Proteomic Profiling in the Spinal Cord and Sciatic Nerve in a Global Cerebral Ischemia-Induced Mechanical Allodynia Mouse Model

Shinichi Harada, Wataru Matsuura, Masaoki Takano, and Shogo Tokuyama

*Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Kobe Gakuin University; 1–1–3 Minatojima, Chuo-ku, Kobe 650–8586, Japan; and Department of Life Sciences Pharmacy, School of Pharmaceutical Sciences, Kobe Gakuin University; 1–1–3 Minatojima, Chuo-ku, Kobe 650–8586, Japan.

Received August 20, 2015; accepted November 17, 2015

Central post-stroke pain (CPSP) is one of the complications of cerebral ischemia and neuropathic pain syndrome. At present, there are few studies of pain in regions such as the spinal cord or sciatic nerve in cerebral ischemic animal models. To identify proteomic changes in the spinal cord and sciatic nerve in global cerebral ischemic model mice, in the present study we performed an investigation using proteomic methods. In a comparison between the intensity of protein spots obtained from a sham and that from a bilateral carotid artery occlusion (BCAO) in spinal cord and sciatic nerve, the levels of 10 (spinal cord) and 7 (sciatic nerve) protein spots were altered. The protein levels in the spinal cord were significantly increased in N2,N2-dimethylarginine dimethylaminohydrolase 1 (DDAH1), 6-phosphogluconolactonase isoform 1, and precursor apoprotein A-1 and decreased in dihydropyrimidinidase-related protein 2 (CRMP-2), enolase 1B, rab guanosine 5’-diphosphate (GDP) dissociation inhibitor beta, septin-2 isoform a, isocitrate dehydrogenase subunit alpha, cytosolic malate dehydrogenase, and adenosine triphosphate synthase. The protein levels in the sciatic nerve were significantly increased in a mimecan precursor, myosin light chain 1/3, and myosin regulatory light chain 2 (MLC2), and decreased in dihydropyrimidinidase-related protein 3 (CRMP-4), protein disulphide-isomerase A3, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1, and B-type creatine kinase. In addition, CRMP-2 and CRMP-4 protein levels were decreased, and DDAH1 and MLC2 protein levels were increased on day 1 after BCAO using Western blotting. These results suggested that changes in these proteins may be involved in the regulation of CPSP.

Key words global ischemia; pain; spinal cord; sciatic nerve; proteome
the spinal cord or sciatic nerve using cerebral ischemic animal models.

In the present study, to identify factors regulating CPSP using global cerebral ischemic model mice in pain-associated regions such as spinal cord or sciatic nerve, we performed a proteomic study.

MATERIALS AND METHODS

Animals Male ddY mice (5-weeks-old; Japan SLC, Inc., Shizuoka, Japan) were housed in an animal room maintained at 24°C and 55±5% humidity on a 12 h light/dark cycle (light phase, 08:00–20:00). Mice were provided with food and water ad libitum. All procedures were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals adopted by the Japanese Pharmacological Society. The study protocol was approved by the Animal Ethics Committee of Kobe Gakuin University, Kobe, Japan (approval number 14–21).

Materials Sodium dodecyl sulfate (SDS), urea, thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dithiothreitol, iodoacetamide, bromophenol blue, and RNase A and DNase I for SDS-polyacrylamide gel electrophoresis (PAGE) or 2-DE were all obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animal Model of Global Cerebral Ischemia Transient global cerebral ischemia was induced by occlusion of the bilateral carotid arteries (BCAO) in mice as described previously.16,27) In brief, mice were anesthetized with pentobarbital (60 mg/kg). The bilateral common carotid arteries (CAs) were occluded for 30 min using Sugita standard aneurysm clips (Mizuho Ikakogyo Co., Ltd., Tokyo, Japan). Sham-operated mice were subjected to the same procedure as above without bilateral carotid artery occlusion. The final number of mice used in the study groups is presented in the figure legends.

