Proteomic Identification of Serum Proteins Associated with Stress-Induced Gastric Ulcers in Fasted Rats

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Several physical and psychological stresses frequently become triggers for gastrointestinal disorders such as ulcer. In this study, we tried to identify serum proteins as potential biomarkers for the evaluation of stress-induced gastric ulcer induced by water immersion and restraint (WIR) stress as an animal model, we found quantitative changes in several serum proteins, including creatine kinase muscle M chain (CK-M) and apolipoprotein A-IV (ApoA4) in the stressed rats. On western blotting and enzyme-linked immunosorbent assay (ELISA), we confirmed that serum CK-M was remarkably increased by WIR stress. However, ApoA4 appeared to be decreased by fasting, but not WIR stress, which is usually applied prior to WIR stress. The findings suggest that these two serum proteins might be useful as biomarkers, CK-M for stress-induced gastric ulcer and ApoA4 for starvation.

Key words: water immersion and restraint stress; creatine kinase; apolipoprotein A-IV; proteomics; rat serum

In today’s so-called stress society, we are regularly exposed to various physical and psychological stresses. It is well-known that these stresses frequently become triggers or modifiers in the clinical course of diverse gastrointestinal disorders, including peptic ulcer, irritable bowel syndrome, and inflammatory bowel disease. In an effort to facilitate the study of such gastric damage, several animal models, including ones induced by water immersion and restraint (WIR) stress in rodents, were developed many years ago, especially to investigate drugs for peptic ulcer.1,2 In recent years, WIR stress-induced gastric lesion animal models have often been used in attempts to prevent gastric damage by taking food or tea ingredients such as polyphenolic compounds.3–7 For the evaluation of suppressive effects due to such components, comparative analysis of gastric hemorrhagic erosion on the mucosal surface in the dissected stomach with and without WIR stress has been accomplished in animal models. However, in human cases, it is hard to evaluate preventive effectiveness. Therefore, the identification of stress-induced gastric ulcer-associated biomarkers in possible specimens such as blood, urine, or saliva from human donors is to be desired.

In WIR stress experiments, the animals are usually fasted for 24 h prior to stress application, because the presence of food and its digests within the stomach frequently blocks the generation of gastric ulcer by WIR stress.3–7 Starvation is also known to induce physiological alterations through several events such as quantitative changes in corticosterone and corticosteroid-binding globulin,8 the modulation of the lipid profile in the blood,8–9 and the differential gene expression of several genes in the gastric mucosa.8–10 Hence, the starved condition also needs to be considered a physical and psychological stress.

In this study, we focused on the identification of stress biomarker candidates for the evaluation of stress-induced gastric ulcer that can be analyzed in human specimens (blood in this study). First, we attempted to identify serum proteins associated with WIR stress-induced gastric ulcer in fasted rats by proteomic analysis. Further analysis revealed that two serum proteins identified might be useful as biomarkers, one for stress-induced gastric ulcer and another for starvation.

Materials and Methods

Animals. Male Sprague-Dawley rats (8 weeks) (Charles River Japan, Kanagawa, Japan) were housed in an air-conditioned room (23 ± 1°C) under a 12 h dark/12 h light cycle (light on 7:00–19:00) with free access to standard laboratory chow (CE-2, Clea Japan, Tokyo) and tap water. The experiments were conducted following to the guidelines for the care and use of laboratory animals of the University of Shizuoka. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Induction of gastric mucosal lesions, preparation of serum samples, and determination of ulcer index. WIR stress-induced gastric lesions were generated in rats by a modification of the procedure of Takagi et al.11,12 Rats were acclimated for 7 d and then divided into three groups (3–5 rats in each group); a control group (rats without fasting or WIR-
**Proteomics of Serum Proteins in Stressed and Fasted Rats**

stress application used as normal rats), a fasting group (rats with fasting for 24 h plus 0.5, 1, 5, 10, or 16 h) with only water supplied, and a WIR stress-treated group (rats with fasting for 24 h followed by WIR stress application for 0.5, 1, 5, 10, or 16 h). Before and after the tests for each group, blood was collected from the tail vein or the heart under the anesthesia with diethyl ether. The blood samples were coagulated at 4 °C overnight. Serum fractions were obtained from all blood samples after centrifugation at 3,000 g for 10 min and then stored at −80 °C until use. The stomachs were removed by opening along the line of greatest curvature, and then washed with physiological saline. In order to evaluate the degree of gastric mucosal lesions, the length (mm) and width (mm) of hemorrhagic erosions in the gastric mucosa were measured using a digital camera and Adobe Photoshope version 6.0 software, and the ulcer index was calculated as the ratio of the gastric ulcer area to the total stomach surface area (mm²) according to the modification of the method of Takekawa et al.13

