

Comparative proteomic analysis of rats subjected to water immersion and restraint stress as an insight into gastric ulcers

ZHENG-RONG ZHOU^{1*}, PAN HUANG^{1*}, GUANG-HAO SONG¹, ZHUANG ZHANG¹,
KE AN¹, HAN-WEN LU¹, XIAO-LI JU¹ and WEI DING²

¹School of Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013; ²Department of Animal Husbandry and Veterinary Medicine, Jiangsu Polytechnic College of Agriculture and Forestry, Jurong, Jiangsu 212499, P.R. China

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Abstract. In the present study, comparative proteomic analysis was performed in rats subjected to water immersion-restraint stress (WRS). A total of 26 proteins were differentially expressed and identified using matrix-assisted laser desorption/ionization time of flight mass spectrometry. Among the 26 differentially expressed protein spots identified, 13 proteins were significantly upregulated under WRS, including pyruvate kinase and calreticulin, which may be closely associated with energy metabolism. In addition, 12 proteins were downregulated under WRS, including hemoglobin subunit β -2 and keratin type II cytoskeletal 8, which may be important in protein metabolism and cell death. Gene Ontology analysis revealed the cellular distribution, molecular function and biological processes of the identified proteins. The mRNA levels of certain differentially expressed proteins were analyzed using fluorescence quantitative polymerase chain reaction analysis. The results of the present study aimed to offer insights into proteins, which are differentially expressed in gastric ulcers in stress, and provide theoretical evidence of a radical cure for gastric ulcers in humans.

Introduction

Gastric ulcers are among the most common diseases affecting humans. They are caused by a variety of elements, including cold stimulation, mental stress, ethanol intake and seasonal variation (1). Relapse of gastric ulcers is common and its

treatment is complex. To determine the mechanism underlying gastric ulcer development, rats exposed to water immersion-restraint stress (WRS) have been used as animal models for investigating gastroduodenal mucosal lesions (2). WRS can cause several diseases, including acute gastroenteritis, peptic ulcers and colitis (3). In our previous studies, a gastric ulcer model was successfully established using rats exposed to WRS, providing a solid foundation for the present study (4,5).

It is reported that gastric ulcers can be induced by several physiological mechanisms, which have been reported as key indicators, including molecular signal transduction, regulation of cell integrity, and induction of the anti-stress defense system (6,7). Among various factors, acute stress is indicated in the formation and pathogenesis of gastroduodenal injury (8). Antioxidants are reported to be effective in protecting against gastric mucosal injury and inhibiting the progression of gastric ulcers (9,10). Previous studies have also suggested a molecular signaling pathway may be associated with the gastrointestinal tract, which is crucial in maintaining gastrointestinal homeostasis and mucosal integrity (11,12). This signal transduction causes a series of intracellular phosphorylation hurdles, disrupting the balance of cell proliferation and reducing the migration of epithelial cells. This signal transduction also decreases blood flow and angiogenesis in the gastric mucosa (9,12). In addition, energy metabolism-associated proteins have been shown to be involved in maintaining intracellular levels of ATP in digestive diseases, regulating the physiological function of mast cells via calreticulin (CALR) (13). Exposure to CALR can determine cancer cell death (14). To the best of our knowledge, although several studies have focused on analyzing the mRNA expression of genes to explain the molecular mechanisms underlying gastric ulcers, few have investigated the effects of proteomic changes in acute gastric ulcers.

In order to identify proteins crucial for stress responsiveness, proteomic analysis is usually performed, which can analyze short-term fluctuations and protein expression patterns in complex physiological processes (15,16). Proteomic techniques have been successfully applied for the functional analysis of several diseases of immune responses, inflammation and cancer proteomics (17,18). However, few studies have utilized mass spectrometry (MS)-based proteomic techniques to investigate gastric ulcers in WRS rats.

Correspondence to: Professor Wei Ding, Department of Animal Husbandry and Veterinary Medicine, Jiangsu Polytechnic College of Agriculture and Forestry, 17 Wenchang Road, Jurong, Jiangsu 212499, P.R. China
E-mail: qishi6598@126.com

*Contributed equally

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The present study performed a comparative proteomic analysis of WRS rats with stress-induced gastric ulcers in order to identify proteins exhibiting distinct changes due to protein synthesis, degradation changes, or post-translational modifications. The aim of the present study was to provide a novel understanding of the role of these proteins in stress gastric ulcers, and provide a theoretical basis for the radical treatment of gastric ulcers.

