**Rapid Communication**

**Differences in expression of retinal proteins between diabetic and normal rats**

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**Abstract**

**Aim:** To compare and identify the differences in expression of retinal proteins between normal and diabetic rats, and to analyze the molecular pathogenetic mechanisms of retinal diseases caused by diabetes.

**Methods:** Changes in protein expression of retinal tissues from diabetic and normal rats were observed using 2-dimensional polyacrylamide gel electrophoresis (2-DE). Some protein spots exhibiting statistically significant variations ($P < 0.05$) were selected randomly and identified by tandem mass spectrometry and analyzed by bioinformatics.

**Results:** 2-DE showed that the expression was up-regulated in 5 retinal proteins, down-regulated in 23 retinal proteins, and disappeared in 8 retinal proteins. Eight spots were identified from the 36 spots by tandem mass spectrometry (MS/MS) and analyzed by bioinformatics. Guanylate kinase 1, triosephosphate isomerase 1, ATP synthase subunit d, albumin and dimethylarginine dimethylaminohydrolase 2 played an important role in signal transduction. Triosephosphate isomerase 1, crystallin alpha B, ATP synthase subunit d and peroxiredoxin 6 were involved in energy metabolism of retinal tissues. Guanylate kinase 1 played an important role in phototransduction of retinal rod photoreceptor cells. Whether crystallin beta A1 plays a role in diabetic retinas is an unknown so far.

**Conclusion:** There are differences in expression of retinal proteins between diabetic and normal rats. These proteins may be involved in the mechanisms and prognosis of retinal diseases caused by diabetes.
ssion profiles in diabetic rat retinas are needed.

Proteomics is defined as the characterization of all proteins encoded by the genome and allows identification of protein-protein interactions and disease-associated proteins. Tools, such as 2-dimensional polyacrylamide gel electrophoresis (2-DE) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), enable the study of disease-associated proteomics. Using this technology, the changes of protein expression in retinal tissue from diabetic rats can also be demonstrated.

The present study was to compare the expression profile of retinal protein in diabetic and normal rats, and to analyze the molecular pathogenetic mechanisms of retinal diseases caused by diabetes.

MATERIALS AND METHODS

Reagents and apparatus
All reagents were purchased from Sigma (St. Louis, MO, USA). Acrylamide, bis-acrylamide, Coomassie brilliant blue R-250, Tris, ammonium persulfate, glycine, EDTA, SDS, bromphenol blue, 2-mercaptoethanol, endonuclease, mineral oil and TEMED were from Amersol (Solon, Ohio, USA). Dithiothreitol (DTT) was from Merck (Darmstadt, Germany). Protein (300 μg for analytical gels, 1500 μg for preparative gels) was utilized for IEF and subsequent 2-D gels.

Establishment of diabetic rat models
The study was approved by the Medical Ethical Committee of North Sichuan Medical College. Thirty healthy adult (eight-week-old) female Sprague-Dawley (SD) rats weighing 180-200 g (provided by Animal Experimental Center, North Sichuan Medical College) were randomly divided into diabetic group and normal control group. Forty rats in diabetic group received a single intra-peritoneal injection of alloxan tetrahydrate (200 mg/kg) in normal sodium, 20 rats in the control group received injection of 1 mmol/L PMSF, and 200 μg/mL DNase I. Samples were incubated at 4°C for 15 min and then centrifuged at room temperature (18-20°C) for 30 min at 16000 r/min to remove DNA, RNA and any particulate materials (tissue and cell debris). As extracted proteins, the supernatant was purified using ReadyPrep cleanup kit (Bio-Rad Laboratories). The protein concentration of samples was measured on a Model 550 microplate reader using a RC DC protein assay kit (Bio-Rad Laboratories) with BSA as standard. Liquid of the final protein solution was stored at -80°C until further use.

First dimensional isoelectric focusing
2-DE was performed according to the manufacturer's instructions. The first dimensional isoelectric focusing (IEF) was performed on precast 17 cm immobilized pH 5-8 gradient (IPG) strips at 17°C using a protein IEF cell. Protein (300 μg for analytical gels, 1500 μg for preparative gels) was utilized for IEF and subsequent 2-D gels.