Two-dimensional difference in-gel electrophoresis (2-D DIGE). Serum samples from normal rats and rats that underwent WIR stress application for 10 h were used in 2-D DIGE following the method of Ibi et al.,15 with some modifications. In brief, albumin was selectively removed from the sera with a Montage Albumin Deplete kit (Millipore, Billerica, MA). The samples were then desalted and concentrated with a 2-D clean-up kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). The protein content was determined using a protein assay kit (Bradford method) purchased from Bio-Rad Laboratories (Hercules, CA) with the manufacturer’s protocol. All the protein samples examined were adjusted to a final protein concentration of 2 mg/ml with 2-D buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris, pH 8.5). The serum proteins in the samples were fluorescently labeled with three cyanine dyes: Cy2 labeling for a normal rat alone (control), Cy3 labeling for a WIR stressed rat alone, and Cy5 labeling for a WIR stressed rat alone. The mixtures of the protein samples and the respective cyanine dyes were individually placed for labeling on ice for 30 min, and then 10 mM of lysine was added to stop the reaction. Equal volumes of three Cye-labeled samples were mixed and used for 2-D DIGE. First-dimensional isoelectric focusing was carried out with the IPGphor IEF system (GE Healthcare) for 7.5–8.45 h (a total of 35–45 kVh, below 50 mA/cm). The strips were then equilibrated with 10 ml of equilibration buffer containing 25 mg/ml of iodoacetamide for 15 min. The equilibrated strips were washed with PBS–PAGE running buffer (25 mM Tris–HCl, pH 8.8, 6.7 M urea, 30% glycerol, and 2% SDS) containing 10 mg/ml of dithiothreitol (DTT) for 15 min, followed by 10 ml of equilibration buffer containing 25 mg/ml of iodoacetamide for 15 min. The equilibrated strips were washed with PBS–PAGE running buffer containing 10% isocratic SDS–PAGE gel (24 cm x 20 cm) and then stored overnight in 1–2 W per gel. The 2-D images on the gel were visualized with a Typhoon 9410 scanner (GE Healthcare), the Cy2 image with a wavelength at 510–522 nm or 470–480 nm for Cy5, 530–550 nm for Cy3, and 580–592 nm for Cy2. The 2-D images were scanned with a Typhoon 9410 scanner for Cy2 and Cy5, and Cy3 images were scanned for Cy3 labeling. The Cy2 labeling was visualized with SureBlue TMB 1-component microwell peroxidase substrate (Pierce, Rockford, IL) and Cy5 labeling with a luminol substrate (Cortex Biochem, San Leandro, CA) using a fluorimetric method. The plate was washed with 0.05% Tween 20 in PBS (PBST) and blocked with blocking solution that was 5-fold diluted N101 (NOF, Tokyo) with PBS at 37 °C for 2 h. After it was washed with PBST, 50 μl of a 5- or 10-fold dilution of the rat serum samples (the control group of normal rats, fasting group, and WIR stress-treated group) were added to each well. The plate was incubated at 37 °C for 2 h and then washed with PBST.

In-gel digestion and MALDI-TOF/TOF-MS analysis. To pick protein spots, 500 μg of protein samples without fluorescent prelabeling was subjected to 2-D DIGE, and the gel after electrophoresis was stained with Deep Purple (GE Healthcare). The gel image was visualized using a Typhoon 9410 scanner with a 532 nm laser and an emission filter of 610 nm BP30. After scanned, the protein spots were marked on the Deep purple-stained gel image by matching them with those on the CyDye-labeled electrophoresis gel images, as described above. The protein spots of interest, selected by comparison of the treatments with and without WIR stress application, were chosen with Ettan Spotpicker (GE Healthcare). Gel pieces were washed in 50 mM ammonium bicarbonate containing 50% ACN for 20 min 3 times. After they were dehydrated with ACN, the gel pieces were reswollen with 20 μl of 100 mM ammonium bicarbonate containing 25 μg/ml of trypsin (Promega, Madison, WI). Then the reaction solution with the gel pieces was incubated overnight at 37 °C. The supernatant was collected, and the gel pieces were extracted once with 80% ACN containing 1% TFA.

