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ARTICLES

Back-Regulation of Six Oxidative Stress Proteins With Grape Seed Proanthocyanidin Extracts in Rat Diabetic Nephropathy

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Abstract Diabetic nephropathy (DN) is a major cause of morbidity and mortality in diabetic patients. To prevent the development of this disease and to improve advanced kidney injury, effective therapies directed toward the key molecular target are required. Grape seed proanthocyanidin extracts (GSPE) have been reported to be effective in treating DN, while little is known about the functional protein changes. In this study, we used streptozotocin (STZ) to induce diabetic rats. GSPE (250 mg/kg body weight/day) were administrated to diabetic rats for 24 weeks. Serum glucose, glycated hemoglobin, and advanced glycation end products were determined. Consequently, 2-D difference gel electrophoresis and mass spectrometry were used to investigate kidney protein profiles among the control, untreated and GSPE treated diabetic rats. Twenty-five proteins were found either up-regulated or down-regulated in the kidneys of untreated diabetic rats. Only nine proteins in the kidneys of diabetic rats were found to be back-regulated to normal levels after GSPE therapy. These back-regulated proteins are involved in oxidative stress, glycosylation damage, and amino acids metabolism. Our findings might help to better understanding of the mechanism of DN, and provide novel targets for estimating the effects of GSPE therapy. J. Cell. Biochem. 104: 668–679, 2008.

Key words: grape seed proanthocyanidins extracts; diabetic nephropathy; proteomics; 2-DE DIGE; mass spectrometry

The incidence of diabetes mellitus (DM) and diabetic complications has increased in developed countries, the main reason being modern life styles, which are too sedentary and lack exercise, with dietary imbalances and irregular eating hours [Fujii et al., 2006; Broumand, 2007]. Chronic hyperglycemia participates in the development of diabetic complications such as atherosclerosis, cardiac dysfunction, and nephropathy [Brownlee, 2001, 2005; Tikoo et al., 2007]. Diabetes now accounts for 40% of patients with kidney disease, and the number of renal failure patients with diabetes is expected to increase in the coming years. Diabetic nephropathy (DN) is one of chronic complications of DM, which is a major cause of morbidity and mortality in diabetic patients [Thongboonkerd et al., 2004a; Merchant and Klein, 2005; Awad et al., 2006].

Grape seed proanthocyanidin extracts (GSPE) derived from grape seeds, have been reported to possess a variety of potent properties including anti-oxidant, anti-inflammation, radical-scavenging and renal protecting activity, anti-tumor and so on [Shao et al., 2003; Vayalil et al., 2004; Houde et al., 2006]. It was reported that GSPE had an effect in protecting the kidneys of diabetic rats [Liu et al., 2006]. Our previous experiments showed that GSPE displayed anti-non-enzymatic glycation, reducing receptor of advanced glycation end products (AGEs) protein expression, subsequently leading to decreased expression of high level vascular cell adhesion molecule 1 induced by AGEs [Zhang et al., 2006a], and reduced the content of AGEs in the kidneys of diabetic rats [Zhou et al., 2005].

Despite decades of intensive research into its pathogenesis, the triggering factors and

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underlying mechanism behind the development of DN remain largely unknown. For such a complicated disease, we took streptozotocin (STZ)-induced diabetic rats as an animal model, collected kidneys of control rats, untreated diabetic rats and GSPE treated diabetic rats, and applied them to 2-D difference gel electrophoresis (2-D DIGE), followed by analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-TOF MS) or liquid chromatography electrospray ionization mass spectrometry/mass spectrometry (LTQ-ESI-MS/MS). We expected to figure out which proteins participated in the DN onset and the efficiency of therapy.

The 2-D DIGE technology was originally developed by Unlu et al. [1997]. It is a method of prelabeling protein samples prior to 2-DE for differential analysis. There are three CyDye DIGE fluors available, Cy2, Cy3, and Cy5. They are covalently attached to proteins via lysine residues prior to electrophoresis. Therefore, up to three different samples can be labeled with the different dyes and separated on a single gel. The Cy dyes are all matched for charge and molecular weight. Consequently, the same protein labeled with any of the CyDye DIGE fluors will migrate to the same position on a 2-D gel. This allows the codetection of individual proteins originating from the different samples in a single spot and a direct comparison of the protein expression levels. Moreover, this technique enables the incorporation of the same internal standard on every 2-D gel, which is a pool of all the samples in the experiment. The internal standard is used to match the protein patterns across gels, thereby negating the problem of inter-gel variation, a common problem with standard 2-D assays [Alban et al., 2003; Shaw et al., 2003]. Thus accurate quantitation of differences between samples could be accomplished with an associated statistical significance. 2-D DIGE technology has been applied widely in comparative proteomic researches to detect protein differences with high reproducibility and reliability [Sharma et al., 2005; Zhang et al., 2006b; Prabakaran et al., 2007].

In our present study, we verified the different expression of some proteins in diabetic rats among which some were back-regulated after treatment with GSPE. The expression of these proteins that was changed gave us clues for DN pathogenesis. These potential functional proteins may benefit early diagnosis, monitor the effects of DN therapy, and provide candidates for therapeutic intervention. Moreover, we examined the underlying mechanisms with the goal of identifying valid multifunctional targets and therapeutic strategies against DN by GSPE in the future.

MATERIALS AND METHODS

Materials

GSPE (proanthocyanidin content exceeds 96%, Lot No: G050412) were provided by Jianfeng, Inc. (Tianjin, China). STZ was obtained from Sigma-Aldrich Corp. (St. Louis, MO). Cy2, Cy3, Cy5, IPG strips, and IPG buffer were from GE Healthcare (Little Chalfont, Bucks, UK). Thiourea was from Fluka (Buchs, Switzerland). Urea, CHAPS, DTT, Bradford assay kit were from BioRad Ltd (Hercules) and complete protease inhibitor cocktail tablet was from Roche (Mannheim, Germany). Modified trypsin (sequencing grade) was obtained from Promega (WI). Goat polyclonal IgG antibody to glutathione *S*-transferase mu (GSTM) was provided by Abcam (UK), secondary antibody anti-mouse IgG was purchased from Santa Cruz Biotechnology (CA). All buffers were prepared with Milli-Q water (Millipore, Bedford, MA). All other chemicals and biochemicals used were of analytical grade.

