# Anti-glycative Effects of Protocatechuic Acid in Kidney of Diabetic Mice

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**ABSTRACT**

Protocatechuic acid (PCA) at 2 or 4% was supplied to diabetic mice for 12 wks. PCA treatments increased its deposit in organs, and significantly reduced plasma HbA1c level, 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 only at 4% significantly enhanced renal glyoxalase I mRNA expression ($P$<0.05). PCA treatments also dose-dependently decreased renal level of type IV collagen, fibronectin and transforming growth factor-beta1 ($P$<0.05), as well as dose-dependently diminished renal protein kinase C (PKC) activity ($P$<0.05); however, only at 4% suppressed renal mRNA expression of PKC-alpha and PKC-beta ($P$<0.05). PCA treatments at 4% significantly restored renal mRNA expression of peroxisome proliferator-activated receptor (PPAR)-alpha and PPAR-gamma, as well as suppressed expression of advanced glycation endproduct receptor ($P$<0.05). These findings suggest that the supplement of protocatechuic acid might be helpful for the prevention or alleviation of glycation associated diabetic complications.

KEYWORDS: protocatechuic acid; diabetes; glycation; aldose reducatse; protein kinase C

## INTRODUCTION

Advanced glycation endproducts (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 occurred in diabetic condition favors glucose metabolism through the polyol pathway and promotes AGEs 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 AGEs production and causes vascular abnormalities (*5*). Glyoxalase I (GLI), part of the glyoxalase system presented in cytosol of cells, could metabolize physiological reactive α-carbonyl compounds such as glyoxal and methylglyoxal, and subsequently decreases the available precursors for AGEs 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 AGEs production.

It is documented that glycated albumin enhances protein kinase C (PKC) activity and stimulates transforming growth factor (TGF)-beta1 expression in kidney, which in turn leads to massive production of 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-gamma activation by its agonists could attenuate AGEs-induced renal ECM accumulation and fibrosis (*9*, *10*). Thus, any agent with capability to lower renal level of glycated albumin and TGF-beta1, and/or promote

PPAR-gamma activation may be more efficacious in attenuating diabetes associated renal injury.

Protocatechuic acid (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 protocatechuic acid provided *in vitro* inhibitory activity for alpha-glucosidase, and suggested that this compound might be able to manage diabetes and hypertension. The study of Harini and Pugalendi (*15*) indicated that protocatechuic acid could alleviate hyperlipidemia in diabetic rats. Our previous animal study reported that this compound could provide anti-oxidative, anti-inflammatory and anti-coagulatory protection against diabetic deterioration (*13*). Although these previous studies support that protocatechuic acid is a potent anti-diabetic agent, it remains known that this compound possesses anti-glycative and renal protective activities.

The major purpose of this study was to examine the anti-glycative effects and possible actions of protocatechuic acid in kidney of diabetic mice. The impact of this compound upon AGEs production, and renal activity and/or mRNA expression of AR, SDH, GLI, PKC and PPARs was determined. Furthermore, the content of protocatechuic acid in organs of mice consumed protocatechuic acid was measured.

## MATERIALS AND METHODS

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

**Animals and Diet.** Male Balb/cA mice, 3-4 wk old, were obtained from 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 China Medical University animal care committee (CMU-97-22-N). To induce diabetes, mice with body weight of 23.0 ± 1.3 g were treated with a single i.v. dose (50 mg/kg) of streptozotocin dissolved in citrate buffer (pH 4.5) into the tail vein. Blood glucose level was monitored on d 5 and 10 from the tail vein using a one-touch blood glucose meter (Lifescan, Inc. Milpitas, CA, USA). 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 powder diet (PMI Nutrition International LLC, Brentwood, MO, USA) to prepare 2 or 4% PCA diet, and supplied to mice for 12 wks. All mice had free access to food and water at all times.

**Experimental Design.** Non-diabetic mice consumed PCA diets were used for determining tissue PCA content only. Diabetic mice consumed PCA diets were used for other measurements, and compared with a group of non-diabetic and a group of diabetic mice consumed normal diet. Consumed water volume, feed intake and body weight were recorded weekly. Twenty-four h urine output collected by metabolic cage was measured at wk 12. After 12 wk supplementation, mice were fasted overnight and sacrificed with carbon dioxide. Blood was collected, and plasma was separated from erythrocytes immediately. Brain, heart, liver and kidney were collected, and 100 mg of each organ was homogenized on ice in 2 mL phosphate buffer saline (PBS, pH 7.2), and the filtrate was collected. The protein concentration of plasma or organ filtrate was determined by the method of 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 protein/L using PBS, pH 7.2.

**Tissue Content of PCA.** The content of PCA in plasma, brain, heart, liver and kidney was measured according to a GC-MS method described in Caccetta et al. (*17*). Briefly, lyophilized sample was mixed with 1-hydroxy-2-naphthoic acid, as 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 equal amount of *bis*(trimethylsilyl)-trifluoroacetamide and dry pyridine. PCA content was quantified by a HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a HP 5970 series mass-selective detector and a HP-1 cross-linked methyl silicone column (12 m x 0.20 mm, 0.33 µm film thickness). The electron-impact mode was used for mass spectrometer, and the mass-to-charge ratio of PCA was 370. The limit of detection and quantification was 0.2 and 0.8 ng/mL, respectively.

