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Inhibition of Advanced Glycation End Product Formation by Pu-erh Tea Ameliorates Progression of Experimental Diabetic Nephropathy

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ABSTRACT: Accumulation of advanced glycation end products (AGEs) has been implicated in the development of diabetic nephropathy. We investigated the effects of Pu-erh tea on AGE accumulation associated with diabetic nephropathy. Although it did not affect blood glucose levels and insulin sensitivy, Pu-erh tea treatment for 8 weeks attenuated the increases in urinary albumin, serum creatinine, and mesangial matrix in *db/db* mice. We found that Pu-erh tea prevented diabetes-induced accumulation of AGEs and led to a decreased level of receptor for AGE expression in glomeruli. Both production and clearance of carbonyl compounds, the main precursor of AGE formation, were probably attenuated by Pu-erh tea in vivo independent of glyoxalase I expression. In vitro, HPLC assay demonstrated Pu-erh tea could trap methylglyoxal in a dose-dependent manner. Our study raises the possibility that inhibition of AGE formation by carbonyl trapping is a promising approach to prevent or arrest the progression of diabetic complications.

KEYWORDS: *Pu-erh tea, rosiglitazone, diabetic nephropathy, db/db mice, methylglyoxal, advanced glycation end products, RAGE, glyoxalase I*

INTRODUCTION

Diabetic nephropathy is emerging as the leading cause of end-stage renal failure and a life-threatening complication of diabetes mellitus.[1] Approximately 30% of diabetic patients suffer from diabetic nephropathy, eventually undergoing renal dialysis or transplantation.[2] The conspicuous pathologic changes of diabetic nephropathy are persistent albuminuria, altered creatinine clearance, mesangial matrix expansion, glomerular basement membrane (GBM) thickening, and glomerular sclerosis.[3,4] These changes are also observed in *db/db* mice, a genetic obesity-induced type 2 diabetic model, which exhibits severe obesity, insulin resistance, hyperglycemia, and diabetic complications.[5,6]

Several mechanisms are thought to be involved in the pathogenesis of diabetic nephropathy and other diabetes-associated complications, all of them originating from prolonged hyperglycemia.[7] Some of these pathways are increased polyol pathway flux, accumulation of advanced glycation end products (AGEs), activation of protein kinase C (PKC), oxidative stress, and also chronic unresolved inflammation due to increased production of inflammatory cytokines.[7] Of them, AGEs have been regarded as major mediators of untoward effects of hyperglycemia.[8] An excessive AGE−RAGE (receptor for AGE) interaction activates intracellular signaling cascades, leading to a plethora of proinflammatory and profibrotic cellular responses through various downstream pathways.[9,10] AGEs arise from nonenzymatic reactions between proteins and carbonyl compounds, including methylglyoxal (MG), glyoxal, and 3-deoxyglucosone.[11] In diabetes, increases in carbonyl compounds derived from autoxidation of carbohydrates, lipids, or amino acids led to accumulation of AGEs.[8] Thus, therapeutic strategies aimed at reducing dicarbonyl compounds or enhancing their clearance and subsequently inhibiting AGE formation would be efficient to prevent the pathogenesis of diabetic nephropathy.

It has been demonstrated that epicatechin (EC), one of numerous catechins present largely in tea, underwent electrophilic aromatic substitution reactions with MG and formed a covalent bonding adduct. This process was called "carbonyl trapping".[12] It indicated the possibility that tea could control AGE-mediated diabetic complications by an inhibiton of protein glycation. Several studies have demonstrated that tea and polyphenols could attenuate the progression of hyper-

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glycemia and diabetic complications.[13−15] The possibility of preventing the onset of diabetic complications using dietary supplements with natural plants or herbal medicines has attracted increasing attention. Pu-erh tea, produced mainly in Yunnan Province, China, is consumed widely in southeastern Asia owing to its unique flavor and potential health benefits. Numerous studies have shown many beneficial health effects of Pu-erh tea, including lowering blood lipids,[16] antiobesity,[17] preventing atherosclerosis,[18] and antioxidation.[19] However, the effect of Pu-erh tea in diabetic nephropathy remains unknown.

In the present study, we primarily investigated the effects of Pu-erh tea on the progression of diabetic nephropathy in a young *db/db* mouse model. Our report provides an experimental basis for daily oral administration of Pu-erh tea in the prevention or treatment of kidney disease in diabetes mellitus.

MATERIALS AND METHODS

Preparation of Pu-erh Tea. Pu-erh tea powder, kindly supplied by the China Pu'er Tea Research Institute, was produced with fermented Pu-erh tea as a raw material using the following extraction technology: ethanol soaking, water extraction (1:8 tea/water rate, 100 °C), filtering, concentrating, and freeze-drying. The powder was resolved in sterile distilled water to make a 20 mg/mL stock solution of Pu-erh tea. The solution was filtered with filter papers and a 0.22 μm Millipore membrane filter (Millipore, Bedford, MA). The sterilized Pu-erh tea solution was aliquoted and stored at −20 °C.

Determination of Catechin Levels in Pu-erh Tea Powder. Determination of the catechins present in Pu-erh tea powder was carried out using reversed-phase high-performance liquid chromatography (RP-HPLC). A Diamonsil-C18 column (250 mm × 4.6 mm, 5 μm) was used. Methanol (solution A) and formic acid aqueous solution (0.1%) (solution B) were used as mobile phases. The flow rate was 1 mL min^{-1}. The column was maintained at room temperature. The catechins were detected by UV at 280 nm. The contents of major catechins, including gallic acid, (+)-catechin (C), (−)-epicatechin (EC), and (−)-epigallocatechin (EGCG) present in Pu-erh tea powder were measured.

