Down-regulation of alpha-2u globulin in renal mitochondria of STZ-induced diabetic rats observed by a proteomic method

Diminution de l’alpha-2u globuline mitochondriale rénale observée par une méthode protéomique chez des rats présentant un diabète induit par la streptozotocine

Shi-He Sun, Shang-Qing Liu, Chang-Ping Cai, Rong Cai, Li Chen, Quan-Bo Zhang

Abstract

Aim. – To identify the changes of mitochondrial protein expression in diabetic renal parenchyma and to characterize their molecular functions and biological processes in diabetes. Methods. – Mitochondrial proteins extracted from renal parenchyma mitochondria of streptozotocin-induced diabetic rats and normal rats were separated by two-dimensional polyacrylamide gel electrophoresis and identified by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry. Results. – Eleven proteins from 533 visualized protein spots displayed significant different expressions in mitochondria of diabetic kidneys compared with those in normal ones. Among these altered proteins, two proteins with the most obvious changes in protein expression were identified as alpha-2u globulin (mature protein, named A2) and its proteolytically modified form (named A2-fragment) respectively. These proteins were found in mitochondria of male rat renal parenchyma and were proved to be down-regulated in diabetic rats simultaneously. Conclusion. – Our results suggest that down-regulation of alpha-2u globulin may be associated with an abnormal β-oxidation of long-chain fatty acids during diabetes. The decreased expression of A2-fragment in renal mitochondria of diabetic nephropathy may reduce fatty acid β-oxidation, which leads to a diminished energy supply from mitochondria to kidney tissue and the deposition of a large number of fatty acids in the kidney, ultimately causing and aggravating kidney damage. In conclusion, these findings may be helpful for understanding the molecular mechanism of diabetic nephropathy.

Résumé

Objectif. – Identifier les modifications de l’expression des protéines mitochondriales du parenchyme rénal diabétique et caractériser leurs fonctions moléculaires et processus biologiques au cours du diabète. Méthodes. – Des protéines mitochondriales extraites du parenchyme mitochondrial rénal de rats présentant un diabète induit par la streptozotocine étaient séparées par électrophorèse bidimensionnelle sur gel de polyacrylamide et identifiées par une spectrométrie de masse au moyen du MALDI-QqTOF. Résultats. – À partir de 533 taches de protéine visualisées, 11 protéines ont été identifiées dont les expressions dans la mitochondrie rénale diabétique étaient différentes de celles observées dans la mitochondrie rénale normale. Deux de ces protéines modifiées présentant des altérations d’expression importantes étaient identifiées comme l’alpha-2u globuline (protéine mature, appelée A2) et sa forme modifiée par protéolyse (appelée fragment A2). La comparaison des taux de protéines mitochondriales rénales chez les rats mâles diabétiques et non diabétiques montrait un taux plus faible lié à la maladie. Conclusion. – Nos résultats suggèrent la possibilité au cours du diabète d’une diminution de l’alpha-2u globuline associée à une bêta-oxydation anormale des acides gras à chaînes longues. Il est possible que la diminution de l’expression du fragment A2 dans la mitochondrie rénale de la néphropathie diabétique diminue la bêta-oxydation des acides gras et par conséquence l’énergie de source mitochondriale disponible pour le tissu rénal, favorisant la déposition d’un grand nombre d’acides gras dans le rein, cause ultime des lésions rénales. En conclusion, ces résultats contribuent à notre compréhension des mécanismes moléculaires de la néphropathie diabétique.

1. Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal failure in the Western world, constituting about
40% of all new end stage renal disease cases in the United States [1–3]. Although type 1 diabetes only accounts for 5 to 10% of diabetic patients, its incidence continues to increase worldwide [4]. Some studies have demonstrated that the onset and course of DN can be significantly improved by intervention in early stage of the development of DN [5]. However, no one type of treatment to DN has been proven effective so far. Hence, a further elucidation of the pathogenesis of DN, in particular, to find a new regulation target and to find an effective drug intervention, is very important and meaningful to the treatment of DN.

