Design. Non-diabetic mice that consumed PCA diets were used for determining tissue PCA content only. Diabetic mice that consumed PCA diets were used for other measurements and compared to groups of non-diabetic and diabetic mice that consumed a normal diet. The consumed water volume, feed intake, and body weight were recorded weekly. A total of 24 h of urine output collected by a metabolic cage was measured at week 12. After 12 weeks of supplementation, mice were fasted overnight and sacrificed with carbon dioxide. Blood was collected, and plasma was separated from erythrocytes immediately. The brain, heart, liver, and kidneys were collected, and 100 mg of each organ was homogenized on ice in 2 mL of phosphate-buffered saline (PBS; pH 7.2). The filtrate was collected. The protein concentration of plasma or organ filtrate was determined by the method by Lowry et al.[16] using bovine serum albumin as a standard. In all experiments, the sample was diluted to a final concentration of 1 g of protein/L using PBS at pH 7.2.

Tissue Content of PCA. The content of PCA in the plasma, brain, heart, liver, and kidneys was measured according to a gas chromatography−mass spectrometry (GC−MS) method described by Caccetta et al.[17] Briefly, the lyophilized sample was mixed with 1-hydroxy-2-naphthoic acid, as an internal standard, and followed by acidification with 6 N HCl and extraction with ethyl acetate and 5% $NaHCO_3$. The ethyl acetate extract was dried and derivatized with an equal amount of *bis*(trimethylsilyl)-trifluoroacetamide and dry pyridine. The PCA content was quantified by a HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a HP 5970 series mass-selective detector and a HP-1 cross-linked methyl silicone column (12 m × 0.20 mm, 0.33 μm film thickness). The electron-impact mode was used for the mass spectrometer, and the mass/charge ratio of PCA was 370. The limits of detection and quantification were 0.2 and 0.8 ng/mL, respectively.

Blood and Urinary Analyses. The plasma glucose level was measured by a glucose HK kit (Sigma Chemical Co., St. Louis, MO). The plasma insulin level was measured using a rat insulin radioimmunoassay kit (Linco Research, Inc., St. Charles, MO). The plasma HbA1c level was measured using a DCA2000 analyzer (Bayer-Sankyo, Tokyo, Japan). Plasma blood urea nitrogen (BUN), plasma creatinine (Cr), and urinary Cr concentrations were detected by a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA). The creatinine clearance rate (CCr) was calculated according to the Cockcroft−Gault formula, [urinary creatinine (mg/dL) × urinary volume (mL)]/[plasma creatinine (mg/dL) × length of urine collection (min)], and expressed as milliliters per minute per 100 g of body weight. Urine albumin was measured by a competitive enzyme-linked immunosorbent assay (ELISA) assay according to the instructions of the manufacturer (Exocell, Philadelphia, PA). Glycated albumin was determined by affinity chromatography on phenylboronate agarose to separate nonglycated (unbound) from glycated (bound) albumin via eluting the bound fraction with 0.3 mol/L sorbitol.

Measurement of CML and Pentosidine. For CML determination, 50 mg of brain or kidney tissue was homogenized and digested with proteinase K (1 mg/mL) for 3 h at 37 °C, and the reaction was stopped by 2 mmol/L phenylmethylsulfonyl fluoride. CML was immunochemically determined with a competitive ELISA kit (Roche Diagnostics, Penzberg, Germany) using the CML-specific monoclonal antibody 4G9 and calibration with 6-(*N*-carboxymethylamino)caproic acid. Absorbance was read in a microtiter ELISA plate reader (Bio-Rad, Hercules, CA) at 405 nm (reference at 603 nm). Intra- and interassay variability of this assay was 5.3 and 6.2%, respectively. The pentosidine level was analyzed by a high-performance liquid chromatograph (HPLC) equipped with a C18 reverse-phase column and a fluorescence detector according to the method described by Miyata et al.[18] Briefly, the sample was lyophilized and acid-hydrolyzed in 500 μL of 6 N HCl for 16 h at 110 °C in screw-cap tubes purged with nitrogen. After neutralization with NaOH and diluted with PBS, the sample was used for HPLC measurement.

Determination of Sorbitol and Fructose Content. A total of 100 mg of brain or kidneys was homogenized with PBS (pH 7.4) containing U-[^{13}C]-sorbitol as an internal standard. After protein was precipitated by ethanol, the supernatant was lyophilized. The content of sorbitol and fructose in each lyophilized sample was determined by liquid chromatography with tandem mass spectrometry, according to the method by Guerrant and Moss.[19]

Activity of Renal AR, SDH, GLI, and PKC. The method by Nishinaka and Yabe-Nishimura[20] was used to measure AR activity in the kidneys by monitoring the decrease in absorbance at 340 nm because of NADPH oxidation. SDH activity was assayed according to the method by Ulrich[21] by mixing 100 μL of homogenate, 200 μL of NADH (12 mM), and 1.6 mL of triethanolamine buffer (0.2 M, pH 7.4) and