Animals and Grouping. Six-week-old BKS.Cg-+ $Lepr^{db}$/+ $Lepr^{db}$/J (*db/db*) mice and BKS.Cg-*Dock7*^{m} +/+ $Lepr^{db}$/J (*db/m*) mice were purchased from MARC (Nanjing, Jiangsu, China). The animals were housed in a standard specific-pathogen-free (SPF) facility (25 ± 2 °C, 40−60% humidity, and 12 h light/dark cycle), with free access to water and rodent chow. *db/db* mice were divided into three groups (n = 4−6 mice per group) which received Pu-erh tea water (1 g kg^{-1} day^{-1}), rosiglitazone (RSG; 4 mg kg^{-1} day^{-1}), or vehicle (sterile distilled water) twice daily (9:00 a.m. and 6:00 p.m.) via oral gavage. Age-matched *db/m* mice (n = 6) were treated with vehicle (sterile distilled water) and used as a normal control. The dose of Pu-erh tea was 1 g kg^{-1} day^{-1}, which was approximately 1/10 the LD_{50}.[20] Rosiglitazone maleate (RSG) (GlaxoSmithKline, Tianjin, China) was used as an established antidiabetic drug. The dose of RSG, 4 mg kg^{-1} day^{-1}, was based on previous reports.[21,22] After 8 weeks of administration, animals were euthanized and various biochemical and histological parameters were evaluated. All animal studies were conducted with the review and approval of the Institutional Animal Care and Use Committee of Beijing Normal University.

Diabetes Assessment. The animals were weighed weekly. For food and water intake measurement, 2−4 mice were housed in a cage. A preweighed amount of food (Beijing HFK Bio-Technology, Beijing, China) was given, and the mass consumed, evaluated as the difference between the original amount and the food left in the cage, including spillage, was measured after 48 h, which was performed every week from the age of 6 weeks to the age of 14 weeks. Similarly, water intake was determined by measuring the difference between the original water and the water left in the bottle after 48 h. Food intake (g/day per mouse) and water intake (mL/day per mouse) were calculated as the average of 9 weeks' values.

Blood glucose levels were determined by a monitoring system and corresponding test strips (LifeScan, Milpitas, CA) from the tail. Fasting blood glucose was measured at 8:00 a.m. after overnight fasting. For the insulin tolerance test (ITT) performed after 8 weeks of treatments, the mice were fasted overnight and injected intraperitoneally with 0.75 U/kg human regular insulin (Humulin R; Lilly, Saint Cloud, France). Blood glucose levels were determined from the tail before and at different time points after insulin injection as indicated in the figures.

Twenty-four hour urine was collected for each animal separately using a metabolic cage every other week (zeroth, second, fourth, sixth, and eighth weeks). Urinary and serum creatinine were measured using the standardized method. The level of urinary albumin was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Cusabio, Wuhan, Hubei, China). Urinary albumin excretion is presented as the ratio of urinary albumin to urinary creatinine (μg/mg).

Renal Morphometric and Immunohistochemistry Analysis. Mice were anesthetized with pelltobarbitalum natricum (30 mg/kg) (Sigma, St Louis, MO). The kidneys were removed, fixed in paraformaldehyde (4%) overnight, and processed for paraffin embedding. Paraffin sections (7 μm thick) were cut, deparaffinized, and stained with hematoxylin and eosin (H&E) for morphometric observation and glomerular cell quantification or with periodic acid Schiff (PAS) (Sigma) for mesangial expansion measurement. For immunoperoxidase staining, deparaffinized sections were preincubated in boiling sodium citrate buffer (pH 6.0) for antigen retrieval. Primary antibody against RAGE (1:100 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA) or glyoxalase I (GLO I; 1:100 dilution; Abcam, Cambridge, MA) was incubated with the sections at 4 °C overnight. The specificity of the labeling was established by negative control staining without the primary antibody. For visualization, the sections were developed with 3,3-diaminobenzidine (DAB) to produce a brown color and counterstained with hematoxylin. For glomerular cell number, glomerular area, and PAS-positive area analysis, the images were visualized and analyzed with a light microscope and the supplied image analysis software (QWin2.8, Leica). Mesangial expansion was calculated as the ratio of PAS-positive area to glomerular area. For each measurement, 20 randomly selected glomeruli per mouse and 4−6 mice per group were analyzed and quantified by the person who was blind to different groups and treatments.

Western Blotting. Tissue samples were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Applygen, Beijing, China) and centrifuged at 13000*g* for 30 min at 4 °C. Protein concentrations were determined using bicinchoninic acid (BCA) assay (CellChip, Beijing, China) according to the manufacturer's instructions. Protein of tissue lysates (50 μg) was loaded into sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and blotted. Primary antibodies against RAGE (1:500, SantaCruz), GLO I (1:2000, Abcam), and β-actin (C4) (1:5000, Santa Cruz) were used. Notably, to detect immunoglobulin G (IgG) in kidney homogenate, a mouse monoclonal antibody was used as the primary antibody against mouse β-actin, and the secondary antibody against mouse IgG was used to identify the sites of both primary antibody and the mouse renal IgG. β-Actin was used as a loading control for general protein contents. Quantitative analysis of the band density was performed using Image J software (National Institutes of Health, Bethesda, MD).

Measurement of Renal AGE Levels. Renal AGEs were determined using the OxiSelect AGE ELISA kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instructions. The AGE protein adducts present in the sample or standard were probed with a polyclonal antibody to N^{ε}-(carboxymethyl)lysine (CML), pentosidine, and other AGE structures. The quantity of AGE adduct in kidney protein samples was determined by comparing its absorbance with that of a known AGE−bovine serum albumin (BSA) standard curve and normalized to total protein content in the kidney.

Measurement of Renal Methylglyoxal Using HPLC. Kidney tissue (approximately 50 mg) was homogenized under liquid nitrogen, then reconstituted in 400 μL of phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.2), and sonicated (5 s on, 5 s pause, 20 times) on ice.