**Blood and Urinary Analyses.** Plasma glucose level was measured by a glucose HK kit (Sigma Chemical Co., St. Louis, MO, USA). Plasma insulin level was measured by using a rat insulin radioimmunoassay kit (Linco Research Inc., St. Charles, MO, USA). Plasma HbA1c level was measured by 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, USA). Creatinine clearance rate (CCr) was calculated according to Cockcroft-Gault formula, [urinary creatinine (mg/dL) x urinary volume (mL)] ÷ [plasma creatinine (mg/dL) x length of urine collection (min)], and expressed as mL/min/100 g body weight. Urine albumin was measured by a competitive ELISA assay according to the manufacturer's instruction (Exocell, Philadelphia, PA, USA). 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 brain or kidney tissue was homogenized and digested with proteinase K (1 mg/mL) for 3 h at 37 °C, and 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, USA) at 405 nm (reference 603 nm). Intra- and interassay variability of this assay were 5.3 and 6.2%, respectively. Pentosidine level was analyzed by a HPLC equipped with a C18 reverse-phase column and a fluorescence detector according to the method described in Miyata et al. (*18*). Briefly, sample was lyophilized and acid hydrolyzed in 500 µL 6 N HCl for 16 h at 110 °C in screw-cap tubes purged with nitrogen. After neutralization with NaOH and diluted with PBS, sample was used for HPLC measurement.

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

**Activity of Renal AR, SDH, GLI and PKC.** The method of Nishinaka and Yabe-Nishimura (*20*) was used to measure AR activity in kidney by monitoring the decrease in absorbance at 340 nm due to NADPH oxidation. SDH activity was assayed according to the method of Ulrich (*21*) by mixing 100 µL homogenate, 200 µL NADH (12 mM) and 1.6

mL triethanolamine buffer (0.2 M, pH 7.4), and monitoring the absorbance change at 365 nm. The method of McLellan and Thornalley (*22*) was used to assay GLI activity by monitoring the increase in absorbance at 240 nm due to the formation of *S*-(D)-lactoylglutathione. The method described in Koya et al. (*23*) was used to measure glomeruli PKC activity. Briefly, kidney were homogenized in ice-cold RPMI1640 media containing 20 mM 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, and then spotted onto P81 phosphocellulose paper and washed four 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-beta1, Fibronectin and Type IV Collagen.** Renal cortex was homogenized with ice-cold PBS containing 0.05% Tween 20. After centrifugating at 9000 xg for 15 min at 4 °C, the supernatants were used for measuring renal TGF-beta1 level (ng/mg protein), which was quantified by a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA). Fibronectin (mg/mg protein) was assayed using rabbit anti-rat fibronectin antibody and quantified by solid-phase immunoenzymic ELISA (*24*). Type IV collagen concentration was measured by a Collagen IV M kit (Exocell Inc, Philadelphia, PA, USA), which measured both intact and fragments of type IV collagen.

**Real Time Polymerase Chain Reaction for mRNA Expression.** Total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). One μg

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, pH 8.4, 50 mM KCl, 200 mM dNTP, 2.5 mM $MgCl_2$, 0.5 mM of each primer) and 2.5 U Taq DNA polymerase. The specific oligonucleotide primers of targets are shown in Table 1. The cDNA was amplified under the following reaction conditions: 95 ºC for 1 min, 55 ºC for 1 min, and 72 ºC for 1 min. 28 cycles were performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene) and 35 cycles were performed for others. Generated fluorescence from each cycle was quantitatively analyzed by using the Taqman system based on real-time sequence detection system (ABI Prism 7700, Perkin-Elmer Inc., Foster City, CA, USA). In this study, mRNA level was calculated as percentage of the control group.

**Statistical Analysis.** The effect of each measurement was analyzed from 10 mice (n=10). All data were expressed as mean ± standard deviation (SD). Statistical analysis was done 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-DM and DM mice. However, DM mice had lower PCA content in plasma and organs than non-DM mice ($P<0.05$). Feed intake, water intake and body weight recorded at wks 2 and 12, and organ weight and urine volume measured at wk 12 are presented in Table 3. Compared with 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 wk 12 ($P<0.05$). As shown in Table 4, compared with 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 ($P<0.05$). These treatments also decreased renal level of CML, pentosidine, sorbitol and fructose ($P<0.05$); however, PCA treatments only at 4% significantly decreased brain level 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, $P<0.05$), 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 concentration, and renal level of fibronectin, type IV collagen and TGF-beta1 ($P<0.05$).

As shown in Figure 2, diabetic condition increased renal PKC activity, and up-regulated expression of three PKC isoforms. PCA treatments dose-dependently declined renal PKC activity ($P<0.05$); however, only at 4% suppressed renal mRNA expression of PKC-alpha and PKC-beta ($P<0.05$). Diabetic condition also down-regulated renal mRNA expression of PPAR-alpha and PPAR-gamma, as well as up-regulated RAGE expression (Figure 3, $P<0.05$); PCA treatments only at 4% significantly restored mRNA expression of PPAR-alpha and PPAR-gamma, and repressed RAGE expression ($P<0.05$).