Table 2. Level (nmol/mL or nmol/g of Tissue) of PCA in the Plasma, Brain, Heart, Liver, and Kidneys from non-DM and DM That Consumed a Normal Diet or 2 or 4% PCA for 12 Weeks[a]

	non-DM	non-DM + 2% PCA	non-DM + 4% PCA	DM	DM + 2% PCA	DM + 4% PCA
plasma	*b*	0.12 ± 0.03 b	0.20 ± 0.08 c	*b*	0.06 ± 0.02 a	0.13 ± 0.05 b
brain	*b*	0.26 ± 0.10 b	0.41 ± 0.14 c	*b*	0.17 ± 0.07 a	0.31 ± 0.11 b
heart	*b*	0.29 ± 0.08 b	0.45 ± 0.12 c	*b*	0.15 ± 0.06 a	0.33 ± 0.09 b
liver	*b*	0.39 ± 0.11 b	0.67 ± 0.17 c	*b*	0.25 ± 0.10 a	0.42 ± 0.13 b
kidneys	*b*	0.40 ± 0.06 b	0.72 ± 0.14 c	*b*	0.26 ± 0.05 a	0.46 ± 0.10 b

[a] Data are the mean ± SD (n = 10). Means in a row without a common letter differ ($p < 0.05$). [b] Means too low to be detected.

Table 3. Water Intake (WI, mL Mouse^{-1} Day^{-1}), Feed Intake (FI, g Mouse^{-1} Day^{-1}), Body Weight (BW, g/Mouse), Organ Weight (g/Mouse), and Urine Output (mL Mouse^{-1} Day^{-1}) of non-DM and DM That Consumed a Normal Diet or 2 or 4% PCA at Weeks 2 and/or 12[a]

	non-DM	DM	DM + 2% PA	DM + 4% PA
WI				
week 2	1.1 ± 0.4 a	3.2 ± 1.0 b	3.5 ± 0.9 b	3.0 ± 1.1 b
week 12	2.0 ± 0.8 a	7.5 ± 1.6 c	5.8 ± 1.2 b	5.1 ± 1.0 b
FI				
week 2	0.9 ± 0.3 a	1.9 ± 0.5 b	2.1 ± 0.6 b	2.2 ± 0.7 b
week 12	2.3 ± 1.1 a	8.0 ± 1.7 c	6.9 ± 1.3 b	5.8 ± 1.0 b
BW				
week 2	22.9 ± 1.4 b	20.3 ± 1.5 a	20.1 ± 1.3 a	20.7 ± 0.9 a
week 12	32.6 ± 3.2 c	11.8 ± 2.1 a	14.8 ± 1.9 b	16.1 ± 1.5 b
brain weight, week 12	0.51 ± 0.09 a	0.47 ± 0.06 a	0.53 ± 0.10 a	0.49 ± 0.08 a
heart weight, week 12	0.25 ± 0.04 a	0.21 ± 0.03 a	0.29 ± 0.05 a	0.22 ± 0.04 a
liver weight, week 12	1.58 ± 0.10 a	1.66 ± 0.13 a	1.49 ± 0.09 a	1.60 ± 0.17 a
kidney weight, week 12	0.43 ± 0.11 a	0.52 ± 0.08 a	0.47 ± 0.06 a	0.54 ± 0.13 a
urine volume, week 12	0.61 ± 0.12 a	6.58 ± 1.08 c	5.04 ± 0.83 b	4.17 ± 0.55 b

[a] Data are the mean ± SD (n = 10). Means in a row without a common letter differ ($p < 0.05$).

monitoring the absorbance change at 365 nm. The method by McLellan and Thornalley[22] was used to assay GLI activity by monitoring the increase in the absorbance at 240 nm because of the formation of *S*-D-lactoylglutathione. The method described by Koya et al.[23] was used to measure glomeruli PKC activity. Briefly, the kidneys were homogenized in ice-cold RPMI1640 media containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Glomeruli were isolated by removing the capsules and passed through sieves of various sizes. After washing twice with RPMI1640 media and once with a mixed salt solution, glomeruli were incubated with a salt solution in the presence or absence of 100 μM PKC-specific substrate, RTLRRL, and followed by adding digitonin and ATP mixed with γ-[^{32}P]ATP (<1500 cpm/pmol). The reaction was stopped by 5% trichloroacetic acid, then spotted onto P81 phosphocellulose paper, and washed 4 times with phosphoric acid and once with acetone. The amount of incorporated radioactivity into the substrate was determined by scintillation counting. Glomerular PKC activity was normalized by the corresponding protein content.

Renal Level of TGF-β1, Fibronectin, and Type-IV Collagen. The renal cortex was homogenized with ice-cold PBS containing 0.05% Tween 20. After centrifugation at 9000*g* for 15 min at 4 °C, the supernatants were used for measuring the renal TGF-β1 level (ng/mg of protein), which was quantified by a commercial ELISA kit (R&D Systems, Minneapolis, MN). Fibronectin (mg/mg of protein) was assayed using rabbit anti-rat fibronectin antibody and quantified by solid-phase immunoenzymic ELISA.[24] The type-IV collagen concentration was measured by a Collagen IV M kit (Exocell, Inc., Philadelphia, PA), which measured both intact and fragments of type-IV collagen.