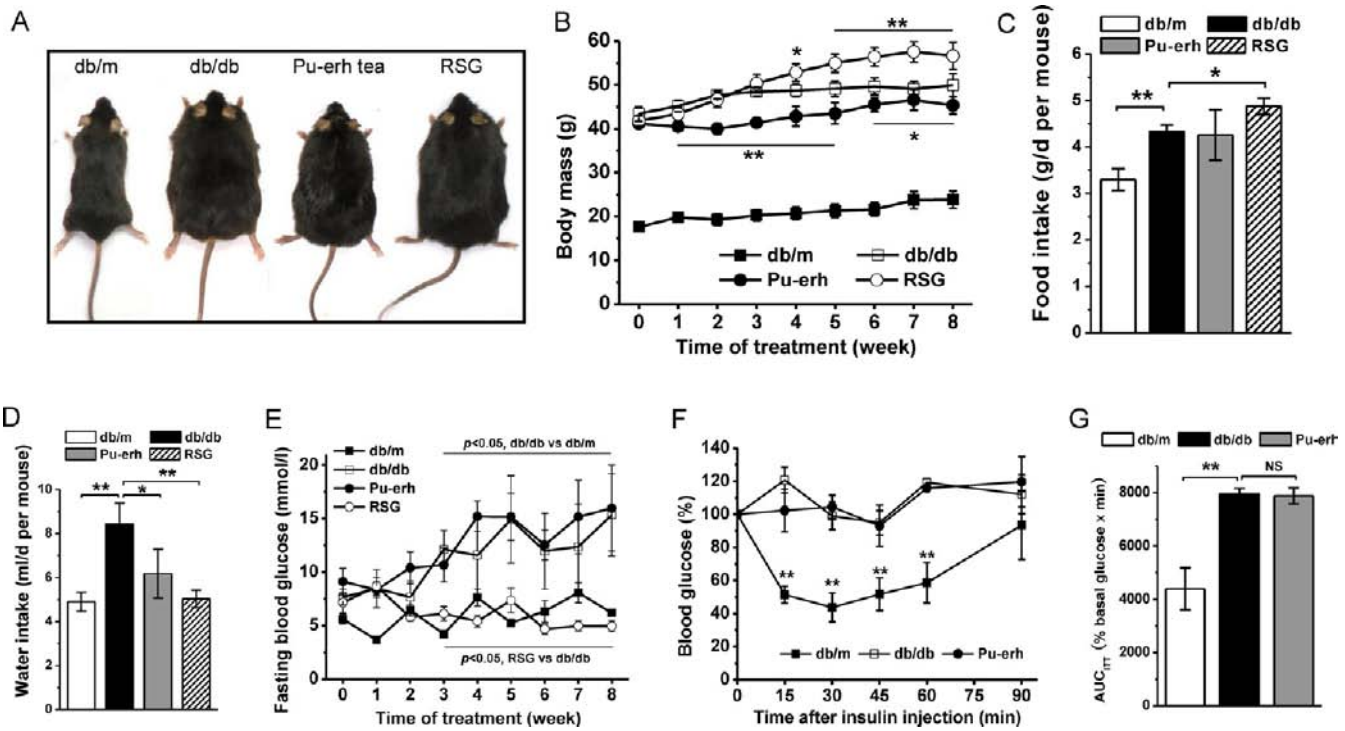

Figure 1. Impacts of Pu-erh tea on metabolic features of diabetic mice. (A) Body appearance of the nondiabetic control (*db/m*), diabetic mice (*db/db*), and *db/db* mice treated with 1 g kg^{-1} day^{-1} Pu-erh tea (Pu-erh) or with 4 mg kg^{-1} day^{-1} rosiglitazone (RSG). (B) Body mass changes in mice with different treatments over an observation of 8 weeks. Average daily food (C) and water (D) intake of mice with the indicated treatment. (E) Fasting blood glucose levels of mice with the indicated treatment. ITT in mice with the indicated treatment was performed at 14 weeks old. Blood glucose levels after insulin injection (0.75 U/kg) (F) and AUC of blood glucose during ITT (G). The data are presented as means ± SEM. n = 4−6 mice per group. Key: *, $p < 0.05$; **, $p < 0.01$; NS, not significant; vs *db/db* group.

The supernatant was collected by centrifugation (13000*g* for 10 min), and the protein concentration was measured using BCA assay. MG derivatization and quantization were performed using the method described in previous papers[23,24] with some modifications and normalized to total protein content in the kidney. Briefly, the supernatant was incubated with perchloric acid (PCA; 0.5 mol/L; Sigma), *o*-phenylenediamine (*o*-PD; 1 mmol/L; Sigma), and 5-methylquinoxaline (5-MQ; 25 μmol/L; Sigma) for 24 h at room temperature in the dark. The sample was further centrifuged at 13000*g* for 10 min, and the supernatant was filtered with a 0.22 μm membrane before addition to the HPLC sample vials. MG was quantified on a Waters HPLC system (1515 pump, 2487 detector, 717 plus autosampler) with a Dikma Diamonsil C-18 column (250 × 4.6 mm, 5 μm). Acetonitrile (40%) was kept for running the samples. Each sample was run for 15 min with a flow rate of 1.0 mL/min. Other analysis conditions were as follows: detector wavelength, 315 nm; sample injection volume, 10 μL; internal standard, 5-MQ.

Determination of MG Trapping of Pu-erh Tea in Vitro Using HPLC. MG (1.0 mmol/L) was incubated with vehicle (PBS) or Pu-erh tea (0.1, 1.0, and 10.0 mg/mL) at 37 °C for 0, 6, and 24 h. EC (0.29 mg/mL or 1.0 mmol/L) and aminoguanidine (AG; 0.74 mg/mL or 1.0 mmol/L; Sigma) were used as positive controls. The amounts of MG in samples with different treatments were measured by HPLC analysis described above. The MG trapping ratio was calculated as the percentage of trapped MG.

Statistical Analysis. All data were expressed as the mean ± standard error of the mean (SEM). The results were analyzed statistically using SPSS 13.0 (SPSS, Chicago, IL). For analysis among experimental and control groups, p values were calculated with an unpaired Student t test (two-tailed test). Statistical significance was accepted at $p < 0.05$.

RESULTS

Levels of Catechins in Tested Pu-erh Tea Powder. We first analyzed the levels of catechins in Pu-erh tea powder. The major catechins present were gallic acid (3.019% ± 0.032%, w/w), C (0.645% ± 0.028%, w/w), EC (0.433% ± 0.002%, w/w), and EGCG (0.069% ± 0.002%, w/w).

