JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Polyphenols of *Hibiscus sabdariffa* Improved Diabetic Nephropathy via Attenuating Renal Epithelial Mesenchymal Transition

Yi-Sun Yang,[†,‡,§] Chau-Jong Wang,[§,∥] Chien-Ning Huang,[†,‡] Mu-Lin Chen,[∥] Ming-Jinn Chen,[⊥] and Chiung-Huei Peng*[,#]

[†]Department of Internal Medicine, Chung-Shan Medical University Hospital, Number 110, Section 1, Chien-Kuo North Road, Taichung 402, Taiwan

[‡]Institute of Medicine, Chung-Shan Medical University, Number 110, Section 1, Chien-Kuo North Road, Taichung 402, Taiwan

[∥]Institute of Biochemistry and Biotechnology, Chung-Shan Medical University, Number 110, Section 1, Chien-Kuo North Road, Taichung 402, Taiwan

[⊥]Taiwan Yu Shing Biotech Co., Ltd. No. 1, Alley 3, Lane 320, Pong Yi Road, Taiping Dist, Taichung City, Taiwan

[#]Division of Basic Medical Science, Hungkuang University, Number 34, Chung Chie Road, Shalu County, Taichung 433, Taiwan

Supporting Information

**ABSTRACT:** We previously reported that *Hibiscus sabdariffa* polyphenol extracts (HPE) are beneficial for diabetic nephropathy. Since an epithelial to mesenchymal transition (EMT) is critical in renal fibrosis, the present study aimed to investigate whether HPE could prevent EMT of tubular cells. Treatment of HPE reduced angiotensin II receptors (AT)-1 and transforming growth factor $\beta$1 (TGF-$\beta$1) evoked by high glucose and recovered the increased vimentin and decreased E-cadherin. HPE decreased fibronectin, thus avoiding EMT and accompanying fibrosis. AT-1 was upstream to TGF-$\beta$1, while there were recruitment signals between AT-1 and TGF-$\beta$1. Scan electron microscopy (SEM) and immunohistochemistry (IHC) revealed that the interacting filaments of tubular cells disappeared when treated with high glucose, and type IV collagen of tubulointerstitial decreased in diabetic kidneys. Treatment of HPE recovered morphological changes of cell junction and basement membrane. We suggest that HPE has the potential to be an adjuvant for diabetic nephropathy by regulating AT-1/TGF-$\beta$1 and EMT.

**KEYWORDS:** *Hibiscus sabdariffa polyphenols, epithelial to mesenchymal transition, diabetic nephropathy*

## INTRODUCTION

Diabetes mellitus is increasing worldwide and has a profound impact on human health.[1] Despite improvements in the diagnosis and management of diabetes, most diabetic patients progress to renal dysfunction, with hyperglycemia and hypertension being the main risk factors. However, many patients still continue to develop diabetic nephropathy despite intensive efforts to achieve optimal control of these parameters. Once renal function begins to deteriorate, there is concern about the dosage of most antidiabetic drugs since the metabolic and pharmacological controls become more difficult and complicated. Hence, new treatment paradigms are needed urgently.[2]

*Hibiscus sabdariffa*, whose calyces are used as a daily drink in North Africa and Middle and South Asia, was shown to be bioeffective in many ways.[3,4] At least 18 phenolic compounds were found in the polyphenol extracts of *H. sabdariffa* L. calyx (HPE).[5] HPE attenuated the high glucose-induced injuries via regulating connective tissue growth factor (CTGF) and receptor of advanced glycation end-products (AGEs).[6] It was suggested that HPE improved oxidative markers and prevented renal damage in type 1 diabetic rats.[7] Using a type 2 diabetic rat exhibiting insulin resistance,[8] we recently demonstrated the effects of HPE on antihyperglycaemia, antihyperlipidaemia, weight recovery, and modulation of pathogenic signals.[5] Besides, we have successfully shown that HPE has benefit in preventing diabetic nephropathy by inhibiting albuminuria and hyperfiltration and reversing renal fibrosis and the expressions of angiotensin II receptors (AT).[9]

Renal fibrosis, characterized by abnormal accumulation of extracellular matrix (ECM) in glomeruli and tubulointerstitium,[10,11] is a common feature occurring in chronic kidney disease and diabetic nephropathy.[12] Although the expanded interstitial fibroblasts result in fibrosis,[13] emerging evidence suggests that epithelial to mesenchymal transition (EMT) of renal tubular epithelial cells plays a critical role in renal fibrosis. Decreasing E-cadherin and increasing vimentin, tubular epithelial cells undergo a phenotypic transformation into matrix-producing fibroblasts.[14] It was reported that high glucose induced EMT and increased the synthesis fibronectin in rat NRK-52E cells. Treatment of AT-1 antagonist attenuated high glucose-induced TGF-$\beta$1 elevation and ERK1/2 phosphorylation thus reduced EMT.[15]

In the present study, we observe the high glucose-induced EMT of human tubular cells—the molecular alteration, signal transduction and morphologic changes with electron-micro-

Received: May 11, 2013
Revised: July 10, 2013
Accepted: July 12, 2013
Published: July 12, 2013

scopic and immunohistological examinations—and investigate whether HPE would prevent or relieve the occurrence of EMT.

## MATERIALS AND METHODS

**Preparation of HPE.** *Hibiscus sabdariffa* L. calyx was purchased from Taitung Area Farmer's association. HPE was prepared as previously reported, and its chemical composition was analyzed with liquid chromatography−tandem mass spectrometry (LC−MS/MS).[5] At least 18 phenolic compounds were found in HPE, including hibiscus acid, hibiscus acid 6-methyl ester, gallic acid, 5-hydroxymethylfurfural, protocatechuic acid, 5-caffeoyl quinic acid, feruloyl derivative, chlorogenic acid, 4-caffeoyl quinic acid, caffeic acid, galloyl ester, feruloyl qunic derivative, kaempferol-3-glucoside, quercetin derivative, tiliroside, delphinidin-3-sambubioside, cyanidin-3-sambubioside and one unidentified compound (shown in the Supporting Information).