Materials and methods

WRS model establishment. A total of 20 male Sprague-Dawley rats at 9–11 weeks old (200–220 g) were used in the present study, which were obtained from an experimental animal-breeding farm, Qing Long Shan (Nanjing, China). The commercial diet for experimental use was also purchased from here. Regular rat feed and tap water were provided, and the rats were allowed to feed freely. The rats were housed individually at room temperature (25°C), on 12-h:12-h light/dark cycles, and at 65–70% humidity. The WRS models were established as described in our previous study (5). The rats in the WRS group were sacrificed after 3 h. Following WRS, the rats in the control group were fed normally, starting 1 h later, and sacrificed. Rat stomach tissues were collected from the two groups (10 rats from each group) and stored at -80°C until analysis. All procedures were designed according to the generally accepted ethical standards of animal experimentation and the guidelines established by the animal protection and use committee of Jiangsu University (Jiangsu, China). All experiments were approved by the Laboratory Animal Management Committee of Jiangsu University and were in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to reduce the suffering of animals.

Protein extraction. All chemical reagents used were of analytical grade. MilliQ water was used in all buffers and solutions. The stomach tissues were immersed in cold buffer and homogenized. The homogenates were then mixed for 30 min and centrifuged for 30 min at 15,000 × g at 4°C. The protein extracts were collected and maintained at -80°C for further analysis. An RC DCTM kit was used to determine protein concentrations, according to the manufacturer's protocol (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Two-dimensional gel electrophoresis (2-DE) and image analysis. The stomach total protein (850 µg) was loaded into a commercial prefabricated IPG strip, which was rehydrated for 13 h at 50 V and 20°C (non-linear, 17 cm; pH 3–10; Bio-Rad Laboratories, Inc.). Isoelectric focusing was performed with a voltage gradient of 250, 500 and 2,000 V for 1 h each, and 8,000 V for 3 h, and then run at 8,000 V to a total of ~60 kVh. Prior to performing SDS-PAGE, the focused strips were equilibrated in 1% (w/v) DTT in equilibration buffer for 15 min, and in alkylating equilibration buffer for another 15 min, which contained 1% (w/v) iodoacetamide. Using a PowerPac HV, the strips were sealed on a 12.5% SDS-PAGE gel for electrophoresis (Bio-Rad Laboratories, Inc.). The gel, visualized using 0.08% CBB G-250, was digitized through a high-precision scanner (Versa Doc 3,000; Bio-Rad Laboratories, Inc.). Using

PDQuest 2-D analysis software, protein spot assessment, measuring and matching were performed (version 8.0; Bio-Rad Laboratories, Inc.). The protein spots were automatically matched and carefully edited. The differentially expressed proteins were considered to be spots showing significant differences in intensity and present on at least two of the three gels in a single treatment ($P \leq 0.05$).

Protein digestion. The selected gel spots were manually excised. The gel was then cleared with 100 µl of 50% acetonitrile and 25 mM NH_4HCO_3 for 30 min, dehydrated with 100% CAN for 10 min, and completely dried in a SpeedVac concentrator. For protein digestion, the spots were soaked in 10 µl of trypsin solution for 1 h at 4°C and incubated overnight at 37°C. Excess trypsin was removed to prevent trypsin autolysis. The gel peptides were dissolved in 30 µl of 50% (v/v) ACN. The supernatants were centrifuged at 2,500 × g for 3 min at room temperature, collected and stored at -20°C for analysis.

Protein identification. The samples were added to 5 µl of 0.1% TFA for re-suspension. The TFA was mixed at a 1:1 ratio with α -cyano-4-hydroxy-trans-cinnamic acid in 50% ACN. The mixed solution (1 µl) was added to a sample target plate. Using an ABI 4800 MALDI TOF/TOF Plus mass spectrometer, peptide MS and MS/MS were performed (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Using GPS Explorer™ software (version 3.6; Applied Biosystems; Thermo Fisher Scientific, Inc.), a search (MS+MS/MS) was performed. The time of flight spectra recorded the positive ion reflector mode in a mass range of 800–4,000 Da. According to the MS and MS/MS spectra, proteins were successfully determined, with a confidence interval of $\geq 95\%$, using the Mascot V2.3 search engine (Matrix Science, London, UK). The other parameters were set as follows: NCBI-Animals database; 100 ppm for precursor ion tolerance; fixed modifications of carbamidomethyl; 0.3 Da for fragment ion tolerance; and partial modifications of acetyl and oxidation.