Over the years, many scholars have done a large number of studies about the genomic and proteomic changes of DN [6–16] and believed that DN occurs as a result of interactions among a number of cytokines, active substances, enzymes, receptors and other factors. In addition to the disorder of glucose metabolism in DN, there are varying degrees of lipid metabolism disorders which can directly damage the kidneys and also activate a number of cytokines and inflammatory factors so as to promote the development of DN. As early as 1936, Kimmelstiel and Wilson found a large amount of lipid deposition in renal arteries, glomerular and tubular of DN patients [17]. Clinical studies have also confirmed that there is a certain correlation exists between the disorder of lipid metabolism and DN. Tilton’s group analyzed the renal cortical proteome of db/db mice using two-dimensional polyacrylamide gel electrophoresis (2-DE) combined with matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF-MS) technology and found 278 high confidence identifications whose expression levels were significantly increased or decreased to greater than two-fold by diabetes, 12% of which involved fatty acid metabolism [18]. Despite recent progress in understanding lipid metabolism, it’s still unclear about the lipid metabolism disorders in DN and its pathogenesis that leads to diabetes-induced renal damage.

Mammalian mitochondria, which are ubiquitous organelles responsible for 90% of ATP production in respiring cells, are best known for housing the oxidative phosphorylation machinery as well as enzymes needed for free fatty acid metabolism and the Krebs cycle [19]. Mitochondria are the major sites of energy production within cells and the hyperglycemia-mediated metabolism which plays a pivotal role in the complex pathogenic mechanisms of DN, so global analysis of alterations in renal mitochondrial protein expression is necessary to better understand the complex pathogenic mechanisms of DN. In this study, we aimed to identify the changes of mitochondrial protein expression in diabetic renal parenchyma and to characterize their molecular functions and biological processes in diabetes.

2. Material and methods

2.1. Reagents and experimental animals

OptiPrep iodixanol was purchased from Axis-Shield (Oslo, Norway), Streptozotocin, ammonium persulfate, urea, low-melt agarose, dithioerythritol, iodoacetamide, PMSF, endonuclease were from Sigma (Sigma, St. Louis, MO). Sucrose, D-mannitol, Hepes, coomassie brilliant blue R250, SDS, acrylamide, bis-acrylamide, Tris, CHAPS, EDTA, bromophenol blue, glycine, thiourea, mineral oil, TEMED, Protease Inhibitor Cocktail were from Amersco (Solon, Ohio, USA). ReadyStrip™ IPG Strips, Bio-Lyte Ampholyte, RC DC protein Assary, Readyprep™ 2-D cleanup kit were from Bio-Rad (Hercules, CA, USA). All other chemicals were commercial products of high purity.

All animal studies were approved by and conducted in accordance with North Sichuan Medical College Institutional Animal Care and Use Committee guidelines according to the licenses for use of experimental animals issued by the Ministry of Justice P.C.R. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Establishment of diabetic models

The establishment of diabetic rat models was performed as described previously [20]. Briefly, thirty male Sprague-Dawley rats (weight, 150 to 190 g) were randomly assigned to a diabetic group and a control group. After an overnight fast, twenty rats in the diabetic group received a single intraperitoneal injection of sterile STZ (65 mg/kg body weight) in 0.1 mol/l sodium citrate buffer (pH 4.2). Ten rats in the control group received an equal volume of sodium citrate buffer. One week after STZ injections, rats with strong positive urine glucose (measured by reagent papers) and blood glucose greater than 16.7 mmol/l were classified as diabetic. Glycaemia was monitored by weekly determination of urine glucose levels. During the study, the rats with weak positive urine glucose or with their blood glucose less or equal to 16.7 mmol/l were excluded. All rats were housed in an air-conditioned room with free access to standard laboratory chow and water in a 12-h light/dark cycle.