The supernatant and extract were combined and concentrated to the required concentrations. The resulting peptide mixtures were desalted and concentrated by using ZipTips C18 column (Millipore). For MS analysis, the matrix solution containing 2.5 mg/ml of CHCA in 50% ACN containing 0.05% TFA was mixed with an equal volume of the tryptic digest and loaded onto a target plate for MALDI-TOF/TOF-MS. Laser irradiation ionized the CHCA-containing peptides, and the sequences of the amino acids were determined by Ultraflex MALDI-TOF/TOF-MS (Bruker Daltonics, Bremen, Germany) in reflector mode and analyzed with flexanalysis software (Bruker Daltonics). Ions specific for each sample were used to interrogate Rattus norvegicus sequences deposited in NCBI data bases using MOWSE algorithms in the MASCOT software (www.matrixscience.com).

Enzyme-linked immunosorbent assay (ELISA). Anti-creatine kinase MM (CR3014M1, Cortex Biochem, San Leandro, CA) and anti-apolipoprotein A-IV (A4-18A3, BML, Tokyo) were used individually as the solid phase antibody. Fifty μl of 5 μg/ml solid-phase antibody in PBS was added to each well and incubated overnight. The plate was washed at 4 °C for 16 h. The plate was washed with 0.05% Tween 20 in PBS (PBST) and blocked with blocking solution that was 5-fold diluted N101 (NOF, Tokyo) with PBS at 37 °C for 2 h. After it was washed with PBST, 50 μl of a 5- or 10-fold dilution of the rat serum samples (the control group of normal rats, fasting group, and WIR stress-treated group) were added to each well. The plate was incubated at 37 °C for 2 h and then washed with PBST.

To detect creatine kinase muscle type isozyme (CK-MM), a biotinylated antibody was prepared by the following procedure: Anti-creatine kinase MM (CR3014M3, Cortex Biochem, San Leandro, CA) was replaced with 50 mM sodium bicarbonate buffer (pH 8.5) with an ultrafiltration membrane, and the concentration of the protein was adjusted 10 μg/ml. Next, 0.3 μl of DMSO containing 3% NHS-Biotin (Pierce, Rockford, IL) was added to 50 μl of the antibody solution and the mixture was incubated on ice for 2 h. The buffer was washed with PBS by ultrafiltration. After biotinylation, 50 μg of 1 μg/ml biotinylated anti-creatine kinase was added to each well of the microwell and incubated at 37 °C for 2 h. On the other hand, anti-apolipoprotein A-IV (A4-11G12, BML, Tokyo) was used as the biotinylated antibody to detect ApoA4.

The plate including serum sample as described above was incubated with the individual biotinylated antibody at 37 °C for 2 h. After washing 5 times with PBST, 50 μl of Streptavidin:HRP (710005, AbD Serotec, Oxford, UK) diluted at 8,000-fold with PBST was added and then incubated at 37 °C for 2 h. After washed with PBST, 50 μl of SureBlue TMB 1-component microwell peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added and the mixture was incubated at room temperature for 15 min. The reaction was quenched with 50 μl of 1 N HCl in each well, and the absorbance measurements were done at 450 nm.

**Results**

WIR stress-induced gastric ulceration in rats

Under application of WIR stress for 0.5, 1, 5, 10, or 16 h, the rats developed gastric mucosal lesions. In particular, the stomachs stressed for more than 10 h, severe
gastric mucosal lesions, including petechial bleeding or streak-like hemorrhage, were observed in the glandular part of the stomach (shown by representatives in Fig. 1). The ulcer index of the rats with gastric mucosal lesions induced by WIR stress for 16 h was estimated to be 1.3%, while no typical gastric mucosal lesions were observed in the rats of the control group or the fasting group without WIR stress.

Proteomic analysis of serum protein biomarker candidates for stress-induced gastric ulcers

