The effects of the Chinese medicine ZiBu PiYin recipe on the hippocampus in a rat model of diabetes-associated cognitive decline: a proteomic analysis

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Abstract

Aims/hypothesis Increasing evidence suggests that diabetes is associated with an enhanced risk of cognitive decline. The precise mechanisms underlying diabetes-associated cognitive decline (DACD) remain unclear. Here we investigated the molecular changes associated with DACD

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using a comparative proteomics study of hippocampus in a rat model of type 2 diabetes. In addition, we tested the effects of the Chinese medicine ZiBu PiYin recipe (ZBPYR) on DACD.

Methods The hippocampus was dissected from control, diabetic and diabetic rats treated with ZBPYR (DM/ZBPYR). Soluble proteins were separated using fluorescence-based difference gel electrophoresis. Protein spots were visualised with fluorescent dyes and spot density was compared between each pair of groups. Proteins of interest were identified using mass spectrometry. Proteins of specific interest were also tested by western blot and real-time PCR analysis.

Results We found 13 spots that were altered between control and diabetes groups, and 12 spots that were changed between diabetes and DM/ZBPYR groups. The identities of nine proteins were determined by mass spectrometry. The identified proteins were largely involved in energy metabolism, cytoskeleton regulation and oxidative stress. The protein alterations observed in the diabetes group were ameliorated to varying degrees following ZBPYR treatment.

Conclusions/interpretation The protein changes identified in hippocampus from a rat model of type 2 diabetes suggest that specific cellular alterations contribute to DACD. The Chinese medicine ZBPYR was found to affect multiple targets and partially repaired the original cellular balance. This study may provide important insights into the molecular events underlying DACD and allow the identification of novel therapeutic targets.

Keywords Chinese medicine ZiBu PiYin recipe · Diabetes-associated cognitive decline · DIGE · MALDI-TOF/TOF mass spectrometry · Proteomics · Type 2 diabetes

Abbreviations

BDNF	Brain-derived neurotrophic factor
DACD	Diabetes-associated cognitive decline
DIGE	Fluorescence-based difference gel electrophoresis
DM/ZBPYR	Diabetic rats treated with ZBPYR
DRP-2	Dihydropyrimidinase-related protein 2
FSI	Fasting serum insulin
G6PD	Glucose-6-phosphate 1-dehydrogenase
GSTM1-1	Glutathione <i>S</i> -transferase Mu 1
ITT	Insulin tolerance test
MALDI	Matrix-assisted laser desorption/ionisation
MS	Mass spectrometry
p34-ARC	Actin-related protein 2/3 complex 34-kDa subunit
PDHE1 α	Pyruvate dehydrogenase E1 component subunit alpha
pI	Isoelectric point
RBG	Random blood glucose
TOF	Time-of-flight
ZBPYR	ZiBu PiYin recipe

Introduction

Diabetes-associated cognitive decline (DACD), also called diabetic encephalopathy [1], represents a complication of the diabetic brain, which manifests as a gradual decline of cognitive function. The global prevalence of diabetes among adults is expected to rise from 285 million persons in 2010 to an estimated 439 million in 2030 [2]. Compelling evidence indicates a link between diabetes and dementia regardless of the underlying vascular pathology [3]. Clinical research has confirmed that patients with type 2 diabetes exhibited an increase in hippocampal atrophy compared with non-diabetic participants [4]. The alterations in hippocampal plasticity and function have also been observed in animal models [5, 6]. The processes that precede the direct effects of diabetes on the hippocampus have yet to be defined. Cognitive decrement in type 2 diabetes has been shown to occur slowly over a prolonged period of time [7], suggesting a possible window for the prevention of DACD. To fully use these possibilities, an understanding of the molecular changes occurring in the hippocampus and of potential pharmacological targets is required.

For thousands of years, traditional Chinese medicine has played an indispensable role in the fight against disease in China. Investigations into the effects of traditional Chinese medicine formulas are now attracting increasing attention around the world [8]. The ZiBu PiYin recipe (ZBPYR) is derived from Zicheng Decoction, a

traditional Chinese medicine formula recorded in the book of Bujuji, written by Wu Cheng in the Qing dynasty and used for clinical treatment of amnesia [9]. Previous studies by our laboratory have shown that ZBPYR treatment improved learning and memory ability in aged rats [10] through anti-oxidation and regulation of energy metabolism [11]. We also found that the treatment protected hippocampal neurons against Abeta amyloid- and glutamate-induced neurotoxicity via blockage of serum-inducible kinase-spine-associated Rap GTPase-activating protein pathway [12, 13]. Existing evidence suggests many similarities among DACD, brain ageing and Alzheimer's disease [4, 14, 15]. However, the effect of ZBPYR on DACD is unclear.