**Cell Culture.** HK-2 cells were cultured in keratinocyte serum free medium (KSFM, Gibco BRL) with 5 ng/mL epidermal growth factor and 40 $\mu$g/mL bovine pituitary extract. The cells were grown in a humidified incubator at 37 °C in an atmosphere of 5% $CO_2$ and 95% air. HK-2 cells were inoculated into a 6 cm dish at a density of $5 \times 10^5$ cells for 24 h and then treated with 30 mM of glucose with or without various concentrations (0.05−5 mg/mL) of HPE for 48 h. Cells were then harvested and processed for Western blot analysis. For testing the signal cascades, Telmisartan, the angiotensin receptor blocker, was purchased from Toronto Research Chemicals, Canada. TGF-$\beta$1 receptor kinase inhibitor was from Calbiochem, Germany.

**Cytotoxicity Assay.** HK-2 cells were seeded at a density of $1 \times 10^6$ cells/mL in a 24-well plate and incubated with compounds at various concentrations for 24 h. Thereafter, the medium was changed and cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) for 4 h. The viable cell was directly proportional to the production of formazan. Following dissolution in isopropanol, the result was read at 563 nm with a spectrophotometer (Hitachi, U-3210).

**Western Blot Analysis.** Cells were harvested into lysis buffer containing 50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 5% mercaptoethanol and then lysed by sonication. Cell lysate was centrifuged at 4 °C, 10000 rpm for 20 min, and the supernatant was collected as the protein sample. After quantifying, equal amounts of protein samples (50 $\mu$g) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulosemembranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk powder with 0.1% Tween-20 in TBS and then incubated with the primary antibody at 4 °C overnight against the following targets: AT-1 (1:1000), AT-2 (1:1000), TGF-$\beta$1 (1:500), vimentin (1:1000), E-cadherin (1:500), and fibronectin (1:1000). Antibodies of AT-1 and AT-2 were from Epitomics, CA. Antibodies of TGF-$\beta$1 and vimentin were from Santa Cruz, CA. Antibodies of fibronectin and E-cadherin were from Novus biological, USA. Afterward, membranes were washed three times with 0.1% Tween-20 in TBS and incubated with the secondary antibody (1:5000) conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Band detection was thereafter revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed in FUJFILM Las-3000 (Tokyo, Japan). Protein quantitative was determined by densitometry using FUJFILM-Multi Gauge V2.2 software.

**Scan Electron Microscopy (SEM).** Cells were inoculated on coverslips in a 24-well plate at a density of $4 \times 10^4$ cells per well. After various treatments, culture media was removed and samples were rinsed with PBS. The samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 30 min, washed with PBS for 10 min, and then dehydrated with alcohol gradient at 70%, 80%, 90% (15 min for each conc.), 95% (10 min, twice), and 100% (10 min, three times) in a critical point dryer (HCP-2, Hitachi Ltd., Tokyo, Japan). After mounted onto aluminum stubs and sputter coated with gold, samples were examined using a scanning electron microscope (Hitachi 2400).

**Diabetic Animals.** The animal experimental project was approved by the Animal Model Experimental Ethics Committee of Chung-Shan Medical University based on the European Community guidelines (IACUC approval No: 1007). A type 2 diabetic model was carried out according to our previous publication.[9] Briefly, male Sprague−Dawley (SD) rats (weight 250 ± 20 g) were divided into several groups ($n$ = 8 for each group), including the control (normal diet), FAT + STZ (high fat diet and streptozocin injection), FAT + STZ + low HPE (with 100 mg/kg HPE added), and FAT + STZ + high HPE (with 200 mg/kg HPE added). Safety evaluation revealed that HPE did no harm to liver, kidney or cardiovascular tissue. After rats were sacrificed, the kidney chops were added to radioimmunoprecipitation assay (RIPA) buffer and protein inhibitors and homogenized at 4 °C. The tissue homogenates were centrifuged (10000× $g$ for 20 min at 4 °C), and the resulting supernatants (whole-tissue extracts) were used for Western blot analysis.

**Immunohistochemistry (IHC).** The kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned into 4 $\mu$m, and processed for IHC examination. The primary antibody of type IV collagen (1:1000) was from Novus biologicals. Immunostain was completed using the UltraSensitive S−P kit (Maxim Co., streptavidin peroxidase method).

**Statistical Analysis.** The statistical software SPSS v.12.0 was used to analyze the data. One-way ANOVA was performed ($p < 0.05$), while Bonferroni's Multiple Comparison was used for post test.

## RESULTS

**HPE Is Nontoxic to HK Cells in the Whole Experiment.** At first, we tried the nontoxic dose range of HPE in HK-2 cells. Cell viability was not significantly altered by 0.05 mg/mL−1 mg/mL. However, nearly 80% of cells died as the dose reached 5 mg/mL. Hence, 1 mg/mL of HPE was used as the maximum dose in the whole experiment (Figure 1).

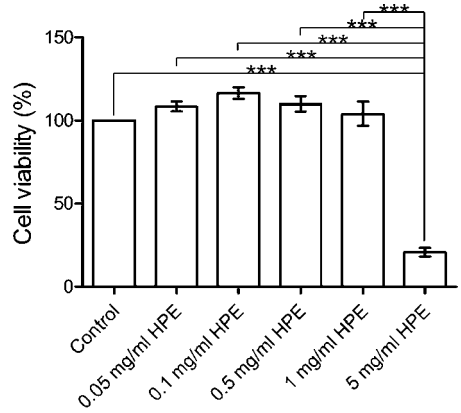


**Figure 1.** Cytotoxicity test of HPE. HK-2 cells were incubated for 24 h with or without different concentrations of HPE. Cell viability was calculated as percentage compared with the control group. Data are presented as means ± SD ($n$ = 3) and analyzed with ANOVA. ***$p < 0.001$, compared with the control.

**HPE Reduces High Glucose-Induced AT-1.** Cells were cultured under normal or high glucose concentrations. Figure 2 shows that AT-1 was elevated 2.7 fold by 30 mM of glucose. Treatment of HPE dose-dependently reduced the expression of AT-1 evoked by high glucose; 0.5 and 1 mg/mL significantly reduced AT-1 by 76 and 87%, respectively, even below the level of the control. The expression of AT-2 was also increased by high glucose and inhibited by HPE. Noticeably, HPE per se did not alter the AT expressions (Figure 2A).

