Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2012;29:603-616

Accepted: January 12, 2012

Alterations in Pancreatic Protein Expression in STZ-Induced Diabetic Rats and Genetically Diabetic Mice in Response to Treatment with Hypoglycemic Dipeptide Cyclo (His-Pro)

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Key Words

Cyclo (His-Pro) • Diabetes • *ob/ob* mice • Pancreatic proteome • STZ-induced diabetic rats

Abstract

To provide insights into the molecular mechanisms underlying diabetes mellitus, we performed a proteomic study on two diabetic animal models, streptozotocin (STZ)-induced diabetic rats (T1DM) and genetically diabetic (C57BL/6J *ob/ob*) mice (T2DM). To better understand the recovery process of those diabetic rodents, we examined the effect of hypoglycemic dipeptide Cyclo (His-Pro) (CHP) treatment on the differential expression of pancreatic proteins in both animal models. Oral administration of CHP had an excellent hypoglycemic effect in both animal models, lowering the average plasma glucose level by over 50%. Pancreatic proteins were separated by two-dimensional gel electrophoresis (2-DE) and identified by MALDI-TOF mass spectrometry. This study allowed, for the first time, the identification of 34 proteins that are related to diabetes and potential targets of CHP, a potent anti-diabetic agent for both T1DM and T2DM. The alterations in the expression of these proteins could indicate a tendency for diabetic animals to overcome their diabetic state. These proteins are involved in cellular functions such as metabolism, cellular structure, oxidative stress, as well as signal and energy transduction. Some have already been linked to diabetes, suggesting that the newly identified proteins might also be significant in the etiology of this pathology and should be further investigated. Furthermore, CHP has emerged as a potent tool for both the treatment and study of the molecular mechanisms underlying diabetes. Thus, the findings presented here provide new insights into the study and potential treatment of this pathology.

Introduction

Diabetes mellitus is a multifactorial disease characterized by the destruction of the insulin-secreting β-cells of the islets of Langerhans in the pancreas (type 1 diabetes mellitus, T1DM) or by insulin resistance, whereby the primary insulin target organs are unresponsive to insulin action (type 2 diabetes mellitus, T2DM) [1]. To date, numerous treatments based on medicinal plants for the management of diabetes have

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been developed due to the serious side effects of chemically synthesized anti-diabetic drugs [2, 3].

Cyclo (His-Pro) (CHP) is a naturally occurring cyclic dipeptide consisting of histidyl and proline, and it is produced directly from amino acid or peptide sources [4]. It has been demonstrated that CHP levels in both animal and human diabetic subjects are significantly lower than those in non-diabetic subjects [5], indicating that CHP metabolism may be related to diabetes. Song et al. [6, 7] previously reported that high levels of CHP in prostate tissue are associated with anti-diabetic activity in animal and human subjects. Recently, soy proteins have become a very important component in the human diet with no evidence of the toxicity or side effects associated with oral administration of them [8-10].

Rodent animal models are widely used to study the underlying mechanisms and consequences of diabetes [11]. One of the animal models of T1DM is the streptozotocin (STZ)-induced model [12]. Regarding T2DM, *ob/ob* obese mouse is an representative animal model possessing a mutated *ob* gene leading to impaired leptin secretion and thereby uncontrolled appetite regulation [13].

Proteomics is a powerful tool used to describe changes in protein expression and modification, and moreover, it holds the promise of providing major contributions to diabetes research as well as other metabolic syndromes due to its potential to dissect complex human disorders [14-20]. A series of proteomic studies have been performed on both whole pancreatic tissues as well as isolated islets of Langerhans from mice [21, 22] and rats [14-27]. However, those pancreatic proteome profiling studies were not aimed to investigate proteomic alterations in response to treatment with anti-diabetic agents or other environmental stimuli. To the best of our knowledge, this is the first proteomic study on the expression of pancreatic proteins in both T1DM and T2DM animal models in response to an anti-diabetic agent.

In the past, diabetic studies have mostly been interested in genes expressed in the pancreas, the site of insulin secretion that regulates blood glucose levels [28-31]. However, there has been little evidence that antidiabetic agents themselves directly modulate gene expression in the cells of diabetes-relevant organs.

Therefore, in the present study, we carried out comparative proteomic analysis on pancreatic tissues from diabetic Sprague-Dawley (SD) rats induced by STZ as well as genetically obese (*ob/ob*) mice. The proteomic profile of the pancreas was assessed by two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry analysis in order to identify differentially expressed proteins with the goal of providing novel insights into the mechanisms of both T1DM and T1DM.

Materials and Methods

Preparation of Cyclo (His-Pro)

For extraction of CHP, 100 g of soybean meal was suspended in 2 L of distilled water (pH 7) and hydrolyzed for 12 h at 50°C with commercial proteases (6% flavourzyme and 2% alcalase). After hydrolysis, the reaction was terminated by inactivating the enzyme at 80°C in a water bath for 30 min. The hydrolysate was then centrifuged at 10,000x*g* for 20 min at 4°C, after which the supernatant was filtered through glass wool. Supernatant (300 µl) was deproteinized by adding three volumes (900 µl) of methanol (HPLC grade), after which the mixture was allowed to stand at 4°C for 15 min. The sample was centrifuged (12,000x*g*) in an Eppendorf centrifuge for 3 min, and the supernatants were filtered through a 0.22 µm membrane filter. The CHP content was measured as described previously [32].

Animals and breeding conditions

Five-week-old male Sprague-Dawley (SD) rats (Daehan Experiment Animals, Seoul, Korea) weighing 130-150 g were used for the T1DM animal model. To induce diabetes, after 1 week of acclimatization, the rats were subjected to a 16-h fast. Streptozotocin (STZ, Sigma, St. Louis, MO, USA) was prepared by dissolving in 0.01 M sodium citrate buffer (pH 4.5) immediately before administration. T1DM was induced by intramuscular injection of STZ (50 mg/kg body weight), whereas control rats were injected with vehicle alone. For T1DM animal experiments, all rats were randomly divided into three groups consisting of seven animals each: normal healthy group received distilled water; STZ-induced diabetic control group (STZ group); and CHP-treated diabetic group (CHP group) treated with CHP at a level of 4 mg/kg of body weight for 21 days. For T2DM animal experiments, all procedures were the same as in the T1DM experiments except that diabetic mice (C57BL/6J *ob/ob*) were treated with CHP at a level of 2 mg/kg of body weight for 24 days. Animals were housed individually in stainless steel cages at a controlled temperature (23±2°C with 55±5% humidity) under a 12:12-h light: dark cycle. A commercial pellet diet (Sam Yang Co., Seoul, Korea) and water were provided during the experiment period. All animals were randomly divided into each group consisting of seven animals each, and mice and rats with a blood glucose level of 300 mg/dl (16.7 mmol/l) were considered to be diabetic. Body weight gain and food intake were periodically measured. After fasting for 4 h, blood glucose levels were measured using one drop of whole blood obtained by making a cut on the underside of the tails of mice or rats with a glucometer (Lifescan, Milpitas, CA, USA). These experiments were approved by the Committee for Laboratory Animal Care and Use of Daegu University. All procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Pancreatic tissue preparation

Pancreatic tissues were removed immediately after sacrifice, after which excessive blood contents of pancreatic tissues were eliminated using cold saline solution. Pancreatic tissues were then pulverized into a powder under liquid nitrogen and stored at -80°C until use. Frozen tissues (40 mg) were solubilized in 200 ml of rehydration buffer solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1 mM PMSF, 20 mM DTT, 2% IPG buffer, and a trace amount of bromophenol blue. An ultrasonic generator was used for 2x30 s with 1 min on ice between each round to aid solubilization. Samples were centrifuged at 13,000x*g* for 15 min, after which the supernatant was transferred into new tubes. Protein from the supernatant was precipitated by the methanol/chloroform method prior to electrophoretic separation. The precipitate was then resuspended in rehydration buffer and kept at -80°C until use.