Effects of Pu-erh Tea on Metabolic Features of Diabetic Mice. Diabetic (*db/db*) mice (n = 6) developed early-onset obesity compared to nondiabetic (*db/m*) mice (n = 5), with a mean body mass of 40 g at 6 weeks of age, and their mass continued to increase during the observation. The *db/db* mice treated with Pu-erh tea were visibly less fat and increased in body mass more slowly than the *db/db* mice treated with vehicle, beginning 1 week after the start of treatment (n = 4, $p <$ 0.01 from the first to the fifth week and $p < 0.05$ from the sixth to the eighth week of treatment), while the *db/db* mice treated with RSG exhibited faster increases in body mass than *db/db* mice treated with vehicle, and after 4 weeks, they were heavier (n = 5, $p < 0.05$ at the fourth week and $p < 0.01$ from the fifth to the eighth week of treatment) (Figure 1A,B).

Compared to *db/m* mice, during the experimental period, *db/db* mice were polyphagic (eating 4.33 ± 0.15 vs 3.30 ± 0.23 g/day, n = 6 and 5, $p < 0.01$) and polydipsic (drinking 8.45 ± 0.94 vs 4.91 ± 0.43 mL/day, n = 6 and 5, $p < 0.01$). Pu-erh tea treatment significantly reduced water consumption (6.18 ± 1.11 vs 8.45 ± 0.94 mL/day, n = 4 and 6, $p < 0.05$), but there was no significant difference in food intake (4.26 ± 0.55 vs 4.33 ± 0.15 g/day, n = 5 and 6) compared to that of vehicle-treated *db/db* mice. RSG treatment increased food intake (4.88 ± 0.18 vs 4.33 ± 0.15 g/day, n = 5 and 6, $p < 0.05$) and reduced the

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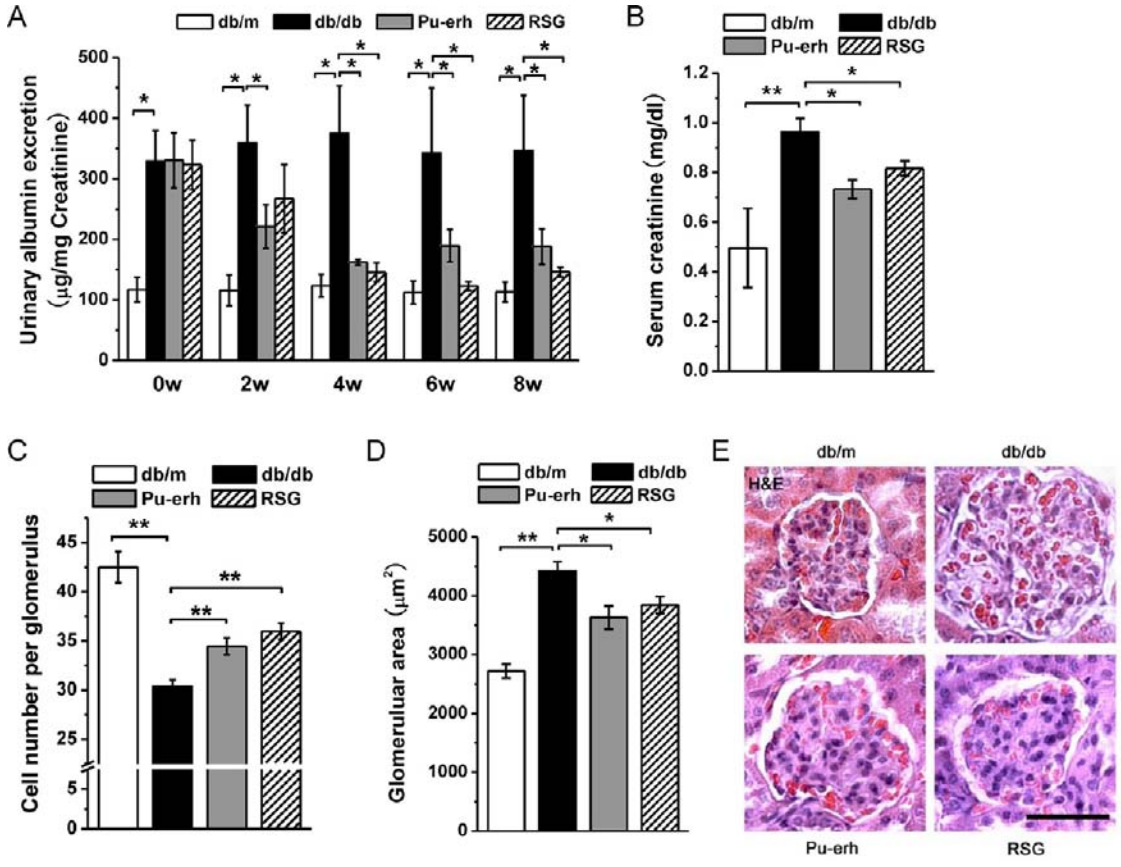

Figure 2. Pu-erh tea or rosiglitazone treatment ameliorates renal dysfunction in diabetic mice. (A) Quantification of urinary albumin excretion (μg of albumin/mg of creatinine) by ELISA in urine collected for 24 h after treatment for 0, 2, 4, 6, and 8 weeks. (B) Serum creatinine was measured after treatment for 8 weeks. Comparisons of the glomerular cell number (C) and glomerular area (μm^2) (D) among groups. The average value was obtained from analyses of 20 glomeruli per mouse. (E) Representative H&E staining images of the glomerulus (scale bar 50 μm). The data are presented as means ± SEM. n = 4−6 mice per group. Key: *, $p < 0.05$; **, $p < 0.01$.

water consumption (5.04 ± 0.39 vs 8.45 ± 0.94 mL/day, n = 5 and 6, $p < 0.01$) in *db/db* mice (Figure 1C,D).

From the third week, *db/db* mice showed higher fasting blood glucose (>12 mmol/L) compared to *db/m* mice (about 6 mmol/L) (n = 6 and 5, $p < 0.05$). There was no significant difference in fasting blood glucose levels between the *db/db* mice treated with Pu-erh tea and the *db/db* mice treated with vehicle. Diabetic *db/db* mice treated with RSG maintained a normal fasting blood glucose level (Figure 1E). Insulin sensitivity measured by the reduction in blood glucose after insulin injection (0.75 U/kg) markedly decreased by ∼50% in *db/db* mice compared to *db/m* mice (7951 ± 213 vs 4393 ± 788 for AUC_{ITT} (AUC = area under the curve), n = 6 and 5, $p < 0.01$). There was no significant difference in insulin sensitivity between Pu-erh tea-treated *db/db* mice (7882 ± 297 for AUC_{ITT}, n = 4) and vehicle-treated mice (7951 ± 213 for AUC_{ITT}, n = 5) (Figure 1F,G).