The development of proteomics has allowed high-throughput discovery of differences in protein abundance in a short period of time. Fluorescence-based difference gel electrophoresis (DIGE) is considered the first real quantitative proteomics differential display method with decreased variation between gels and increased confidence [16]. Here, we investigated alterations in the hippocampal protein profile in DACD and in ZBPYR treated rats using DIGE-based proteomics.

Methods

Animals Adult male Sprague–Dawley rats (6 weeks of age, 180–200 g) were obtained from the Experimental Animal Center, Dalian Medical University, Dalian, China. Rats were housed in a 12 h light–dark cycle and had free access to chow and water. All animal experiments were conducted in accordance with the NIH Principles of Laboratory Animal Care and the institutional guidelines for the care and use of laboratory animals at Dalian Medical University.

Animals were assigned to the following groups: control, diabetes and diabetic rats treated with ZBPYR (DM/ZBPYR). The control group was fed a standard diet and the two diabetic groups received a high-fat diet (40% energy from fat; Anlimo Technology, Nanjing, China) [17]. After 4 weeks, the two diabetic groups were treated with streptozotocin (30 mg/kg i.p.) that had been freshly dissolved in citrate buffer (Sigma, St Louis, MO, USA). The control group animals were administered vehicle citrate buffer. Tail random blood glucose (RBG) was measured 72 h later using a strip-operated blood glucose sensor (Accucheck; Roche, Mannheim, Germany) and monitored every 2 weeks until drug intervention. Rats with RBG levels less than 16.7 mmol/l were excluded from the diabetic groups. At 7, 12 and 16 days after streptozotocin injection, the fasting serum insulin (FSI), OGTT and insulin tolerance test (ITT) were performed (design of animal

experiment, see Fig. 1). The samples for the FSI were taken from the angular vein and analysed using a radioimmunoassay kit (Atom High-tech, Beijing, China). Glucose (50% [wt/wt]; 2 g/kg, given orally) and regular human insulin (0.75 U/kg; Novo Nordisk, Tianjin, China) were used in the OGTT and ITT, respectively. At 7 weeks after the streptozotocin injection, ZBPYR was administered to DM/ZBPYR group by gavage at a dose of 10 ml/kg (1 ml equivalent to 3.29 g of the crude drug) per day for 16 days. The control and non-treated diabetes groups received physiological saline at the same dose during this time. Details on ZBPYR preparation have been previously described [13].

Morris water maze Spatial learning and memory performance was assessed using the Morris water maze test at 11 days after ZBPYR administration. The apparatus consisted of a round stainless steel water tank (120 cm in diameter, 50 cm in height and filled to a depth of 30 cm with water maintained at $28 \pm 1^\circ\text{C}$), a transparent platform (9 cm in diameter, 29 cm in height and located 30 cm from the edge of the tank) that was hidden under water and an automatic photographic recording and analysis system (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China). Black and white spatial cues on the walls of the room provided rats with orientation. The test was carried out for 5 days by operators blinded to the treatment. On the first day, rats were permitted to swim freely in the tank for 120 s without the platform to adapt to the new condition. Over the following 4 days, rats were trained with four trials per day at intervals of 60 s. The platform

location was fixed and the starting points were changed every trial. Rats had to swim until they reached the platform and were allowed to rest for 20 s. If the rat failed to reach the platform within 120 s, it was gently guided to the platform and stayed there for 20 s. The time taken to reach the platform (latency in s) was measured automatically.

Sample preparation For sample preparation, rats were anaesthetised with chloral hydrate (4% [wt/wt], i.p.) and decapitated. Hippocampus was rapidly dissected via surgery on ice. All samples were immediately frozen in liquid nitrogen and stored at -80°C until required.