Two-dimensional electrophoresis (2-DE)

2-DE was carried out in triplicate on six rats or mice from each experimental group. IEF was performed on IPG DryStrip gels (17 cm, pH 4-7) in a PROTEIN IEF cell (Bio-Rad) by following the protocol recommended by the manufacturer. Prior to IEF, 400 µg (~3 µl) of the pancreatic protein was solubilized in 347 µl of rehydration solution containing 7 M urea, 2 M Thiourea, 4% CHAPS, 1 mM PMSF, 20 mM DTT, and 2% IPG buffer. IPG strips were rehydrated passively overnight to ensure maximal diffusion of the proteins within the strip in the strip holders. IEF was carried out under a layer of Drystrip cover fluid as follows: 15 min at 250 V, 3 h at 250-10,000 V, 6 h at 10,000 V, and then holding at 500 V until ready to run the second dimension. After focusing, IPG strips were equilibrated in 10 ml of reducing buffer containing 6 M urea, 2% SDS, 1% DTT, 30% glycerol, and 50 mM Tris-HCl (pH 6.8) for 15 min, followed by 15 min equilibration in the same solution, except for DTT was replaced with 2.5% iodoacetamide. After equilibration, the gel strips were placed on 20x20 cm 12% polyacrylamide gels and then overlaid at the top of the gels with 0.75% w/v low-melting-point agarose in running buffer. Electrophoresis was run using the Laemmli SDS-discontinuous system at a constant voltage of 20 mA/gel until the dye front had run off the bottom of the gels. Subsequently, the gels were visualized using silver staining.

Digestion of protein in gel

In-gel digestion was performed using modified porcine trypsin according to the methods outlined by our previous method [27]. Each of the silver-stained gels was washed with 50% acetonitrile to remove SDS, salts, and the stain. After drying to remove solvent, the gels were rehydrated with trypsin (8-10 ng/µl) and then incubated for 8-10 h at 37°C. The proteolytic reaction was terminated by adding 5 µl of 0.5% trifluoroacetic acid. Tryptic peptides were then recovered by pooling the aqueous phases from the repeated gel extractions with 50% acetonitrile. After concentration, the peptide mixture was redissolved in buffer and desalted using C_{18}ZipTips (Millipore, Watford, Herts, UK), after which the peptides were eluted with 1-5 µl of acetonitrile. An aliquot of this solution was then mixed with an equal volume of saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 1 µl of the mixture was spotted onto a target plate.

Image analysis

Imaging gels produced by a UMAX PowerLook 1120 (Maxium Technologies, Akron, OH, USA) were analyzed by modified ImageMaster 2-D software V4.95 (GE Healthcare). Relative optical density and relative volume were calculated for correction of differences in gel staining. Each spot intensity volume (%) was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison between the groups.

Protein identification

Differentially regulated spots between the experimental groups were identified by PMF. Excised spots were digested with trypsin (Promega, Madison, WI, USA), followed by mixing with α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid. Peptides were then analyzed using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF; Ettan MALDI-TOF Pro, GE Healthcare). Spectra were collected from 350 shots per spectrum over an *m/z* range of 600-3000 and then calibrated by two point internal calibration using trypsin auto-digestion peaks (*m/z* 842.5099, 2211.1046). Peak lists were generated using the Ettan MALDI-TOF Pro Evaluation Module (version 2.0.16). The threshold used for peak-picking was as follows: 5,000 for minimum resolution of monoisotopic mass; 2.5 for S/N. The search program MASCOT (Mascot Sever 2.3) developed by The Matrixscience (http://www.matrixscience.com) was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of 1 missed cleavage, iodoacetamide (Cys) as a fixed modification, oxidation (Met) as a variable modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. MASCOT probability-based MOWSE (molecular weight search) score was calculated for PMF. Protein score was calculated as -10*Log (P), where P is the probability that the observed match is a random event, and scores >61 were regarded as significant (p<0.05).

Western blot analysis

Levels of four proteins identified by the 2-DE experiments were validated by immunoblot analysis. The pancreas sample (100 mg) was diluted in 2 x sample buffer (50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% b-mercaptoethanol) and then heated for 5 min at 95°C before SDS-PAGE (12%). Subsequently, proteins were transferred to a PVDF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) membrane and then incubated overnight with 5% blocking reagent (GE Healthcare) in Tris-buffered salt (TBS) containing 0.1% Tween-20 at 4°C. The membrane was then rinsed in four changes of TBS with Tween-20 (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0), incubated twice for 5 min and twice for 10 min in fresh washing buffer, and then incubated for 1 h in blocking solution containing a 1:1000 dilution of primary antibody (anti-PRDX3, anti-HBA2, anti-APEH, anti-MDH, anti-β-actin; Santa Cruz Biotechnology). After four washes, the membrane was incubated for 1 h with HRP-conjugated anti-mouse IgG secondary antibody (1:1000, Santa Cruz