## DISCUSSION

Our previous animal study reported that dietary supplement of protocatechuic acid at 2 and 4% improved glycemic control, decreased oxidative and inflammatory stress in heart and

kidney, 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 AGEs production in plasma, kidney and brain, as well as regulated enzymes involved in 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-beta1 levels. The impact of protocatechuic acid upon mRNA expression of PKCs and PPARs implied that it could act on transcriptional level. These findings support that protocatechuic acid 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 protocatechuic acid effectively lowered AGEs levels in organs and circulation, which in turn alleviated systemic glycative stress. It has been documented that free radicals and inflammatory cytokines promote AGEs production (*26*, *27*). Our previous study (*13*) indicated that protocatechuic acid could provide anti-oxidative and anti-inflammatory protection for diabetic mice. Thus, the anti-glycative actions of this compound as we observed in our present study could be partially ascribed to this agent already attenuate oxidative and inflammatory stresses. On the other hand, increased activity and expression of aldose reductase and sorbitol dehydrogenase facilitate the generation of sorbitol and fructose in polyol pathway (*3*, *28*), and consequently enhances AGEs formation and glycative injury in kidney and other organs (*4*, *29*). We found that protocatechuic acid 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 AGEs formation via suppressing polyol pathway. Glyoxalase I catalyses the

detoxification of alpha-oxoaldehydes to corresponding aldonic acids in tissues and decreased the available precursors for AGEs production (*6, 30*). Our present study also found that protocatechuic acid enhanced the activity and mRNA expression of glyoxalase I, which further metabolized AGEs precursors. Therefore, the observed lower renal AGEs content in 4% protocatechuic acid-treated mice could be partially due to this compound elevate the activity and expression of glyoxalase I. In addition, the lower CML, pentosidine, fructose and sorbitol levels in brain from 4% protocatechuic acid-treated mice implied that this compound attenuated glycative stress in 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-beta1 expression, which promotes ECMs synthesis in kidney (*7, 8*). Our present study found that protocatechuic acid markedly decreased renal level of glycated albumin, which subsequently diminished PKC activity and TGF-beta1 expression, and led to lower production of type IV collagen and fibronectin in kidney, 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 protocatechuic acid improved renal functions. In addition, it has been reported that AGEs could activate PKC-beta in kidney cells (*31*); and the activation of PKC isoforms such as alpha and beta could facilitate TGF-beta1 signalings and enhance the development of diabetic nephropathy (*32, 33*). The results of our present study revealed that protocatechuic acid treatments diminished renal mRNA expression of PKC-alpha and PKC-beta. It is possible that protocatechuic acid decreased renal AGEs formation, and further lowered the stimulation of AGEs upon activity and/or expression of PKCs. The other

possibility is that protocatechuic acid directly acted on the expression of PKC-alpha and PKC-beta, which subsequently decreased the stimulation from PKC upon TGF-beta1. Since PKC activity and TGF-beta1 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 anti-fibrogenic agent against diabetic renal injury.

Both PPAR-alpha and PPAR-gamma are nuclear receptors and share several regulatory properties such as inflammation and glucose homeostasis (*34*). It has been reported that activation of PPAR-gamma by its agonist ameliorated proximal tubular cell injury and suppressed renal RAGE expression (*9*, *10*). The study of Koh et al. (*35*) also revealed that PPAR-alpha activation could attenuate diabetic nephropathy via enhancing insulin action. The results of our present study found that 4% protocatechuic acid treatments markedly reversed diabetes induced down-regulation of PPAR-alpha and PPAR-gamma, and declined RAGE expression in kidney. Although it is hard to conclude that protocatechuic acid is a novel PPAR-gamma agonist, it is highly possible that protocatechuic acid could maintain renal PPARs expression under 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 PPAR-gamma protein level in neural stem cells. Thus, the restored PPAR-gamma expression in kidney resulted from protocatechuic acid treatments as we observed might be partially due to this compound lower renal AGEs formation. These findings supported that the anti-glycative and renal-protective effects of protocatechuic acid seemingly involved the regulation of this compound upon renal PPARs.

In summary, the intake of protocatechuic acid increased its deposit in organs of mice. Protocatechuic acid treatments at 2 and 4% provided renal protection for diabetic mice against

glycative and fibrogenic stresses via decreasing AGEs, glycated albumin, fibronectin, type IV collagen and TGF-beta1 levels. Protocatechuic acid at 4% also repressed renal activity and/or expression of AR, SDH, GLI, PKC, PPAR-alpha, PPAR-gamma and RAGE. Therefore, the supplement of protocatechuic acid or foods rich in this compound might be helpful for the prevention or alleviation of diabetic nephropathy.

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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-alph | 5’-CTG CAG AGC AAC CAT CCA GAT-3’ | 5’-GCC GAA GGT CCA CCA TTT T-3 |
| PPAR-gamma | 5’-TCC GTG ATG GAA GAC CAC TC-3’ | 5’-CCC TTG CAT CCT TCA CAA GC-3’ |
| PKC-alpha | 5’-GAA CCA TGG CTG ACG TTT AC-3’ | 5’-GCA AGA TTG GGT GCA CAA AC-3’ |
| PKC-beta | 5’-TTC AAG CAG CCC ACC TTC TG-3’ | 5’-AAG GTG GCT GAA TCT CCT TG-3’ |
| PKC-gamma | 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’ |

**Table 2.** Level (nmol/mL or nmol/g tissue) of protocatechuic acid (PCA) in plasma, brain, heart, liver and kidney from non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% PCA at 12 week. Data are mean ± SD, n=10.