Renal Dysfunction of Diabetic Mice Was Markedly Ameliorated by Pu-erh Tea Despite Persistent Hyperglycemia. Diabetic renal impairment was markedly attenuated by Pu-erh tea administration (Figure 2). We detected a 2−4-fold increase in urinary albumin excretion in *db/db* mice compared to *db/m* mice during observation. After 2 weeks of administration with Pu-erh tea or 4 weeks with RSG, the diabetic mice exhibited a significant reduction in albuminuria (Figure 2A).

Similarly, the serum creatinine level significantly increased in *db/db* mice (0.96 ± 0.05 mg/dL, n = 6) compared with that in *db/m* mice (0.50 ± 0.06 mg/dL, n = 5, $p < 0.01$). Treatment with Pu-erh tea as well as RSG for 8 weeks reduced the elevated serum creatinine levels in diabetic *db/db* mice (0.73 ± 0.04 mg/dL for Pu-erh tea treatment and 0.82 ± 0.03 mg/dL for RSG treatment, n = 4 and 5, $p < 0.05$ vs vehicle-treated *db/db* mice) (Figure 2B).

As glomerular endothelial cells have greatly contributed to the functional maintenance of glomerular filtration,[25] we examined the cell number in glomeruli using kidney sections stained with H&E. The glomerular cell population was significantly reduced in *db/db* mice compared to *db/m* mice (30.35 ± 0.68 vs 42.51 ± 1.58 cells per glomerulus, n = 6 and 5, $p < 0.01$). However, treatment with Pu-erh tea as well as RSG noticeably suppressed glomerular cell loss in *db/db* mice (34.45 ± 0.84 cells per glomerulus for Pu-erh tea treatment and 35.97 ± 0.83 cells per glomerulus for RSG treatment, n = 4 and 5, $p < 0.01$ vs vehicle-treated *db/db* mice) (Figure 2C,E). In addition, treatment with Pu-erh tea as well as RSG significantly reduced glomerular hypertrophy (3629.63 ± 195.91 μm^2 for Pu-erh tea, n = 4; 3842.60 ± 141.87 for RSG, n = 5; 4415.99 ± 165.13 for *db/db*, n = 6; 2721.63 ± 119.26 for *db/m*, n = 5; $p < 0.05$) (Figure 2D,E).

Mesangial Matrix Expansion and Immunoglobulin IgG Deposition in Kidney of *db/db* Mice Were Reduced by Pu-erh Tea. Diffuse expansion of the mesangial matrix is

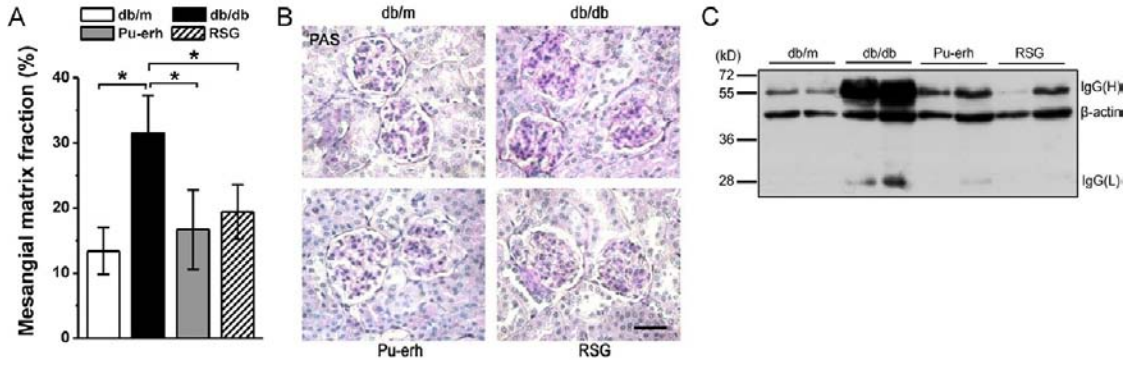

Figure 3. Pu-erh tea imparted protection from the structural abnormalities of diabetic nephropathy. (A) Quantitative measurement of extracellular mesangial matrix expansion. The mesangial matrix fraction was calculated as the ratio of mesangial area to glomerular area. The average value was obtained from analyses of 20 glomeruli per mouse. The data are presented as means ± SEM. $n = 4–6$ mice per group. The asterisk indicates $p < 0.05$. (B) Representative images of the glomerulus with PAS staining (scale bar 50 μm). (C) Western blot analysis of IgG in mouse renal tissue using a secondary antibody which recognizes the mouse IgG and the mouse antiactin IgG. The IgG heavy chain (IgG(H), top bands) and light chain (IgG(L), bottom bands) were detected. β-Actin (middle bands) was used as a loading control.