Methods

Induction of experimental diabetes. Ten-week-old 200–220 g male Wistar rats ($n = 60$) were purchased from Laboratory Animal Center of Shandong University (Shandong, China). The animals were housed in cages and received normal rat chow and tapwater ad libitum in a constant environment (room temperature $22 \pm 1.5^{\circ}C$, room humidity $55 \pm 5\%$) with a 12-h light, 12-h dark cycle. The animals were kept under observation for one week prior to the start of the experiments. All procedures were approved by the Animal Ethics Committee of Shandong University. Twelve rats were randomly selected as control group (vehicle, C, $n = 12$), which received a single tail vein injection of 0.1 mol/L citrate buffer only. The other 48 rats received a single dose of STZ ($55\ mg\,kg^{-1}$, injected into tail veins) freshly dissolved in 0.1 mol/L sodium citrate buffer (pH 4.5) after a 12 h overnight fasting [Pinent et al., 2004; Kamata et al., 2006; Nemoto et al., 2006].

Only rats with blood glucose higher than 16.7 mmol/L after 5 days were considered as being diabetic in the fasting state, by using One Touch II Glucose Analyzer (Johnson & Johnson). Eight rats with blood glucose levels of less than 16.7 mmol/L were excluded from the study. All studies were carried out one week after STZ had been injected.

Experimental protocols. All rats were divided into three groups. The groups were divided as follows: group 1, control rats (vehicle, C, n = 12); group 2, untreated diabetic rats (STZ induced then vehicle, DM, n = 20); group 3, treated diabetic rats (GSPE, 250 mg/kg body weight/day, DM + GSPE, n = 20). The GSPE was given in normal saline solution by intragastric administration for 24 weeks. At the end of the experiments, the animals were fasted overnight (18 h) and then anesthetized intraperitoneally with 10% chloral hydrate (350 mg/kg body weight). The kidneys were perfused with ice-cold PBS via abdominal aorta according to an amended method described previously. The distal aorta was cannulated with polyethylene tubing (Portex, Germany; id 0.28 mm) and perfused in situ at a constant rate of 7.5 ml/min/g kidney. The proximal aorta was ligated. To ensure venous drainage, a hole was cut into the inferior cava vein. Initially, kidneys were perfused with ice-cold PBS to free the blood vessels from any remaining blood. After preparation, kidneys were dissected out and kept at −80°C until further analysis.

Estimation of body weight, fasting blood glucose (FBG), glycated hemoglobin (HbAlc), AGEs, blood urea nitrogen (BUN), and creatinine (Cr). Prior to sacrifice after 24 weeks of diabetes, animals were weighted. FBG was determined using a commercially available kit (Adamco Ltd). Serum HbAlc level was determined with a commercially available kit (BioRad Ltd). Serum AGEs specific fluorescence determinations were performed by measuring emission at 440 nm on excitation at 370 nm using a fluorescence spectrophotometer (HITACHI 850, Japan) [Soulis-Liparota et al., 1991; Candido et al., 2003]. Plasma BUN and Cr were determined by DVI-1650 Automatic Biochemistry and Analysis Instrument (Bayer, Germany).

Light microscopy. The kidneys were excised, part of them was fixed in 10% formaldehyde, and embedded in paraffin and cut into 4 μm-thick sections for light microscopy. Then they were stained with hematoxylin-eosin under a light microscope at a magnification of 200×.

Protein sample preparation. To perform a differential proteome analysis, left kidney from group C (n = 3), group DM (n = 3) and group DM + GSPE (n = 3) were dissected and rinsed thoroughly with ice-cold phosphate-buffered saline to remove blood components. Later they were blotted dry and frozen immediately in liquid nitrogen. Every kidney tissue was cut into small pieces and washed by phosphate-buffered saline. Afterward it was ground in a Dounce homogenizer for 20 min on ice in 1 ml DIGE lysis buffer containing: 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 0.2% carrier ampholyte, pH 3–10 NL (IPG buffer) and a complete proteinase inhibitor cocktail. For improved cell lysis, the solution was sonicated on ice for 1 min with 1 s pulse-on and 1 s pulse-off to prevent overheating. The suspension was sonicated for 30 s followed by centrifugation at 25,000*g* for 1 h. The supernatant was aliquoted and stored at −80°C. Protein concentration was determined with the Bradford assay kit (BioRad Ltd) using albumin diluted in lysis buffer as standard.

Protein labeling with CyDye DIGE fluor. Kidney tissue lysates were labeled with Cy2, Cy3, and Cy5 following the protocols described in the Ettan DIGE User Manual (GE Healthcare). The DIGE experimental design was shown in Table I. Typically, 50 μg of lysates (PH 8.5) was labeled with 400 pmol of Cy3 or Cy5, while the same amount of the internal standard that contained equal quantities of all the samples was labeled with Cy2. Labeling reactions were carried out in the dark on ice for 30 min before quenching with 1 μl of 10 mM lysine for 10 min on ice. These labeled samples were then combined for 2-D DIGE analysis.

Two-dimensional gel electrophoresis. Two-dimensional electrophoresis (2-DE) was performed as previously described with some modifications [Liang et al., 2005]. 2-DE was performed. IPG strips (13 cm, pH 3–10, and NL) were rehydrated with labeled samples in the dark and kept overnight with rehydrated buffer (8 M urea, 4% w/v CHAPS, 20 mM DTT, and 1% v/v IPG buffer and trace amount of bromophenol blue). First-dimension IEF was performed using an Ettan IPGphor System (GE Healthcare) for a total of 60 kV h 20°C. The strips were then treated with a two-step reduction and

TABLE I. DIGE Experimental Design for Nine Kidney Samples of C, DM, and DM + GSPE

	Cy2	Cy3	Cy5
Gel 1	Internal standard	C 3	DM 2
Gel 2	Internal standard	DM + GSPE 1	C 2
Gel 3	Internal standard	DM + GSPE 2	DM 1
Gel 4	Internal standard	C 2	DM + GSPE 3
Gel 5	Internal standard	DM 3	C 1

C, untreated control group; DM, untreated diabetic group; DM + GSPE, GSPE treated diabetic group.

alkylation step prior to the second dimension (SDS–PAGE). After equilibration with a solution containing 6 M urea, 30% glycerol, 2% SDS, 50 mM of Tris–Cl, pH 8.8, and 0.5% w/v DTT, the strips were treated with the same solution containing 4.5% w/v iodoacetamide instead of DTT. The strips were overlayered onto 12.5% polyacrylamide gels (16 cm × 16 cm), immobilized to a low-fluorescent glass plate and electrophoresed for 5 h at 30 mA per gel using a Hofer SE 600 Series standard dual-cooled gel electrophoresis unit (GE Healthcare). The Cy2-, Cy3-, and Cy5-labeled images were acquired on a Typhoon 9400 scanner (GE Healthcare) at the excitation/emission values of 488/520, 532/580, 633/670 nm, respectively.