Table 4. Plasma Levels of Glucose (mmol/L), Insulin (nmol/L), BUN (mg/dL), and CCr [mL min^{-1} (100 g of Body Weight)$^{-1}$] of non-DM and DM That Consumed a Normal Diet or 2 or 4% PCA at Week 12[a]

	non-DM	DM	DM + 2% PCA	DM + 4% PCA
glucose	10.8 ± 1.0 a	27.6 ± 2.1 d	22.1 ± 1.3 c	17.4 ± 1.2 b
insulin	13.1 ± 1.4 d	4.5 ± 0.6 a	6.9 ± 0.9 b	9.5 ± 0.7 c
BUN	6.1 ± 0.8 a	58.3 ± 4.5 d	49.7 ± 3.9 c	32.6 ± 2.3 b
CCr	1.60 ± 0.10 d	0.41 ± 0.05 a	0.68 ± 0.08 b	1.03 ± 0.12 c

[a] Data are the mean ± SD (n = 10). Means in a row without a common letter differ ($p < 0.05$).

Real-Time Polymerase Chain Reaction (PCR) for mRNA Expression. Total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA). A total of 1 μg of RNA was used to generate cDNA, which was amplified using Taq DNA polymerase. PCR was carried out in 50 μL of reaction mixture containing Taq DNA polymerase buffer (20 mM Tris-HCl at pH 8.4, 50 mM KCl, 200 mM dNTP, 2.5 mM $MgCl_2$, and 0.5 mM of each primer) and 2.5 units of Taq DNA polymerase. The specific oligonucleotide primers of targets are shown in Table 1. The cDNA was amplified under the following reaction conditions: 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. A total of 28 cycles were performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene), and a total of 35 cycles were performed for others. Generated fluorescence from each cycle was quantitatively analyzed using the Taqman system based on a real-time sequence detection

Table 5. Level of Plasma HbA1c (%), Urinary Glycated Albumin (μg/mL), and Renal and Brain Levels of CML (pmol/mg of Protein), Pentosidine (pmol/mg of Protein), Sorbitol (nmol/mg of Protein), and Fructose (nmol/mg of Protein) in non-DM and DM That Consumed a Normal Diet or 2 or 4% PCA for 12 Weeks[a]

	non-DM	DM	DM + 2% PCA	DM + 4% PCA
plasma				
HbA1c	3.3 ± 0.6 a	12.1 ± 1.5 d	9.8 ± 1.0 c	7.1 ± 0.8 b
urinary				
glycated albumin	134 ± 24 a	1308 ± 215 d	1087 ± 164 c	608 ± 103 b
renal				
CML	16 ± 4 a	102 ± 13 d	83 ± 9 c	56 ± 10 b
pentosidine	0.45 ± 0.08 a	2.14 ± 0.31 d	1.70 ± 0.22 c	1.18 ± 0.14 b
sorbitol	7.2 ± 0.7 a	29.4 ± 2.0 d	24.5 ± 1.6 c	18.6 ± 1.8 b
fructose	19.1 ± 1.1 a	101.7 ± 8.4 d	76.1 ± 5.6 c	46.2 ± 2.7 b
brain				
CML	5 ± 2 a	53 ± 6 c	50 ± 4 c	39 ± 5 b
pentosidine	0.19 ± 0.06 a	1.66 ± 0.23 c	1.53 ± 0.14 c	1.21 ± 0.10 b
sorbitol	3.1 ± 0.5 a	8.7 ± 1.7 c	7.9 ± 1.0 c	5.9 ± 0.8 b
fructose	13.2 ± 1.3 a	73.1 ± 6.3 c	70.8 ± 4.4 c	54.7 ± 5.0 b

[a] Data are the mean ± SD (n = 10). Means in a row without a common letter differ ($p < 0.05$).

Table 6. Renal Activity of AR [nmol min^{-1} (mg of Protein)$^{-1}$], SDH (units/g of Protein), and GLI [nmol min^{-1} (mg of Protein)$^{-1}$] in non-DM and DM That Consumed a Normal Diet or 2 or 4% PCA for 12 Weeks[a]

	non-DM	DM	DM + 2% PCA	DM + 4% PCA
AR	1.04 ± 0.22 a	2.77 ± 0.34 d	2.41 ± 0.23 c	1.98 ± 0.19 b
SDH	4.0 ± 0.3 a	8.9 ± 1.0 c	7.6 ± 0.6 b	7.3 ± 0.4 b
GLI	285 ± 27 c	117 ± 15 a	131 ± 18 a	176 ± 20 b

[a] Data are the mean ± SD (n = 10). Means in a row without a common letter differ ($p < 0.05$).

system (ABI Prism 7700, Perkin-Elmer, Inc., Foster City, CA). In this study, the mRNA level was calculated as a percentage of the control group.

Statistical Analysis. The effect of each measurement was analyzed from 10 mice (n = 10). All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), and post-hoc comparisons were carried out using Dunnett's t test. Statistical significance is defined as $p < 0.05$.