Equilibration of IPGs using DTT and iodoacetamide
Following IEF, the gel strip was equilibrated for 15 min in the equilibration buffer containing 8 mol/L urea, 4% (w/v) CHAPS, 65 mmol/L DTT, 0.2% (v/v) bio-lytes, and a trace of bromphenol blue, to a total volume of 300 μL. The mixture was loaded onto the IEF strip. After rehydration for 12 h, IEF was carried out using the following conditions: (1) 250 V, slow, 25 min; (2) 1000 V, rapid, 2 h; (3) 8000 V, linear, 5 h; (4) 8000 V, rapid, 60000 Vh; (5) 500 V, rapid, anytime. After IEF, the strips were stored at -80°C until required for the second dimension.

Equilibration of IPGs using DTT and iodoacetamide
Following IEF, the gel strip was equilibrated for 15 min in the equilibration buffer containing 130 mmol/L DTT, 6 mol/L urea, 2% SDS, 0.375 mol/L Tris HCl, pH 8.8, and 20% glycerol. Then the gel strip was equilibrated for another 15 min in the same equilibration buffer except that DTT was replaced with 135 mmol/L iodoacetamide.

Second-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis
The second dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% acrylamide vertical slab gels using the protein II xi cell system. The equilibrated strip was sealed at the top of the 1.0 mm thick second-dimensional gel with the help of 0.5% low-melting agarose in stacking buffer (0.1% SDS, 0.25 mol/L Tris, 0.192 mol/L glycine, supplemented with bromphenol blue as a tracking dye). Gels were run at constant voltage of 60 V for 20 min and then 250 V until the bromphenol blue front traversed the gel. Proteins were visualized by staining with Coomassie brilliant blue R-250 or silver nitrate. The completed analytical 2-D gels were stained with silver nitrate, the preparative gels were stained for 4 h in 0.1% w/v Coomassie brilliant blue R-250 in 45% v/v methanol,
10% v/v acetic acid, destained in 45% v/v methanol, 10% v/v acetic acid.

**Image acquisition and analysis**

The stained analytical gels were scanned by Umax Powerlook1120 scanner (UMAX, Taiwan, China), and the image analysis was carried out using the two-dimensional analysis software package PDQuest 7.2 (Bio-Rad, Hercules, CA, USA), including image editing, spot finding, quantitation and matching. The PDQuest software was used to calculate the 2-D spot intensity by integrating the optical density over the spot area. Proteins separated by 2-D gels were quantitated in terms of their normalized quantity. The individual protein spot quantity was normalized as a percentage of the total quantity of valid spots present in the gel. The two characteristics (normal vs diabetic tissue) were assessed using the Student’s t-test and P < 0.05 was considered statistically significant. The confidence level was 95% and the results were exported to Excel 2000 (Microsoft, Seattle, CA, USA) for further analysis.

**In-gel digestion by trypsin, tandem mass spectrometry and bioinformatics analysis**

Differentially expressed protein spots were excised from the preparative gels according to the result of image analysis and transferred to a siliconized 1.5 mL Eppendorf tube (Sigma). Fifty mmol/L ammonium bicarbonate/50% acetonitrile (1:1) (Sigma) was added to the tube to cover the gel piece, and the tube was incubated at room temperature for 20 min. The liquid was discarded and washing was repeated. After shrink by dehydration in 100 μL of 100% acetonitrile, which was then removed, the gel pieces were dried in a vacuum centrifuge. The gel pieces were swollen in 10 μL of 12.5 ng/μL trypsin (Roshe, Mannheim, Germany) and incubated at 4°C for 30 min, then at 37°C overnight. Mass spectra were obtained using 4700 proteomics analyzer (TOF/TOFTM) mass spectrometer (Applied Biosystems, USA). Database searches were carried out with GPS Explorer TM software (Applied Biosystems, USA)-MASCOT (Matrix Science, London, UK) against the NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov/).