Bioinformatics analysis. A Basic Local Alignment Search Tool homology protein search was performed to match the Rat protein database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The 10 highest hits per query sequence were recorded. Among the top 10 hits, the highest matching query identity was selected as the Rat homolog. Gene ontology (GO) enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (<https://david.ncifcrf.gov/>) (3). DAVID calculates the exact P-values of the modified Fisher to determine whether a GO is overexpressed or underexpressed in a protein database. The three GO categories, cellular component, molecular function and biological process, were also identified in the enrichment analysis. It was found that ~90% of the Rat homolog databases was matched. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was also performed (<http://www.kegg.jp/kegg/pathway.html>).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration and purity were measured

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction analysis of the differentially expressed genes in water immersion-restraint stress rats.

Gene name	NCBI accession no.	Primer sequence (5'-3')	Product size (bp)
Fructose-bisphosphate aldolase A	GI6978487	F: ACCGTCACAGCACTTCGT R: TCGCTTGATGTACTCCTCC	237
Pyruvate kinase	GI402745143	F: ACCTGGTGACAGAAGTGGA R: GGATGAAAGACGCAAACA	162
β -enolase	GI126723392	F: GGGGAGACCGAAGACACT R: GCCTTTGGATTACGGAAC	179
Heat shock protein β -1	GI752993026	F: AACTCAGCAGCGGTGTCTC R: CACGCCTTCCTTGGTCTTA	113
14-3-3 protein ζ	GI1051269	F: CGTTGTAGGAGCCCGTAG R: AACCTCAGCCAAGTAGCG	247
Calreticulin	GI166064019	F: TCTTCAGCCCTCACCTCC R: TTCCTCCATACCTGTTCCTA	121
Hypoxanthine phosphoribosyltransferase 1	X62085	F: AGTGATGATGAACCAGGTTA R: ATTATAGTCAAGGGCATATC	556

by spectrophotometry (NanoVue; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and its integrity was determined using 1.2% agarose gels with 0.1% ethidium bromide. According to the manufacturer's protocol, 1 μ g of the total RNA was reverse transcribed using a reverse transcription reagent kit with a gDNA Eraser (TransGen Biotech, Beijing, China). The primers were designed according to the corresponding gene sequences (Table I). The RT-qPCR procedure was performed on an ABI 7300 instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a total volume of 20 μ l, consisting of SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd., Dalian, China), ROX reference dye, 200 nM of each forward and reverse primer and 100 ng cDNA. The amplification conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 45 sec. The reaction was performed three times for all genes, including the hypoxanthine phosphoribosyltransferase 1 housekeeping gene. Using the $2^{-\Delta\Delta C_q}$ method (19), the relative expression levels of the genes of interest were calculated.

Statistical analysis. Data are expressed as the mean \pm standard error of the mean. The statistical significance was determined using Student's t-test with two-tailed P-values. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Protein identification. The present study performed comparative visual and software analyses of representative 2-DE proteome profiles of the gastric tissue of rats subjected to WRS. The results are shown in Fig. 1. Quantitative image analysis using PDQuest software showed that 26 protein spots were differentially expressed, compared with the control group, with a differential expression value 2-fold higher. The

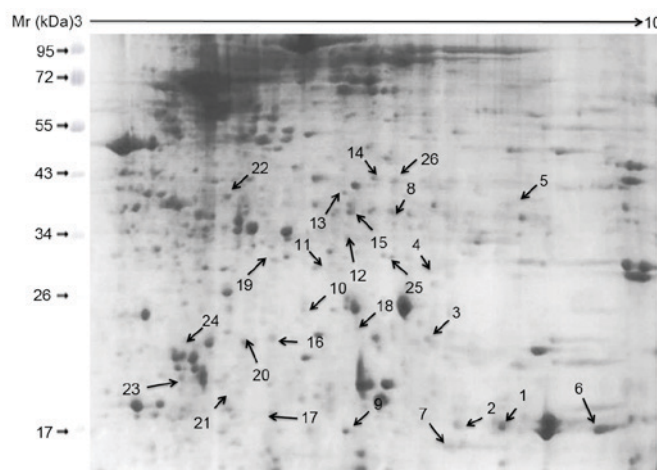


Figure 1. Two-dimensional gel electrophoresis pattern of proteins in WRS rats. A total of 26 identified proteins (arrows) showed significant spot intensity changes under WRS. The proteins are listed in Table II. The experiment was repeated three times. WRS, water immersion-restraint stress.

spots containing the differentially expressed proteins were excised, digested in trypsin and analyzed using MS/MS. All 26 proteins were identified in the NCBI database (Table II). Two major protein expression patterns were observed in the present study; of the 26 differentially expressed proteins, ~50% were significantly upregulated under WRS. These proteins, including pyruvate kinase (PK) and CALR, may be closely associated with stress. The other half of the proteins were downregulated, and may be key in protein metabolism and cell death, including hemoglobin subunit β -2 and keratin type II cytoskeletal 8 (K2C8).

