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LABORATORY STUDY

Potential biomarkers associated with diabetic glomerulopathy through proteomics

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Abstract

Diabetic nephropathy (DN) is characterized by the development of progressive glomerulosclerotic lesions gradually leading to an increasing loss of functioning kidney parenchyma. Relatively little proteomic research of isolated glomeruli of experimental animal models has been done so far. Isolated glomerular proteomics is an innovative tool that potentially detects simultaneous expressions of glomeruli in diabetic pathological contexts. We compared the isolated glomerular profiles of rats with and without diabetes. The proteins in the aliquots of glomeruli were subjected to two-dimensional gel electrophoresis. The protein spots were matched and quantified using an imaging analysis system. The peptide mass fingerprints were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and a bioinformation search. We found that diabetes increased collagen type I and collagen type IV levels in diabetic glomeruli when compared to normal control group using Dynabeads. We found that rats with diabetes had significantly higher abundance of the Protein disulfide isomerase associated 3, Aspartoacylase-3,3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase, Lactamase beta 2 and Agmat protein. However, diabetic glomeruli in rats had significantly lower levels of the Regucalcin, rCG52140, Aldo-keto reductase family 1, Peroxiredoxin 1, and L-arginine: glycine amidinotransferase. These proteins of interest were reported to modulate disturbances in the homeostasis of endoplasmic reticulum stress, disturbance of inflammatory and fibrinogenic activities, impairing endothelial function, and dysregulation in the antioxidation capacity/oxidative stress in several tissue types under pathological contexts. Taken together, our high-throughput isolated glomerular proteomic findings indicated that multiple pathological reactions presumably occurred in DN.

Keywords

Biomarker, diabetic nephropathy, proteomics, proteinuria, Two-D

History

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Introduction

Chronic kidney disease has emerged as a global public health burden. Taiwan has the highest incidence and prevalence rates of end-stage renal disease (ESRD) in the world.[1] Diabetic nephropathy (DN) is the most common cause of chronic kidney failure and end-stage kidney disease in Taiwan. DN, also known as Kimmelstiel-Wilson syndrome, or nodular diabetic glomerulosclerosis[2] and intercapillary glomerulonephritis, is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. DN is characterized by the development of progressive glomerulosclerotic and tubulointerstitial kidney lesions gradually leading to an increasing loss of functioning kidney parenchyma.[3] Hence, the need for reliable diagnostic tools and treatment strategies grows.

Proteomics is a multidisciplinary science of cell/molecular biology, protein engineering and bio-information.[4] Proteome technology integrated with isoelectric focusing (IEF), gel electrophoresis, mass spectrometry and bioinformation is an emerging tool useful for high throughput detecting simultaneous expression of proteins in cell cultures and tissues in physiological and pathological contexts.[5–8] Renal tissue proteomics and urinary proteomes are recently employed to associate the candidate proteins with the pathogenesis of renal disorders in human subjects.[9–11] Since glomerular damage denotes the initial event in the pathogenesis of DN and plays a pivotal role for its progression to ESRD,[12] the glomerulus represents a particularly relevant analytical target of proteomic investigation of DN. However, compared to the total number of proteomic studies in DN research, only few proteomic investigations of isolated glomeruli of DN patients or experimental animal models of DM-related nephropathy

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have been published so far. Therefore, we hypothesize that diabetes induction of specific glomerular damage may be regulated by many bioactive proteins in glomerular tissue. Analyzing the glomerular proteome at early stage of the DN could be helpful to identifying potential proteins to serve as diabetic glomerular sclerosis-related tissue biomarkers. This approach has been applied to different stages of diabetic glomerulonephropathy in the future.

The aims of this study were to investigate the proteome-wide expression of diabetic renal tissue in streptozotocin-induced diabetic model. In this project, we attempted to use two-dimensional polyacrylamide gel electrophoresis/matrix-assisted laser desorption ionization time-of-flight mass spectrometry (2D gel/MALDI-TOF MS) to identify proteins associated with DN. Additionally, all candidate proteins were classified, and a few selected proteins were chosen to confirm the MS identification and alteration in expression.

Materials and methods

Diabetes animal model

We caged 4-month-old male Wistar rats in pairs and maintained them on rodent chow and water *ad libitum.* We induced diabetes as previously described.[13] Briefly, we used a single intraperitoneal injection of 50 mg/kg streptozotocin (Sigma Chemical Inc., St Louis, MO). We considered rats with blood glucose >300 mg/dL as diabetic and used these rates for subsequent experiments. We adjusted the dose of insulin to reach a target blood glucose level of 200–250 mg/dL. We used six rats without streptozotocin injections as normal controls and six rats with streptozotocin injections as diabetic group. On day 28, we collected urine using metabolic cage systems and measured urinary albumin and creatinine levels using commercial assay kits (Sigma-Aldrich, St. Louis, MO). We sacrificed rats and harvested kidneys for immunohistochemical analysis.