| | non-DM | non-DM+PCA, 2% | non-DM+PCA, 4% | DM | DM+PCA, 2% | DM+PCA, 4% |
|---|---|---|---|---|---|---|
| Plasma | _* | $0.12\pm0.03^{b}$ | $0.20\pm0.08^{c}$ | _ | $0.06\pm0.02^{a}$ | $0.13\pm0.05^{b}$ |
| Brain | _ | $0.26\pm0.10^{b}$ | $0.41\pm0.14^{c}$ | _ | $0.17\pm0.07^{a}$ | $0.31\pm0.11^{b}$ |
| Heart | _ | $0.29\pm0.08^{b}$ | $0.45\pm0.12^{c}$ | _ | $0.15\pm0.06^{a}$ | $0.33\pm0.09^{b}$ |
| Liver | _ | $0.39\pm0.11^{b}$ | $0.67\pm0.17^{c}$ | _ | $0.25\pm0.10^{a}$ | $0.42\pm0.13^{b}$ |
| Kidney | _ | $0.40\pm0.06^{b}$ | $0.72\pm0.14^{c}$ | _ | $0.26\pm0.05^{a}$ | $0.46\pm0.10^{b}$ |

*Means too low to be detected.

$^{a\text{-}c}$Means in a row without a common letter differ, $P<0.05$

**Table 3**. Water intake (WI, mL/mouse/d), Feed intake (FI, g/mouse/d), body weight (BW, g/mouse), organ weight (g/mouse) and urine output (mL/mouse/d) of non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% protocatechuic acid (PCA) at wks 2 and/or 12. Data are mean ± SD, n=10.

| | non-DM | DM | DM+PA, 2% | DM+PA, 4% |
|---|---|---|---|---|
| WI | | | | |
| wk 2 | 1.1±0.4[a] | 3.2±1.0[b] | 3.5±0.9[b] | 3.0±1.1[b] |
| wk12 | 2.0±0.8[a] | 7.5±1.6[c] | 5.8±1.2[b] | 5.1±1.0[b] |
| FI | | | | |
| wk 2 | 0.9±0.3[a] | 1.9±0.5[b] | 2.1±0.6[b] | 2.2±0.7[b] |
| wk 12 | 2.3±1.1[a] | 8.0±1.7[c] | 6.9±1.3[b] | 5.8±1.0[b] |
| BW | | | | |
| wk 2 | 22.9±1.4[b] | 20.3±1.5[a] | 20.1±1.3[a] | 20.7±0.9[a] |
| wk 12 | 32.6±3.2[c] | 11.8±2.1[a] | 14.8±1.9[b] | 16.1±1.5[b] |
| Brain weight, wk 12 | 0.51±0.09[a] | 0.47±0.06[a] | 0.53±0.10[a] | 0.49±0.08[a] |
| Heart weight, wk 12 | 0.25±0.04[a] | 0.21±0.03[a] | 0.29±0.05[a] | 0.22±0.04[a] |
| Liver weight, wk 12 | 1.58±0.10[a] | 1.66±0.13[a] | 1.49±0.09[a] | 1.60±0.17[a] |
| Kidney weight, wk12 | 0.43±0.11[a] | 0.52±0.08[a] | 0.47±0.06[a] | 0.54±0.13[a] |
| Urine volume, wk 12 | 0.61±0.12[a] | 6.58±1.08[c] | 5.04±0.83[b] | 4.17±0.55[b] |

[a-c]Means in a row without a common letter differ, $P<0.05$.

**Table 4.** Plasma level of glucose (mmol/L), insulin (nmol/L), BUN (mg/dL) and CCr (mL/min/100 g body weight) of non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% protocatechuic acid (PCA) at week 12. Data are mean ± SD, n=10.

| | non-DM | DM | DM+PCA, 2% | DM+PCA, 4% |
|---|---|---|---|---|
| Glucose | $10.8\pm1.0^{a}$ | $27.6\pm2.1^{d}$ | $22.1\pm1.3^{c}$ | $17.4\pm1.2^{b}$ |
| Insulin | $13.1\pm1.4^{d}$ | $4.5\pm0.6^{a}$ | $6.9\pm0.9^{b}$ | $9.5\pm0.7^{c}$ |
| BUN | $6.1\pm0.8^{a}$ | $58.3\pm4.5^{d}$ | $49.7\pm3.9^{c}$ | $32.6\pm2.3^{b}$ |
| CCr | $1.60\pm0.10^{d}$ | $0.41\pm0.05^{a}$ | $0.68\pm0.08^{b}$ | $1.03\pm0.12^{c}$ |

[a-d]Means in a row without a common letter differ, $P<0.05$

**Table 5.** Level of plasma HbA1c (%), urinary glycated albumin (μg/mL), renal and brain levels of CML (pmol/mg protein), pentosidine (pmol/mg protein), sorbitol (nmol/mg protein) and fructose (nmol/mg protein) in non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% protocatechuic acid (PCA) at 12 week. Data are mean ± SD, n=10.

| | non-DM | DM | DM+PCA, 2% | DM+PCA, 4% |
|---|---|---|---|---|
| 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] |