**HPE Reduces High Glucose-Induced TGF-$\beta$1.** Compared with the control, high glucose increased the level of TGF-


dx.doi.org/10.1021/jf4020735 | J. Agric. Food Chem. 2013, 61, 7545−7551

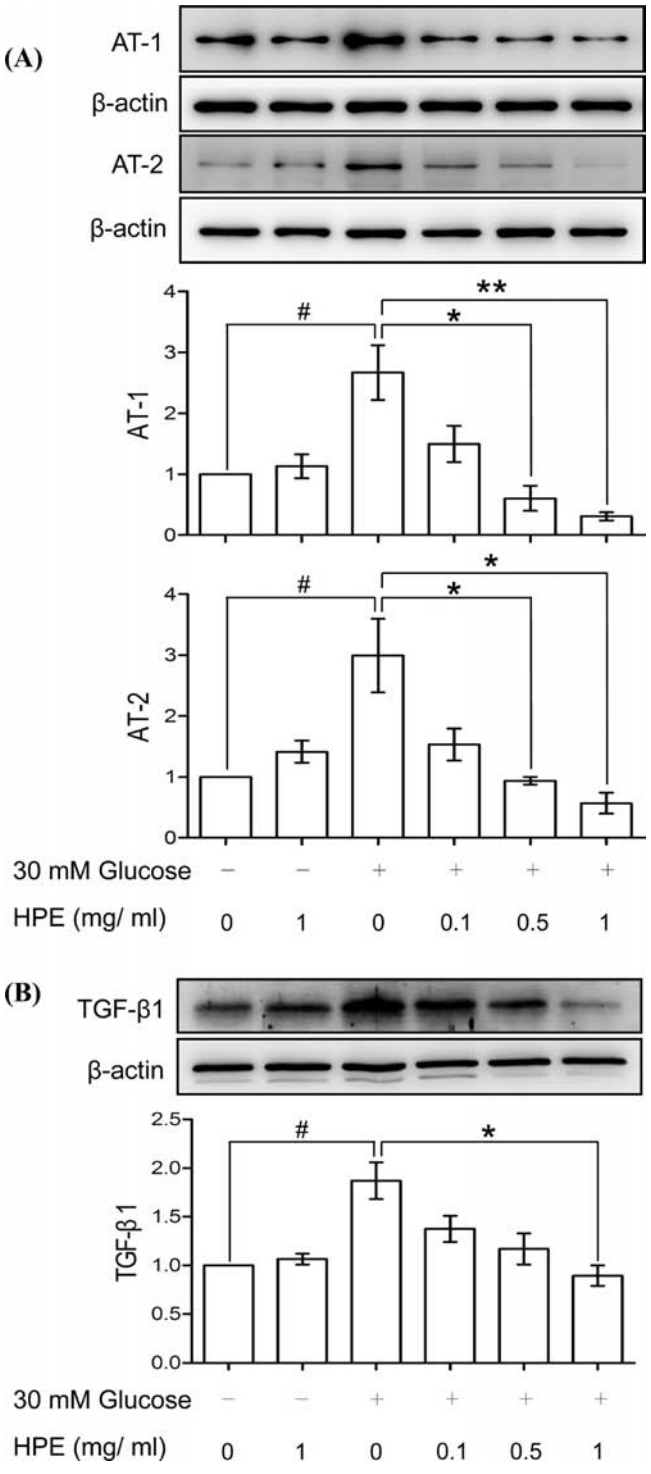


**Figure 2.** Effect of HPE on AT and TGF-$\beta$1. HK-2 cells were incubated for 24 h under normal or high glucose (30 mM) condition with or without different concentrations of HPE. Cell proteins were extracted and analyzed with Western blot. (A) AT-1 and AT-2, (B) TGF-$\beta$1. Protein levels were calculated as fold compared with the control. Data are presented as means ± SD ($n$ = 3) and analyzed with ANOVA. #$p$ < 0.05, compared with the control. *$p$ < 0.05, **$p$ < 0.01, compared with the high glucose-treated.

$\beta$1 to 1.85 folds, and treatment of HPE dose-dependently reduced the level of TGF-$\beta$1. Especially at the dose of 1 mg/mL, HPE completely abolished high glucose-stimulated TGF-$\beta$1 elevation (Figure 2B).

**HPE Inhibits High Glucose-Induced EMT.** Figure 3A shows that high glucose increased vimentin to 3 folds and decreased E-cadherin by half. The expressions of vimentin and E-cadherin were recovered dose-dependently by HPE, indicating the effect of HPE on preventing EMT.