2.3. Preparation of samples

After twelve weeks of diabetes, the remaining eleven diabetic rats and ten normal rats were used for subsequent experiments. Six diabetic rats and six normal rats were sacrificed by cervical dislocation and kidneys were promptly removed. Longitudinal incision of the left kidneys was made, fixed in 10% neutral formalin for hematoxylin-eosin staining. Then the morphological changes were observed by light microscopy. For electron microscopy analysis, fresh tissue cut from the upper pole of the left kidneys by cold blade were immediately fixed with 3% glutaraldehyde in 0.075 M phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in 0.24 M phosphate buffer (pH 7.4). After postfixation, the specimens were dehydrated through an ethanol, acetone series, and embedded in Epon. The thin sections were stained with uranylacetate and lead citrate, and examined in a Hitachi-7500 electron microscope.

2.4. Isolation and purification of mitochondria

Mitochondrial preparations were made as described [21,22] with minor modification. The rats were sacrificed by cervical dislocation. Twelve right kidneys from six diabetic rats and six normal rats were then removed and immediately placed...
in ice-cold buffer A (200 mM mannitol, 50 mM sucrose, 1 mM EDTA, and 20 mM Hepes-KOH, 1 mM PMSF) at pH 7.4. After mincing with scissors and washing to remove blood, the renal parenchyma was separately homogenized in a Potter-Elvejhem homogenizer with buffer A (10 ml homogenization buffer A for every 2.5 g tissue). 100 μl homogenate was subjected to marker enzyme assays to determine the purity of mitochondria. The remaining homogenate was centrifuged at 1000 g for 20 h at 17°C in a focusing chamber. IEF with a Ettan™ IPG Phor II™ (Amersham Biosciences, Piscataway, NJ, USA) was performed at 17°C with the following parameters: 250 V for 1 h, 1000 V gradient for 2.5 h, 8000 V gradient for 5 h, then 8000 V for a total 108000 Vh. Second-dimension separation was undertaken using the Ettan™ DALTSix system (Amersham Biosciences, Piscataway, NJ, USA). IPG strips were equilibrated for 15 min in 6 ml of equilibration buffer [6 M urea, 2% (w/v) SDS, 0.375 M Tris HCl, pH 8.8, 20% (w/v) glycerol, 2% (w/v) DTT], and then for a further 15 min in the same equilibration buffer to which 2% (w/v) DTT was replaced with 2.5% (w/v) iodoacetamide. IPG strips were loaded as per the manufacturer’s instructions onto 12% polyacrylamide gels in Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3.) Gels were run at 50 V for 2 h and then 200 V at 17°C. Analytical gels were stained with silver nitrate as previously described [23] and preparative gels were stained with Coomassie Brilliant Blue R250. Silver-stained gels were scanned by Image Scanner, the images were analyzed using ImageMaster™ 2D Platinum 7.0 software (Amersham Biosciences, Geneva, Switzerland). To evaluate the reproducibility and account for experimental variation, three gels from diabetic group and three gels from control group were prepared. Valid spots detected by ImageMaster™ 2D Platinum 7.0 were matched between the individual gel images (two gels from diabetic group and three gels from control group) and the reference gel image (one gel from diabetic group) which was a representative of all the gel images from diabetic group. Protein spots were quantitated in terms of their normalized quantity, which was normalized as a percentage of the total quantity of valid spots present in the gel. Quantification values (% Volume) remained relatively independent of variations due to protein loading and staining by considering the total volume over all the spots in the image.

2.5. Mitochondrial purity assessment and preparation of mitochondrial proteins

The purity of the freshly purified mitochondria from normal rats was assessed by electron microscopy analysis. Specimen preparation for electron microscopy was as mentioned above. A small amount of mitochondrial fraction was subjected to marker enzyme assays to evaluate the purity of mitochondria. The 5'-nucleotidase activity was assayed by the hydrolysis of adenosine-5'-nucleotidase acid to adenosine and inorganic phosphorus, and the content of inorganic phosphorus was evaluated by reading its absorbance at 680 nm.