Urine and blood biochemistry

HbA1c (Primus Diagnostics, Kansas City, MO) and blood glucose levels in serum and albumin (Dade Behring Inc., Newark NJ), protein and creatinine concentrations (Formosa Biomedical Technology Corp, Taipei, Taiwan) in urine were detected according to the manufacturer's instructions.

Immunohistochemistry

Immunostaining in sections was probed by collagen type I and collagen type II monoclonal antibodies (BioWord, Dublin, OH) and an immunohistochemical staining kit (BioGenex, San Ramon, CA). Sections probed by IgG were used as negative controls. The positive immunolabeled and total cells per high power field in each section were counted, and the percentage of positive labeled cells was calculated.

Glomerular purification

Rat glomeruli were isolated based on a previously published method.[14] Briefly, after rats were perfused with deactivated Dynabeads M-450, the perfused kidneys were collected and cut into small pieces, which were ground on a 100-mesh sterile stainless sieve. The bead strapping glomeruli were collected with a magnetic bar. After digestion with collagenase and washes, glomeruli were recollected with a magnetic bar. The purity of glomeruli obtained by this method was >95% with no renal tubule contamination. The purified glomeruli were used for 2D gel electrophoresis.

IEF and 2D gel electrophoresis

IEF and 2D electrophoresis of glomerular tissue extracts are according to the manufacturer's instructions and previous description.[15–17] Briefly, aliquots of renal glomerular tissue extracts (150 µg) dissolved in 250 µL rehydration buffer containing 8 M urea, 2% CHAPS, 1% dithiothreitol, 0.5% IPG (pH 4–7) and bromophenol blue are loaded onto immobiline™ strips (pH 4–7, 13 cm; GE healthcare Bio-Sciences AB, Uppsala, Sweden). The strips are hydrated and IEF at 32,000 V/h, 20°C using an Ettan™ IPGphor II/3 isoelectric focusing unit (GE Healthcare Bio-Sciences AB). After IEF, the strips are equilibrated in buffer containing 50 mM Tris–HCl (pH 8.8), 8 M urea, 30% glycerol, 2% SDS and 0.25% iodoacetamide then 12.5% SDS–polyacrylamide gel electrophoreses at 110 V for 16 h. Each specimen is subjected to IEF and gel electrophoresis in triplicate.

Silver staining and gel imaging

After electrophoresis, gels are silver stained according to the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Briefly, gels are fixed, sensitized, rinsed with de-ionized water then silver stained (0.25% silver nitrate and 0.01% formaldehyde). Protein spots in the gels are developed in 0.25% sodium carbonate and 0.01% formaldehyde. Stained polyacrylamide gels are scanned using an Amersham Image Scanner (Amersham Bioscience Inc., Piscataway, NJ). The images and spot match are analyzed using Bio-Rad Proteoweaver 2-D Analysis Software Version 4.0 (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instructions.

MALDI-TOF/MS

Spots of interest are manually excised, washed with de-ionized water, de-stained (1% $K_3Fe(CN)_6$ and 1.6% $Na_2S_2O_3$), rehydrated, reduced then trypsin (20 ng/mL) digested at 37°C for 16 h. Digests are extracted with 1% trifluoroacetate in acetonitrile. Aliquots of the digest are loaded onto AnchorChip and followed by MALDI-TOF assessment using an Ultraflex™ TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) according to the manufacturer's instructions. Peptide mass data are submitted to NCBI or Swiss-Port database using MASCO search engines. The MASCOT score $\geq$65 and the peptide sequence coverage $\geq$20% are considered as a potent candidate.[18]

Statistical analysis

All values were expressed as means ± standard errors. An independent-sample *t*-test was used to analyze the difference between the wild-type and diabetes-treatment groups.