Protein samples for DIGE were isolated from the hippocampus ($n=3$). The tissue samples were homogenised (Dounce's homogeniser) in 1 ml of DIGE lysis buffer (GE Healthcare, Munich, Germany) containing 7 mol/l urea, 2 mol/l thiourea, 65 mmol/l Tris, 4% (wt/vol.) CHAPS, 0.2% (vol./vol.) IPG buffer and protease inhibitors. The suspension was further disrupted by sonication (80 W, 10 s, five repeats with an interval of 15 s between repeats). All these steps were undertaken on ice. The mixture was then centrifuged at $15,000 \times g$ for 45 min at 4°C . Protein quantification was performed using Coomassie protein assay reagent (Pierce Biotechnology, Rockford, IL, USA) and absorbance measured at 595 nm with a Bradford protein assay and using bovine serum albumin as the protein standard.

Fluorescence-based difference gel electrophoresis For DIGE, 50 μg protein sample was labelled with 400 pmol Cy3 or Cy5 minimal dye according to the experimental

Fig. 1 Details of the animal study period. The control group was fed a standard diet during the whole study. The two diabetic groups received a high-fat diet until drug intervention (ZBPYR). In week 4 of the procedure, the two diabetic groups received streptozotocin, while the control group was administered vehicle citrate buffer. At 72 h, and 7, 12 and 16 days after streptozotocin injection, RBG, FSI,

OGTT and ITT were determined/performed. From 7 weeks after the streptozotocin injection, ZBPYR was administered to the DM/ZBPYR group daily for 16 days. The control and non-treated diabetes groups received physiological saline. The Morris water maze test was performed for 5 days before animals were killed

design (Table 1). Dye swap among groups was designed so that artefacts caused by preferential labelling were avoided. A pool consisting of equal amounts of each sample analysed in DIGE was used as an internal standard to control for quantitative comparisons and labelled with Cy2. All the individual samples were biological replicates, but a control one was used on gels 1 and 5. It was a technical replicate of the control animal.

For each gel, 50 µg Cy2-, Cy3- and Cy5-labelled proteins were combined, and an equal volume of rehydration buffer containing 8 mol/l urea, 4% (wt/vol.) CHAPS, 130 mmol/l dithiothreitol and 2% (vol./vol.) Pharmalyte pH 3–10 was added. Isoelectric focusing was carried out on non-linear IPG strips (13 cm long, pH 3–10) with an Ettan IPG-phor apparatus for a total of 40,000 V h at 20°C and at a maximum current setting of 50 µA per strip. After isoelectric focusing, individual strips were incubated in equilibration buffer containing 50 mmol/l Tris-HCl, 6 mol/l urea, 30% (vol./vol.) glycerol and 2% (wt/vol.) SDS supplemented with 1% (wt/vol.) dithiothreitol, and then in 2.5% (wt/vol.) iodoacetamide. The proteins were resolved in 12.5% SDS-PAGE gels using a device (SE 600 Ruby; Hoefer) at 15 mA for 15 min and then 30 mA at 20°C to the end. To facilitate mass spectrometry (MS) analysis, 800 µg unlabelled pooled protein sample of each group was separated on a preparative gel in parallel and stained with Coomassie blue (Colloidal Blue stain kit; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DIGE was performed using reagents and equipments from GE Healthcare.

Gel images for analysis were obtained using an imager (Typhoon 9700; Amersham Bioscience, Piscataway, NJ, USA). Relative protein quantification was performed using an in gel and biological variance analysis system (DeCyder Differential In gel Analysis and Biological Variance Analysis software, version 6.5; Amersham Biosciences). The in gel analysis module was used for pairwise comparisons of each gel and for calculation of normalised spot volumes/protein abundance. The presence of a Cy2-

labelled pooled internal standard on every gel allowed for accurate relative quantification of protein spot features across the different gels. The Student's *t* test was used to calculate any significant differences in the relative abundance of protein spot features between each pair of groups. Protein spots of interest with significant difference (≥ 1.2 -fold, $p < 0.05$) were isolated from the stained preparative gels.