Mitochondrially enriched pellets were rehydrated in a Lysis buffer (7 M Urea, 2 M thiourea, 4%(w/v) CHAPS, 40 mM DTT, 0.2%(w/v) Bio-Lyte pH 3–10, 0.001%(w/v) bromphenol blue] for silver-stained gels and 3000 μg protein in 334 μl rehydration buffer for Coomassie-stained gels. The strips (pH3–10NL, 17 cm) were loaded with rehydration buffer for Coomassie-stained gels. The strips (pH3–10NL, 17 cm) were allowed to rehydrate for 1 h before adding mineral oil. The passive hydration of the gels was carried out overnight for 20 h at 17°C in a focusing chamber. IEF with a Ettan™ IPG Phor II™ (Amersham Biosciences, Piscataway, NJ, USA) was performed at 17°C with the following parameters: 250 V for 1 h, 1000 V gradient for 2.5 h, 8000 V gradient for 5 h, then 8000 V for a total 108000 Vh. Second-dimension separation was undertaken using the Ettan™ DALTSix system (Amersham Biosciences, Piscataway, NJ, USA). IPG strips were equilibrated for 15 min in 6 ml of equilibration buffer [6 M urea, 2% (w/v) SDS, 0.375 M Tris HCl, pH 8.8, 20% (w/v) glycerol, 2% (w/v) DTT], and then for a further 15 min in the same equilibration buffer to which 2% (w/v) DTT was replaced with 2.5% (w/v) iodoacetamide. IPG strips were loaded as per the manufacturer’s instructions onto 12% polyacrylamide gels in Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3.) Gels were run at 50 V for 2 h and then 200 V at 17°C. Analytical gels were stained with silver nitrate as previously described [23] and preparative gels were stained with Coomassie Brilliant Blue R250. Silver-stained gels were scanned by ImageScanner, the images were analyzed using ImageMaster™ 2D Platinum 7.0 software (Amersham Biosciences, Geneva, Switzerland). To evaluate the reproducibility and account for experimental variation, three gels from diabetic group and three gels from control group were prepared. Valid spots detected by ImageMaster™ 2D Platinum 7.0 were matched between the individual gel images (two gels from diabetic group and three gels from control group) and the reference gel image (one gel from diabetic group) which was a representative of all the gel images from diabetic group. Protein spots were quantitated in terms of their normalized quantity, which was normalized as a percentage of the total quantity of valid spots present in the gel. Quantification values (% Volume) remained relatively independent of variations due to protein loading and staining by considering the total volume over all the spots in the image.

2.7. Protein identification and bioinformatic analyses

Protein spots with significant different expressions were excised from the CBB R-250 stained 2-DE gels for matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF-TOF MS) analysis. Briefly, the selected gel pieces were excised from the Coomassie Brilliant Blue-stained gels and then washed with distilled water for 15 min. The destaining procedure was carried out by washing the spots with 50% acetonitrile (ACN) and 50 mM ammonium bicarbonate (1:1) for 20 min at room temperature. After dehydrating the gel pieces with 100 μl of 100% ACN, they were dried in a vacuum centrifuge. Then, the gel pieces were rehydrated for digestion with 10 μl of 12.5 ng/μl trypsin and incubated at 4°C for 30 min, and at 37°C overnight. The peptide samples were extracted with different concentrations of ACN and trifluoroacetic acid (TFA). Typical dried peptide was
Fig. 1. Light micrographs and electron micrographs of kidneys from normal (a,c) and one diabetic rats (b,d,e,f). a: a normal glomerulus. Magnification \( \times 400 \); b: proximal tubule epithelial cell edema and hypertrophy (arrowheads). Magnification \( \times 400 \); c: the glomerular microstructure. Magnification \( \times 12000 \); d: thickened glomerular basement membrane (arrowheads). Magnification \( \times 12000 \); e: foot process effacement or fusion (arrowheads). Magnification \( \times 15000 \); f: mesangial regions proliferation and expansion (heart-shaped). Magnification \( \times 5000 \). c: capillary lumen; En: endothelial cell; Ep: visceral epithelial cell (podocyte); PT: proximal tubule; GBM: glomerular basement membrane; FGC: fenestrae of glomerular capillaries; FP: foot process; Me: mesangium; US: urinary space.