Proteomic analyses. Compared with the control group, six types of protein were detected (Fig. 2). The hemoglobin subunit-associated proteins (spots 1, 2 and 7), which are involved in metabolism, were downregulated following WRS.

Table II. MALDI-TOF/TOF MS identification of differentially expressed proteins in the gastric mucosa of rats under water immersion-restraint stress.

Spot ^a	Protein name	Species	NCBI Accession no.	Cov. (%)	H.pI/Mr (kDa)	MS	Fold change
Protein metabolism							
1	Hemoglobin subunit β -2	<i>R. norvegicus</i>	GII16444868	39	8.91/16.1	257	0.27±0.05
2	Hemoglobin subunit α -1	<i>R. norvegicus</i>	GII6981010	50	7.82/15.5	430	0.34±0.07
7	Hemoglobin subunit β -1	<i>R. norvegicus</i>	GII17985949	56	7.88/16.1	395	0.23±0.06
14	Serotransferrin	<i>R. norvegicus</i>	GII122066515	17	7.14/78.5	592	0.27±0.09
16	2-oxoglutarate dehydrogenase	<i>R. norvegicus</i>	GII62945278	10	6.30/11.7	249	3.60±0.31
Energy metabolism							
3	ATP synthase subunit γ	<i>R. norvegicus</i>	GII39930503	21	8.87/30.2	218	2.35±0.23
4	Fructose-bisphosphate aldolase A	<i>R. norvegicus</i>	GII408772019	38	8.31/39.8	662	3.12±0.31
5	ATP synthase subunit α	<i>R. norvegicus</i>	GII40538742	24	9.22/59.8	884	2.17±0.03
8	Pyruvate kinase	<i>R. norvegicus</i>	GII16757994	17	6.63/58.3	438	2.85±0.16
11	Creatine kinase M-type	<i>R. norvegicus</i>	GII6978661	24	6.58/43.2	677	0.22±0.02
12	β -enolase	<i>R. norvegicus</i>	GII126723393	31	7.08/47.3	668	3.01±0.32
15	Aldehyde dehydrogenase, mitochondrial	<i>R. norvegicus</i>	GII118505	21	6.63/57.0	618	3.12±0.39
Signal transduction							
6	Histone H2B type 1	<i>R. norvegicus</i>	GII12025526	36	10.36/14.0	256	1.96±0.31
9	Phospholipase A2	<i>R. norvegicus</i>	GII56931	41	7.89/17.2	353	0.38±0.03
17	Heat shock protein β -1	<i>R. norvegicus</i>	GII752993027	37	6.12/22.9	417	4.01±0.49
19	Protein disulfide-isomerase A3	<i>R. norvegicus</i>	GII8393322	18	5.88/57.0	721	0.36±0.01
20	Actin, aortic smooth muscle	<i>R. norvegicus</i>	GII110625958	23	5.23/42.4	370	0.40±0.00
22	78 kDa glucose-regulated protein	<i>R. norvegicus</i>	GII554440	14	5.07/72.3	859	3.61±0.77
23	14-3-3 protein ζ	<i>R. norvegicus</i>	GII62990183	28	4.73/27.9	245	4.36±0.57
24	Myosin light chain 3	<i>R. norvegicus</i>	GII6981240	43	5.03/22.2	451	0.20±0.05
25	Calreticulin	<i>R. norvegicus</i>	GII488841	23	4.33/48.2	460	5.01±0.38
Cell death							
10	Aflatoxin B1 aldehyde reductase member 3	<i>R. norvegicus</i>	GII7106240	25	6.79/37.1	343	2.17±0.17
13	Catalase	<i>R. norvegicus</i>	GII203335	18	7.07/60.1	458	2.71±0.29
18	Keratin, type II cytoskeletal 8	<i>R. norvegicus</i>	GII40786432	23	5.83/54.0	636	0.41±0.04
21	40S ribosomal protein SA	<i>R. norvegicus</i>	GII8393693	10	4.80/32.9	162	0.48±0.02
26	Microtubule-associated tumor suppressor 1	<i>R. norvegicus</i>	GII125630382	2	6.89/51.1	36	0.41±0.04

MALDI-TOF/TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; *R. norvegicus*, *Rattus norvegicus*; ^aSpot, spot number as presented in Fig. 1; Cov, sequence coverage, i.e., number of query matched peptides; H.pI/Mr, isoelectric point of predicted protein/molecular mass of predicted protein; MS, Mowse protein score, as identified with MALDI-TOF/TOF MS. Proteins with a statistically significant score >48 (P<0.05) were identified; fold change, the ratio of the absolute intensities of protein spots in the treatment and control groups.