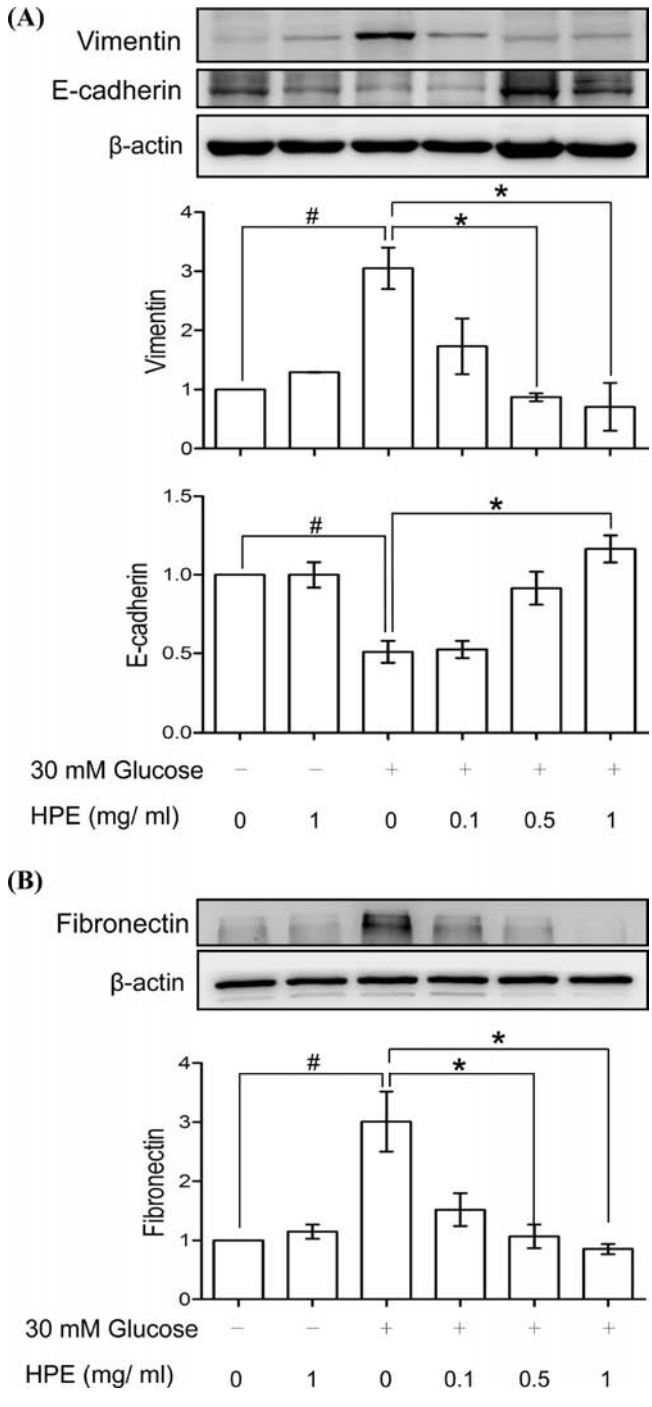


**Figure 3.** Effect of HPE on vimentin, cadherin, and fibronectin. HK-2 cells were incubated for 24 h under normal or high glucose (30 mM) condition with or without different concentrations of HPE. Cell proteins were extracted and analyzed with Western blot. (A) Vimentin and cadherin, (B) fibronectin. Protein levels were calculated as fold compared with the control. Data are presented as means ± SD ($n$ = 3) and analyzed with ANOVA. # $p$ < 0.05, compared with the control. * $p$ < 0.05, compared with the high glucose-treated.


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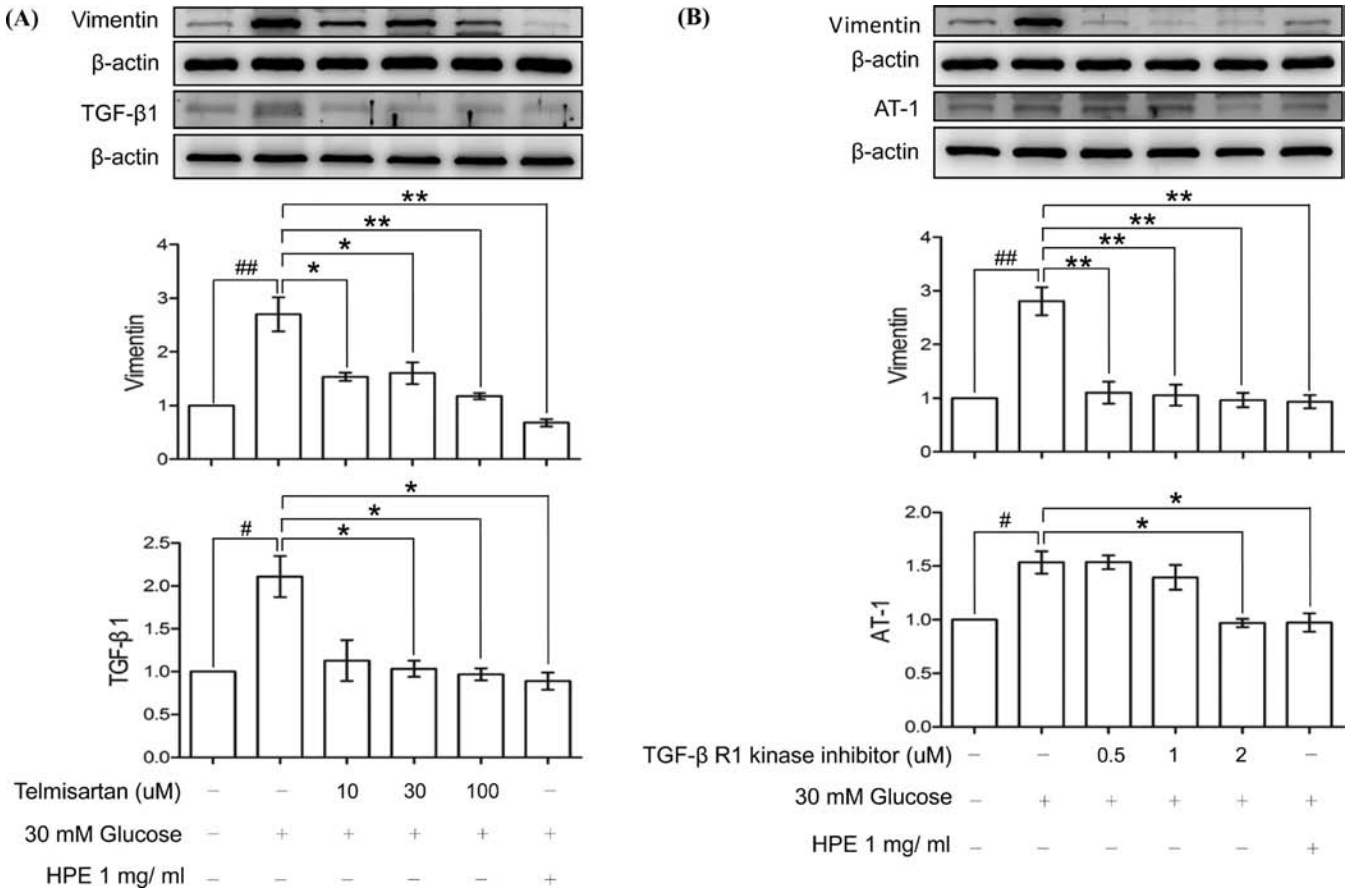


**Figure 4.** Signal cascades of AT-1, TGF-$\beta$1 and vimentin. (A) HK-2 cells were incubated for 24 h under high glucose with or without different concentrations of Telmisartan, (B) HK-2 cells were incubated for 24 h under high glucose with or without different concentrations of TGF-$\beta$ R1 kinase inhibitor. Cell proteins were extracted and analyzed with Western blot. Protein levels were calculated as fold compared with the control. Data are presented as means ± SD ($n$ = 3) and analyzed with ANOVA. # $p < 0.05$, ## $p < 0.01$, compared with the control. * $p < 0.05$, ** $p < 0.01$, compared with the high glucose-treated.