Redissolved in α-cyano-3-hydroxy-cinnamic acid saturated solution (in 50% ACN aqueous solution containing 0.1% TFA) on a well on the MALDI plate, and air dried. MALDI-TOF-TOF MS were performed on a 4700 Proteomics Analyzer (TOF/TOFTM) (Applied Biosystems, CA, USA) including a 200 Hz laser and acquisition system. MASCOT Software (Matrix Science, London, UK) was used to search the NCBI non-redundant protein database in order to obtain a match and identify the protein spot.
2.8. Western Blotting

Equal amounts of mitochondrial proteins from control and diabetic groups were mixed with Laemmli buffer, boiled for 5 min, and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These proteins were transferred to a nitrocellulose membrane and blocked with 5% milk/TTBS. The target protein was detected with a specific, polyclonal goat antibody (AF586, R&D Systems, Minneapolis, US) raised against alpha-2u globulin in rat (1:1000 dilution). The membranes were washed four times for 5 min each with TTBS and then incubated in horseradish peroxidase-conjugated polyclonal rabbit anti-goat antibody (1:1500 dilution, NB7376, Novus Biologicals, Colorado, USA). The membranes were washed four times for 15 min each with TTBS and developed with an enhanced chemiluminescence kit (pro#32109, Thermo Scientific, USA). Anti-β-actin antibody (bs-0061R, Bios, Beijing, China) was used as a loading control.

2.9. Statistical analysis

Blood glucose data was analyzed using the Wilcoxon rank sum test. 5′-nucleotidase activity data was analyzed using one-way ANOVA. Three replicate gels were run for each pooled protein extract from diabetic and control groups, a mean and SD were calculated for the normalized volume of each protein spot, and statistical comparisons of the % Volume between diabetic and control group were performed using one-way ANOVA (P < 0.05 considered significant).

3. Results

3.1. Characteristics of diabetic rat models

STZ-induced diabetes was a well-documented model of experimental diabetes. Urine glucose was measured weekly after STZ injections, which was negative (−) in the control group, while the diabetic group was strongly positive (+++). The blood glucose was measured at the first and twelfth week after STZ injections. Comparing with those in the control group, the diabetic group showed significant hyperglycemia after STZ injections. Body weight decreased in some diabetic rats but increased in all the normal rats (not shown). In addition, some pathological changes in the glomerular of diabetic rats were evident, such as proximal tubule epithelial cell edema and hypertrophy, thickening of the glomerular basement membrane, and mesangial regions proliferative and expansion (Fig. 1). In addition, some pathological changes in the glomerular of diabetic rats were evident, including proximal tubule epithelial cell edema and hypertrophy, thickening of the glomerular basement membrane, and mesangial regions proliferation and expansion (Fig. 1).

3.2. Assessment of mitochondrial enrichment

Electron micrographs were used to show the structural features of mitochondria and detect contamination. The structure integrity of the mitochondria prepared from normal rats was remarkably preserved. Although some mitochondria became swollen, the mitochondrial cristae were fine and the membranes were continuous. Most importantly, little contamination can be observed. Moreover, the result of 5′-nucleotidase analysis demonstrated that suspension of purified mitochondria had a very small amount of contamination, indicating they might be from the organelles in which the latent activity of 5′-nucleotidase can be present at Golgi, vacuoles, vesicles and ER. However, activity of 5′-nucleotidase in mitochondrial proteins was almost zero (Fig. 2).

3.3. Proteome map of rat renal mitochondria

Mitochondrial proteins from control and diabetic renal parenchyma were separated by 2-DE. Six proteome maps of rat renal mitochondria are shown in Fig. 3. Approximately 540 protein spots were visualized on each 2-D gel by silver nitrate staining. ANOVA was used to analyze the difference in protein expression between the two groups. Protein spots with significant different expressions (changes of volume ≥ 1.5 fold and P < 0.05) were marked by their respective matched ID in 2-D gels (Fig. 3). Compared to those in the control group, 11 protein spots in the diabetic group were up-regulated (9 protein spots) or down-regulated (2 protein spots). Protein spots of number 35 and 45 were excised from gels and submitted for MALDI-TOF-TOF MS.