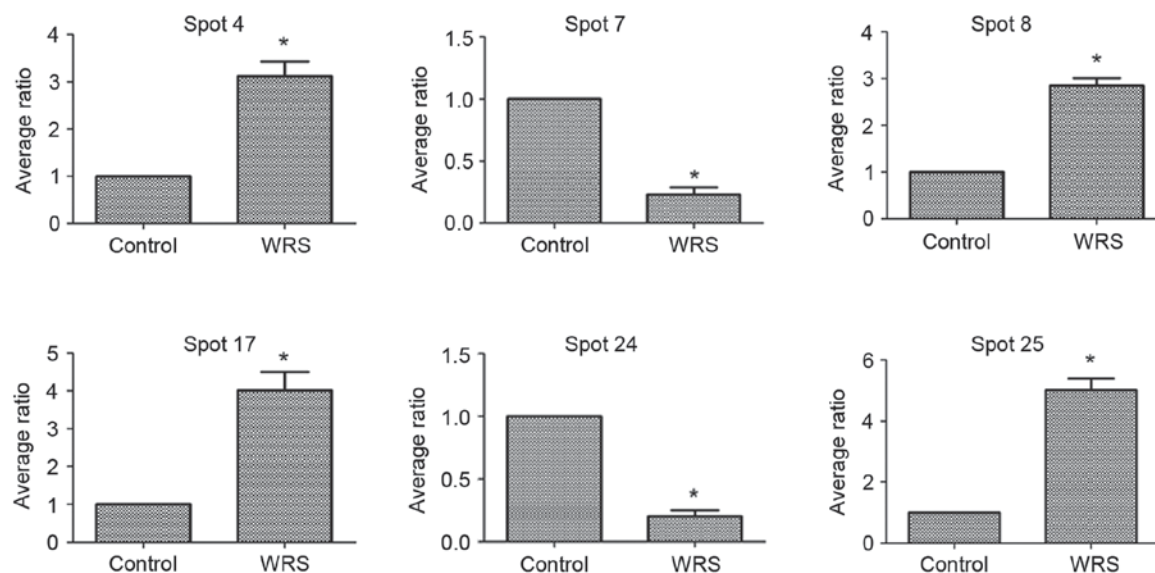


Figure 2. Expression patterns of six differentially expressed protein spots under WRS, compared with the control group. Data are expressed as the mean \pm standard error of the mean. Statistical significance was determined using a two-tailed Student's t-test. * $P < 0.05$. Spot 4, fructose-bisphosphate aldolase A; spot 7, hemoglobin subunit β -1; spot 8, pyruvate kinase; spot 17, heat shock protein β -1; spot 24, myosin light chain 3; spot 25, calreticulin; WRS, water immersion-restraint stress.

Proteins associated with energy metabolism were upregulated following WRS, including protein fructose-bisphosphate aldolase A (ALDOA; spot 4), PK (spot 8) and β -enolase (ENOB; spot 12). However, creatine kinase M-type (CKM; spot 11), which is a protein involved in energy metabolism, was downregulated after WRS. In terms of signal transduction, heat shock protein β -1 (HSPB1; spot 17), 14-3-3 protein (1433Z; spot 23) and CALR (spot 25) showed upregulated expression under WRS. The expression levels of protein phospholipase A2 (PA21B; spot 9) and myosin light chain 3 (spot 24) were significantly decreased. The K2C8 (spot 18) and microtubule-associated tumor suppressor 1 (spot 26) proteins are involved in cell death, which were downregulated under WRS in the rats, whereas the other 2 proteins in the cell death group, aflatoxin B1 aldehyde reductase member 3 (ARK73; spot 10) and catalase (CATA; spot 13), were upregulated under stress.