RESULTS

The content of PCA in mice tissue is shown in Table 2. The intake of PCA increased PCA deposit in plasma and organs in both non-diabetic mice (non-DM) and diabetic mice (DM). However, DM had lower PCA content in plasma and organs than non-DM ($p < 0.05$). The feed intake, water intake, and body weight recorded at weeks 2 and 12 and organ weight and urine volume measured at week 12 are presented in Table 3. In comparison to the diabetic control group, mice with 2 and 4% PCA treatments had significantly lower water intake, lower feed intake, higher body weight, and less urine output at week 12 ($p < 0.05$). As shown in Table 4, in comparison to the diabetic control group, mice with 2 and 4% PCA treatments had significantly lower glucose and BUN levels and higher insulin and CCr levels ($p < 0.05$).

As shown in Table 5, PCA treatments at 2 and 4% significantly reduced plasma HbA1c and urinary glycated albumin levels

Table 7. Urinary Level of Albumin (μg/Day) and Renal Levels of Fibronectin (mg/mg of Protein), Type-IV Collagen (ng/mg of Protein), and TGF-β1 (ng/mg of Protein) in non-DM and DM That Consumed a Normal Diet or 2 or 4% PCA for 12 Weeks[a]

	non-DM	DM	DM + 2% PCA	DM + 4% PCA
urinary				
albumin	22 ± 4 a	176 ± 10 d	121 ± 8 c	71 ± 6 b
renal				
fibronectin	2.64 ± 0.25 a	6.17 ± 0.47 d	5.11 ± 0.32 c	3.95 ± 0.29 b
type-IV collagen	9 ± 3 a	84 ± 9 d	61 ± 6 c	37 ± 5 b
TGF-β1	13 ± 4 a	73 ± 7 d	52 ± 5 c	31 ± 3 b

[a] Data are the mean ± SD (n = 10). Means in a row without a common letter differ ($p < 0.05$).

($p < 0.05$). These treatments also decreased the renal levels of CML, pentosidine, sorbitol, and fructose ($p < 0.05$); however, PCA treatments only at 4% significantly decreased brain levels of CML, pentosidine, sorbitol, and fructose ($p < 0.05$). PCA treatments at 2 and 4% significantly lowered renal activity and mRNA expression of AR and SDH (Table 6 and Figure 1; $p < 0.05$). A dose-dependent effect was presented in AR activity and expression ($p < 0.05$). PCA treatments at 4% significantly elevated renal GLI activity and expression ($p < 0.05$). As shown in Table 7, PCA treatments dose-dependently decreased urinary albumin concentrations and renal levels of fibronectin, type-IV collagen, and TGF-β1 ($p < 0.05$).

As shown in Figure 2, the diabetic condition increased renal PKC activity and upregulated expression of three PKC isoforms. PCA treatments dose-dependently declined renal PKC activity ($p < 0.05$); however, PCA treatments only at 4% suppressed renal mRNA expression of PKC-α and PKC-β ($p < 0.05$). The diabetic condition also downregulated renal mRNA expression of PPAR-α and PPAR-γ, as well as upregulated RAGE expression (Figure 3; $p < 0.05$); PCA treatments only at 4% significantly restored mRNA expression of PPAR-α and PPAR-γ and repressed RAGE expression ($p < 0.05$).

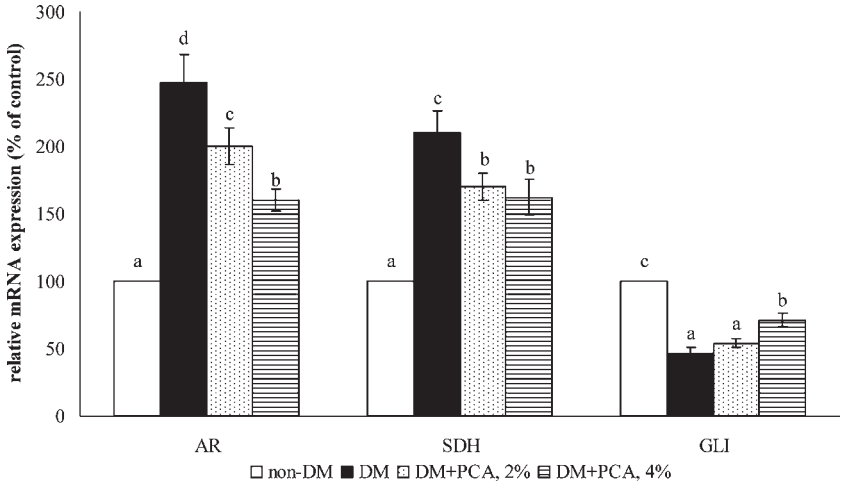

Figure 1. mRNA expression of renal AR, SDH, and GLI in non-DM and DM that consumed a normal diet or 2 or 4% PCA for 12 weeks. Data are the mean ± SD (n = 10). Means among bars without a common letter differ ($p < 0.05$).