3.4. Protein Identification

The identified proteins are summarized in Figs. 4–6 and Table 1. Compare to the control group, expression of protein spots 35 and 45 decreased in the diabetic group significantly. The sequences of the identified peptides from protein spot 35 and 45 are both matched with parts of peptides in alpha-2u
Fig. 3. Representative 2-D gels for mitochondrial proteins extracted from renal parenchyma of control and diabetic rats. IEF was performed on IPG strips (pH 3–10 NL, 17 cm), the second-dimensional separation by SDS-PAGE (12%). The separated proteins were visualized by silver nitrate staining. Protein spots with the corresponding match IDs represent significantly differently expressed proteins.
Fig. 4. Proteomic analysis of protein spot 35 and 45 expression in diabetic rats. Higher magnification images of protein spot 35 and 45 from normal and diabetic 2-D gels (a and b). Class Analysis Histograms with Center values (central tendency) displayed (c and d). The protein spot 35 and 45 values (% volume) were displayed in each match, separated for each class by vertical gray lines; they were significantly decreased in diabetic rats (one-way ANOVA, ImageMaster™ 2D Platinum 7.0). *Significant to \( P = 0.003 \), **Significant to \( P = 0.002 \). The classes were characterized by their central tendency (blue horizontal line) and dispersion interval (bounded by the outer red lines). The Match IDs appear below each histogram.

globulin of rat simultaneously (sequence coverage was 40% and 60% respectively).

3.5. Validation of down-regulated proteins

Equal quantities of protein were used for Western blotting analysis of the differentially expressed proteins. Actin was used as an internal control. As shown in Fig. 6, the changes in the expression levels of alpha-2u globulin decreased significantly in diabetes, which was consistent with the 2-DE results.

4. Discussion

DN is known as a microvascular complication of diabetes mellitus, a metabolic disorder, which induces dysfunction in various types of cells in kidney, eventually leading to progressive renal failure. As the powerhouses of the cell, mitochondria are involved in numerous cellular functions, including ATP production, free fatty acid metabolism, pyrimidine biosynthesis, and calcium homeostasis. Proteomics has been proven as an efficient tool to identify a large number of proteins and to compare the expression of these proteins between complicated diseases and normal organisms, so that the potential pathogenesis of these
Fig. 5. Matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF-TOF MS) and peptide mass fingerprinting. a and b: peptide masses were obtained by MALDI-TOF-TOF MS after in-gel tryptic digestion of spot 35 and 45 in Fig. 4. (c and d) The sequences of the identified peptides from protein 35 and 45 and the sequence of alpha-2u globulin (20.7 kDa), matched peptides shown in Underlined.
complicated diseases can be better analyzed. The current study focused on the identification of mitochondrial proteins of renal parenchyma in STZ-induced diabetic rats and the evaluation of the role of signaling pathways involved in the pathogenesis of DN.

In this study, we performed a proteomic approach based on 2-DE and MALDI-TOF-TOF MS to screen for global changes of mitochondrial protein expression in renal parenchyma of STZ-induced diabetic rats. Our study showed that STZ-induced diabetic rats had a significant hyperglycemia and a strong positive of urine glucose. Subsequently, remarkable pathological changes in the kidneys were proven by light micrographs and electron micrographs (Fig. 1). High purity mitochondria were obtained from renal parenchyma. Electron micrographs gave us an intuitional survey of organelles’ integrity and purity; there is no visible vesicle or lysosome in the electron micrographs of purified mitochondrial precipitate. 5′-nucleotidase is usually considered to be a plasma membrane marker and is widely used to evaluate the purity of the mitochondria; cytochemical studies in cultured rat fibroblasts showed that the 5′-nucleotidase patent activity was located on the cell surface and that latent activity was present in cytoplasmic vacuoles and vesicles, and in the Golgi complex [24]; this enzyme was also shown to be indigenous to endoplasmic reticulum membranes as well as plasma membranes in the hepatocyte [25] and Golgi membranes in rat liver [26]; however, to our knowledge, there is no published report concerning its location in the mitochondria. The 5′-nucleotidase activity in mitochondrial protein was almost zero in our study (Fig. 2), which means that there is almost no contamination of vacuoles and vesicles.