Image analysis. DIGE images were analyzed with DeCyder software (GE Healthcare) as described in the Ettan DIGE User Manual. The intra-gel spot detection and quantification was performed using the differential in-gel analysis (DIA) module of the DeCyder software. Images from different gels were matched using the biological variation analysis (BVA) module of DeCyder software. The best internal standard image was assigned as the "Master," which was used as a template. The protein spots on the remaining internal standard images were all matched to the master gel to ensure that the same protein spots were compared between gels. We defined $P < 0.05$ as significant difference when analyzing the parallel spots between two groups with Student's *t*-test.

In-gel trypsin digestion. Separate prepared gels were run to obtain enough amount of proteins for MS analysis [Candiano et al., 2004]. These gels were fixed and stained with colloidal Coomassie brilliant blue (CBB). Proteins of interest, as defined by the 2-D DIGE/DeCyder analysis, were excised from the cCBB-stained gels for a modified in-gel trypsin digestion procedure. Gel sections were first discolored in 30% ACN and 100 mM of ammonium bicarbonate and then subjected to reduction and alkylation in 100 mM of DTT and 200 mM of iodoacetic acid, respectively. Following vacuum drying, the gel sections were incubated with sequencing-grade modified trypsin (Promega) at a final concentration of 0.01 mg/ml in 25 mM of ammonium bicarbonate for 20 h at 37°C. one percent aqueous formic acid was added to stop the reaction; digests were desalted using Zip-Tips C18 (Millipore) and was eluted in 50% and 100% acrylonitrile step-by-step. The desalted peptide mixture was concentrated into 2 ml consisting of 1% aqueous formic acid. The extracted solutions were mixed in an Eppendorf tube, and dried in a vacuum concentrator. The peptide mixture was solubilized with 1% TFA for mass spectrometry analysis.

MS and protein identification. Peptides were mixed with MALDI matrix (7 mg/ml CHCA, 0.1% TFA, and 30% ACN) and was spotted on to stainless steel MALDI target plates. Samples on the MALDI target plates were then analyzed using a Bruker Reflex III MALDI-TOF mass spectrometer (Karlsruhe, Germany). Protein database searching was performed with the MASCOT search engine (http://www.matrixscience.com; Matrix Science, London, UK) using monoisotopic peaks against the NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov/). The species were *Rattus norvegicus*. Mass tolerance was allowed within 0.05%. Proteins matching more than two peptides and with a MASCOT score higher than 30 were considered significant ($P < 0.05$). Unidentified peptide mixtures by MALDI-TOF MS were measured using on an LTQ-ESI-MS/MS (ThermoFinnigan, San Jose, CA) and a surveyor high-performance liquid chromatography (HPLC) system connected through PepFinder kit (with peptide trap and 99: 1 flow splitter). The ion transfer

capillary temperature was set at 170°C. Mass data collected during the LTQ-ESI-MS/MS were processed and converted into a file using the Masslynx™ software (Micromass) to be submitted to the Mascot search software (http://www.matrixscience.com). Identifications were obtained by comparison of experimental data with the NCBI non-redundant mammalian database and were validated when there were at least two peptide sequences per protein presented.

Western blot analysis of GSTM. Western blot analysis was performed on samples of kidneys obtained from three groups of the rats. Equal amount of proteins were separated by electrophoresis in a 12% SDS–polyacrylamide gel. After the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore), the blot was blocked with 5% (w/v) non-fat milk in TBST (Tris-buffered saline and 0.05% Tween-20) for 1 h at room temperature and then probed with anti-GSTM (1:1,000) polyclonal antibody and anti-GAPDH (1:500), followed by incubation with secondary antibody, horseradish peroxidase conjugated affinity anti-rabbit IgG (1:7,500) for 1 h. Signal detection was performed via exposing the blots to enhanced DAB color reagenta for 5 min. Quantification of the luminosity of each identified protein band was performed using a densitometric analysis (Digital Protein DNA Imagineware, Huntington Station, NY).

Statistical Analysis

Data were expressed as mean ± standard deviation. Statistical significance of the difference among experimental groups were calculated by unpaired Student's *t*-test and one way ANOVA. *P*-value <0.05 was considered statistically significant.

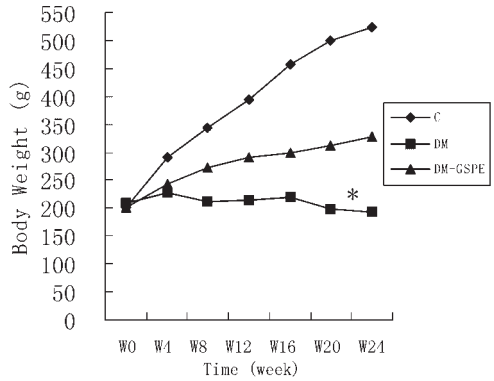

Fig. 1. Weight changes of the rats. C: untreated control group (n = 10); DM: untreated diabetic group (n = 9); DM + GSPE: GSPE treated diabetic group (n = 13).

RESULTS

Effects of GSPE on Body Weight, PBG, HbAlc, AGEs, BUN, and Cr

Diabetes was maintained over the length of this study without any hypoglycemic treatments. The number remained alive at the end of the research in the three groups was 10, 9, and 13 respectively of group C, DM, and DM + GSPE. Figure 1 showed the results of comparisons of body weight findings among group C, DM, and DM + GSPE. The body weight of diabetic rats was significantly smaller than that of the control rats at 8 weeks, 16 weeks, 20 weeks, and 24 weeks ($P < 0.01$). GSPE significantly improved the body weight of diabetic rats at 8 weeks, 16 weeks, 20 weeks, and 24 weeks ($P < 0.01$). Table II showed the comparisons of the ratio of kidneys weight to body weight (Kw/Bw ratio), FBG, HbAlc, AGEs, BUN, and Cr findings between control, untreated diabetic and GSPE treated diabetic rats. The Kw/Bw ratio, FBG, HbAlc, AGEs, BUN, and Cr of diabetic rats were significantly higher than those of the control rats ($P < 0.01$). GSPE significantly reduced the Kw/Bw ratio,