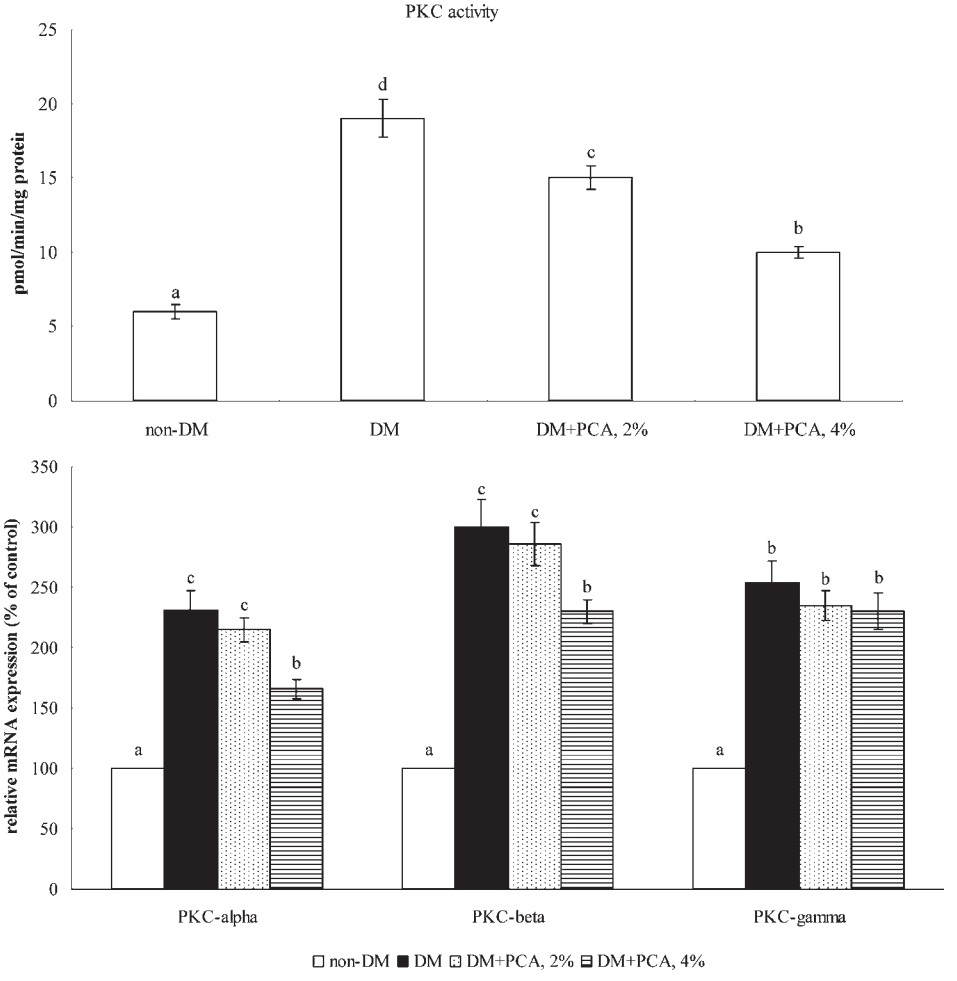

Figure 2. Renal activity [pmol min^{-1} (mg of protein)$^{-1}$] and mRNA expression of PKC-α, PKC-β, and PKC-γ in non-DM and DM that consumed a normal diet or 2 or 4% PCA for 12 weeks. Data are the mean ± SD (n = 10). Means among bars without a common letter differ ($p < 0.05$).

DISCUSSION

Our previous animal study reported that the dietary supplement of PCA at 2 and 4% improved glycemic control, decreased oxidative and inflammatory stress in the heart and kidneys, and attenuated hemostatic disorder in diabetic mice.[13] Our present study further found that the intake of this compound effectively increased its deposit in organs, reduced AGE production in the plasma, kidneys, and brain, as well as regulated enzymes involved