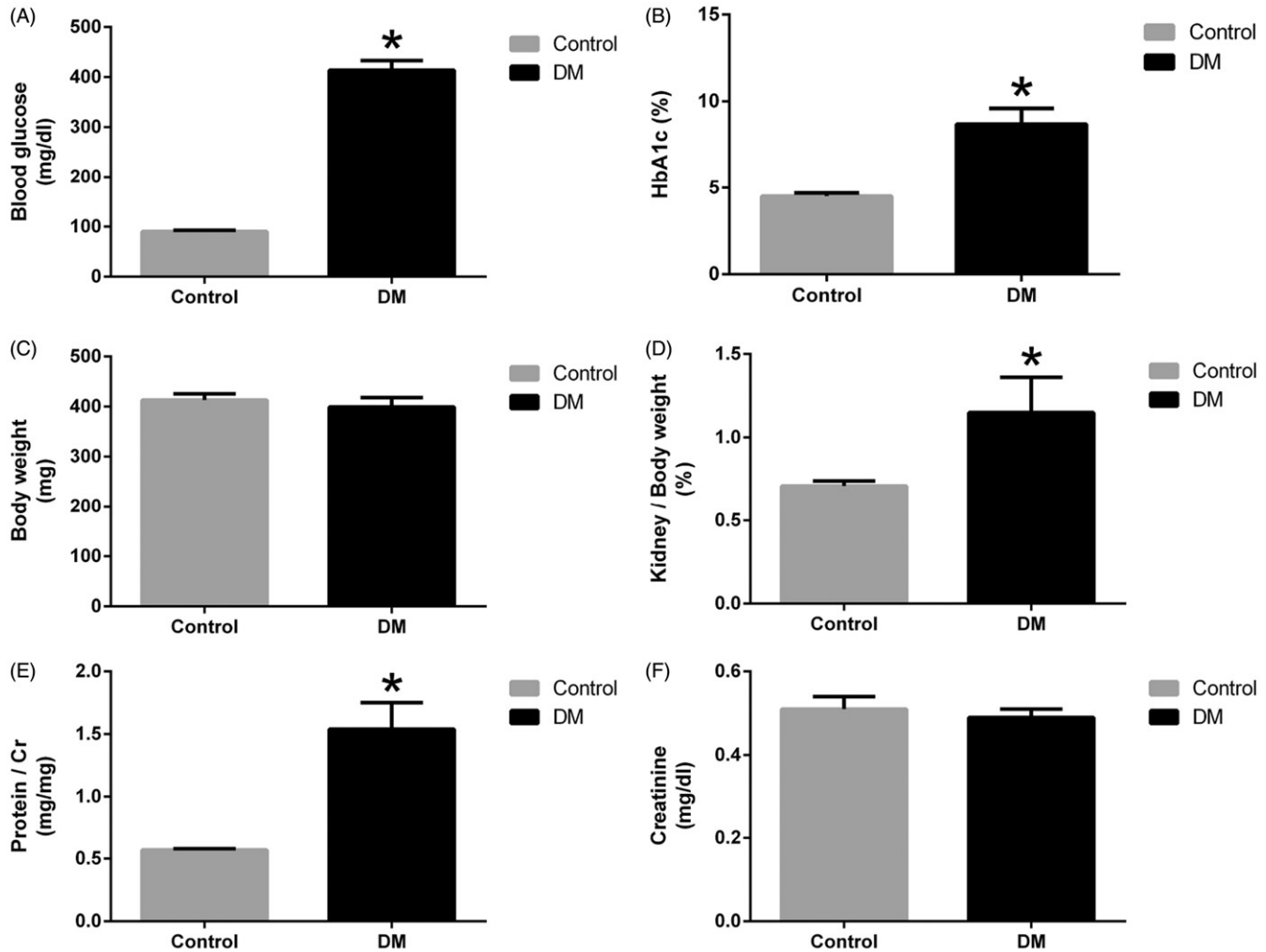

Figure 1. Effect of diabetes on biochemical parameters *in vivo*. Diabetes increased levels of blood sugar (A), HbA_{1c} (B), kidney/body weight (%) (D) and urinary protein/Cr (E) in experimental animals. Diabetes mildly but no significantly attenuated body weight (C) and no significantly difference in serum Cr level (F) between these two groups. Rats were given STZ to induce diabetes. Symbol * indicates significant difference from the normal group ($p < 0.05$).

Results

Biochemical properties between normal and diabetic rats

In comparison with the normal control group, diabetes significantly increased systemic blood sugar level (Figure 1A) coincided with the level of HBA1C (Figure 1B). Furthermore, diabetes significantly increased body weight (Figure 1C) and ratio of kidney weight/body weight (Figure 1D). Quantitative renal functional analyses showed that diabetic rats had higher total urinary excretion (Figure 1E) but the same level of serum Cr (Figure 1F) when compared to the wild-type group.

Renal fibrosis in diabetic kidney and isolation of glomeruli from kidney

Immunoblotting showed that diabetes increased collagen type I and collagen type IV levels in diabetic glomeruli (Figure 2A and B) when compared to normal control group. Collagenase digestion of the kidney had little effect on the glomerular structure. Dynabeads accumulated in the glomerular vessels, making the glomeruli easy to isolate using a magnet with a low degree of contaminating tissues (Figure 2C).

Comparative proteome 2-DE profiles of rat renal tissue with and without diabetes

As shown in Figure 3(A), we investigated the proteomic profiles of rat renal glomerular tissue with and without diabetes. Renal glomerular tissues were ground, lysis with protein extraction solution and de-salted. Aliquots of renal glomerular tissue extract were subjected to IEF (pH 3–10, 13-cm) and two-dimensional electrophoresis. In silver-stained gels, spot of renal glomerular tissue extracts was matched. Image match analysis showed that 10 proteins were prominently altered in diabetic renal glomerular tissues.