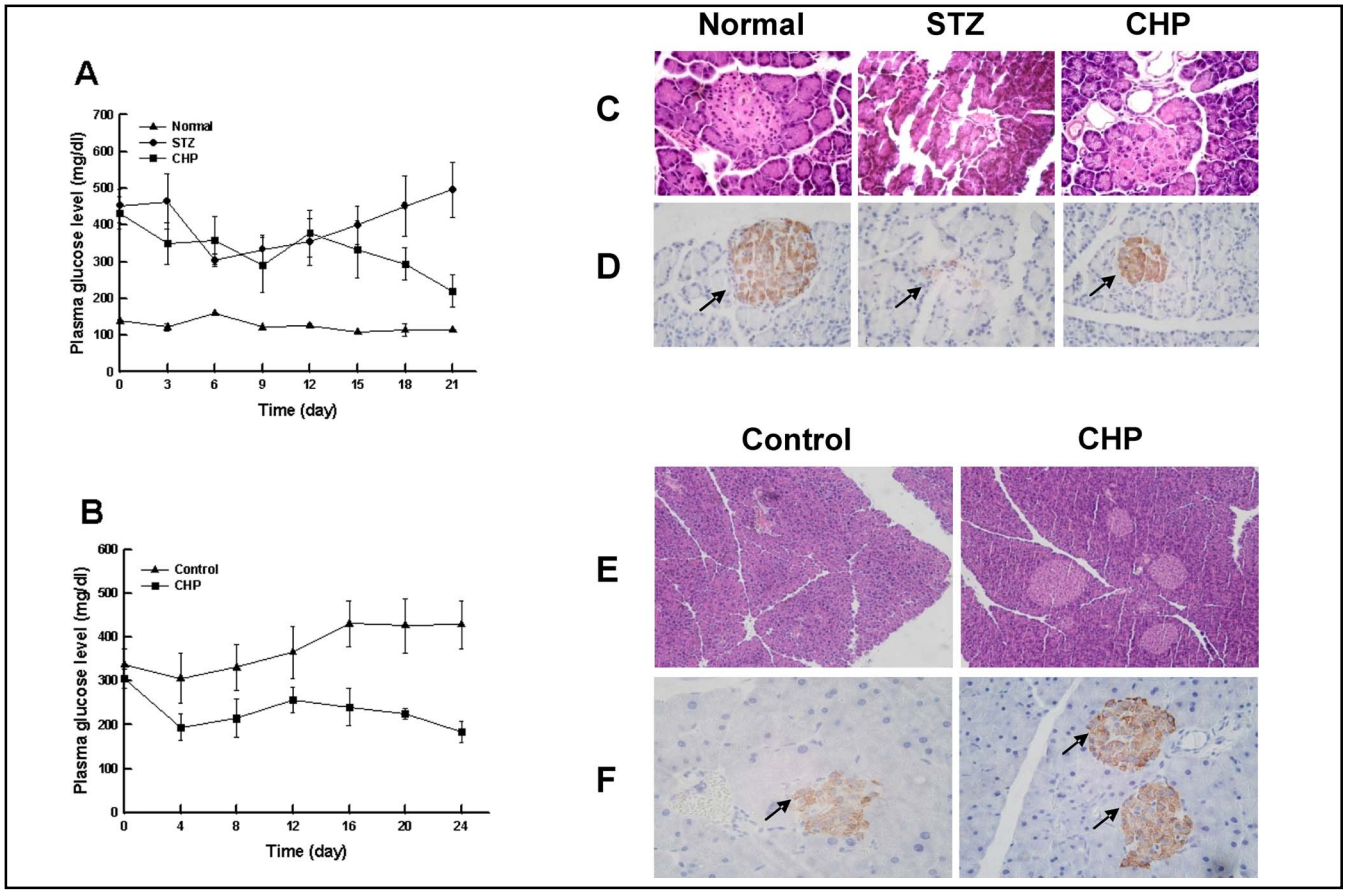

Fig. 1. Time profiles of blood glucose levels in STZ-induced diabetic rats (A) and genetically diabetic mice (B) before and after treatments of hypoglycemic dipeptide cyclo (His-Pro) (CHP). Values are mean ± SD for seven rats in each group. H&E staining (C, E) and immunohistochemical staining of insulin in pancreatic tissue (D, F), where islets were indicated by arrows (magnification, x 400).

Biotechnology) and developed by ECL (Western blot analysis system kit, GE Healthcare). The Western blot was analyzed by scanning with a UMAX PowerLook 1120 (Maxium Technologies) and digitalizing using image analysis software (KODAK 1D, Eastman Kodak Company, NY, USA).

Histological study

For the histological study, pancreatic tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Tissues were next dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax. Sections were cut at 4-µm thickness, deparaffinized, and rehydrated. Tissue blocks were chosen carefully after histological assessment of the sections stained with hematoxylin and eosin. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded pancreatic tissue derived from control and diabetic rats or mice. Anti-insulin antibody (working dilution 1:100; ab7842, Abcam, Cambridge, MA) was immunoreacted against samples and visualized with 3,3´-diaminobenzidinetetrahydro-chloride (DAKO, CA, USA) using Histofine Simple Stain MAX PO Multi (Nichirei Ltd, Tokyo, Japan). The histopathological findings were observed using an image analyzer (Matrox Electronic System Ltd, Quebec, Canada) with software coupled to an Olympus light microscope (Olympus Optical Co., Ltd, Tokyo, Japan) through a CCD camera (Matsushita Communication Industrial Co., Ltd, Yokohama, Japan).

Statistical Analysis

The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) program. All data are expressed as the mean ± SD values. In all analyses, a $p<0.05$ was considered statistically significant.

Results

Hypoglycemic effect of CHP on STZ-induced diabetic rats and genetically obese (ob/ob) mice

Prior to the proteomics study, the effect of CHP on plasma glucose level was investigated in STZ-induced diabetic rats. The results revealed that orally administrated CHP (4 mg/kg body weight) administered for 21 days had an excellent hypoglycemic effect, lowering the