Protein identification Spots of interest were manually cut out and in-gel digested with porcine trypsin (Promega, Madison, WI, USA) as previously described [18]. The purified peptides were then spotted on to a matrix-assisted laser desorption/ionisation (MALDI) plate and covered with 0.7 ml of 2 mg/ml 3,5-dimethoxy-4-hydroxycinnamic acid matrix (Sigma) containing 10 mmol/l $\text{NH}_4\text{H}_2\text{PO}_4$ in 60% (vol./vol.) acetonitrile. Samples were analysed using MALDI-time-of-flight (TOF)/TOF MS with a proteomics analyser (4800 P; Applied Biosystems, Foster City, CA, USA). Mono-isotopic peak masses were acquired in a mass range of 800 to 4,000 Da, with a signal : noise ratio (S/N) of 50. We selected five of the most intense ion signals, excluding common trypsin autolysis peaks and matrix ion signals, as precursors for MS/MS acquisition. The peptide mass fingerprint combined MS/MS data were submitted to Mascot version 2.1 (Matrix Science, London, UK) for identification according to Swiss Prot database (200911 release, Rattus proteins, www.expasy.org/databases/uniprot/knowledgebase/). The search variables were set as follows: Rattus, trypsin cleavage (one missed cleavage allowed), carbamidomethylation as fixed modification, methionine oxidation as variable modification, peptide mass tolerance set at 100 ppm and fragment tolerance set at 0.4 Da. The criteria for successfully identified proteins were as follows: ion score CI for peptide mass fingerprint and MS/MS data was $\geq 95\%$.

Western blot and quantitative real-time RT-PCR analysis The protein and mRNA levels of pyruvate dehydrogenase E1 component subunit alpha (PDHE1 α), dihydropyrimidinase-related protein 2 (DRP-2) and glucose-6-phosphate 1-dehydrogenase (G6PD) that had been identified by MS were further investigated by western blotting and quantitative real-time RT-PCR, respectively. The details are described in the electronic supplementary material (ESM), including primer sequences for RT-PCR (ESM Table 1).

Statistical analysis Values are expressed as means $\pm$ SD. Statistical significance was analysed using ANOVA, followed by least-significant difference post hoc with SPSS 13.0 (SPSS, Chicago, IL, USA), with exception of protein

Table 1 Experimental design for DIGE

Gel number	Cy2-labelled	Cy3-labelled	Cy5-labelled
1	Pool	Control 1	Diabetes 2
2	Pool	Diabetes 1	Control 2
3	Pool	Control 3	DM/ZBPYR 1
4	Pool	Diabetes 3	DM/ZBPYR 2
5	Pool	DM/ZBPYR 3	Control 1

Pool served as an internal standard and consisted of equal amounts of each sample analysed in DIGE

abundance in DIGE. A value of $p < 0.05$ was considered to be statistically significant.

Results

Type 2 diabetes RBG, FSI and blood glucose levels following OGTT were significantly higher in the diabetic than in control rats (Table 2 and Fig. 2a). Insulin sensitivity detected by ITT was also impaired in the diabetic rats (Fig. 2b). The body weight of diabetic rats increased rapidly due to the high-fat diet administered prior to the streptozotocin injection. Following the injection, weight gain was slowed. When compared with control group, body weight in diabetic rats was increased, however, not significantly ($p > 0.05$, data not shown). Administration of ZBPYR had no obvious effects on blood glucose or body weight.

Cognitive performance in the Morris water maze In the Morris water maze test, the latency of the control group was short and gradually decreased over the 4 days of training. In contrast, the diabetes group required a significantly longer latency than the controls (days 3 to 5, $p < 0.05$). The performance of the DM/ZBPYR group was found to be similar to control group between days 3 to 5 and was significantly enhanced compared with the diabetes group ($p < 0.05$ day 3, $p < 0.01$ day 5). This finding indicates that the decline in cognitive function observed in the diabetes group evidently improved following ZBPYR administration (Fig. 2c).

Fluorescence-based difference gel electrophoresis A mean of $1,655.80 \pm 166.89$ spots were detected in the gel analysis. Using the biological variation analysis module of the DeCyder software, we identified 13 spots that showed significant differences between the control and diabetes groups, 12 between the diabetes and DM/ZBPYR groups, and six between the DM/ZBPYR and control groups (Table 3). We identified five spots that were upregulated

and eight spots that were downregulated in the diabetes group when compared with control group (Table 3). In the DM/ZBPYR group, seven spots upregulated and five downregulated when compared with the diabetes group (Table 3). The positions of the spots in the representative DIGE images are shown in Fig. 3. Changes of these spots were found to be ameliorated by treatment of ZBPYR (Fig. 4).