MALDI is a soft ionization technique allowing the analysis of biomolecules and large organic molecules. We elucidated protein expressions in renal glomerular tissue exposed to high glucose stress and these proteins were chosen for protein sequence identification by MALDI-TOF/MS. In our study, 10 different proteins sequence was identified between rat with or

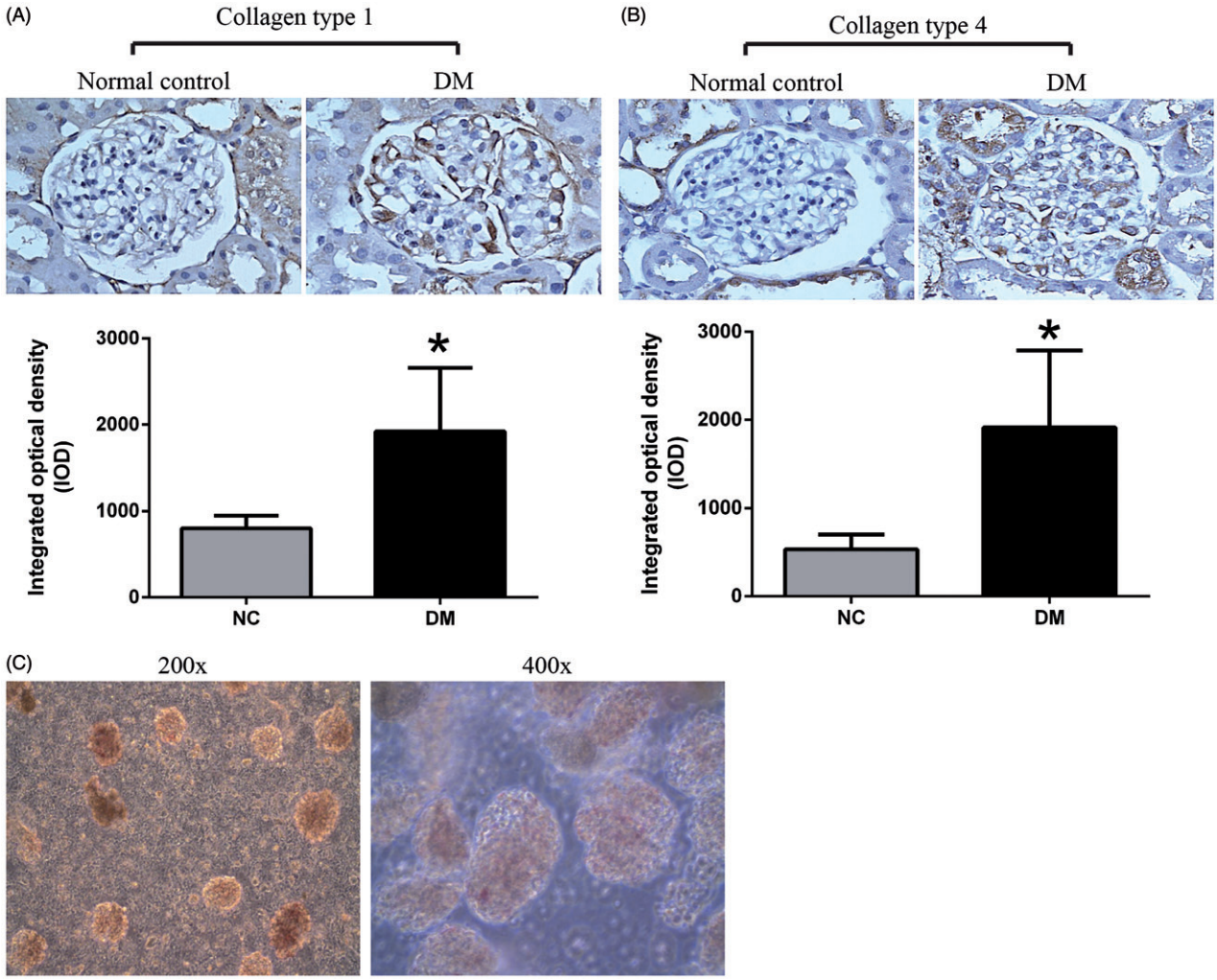

Figure 2. (A) Immunohistochemical photograph of glomeruli in rats with and without diabetes. The diabetic group expressed stronger type I collagen (A) and type IV collagen (B) expression when compared with the normal group. Semi-quantitative evaluation of the positive immune-labeled cells was shown below for type I and IV collagen staining. Data were expressed as mean ± SE. *$p < 0.05$ versus control group. Specimens were observed under magnification 400×. (C) Histological examination of rat glomeruli subjected to magnetic bead perfusion. An adult rat was perfused with magnetic beads through the heart and glomeruli were isolated.

without diabetes. These different proteins could be the targets or biomarkers in the pathogenesis of DN.