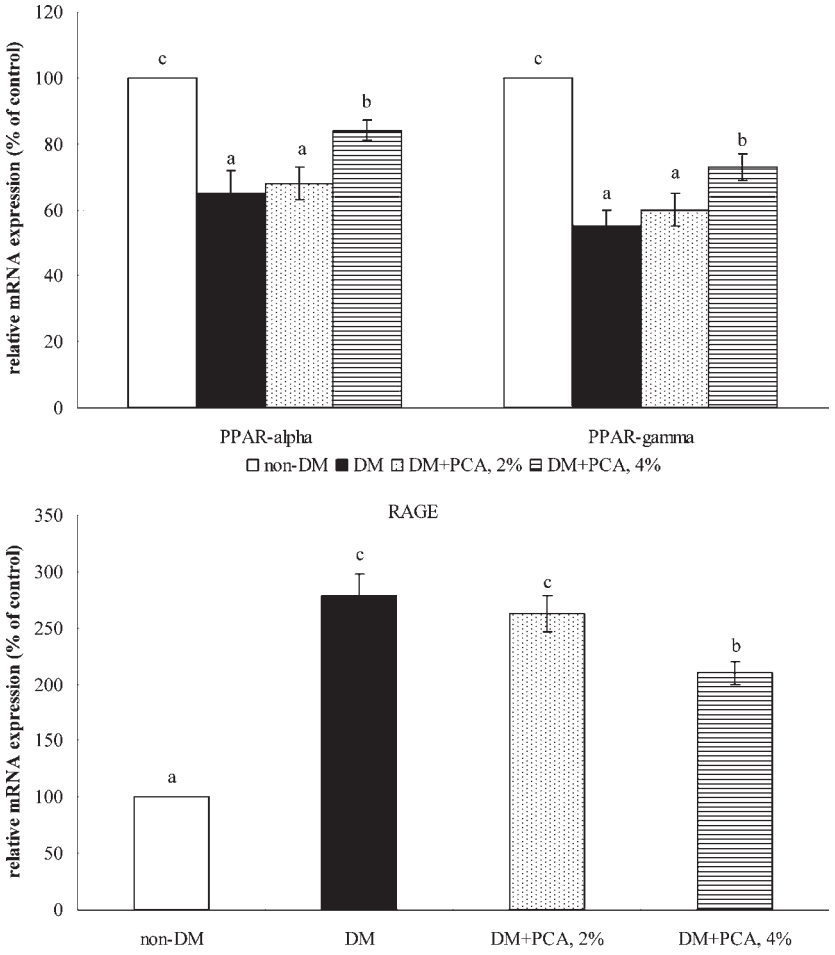

Figure 3. mRNA expression of renal PPAR-α, PPAR-γ, and RAGE in non-DM and DM that consumed a normal diet or 2 or 4% PCA for 12 weeks. Data are the mean ± SD (n = 10). Means among bars without a common letter differ ($p < 0.05$).

in the polyol pathway. In addition, we notified that this compound also provided renal protection via improving CCr, diminishing PKC activity, and lowering type-IV collagen and TGF-β1 levels. The impact of PCA on mRNA expression of PKCs and PPARs implied that it could act on the transcription level. These findings support that PCA is a potent agent against diabetes-associated glycative and renal injury.

The accumulation of AGEs in plasma or organs means diabetic deterioration and favors the development of diabetic complications.[2,25] The results of our present study revealed that PCA effectively lowered AGE levels in organs and circulation, which in turn alleviated systemic glycative stress. It has been documented that free radicals and inflammatory cytokines promote AGE production.[26,27] Our previous study[13] indicated that PCA could provide antioxidative and anti-inflammatory protection for diabetic mice. Thus, the antiglycative actions of this compound as we observed in our present study could be partially ascribed to this agent already attenuating oxidative and inflammatory stresses. On the other hand, an increased activity and expression of aldose reductase and sorbitol dehydrogenase facilitate the generation of sorbitol and fructose in the polyol pathway[3,28] and, consequently, enhance AGE formation and glycative injury in kidneys and other organs.[4,29] We found that PCA diminished renal activity and mRNA expression of these enzymes, which subsequently decreased the production of sorbitol and fructose. Thus, the observed lower renal level of AGEs, including CML and pentosidine, could be partially explained. These findings suggested that this compound could inhibit renal AGE formation via suppressing the polyol pathway. Glyoxalase I catalyzed the detoxification of α-oxoaldehydes to corresponding aldonic acids in tissues and decreased the available precursors for AGE production.[6,30] Our present study also found that PCA enhanced the activity and mRNA expression of glyoxalase I, which further metabolized AGE precursors. Therefore, the observed lower renal AGE content in 4% PCA-treated mice could be partially due to this compound elevating the activity and expression of glyoxalase I. In addition, the lower CML, pentosidine, fructose, and sorbitol levels in the brain from 4% PCA-treated mice implied that this compound attenuated glycative stress in the neuronal system. These findings suggested that this compound might be able to delay the progression of glycation-associated neurodegenerative diseases, such as Alzheimer's disease.

It is reported that glycated albumin enhances PKC activity and TGF-β1 expression, which promotes ECM synthesis in the kidneys.[7,8] Our present study found that PCA markedly decreased the renal level of glycated albumin, which subsequently diminished PKC activity and TGF-β1 expression, led to a lower production of type-IV collagen and fibronectin in the kidneys, and finally, alleviated albuminuria. Obviously, the reduced

glycated albumin contributed to alleviate the progression of renal injury via declining the activity and expression of fibrogenic factors. The decreased BUN and increased CCr in these mice agreed that PCA improved renal functions. In addition, it has been reported that AGEs could activate PKC-β in kidney cells,[31] and the activation of PKC isoforms, such as α and β, could facilitate TGF-β1 signalings and enhance the development of diabetic nephropathy.[32,33] The results of our present study revealed that PCA treatments diminished renal mRNA expression of PKC-α and PKC-β. It is possible that PCA decreased renal AGE formation and further lowered the stimulation of AGEs on activity and/or expression of PKCs. The other possibility is that PCA directly acted on the expression of PKC-α and PKC-β, which subsequently decreased the stimulation from PKC on TGF-β1. Because PKC activity and the TGF-β1 level had been declined, the lower renal production of type-IV collagen and fibronectin could be explained. These findings implied that this compound was an effective antifibrogenic agent against diabetic renal injury.

Both PPAR-α and PPAR-γ are nuclear receptors and share several regulatory properties, such as inflammation and glucose homeostasis.[34] It has been reported that activation of PPAR-γ by its agonist ameliorated proximal tubular cell injury and suppressed renal RAGE expression.[9,10] The study by Koh et al.[35] also revealed that PPAR-α activation could attenuate diabetic nephropathy via enhancing insulin action. The results of our present study found that 4% PCA treatments markedly reversed diabetes-induced downregulation of PPAR-α and PPAR-γ and declined RAGE expression in kidney. Although it is hard to conclude that PCA is a novel PPAR-γ agonist, it is highly possible that PCA could maintain renal PPAR expression under the diabetic condition, which in turn improved glucose homeostasis, mitigated renal cell injury, and abated the interaction of RAGE and AGEs. In addition, Wang et al.[36] indicated that AGEs could reduce the PPAR-γ protein level in neural stem cells. Thus, the restored PPAR-γ expression in kidneys resulted from PCA treatments, which, we observed, might be partially due to the lower renal AGE formation of this compound. These findings supported that the antiglycative and renal-protective effects of PCA seemingly involved the regulation of this compound on renal PPARs.