To identify serum proteins quantitatively changed between normal rats (control) and WIR-stressed rats, proteomics using 2-D DIGE and MALDI-TOF/TOF-MS was performed, as described in “Materials and Methods” above. Between 2-D DIGE gel images of the rats in the control group and these in the WIR-stressed group, 2,316 protein spots (spot volume 100,000–100,000,000) were matched (Fig. 2). By differential analysis using Decyder-DIA and Decyder-BVA software, 52 and 59 of those spots were significantly increased (average ratio >1.5) and decreased (average ratio <1.5, corresponding to a 0.67-fold change) respectively in the stressed rats. The protein spots that were significantly changed were picked out of the gels, digested with trypsin, and subjected to MALDI-TOF/TOF-MS analysis to identify their protein species. As shown in Table 1, several serum proteins were identified. In particular, two protein spots, nos. 1778 and 1783, which were identified as creatine kinase muscle M chain (CK-M), were remarkably increased in the serum of the rats under WIR stress exposure (average ratios of +12.71 and +15.98 respectively, Fig. 2 and Table 1). Three protein spots, nos. 1913, 1921, and 1931, which were identified as haptoglobin, were markedly decreased in the serum of the rats with WIR stress, and their average ratios were −26.99, −15.71, and −10.13 respectively. Three spots, nos. 1967, 1979 and 2001, were identified as apolipoprotein E (ApoE), and they were increased (average ratios of 2.89, 2.33 and 1.97 respectively) by the stress. Moreover, two spots, nos. 1754 and 1764, were identified as apolipoprotein A-IV: one of the two spots was decreased and another was increased (average ratios of −1.96 and 1.5 respectively) in the stressed rats.

Possibilities of the identified serum proteins as biomarkers of stress-induced gastric ulcer in fasted rats

To confirm the proteomics results, first we examined CK-M in the sera from the normal rats and the WIR-stressed rats by western blotting. As shown in Fig. 3, CK-M was strongly detected in the serum of the WIR-stressed rats as expected, but was not observed in the serum obtained from the normal rats. Furthermore, ELISA was conducted to confirm the proteomics results.
Two proteins, ApoA4 and CK-M, were selected for this examination, because they were markedly changed by stress in the proteomic analysis, and their antibodies are commercially available. As shown in Fig. 4A, the amounts of CK-M in the serum of the rats under WIR stress for 16 h was significantly increased as compared to the normal rats and the fasted rats (fasting). In ApoA4, the amount of the protein in the serum showed no significant difference as between the normal rats (control) and the WIR-stressed rats, but the ApoA4 level in the fasted rats was significantly decreased compared to the normal rats (Fig. 4B).

Discussion

This study indicated for the first time that the amounts of serum CK-M increases in rats with gastric ulcer induced by WIR stress, while ApoA4 decreases under fasting, suggesting that these serum proteins might be useful as biomarkers, one for stress-induced gastric ulcer and the other for starvation.

CK is an enzyme that catalyzes the reversible phosphorylation of ATP and creatine to ADP and phosphocreatine, and it plays an important role in the regulation and maintenance of cellular energy metabolism. It is well-known that blood contains predominantly CK-MM isozyme, followed by CK-MB, but does not CK-BB. Previous studies have shown that total CK enzyme activity in the plasma is increased by WIR stress imposed for 6 h, and that CK-MB isozyme in serum is also increased by WIR

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"Numbers correspond to those in Fig. 2."
"Av. ratio (average ratio) means fold changed spot intensity ratio between sera from normal rats and WIR-stressed rats. Positive value means that increased and negative value means decreased."
"From NCBI Entrez Gene."
"Theoretical molecular mass"
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stress imposed for 4 h. However, little is known about serum CK-MM in WIR-stressed rats. In this study, proteomic analysis and western blotting revealed an increase in CK-M in rats with WIR stress-induced gastric ulcers. Additionally, ELISA using an antibody specific for CK-MM suggested that the isozyme increased by WIR stress is probably CK-MM.

It has been reported that the exposure of rats to WIR stress for 4 h or 6 h causes increases in the plasma and serum activities of several enzymes, alanine aminotransferase (ATL), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), CK, and LPO in plasma and serum induced by WIR stress. Of those enzymes, serum CK-MM was found to be extremely increased in rats with severe gastric lesions induced by WIR stress much longer than the 10 to 16 h in this study. Because it is known that CK is expressed in gastric mucosal cells as well, serum CK-MM in part be released directly from gastric mucosa oxidative-damaged by WIR stress.

Toru et al. have reported that serum CK activity is markedly elevated in patients with neuroleptic malignant syndrome, and specific isozyme, CK-MM, is thought to be especially increased. This elevation of serum CK activity in the patients is probably due to cellular damage in the skeletal muscles, because skeletal muscle possesses the CK-M chain subunit exclusively. Similar results have been reported in Parkinsonian patients. The patients with Parkinson’s disease are sensitive to physical activity resulting in leakage of CK-MM from skeletal muscles. This elevation is considered to be a neural mechanism mediated by autonomic nervous system. In patients with depressive illness, gross motor activity, which is probably evoked via disruption of the nervous system is known to be lower than that of normal persons. Recently, Segal and co-workers have investigated the total activity level of serum CK enzyme in various forms of depression, major depression with and without psychotic symptoms, bipolar depression, and schizoaffective depression. The results have shown large differences in CK activities among the types of depression and have suggested that the serum CK activity level reflects biological differences between nonpsychotic major depression and the psychotic cluster of depressive syndromes. Quantitative detection of serum CK-MM is expected to be more sensitive than the total enzyme activity of serum CK. Hence, serum CK-MM may be useful as a potential biomarker for diseases involving with disruption of the nervous systems including depression and stress-induced gastric ulcer.