Protein identification

The spots of interest were subjected to in-gel trypsin digestion and MALDI-TOF mass spectrometry in order to elucidate the peptide mass fingerprints. Figure 3(C) shows the representative mass spectrum of Spot 15863 (Protein disulfide isomerase associated 3). The peptide mass data of each spot were submitted to the NCBI and SwissPort bioinformation stations using MASCOT search engines. All spots of interest in Figure 3(B) were positively identified as Protein disulfide isomerase associated 3 (Spot 15863), Aspartoacylase-3 (Spot 15674), 3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase (Spot 15523), Lactamase beta 2 (Spot 15513), Agmat protein (Spot 15666), Regucalcin (senescence marker protein-30) (Spot 15539), rCG52140 (Spot 15753), Aldo-keto reductase family 1 (AKR1) (Spot 15642), Peroxiredoxin 1 (Spot 15622), and L-arginine: glycine amidinotransferase (Spot 15627), respectively. The mass spectrometric identified proteins are summarized in Table 1.

Compared with the control group, rats with diabetes had significantly higher abundance of the Protein disulfide isomerase associated 3 (Figure 4A), Aspartoacylase-3 ($181.2 \pm 76.7\%$; $p < 0.01$) (Figure 4B), 3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase ($232.2 \pm 76.7\%$; $p < 0.01$) (Figure 4C), Lactamase beta 2 ($228.4 \pm 76.7\%$; $p < 0.01$) (Figure 4D) and Agmat protein ($171.2 \pm 76.7\%$; $p < 0.01$) (Figure 4E). On the other hand, diabetic glomeruli in rats had significantly lower levels of the Regucalcin ($50.9 \pm 12.1\%$; $p < 0.01$; Figure 5A), rCG52140 ($44.0 \pm 12.1\%$; $p < 0.01$; Figure 5B), AKR1 ($11.9 \pm 9.1\%$; $p < 0.01$; Figure 5C), Peroxiredoxin 1 ($43.0 \pm 11.9\%$; $p < 0.01$; Figure 5D), and L-arginine: glycine amidinotransferase ($51.1 \pm 9.9\%$; $p < 0.01$; Figure 5E).

Discussion

In this study, the abundances of Protein disulfide isomerase associated 3, Aspartoacylase-3,3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase, Lactamase beta 2, Agmat protein, Regucalcin, rCG52140, AKR1, Peroxiredoxin 1, L-arginine: glycine amidinotransferase in