**RESULTS**

**Establishment of diabetic rat models**

After eight weeks, the glucose levels in urine and blood and the body weights of 34 rats including 16 diabetic rats and 18 non-diabetic control rats were measured. The urine glucose levels in all normal rats were negative (−) while the urine glucose levels in all diabetic rats were strongly positive (from +++ to ++++). The blood glucose levels in all normal rats were much lower than 16.7 mmol/L (about 4-5 mmol/L), while the blood glucose levels in all diabetic rats were 19.2 mmol/L-31.05 mmol/L. The body weight was reduced in some diabetic rats but increased in normal rats.

**2-DE protein separation and image analysis**

Proteins obtained from normal and diabetic retinal tissues were applied to a 2-D gel. The protein spots were visualized by staining with Coomassie brilliant blue R-250 or silver nitrate. Examples of silver stained 2-D gels representing the proteome collected from diabetic and normal retinal tissues are shown in Figure 1. 2-DE was executed three times for protein sample from each rat and the best stained gel was picked out for image analysis. The normalized quantity of protein spots was compared between 3 normal vs 3 diabetic retinal tissue samples using the Student’s t-test. Only the protein spots (P < 0.05) were selected for MS. The expression was up-regulated in 5 proteins, down-regulated in 23 proteins and disappeared in 8 proteins of diabetic rat retinae. Figure 2 shows these 3 patterns of protein spots on a 2-D gel.

**Protein identification by tandem mass spectrometry**

Eight spots were selected randomly from 36 spots and identified by tandem mass spectrometry. The identified proteins are listed in Table 1 and Figure 2. The expression of crystallin alpha B and crystallin beta A1 increased in diabetic retinal tissues. The expression of albumin, guanylate kinase 1, dimethylarginine dimethylaminohydrolase 2,
ATP synthase subunit d and triosephosphate isomerase 1 was down-regulated in diabetic retinal tissues. However, expression of peroxiredoxin 6 could hardly be found in diabetic retinal sample.

**DISCUSSION**

It is well known that retinal disease is the most common microvascular complication in patients with long-standing diabetes. However, the underlying molecular
pathways that lead to initiation and progression of this complication remain unresolved. There is little doubt that the pathogenesis of retinal disease is highly complicated and there is a pressing need to establish new therapeutic regimens that can effectively prevent or limit retinal microvascular cell dysfunction. Some investigations of functional changes in diabetic retina indicate that impairment of retinal function precedes the earliest signs of vascular complications[16,17]. The electroretinogram (ERG) responses of diabetic rats were reduced in amplitude compared to the responses of control rats 2 wk after onset of diabetes. Early functional changes of the inner retina are evident in diabetic patients before impairment of the outer retina is observed[18]. Joussen et al[9] and Mei et al[19] also reported that retinal gene expression is changed in early diabetes in the present study. These observations indicate that the functional and molecular retinal changes occur earlier than morphological change in experimental diabetic rats. The results of this study confirm this viewpoint.

**Up-regulation of protein expression**

Crystallin alpha B and crystallin beta A1 were over-expressed in diabetic tissues in comparison with normal tissues. Crystallin alpha B, one of the small heat-shock proteins (sHSPs), is the main structural protein of eye lens that contribute to the transparency and refractive properties of mammalian eye lens. It was recently reported that crystallin alpha B is highly expressed in multiple non-lenticular normal and diseased human tissues, such as brain, gliomas, renal carcinomas, breast carcinomas, having an extensive tissue distribution[20-23]. In the present study, a higher expression of crystallin alpha B was found in retinae of diabetic rats than in those of normal rats. Crystallin alpha B can effectively inhibit both formation of reactive oxygen species (ROS) and hyperglycemia-induced apoptosis, and has anti-apoptotic function and a cardioprotective role[24]. Crystallin alpha B also meets one of the “gold standards” for classification of oncogenic protein and may be a useful biochemical marker for studying the pathogenesis and prognosis of various human tumors[25,26]. In the present study, high blood glucose levels in diabetic rats (eight-week duration) enhanced the expression of crystallin alpha B in retinae, resulting in inhibition of apoptosis and ROS, suggesting that crystallin alpha B weakens the retinal tissue damage through cytoprotective action, which may provide a basis for future therapeutic interventions in diabetic vascular complications. In future study, we may compare the expression of crystallin alpha B between diabetic and normal rats to determine if it may be regarded as a powerful biochemical marker for diagnosis, treatment and prognosis of diabetes. Beta-crystallins have previously been regarded as lens-specific proteins[27]. However, beta B2-and beta A3/A1-crystallin RNAs are transcribed and beta-crystallin polypeptides are detectable in developing chick retinae[28]. In our study, crystallin beta A1 was over-expressed in diabetic rat retinae in comparison with normal retinal tissues, implying a function for extra-lenticular-beta-crystallins. However, the function of crystallin beta A1 in non-lenticular tissues remains equivocal so far.