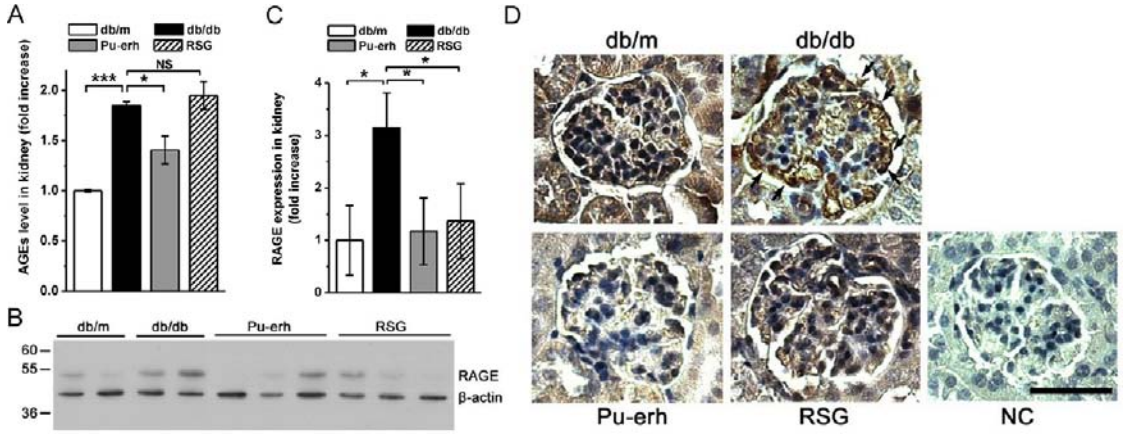

Figure 4. Pu-erh tea prevented diabetes-induced accumulation of AGEs and led to a decreased RAGE level in glomeruli. (A) Detection of the renal AGE level (fold increase vs that of *db/m*) by ELISA. The AGE level was normalized to total protein concentration in the tissue sample. $n = 4–6$ mice per group. (B) Western blot analysis of RAGE protein levels in mouse kidney and (C) densitometric quantification of them ($n = 3$ independent experiments). (D) Immunohistochemical staining for RAGE in kidney sections. The changes in the amount of RAGE staining (arrow) appeared most evident in glomeruli. NC = negative control stained in the absence of primary antibody. The scale bar represents 50 μm. The data are presented as means ± SEM. Key: *, $p < 0.05$; ***, $p < 0.001$; NS, not significant.

considered a hallmark pathological feature of diabetic nephropathy.[6] *db/db* mice showed accelerated mesangial matrix expansion, characterized by a ~3.5-fold increase in PAS-stained area compared to that of *db/m* mice (31.5% ± 5.8% vs 13.4% ± 3.6%, $p < 0.05$). However, treatment with Pu-erh tea as well as RSG reduced mesangial expansion in *db/db* mice (16.7% ± 6.1% for Pu-erh, 19.4 ± 4.2% for RSG, $p < 0.05$ vs vehicle-treated *db/db* mice) (Figure 3A,B).

Immune complex deposition in glomeruli has been associated with the development of albuminuria and could promote tissue injury.[26,27] We examined whether Pu-erh tea prevented glomerular IgG deposition in diabetic mice. Western blot analysis of IgG in mouse renal tissue showed significantly increased accumulation in *db/db* mice compared to that in *db/m* mice, while this phenomenon was largely prohibited by both Pu-erh tea and RSG treatment (Figure 3C).

Pu-erh Tea Attenuated Renal AGE Accumulation and Re-Established Normal Expression of RAGE. AGEs are known to accumulate in diabetic subjects and may be important mediators of untoward effects of hyperglycemia.[8] Glomerular IgG deposition may be due to the increased levels of circulating antibodies targeting the modified proteins in diabetes.[27] Therefore, alternation of AGE accumulation possibly underlies the beneficial effects of Pu-erh tea on diabetic nephropathy. Measurements of AGEs using ELISA revealed an increase in the kidneys from *db/db* mice compared to kidneys from *db/m* littermate mice (1.85 ± 0.04-fold increase compared to that of *db/m* mice, $n = 5$ (*db/m* mice) and 6 (*db/db* mice), $p < 0.001$). The diabetes-induced accumulation of AGEs in kidneys was attenuated by Pu-erh tea (1.41 ± 0.14-fold increase compared to that of *db/m* mice, $n = 4$, $p < 0.05$ vs *db/db* mice) but not by RSG (1.95 ± 0.14-fold increase compared to that of *db/m* mice, $n = 5$, not significant (NS)) (Figure 4A).

Given that AGEs exert inflammatory and oxidative stress insults inducing diabetic nephropathy mainly through the receptor RAGE,[28] we examined the expression of RAGE in the kidneys. Compared with normal controls, expression of RAGE was significantly increased in the diabetic kidney, the levels of which were decreased with treatment with Pu-erh tea or RSG (Figure 4B,C). The changes in the amount of RAGE staining appeared most evident in the glomerulus, as shown by immunohistochemistry (Figure 4D).

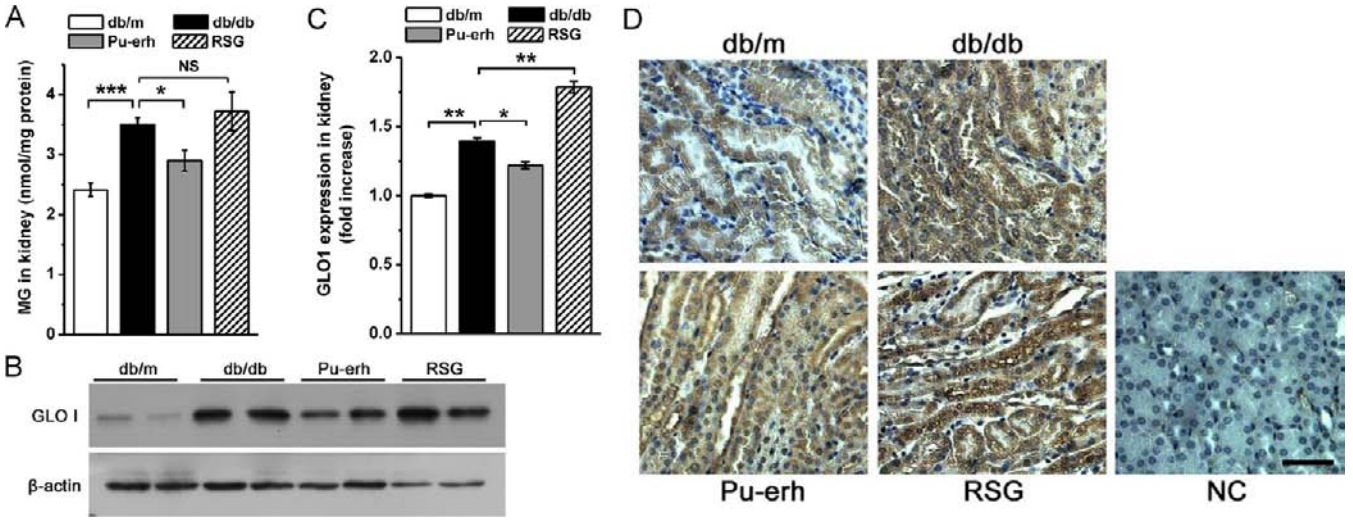

Figure 5. Pu-erh tea decreased renal MG content independent of GLO I. (A) Quantification of MG content in kidney using HPLC (nmol/mg, normalized to the total protein concentration in tissue samples). n = 4−6 mice per group. (B) Western blot analysis of GLO I protein levels in mouse kidney and (C) densitometric quantification of them (n = 3 independent experiments). (D) Immunohistochemical staining for GLO I in kidney sections. The changes in the amount of GLO I staining appeared most evident in the tubulointerstitium. NC = negative control stained in the absence of primary antibody. The scale bar represents 50 μm. The data are presented as means ± SEM. Key: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant.