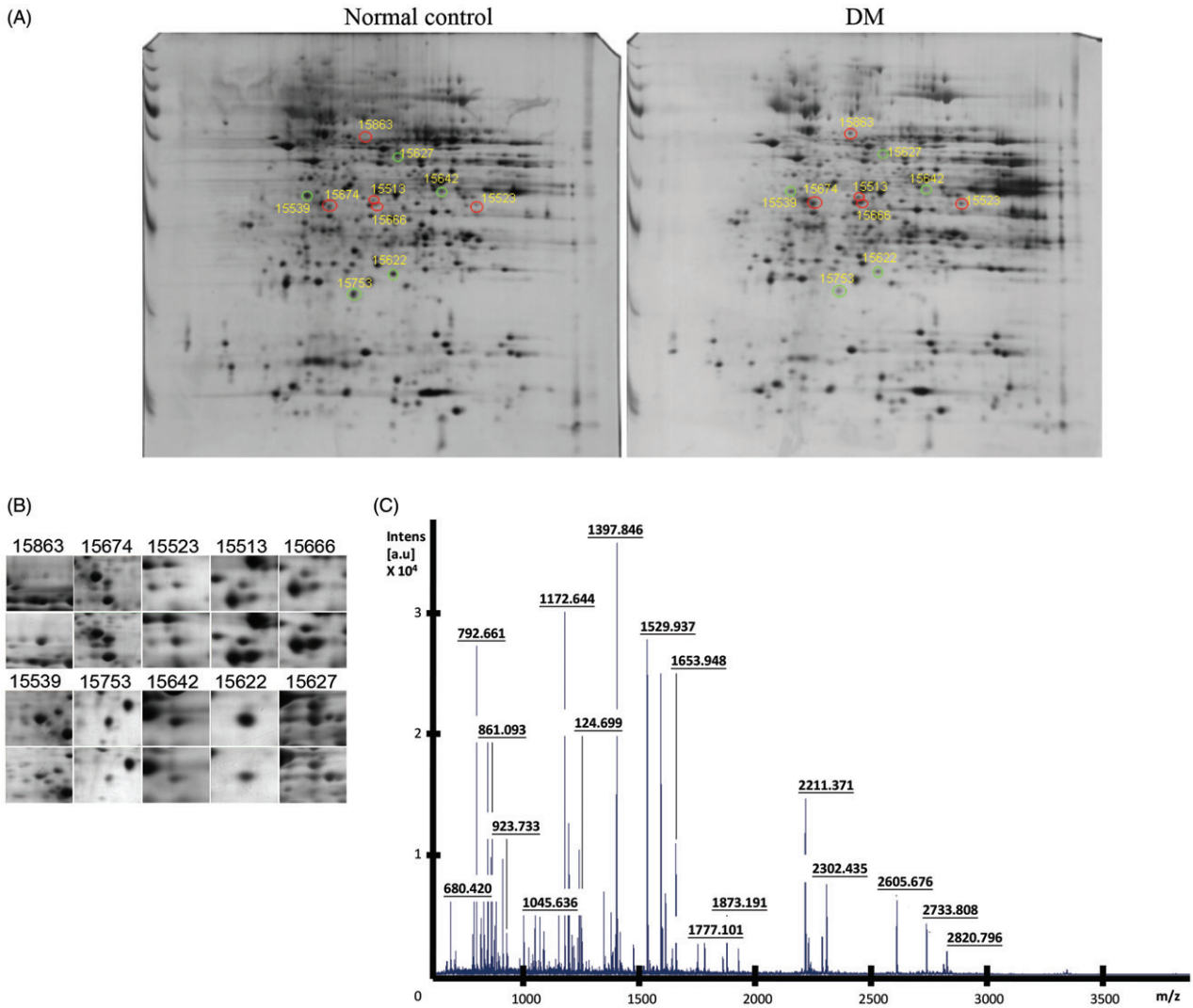

Figure 3. (A) Representative 2D gel electrophoretograms of glomerular proteins in rats with and without diabetes; 250 μg of glomerular lysate was subjected to IEF (pH 4–7), SDS–polyacrylamide gel separation and silver staining. (B) Enlarged regions of the 10 spots of interest in the silver-stained SDS-polyacrylamide gels. The spots in the gels of normal glomeruli and diabetic glomeruli from rats were matched using Bio-Rad Proteoweaver 2D Analysis Software Version 4.0. The arrows in the images indicate the spots of interest in both groups. (C) Representative mass spectrum of Spot 15863 (Protein disulfide isomerase associated 3). In-gel trypsin digestion products were assessed using an Ultraflex™ TOF/TOF mass spectrophotometer. Peptide mass fingerprints were submitted to the NCBI or the SwissPort bioinformation stations using MASCOT search engine. The *X*- and *Y*-axes in the mass spectrum indicate *m*/*z* and intensities of peptides (arbitrary unit × 104), respectively.

Table 1. The mass spectrometric identified proteins.

Spots	Protein sequence identified by MALDI-TOF/MS
15863	Protein disulfide isomerase associated 3
15674	Aspartoacylase-3
15523	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase
15513	Lactamase beta 2
15666	Agmat protein
15539	Regucalcin (senescence marker protein-30)
15753	rCG52140
15642	Aldo-keto reductase family 1
15622	Peroxiredoxin 1
15627	L-arginine: glycine amidinotransferase

renal glomeruli were prominently different in diabetic rats. Previous studies have demonstrated Early Urinary and Plasma Biomarkers in Experimental Diabetic Nephropathy.[19,20] Since glomerular sclerosis represents the primary event in the pathogenesis of DN and plays a key role for its progression to ESRD,[21] the glomerulus represents a particularly relevant analytical target of proteomic investigation of DN. However, compared to the previous proteomic studies in DN research, relatively little proteomic research of isolated glomeruli of experimental animal models has been done so far. This study reports the prominent evidence that the 10 differential renal glomerular tissue proteins reflected the occurrence of diabetic glomerular extracellular matrix accumulation. The differential renal glomerular proteomes also provide new insights into renal local expression of diabetic glomerular fibrotic reactions occurred in diabetic animal.

The diabetic glomeruli showed higher abundances of Protein disulfide isomerase associated 3 (Pdia3). Very few studies have focused on the pathological role of Pdia3 in regulating diabetic renal fibrosis. Pdia3 has been intensively studied as a chaperone protein in glycoprotein folding[22] and major histocompatibility complex I loading.[23] Unlike other