TABLE II. Mean ± SD Effect of GSPE on FBG, HbA_{1C}, AGEs, BUN, Cr, and Kw/Bw of Diabetic Rats

Group	n	FPG (mmol/L)	HbA_{1C} (%)	AGEs (AU/mg)	BUN (mmol/L)	Cr (μmol/L)	Kw/Bw (mg/g)
C	10	6.99 ± 2.74	5.61 ± 0.73	0.017 ± 0.004	7.59 ± 2.98	45.67 ± 6.31	7.95 ± 1.14
DM	9	23.03 ± 3.38**	12.40 ± 2.35**	0.035 ± 0.012**	16.89 ± 5.52**	66.00 ± 10.24**	11.84 ± 2.01**
DM + GSPE	13	21.23 ± 3.92**	11.41 ± 2.14**	0.023 ± 0.008*#	11.56 ± 3.75*#	56.33 ± 7.83**#	9.16 ± 1.05*##

*$P < 0.05$, **$P < 0.01$: vs. C; #$P < 0.05$, ##$P < 0.01$: DM + GSPE vs. DM.
C, untreated control group; DM, untreated diabetic group; DM + GSPE, GSPE treated diabetic group; FBG, fasting blood glucose; HbA_{1C}, glycated hemoglobin; AGEs, advanced glycation end products; BUN, blood urea nitrogen; Cr, creatinine; Kw/Bw, kidneys/body weight ratio.

AGEs, BUN, and Cr of diabetic rats ($P < 0.05$). But GSPE could not decrease FBG and HbAlc of diabetic rats.

Histological Findings of DN

In the diabetic rats, glomerular hypertrophy and hypercellularity, glomerular and interstitial fibrosis, tubular atrophy and interstitial myofibroblast accumulation were observed in the kidney. Moreover, in the diabetic rats, treatment with GSPE significantly suppressed the glomerular hypertrophy, decreased interstitial fibrosis, and led to light microscopic findings similar to those of the control rats (Fig. 2).

DIGE and Mass Spectrometry

To further investigate the actions of GSPE in rat DN, we used 2-D DIGE to generate the protein expression profiles of three kidney samples per group (Fig. 3). Spots detected by cCBB staining were excised and used to for identification. In total, up to 1,368–1,532 different spots were detected on the five gels as determined by the DeCyder Differential Analysis Software. Inter-gel matching was performed through the inclusion of the internal standard on each gel. MALDI-TOF MS was first used to analyze the peptides after in-gel digestion of each spot. The spots which could not be identified with MALDI-TOF MS were then analyzed by LTQ-ESI-MS/MS. A total of 25 protein spots included 13 up-regulated and 12 down-regulated spots in kidney tissue of diabetic rats, and were successfully identified with these two types of MS. Table III showed the proteins identified, including standard spot (SSP) number on 2-DE DIGE gels, NCBI accession number, protein description, theoretical Mr/PI, protein coverage and identification method, etc.

In the kidney of diabetic rats, 25 proteins were found to be significantly changed in comparison to normal. Among them, nine proteins were also found back-regulated to normal level after treatment with GSPE, as shown in Table IV. In brief, the differentially expression of proteins were related to many important biological functions including metabolism, oxidative stress, signal transduction, cell proliferation, cell growth, apoptosis, and heat shock. Among these proteins, in comparison with the kidney tissue of diabetic rats, the differential proteomic analysis of the kidney tissue of diabetic rats, treated by GSPE further revealed the variation of nine proteins, namely,

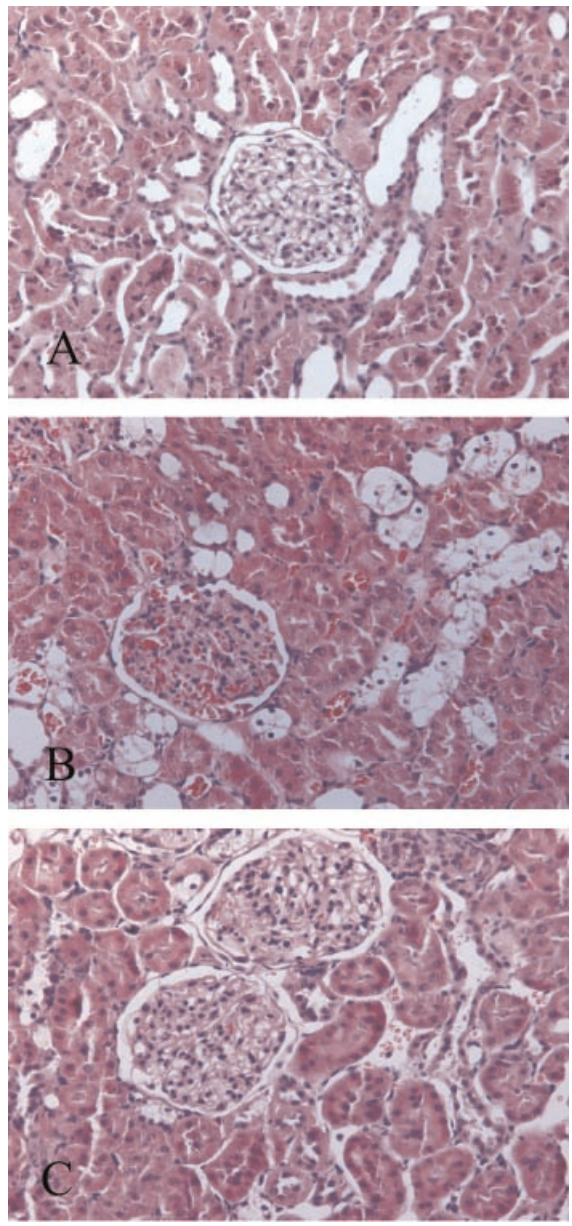

Fig. 2. Representative light micrographs of the glomerulus and nephric tubule (hematoxylin-eosin; 200×). **A**: Untreated control group; (**B**) untreated diabetic group; (**C**) GSPE treated diabetic group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

NADH-ubiquinone oxidoreductase (complex I), Aflatoxin B1 aldehyde reductase (AFAR), GSTM, selenium binding protein 2 (SBP2), F1-ATPase beta subunit, glutamate carboxypeptidase (GCP), LOC500183 protein, phosphotriesterase related protein, and beta actin.