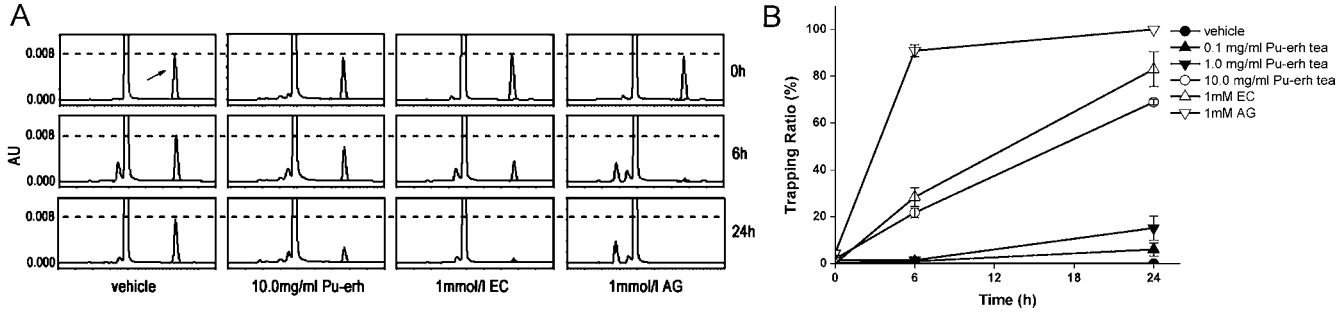

Figure 6. MG trapping of Pu-erh tea detected by the HPLC method. (A) Representative chromatograms of samples with different treatments. The arrow indicates the peak of 2-MQ, a specific stable product formed by derivatization of MG when the sample is incubated with o-PD. (B) The trapping ratio (%) was calculated as the percentage of trapped MG. MG (1 mmol/L) was incubated with vehicle, Pu-erh tea (0.1, 1.0, and 10.0 mg/mL), EC (0.29 mg/mL or 1.0 mmol/L), or AG (0.74 mg/mL or 1.0 mmol/L) at 37 °C for 0, 6, and 24 h. The data are presented as means ± SEM. n = 4 independent experiments.

Pu-erh Tea Decreased Renal MG through Both Reducing MG Production and Enhancing MG Clearance Independent of GLO I Activation. To gain insight into the mechanisms underlying the decrease of AGE accumulation induced by Pu-erh tea, we examined any changes in MG levels, the main precursor of AGE formation. We found that the content of MG in the kidneys was higher in diabetic *db/db* mice (3.50 ± 0.11 nmol/mg of protein, n = 6) compared to *db/m* mice (2.42 ± 0.11 nmol/mg of protein, n = 5, $p < 0.001$). However, the increases in the MG content were attenuated in *db/db* mice treated with Pu-erh tea (2.90 ± 0.17 nmol/mg of protein, n = 4, $p < 0.05$). We found no significant change in MG content in *db/db* mice treated with RSG (3.72 ± 0.72 nmol/mg of protein, n = 5) (Figure 5A).

A possibility is that the clearance of MG in tissue would be enhanced by Pu-erh tea. As GLO I is the key enzyme of the glyoxalase system which catalyzes the conversion of MG to D-lactate,[29] we first examined the protein expression of GLO I in kidney. We found that the expression of GLO I was higher in *db/db* mice compared to *db/m* mice, the level of which was reversed by Pu-erh tea but aggravated by RSG treatment (Figure 5B,C). Immunohistochemisty analysis showed that GLO I was universally expressed in the tubulointerstitium (Figure 5D). These results showed a good proportion between the MG level and GLO I expression in all groups.

Pu-erh Tea Enhanced MG Clearance by MG Trapping in Vitro. A previous study showed that EC, present largely in tea, could trap MG and form an adduct.[12] Given this, we examined whether Pu-erh tea could directly trap MG using an in vitro analysis system. In vitro, the concentration of MG was 1 mmol/L, which is approximately 10^3-fold more than the physiologic concentration.[23] Thus, a higher concentration of Pu-erh tea was used to detect the MG trapping effect of Pu-erh tea. We found that incubation of MG (1.0 mmol/L) with Pu-erh tea led to a decreased amount of MG detected by HPLC in a time- and dose-dependent manner. Pu-erh tea at 10 mg/mL for 24 h was found to trap MG by 68.98% ± 1.18%. These values suggested that Pu-erh tea could directly trap MG and form a stable adduct. As a previous study showed EC was able to trap MG, we further measured the trapping effect of EC by the HPLC method. Similar to 10 mg/mL Pu-erh tea, EC could

trap MG by 82.99% ± 7.46% at a 0.29 mg/mL (1 mmol/L) concentration (Figure 6A,B).

DISCUSSION

Although Pu-erh tea did not influence the blood glucose level or insulin sensitivity in *db/db* mice, diabetic mice treated with Pu-erh tea showed decreased urinary albumin and serum creatinine and attenuated pathologic renal damage characterized by reduced mesangial expansion, glomerular hypertrophy, and glomerular cell loss. These effects were similar to those of RSG, a well-known antidiabetic drug through insulin sensitization and reduction in the level of blood sugar. RSG, however, improved glycemic control and diabetes-induced renal impairment at the expense of body mass and body fat gain, which was demonstrated recently to be correlated with the activation of central nervous system (CNS) nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ).[30,31] These values suggest that Pu-erh administration markedly ameliorated diabetic renal dysfunction without the side effects of thiazolidinedione drugs.