Identification of proteins We identified nine proteins in the spots of interest analysed by MALDI-TOF/TOF MS (Table 4). Of these, four (G6PD, DRP-2, PDHE1 α and NADH-ubiquinone oxidoreductase 42-kDa subunit) were decreased in the diabetes group when compared with the control group. The three proteins syntaxin-binding protein 1, actin-related protein 2/3 complex 34-kDa subunit (p34-ARC) and glutathione *S*-transferase Mu 1 (GSTM1-1) were downregulated and three proteins (PDHE1 α , ATP synthase subunit α and tubulin α -1B chain) were upregulated in the DM/ZBPYR group when compared with the diabetes group. The experimental molecular mass and isoelectric point were similar to the theoretical values for each protein, with the exception of spot 601. Spot 601, identified as G6PD and with a theoretical isoelectric point of 5.97, migrated to an alkaline isoelectric point (Fig. 3a).

The identified proteins were categorised according to their function based on published literatures [19–22] and Swiss Prot database (www.expasy.org/databases/uniprot/knowledgebase/). The functional groups were found to be mainly involved in the regulation of energy metabolism, cytoskeleton dynamics and oxidative stress. The majority of proteins identified were located in the cytoplasm and mitochondrion (Table 4).

Western blot analysis The levels of proteins whose abundance was different (PDHE1 α , DRP-2 and G6PD) were further tested via western blot analysis. PDHE1 α was significantly downregulated in the diabetes group when compared with control and DM/ZBPYR groups ($46.46 \pm 22.36\%$ vs $103.93 \pm 18.77\%$, $p = 0.002$ and vs $84.87 \pm 23.37\%$, $p = 0.022$, respectively; Fig. 5a). Similar changes were also observed for DRP-2 between diabetes and control groups ($68.9 \pm 8.79\%$ vs $103.1 \pm 13.19\%$, $p = 0.002$; Fig. 5b). Western blot results for the two proteins were consistent with the proteomic analysis, confirming the reliability of the proteomics method. The total protein abundance of G6PD in the diabetes group as detected by western blot was not statistically significant compared with the other two groups (Fig. 5c).

Quantitative real-time RT-PCR Levels of *Pdha1*, *Dpysl2* and *G6pd* (corresponding to PDHE1 α , DRP-2 and G6PD,

Table 2 Random blood glucose and FSI of rats after streptozotocin treatment

Group	RBG (mmol/l)	FSI (pmol/l)
Control	4.92 ± 0.7	73.27 ± 32.25
Diabetes	$23.06 \pm 5.7^{**}$	$127.05 \pm 31.99^{**}$
DM/ZBPYR	$22.14 \pm 3.24^{**}$	$146.30 \pm 30.02^{**}$

Data are expressed as mean $\pm$ SD; RBG was determined 72 h and FSI 7 days after streptozotocin injection.

$^{**}p < 0.01$ vs control group ($n = 5$ in each group)

Fig. 2 Diabetic rats displayed impaired glucose tolerance, insulin sensitivity and cognitive function. **a** Blood glucose levels at the indicated times of OGTT after 40 days on a high-fat diet and 12 days after streptozotocin. **b** The decrement of blood glucose in ITT after 44 days on a high-fat diet and 16 days after streptozotocin. **c**

Cognitive performance assessed by Morris water maze. **a–c** Black circles, control ($n=5$); black triangles, diabetes group ($n=5$); black squares DM/ZBPYR ($n=4$, except [c] $n=5$). Data are expressed as the means $\pm$ SD; * $p<0.05$ and ** $p<0.01$ vs control group; † $p<0.05$ and †† $p<0.01$ vs diabetes group

respectively) mRNA among control, diabetes and DM/ZBPYR groups are presented in Fig. 5d. Those of *Dpysl2* and *G6pd* were lower in the diabetes hippocampal samples than in control and DM/ZBPYR samples; however, these changes were not statistically significant. *Pdhal* mRNA levels were similar between the three groups.

Discussion

Given our ageing society, hundreds of studies have concentrated on DACD. Despite this, scant direct attention has been given to comparative proteomic analysis of the

brain in DACD. The hippocampus plays an important role in cognitive capability and is one of the most vulnerable regions of the brain [23]. The present study performed an analysis of hippocampal proteome in DACD and ZBPYR-treated rats. Analysis of more than 1,600 spots on DIGE gels identified 13 spots that were different between diabetes and control groups and 12 that were different between diabetes and DM/ZBPYR groups. For nine proteins identities were determined by MALDI-TOF/TOF MS. The functions of these proteins were found to mainly include regulation of energy metabolism, cytoskeleton and oxidative stress.