**HPE Inhibits High Glucose-Induced Fibroblast Differentiation.** We further observe the expression of fibronectin, a typical marker of fibroblast. Figure 3B shows that high glucose increased fibronectin up to 3 folds, and HPE dose-dependently decreased fibronectin. These results implicate potentials of HPE to avoid fibroblast generation and the accompanying fibrosis.

**HPE Inhibits the Cascades of AT-1−TGF-$\beta$1−Vimentin Signals.** Our pretest results revealed that AT-1antagonist Telmisartan and TGF-$\beta$ R1 kinase inhibitor per se did not affect the changes of EMT (shown in the Supporting Information). Blocking AT-1 with Telmisartan inhibited the high glucose-induced elevation of TGF-$\beta$1 and vimentin. AT-1 seems to be the upstream of TGF-$\beta$1 (Figure 4A). In contrast, low dose of TGF-$\beta$ R1 kinase inhibitor did not interfere AT-1 expression. The interference can only be found by 2 $\mu$M of the inhibitor (Figure 4B). Hence, TGF-$\beta$1 is the downstream of AT-1. Compared with AT-1, blocking TGF-$\beta$1 more effectively inhibits vimentin expression, implicating the pivotal role of TGF-$\beta$1 on EMT.

**HPE Improves the Junction of Renal Tubular Cells and Intact of Basement Membrane.** Cell−cell junction is an important feature for endothelial cells. It was shown that cell interacting filaments were abundant in the control but disappear under the high glucose treatment. Addition of HPE recovered the generation of filaments, thus improving the junctions among renal tubular cells (Figure 5).

Type IV collagen is the foundation for basement membrane. Using a type 2 diabetic rat, we demonstrated that type IV collagen decreased in diabetic kidneys (Figure 6A). Treatment of low HPE is sufficient to recover the expression, and high HPE even significantly increased type IV collagen. The observation of IHC revealed that the outline of the tubular region was destructed and expression of type IV collagen was reduced (Figure 6B). Besides, as our recent publication indicated, vacuole fusions and lipid accumulation were found in the diabetic tubular region. Treatment of HPE restored the tubular structure and completeness of the basement membrane.

## DISCUSSION

In the present study, we use the concentration 30 mM to mimic high glucose status. Clinically the serum glucose level of diabetic patients ranges from normal (80−100 mg/mL, which is equivalent to 4.44−5.55 mM) to extremely high. Some patients even reach 300−600 mg/mL persistently yet without symptoms. According to the literature, 30 mM could be expected to induce cellular changes at the indicated time. We demonstrated that high glucose induces EMT of human tubular cells, thus promoting the fibroblast generation via up-regulating AT-1 and downstream TGF-$\beta$1. HPE inhibited the expressions of AT-1, TGF-$\beta$1, and vimentin, while it recovered E-cadherin


dx.doi.org/10.1021/jf4020735 | J. Agric. Food Chem. 2013, 61, 7545−7551

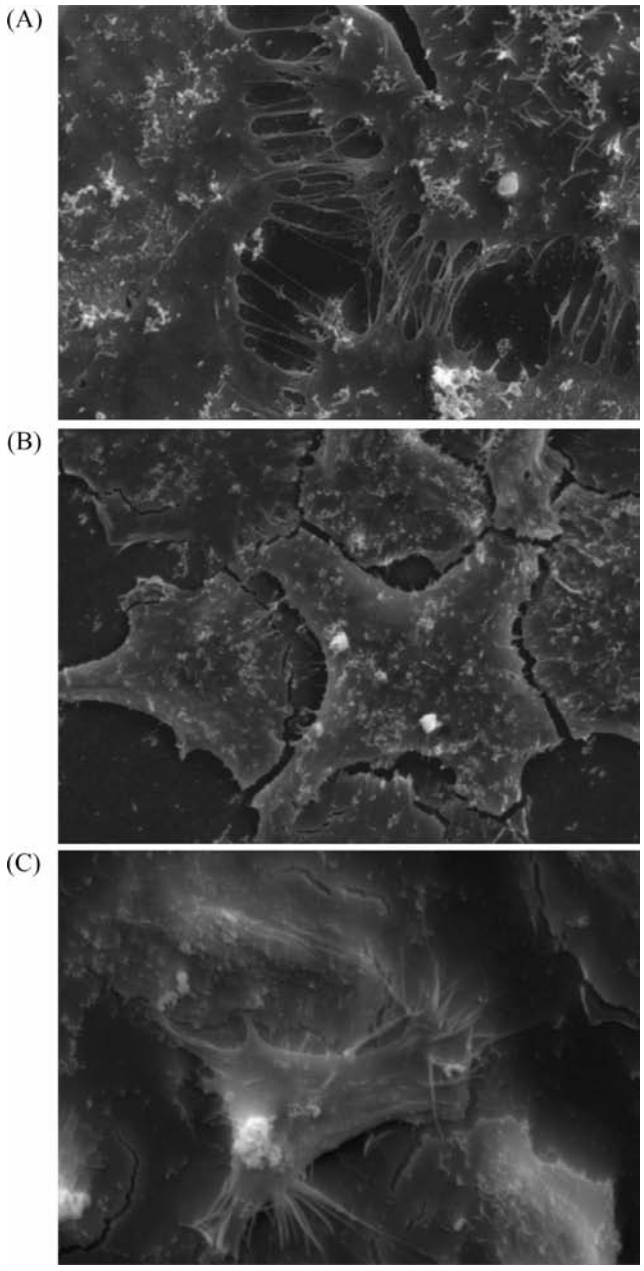


**Figure 5.** Effect of HPE on cell interacting filament. Cells under different treatment were examined by SEM (40000×). (A) Control, (B) high glucose-treated, (C) high glucose with 1 mg/mL of HPE.

loss induced by high glucose. The observation of SEM showed that HPE prevents the filament loss and restores cell junctions in renal tubular cells. Histological finding discovered that HPE restored the tubular outline and type IV collagen expression in diabetic kidneys.