To investigate proteins regulated diabetes, we separated and compared mitochondrial proteins expression in renal parenchyma from STZ-induced diabetic and normal rats by 2-DE method. Only two protein spots (Match ID: 45 and 35, Fig. 3) were identified by MALDI-TOF-TOF MS, whereas the other nine protein spots were not identified in the present study due to the low expression levels of these proteins in diabetic rats and normal rats. Protein spots 45 and 35 with the great magnitude of change were identified as alpha-2u globulin simultaneously. Alpha-2u globulin corresponding to match ID 45 was the mature form of alpha-2u globulin. The molecular weight of this mature protein was reduced to 18.7 kDa due to the signal peptide of the rat liver protein alpha-2u globulin (20.7 kDa) being removed during the secretion process [27]. Presumably alpha-2u globulin corresponding to match ID 35 should be a processed form of the mature alpha-2u globulin [28], which was termed 15.5 kDa fatty acid-binding protein (15.5 kDa FABP) in NCBI (http://www.ncbi.nlm.nih.gov/protein/gi%7C127533), this speculation was confirmed by Western blotting analysis (Fig. 6).

Rat alpha-2u globulin is a low molecular weight protein and belongs to the lipocalin protein family, which is synthesized in the liver of adult male rats, secreted into the blood stream, and excreted in the urine [29]. Alpha 2u-globulin expressed in a tissue-, age-, and sex-specific fashion. They are completely absent from the urine of female rats and immature male rats. Several other tissues, in both males and females, also express alpha-2u globulins. These tissues are primarily secretory in nature and include preputial, lachrymal, meibomian, submaxillary, perianal, and mammary glands, kidney, and brain [30]. Several hepatomas [31], the female liver, and male kidney do not synthesize alpha-2u globulin and have no detectable alpha-2u globulin mRNA sequences [32]. Alpha-2u globulin is first synthesized as the 20.7-kDa alpha-2u globulin precursor, which is known to be synthesized by membrane-bound polysomes and then rapidly secreted [33]. During secretion, a signal peptide sequence is cleaved from the 20.7-kDa alpha-2u globulin precursor. The cleaved alpha-2u globulin species (18.7 kDa mature protein) circulate in the blood stream [34], is filtered at the glomerulus, and a portion of them were resorbed into proximal tubule epithelial cells, where the alpha-2u globulin is converted to 15.5 kD cleavage product of alpha-2u globulin (named “15.5 kDa FABP” in NCBI) by lysosomal proteinases [35,36]. In this study, 18.7 kDa alpha-2u globulin (named A2) and 15.5 kD cleavage product (named A2-fragment) identified by MALDI-TOF-TOF MS and western blotting analysis appeared simultaneously in the kidney mitochondria of normal rats. These present observations differed slightly from previous reports with regard to the intracellular distribution of A2/A2-fragment [28,36]. Hepatic origin alpha-2u globulin is secreted into the blood; a portion of alpha-2u globulin excreted in the urine of male rats, and another part of alpha-2u globulin undergoes endocytosis by the proximal tubule epithelial cells. Alpha-2u globulin captured by membrane-bound vesicles is transported to lysosomes, where part of alpha-2u globulin will be converted to A2-fragment by hydrolytic enzymes within
lysosomes [35,36]. After the limited proteolysis of alpha-2u globulin to A2-fragment, a portion of A2-fragment escaped lysosomal degradation and entered the cytosol of the proximal tubule epithelial cells, and the remaining A2-fragment was eventually digested.