Proteomic analysis of hippocampal samples from human patients with type 2 diabetes would have been the preferred

Table 3 DIGE analysis of changed protein spots

Diabetes vs control			DM/ZBPYR vs diabetes			DM/ZBPYR vs control		
Master ^a	<i>t</i> test ^b	Average ratio	Master ^a	<i>t</i> test ^b	Average ratio	Master ^a	<i>t</i> test ^b	Average ratio
1385	0.015	1.56	1,324	0.028	−1.61	1,617	0.023	−1.49
1577	0.0024	1.46	1,780	0.032	−1.40	773	0.012	−1.32
495	0.035	1.31	1,682	0.038	−1.26	1,403	0.039	−1.29
497	0.0028	1.24	1,169	0.034	−1.23	1,292	0.015	−1.24
1172	0.044	1.20	524	0.0095	−1.21	708	0.043	1.21
607	0.013	−1.20	702	0.036	1.21	837	0.012	1.57
549	0.023	−1.24	606	0.0032	1.23			
620	0.049	−1.27	572	0.046	1.34			
969	0.0039	−1.38	827	0.037	1.39			
1586	0.043	−1.41	571	0.0085	1.43			
826	0.0030	−1.63	826	0.0073	1.50			
601	0.016	−1.64	1,199	0.049	1.51			
597	0.011	−2.00						

^a Master number; ^b *p* value

Fig. 3 Distribution of spots that were changed on DIGE image of DACD rat hippocampus. **a** The arrows point to the spots that were changed between diabetes and control groups. The master number of corresponding spots is shown on the image. **b** The arrows point to the spots that were changed between DM/ZBPYR and diabetes group. The master number of corresponding spots is also presented. The range of the horizontal dimension is isoelectric point 3 (*left*) to isoelectric point 10 (*right*). The vertical dimension ranges from

~15 kDa (*bottom*) to ~100 kDa (*top*). **c** Superimposed images in pseudocolour from samples labelled with Cy3 (green, control group) and Cy5 (red, diabetes group). The three labelled spots were identified as DRP-2 (607), G6PD (601) and PDHE1 α (826) using MALDI-TOF/MS. Flanking the two-dimensional gel image are the three-dimensional depictions of abundance of the three spots, corresponding to Cy3 image (control group), the Cy5 image (diabetes group) and the Cy3 image (DM/ZBPYR group) as indicated. $n=3$ in each group

choice in this study. However, this was not feasible due to a lack of tissue sources. Here we selected the high-fat-fed and streptozotocin-treated rat model that is commonly used in pharmacological studies of diabetes [24, 25]. During model construction, the animals showed hyperinsulinaemia and hyperglycaemia. For successful intervention, it has been suggested that the sooner treatment is established, the better the treatment outcome. We therefore performed ZBPYR intervention after a relative short period of 2 months after initiation of cognitive impairment in the diabetic animals [26–29].

There are two limitations to the present study. First, although DIGE permits accurate quantitative comparisons, some technical constraints remain for use of two-dimensional gels. For example, membrane proteins, extremely low-abundance proteins, proteins outside the pH range of 3 to 10, and very high or low molecular mass proteins are likely to be under-represented using this method [30, 31]. Second, some spots were analysed by MS, but failed to reach the standards required for successful identification. Thus, the nine proteins identified here reflect a conservative estimate. There may have been some

Fig. 4 Abundance of changes in changed spots among control (black circles), diabetes (black triangles) and DM/ZBPYR (black squares) groups. Standardised log abundance for spots (a) 826, (b) 601, (c) 607, (d) 497, (e) 495 and (f) 1172. These graphs demonstrate significant changes in diabetes group when compared with control. The changes were ameliorated in DM/ZBPYR group, although only data for spot 826 reached the statistically significant level. Standardised log abundance for spots (g) 1682, (h) 1169, (i) 1324, (j) 1199, (k) 606 and (l) 702. These graphs demonstrate significant changes in

the DM/ZBPYR group compared with diabetes group. The changes in diabetes groups were not statistically significant when compared with the control. a–l Each graph displays the data observed for the spot in each of the five gel images after standardising the values using the internal standard pool images (Cy2) of each of the five gels. The lines link the mean values for each group of samples (crosses). Calculation by Student's *t* test for the difference in log abundance between each pair of groups resulted in * $p < 0.05$ vs control group and † $p < 0.05$ vs diabetes group for each of the spots presented

meaningful changes that remained undetected in this study. Despite these caveats, the present study identified many novel changes related to DACD.