Bioinformatics analysis. GO analysis with the 26 proteins was performed, using the DAVID toolkit. In terms of cellular distribution, the analysis revealed that 11 of the identified proteins were cytosol proteins, 8 proteins were present in the mitochondria, and 5 were present in the endoplasmic reticulum. The analysis also revealed the molecular function of the identified proteins was protein binding (6 proteins). The biological processes were divided into two categories: Response to hypoxia (4 proteins) and glycolysis (4 proteins), as shown in Fig. 3. For functional analysis of the differentially expressed proteins, KEGG pathway enrichment analysis is the most suitable method. In the WRS rats, nine differentially expressed proteins were enriched in pathways, which were involved in multiple biological processes, as shown in Table III. Among the 9 proteins, 4 were associated with the glycolysis pathway: ALDOA (spot 4), PK (spot 8), ENOB (spot 12) and aldehyde dehydrogenase (spot 15); 3 proteins were involved in antigen processing and presentation: Protein disulfide-isomerase A3 (spot 19), 78 kDa glucose-regulated protein (spot 22) and CALBP (spot 25). The other 3 proteins

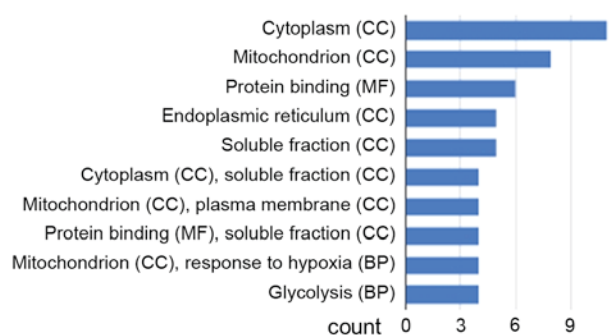


Figure 3. Gene Ontology enrichment analysis of the identified proteins. BP, biological process; MF, molecular function; CC, cellular component.

were involved in tryptophan metabolism: CATA (spot 13), and 2-oxoglutarate dehydrogenase (spot 16).

Protein transcription. To determine the association between the changes in the identified protein levels and the transcription levels of the corresponding genes under WRS, the present study investigated the six genes encoding the identified proteins using RT-qPCR analysis. The results indicated that the gene expression levels of the identified proteins (ALDOA, spot 4; PK, spot 8; ENOB, spot 12; HSPB1, spot 17; 1433Z, spot 23; and CALR, spot 25) were consistent with the mRNA expression levels, as shown in Fig. 4, suggesting their upregulation in rats under WRS.

Discussion

Energy metabolism-associated proteins. Disturbance of mitochondrial energy metabolism is a common result of multiple complex factors, particularly when the body suffers from stress (20,21). In the present study, CKM, which is involved in the electron transport chain, was found to be downregulated under WRS. The primary physiological function of creatine

Table III. Enriched Kyoto Encyclopedia of Genes and Genomes pathway-based sets of differentially expressed proteins in rats under water immersion-restraint stress.

Pathway	n	Protein
Glycolysis pathway	4	ALDOA (spot 4); PK (spot 8); ENOB (spot 12); ALDH2 (spot 15)
Antigen processing and presentation	3	PDIA3 (spot 19); GRP78 (spot 22); CALBP (spot 25)
Tryptophan metabolism pathway	2	CATA (spot 13); ODO1 (spot 16)

ALDOA, protein fructose-bisphosphate aldolase; PK, pyruvate kinase; ENOB, β -enolase; ALDH2, aldehyde dehydrogenase; PDIA3, protein disulfide-isomerase A3; GRP78, 78 kDa glucose-regulated protein; CATA, catalase; ODO1, 2-oxoglutarate dehydrogenase.