In summary, the intake of PCA increased its deposit in organs of mice. PCA treatments at 2 and 4% provided renal protection for diabetic mice against glycative and fibrogenic stresses via decreasing AGE, glycated albumin, fibronectin, type-IV collagen, and TGF-β1 levels. PCA at 4% also repressed renal activity and/or expression of AR, SDH, GLI, PKC, PPAR-α, PPAR-γ, and RAGE. Therefore, the supplement of PCA or foods rich in this compound might be helpful for the prevention or alleviation of diabetic nephropathy.

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REFERENCES

(1) Ziyadeh, F. N.; Mogyorosi, A.; Kalluri, R. Early and advanced non-enzymatic glycation products in the pathogenesis of diabetic kidney disease. *Exp. Nephrol.* **1997**, *5*, 2–9.

(2) Schleicher, E. D.; Wagner, E.; Nerlich, A. G. Increased accumulation of the glycoxidation product N^{ϵ}-(carboxymethyl)lysine in human tissues in diabetes and aging. *J. Clin. Invest.* **1997**, *99*, 457–468.

(3) Maekawa, K.; Tanimoto, T.; Okada, S.; Suzuki, T.; Suzuki, T.; Yabe-Nishimura, C. Expression of aldose reductase and sorbitol dehydrogenase genes in Schwann cells isolated from rat: Effects of high glucose and osmotic stress. *Mol. Brain Res.* **2001**, *87*, 251–256.

(4) Dunlop, M. Aldose reductase and the role of the polyol pathway in diabetic nephropathy. *Kidney Int.* **2000**, *58*, 3–12.

(5) Sato, T.; Iwaki, M.; Shimogaito, N.; Wu, X.; Yamagishi, S.; Takeuchi, M. TAGE (toxic AGEs) theory in diabetic complications. *Curr. Mol. Med.* **2006**, *6*, 351–358.

(6) Shinohara, M.; Thornalley, P. J.; Giardino, I.; Beisswenger, P.; Thorpe, S. R.; Onorato, J.; Brownlee, M. Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. *J. Clin. Invest.* **1998**, *101*, 1142–1147.

(7) Chen, S.; Cohen, M. P.; Ziyadeh, F. N. Amadori-glycated albumin in diabetic nephropathy: Pathophysiologic connections. *Kidney Int. Suppl.* **2000**, *77*, 40–44.

(8) Hayashida, T.; Schnaper, H. W. High ambient glucose enhances sensitivity to TGF-β1 via extracellular signal-regulated kinase and protein kinase Cδ activities in human mesangial cells. *J. Am. Soc. Nephrol.* **2004**, *15*, 2032–2041.

(9) Tang, S. C.; Leung, J. C.; Chan, L. Y.; Tsang, A. W.; Lai, K. N. Activation of tubular epithelial cells in diabetic nephropathy and the role of the peroxisome proliferator-activated receptor-γ agonist. *J. Am. Soc. Nephrol.* **2006**, *17*, 1633–1643.

(10) Matsui, T.; Yamagishi, S.; Takeuchi, M.; Ueda, S.; Fukami, K.; Okuda, S. Nifedipine inhibits advanced glycation end products (AGEs) and their receptor (RAGE) interaction-mediated proximal tubular cell injury via peroxisome proliferator-activated receptor-γ activation. *Biochem. Biophys. Res. Commun.* **2010**, *398*, 326–330.

(11) Lin, W. L.; Hsieh, Y. J.; Chou, F. P.; Wang, C. J.; Cheng, M. T.; Tseng, T. H. Hibiscus protocatechuic acid inhibits lipopolysaccharide-induced rat hepatic damage. *Arch. Toxicol.* **2003**, *77*, 42–47.

(12) Pacheco-Palencia, L. A.; Mertens-Talcott, S.; Talcott, S. T. Chemical composition, antioxidant properties, and thermal stability of a phytochemical enriched oil from Acai (*Euterpe oleracea* Mart.). *J. Agric. Food Chem.* **2008**, *56*, 4631–4636.

(13) Lin, C. Y.; Huang, C. S.; Huang, C. Y.; Yin, M. C. Anticoagulatory, antiinflammatory, and antioxidative effects of protocatechuic acid in diabetic mice. *J. Agric. Food Chem.* **2009**, *57*, 6661–6667.

(14) Kwon, Y. I.; Vattem, D. A.; Shetty, K. Evaluation of clonal herbs of *Lamiaceae* species for management of diabetes and hypertension. *Asia Pac. J. Clin. Nutr.* **2006**, *15*, 107–118.

(15) Harini, R.; Pugalendi, K. V. Antioxidant and antihyperlipidaemic activity of protocatechuic acid on streptozotocin-diabetic rats. *Redox Rep.* **2010**, *15*, 71–80.