Effects of GSPE on the Expression of GSTM

In order to conform the results of 2-D DIGE, the expression of GSTM in the kidney of control,

Fig. 3. A representative 2-D DIGE image of kidney lysates from DM + GSPE 2 and DM 1 (13 cm, pH 3–10, NL). Cy2 (blue) image of proteins from an internal standard is the pool of all the samples, Cy3 (green) image of proteins from DM + GSPE 2, and Cy5 (red) image of proteins from DM 1. Spot numbers correspond to those in Supplementary Table III.

untreated diabetic, and GSPE treated diabetic rats was carried out by Western blot analysis. The expression of GSTM of diabetic rats were significantly higher than that of control rats ($P < 0.01$); the expression of GSTM decreased after treated with GSPE, which was accordance with the result of 2-D DIGE (Fig. 4).

DISCUSSION

Diabetes is the most common cause of end-stage renal diseases (ESRD), and accounts for more than 40% of patients who receive renal replacement therapy [Thongboonkerd et al., 2004b; Merchant and Klein, 2005; Awad et al., 2006]. Current therapies aiming to halt the progression of renal damage in established DN is limited to anti-hypertensive drugs, such as angiotensin-converting enzyme inhibitors and angiotensin receptor blocker. Successful reduction of proteinuria slows the rate of progression of diabetic renal injury, renal failure remains a major diabetic complication [Cooper, 1998; Actis-Goretta et al., 2003; Langham et al., 2006]. Defining the pathophysiologic mechanisms of DN is necessary to identify new targets for therapeutic intervention. The aim of this study was to identify proteins with altered expression in diabetic kidney and to disclose if could GSPE treat diabetic kidney by proteomic techniques.

Conventionally, 2-DE has relied on Coomassie blue or silver staining combined with image analysis to visualize gel separated proteins. In this study, 2-D DIGE exhibits more accurate qualitative and quantitative analysis by running multiple sample lysates prelabeled with three matched cyanine dyes within the same gel. The use of pre-electrophoretic label with fluorescent dyes and an internal standard created by pooling aliquots of all the samples in the experiment improves the sensitivity and dynamic range of protein detection [Tonge et al., 2001; Prabakaran et al., 2007].

The STZ induced diabetic rats used in this study developed degenerative changes. These changes were similar to those seen in diabetic process, when high glucose level was maintained without any hypoglycemic agents. The renal lesion of diabetic rats was characterized previously by showing renal histologic changes commonly seen in human DN, including mesangial expansion and increased thickness of the glomerular basement membrane (GBM) [Matsubara et al., 2006; Dixon et al., 2007]. The present study also showed that STZ-induced diabetic rats had a dramatic increase in urinary albumin excretion. Thus, STZ-induced diabetic rats presented many of the features observed in human DN. First was the increase of serum glycosylated protein, which was caused by hyperglycemia and other reducing sugars such as ribose and fructose reacting with the amino residues of proteins to form Amadori products, for instance, HbA1c and AGEs are also generated in the process of AGE formation [Basta et al., 2002; Smit and Lutgers, 2004; Yamagishi et al., 2005].

Our results indicated that diabetic rats induced by STZ showed body weight reduction during the 24 weeks experimental period, suggesting that these animals were undergoing growth retardation due to the obstruction of glucose uptake caused by the lack of insulin following STZ injection, but treatment with GSPE increased the body weight from the initial value. It had been reported GSPE have

TABLE III. Characterization of Differentially Displayed Proteins

SSP no.	Accession number	Protein name	Mr(Da)	pI	Protein score	Sequence coverage (%)	Identification method	DM vs. C	DM + GSPE vs. DM
1252	gi|28933457	Glutathione *S*-transferase mu	25,857	6.90	81	12	MALDI-TOF/TOF	U	D
416, 422	gi|18266692	Selenium binding protein 2	53,069	6.10	87	4	MALDI-TOF/TOF	U	D
1171	gi|203033	F1-ATPase beta subunit	38,747	5.07	35	3	MALDI-TOF/TOF	U	D
443	gi|7108713	Glutamate carboxypeptidase	52,036	5.99	37	8	MALDI-TOF/TOF	U	D
944	gi|34863072	Hypothetical protein	34,785	8.48	62	9	MALDI-TOF/TOF	U	D
913	gi|57429	Unnamed protein product	50,387	4.79	71	14	MALDI-TOF	U	D
1280	P19234	NADH-ubiquinone oxidoreductase	27,378	6.23		49	LTQ-ESI-MS/MS	U	D
1293	Q4KM66	LOC500183 protein	25,692	5.34		28	LTQ-ESI-MS/MS	U	D
742	gi|3914481	Phosphotriesterase-related protein	39,249	6.06	68	5	MALDI-TOF/TOF	U	nsd
1308	gi|8393910	Phosphatidylethanolamine binding protein	20,902	5.48	70	18	MALDI-TOF/TOF	U	nsd
1188	gi|62650520	Hypothetical protein	28,386	5.63	68	36	MALDI-TOF/TOF	U	nsd
1336	P04041	Glutathione peroxidase 1	22,258	7.65		30	LTQ-ESI-MS/MS	U	nsd
1287	P10719	ATP synthase beta chain	56,354	5.18		28	LTQ-ESI-MS/MS	U	nsd
848	gi|433611	Aflatoxin B1 aldehyde reductase	37,117	6.83	83	7	MALDI-TOF/TOF	D	U
772	gi|61889077	Resiniferatoxin-binding, phosphotriesterase-related protein	39,462	6.40	82	7	MALDI-TOF/TOF	D	U
420	gi|4501885	Beta actin	42,052	5.29	81	24	MALDI-TOF	D	U
372, 348	gi|56905	Unnamed protein product	57,044	6.38	79	5	MALDI-TOF/TOF	D	U
591	gi|56200	Unnamed protein product	61,731	8.05	94	5	MALDI-TOF/TOF	D	U
552, 538	gi|13591949	L-arginine: glycine amidino-transferase	48,724	7.17	92	7	MALDI-TOF/TOF	D	nsd
605	gi|40254781	GDP dissociation inhibitor 2	51,018	5.93	84	25	MALDI-TOF	D	nsd
589	gi|11968102	Ornithine aminotransferase	48,701	6.53	95	5	MALDI-TOF/TOF	D	nsd
598	gi|4139571	*S*-adenosylhomocystein hydrolase	47,889	6.08	102	6	MALDI-TOF/TOF	D	nsd
279	gi|266504	NADP-dependent malic enzyme	64,589	6.49	32	3	MALDI-TOF/TOF	D	nsd
629	gi|13928690	Isocitrate dehydrogenase 1 (NADP+)	47,047	6.53	37	3	MALDI-TOF/TOF	D	nsd
446	P63039	60 kDa heat shock protein	60,955	5.91		26	LTQ-ESI-MS/MS	D	nsd

SSP no., standard spot (SSP) number. SSP numbers are unique numbers assigned to each spot in the matchset standard of PDQuest; Theoretical Mr(Da)/pI are based on the amino acid sequence of the identified proteins and calculated with the Ex-PASy program; U means protein of diabetic group up-regulated compared to that of control group; D means down-regulated; nsd means no significant difference between protein of GSPE treated diabetic group and that of untreated diabetic group; U means up-regulated and D means down-regulated.