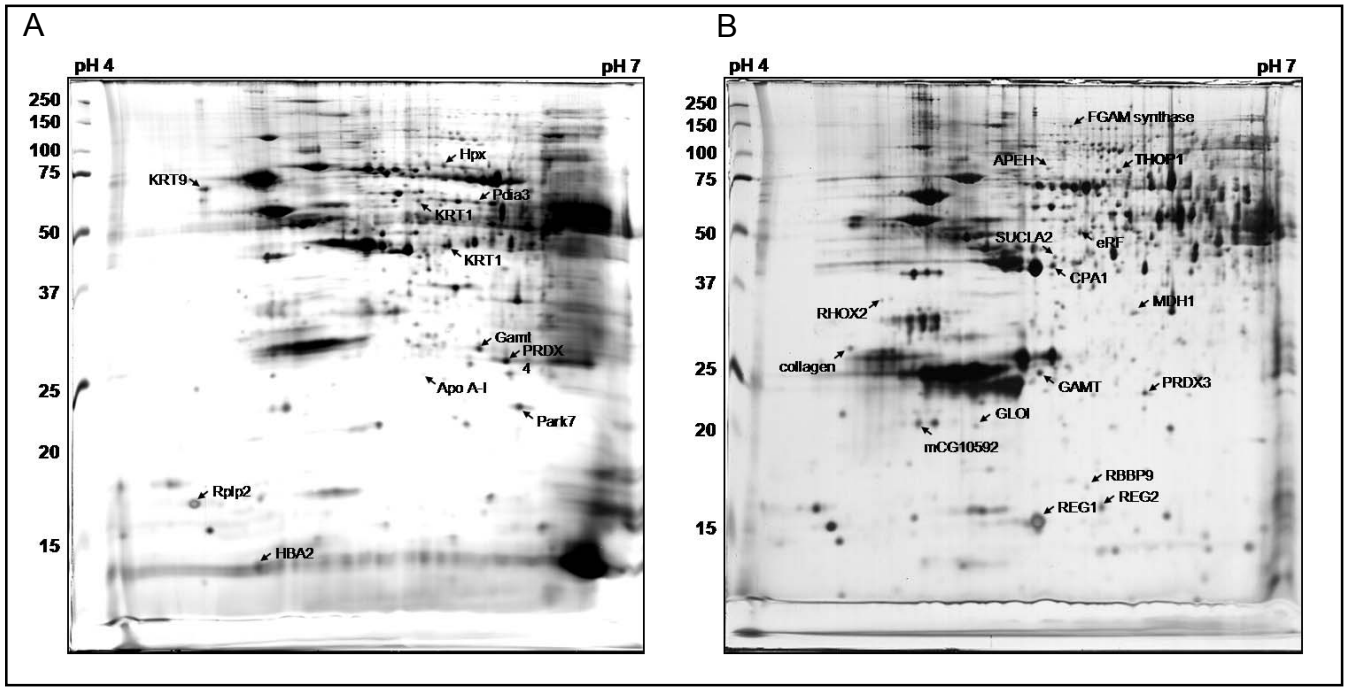

Fig. 2. A representative silver-stained 2-DE image of pancreatic tissues in STZ-induced diabetic rat (A) and genetically obese (*ob/ob*) mouse (B), where differentially regulated proteins were marked with arrows together with identified major rat plasma proteins. For abbreviation of each protein name, see abbreviations.

average plasma glucose level in CHP-fed rats to about 56% of that in STZ-treated control rats (Fig. 1A). Further, CHP-treated genetically obese mice (C57BL/6J *ob/ob*) displayed decreased blood glucose levels (about 57%) when administrated at 2 mg/kg for 24 days (Fig. 1B). These results suggest that CHP had a hypoglycemic effect in both STZ-induced diabetic rats and genetically obese mice. Histological study revealed less degenerative and necrotic changes along with visible shrinkage of the islets of Langerhans in sections of pancreatic tissues stained with H&E from STZ-induced diabetic control rats (Normal rats in Fig. 1C). The nuclei of necrotic cells indicated either pyknosis or marginal hyperchromasia (STZ rats in Fig. 1C). There was apparent hydropic degeneration and degranulation in the cytoplasm of degenerative and necrotic cells, whereas other cells with pyknotic nuclei had a dark eosinophilic cytoplasm (STZ rats in Fig. 1C). Surprisingly, CHP treatment protected the majority of Langerhans islet cells (CHP rats in Fig. 1C). This result suggests that CHP, at least in part, regenerated β-cells in islets that were destroyed by STZ. A similar result was observed in genetically diabetic mice before and after CHP treatment (Fig. 1E). The results of the immunohistochemical staining of pancreatic tissues showed strong insulin antigen positivity in the β-cells of islets of healthy rats (Normal rats in Fig. 1D). In contrast, there was weak insulin immunoreactivity in a few of the β-cells of Langerhans islets of diabetic control rats (STZ rats in Fig. 1D). CHP treatment considerably increased the insulin antigenecity of diabetic islet β-cells, suggesting the possibility of β-cell proliferation or regeneration by CHP therapy (CHP rats in Fig. 1D). Moreover, CHP treatment significantly increased the area of insulin-immunoreactive β-cells (see the arrows for CHP rats in Fig. 1D). Similarly, genetically diabetic mice that received CHP also showed increased insulin immunoreactivity (CHP mice in Fig. 1F) compared with diabetic control mice.

2-DE separation and identification of proteins

To investigate the mechanism underlying the hypoglycemic effect of CHP, we used thiourea-containing lysis buffer to extract proteins from the pancreas. Pancreatic proteins were arrayed by 2-DE in a pH 4-7 IEF strip, and protein expression was comparatively analyzed using image analysis software. Following this, the proteins were identified by PMF using MALDI-TOF mass spectrometry. As shown in Fig. 2A, up to 500 protein spots were visualized on each 2-DE gel upon sliver staining. We applied 12 spots that were differentially expressed between normal healthy and STZ-induced diabetic rats for identification by MALDI-TOF analysis

Protein	Function	Acc. No.[a]	Expression[b]	Mw[c]	pI[d]	Coverage (%)[e]
Hemopexin (Hpx)	Transport	GI:122065203	↓	52.06	7.58	24
keratin, type I cytoskeletal 9 (KRT9)	Protein binding	GI:55956899	↓	62.26	5.14	22
keratin, type II cytoskeletal 1 (KRT1)	Rsponse to oxidative stress	GI:160961491	↓	65.62	7.62	47
protein disulfide isomerase associated 3, isoform CRA_a (Pdia3)	Cell redox homeostasis Protein disulfide isomerase activity	GI:149023097	↓	54.12	7.10	34
keratin 1 (KRT1)	Sructural molecule activity	GI:11935049	↓	66.20	8.16	17
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 (Ddah1)	Aginine metabolic process Positive regulation of angiogenesis Positive regulation of Nitric oxide biosynthetic process Dmethylargininase activity Metal ion binding	GI:11560131	↓	31.81	5.75	39
Chain A, Guanidinoacetate Methyltransferase Containing S- Adenosylhomocysteine and Guanidinoacetate (Gamt)	S-adenosylhomocysteine metabolic process, S-adenosylmethionine Metabolic process, creatine biosynthetic process		↓	26.51	5.83	61
Peroxiredoxin-4 (PRDX4)	Cell redox homeostasis Oxidation reduction Peroxidase activity Peroxiredoxin activity	Gi:16758274	↓	31.22	6.18	52
Apolipoprotein A-I preproprotein (Apo A-I)	Metabolism & Transport	Gi:6978515	↑	30.10	5.52	52
Protein DJ-1 (Park7)	Rsponse to drug Rsponse to oxidative stress	Gi:16924002	↓	20.19	6.32	61
60S acidic ribosomal protein P2 (Rplp2)	Tanslational elongation Sructural constituent of ribosome	Gi:71795613	↓	11.69	4.44	78
Hemoglobin alpha 2 chain (HBA2)	Oxygen transport & binding Heme binding	Gi:60678292	↓	15.45	8.45	58

Table 1. Identification of pancreatic proteins by PMF in STZ-induced rats in response to CHP treatment. [a]NCBI database accession number. [b]STZ-induced diabetic group compared with diabetic control group. [c]Theoretical molecular weight of the matching protein in kDa. [d]Theoretical isoelectric point of the matching protein. [e]Percent of identified sequence to the complete sequence of the known protein.

(see the marked proteins in Fig. 2A). Identified proteins on 2-D gels were detected in their expected ranges of theoretical isoelectric points (pI) and molecular weights (Mw). The altered expression patterns of the identified proteins were detected. All identified proteins in the proteome map are summarized in Table 1. The expression levels of these proteins remarkably changed upon diabetes induction, but they returned to approximately normal after CHP treatment. Moreover, the orders of magnitude of the expression changes widely varied. Most of identified proteins were down-regulated by STZ treatment, including keratin (KRT)1, KRT2, protein disulfide isomerase 3 (PDIA3), guanidinoacetate methyltransferase (GAMT), peroxiredoxin 4 (PRDX4), protein DJ-1, and 60S acidic ribosomal protein P2 (RPLP2) (Fig. 3). The expression levels of these reverted back to those of normal healthy rats upon treatment with CHP.