The significant findings of this study are numerous. For the first time, we have employed DIGE to analyse the DACD hippocampal proteome. In addition, traditional Chinese medicine ZBPYR was administered to DACD rats, revealing the effective molecular targets of ZBPYR. Western blot analysis further confirmed the results of the proteomic analysis and real-time RT-PCR analysis of several proteins revealed whether the changes in protein

abundance were regulated at mRNA level. Finally, the short duration and improved cognition in the treated animals support the notion that early pathological changes in DACD respond to intervention.

ZBPYR was created according to the ancient Chinese medical theory in order to improve cognitive function by restoring the initial balance. One obvious difference between traditional Chinese medicine formulas and modern drugs is the complexity of the formula components and the broad-spectrum effects of their active ingredients. The underlying disease is also complicated and can involve an

Table 4 Identification of the changed proteins by MALDI-TOF/TOF MS

Master number	Protein name	Subcellular location ^a	Function ^a	Accession number ^a	M (Da) ^b	Protein pI ^c	Protein score	Protein score (CI%)	Best ion score	Peptide count
601	G6PD	Cytoplasm; microsome; nucleus	Stress-related proteins	P05370	59,337.8	5.97	247	100	26	24
607	DRP-2	Cytoplasm	Cytoskeleton protein	P47942	62,238.6	5.95	221	100	72	12
826	PDHE1 α	Mitochondrion matrix	Energy metabolism	P26284	43,198.6	8.49	295	100	82	16
969	CI-42kD	Mitochondrion matrix	Energy metabolism	Q561S0	40,467.6	7.64	225	100	41	16
524	STXBP1	Cytoplasm; membrane	Protein transport	P61765	67,525.9	6.49	93	100	44	10
606	TUBA1B	Microtubule	Cytoskeleton protein	Q6P9V9	50,119.6	4.94	187	100	53	12
702	MATPS α	Mitochondrion inner membrane	Energy metabolism	P15999	59,716.6	9.22	276	100	78	17
1324	GSTM1-1	Cytoplasm; extracellular region	Stress-related proteins	P04905	25,897.1	8.27	143	100	24	14
1169	p34-ARC	Cytoplasm; cytoskeleton; cell projection	Cytoskeleton protein	P85970	34,369.5	6.84	112	100	39	9

CI-42kD, NADH-ubiquinone oxidoreductase 42-kDa subunit; MATPS α , mitochondrial ATP synthase subunit alpha; STXBP1, syntaxin-binding protein 1; TUBA1B, tubulin alpha-1B chain

^a Swiss Prot; ^b M, molecular mass; ^c pI, isoelectric point

entire cellular network. Notably, ZBPYR was found to ameliorate the changes of most of the spots that were different in the diabetic group (Fig. 4), suggesting that ZBPYR might correct disorders back towards the normal direction by playing a two-way adjustable multi-target role.

Disturbance in energy metabolism has been demonstrated in diabetes-induced brain injury [32]. We found that three proteins identified in this study participate in energy metabolism. Pyruvate dehydrogenase is the rate-limiting enzyme in the synthesis of acetyl coenzyme A from pyruvate in mammals and couples the reaction of the cytosolic glycolysis to the mitochondrial citric acid cycle. PDHE1 α is a pivotal component of pyruvate dehydrogenase [19] and regulates its activity [33]. NADH-ubiquinone oxidoreductase 42-kDa subunit and ATP synthase subunit α are involved in electron transport in the respiratory chain and in ATP synthesis in the mitochondria. These three mitochondrial proteins were all decreased in our diabetes group. Similar findings have been reported in insulin-resistant muscle [34]. Based on evidence that insulin regulates mitochondrial proteins [35], we speculate that the changes here may be related to dysfunction of insulin signalling. The reduced mitochondrial proteins might reflect a defect in energy metabolism within the cells, thus affecting the function of brain, which is highly dependent on glucose to produce ATP. In particular, a decrease in PDHE1 α protein has been reported in the early stage of

Alzheimer's disease in mouse and in traumatic brain injury in rats, demonstrating that energetic deficit preceded brain dysfunction [33, 36, 37]. Levels of *Pdha1* mRNA were not altered in our diabetes group, making it likely that the protein change could have been due to altered protein degradation or post-translational modification. PDHE1 α and ATP synthase subunit α in the DM/ZBPYR group were significantly upregulated, suggesting that the two proteins may serve as molecular targets of ZBPYR.