**Down-regulation of protein expression**

In this study, five proteins were down-regulated in diabetic retinae, including albumin, dimethylarginine dimethylaminohydrolase 2, triosephosphate isomerase 1, ATP synthase subunit d and guanylate kinase 1. Albumin may regulate the colloidal osmotic pressure, increase the internal diameter of blood vessels, especially arterioles or capillaries, and also play a role in nitric oxide signaling by mediating formation of S-nitrosothiols (RS-NO) [29,30]. Albumin has antioxidant activity, which may inhibit the reactions induced by dioxygen (O₂) or peroxydes. One of the most common characteristics of inflammatory diseases is a severe reduction in plasma albumin concentration and diabetic retinopathy is now recognized as an inflammatory disease in which albumin reduction is associated with physiological disorders, such as modification of the intravascular oncotic pressure or alterations in the transport of exogenous and endogenous substrates[31]. The result of our study indicate that diabetes results in the low-expression of albumin in retinae, which affects blood colloidal osmotic pressure and nitric oxide signaling, increases vascular resistance and permeability, and aggravates retinal cell death and causes blindness. Dimethylarginine dimethylaminohydrolase 2 (DDAH II) belongs to the DDAH family and has dimethylargininase activity which hydrolyzes N(G), N(G)-dimethyl-L-arginine (asymmetrical dimethylarginine; ADMA) and N(G)-monomethyl-L-arginine (MMA). Since ADMA and MMA

### Table 1  Identified proteins with different expressions in diabetic and normal retinae

| SSP No. | Protein name                        | NCBI accession No. | Protein Best score | Mean | SD   | 100% PM | 0.05 | 100% PM | 0.05 |
|---------|------------------------------------|--------------------|--------------------|------|------|---------|------|---------|------|
| 7106    | Crystallin alpha B                  | NP_037067          | 201                | 59   |      |         |      |         |      |
| 7103    | Predicted: crystallin, beta A1     | NP_340247          | 480                | 132  |      |         |      |         |      |
| 3103    | Predicted: guanylate kinase 1      | NP_09101333        | 174                | 85   |      |         |      |         |      |
| 4003    | Dimethylarginine dimethyaminohydrolase 2 | NP_997697     | 460                | 191  |      |         |      |         |      |
| 5802    | Albumin                           | NP_599153          | 171                | 73   |      |         |      |         |      |
| 7202    | Triosephosphate isomerase 1        | NP_075211          | 326                | 128  |      |         |      |         |      |
| 6101    | ATP synthase, H+ Transporting, mitochondrial FO complex, subunit d | NP_062256 | 251 | 134 | 18573.6 | 6.17 | 0.005 | 0.014 | 1.06 | 0.445 | 0.105 | 0.016 |
| 5201    | Peroxiredoxin 6                    | NP_446028          | 576                | 196  |      |         |      |         |      |

**SSP:** standard spots number; **MW:** molecular weight; **pl:** isoelectric point; **DM:** diabetes mellitus; **Norm Qty:** normal quantity; **SD:** standard deviation.
act as inhibitors of NO synthases (NOS). DDAH II plays an important role in nitric oxide generation. DDAH II has a more widespread distribution and its expression correlates with the distribution of eNOS[32]. According to the present study, down-regulated expression of DDAH II in diabetic rat retinæ increases plasma ADMA and decreases NO synthesis and may protect retinal cells from injury induced by hyperglycemia. Therapeutic modulation of ADMA levels, possibly via pharmacological or genetic modification of DDAH expression or activity, may represent a new strategy for the treatment of diabetes.