In the case of genetically diabetic mice, as shown in Fig. 2B, up to 500 protein spots were visualized on each 2-DE gel by sliver staining. We applied 22 spots for identification by MALDI-TOF analysis (see the marked proteins in Fig. 2B). Most of the identified proteins showed higher expression levels in diabetic mice than CHP-treated mice. CHP treatment induced down-regulation

Protein	Function	Acc. No.[a]	Expression[b]	Mw[c]	pI[d]	Coverage (%)[e]
Phosphoribosylformylglycinamidine synthase (FGAM synthase)	Purine biosynthesis Nucleotide transport and metabolism	Gi:226958458	↓	146.25	5.43	13
Acylpeptide hydrolase, isoform CRA_d (APEH)	Hydrolase	Gi:148689322	↓	80.93	5.32	12
Thimet oligopeptidase (THOP1)	Hydrolase, Metalloprotease Protease	Gi:239916005	↓	78.78	5.67	29
unnamed protein product		Gi:12846632	↓	50.43	5.55	25
unamed protein product		Gi:74177659	↑	56.73	5.57	23
Eukaryotic peptide chain release factor subunit 1 (eRF)	Translation release factor activity, codon specific	Gi:4759034	↓	49.23	5.51	39
unnamed protein product		Gi:74150721	↑	58.27	8.18	58
ATP-specific succinyl-CoA synthetase beta subunit (SUCLA2)	Ligase, ATP binding	Gi:3766201	↓	46.56	5.65	21
Carboxypeptidase A1 precursor (CPA1)		Gi:54312076	↓	47.47	5.41	37
unnamed protein product		Gi:26350123	↑	33.01	4.80	40
Reproductive homeobox 2A (RHOX2)	Regulation of transcription	Gi:13386376	↓	21.21	4.89	28
Malate dehydrogenase, cytoplasmic (MDH1)	Glycolysis Oxidation reduction	Gi:254540027	↑	36.66	6.16	34
Protein phosphatase 1, catalytic subunit, beta isoform		Gi:148705446	↑	36.09	6.59	30
Collagen	Cell adhesion Protease inhibitor Serineproteaseinhibitor	Gi:467517	↓	115.37	6.11	14
Guanidinoacetate N-methyltransferase (GAMT)	Methyltransferase Transferase	Gi:6753944	↓	26.60	5.43	57
Thioredoxin-dependent peroxid reductase, mitochondrial precursor (PRDX3)	Antioxidant Oxidoreductase Peroxidase	Gi:6680690	↓	28.34	7.15	28
unnamed protein product		Gi:26344461	↓	22.99	5.20	45
mCG10592, isoform CRA_c		Gi:148703873	↓	16.58	5.23	33
Lactoylglutathione lyase (GLO1)	Lyase Anti-apoptosis Metal ion binding	Gi:165932331	↓	20.97	5.24	35
Putative hydrolase RBBP9 (RBBP9)	Hydrolase activity	Gi:86439977	↓	21.07	5.64	45
Lithostathine-2 precursor (REG2)	Sugar binding	Gi:6677705	↓	19.79	5.89	45
Good peak but no identification			↓			
Lithostathine-1 precursor (REG1)	Sugar binding	Gi:6677703	↓	18.91	6.08	56
unnamed protein product		Gi:74220823	↑	44.74	7.53	24

Table 2. Identification of pancreatic proteins by PMF in *ob/ob* mice in response to CHP treatment. [a]NCBI database accession number. [b]STZ-induced diabetic group compared with diabetic control group. [c]Theoretical molecular weight of the matching protein in kDa. [d]Theoretical isoelectric point of the matching protein. [e]Percent of identified sequence to the complete sequence of the known protein.

of acylpeptide hydrolase (APEH), thimet oligopeptidase 1 (THOP1), peroxireductase mitochondrial precursor (PRDX3), lactoylglutathione lyase (GLO1), hydrolase RBBP9 (RBBP9), lithostathine-1 precursor (REG1), and lithostathine-2 precursor (REG2), as well as up-regulation of malate dehydrogenase cytoplasmic (MDH1) (Fig. 4).