Cytoskeleton proteins comprised another major functional category of proteins identified in this study. The dynamics of the cytoskeleton allow for changes in dendritic spine morphology, a process that participates in the storage of information in neurons [38]. Brain-derived neurotrophic factor (BDNF) is an essential protein for maintenance of neuronal function and morphology. In diabetic brains, BDNF was reduced with abnormal dendritic spine and decreased cytoskeleton proteins [39, 40]. DRP-2, also known as collapsin response mediator protein 2, plays a role in axon transport, morphology maintenance and dynamics of microtubules [41, 42], and mediates BDNF signalling [43]. Significantly decreased DRP-2 in our diabetes group may have interacted with BDNF, with both participating in the alteration of diabetic neuron cytoskeleton. Levels of *Dpysl2* mRNA tended to be reduced in the diabetes group, indicating that changes in protein abundance may partly reflect alterations at the mRNA level.

Fig. 5 **a–c** Western blot and **(d)** real-time RT-PCR analysis of the three proteins whose abundance differed in hippocampus from the control, diabetes and DM/ZBPYR rats. The changes in PDHE1α (**a**) and DRP-2 (**b**) detected by western blotting were in good agreement with the proteomic results. **c** The G6PD changes in the diabetes group did not significantly differ from the control and DM/ZBPYR groups ($p > 0.05$). Blots were digitised and the bands were quantified using an

image analysis system. Bars represent means $\pm$ SD from four to five independent experiments. **a**, **b** † $p < 0.05$ vs diabetes group; ** $p < 0.01$ vs control group. **d** Levels of *Pdhal*, *Dpysl2* and *G6pd* mRNA in hippocampus of rats as labelled. Gene expression data are presented following normalising with the gene for β-actin. Bars represent means $\pm$ SD from three independent experiments

Actin cytoskeleton plays a role in insulin-induced translocation of glucose transporter 4 [44]. p34-ARC functions as an actin-binding component of the Arp2/3 complex [45], which plays a critical role in the precise control of actin cytoskeleton dynamics [20]. Thus, an increase in p34-ARC levels in our diabetes group may have been a compensatory result of abnormal insulin signalling. ZBPYR treatment significantly reduced the level of p34-ARC when compared with untreated diabetic rats. Tubulin alpha-1B chain was found at a lower level in our diabetes group, but was significantly elevated following ZBPYR treatment. The decreased level of tubulin alpha-1B may be related to the elevated glycosylation of tubulin under conditions of hyperglycaemia, which forms high molecular mass aggregates and fails to penetrate acrylamide gels [46]. Tubulin glycosylation inhibits GTP-dependent tubulin polymerisation, which could compromise some neuronal functions that are dependent on microtubule formation [46].

Studies have indicated that oxidative stress was markedly increased in the brain of diabetic animals and that administration of antioxidants ameliorated cognitive impairment [47, 48]. Two proteins identified in this study are

known to be involved in oxidative stress reactions. G6PD, which was significantly reduced in the diabetes group, serves as a rate-limiting enzyme of the oxidative branch of the pentose phosphate pathway. Activation of the pentose phosphate pathway in neurons results in production of NADPH [49]. It should be noted that the position of G6PD on the DIGE deviated from its theoretical isoelectric point and that its western blot results were not quite consistent with the proteomic analysis. Post-translational modifications have been shown to lead to isoelectric point alterations in G6PD [22]. These findings suggest that proteomic changes in G6PD may only represent a subset of G6PD with unknown post-translational modifications. Glutathione *S*-transferase is an important antioxidant and detoxification enzyme that catalyses the conjugation of oxidised products with glutathione, the major antioxidant in the brain, to form non-toxic products [50]. GSTM1-1 is an isoenzyme of glutathione *S*-transferase in brain [21]. Here, GSTM1-1 was markedly downregulated following administration of ZBPYR to levels that were similar to those observed in the control group. We speculate that oxidative stress may result in a compensatory increase of GSTM1-1

in DACD rats and that treatment with ZBPYR reduced the stress and levels of GSTM1-1.

In summary, using a DIGE-based proteomic method, we have successfully identified novel candidate proteins involved in the development of DACD and defined potential intervention targets of ZBPYR in hippocampus from a rat model of type 2 diabetes. Further studies are required to investigate the exact role of these proteins in the development of DACD and their potential as therapeutic targets.

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