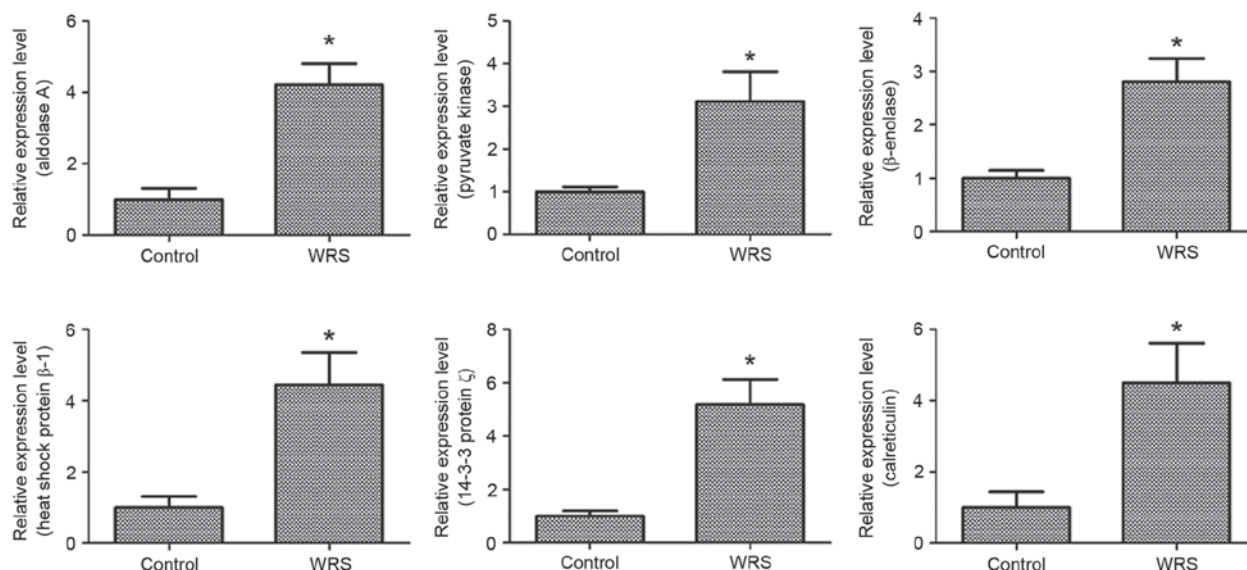


Figure 4. Verification of the six differentially expressed proteins at the mRNA level. Data are expressed as the mean \pm standard error of the mean. Statistical significance was determined using a two-tailed Student's t-test. * $P < 0.05$. WRS, water immersion-restraint stress.

kinase is to provide energy for muscle contraction through the creatine phosphate shuttle mechanism, which is important in mitochondrial respiratory control (22). The results of the present study suggested that WRS decreased mitochondrial respiration. Previous transcriptomic investigations have reported the downregulation of CKM in gastric cancer, whereas others showed the upregulation of CKM (23,24). These results suggest a link between CKM and gastric disease, which requires further investigation.

The present study found that other proteins associated with energy metabolism were upregulated under WRS, including ALDOA, PK and ENOB. It has been shown that the upregulation of ALDOA can accelerate the process of glycolysis, promote cell proliferation and increase intracellular ATP content (25). ALDOA not only functions in glycolysis, but is also involved in tumorigenesis (26). It has been found that high expression levels of ALDOA are correlated with the occurrence and progression of several types of tumor (27). Another key enzyme in glycolysis is PK, which was also elevated under WRS in the rats. This regulation suggests that increased glycolysis results in increased glucose and ATP, indicating that rats may produce more energy when subjected to WRS.

ENOB is a glycolytic enzyme, which can reversibly catalyze the conversion of 2-phosphoglycerate to phosphoenolpyruvate.

This enzyme is found in muscle cells, where it may be involved in muscle development and regeneration (28). α -enolase (ENOA), another enolase, can switch to ENOB in rodents during muscle tissue development. Whether ENOA, which is involved in myocardial cells exposed to ischemic hypoxia, can maintain intracellular ATP levels has been investigated previously, which demonstrated that ENOA is closely associated with cell survival (29). Previous proteomic studies have observed changes in enolase in response to stress (30), and ENOB has been associated with energy metabolism in cancer cells (31). These findings indicate that ENOB has several functions, including as an energy regulator or a stress protein. In the present study, ENOB was evaluated under WRS, which may result in more energy available to combat the epidermal damage and mucosal ulceration caused by stress, and to regulate apoptosis in the rat stomach. However, this requires further validation.

Cell signaling-associated proteins. Molecular chaperones, including HSPs, have several cellular functions, including protein binding (32). In previous studies, HSPs have been considered to be an additional factor in the defense of gastric cells (33,34). HSPs are generally considered to assist with cell recovery, by refolding functional damaged proteins or

increasing the transport of precursor proteins in the mitochondria and endoplasmic reticulum. HSPs may be effective for gastric mucosal defense and ulcer healing, and may also protect certain important enzymes involved in cytoprotection (35). In the present study, HSPB1 was upregulated under WRS. The evaluation of HSPB1 indicated that it is involved in the defense mechanism against WRS.

Another signaling protein, 1433Z, was upregulated in the WRS rats. A notable feature of the 14-3-3 protein family is the ability to bind a variety of signaling proteins. It has been reported that the 14-3-3 protein family has certain functions in cell cycle control, migration and apoptosis (36). In addition, it has been shown that the proteins bound to 14-3-3 proteins are either proto-oncogene or oncogene products (37,38). A study by Nagappan *et al* (39) showed that *Helicobacter pylori*, which is the primary cause of chronic gastritis, induces the expression of 14-3-3 protein in gastric epithelial cells. Pillinger *et al* (11) reported that *H. pylori* stimulated the activation of gastric epithelial cell extracellular signal-regulated protein kinase (ERK). In the present study, the upregulation of 14-3-3 protein may be involved in ERK and affect cell signaling pathways; further investigations are required to investigate the specific mechanism.