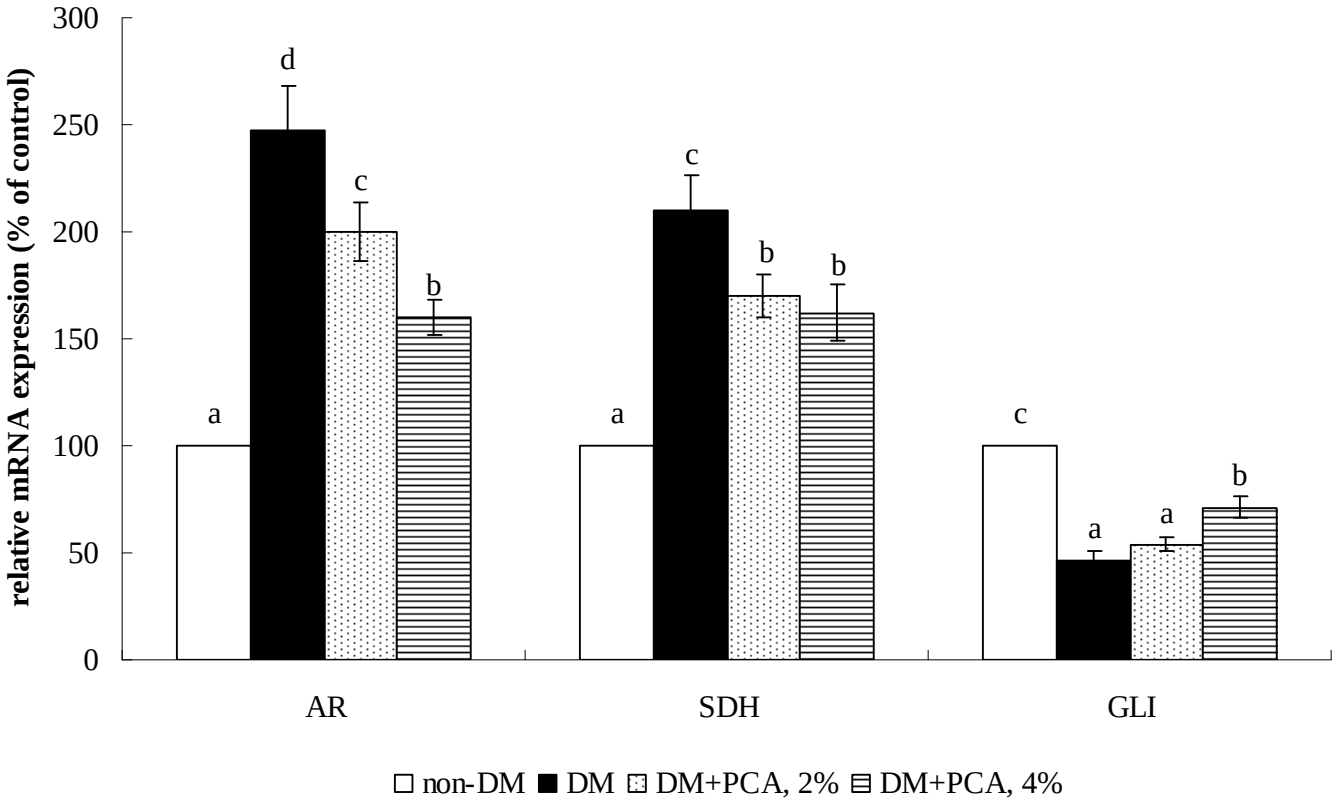
[a-d]Means in a row without a common letter differ, $P<0.05$

**Table 6.** Renal activity of AR (nmol/min/mg protein), SDH (U/g protein) and GLI (nmol/min/mg protein) in non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% protocatechuic acid (PCA) at 12 week. Data are mean ± SD, n=10.

| | non-DM | DM | DM+PCA, 2% | DM+PCA, 4% |
|---|---|---|---|---|
| AR | $1.04\pm0.22^{a}$ | $2.77\pm0.34^{d}$ | $2.41\pm0.23^{c}$ | $1.98\pm0.19^{b}$ |
| SDH | $4.0\pm0.3^{a}$ | $8.9\pm1.0^{c}$ | $7.6\pm0.6^{b}$ | $7.3\pm0.4^{b}$ |
| GLI | $285\pm27^{c}$ | $117\pm15^{a}$ | $131\pm18^{a}$ | $176\pm20^{b}$ |

[a-d]Means in a row without a common letter differ, $P<0.05$.

**Figure 1.** mRNA expression of renal AR, SDH and GLI in non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% protocatechuic acid (PCA) at 12 week. Data are mean ± SD, n=10. [a-d]Means among bars without a common letter differ, $P<0.05$.


relative mRNA expression (% of control)
300
250
200
150
100
50
0
d
c
b
a
c
b
b
a
c
a
a
b
AR
SDH
GLI
non-DM
DM
DM+PCA, 2%
DM+PCA, 4%