TABLE IV. Summary of Average Ratio and Function About Nine Proteins

Protein identification	Average ratio DM/C	Average ratio DM + GSPE/ DM	Average ratio DM + GSPE/C	Function
NADH-ubiquinone oxidoreductase	1.74	−1.78	−1.03	Input to the respiratory chain from the NAD-linked dehydrogenases of the citric acid cycle
Glutathione *S*-transferase	1.83	−1.22	1.46	Detoxify endobiotic and xenobiotic compounds by covalent linking of glutathione to hydrophobic substrate
Selenium binding protein 2	1.68	−1.37	1.19	Selenium and acetaminophen binding, detoxification
F1-ATPase beta subunit	1.51	−1.67	−1.14	Membrane-bound enzyme complexes/ion transporters that combine ATP synthesis and/or hydrolysis with the transport of protons across a membrane
Glutamate carboxypeptidase	1.38	−1.47	−1.06	Catalyzes the hydrolysis of the neurotransmitter N-acetyl-L-aspartyl-L-glutamate (NAAG) to N-acetyl-L-aspartate and L-glutamate
LOC500183 protein	1.68	−1.29	1.27	Similar to NGF-binding Ig light chain
Aflatoxin B1 aldehyde reductase	−2.57	1.50	−1.63	Local scavengers of osones and methylglyoxal
Resiniferatoxin-binding, phosphotriesterase-related protein	−1.66	1.33	−1.25	Catalyze the hydrolysis of a range of phosphotriester compounds including organophosphate insecticides and nerve gase
Beta actin	−1.37	1.32	−1.04	Maintain the functions of cell structure, intracellular movement and cell division

C, untreated control group; DM, untreated diabetic group; DM + GSPE, GSPE treated diabetic group.

anti-oxidant properties as evidenced by the increase in pancreatic glutathione and reduction of lipid peroxidation, so it might improve the function of β cell in the diabetic rats, and influenced insulin effects by directly acting on specific components of the insulin-signaling transduction pathway [Pinent et al., 2004; El-Alfy et al., 2005]. On the contrary, the levels of serum glucose, HbA1c, and AGEs observed in untreated diabetic rats were increased beyond those of untreated control rats. However, GSPE treated diabetic rats showed significant reductions in AGEs levels compared with untreated diabetic rats. Therefore, we supposed that GSPE could have an anti-non-enzymatic glycation effect, having a correlation with body weight gain. By histological study, it was found that GSPE had renoprotective effects on the kidney of diabetic rats.

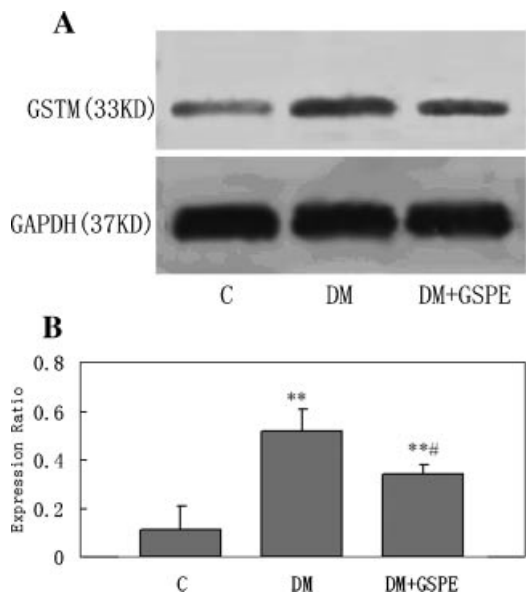

Fig. 4. Effects of GSPE on the expression of GSTM (**A**,**B**). Data were expressed as the expression ratio of GSTM/GAPDH and given as mean ± SD from three experiments. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with C; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ compared with DM. Photographs in **C**: untreated control group; DM: untreated diabetic group; DM + GSPE: GSPE treated diabetic group.

2-DE DIGE and MS were used to investigate kidney protein profiles among control, untreated, and GSPE treated diabetic rats. The results showed that many proteins changed in diabetic rats, wherein the expression of nine proteins was restored after GSPE therapy. The back-regulated proteins were concomitant with the recovered AGEs and HbAlc; accordingly we inferred these proteins function during DM onset or recovery. This, to the best of our knowledge, is the very first report about proteomic analysis of DM before and after GSPE therapy.

In the present study, complex I was found up-regulated in the kidney of diabetic rats and back-regulated to normal after GSPE therapy.

It is well known that increased oxidative stress has been proposed to be implicated in the pathogenesis of DM and DN [King et al., 1996; Brownlee, 2001; Tomohiro et al., 2007]. By this, it was showed that several proteins were involved in the oxidation and reduction changes in DN. Complex I is the point of entry for the major fraction of electrons that traverse the respiratory chain eventually resulting in the reduction of oxygen. Recently, it has been reported that the inhibition of complex I leads to a blockage of more than 95% of activation-induced ROS production [Kaminski et al., 2007]. Moreover, complex I is not only the source of mitochondria-derived ROS but also its activity seems to be a prerequisite for subsequent ROS production via the NADPH oxidase. GSPE is a commonly available dietary supplement taken for the anti-oxidant activity that is attributed to its proanthocyanidin (oligomers of monomeric polyphenols) content. It has also reported that GSPE provides significantly greater protection against ROS and free radical-induced lipid peroxidation and DNA damage than vitamins E, C, and beta-carotene, as well as a combination of vitamins E plus C [Bagchi et al., 2000]. The results suggested that the inhibition effect of GSPE on complex I at least partly contributed to the improvement of DN.