EMT is an important process for embryonic development. Moreover, it occurs under pathological conditions, including cancer cell invasion and renal fibrosis.[16−18] EMT involves a loss of epithelial cell adhesion, disrupted basement membrane, and enhanced cell migration and invasion.[19] In addition to measurement of protein markers, we performed the morphologic observation to identify EMT. Analysis of SEM and IHC proved that high glucose induced typical features of EMT, while HPE prevented the alteration. In a model of chronic kidney disease, destruction of basement membrane was observed in moderate and severe tubulointerstitial damage.[18] In diabetes, transitioned tubular epithelial cells are associated with interstitial fibrosis and renal dysfunction.[20] Our previous report demonstrated the effect of HPE on inhibiting renal fibrosis of type 2 diabetic rats,[9] which should partly attribute to the improvement of EMT.

Hypertension is a critical risk factor for kidney disease. Coexistence of hypertension and diabetes increases the morbidity of renal damage. Nevertheless, in addition to elevating blood pressure, angiotensin II adversely affects renal perfusion and increases oxidative stress through its proliferative, inflammatory and thrombotic effects, thus promoting the progression of kidney disease.[21] Angiotensin II enhanced mitogenesis and type I collagen production of NRK-49F cells,[22] whereas AT-1 blockers reduced oxidative stress and inflammation in diabetic patients and attenuated early diabetic nephropathy by improving microalbuminuria and related pathogenic markers.[21] In hypertensive type 2 diabetic patients with elevated albumin excretion, losartan reduced TGF-$\beta$ urinary excretion, suggesting the protective effect of angiotensin receptor blockade may include a reduction in renal TGF-$\beta$ production.[23] Local renin-angiotensin system mediates high glucose-induced EMT by stimulating AT-1 and the downstream TGF-$\beta$ and then increasing extracellular matrix secretion and renal interstitial fibrosis.[15] The present study showed that HPE improved EMT by regulating AT-1 and the inflammatory cytokine TGF-$\beta$1. However, apart from the previous reports, there existed recruitments between AT-1 and TGF-$\beta$1. Blockade of TGF-$\beta$1 still attenuated the upstream AT-1 (Figure 4). In this experiment, we also found the similar performance of AT-2. As reported, AT-2 could accompany but counteract AT-1, thus regulating the blood pressure.[24] Whether HPE can directly influence blood pressure needs further investigation.

Many phenolics identified in HPE were reported to be antidiabetic. Caffeic acid promoted glycolysis and inhibited gluconeogenesis in insulin-resistant hepatocytes. Caffeic acid increased glycogen synthase but inhibited glycogen synthase kinase, hepatic nuclear factor-4 and phosphoenol-pyruvate carboxykinase.[25] Caffeic acid protected the kidney from diabetic injuries, reducing blood glucose, blood urine nitrogen (BUN) and urine output, and improved clearance of creatinine (CCr). Caffeic acid decreased plasma glycated hemoglobin (HbA1c), urinary glycated albumin, renal AGEs, and renal levels of interleukin (IL)-6, IL-1beta, tumor necrosis factor (TNF)-alpha and MCP-1.[26] Treatment of protocatechuic acid also reduced plasma HbA1c, urinary glycative albumin, renal AGEs and receptor of AGE (RAGE).[27] Quercetin attenuated fasting and postprandial hyperglycaemia, and reduced plasma HbA1c.[28] Chlorogenic acid lowered fasting plasma glucose, TBARS and lipid hydroperoxides in diabetic rats.[29] Gallic acid was antihyperglycaemic by decreasing hepatic hexokinase and increasing glucose-6-phosphatase and fructose-1,6-bisphosphatase. It reduced oxidative stress by inhibiting superoxide dismutase, catalase and glutathione peroxidase.[30] Gallic acid stimulated insulin secretagogue by regenerating pancreas $\beta$-islets, thus increasing plasma insulin and glucose tolerance.[31] Galloyl ester decreased oxidative stress and AGEs and improved hepatic and renal function in type 2 diabetic db/db mice, as well as down-regulating sterol regulatory element-binding protein-1.[32] Tiliroside derivative also possessed the antidiabetic ability.[33] All the above revealed that HPE and its compositions have benefits to prevent diabetic damage. In the