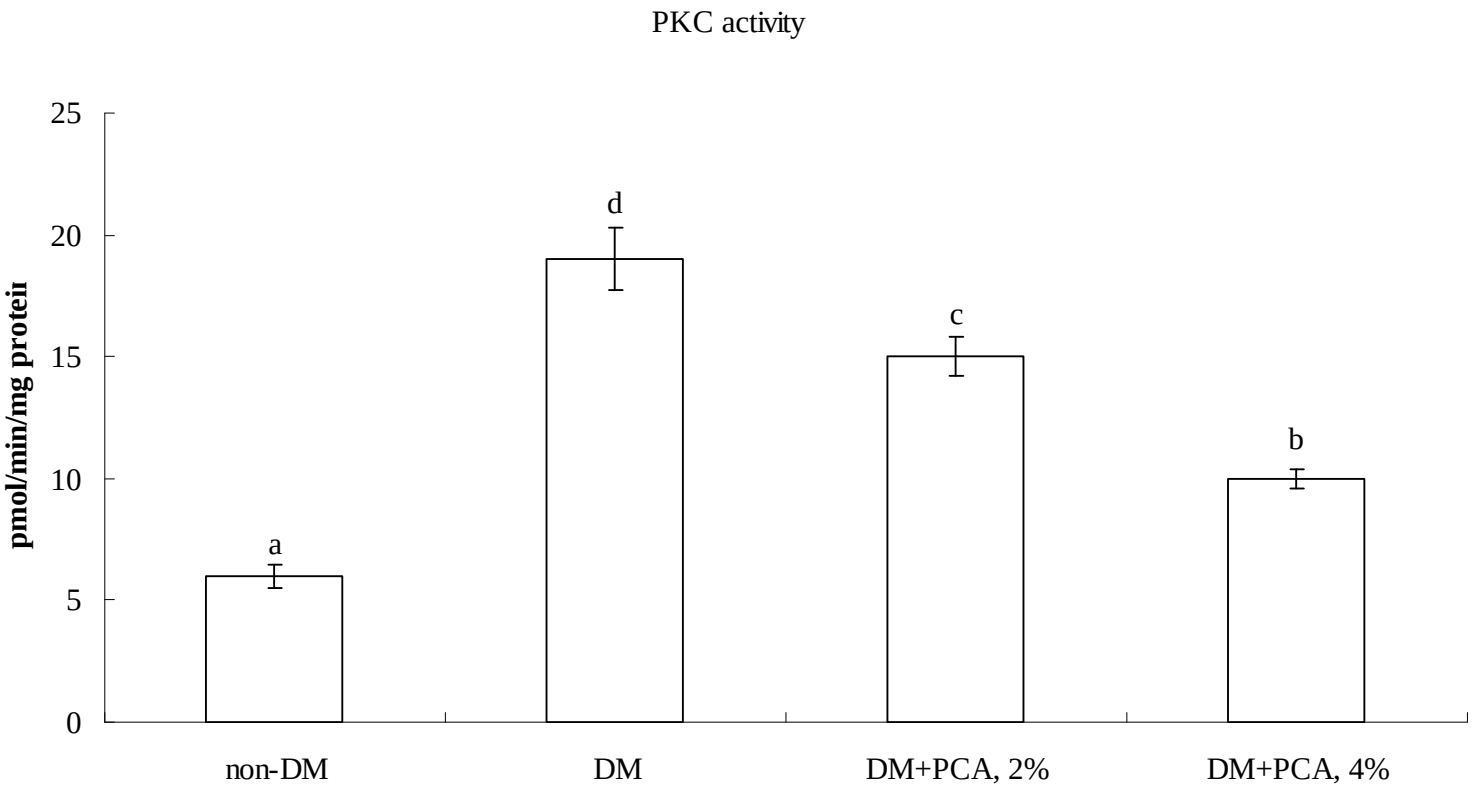
1

**Table 7.** Urinary level of albumin (μg/day), renal level of fibronectin (mg/mg protein), type IV collagen (ng/mg protein) and TGF-beta1 (ng/mg protein) in non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% protocatechuic acid (PCA) at 12 week. Data are mean ± SD, n=10.

| | non-DM | DM | DM+PCA, 2% | DM+PCA, 4% |
|---|---|---|---|---|
| Urinary | | | | |
| albumin | $22\pm4^{a}$ | $176\pm10^{d}$ | $121\pm8^{c}$ | $71\pm6^{b}$ |
| Renal | | | | |
| fibronectin | $2.64\pm0.25^{a}$ | $6.17\pm0.47^{d}$ | $5.11\pm0.32^{c}$ | $3.95\pm0.29^{b}$ |
| type IV collagen | $9\pm3^{a}$ | $84\pm9^{d}$ | $61\pm6^{c}$ | $37\pm5^{b}$ |
| TGF-beta1 | $13\pm4^{a}$ | $73\pm7^{d}$ | $52\pm5^{c}$ | $31\pm3^{b}$ |