(16) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L. Protein determination with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(17) Caccetta, R. A.; Croft, K. D.; Beilin, L. J.; Puddey, I. B. Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely affect ex vivo lipoprotein oxidizability. *Am. J. Clin. Nutr.* **2000**, *71*, 67–74.

(18) Miyata, T.; Taneda, S.; Kawai, R.; Ueda, Y.; Horiuchi, S.; Hara, M.; Maeda, K.; Monnier, V. M. Identification of pentosidine as a native structure for advanced glycation end products in β2-microglobulin-containing amyloid fibrils in patients with dialysis-related amyloidosis. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2353–2358.

(19) Guerrant, G.; Moss, C. W. Determination of monosaccharides as aldononitrile, *O*-methyoxime, alditol, and cyclitol acetate derivatives by gas chromatography. *Anal. Chem.* **1984**, *56*, 633–638.

(20) Nishinaka, T.; Yabe-Nishimura, C. EGF receptor-ERK pathway is the major signaling pathway that mediates upregulation of aldose reductase expression under oxidative stress. *Free Radical Biol. Med.* **2001**, *31*, 205–216.

(21) Ulrich, H. B. In *Methods of Enzyme Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; Vol. 2, pp 567−573.

(22) McLellan, A. C.; Thornalley, P. J. Glyoxalase activity in human red blood cells fractioned by age. *Mech. Ageing Dev.* **1989**, *48*, 63–71.

(23) Koya, D.; Haneda, M.; Nakagawa, H.; Isshiki, K.; Sato, H.; Maeda, S.; Sugimoto, T.; Yasuda, H.; Kashiwagi, A.; Ways, D. K.; King, G. L.; Kikkawa, R. Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC β inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J.* **2000**, *14*, 439–447.

(24) Sakata, N.; Meng, J.; Takebayashi, S. Effects of advanced glycation end products on the proliferation and fibronectin production of smooth muscle cells. *J. Atheroscler. Thromb.* **2000**, *7*, 169–176.

(25) Gugliucci, A.; Bendayan, M. Renal fate of circulating advanced glycated end products (AGE): Evidence for reabsorption and catabolism of AGE-peptides by renal proximal tubular cells. *Diabetologia* **1997**, *39*, 149–160.

(26) Lee, H. B.; Yu, M. R.; Yang, Y.; Jiang, Z.; Ha, H. Reactive oxygen species-regulated signaling pathways in diabetic nephropathy. *J. Am. Soc. Nephrol.* **2003**, *14*, S241–245.

(27) Cameron, N. E.; Cotter, M. A. Pro-inflammatory mechanisms in diabetic neuropathy: Focus on the nuclear factor κB pathway. *Curr. Drug Targets* **2008**, *9*, 60–67.

(28) Mizisin, A. P.; Li, L.; Perello, M.; Freshwater, J. D.; Kalichman, M. W.; Roux, L.; Calcutt, N. A. Polyol pathway and osmoregulation in JS1 Schwann cells grown in hyperglycemic and hyperosmotic conditions. *Am. J. Physiol.* **1996**, *270*, 90–97.

(29) Takeuchi, M.; Yamagishi, S. Alternative routes for the formation of glyceraldehyde-derived AGEs (TAGE) in vivo. *Med. Hypotheses* **2004**, *63*, 453–455.

(30) Thornalley, P. J. Glyoxalase I—Structure, function and a critical role in the enzymatic defence against glycation. *Biochem. Soc. Trans.* **2003**, *31*, 1343–1348.

(31) Scivittaro, V.; Ganz, M. B.; Weiss, M. F. AGEs induce oxidative stress and activate protein kinase C-β_{II} in neonatal mesangial cells. *Am. J. Physiol.* **2000**, *278*, 676–683.

(32) Wei, X. F.; Zhou, Q. G.; Hou, F. F.; Liu, B. Y.; Liang, M. Advanced oxidation protein products induce mesangial cell perturbation through PKC-dependent activation of NADPH oxidase. *Am. J. Physiol.* **2009**, *296*, 427–437.

(33) Wu, D.; Peng, F.; Zhang, B.; Ingram, A. J.; Kelly, D. J.; Gilbert, R. E.; Gao, B.; Krepinsky, J. C. PKC-β1 mediates glucose-induced Akt activation and TGF-β1 upregulation in mesangial cells. *J. Am. Soc. Nephrol.* **2009**, *20*, 554–566.

(34) Delerive, P.; Fruchart, J. C.; Staels, B. Peroxisome proliferator-activated receptors in inflammation control. *J. Endocrinol.* **2001**, *169*, 453–459.

(35) Koh, E. H.; Kim, M. S.; Park, J. Y.; Kim, H. S.; Youn, J. Y.; Park, H. S.; Youn, J. H.; Lee, K. U. Peroxisome proliferator-activated receptor (PPAR)-α activation prevents diabetes in OLETF rats: Comparison with PPAR-γ activation. *Diabetes* **2003**, *52*, 2331–2337.

(36) Wang, S. H.; Sun, Z. L.; Guo, Y. J.; Yuan, Y.; Li, L. PPARγ-mediated advanced glycation end products regulation of neural stem cells. *Mol. Cell. Endocrinol.* **2009**, *307*, 176–184.