GSTM is a biotransformation enzyme and has functions in the elimination of free radicals, peroxides, electrophilic reagents and heavy metals, the participation of cell protection and the regulation of cell growth. The expression of GSTM of diabetic rats was significantly higher than those of control rats by Western blot and 2-D DIGE. This might be the mechanism of compensation in diabetes rats. However, it was reported that the presence of the GSTM1 gene was associated with a susceptibility to 1 type DM [Bekris et al., 2005]. Therefore, we need do further research on the function of GSTM in diabetes. GCP was found up-regulated in the kidney of diabetic rats and back-regulated to normal after GSPE therapy. GCP is a membrane peptidase expressed in the prostate, central and peripheral nervous system, kidney, small intestine, and tumor-associated neovasculature. It has been reported that the inhibition of GCP has beneficial effects on hyperalgesia, nerve function, and structural degenerative changes in diabetic polyneuropathy [Zhang et al., 2002].

Moreover, AFAR and beta actin were found down-regulated in the kidney of diabetic rats and back-regulated to normal after GSPE therapy. It was reported that AFAR may act as local scavengers of osones and methylglyoxal, and may protect cells and tissues against sugar-mediated damage [O'connor et al., 1999]. The ability of AFAR to reduce the lipid peroxidation product 4-hydroxynonenal suggests that the reductase serves as a protection mechanism against oxidative stress. Previous studies showed that beta actin is a member of the actins family, which maintains the functions of cell structure, intracellular movement, and cell division. Beta actin disassembly is a prominent feature of DN [Clarkson et al., 2002]. It suggested that the activation effect of GSPE on AFAR at least partly contributed to increasing the activity of scavengers of AGEs in the kidneys tissue.

Meanwhile, we got the variation of glutathione peroxidase 1, SBP2 and phosphotriesterase related protein. The proteins were related to the functions of deintoxication and anti-oxidant. Moreover, we also got the variation of glutamine synthetase, ornithine aminotransferase, *S*-adenosylhomocystein hydrolase, L-arginine glycine amidino-transferase, isocitrate dehydrogenase 1, and NADP-dependent malic enzyme. The proteins were related to the metabolism of amino acids and glucose.

In conclusion, these findings strongly supported that those back-regulated proteins might play a key role in the development and recovery of DN. Reversible proteins were mostly relevant to oxidative stress, glycosylation damage, and amino acids metabolism. It indicated that GSPE therapy caused high AGEs recovery mainly by regulating the above processes. Those involved proteins might form the basis of functional regulation. Meanwhile, the expression of some proteins in diabetic rats did not back-regulate after GSPE therapy. So whether those proteins are contributing factors in DN pathogenesis demands further research.

To the best of our knowledge, we explored the proteomic changes after natural medicine therapy for the very first time, and revealed the expression of some proteins back-regulated to normal level after GSPE therapy. These proteins are most likely to participate in the deterioration and restoration as important functional proteins, and to provide with target candidate for treatment.

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REFERENCES

Actis-Goretta L, Ottaviani JI, Keen CL, Fraga CG. 2003. Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins. FEBS Lett 555:597–600.

Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I. 2003. A novel experimental design for comparative two-dimensional gel analysis: Two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3:36–44.

Awad AS, Huang L, Ye H, Duong ET, Bolton WK, Linden J, Okusa MD. 2006. Adenosine A2A receptor activation attenuates inflammation and in jury in diabetic nephropathy. Am J Physiol Renal Physiol 290:F828–F837.

Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG. 2000. Free radicals and grape seed proanthocyanidin extract: Importance in human health and disease prevention. Toxicology 148:187–197.

Basta G, Lazzerini G, Massaro M, Simoncini T, Tanganelli P, Fu C, Kislinger T, Stern DM, Schmidt AM, De Caterina R. 2002. Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: A mechanism for amplification of inflammatory responses. Circulation 105:816–822.

Bekris LM, Shephard C, Peterson M, Hoehna J, Van Yserloo B, Rutledge E, Farin F, Kavanagh TJ, Lernmark A. 2005. Glutathione-s-transferase M1 and T1 polymorphisms and associations with type 1 diabetes age-at-onset. Autoimmunity 38:567–575.

Broumand B. 2007. Diabetes: Changing the fate of diabetics in the dialysis unit. Blood Purif 25:39–47.

Brownlee M. 2001. Biochemistry and molecular cell biology of diabetic complications. Nature 414:813–820.

Brownlee M. 2005. The pathobiology of diabetic complications: A unifying mechanism. Diabetes 54(6):1615–1625.

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. 2004. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25:1327–1333.

Candido R, Forbes JM, Thomas MC, Thallas V, Dean RG, Burns WC, Tikellis C, Ritchie RH, Twigg SM, Cooper ME, Burrell LM. 2003. A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes. Circ Res 92:785–792.

Clarkson MR, Murphy M, Gupta S, Lambe T, Mackenzie HS, Godson C, Martin F, Brady HR. 2002. High glucose-altered gene expression in mesangial cells. Actin-regulatory protein gene expression is triggered by oxidative stress and cytoskeletal disassembly. J Biol Chem 277: 9707–9712.

Cooper ME. 1998. Pathogenesis, prevention, and treatment of diabetic nephropathy. Lancet 352:213–219.

Dixon A, Wells CC, Singh S, Babayan R, Maric C. 2007. Renoprotective effects of a selective estrogen receptor modulator, raloxifene, in an animal model of diabetic nephropathy. Am J Nephrol 27:120–128.

El-Alfy AT, Ahmed AA, Fatani AJ. 2005. Protective effect of red grape seeds proanthocyanidins against induction of diabetes by alloxan in rats. Pharmacol Res 52:264–270.

Fujii H, Yokozawa T, Kim YA, Tohda C, Nonaka G. 2006. Protective effect of grape seed polyphenols against high glucose-induced oxidative stress. Biosci Biotechnol Biochem 70:2104–2111.

Houde V, Grenier D, Chandad F. 2006. Protective effects of grape seed proanthocyanidins against oxidative stress induced by lipopolysaccharides of periodontopathogens. J Periodontol 77(8):1371–1379.

Kamata K, Hosokawa M, Matsumoto T, Kobayashi T. 2006. Altered arachidonic acid-mediated responses in the perfused kidney of the streptozotocin-induced diabetic rat. J Smooth Muscle Res 42:171–187.

Kaminski M, Kiessling M, Suss D, Krammer PH, Gülow K. 2007. Novel role for mitochondria: Protein kinase C{theta}-dependent oxidative signaling organelles in activation-induced T-cell death. Mol Cell Biol 27:3625–3639.