CALR is a Ca^{2+} -binding protein. CALR has several significant biological functions, including maintaining Ca^{2+} homeostasis, regulating Ca^{2+} signaling, and modulating gene expression and cellular stress responses (13). It has been reported that CALR is important in the peritoneal dissemination and tumor growth of gastric cancer cells, which can induce endoplasmic reticulum stress (40). The expression of CALR differs in various mammalian cell types, which suggests specific roles in tissues. In the present study, CALR was significantly upregulated under WRS. A reasonable explanation for this result may be that the increase in calreticulin disrupts Ca^{2+} regulation, causing mitochondrial damage and cell apoptosis.

The level of PA21B was significantly decreased in the rats under WRS. PA21B can catalyze fatty acid cleavage to generate free fatty acids, including arachidonic acid, which are key secondary messengers in signal transduction (41). PA21B is also involved in regulating cell membranes and host defenses (42). The overexpression of PA21B causes spontaneous dermatitis in pla2-transgenic mice (43). It has also been demonstrated that PA21B has a key function in mast cell maturation *in vivo*, suggesting that Pla2 regulates the gene expression profile in certain cells (44). In conditions of inflammation, glucocorticoids upregulate the production of the protein lipocortin, which inhibits PLA2 and reduces the inflammatory response. In the present study, PLA2 was downregulated in order to relieve stress and combat the gastric ulcers caused by WRS in rats.

Cell death-associated proteins. Keratin is an important component of the cytoskeleton. Its primary function is to maintain the integrity and continuity of epithelial cells (45). Previous studies have focused on the diagnostic value of carcinoma, whereas investigations of inflammation remain at the initial stages. K2C8 is one of the predominantly expressed keratins in the intestinal epithelium, and it has been reported that keratin-knockout mice exhibit colitis (46) and has been

shown that abnormal keratin mutations are associated with inflammatory bowel disease. In the present study, K2C8 was downregulated when the rats were subjected to WRS. The decrease in K2C8 may affect the intermediate filaments of maintenance and reconstruction. In cell differentiation, keratin cytoskeleton reconstruction may lead to intestinal mucosal epithelial fragility and eventually cause intestinal mucosal barrier damage, suggesting that K2C8 may mediate damage to the intestinal mucosal surface (47).

Previous studies have demonstrated that oxidative stress is involved in the mechanism of stress-induced gastric ulcers. During the pathogenesis of gastric mucosal lesions, oxygen-derived free radicals may be involved, which are cytotoxic and can injure cell membranes to cause the release of intracellular components (48). CATA is an enzyme, which is involved in antioxidant defense and cell development, and in tumor processes (49). The present study showed that the expression of CATA was upregulated under WRS, which corresponds to a report by Ohta and Nishida (8). It was shown that CATA protected against gastric mucosal lesions of rats under WRS and indicated that the protective role of CATA may be due to its ability to scavenge active oxygen species. In the present study, ARK73 was also upregulated following 3 h of WRS. ARK73 is an effective liver toxin, and a carcinogen against antioxidants and experimental drugs to prevent acute toxicity (50). The results of the present study suggested that ARK73 was involved in the protection against acute stress and cytotoxic injury.

The GO enrichment analysis performed in the present study identified three functional terms, namely protein binding, glycolysis, and response to hypoxia. KEGG pathway enrichment analysis indicated that there were three pathways involved in various biological processes, namely glycolysis, antigen processing and presentation, and tryptophan metabolism. These results assist in improving current understanding of gastric ulcers in rats, which is further supported by the consistent results of the protein and mRNA expression levels, indicating significant target genes for RNA interference.

In conclusion, the present study provided insights into differential protein expression in the stomach of rats with gastric ulcers. The proteins identified were associated with multiple functions, including protein binding, energy metabolism and cell death. Certain proteins may be important in causing acute gastric ulcers. The identified proteins were analyzed at the genetic level; the results suggested that ENOB acted against WRS, and that the expression of ENOB may increase the energy available to combat epidermal damage and mucosal ulceration caused by stress, and regulate apoptosis in the rat stomach. However, these conclusions are based on a small sample size. In order to better understand the precise function of these proteins, further investigations using molecular biological and proteomic investigations are required to assess the relevance of the specific functions of these proteins and their roles in regulating gastric ulcers.

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