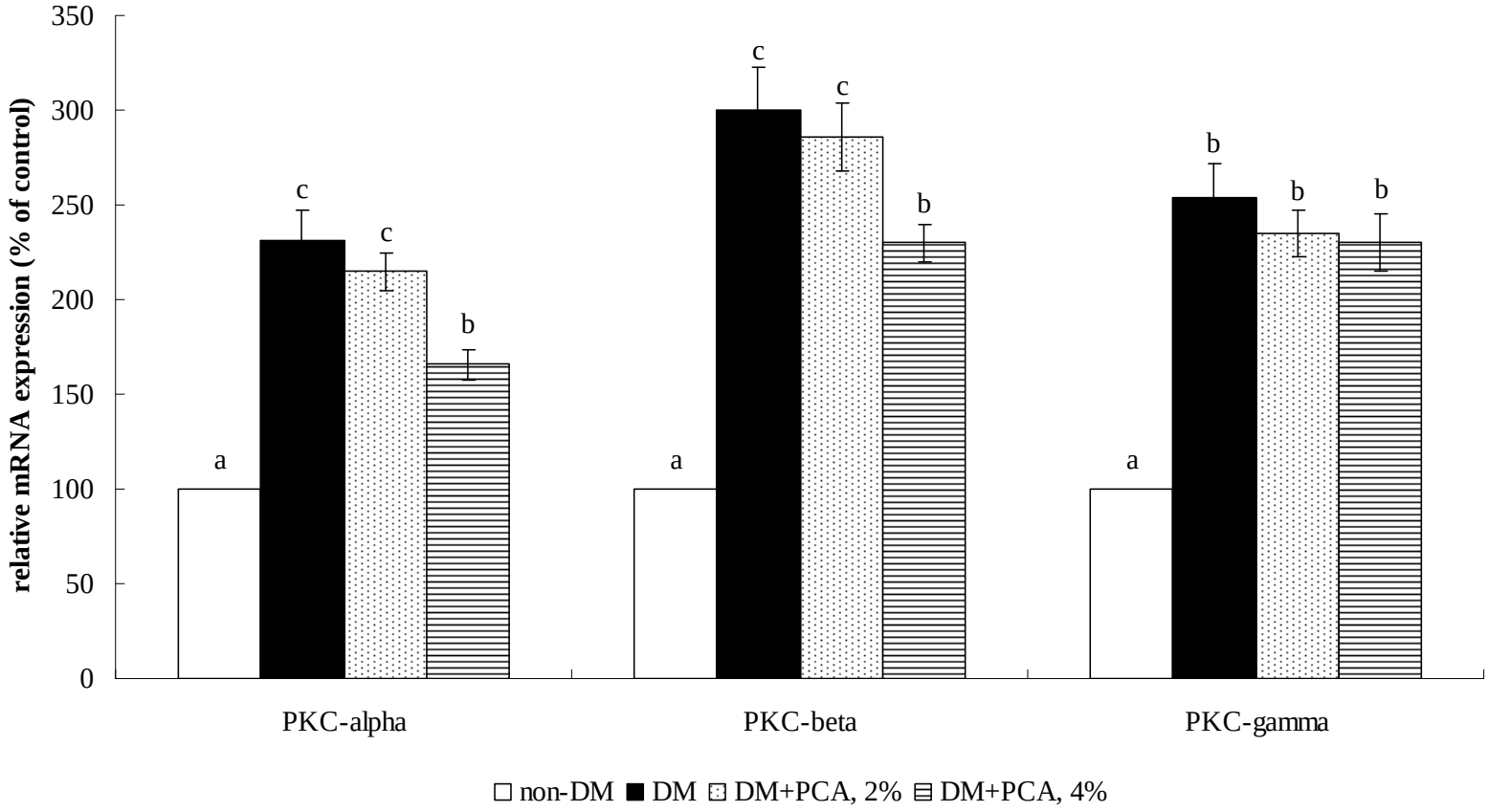
[a-d]Means in a row without a common letter differ, $P<0.05$

**Figure 2.** Renal activity (pmol/min/mg protein) and mRNA expression of PKC-alpha, PKC-beta and PKC-gamma in non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% protocatechuic acid (PCA) at 12 week. Data are mean ± SD, n=10. $^{a-c}$Means among bars without a common letter differ, $P<0.05$.


PKC activity
pmol/min/mg protein
25
20
15
10
5
0
a
d
c
b
non-DM
DM
DM+PCA, 2%
DM+PCA, 4%