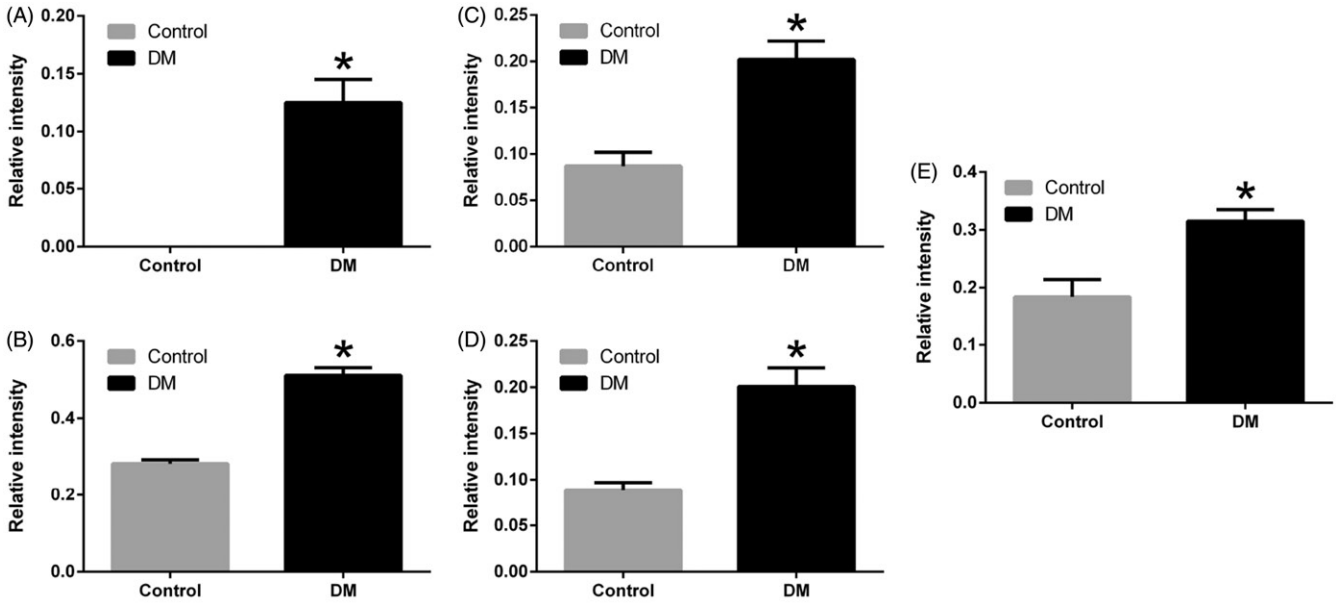

Figure 4. Relative intensities of the positively identified proteins. Rats with diabetic glomeruli displayed higher levels of the (A) protein disulfide isomerase associated 3, (B) aspartoacylase-3, (C) 3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase, (D) lactamase beta 2 and (E) Agmat protein ($171.2 \pm 76.7\%$; $p < 0.01$). The intensity of protein disulfide isomerase associated 3 remained undetectable in six subjects of the normal group. The relative density was calculated by dividing the density of matched spot by the density of all the matched spots in the respective gel. * indicates significant difference from the normal group, $p < 0.05$.

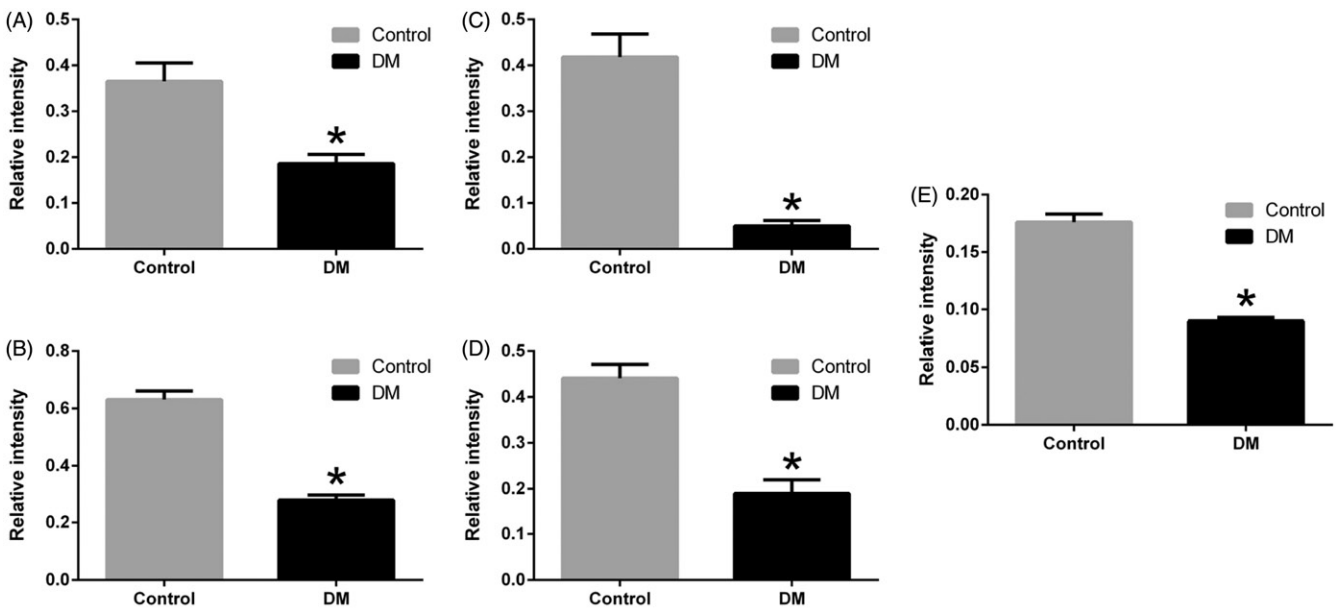

Figure 5. Rats with diabetic isolated glomeruli had lower levels of (A) regucalcin, (B) rCG52140, (C) aldo-keto reductase family 1, (D) peroxiredoxin 1, and (D) L-arginine: glycine and (E) amidinotransferase. The relative density was calculated by dividing the density of matched spot by the density of all the matched spots in the respective gel. * indicates significant difference from the normal group, $p < 0.05$.

protein disulfide isomerase family members, Pdia3 exists not only in the endoplasmic reticulum, but also in the nucleus, extracellular matrix, and plasma membrane, suggesting additional functions.[24] The presence of endoplasmic reticulum (ER) stress in diabetic kidneys reportedly resulted in up-regulation of C/EBP homologous protein (CHOP), which may play a role in increasing kidney lesions and CHOP-deficient mice were protected from DN.[25] Furthermore, MHC antigen expression is a common pathway in renal scarring, and may be important in renal scarring in primary as well as systemic renal diseases.[26] In our study, changes in the abundances of Pdia3 presumably indicated that disturbances in the homeostasis of ER stress, glycoprotein folding, or MHCI-mediated immunological reactions may act as a multiple functional factor that modulates diabetic glomerular extracellular matrix accumulation.