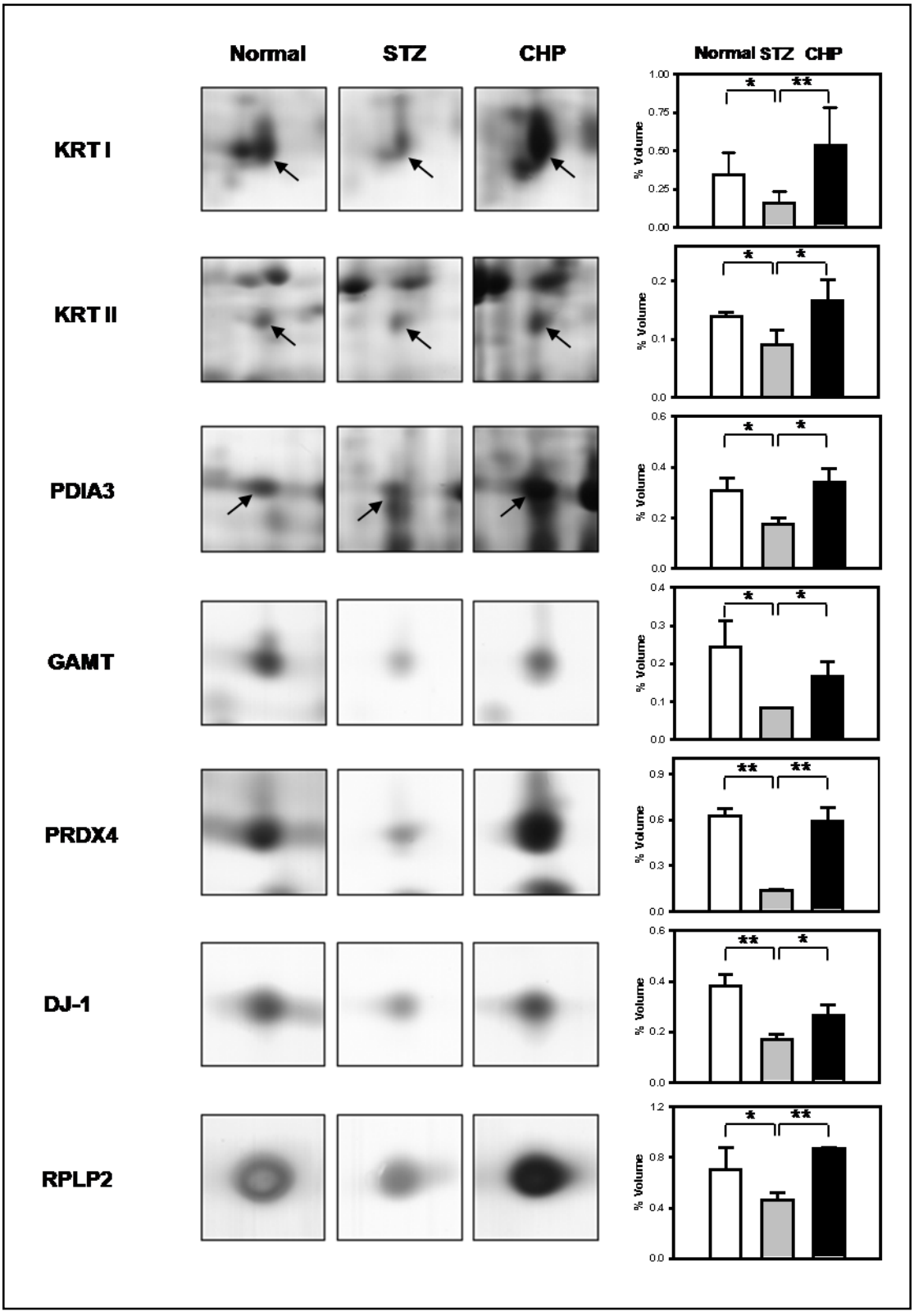

Fig. 3. Differentially regulated pancreatic tissue proteins among normal (white bars), in STZ-induced diabetic control rats (gray bars), and CHP-treated diabetic rats (black bars). Data are exhibited as mean values ± SD of volume density (%) of the changed spot in 3 individual gels from 6 rats. Statistical significance between each group was determined by an ANOVA test, where p value is $^{*}p<0.05$ and $^{**}p<0.01$. For abbreviation of each protein name, see abbreviations.

Validation of proteomic data by immunoblot analysis

To eliminate possible technical errors and artificial effects in the proteomic analysis, the levels of four proteins of interest (PRDX4 and HBA2 from rats; APEH and MDH1 from mice) identified in the proteomic experiments were verified by immunoblot analysis. The results supported our proteomic data, in that the regulation

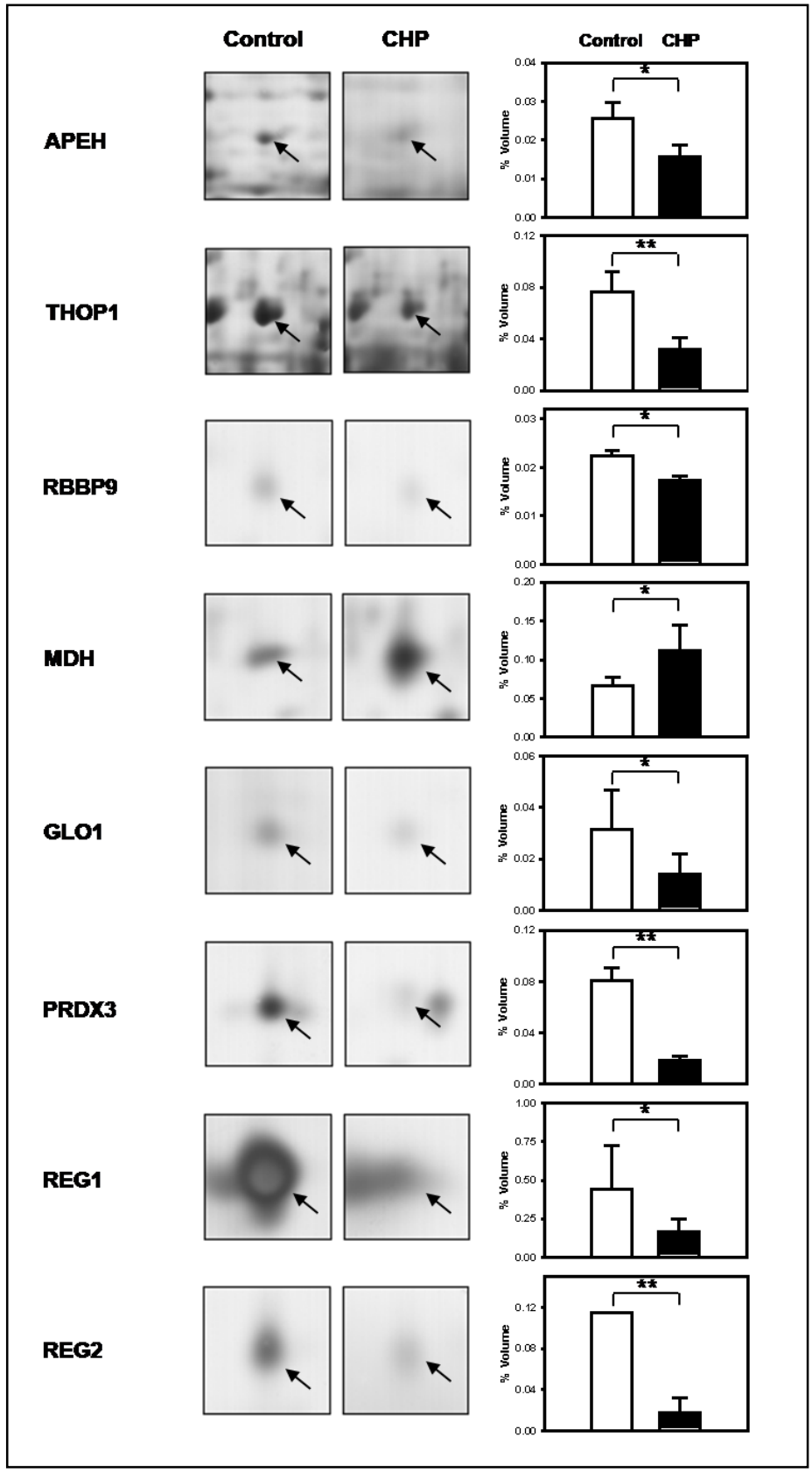

Fig. 4. Differentially regulated pancreatic tissue proteins between genetically diabetic control mice (white bars) and CHP-treated diabetic mice (black bars). Data are exhibited as mean values ± SD of volume density (%) of the changed spot in 3 individual gels from 6 rats. Statistical significance between each group was determined by an ANOVA test, where p value is $^{*}p<0.05$ and $^{**}p<0.01$. For abbreviation of each protein name, see abbreviations.

patterns of two proteins from STZ-induced diabetic rats and two proteins from genetically diabetic mice were almost identical between the two analytical methods (Fig. 5).