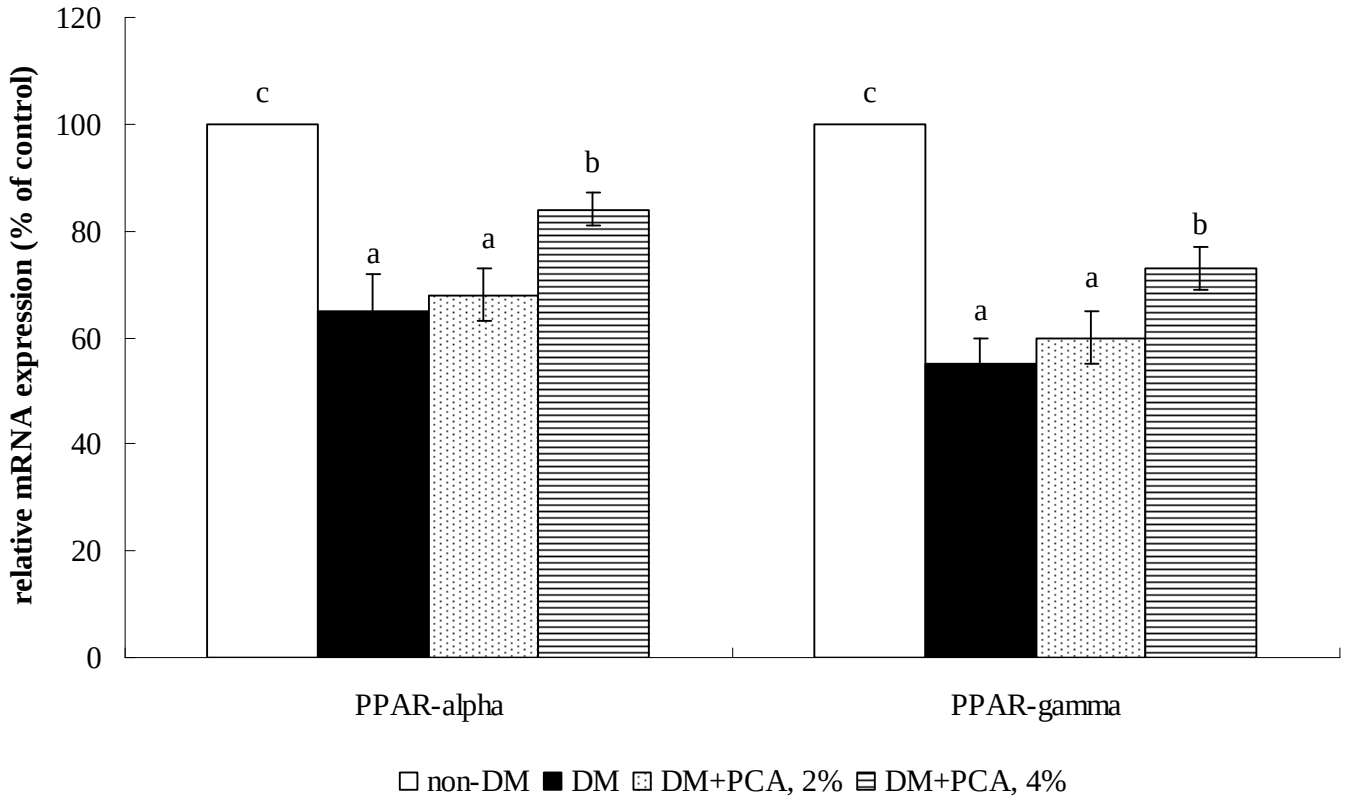
1

2


relative mRNA expression (% of control)
350
300
250
200
150
100
50
0
a
c
c
b
a
c
c
b
a
b
b
b
PKC-alpha
PKC-beta
PKC-gamma
non-DM
DM
DM+PCA, 2%
DM+PCA, 4%

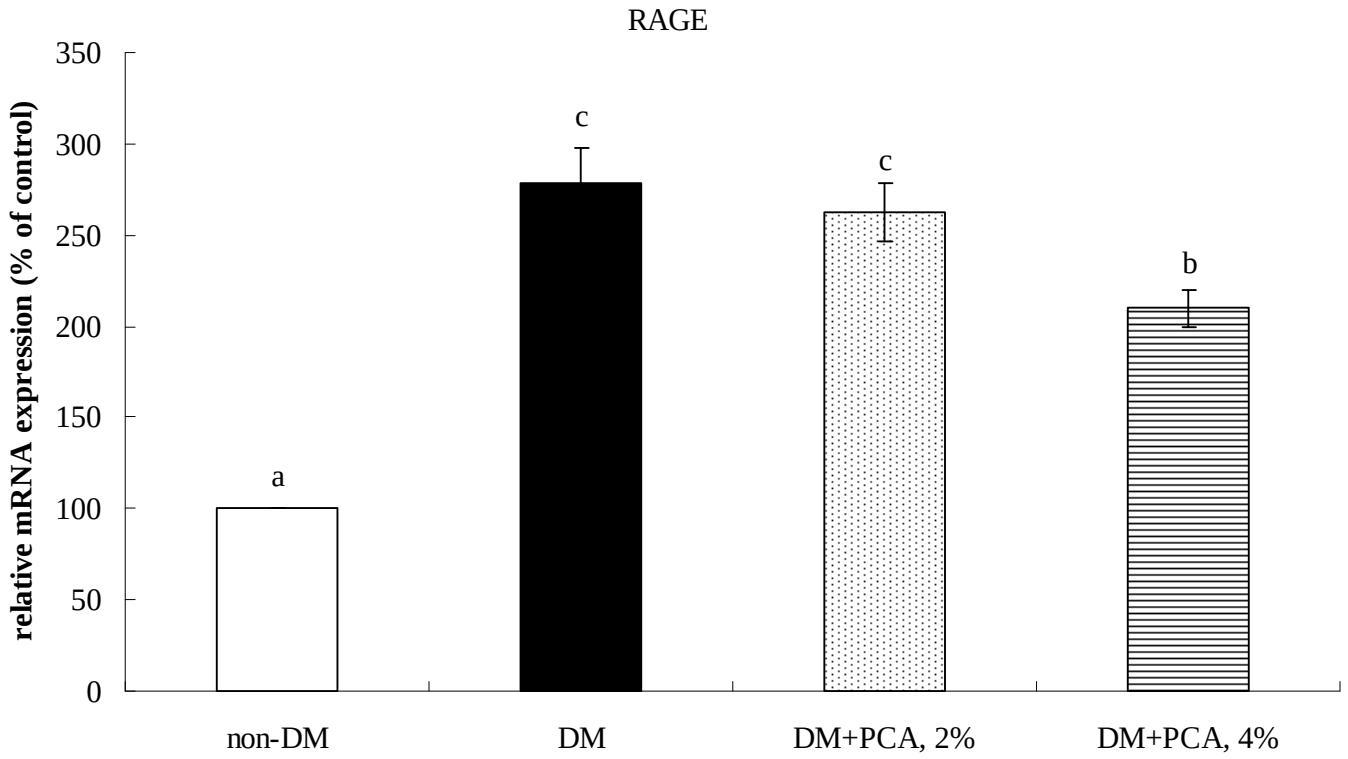
1

**Figure 3.** mRNA expression of renal PPAR-alpha, PPAR-gamma and RAGE in non-diabetic (non-DM), diabetic mice (DM) consumed normal diet, or 2 or 4% protocatechuic acid (PCA) at 12 week. Data are mean ± SD, n=10. [a-c]Means among bars without a common letter differ, $P<0.05$.


relative mRNA expression (% of control)
120
100
80
60
40
20
0
c
a
a
b
c
a
a
b
PPAR-alpha
PPAR-gamma
non-DM
DM
DM+PCA, 2%
DM+PCA, 4%

1

2


RAGE
relative mRNA expression (% of control)
350
300
250
200
150
100
50
0
a
c
c
b
non-DM
DM
DM+PCA, 2%
DM+PCA, 4%

1