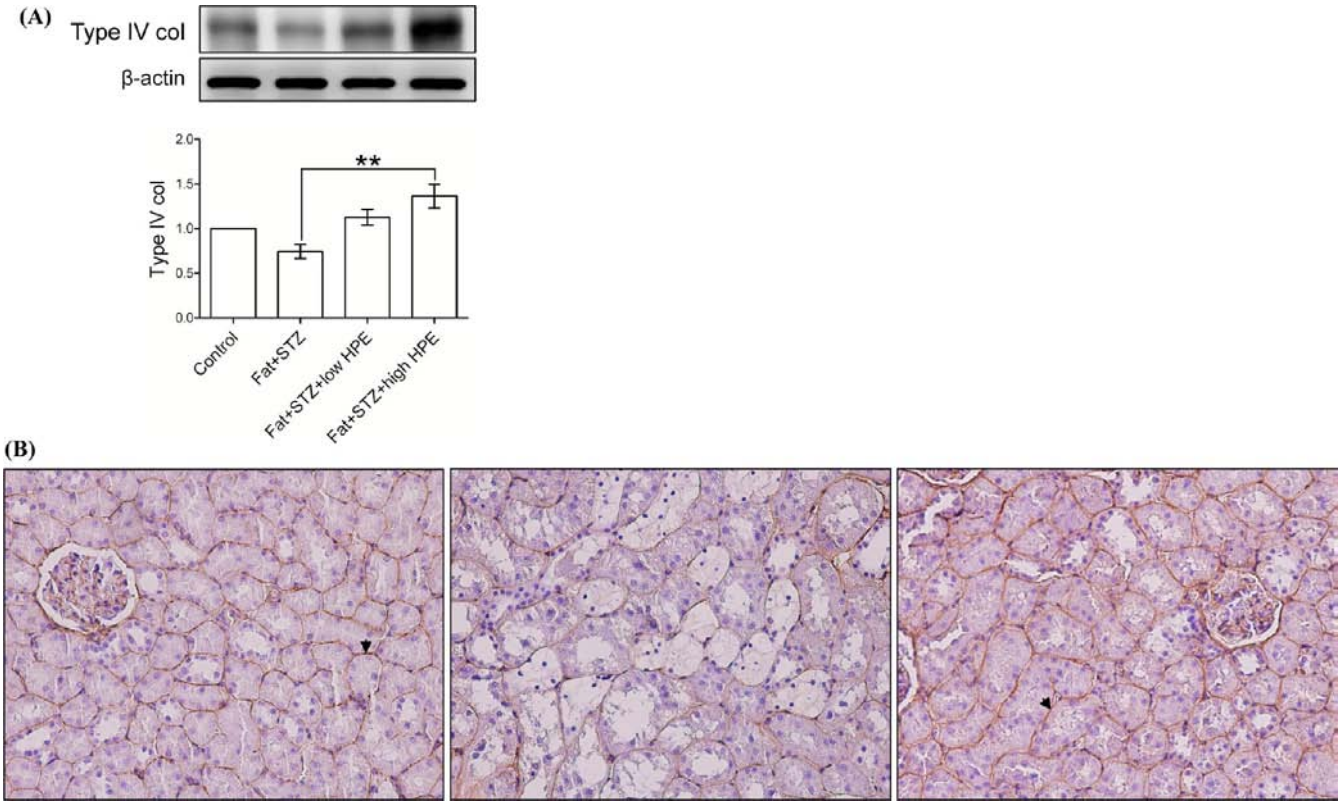


**Figure 6.** Effect of HPE on type IV collagen. Kidneys of normal and type 2 diabetic rats with or without HPE treatment were examined by Western blot and IHC (200×). (A) Protein levels of type IV collagen. ** $p < 0.01$, compared with the diabetic group. (B) Histological expressions of type IV collagen (brown staining as arrow-indicated). From left to right are normal, diabetic, and 200 mg/kg of HPE-treated diabetic groups, respectively.

present study, we have tested some of the HPE components. It is suggested that gallic acid could especially prevent the high-glucose-induced EMT of renal tubular cells (shown in the Supporting Information). In addition, cholorogenic acid could exert similar effect.

HPE potentially can be beneficial to prevent diabetic renal damages. The action mechanism is suggested by regulating AT-1/TGF-$\beta$1 and EMT of tubular cells.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

S1, S2, and S3. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Tel: 866-4-26318652 ext 7065. Fax: 866-4-23764958. E-mail: a222907@sunrise.hk.edu.tw.

### Author Contributions

§Y.-S.Y. and C.-J.W. contributed equally to this work and, therefore, share the first authorship.

### Funding

Funding for this research was provided by Taiwan Yushing Biotech Co. Ltd. under HK101−025.

### Notes

The authors declare no competing financial interest.

## ■ REFERENCES

(1) Venables, M. C.; Jeukendrup, A. E. Physical inactivity and obesity: Links with insulin resistance and type 2 diabetes mellitus. *Diabetes Metab. Res. Rev.* **2009**, *25* (Suppl 1), S18−S23.

(2) Chaykovska, L.; von Websky, K.; Rahnenführer, J.; Alter, M.; Heiden, S.; Fuchs, H.; Runge, F.; Klein, T.; Hocher, B. Effects of DPP-4 inhibitors on the heart in a rat model of uremic cardiomyopathy. *PLoS ONE* **2011**, *6*, e27861.

(3) Lin, H. H.; Chen, J. H.; Wang, C. J. Chemopreventive properties and molecular mechanisms of the bioactive compounds in *Hibiscus Sabdariffa* Linne. *Curr. Med. Chem.* **2011**, *18*, 1245−1254.

(4) Lin, C. Y.; Tsai, S. J.; Huang, C. S.; Yin, M. C. Antiglycative effects of protocatechuic acid in the kidneys of diabetic mice. *J. Agric. Food Chem.* **2011**, *59*, 5117−5124.

(5) Peng, C. H.; Chyau, C. C.; Chan, K. C.; Chan, T. H.; Wang, C. J.; Huang, C. N. *Hibiscus sabdariffa* polyphenolic extract inhibits hyperglycemia, hyperlipidemia, and glycation-oxidative stress while improving insulin resistance. *J. Agric. Food Chem.* **2011**, *59*, 9901−9909.

(6) Huang, C. N.; Chan, K. C.; Lin, W. T.; Su, S. L.; Wang, C. J.; Peng, C. H. *Hibiscus sabdariffa* inhibits vascular smooth muscle cell proliferation and migration induced by high glucoses—A mechanism involves connective tissue growth factor signals. *J. Agric. Food Chem.* **2009**, *57*, 3073−3079.

(7) Lee, W. C.; Wang, C. J.; Chen, Y. H.; Hsu, J. D.; Cheng, S. Y.; Chen, H. C.; Lee, H. J. Polyphenol extracts from *Hibiscus sabdariffa* Linnaeus attenuate nephropathy in experimental type 1 diabetes. *J. Agric. Food Chem.* **2009**, *57*, 2206−2210.

(8) Peng, C. H.; Ker, Y. B.; Weng, C. F.; Peng, C. C.; Huang, C. N.; Lin, L. Y.; Peng, R. Y. Insulin secretagogue bioactivity of finger citron fruit (*Citrus medica* L. var. *Sarcodactylis* Hort, Rutaceae). *J. Agric. Food Chem.* **2009**, *57*, 8812−8819.