In the present study, diabetic glomerular fibrosis showed higher lactamase beta 2 levels and aspartoacylase-3 levels. Very few previous reports demonstrate the expression of these two molecules in diabetic glomerular fibrosis. High levels of

β-lactamase activity have been found in sputum of patients with cystic fibrosis. This study has clearly demonstrated high-level beta-lactamase activity in sputum samples from cystic fibrosis patients during antipseudomonal treatment.[27] This study further propose and support the hypothesis that β-lactamase production is an important *in vivo* resistance mechanism in infected patients which could subsequently lead to more severe inflammation and scaring formation. Aspartoacylase-3 reported to localize to the cytoplasm of S2 and S3 proximal tubules and to the apical domain of S1 proximal tubules[28] and may function as an hepatitis C virus (HCV) core binding protein which may play a role in the development of HCV-associated diseases.[29] To our interest, aspartoacylase-3 was identified in the kidney tissues from proteomics investigations on aristolochic acid nephropathy (AAN): a case study on rat kidney tissues.[30] To our knowledge, the histological features of AAN consist of paucicellular interstitial fibrosis, severe tubular atrophy, and almost intact glomeruli with media lesions of interlobular arteries.[31] Therefore, the role of aspartoacylase-3 in diabetic glomerulopathy would be virgin place to be investigated in the future. In our study, the altered lactamase beta 2 levels and aspartoacylase-3 levels may reflect the disturbance of inflammatory and fibrinogenic activities in diabetic glomerular fibrosis.

Glomeruli with diabetes showed lower AKR1 levels. The biological role of AKR1 levels in diabetic glomerular fibrosis is unclear. Previous study clearly demonstrated that the fibrotic liver had the decreased expression levels of AKR1 when compared to the normal liver[32] indicating that AKR1 responsible for fibrogenesis in liver. Interesting, AKR1 was up-regulated in association with serum AFP, and was an independent risk factor for HCC in chronic hepatitis C patients, suggesting its possible involvement at a very early stage of hepatocarcinogenesis.[33] Furthermore, Microarray and bioinformatics analysis study suggested that overexpression of AKR1 in PC-3 cells modulates estrogen and androgen metabolism, activates insulin-like growth factor-1 and Akt signaling pathways, as well as promotes tumor angiogenesis and aggressiveness.[34] As we all know, impaired angiogenesis in the post-myocardial infarction heart contributes to the progression to cardiac fibrosis.[35] Recent studies have also shown that chronic endothelial dysfunction can impair multiple aspects of renal physiology and, in turn, contribute to renal fibrosis.[36] These results suggest that lower level of AKR1 may have an important role in the development and progression of diabetic glomerular sclerosis by impairing endothelial function in glomeruli.

The diabetic rats in the current study had lower Peroxiredoxin 1 levels. Peroxiredoxins (Prdxs) are a family of small nonseleno peroxidases currently known to possess six mammalian isoforms. Although their individual roles in cellular redox regulation and antioxidant protection are quite distinct, they all catalyze peroxide reduction of H_2O_2, organic hydroperoxides and peroxynitrite.[37,38] Prdx1 is the first antioxidant protein reported protecting protein function from inactivation through interaction.[39] Prdx1 is capable of scavenging H_2O_2, where it is produced, for example, by NADPH oxidases and the mitochondria. Since NADPH oxidases are in close proximity to growth factor receptors which play an important role in diabetic glomerular fibrosis[40] and given that Prdx have a high susceptibility to inactivation by oxidation, Prdxs function as ''fine tuner'' of cellular H_2O_2-signaling. Previous interesting study shows that diabetes-induced renal injury in rats is attenuated by suramin. In this case, they clearly demonstrated that liquid chromatography–tandem mass spectroscopy-based analysis revealed increases in urinary proteins that are early indicators of DN (e.g., cystatin C and peroxiredoxin-1) in the STZ group, which were blocked by suramin.[41]

In conclusion, we focus on renal tissue sample but not urine sample. This is the reason why the opposite trend in Prdx1 expression between renal tissue and urine. In our study, down-regulation of Prdx1 levels in the diabetic glomerular tissue may imply dysregulation in the antioxidation capacity/oxidative stress in diabetic glomerular sclerosis.

Declaration of interest

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