The biosynthesis and modification of alpha-2u globulin is under control by many factors [37–40]. Some researchers thought that the rate of hepatic alpha-2u globulin biosynthesis parallels the level of hepatic alpha-2u globulin mRNA sequences, which parallels the level of the protein in liver, serum, and urine [31,32,41]. Earlier studies have shown that alloxan-induced diabetes in male rats led to greater than 80% decrease in the urinary output of alpha-2u globulin [42]. Subsequent studies showed that STZ-induced diabetes resulted in greater than 80% reduction in the hepatic concentration of alpha-2u globulin, which indicates that the hepatic synthesis of alpha-2u globulin in diabetic rats would reduce. One study reported that 36 h after turpentine injection, the serum concentration of alpha-2u globulin decreased by two- to three-fold and remained low thereafter [43]. Kim examined proteomic changes in the serum of rats housed in winter in concrete cages using 2-DE and MS and found alpha-2u globulin is down-regulated in the concreteshoused rats [44]. Thongboonkerd found that alpha-2u globulin in rat urine increased after acute sodium loading [45]. Roy thought that the rate of hepatic alpha-2u globulin biosynthesis parallels the level of hepatic alpha-2u globulin mRNA sequences, which parallels the level of the protein in liver, serum, and urine [31,32,41]. Earlier studies have shown that alloxan-induced diabetes in male rats led to greater than 80% decrease in the urinary output of alpha-2u globulin [42]. Subsequent studies showed that STZ-induced diabetes resulted in greater than 80% reduction in the hepatic concentration of alpha-2u globulin, which indicates that the hepatic synthesis of alpha-2u globulin in diabetic rats would reduce. One study reported that 36 h after turpentine injection, the serum concentration of alpha-2u globulin decreased by two- to three-fold and remained low thereafter [43]. Kim examined proteomic changes in the serum of rats housed in winter in concrete cages using 2-DE and MS and found alpha-2u globulin is down-regulated in the concreteshoused rats [44]. Thongboonkerd found that alpha-2u globulin in rat urine increased after acute sodium loading [45].

In summary, we applied proteomics technology to identify alterations in mitochondrial protein expression in the renal parenchyma of the STZ-induce diabetic rat. Two proteins with significant decreased expression were identified as alpha-2u globulin and the cleavage product of alpha-2u globulin by MALDI-TOF-TOF MS and western blotting analysis. Proteomic analysis of mitochondrial proteins in renal parenchyma proved the down-regulation of alpha-2u globulin and “15.5 kDa FABP” in STZ-induced diabetic rats, which may be associated with an abnormal β-oxidation of long-chain fatty acids during diabetes. These findings may be helpful for understanding the molecular mechanism of diabetic nephropathy.

### Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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### Table 1

Down-regulated proteins in diabetic rats identified by proteomic analysis.

<table>
<thead>
<tr>
<th>Match ID</th>
<th>Protein name</th>
<th>NCBI accession no.</th>
<th>Gene</th>
<th>Nominal MW (kDa)</th>
<th>Calculated pI</th>
<th>Wilcoxon P-value</th>
<th>DM versus Normal</th>
<th>Alteration (Normal/DM)</th>
<th>Normal (%) Volume</th>
<th>DM (%) Volume</th>
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<tbody>
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<td>35alpha-2u Globulin</td>
<td>gi 127533</td>
<td>PGCL1</td>
<td>20.724</td>
<td>5.43</td>
<td>0.003</td>
<td>↓</td>
<td>3.808</td>
<td>0.754</td>
<td>0.022</td>
<td>0.198</td>
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<td>PGCL1</td>
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<td>0.351</td>
<td>0.018</td>
<td>0.138</td>
</tr>
</tbody>
</table>

% volume representing the quantity of protein was analyzed using ImageMaster™ 2D Platinum 7.0 software. MW: molecular weight; pI: isoelectric point; DM: diabetes mellitus; SD: standard deviation.

* Nominal molecular masses and Calculated pI value are from NCBI.
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