Triosephosphate isomerase 1 (EC 5.3.1.1, TIM1) belongs to the triosephosphate isomerase family, plays an important role in glycolysis by catalysing the reversible interconversion of D-glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP)[33]. Glucose is broken down to pyruvate via a chemical pathway that involves 10 enzymes and TIM1 is the fifth enzyme in this reaction chain that converts DHAP into GAP. High blood glucose level in diabetic rats may increase nitration of TIM1, leading to low expression of TIM1[34], which is consistent with the results of the present study. Low level of TIM1 decreases the synthesis of ATP. Further studies are needed to determine the feasibility of TIM1 replacement therapies. Guanylate kinase 1 (predicted) (EC: 2.7.4.8, GK) catalyzes the ATP-dependent phosphorylation of GMP into GDP, and is essential for recycling GMP and indirectly, cGMP. Photoreceptor cells involve the activation of cGMP enzyme cascade[35]. In the retina of diabetic rats, low expression of GK reduces the phosphorylation of GMP into GDP, thus increasing GMP level. The increased GMP level inhibits the hydrolyzation of cGMP to GMP, resulting in accumulation of cGMP, which does not close the cation-specific channels. As a result, the increased cytosolic calcium level interrupts hyperpolarization of plasma membrane and generation of neural signals. Maybe, it is one of the mechanisms of blindness in diabetic patients with retinopathy. ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d (ATP synthase subunit d) are the subunit d of mitochondrial H (+) -ATP synthase, which catalyzes ATP synthesis from ADP and phosphate utilizing the energy derived from an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. However, the exact function of ATP synthase subunit d is unknown. The data obtained in this study revealed that expression of ATP synthase subunit d was scarcely found in the retina of 8-wk old diabetic rats, suggesting that reduced ATP synthesis in retinae affects the function of retinal cells.

**Disappeared expression of proteins**

Peroxiredoxin 6 matches pattern 3. In this pattern, peroxiredoxin 6 is acarily expressed in the diabetic samples. Peroxiredoxin 6 (Prx6, EC 1.11.1.15) is a unique, bifunctional protein with both GSH peroxidase and phospholipase A2 activities, and is the only mammalian 1-Cys member of the peroxiredoxin family of antioxidant proteins which protect cells from oxidative damage by reducing cellular peroxides[36]. Knockout studies and transgenic over-expression of Prx6 in mice have demonstrated an important role of this protein in protection against oxidative stress which contributes to the pathogenesis of the long-term complications of diabetes[37]. Disappearance of peroxiredoxin 6 in the retinae of diabetic rats may cause excess reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion and hydroxyl radicals, contributing to accumulation of cellular peroxides-convert, and accentuating tissue oxidative injury.

In conclusion, there are differences in expression of retinal protein between diabetic and normal rats. These proteins, up-regulated or down-regulated, may be involved in the pathogenetic mechanisms and prognosis of retinal diseases caused by diabetes, or in the maintenance of survival of retinal cells. Of these proteins, guanylate kinase 1, TIM1, ATP synthase subunit d, albumin and DDAH II may play an important role in signal transduction. TIM1, crystallin alpha B, ATP synthase subunit d and peroxiredoxin 6 are involved in energy metabolism of retinal tissues. The role of crystallin beta A1 in diabetic retinæ has not yet been found. Further study is necessary to determine if some proteins such as crystallin alpha B, DDAH II and TIM1 can be regarded as candidates of biochemical markers for diagnosis and treatment of diabetes. Our findings provide direct and visible evidence for analyzing the molecular pathogenetic mechanisms of retinal diseases caused by diabetes.

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