Poor glycemic control plays a key role in the development of severe complications.[32] AGEs have been considered as important mediators of untoward effects of hyperglycemia,[8] because the progression of diabetic complications could be prohibited by inhibitors of glycoxidation products that do not alter glycemia.[33] AGEs are generated by the Maillard reaction through nonenzymatic glycation of protein amino groups.[8] AGEs stimulate a variety of cellular responses, including proinflammatory, matrix production, and profibrotic responses, via a specific cell-surface receptor, RAGE, on several cell types, including glomerular mesangial cells.[9,10] In addition, immune complexes deposited in glomeruli have been associated with an increased level of circulating antibodies to AGEs and may play a role in albuminuria and tissue injury.[26,27] Our study found that Pu-erh tea inhibited AGE accumulation and led to a decreased RAGE expression and glomerular IgG deposit though there was no significant effect on hyperglycemia. Thus, the inhibition of AGE formation by Pu-erh tea may largely contribute to the amelioration of diabetic nephropathy. It has been shown that genetic deletion or pharmacological blockade of RAGE prevents renal dysfunction and early structural changes in the glomerulus associated with diabetic nephropathy.[34,35] RSG could reduce AGE-induced renal toxicity by down-regulating RAGE expression and NFkB activation in the early phase of diabetic nephropathy.[36] Consistent with this, we observed that RSG decreased RAGE expression and ameliorated the progression of diabetic nephropathy but did not alter AGE accumulation. These results indicated that Pu-erh tea prevented diabetes-induced accumulation of AGEs and re-established a normal RAGE level, effects which are distinct from those of RSG. The mechanism underling the effects of RSG on diabetic nephropathy might involve the blockade of downstream pathways activated by excessive AGE−RAGE interaction[36] (Figure 7).

Furthermore, we found a decrease in the amount of MG (probably extensive carbonyl compounds), the main precursor of AGE formation.[8] Carbonyl compounds have been proposed to be generated from the glycation process and lipid peroxidation.[37] Pu-erh tea could reduce body mass gain in *db/db* mice in our study and attenuate visceral fat accumulation and hyperlipidemia in a rat model of high fat diet-induced obesity described in previous reports.[16,17] Therefore, prevention of MG production from lipid metabolism by Pu-erh tea

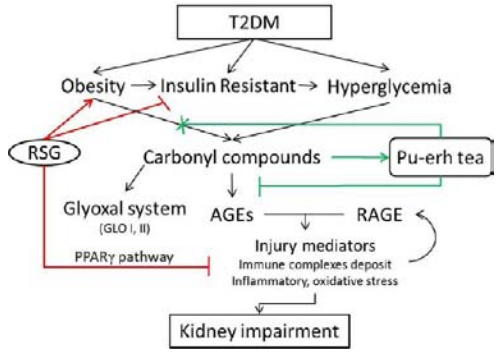

Figure 7. Schematic diagram showing distinct molecular mechanisms of Pu-erh tea and rosiglitazone in alleviating diabetic nephropathy. Inhibition of AGE formation by Pu-erh tea through MG trapping disrupted the carbonyl pathway, re-established a normal expression of GLO I and RAGE, and finally protected against kidney impairment in diabetes. Distinctly, RSG attenuated renal dysfunction by improving the insulin sensitivity and inhibiting RAGE signaling through the PPAR-γ pathway, but aggravated obesity.

administration may in part contribute to the decrease of MG accumulation in diabetic tissue. On the other hand, possibly more important, it may be due to the enhancement of MG clearance. Given that GLO I is the key enzyme of the glyoxalase system in the process of carbonyl compound clearance,[29] we further examined whether GLO I activation was stimulated by Pu-erh tea and subsequently enhanced the clearance of MG. Consistent with the findings that GLO I increases with the accumulation of MG in the early stage of diabetic nephropathy[38] or in the process of aging,[39] we found a higher level of GLO I expression in the tubulointerstitium of *db/db* mice compared to the nondiabetic control, while Pu-erh tea re-established a normal expression of GLO I. This indicated that the enhancement of MG clearance induced by Pu-erh tea might be independent of GLO I. In vitro, the result that fewer molecules of MG were detected by HPLC when incubated with Pu-erh tea indicated that Pu-erh tea components could directly react with MG. A previous study showed a covalent linkage could be formed between the C1 position of the MG and either the C6 or the C8 position of the EC A ring.[12] This process was defined as "carbonyl trapping". The carbonyl trapping effect of EC was also detected by the HPLC method in our study. EC, C, EGCG, and gallic acid are the major catechins present in Pu-erh tea powder. The C6 or C8 position of the A ring of C, EC, and EGCG and the C3 or C5 position of gallic acid have similar chemical environments. Therefore, the clearance of MG might be enhanced by the carbonyl trapping effect of catechins present in Pu-erh tea, such as EC, C, EGCG, and gallic acid. In summary, these values implied that Pu-erh tea inhibited AGE formation through MG trapping, and catechins may be the active components that contributed to the benefits of Pu-erh tea on diabetic nephropathy.

In conclusion, we demonstrated that Pu-erh tea attenuated renal dysfunction and glomerular lesions associated with diabetic nephropathy in an animal model, which was, in part, due to the inhibition of AGE formation through both prevention of carbonyl production and carbonyl trapping (Figure 7). We could not exclude the other possible effects of Pu-erh tea, such as antioxidant effects, anti-inflammatory effects, or modulation of blood pressure, that may ameliorate diabetic nephropathy. Nevertheless, our study implies that inhibition of AGE formation rather than glymeric control alone is important

for the prevention for diabetic complications. Finally, our findings provide a promising dietary supplementary approach for diabetic patients with renal complication.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AG, aminoguanidine; AGE(s), advanced glycation end product(s); C, (+)-catechin; EC, (−)-epicatechin; EGCG, (−)-epigallocatechin; GLO I, glyoxalase I; ITT, insulin tolerance test; MG, methylglyoxal; RAGE, receptor for advanced glycation end products; RSG, rosiglitazone maleate

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