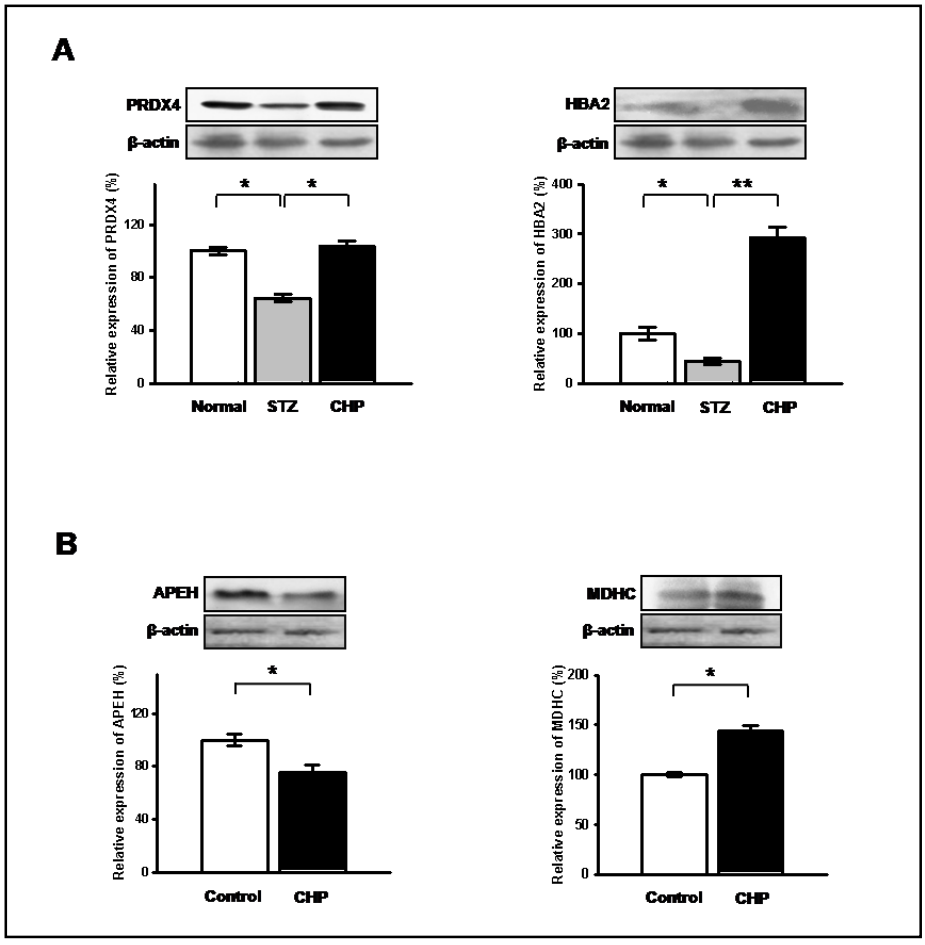

Fig. 5. Validation of differentially regulated pancreatic tissue proteins in STZ-induced diabetic rats (A) and genetically diabetic mice (B). Levels of 4 identified proteins from 2-DE analysis were investigated using 3 individual gels from 6 rats and mice, respectively. Statistical significance between each group was determined by an ANOVA test, where p value is $^{*}p<0.05$ and $^{**}p<0.01$. For abbreviation of each protein name, see abbreviations.

Discussion

To date, two animal models of STZ-induced T1DM and genetically rendered T2DM are commonly employed in the field of diabetes research. In the present study, these diabetic models were successfully established, as demonstrated by the observed hyperglycemia, damage to pancreatic islets, as well as insulin resistance. We compared the proteome maps of the pancreatic tissues from both STZ-induced diabetic rats and genetically diabetic mice with those of normal healthy animals or controls, and we identified 12 and 22 differentially expressed protein spots, respectively.

We observed that a consequence of STZ-induced cellular stress was decreased expression of most of the proteins identified in this study, and their expression levels were up-regulated again by CHP treatment to levels mimicking those of normal healthy rats. Due to space limitations, only selected proteins of physiological significance showing differential expression are discussed here.

One of the interesting results in this proteomic study was that two keratin isoforms (KRT 1 and KRT9) were down-regulated by STZ, but their protein expression levels reverted back to those of normal healthy rats upon treatment with CHP (Fig. 3). Pancreatic keratins have been known to be dispensable, unlike liver keratins that are essential cytoprotective proteins [33]. However, several emerging lines of evidence support a role for keratins in pancreatic tissues, demonstrating that keratin-deficient endodermal cells exhibit increased nuclear factor (NF)-κB and c-Jun NH_2-terminal kinase activation in response to TNF-α production during acute pancreatitis [34, 35]. Therefore, reduced levels of pancreatic keratins could be a sign of pancreatic injury mediated by STZ treatment in rats. Moreover, increased levels of keratins in CHP-treated rats imply a protective role for CHP in the pancreas.

Another interesting result was differential expression of protein disulfide isomerase associated 3 (PDIA3) in the pancreas between diabetic and CHP-treated rats. The PDIA3 protein possesses protein disulfide isomerase activity [36]. PDIA3 is also a part of the major histocompatibility complex (MHC) class I peptide-loading complex, which is essential in the formation of the final antigen conformation as well as export from the

endoplasmic reticulum to the cell surface [37]. An earlier study demonstrated that there is a causal relationship between elevated *Pdi* gene expression and the diabetes phenotype in BTBR-*ob/ob* mice: *Pdi* is expressed ~20-fold higher in the diabetes-susceptible BTBR mouse strain relative to the diabetes-resistant C56BL/6 (B6) strain [38]. Nonetheless, our proteomic study indicated that STZ decreased PDIA3 expression while CHP increased its levels. This result led us to hypothesize that there may exist differences between PDIA3 and its isoforms regarding physiological role. Further studies are required to directly test this hypothesis.

Reduced expression of guanidinoacetate methyltransferase (GAMT) in STZ-treated rats in contrast to increased GAMT expression in CHP-treated rats was also an interesting finding, in that this result likely reflects a disorder in energy production in the STZ-treated rat pancreas as a result of disrupted creatine metabolism [39]. Moreover, the *GAMT* gene provides instructions for making an enzyme that participates in the two-step synthesis of the compound creatine from the protein building blocks (amino acids) glycine, arginine, and methionine. Further, GAMT gene mutations impair the ability of GAMT enzyme to participate in creatine synthesis. Therefore, creatine is needed for many tissues in the body to store and use energy properly, and the effects of GAMT deficiency are most severe in organs and tissues that require large amounts of energy, such as the brain and muscles [39]. Collectively, we postulated here that CHP might play a role in controlling energy regulation in the rat pancreas based on the fact that GAMT deficiency results in disrupted creatine biosynthesis, leading to depletion of creatine and phosphocreatine.

Expectedly, increased susceptibility of STZ rats to oxidative stress was confirmed by the decreased expression of peroxiredoxin 4 (PRDX4), which plays a role in protecting the free thiol groups of proteins against oxidative damage and thioredoxin-dependent peroxidase activity [40]. A similar result has also been reported from the proteomic study by Jiang and coworkers [14]. Bast et al. [41] demonstrated that expression of PRDX I and II is up-regulated in cultured insulinoma cells exposed to various stress agents, including diabetogenic substances such as alloxan and streptozotocin. Further, PRDX4 can protect pancreatic islet β-cells against injury caused by STZ-induced insulitis, which strongly suggests that oxidative stress plays an essential role in STZ-induced diabetes [40]. Interestingly, our proteomic data revealed that CHP significantly reverted expression levels of PRDX4 back to those of normal rats, suggesting its preventive role against oxidative stress induced by STZ treatment.