ApoA4 is a 46-kDa glycoprotein that is a component of triglyceride-rich chylomicrons and high density lipoproteins (HDL). It has been suggested that synthesis of ApoA4 in the small intestine occurs in response to the ingestion of fat. In humans, most circulating plasma ApoA4 is thought to be associated with HDL, some of which is probably transferred from chylomicrons and very low density lipoproteins. In rodents, ApoA4 is known to be present in the plasma in HDL particles, 59% of the ApoA4 being synthesized in the intestine and the remainder in the liver. A number of physiological functions of ApoA4 have been proposed. These include acting as a satiety signal, aiding in ApoC-II transfer to triglyceride-rich lipoproteins, increasing lipoprotein lipase activity, and stimulating lecithin:cholesterol acyltransferase activity. Lohese et al. have identified two isoforms of plasma ApoA4, designated ApoA4-1 and ApoA4-2, detected by isoelectric focusing followed by immunoblotting. Specifically, the difference be-
between ApoA4-1 and ApoA4-2 is due to a single nucleotide change, a G-to-T substitution leading to a conversion of glutamine-360 to histidine in the mature protein. In the present study, we identified two protein spots as ApoA4, probably ApoA4-1 and ApoA4-2 isoforms, in the WIR-stressed rats by proteomic analysis; one increased and the other decreased. To determine serum ApoA4 levels in the WIR-stressed rats, ELISA was performed with sera from 3–5 rats in the control group (normal rats), fasting group (fasting alone), and WIR stress-treated group (fasting followed by WIR stress). Unexpectedly, there were no big difference in serum ApoA4 levels between the control group and the WIR stress-treated group, while a significant difference was found between the control group and the fasting group. This suggests that ApoA4 is decreased by fasting, and that WIR stress after fasting tends to increase ApoA4 reduced by fasting. However, Hanniman et al. have reported that ApoA4 increases in fasted mice (C57BL/6j) as compared to fed mice. It is unclear why this discrepancy occurred in a previous study by Hanniman et al. (increases in serum ApoA4 levels in fasted mice) and the present study (decreases in serum ApoA4 levels in fasted rats). Cohen et al. have produced transgenic mice with inserts of several copies of the murine ApoA4 gene. They found 3-fold increases in plasma ApoA4 levels in mice fed a chow diet and also increases in plasma triglycerides, total cholesterol, HDL cholesterol, and free fatty acids. The findings reported by Cohen et al. appear to support our result that if rodents are unfed (fasting), serum ApoA4 levels decrease.

Besides CK-MM and ApoA4, haptoglobin, VDBP, and ApoE, which were identified in the WIR-stressed rats by proteomic analysis, are suggested to be useful as potential biomarkers for stress or fasting based on the existence of serum protein levels and their protein functions. Unfortunately, the antibodies for those 3 rodent proteins are not commercial available at present, and hence, we could not confirm the possibility of biomarker candidates by ELISA in this study. Haptoglobin functions to bind free plasma hemoglobin, which allows degradative enzymes to gain access to the hemoglobin, at the same time preventing a loss of iron through the kidneys and protecting the kidneys from damage by hemoglobin. VDBP, also called group-specific component, is a multifunctional protein in the plasma, ascetic fluid, and cerebrospinal fluid and on the surfaces of many cell types. It binds to vitamin D and its plasma metabolites and transports them to target tissues. ApoE, a main apoprotein of the chylomicron, binds to a specific receptor on liver cells and peripheral cells. Chylomicron remnants and VLDL remnants are rapidly removed from circulation by receptor-mediated endocytosis in the liver. ApoE is essential for the normal catabolism of triglyceride-rich lipoprotein constituents.

In conclusion, we identified serum proteins quantitatively changed by WIR stress in rats by proteomic analysis. Of these, serum CK-MM and ApoA4 are suggested to be useful as protein biomarkers for stress-induced gastric ulcer and fasting respectively. Based on this study, in WIR stress experiments using a rodent model, the results obtained should be considered to reflect fasting before WIR stress application.

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