(9) Yang, Y. S.; Huang, C. N.; Wang, C. J.; Lee, Y. J.; Chen, M. L.; Peng, C. H. Polyphenols of *Hibiscus sabdariffa* improved diabetic

nephropathy via regulating the pathogenic markers and kidney functions of type 2 diabetic rats. *J. Funct. Foods* **2013**, *5*, 810−819.
(10) Wolf, G.; Ziyadeh, F. N. The role of angiotensin II in diabetic nephropathy: Emphasis on nonhemodynamic mechanisms. *Am. J. Kidney Dis.* **1997**, *29*, 153−163.
(11) Yamamoto, T.; Noble, N. A.; Cohen, A. H.; Nast, C. C.; Hishida, A.; Gold, L. I.; Border, W. A. Expression of transforming growth factor-beta isoforms in human glomerular diseases. *Kidney Int.* **1996**, *49*, 461−469.
(12) Remuzzi, G.; Benigni, A.; Remuzzi, A. Mechanisms of progression and regression of renal lesions of chronic nephropathies and diabetes. *J. Clin. Invest.* **2006**, *116*, 288−296.
(13) Strutz, F. M. EMT and proteinuria as progression factors. *Kidney Int.* **2009**, *75*, 475−481.
(14) Li, J.; Qu, X.; Bertram, J. F. Endothelial−myofibroblast transition contributes to the early development of diabetic renal interstitial fibrosis in streptozotocin-induced diabetic mice. *Am. J. Pathol.* **2009**, *175*, 1380−1388.
(15) Zhou, L.; Xue, H.; Yuan, P.; Ni, J.; Yu, C.; Huang, Y.; Lu, L. M. Angiotensin AT1 receptor activation mediates high glucose-induced epithelial−mesenchymal transition in renal proximal tubular cells. *Clin. Exp. Pharmacol. Physiol.* **2010**, *37*, e152−e157.
(16) Xu, J.; Zhu, W.; Xu, W.; Yao, W.; Zhang, B.; Xu, Y.; Ji, S.; Liu, C.; Long, J.; Ni, Q.; Yu, X. Up-Regulation of MBD1 promotes pancreatic cancer cell epithelial-mesenchymal transition and invasion by epigenetic down-regulation of E-cadherin. *Curr. Mol. Med.* **2013**, *13*, 387−400.
(17) Dai, X.; Ge, J.; Wang, X.; Qian, X.; Zhang, C.; Li, X. OCT4 regulates epithelial-mesenchymal transition and its knockdown inhibits colorectal cancer cell migration and invasion. *Oncol. Rep.* **2013**, *29*, 155−160.
(18) Aresu, L.; Benali, S.; Garbisa, S.; Gallo, E.; Castagnaro, M. Matrix metalloproteinases and their role in the renal epithelial mesenchymal transition. *Histol. Histopathol.* **2011**, *26*, 307−313.
(19) Neilson, E. G. Mechanisms of disease: Fibroblasts– A new look at an old problem. *Nat. Clin. Pract. Nephrol.* **2006**, *2*, 101−108.
(20) Guarino, M.; Tosoni, A.; Nebuloni, M. Direct contribution of epithelium to organ fibrosis: Epithelial−mesenchymal transition. *Hum. Pathol.* **2009**, *40*, 1365−1376.
(21) Kalaitzidis, R.; Bakris, G. L. Effects of angiotensin II receptor blockers on diabetic nephropathy. *J. Hypertens. Suppl.* **2009**, *27*, S15−S21.
(22) Li, J.; Liu, N. F.; Wei, Q. Effect of rosiglitazone on cardiac fibroblast proliferation, nitric oxide production and connective tissue growth factor expression induced by advanced glycation end-products. *J. Int. Med. Res.* **2008**, *36*, 329−335.
(23) Houlihan, C. A.; Akdeniz, A.; Tsalamandris, C.; Cooper, M. E.; Jerums, G.; Gilbert, R. E. Urinary transforming growth factor-beta excretion in patients with hypertension, type 2 diabetes, and elevated albumin excretion rate: effects of angiotensin receptor blockade and sodium restriction. *Diabetes Care* **2002**, *25*, 1072−1077.
(24) Stegbauer, J.; Coffman, T. M. New insights into angiotensin receptor actions: From blood pressure to aging. *Curr. Opin. Nephrol. Hypertens.* **2011**, *20*, 84−88.
(25) Huang, D. W.; Shen, S. C. Caffeic acid and cinnamic acid ameliorate glucose metabolism via modulating glycogenesis and gluconeogenesis in insulin-resistant mouse hepatocytes. *J. Funct. Foods* **2012**, *4*, 358−366.
(26) Chao, C. Y.; Mong, M. C.; Chan, K. C.; Yin, M. C. Antiglycative and anti-inflammatory effects of caffeic acid and ellagic acid in kidney of diabetic mice. *Mol. Nutr. Food Res.* **2010**, *54*, 388−395.
(27) Lin, C. Y.; Tsai, S. J.; Huang, C. S.; Yin, M. C. Antiglycative effects of protocatechuic acid in the kidneys of diabetic mice. *J. Agric. Food Chem.* **2011**, *59*, 5117−5124.
(28) Kim, J. H.; Kang, M. J.; Choi, H. N.; Jeong, S. M.; Lee, Y. M.; Kim, J. I. Quercetin attenuates fasting and postprandial hyper glycemia in animal models of diabetes mellitus. *Nutr. Res. Pract.* **2011**, *5*, 107−111.
(29) Karthikesan, K.; Pari, L.; Menon, V. P. Protective effect of tetrahydrocurcumin and chlorogenic acid against strep tozotocin−nicotinamide generated oxidative stress induce d diabetes. *J. Funct. Foods* **2010**, *2*, 134−142.
(30) Punithavathi, V. R.; Prince, P. S.; Kumar, R.; Selvakumari, J. Antihyperglycaemic, antilipid peroxidative and antioxidant effects of gallic acid on streptozotocin induce d diabetic Wistar rats. *Eur. J. Pharmacol.* **2011**, *650*, 465−471.
(31) Latha, R. C.; Daisy, P. Insulin-secretagogue, antihyperlipidemic and other protective effects of gallic acid isolated from *Terminalia bellerica* Roxb. in streptozotocin-induced diabetic rats. *Chem. Biol. Interact.* **2011**, *189*, 112−118.
(32) Park, C. H.; Noh, J. S.; Yamabe, N.; Kang, K. S.; Tanaka, T.; Yokozawa, T. Beneficial effect of 7-O-galloyl-D-sedoheptulose on oxidative stress and hepatic and renal changes in type 2 diabetic db/db mice. *Eur. J. Pharmacol.* **2010**, *640*, 233−242.
(33) Zhu, Y.; Zhang, Y.; Liu, Y.; Chu, H.; Duan, H. Synthesis and biological activity of trans-tiliroside derivatives as potent anti-diabetic agents. *Molecules* **2010**, *15*, 9174−9183.