Similarly, protein DJ-1 was also significantly down-regulated in STZ-induced diabetic rats. The physiological role of DJ-1 protein is mainly in the brain, where it protects cells from oxidative stress that occurs when free radicals accumulate to dangerous levels [42]. Further, DJ-1 plays a pivotal role in protecting erythroid cells from oxidative damage [43]. A recent study found that the protein levels of DJ-1 in adipose tissue of obese rats fed a high fat diet are significantly reduced [18]. However, the metabolic function of DJ-1 protein in pancreatic tissue has not yet been addressed. In the current study, interestingly, CHP treatment significantly enhanced the expression of these two proteins to levels around those of normal healthy rats, suggesting that CHP plays a role in the alleviation of oxidative stress in the rat pancreas induced by STZ.

Surprisingly, we observed significant differential expression of two peptidases (APEH and THOP1) between genetically diabetic (*ob/ob*) control mice and their CHP-treated counterparts. Acylpeptide hydrolase (APEH) is an acylaminoacyl peptidase that catalyzes the removal of an *N*-acylated amino acid from blocked peptides. Recent studies have demonstrated that APEH activity is associated with the degradation of amyloid-beta peptide (Aβ), which is an important protein in the pathological onset of Alzheimer's disease [44, 45]. Thimet oligopeptidase (THOP) is a thiol-dependent metallopeptidase that can cleave and thereby modulate the activity of many neuropeptides. THOP1 can degrade β-amyloid precursor protein and generate amyloidogenic fragments [46]. In the current proteomic study, higher expression levels of these two proteins were observed in diabetic control mice than in their CHP-treated counterparts, suggesting that the increased expression of these proteins might be a part of a compensatory defense mechanism the pancreas employs against an increased Aβ load. This finding led us to postulate that T2DM is likely to be a risk factor for Alzheimer's disease.

Of particular interest was the down-regulation of hydrolase RBBP9 (RBBP9) after CHP treatment. RBBP9 is a retinoblastoma-binding protein that plays a role in the regulation of cell proliferation and differentiation [47]. Recently, biochemical studies on pancreatic cancer cells have confirmed that this protein is a serine protease, acting on a yet-unknown target in the TGF-β1 signaling pathway [48]. RBBP9 was also suggested to play a role in gender-related differential responses to radiation-induced cell proliferation [49]. We found for the first time here that RBBP9 was highly expressed in a diabetic

state, and its expression levels decreased when treated with the hypoglycemic agent CHP. As a result, the identification of compounds that selectively inhibit RBBP9 activity may provide valuable probes for the study of apoptosis, cell cycle, tumorigenesis, as well as diabetes.

Our proteomic data also revealed that malate dehydrogenase (MDH) expression remarkably increased (3.9-fold) in genetically diabetic mice upon CHP treatment. MDH is an important NAD^+-dependent enzyme involved in glycometabolism that catalyzes the formation of oxaloacetate and NADH from L-malate and NAD^+ [50]. MDH is highly expressed in the brain, heart, and skeletal muscle, where it plays a role in aerobic energy production for many cellular events [51]. A decrease in MDH activity may therefore induce hyperglycemia due to decreased use of glucose, such as glucose uptake and glycolysis, in peripheral tissues [52]. Taken together, the glucose-clearing activity of genetically diabetic mice was significantly enhanced after CHP treatment, and this was also confirmed by immunoblot analysis (Figs. 4 and 5).

Our current proteomic data has shown, for the first time, up-regulation of lactoylglutathione lyase (GLO) in diabetic mice. GLO1 is a ubiquitous cellular defense enzyme involved in the detoxification of methylglyoxal, a cytotoxic byproduct of glycolysis [12], and its expression is known to be up-regulated in various human malignant tumors such as metastatic melanoma [53, 54]. Accumulative evidence suggests an important role for GLO1 expression in protecting against methylglyoxal-dependent protein adduction and cellular damage associated with diabetes, cancer, and chronological aging [55]. Taken together with our data on down-regulation of PRX3, we postulate that CHP-treated mice strikingly recovered from oxidative stress mediated by a diabetic state.

Yet another exciting finding in the present study was the differential expression of two regenerating (REG) family proteins (REG I and REG II). These small, secreted proteins have been implicated in a range of physiological processes, including acting survival/growth factors for insulin-producing pancreatic β-cells, neural cells, and epithelial cells of the digestive system [56]. Members of the REG family are divided into four subclasses (types I, II, III, and IV). Previous evidence has linked their overexpression in islets with new-onset T1DM [56]. REG II, also known as lithostathine 2, has been implicated in the regulation of cell growth, tumorigenesis, and cancer progression [57]. In this study, REG I and REG II showed remarkably decreased expression levels in CHP-treated mice (2.9-fold and 5.2-fold, respectively), and these changes were statistically significant ($p < 0.05$) compared to control (diabetic group). This result suggests that REG proteins are established stress-inducible proteins that ameliorate pancreatic injury in diabetic mice but not in CHP-treated mice [33].

This proteomic study has identified, for the first time, 34 proteins that are related to diabetes and potential targets of CHP, a potent anti-diabetic agent for both T1DM and T2DM. However, unfortunately, we could not find expected numbers of proteins that show differential expression common in T1DM and T2DM animal models, presumably due their different pathophysiological significances. For example, PRDX4 was markedly down-regulated in STZ-induced diabetic rat pancreas (Fig. 3), whereas PRDX3 was up-regulated in diabetic state (Fig. 4). Moreover, due to limited number of published data as well as differences in animal models used, we were not able to directly compare the results of current study with other proteomic data.

Alterations in the expression levels of these proteins could indicate a tendency for diabetic animals to undergo overcome their diabetic state. These proteins are involved in cellular functions such as metabolism, cellular structure, oxidative stress, as well as signal and energy transduction. Some have already been linked to diabetes, suggesting that the newly identified proteins might also be significant in the etiology of this pathology and should be further investigated. Furthermore, CHP has emerged as a potent tool for both the treatment and study of the molecular mechanisms underlying diabetes. Thus, the findings presented here provide new insights into the study and potential treatment of this pathology.

Abbreviations

APEH (acylpeptide hydrolase); CHP (Cyclo (His-Pro)); 2-DE (two-dimensional gel electrophoresis); GAMT (guanidinoacetate methyltransferase); GLO1 (lactoylglutathione lyase); KRT (keratin); MDH1 (malate dehydrogenase cytoplasmic); PDIA3 (protein disulfide isomerase 3); PMF (peptide mass finger printing); PRDX (peroxiredoxin); RBBP9 (hydrolase RBBP9); REG (lithostathine precursor); RPLP2 (60S acidic ribosomal protein P2); T1DM (type 1 diabetes mellitus); T2DM (type 2 diabetes mellitus); THOP1 (thimet oligopeptidase 1).

Acknowledgements

This study was supported by the Technology Development Program for Food, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (Project No.: 109144-03-3).

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