King GL, Kunisaki M, Nishio Y, Inoguchi T, Shiba T, Xia P. 1996. Biochemical and molecular mechanisms in the development of diabetic vascular complications. Diabetes 45:S105–S108.

Langham RG, Kelly DJ, Gow RM, Zhang Y, Cordonnier DJ, Pinel N, Zaoui P, Gilbert RE. 2006. Transforming growth factor-beta in human diabetic nephropathy: Effects of ACE inhibition. Diabetes Care 29:2670–2675.

Liang CR, Leow CK, Neo JC, Tan GS, Lo SL, Lim JW, Seow TK, Lai PB, Chung MC. 2005. Proteome analysis of human hepatocellular carcinoma tissues by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 5:2258–2271.

Liu YN, Shen XN, Yao GY. 2006. Effects of grape seed proanthocyanidins extracts on experimental diabetic nephropathy in rats. Wei Sheng Yan Jiu 35:703–705.

Matsubara T, Abe H, Arai H, Nagai K, Mima A, Kanamori H, Sumi E, Takahashi T, Matsuura M, Iehara N, Fukatsu A, Kita T, Doi T. 2006. Expression of Smad1 is directly associated with mesangial matrix expansion in rat diabetic nephropathy. Lab Invest 86:357–368.

Merchant ML, Klein JB. 2005. Proteomics and diabetic nephropathy. Curr Diab Rep 5:464–469.

Nemoto O, Kawaguchi M, Yaoita H, Miyake K, Maehara K, Maruyama Y. 2006. Left ventricular dysfunction and remodeling in streptozotocin-induced diabetic rats. Circ J 70:327–334.

O'connor T, Ireland LS, Harrison DJ, Hayes JD. 1999. Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. Biochem J 343(Pt 2):487–504.

Pinent M, Blay M, Bladé MC, Salvadó MJ, Arola L, Ardévol A. 2004. Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. Endocrinology 145:4985–4990.

Prabakaran S, Wengenroth M, Lockstone HE, Lilley K, Leweke FM, Bahn S. 2007. 2-D DIGE analysis of liver and red blood cells provides further evidence for oxidative stress in schizophrenia. J Proteome Res 6:141–149.

Shao ZH, Becker LB, Vanden Hoek TL, Schumacker PT, Li CQ, Zhao D, Wojcik K, Anderson T, Qin Y, Dey L, Yuan CS. 2003. Grape seed proanthocyanidin extract attenuates oxidant injury in cardiomyocytes. Pharmacol Res 47:463–469.

Sharma K, Lee S, Han S, Lee S, Francos B, McCue P, Wassell R, Shaw MA, RamachandraRao SP. 2005. Two-dimensional fluorescence difference gel electrophoresis analysis of the urine proteome in human diabetic nephropathy. Proteomics 5:2648–2655.

Shaw J, Rowlinson R, Nickson J, Stone T, Sweet A, Williams K, Tonge R. 2003. Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. Proteomics 3:1181–1195.

Smit AJ, Lutgers HL. 2004. The clinical relevance of advanced glycation end products (AGE) and recent developments in pharmaceutics to reduce AGE accumulation. Curr Med Chem 11:2767–2784.

Soulis-Liparota T, Cooper M, Papazoglou D, Clarke B, Jerums G. 1991. Retardation by aminoguanidine of development of albuminuia, mesangial expansion, and tissue fluorescence in streptozocin-induced diabetic rat. Diabetes 40:1328–1334.

Thongboonkerd V, Barati MT, McLeish KR, Benarafa C, Remold-O'Donnell E, Zheng S, Rovin BH, Pierce WM, Epstein PN, Klein JB. 2004a. Alterations in the renal elastin-elastase system in type 1 diabetic nephropathy identified by proteomic analysis. J Am Soc Nephrol 15:650–662.

Thongboonkerd V, Barati MT, McLeish KR, Pierce WM, Epstein PN, Klein JB. 2004b. Proteomics and diabetic nephropathy. Contrib Nephrol 141:142–154.

Tikoo K, Tripathi DN, Kabra DG, Sharma V, Gaikwad AB. 2007. Intermittent fasting prevents the progression of type I diabetic nephropathy in rats and changes the expression of Sir2 and p53. FEBS Lett 581:1071–1078.

Tomohiro T, Kumai T, Sato T, Takeba Y, Kobayashi S, Kimura K. 2007. Hypertension aggravates glomerular dysfunction with oxidative stress in a rat model of diabetic nephropathy. Life Sci 80:1364–1372.

Tonge R, Shaw J, Middleton B, Rowlinson R, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M. 2001. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1:377–396.

Unlu M, Morgan ME, Minden JS. 1997. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077.

Vayalil PK, Mittal A, Katiyar SK. 2004. Proanthocyanidins from grape seeds inhibit expression of matrix metalloproteinases in human prostate carcinoma cells, which is associated with the inhibition of activation of MAPK and NF kappa B. Carcinogenesis 25:987–995.

Yamagishi S, Matsui T, Nakamura K, Takeuchi M. 2005. Minodronate, a nitrogen-containing bisphosphonate, inhibits advanced glycation end product-induced vascular cell adhesion molecule-1 expression in endothelial cells by suppressing reactive oxygen species generation. Int J Tissue React 27:189–195.

Zhang W, Slusher B, Murakawa Y, Wozniak KM, Tsukamoto T, Jackson PF, Sima AA. 2002. GCPII (NAALADase) inhibition prevents long-term diabetic neuropathy in type 1 diabetic BB/Wor rats. J Neurol Sci 194:21–28.

Zhang FL, Gao HQ, Wu JM, Ma YB, You BA, Li BY, Xuan JH. 2006a. Selective inhibition by grape seed proanthocyanidin extracts of cell adhesion molecule expression induced by advanced glycation end products in endothelial cells. J Cardiovasc Pharmacol 48(2):47–53.

Zhang X, Guo Y, Song Y, Sun W, Yu C, Zhao X, Wang H, Jiang H, Li Y, Qian X, Jiang Y, He F. 2006b. Proteomic analysis of individual variation in normal livers of human beings using difference gel electrophoresis. Proteomics 6:5260–5268.

Zhou Y, Ma YB, Gao HQ, Cheng M. 2005. Experiment study on anti-nonenzyme glycosylation effect of grape seed proanthocyanidin extract in diabetic rats. Chin J Geriatr 24:49–52.