Assessment of Mechanical Allodynia Mechanical allodynia was evaluated using von Frey filaments (Neuroscience Inc., Tokyo, Japan) as previously described.28) Mice were placed on a 5×5-mm wire mesh grid floor, covered with an opaque cup to avoid visual stimulation, and allowed to adapt for 3 h prior to testing. The von Frey filament was then applied to the middle of the plantar surfaces of both hind paws with a weight of 0.4 g. On the indicated days, withdrawal responses following hind paw stimulation were measured 10 times, and the bilateral mechanical allodynia was defined as an increase in the number of withdrawal responses to the stimulation. Before sham or BCAO operation and on day 1 after BCAO, the von Frey test was performed on both paws of the BCAO mice.

Sample Preparation The spinal cord and sciatic nerve isolated on day 1 after BCAO were transferred to a 1.5-mL tube, centrifuged (15000×g, 5 min at 4°C), resuspended in 100 µL of lysis buffer (7 M urea, 2 M thiourea, 5% CHAPS, 2% immobilized pH gradient (IPG) buffer [GE Healthcare UK Ltd., U.K.], 50 mM 2-mercaptoethanol, 2.5 µg/mL DNase I, 2.5 µg/mL RNase A), and disrupted by sonication for 30 s. The lysate was again centrifuged (15000×g, 30 min) to remove cellular debris and the supernatant was recovered for use in 2-DE.

2-DE 2-DE was performed as previously described, with some modification.29,30) Protein (300 µg) was applied to ImmobilineDryStrip pH 3–10 NL (7 cm) gels (GE Healthcare UK Ltd.) and separated at 50 V for 6 h, at 100 V for 6 h, and at 2000 V for 6 h. The IPG strips were equilibrated for 15 min in 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 1% SDS, and 1% (v/v) diethiothreitol (DTT), and then for 15 min in the same buffer with 2.5% (w/v) iodoacetamide instead of DTT. After equilibration, the IPG strips were placed onto a 12.5% acrylamide gel and SDS-PAGE was performed at 5 mA/gel for 7 h.

SYPRO Ruby Staining Proteins on the SDS-polyacrylamide gels were detected using SYPRO Ruby Protein Gel Stain (Molecular Probes) as previously described, with some modification.29,30) Gels after 2-DE were fixed in a solution containing 10% acetic acid/50% methanol for 30 min, then 7% acetic acid/10% methanol for 30 min. After fixation, the gels were incubated in undiluted stock solution of SYPRO Ruby for 90 min and then destained with 7% acetic acid/10% methanol for 30 min. After rinsing with H2O for 10 min, digital images were acquired using a Fluorophorestar 3000 image capture system (Anatech, Japan) with 470-nm excitation and 618-nm emission for SYPRO Ruby detection.

Image Analysis Following image acquisition, 2-D gel imaging and analysis software Prodigy SameSpots version 1.0 (Nonlinear Dynamics, U.K.) was used for gel-to-gel matching and identifying differences between sham and BCAO mouse samples as previously described, with some modifications.29,30) Each of three sets of samples was represented by two independent biological replicates of 2-DE gels. The gel images were normalized with the Prodigy SameSpots software to compensate for differences in the staining intensity among the gels.

In-Gel Digestion and Peptide Extraction In-gel digestion was performed by the previously reported method.29,30) Protein spots in the gels stained with SYPRO Ruby was cut out and subjected to trypsin digestion with porcine trypsin (Promega, Madison, WI, U.S.A.). Briefly, gel pieces were washed, dehydrated, and diluted in 200 µL of 25 mM ammonium hydrogen carbonate with 5% acetonitrile (ACN) (v/v). Trypsin (5 µL, 10 ng/µL) was added and the digest was incubated for 10 h at 37°C. After separation of supernatant, the gel pieces were washed again and then extracted with 50% ACN/0.03% (v/v) trifluoroacetic acid for 10 min by sonication. The supernatant was once again collected, mixed with the two fractions, and evaporated under vacuum. The extracted peptides were then diluted in 5 µL of 50% ACN/0.3% (v/v) trifluoroacetic acid.

Mass Spectrometry Analysis and Protein Identification Mass spectra were recorded in positive reflection mode using a matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS/MS analyzer (ABI PLUS 4800, Applied Biosystems), equipped with delayed ion technology, as previously described.29,30) The samples were dissolved in 5 µL of 50% ACN/0.3% (v/v) trifluoroacetic acid. For the matrix, α-cyano-4-hydroxycinnamic acid (1 µg/µL: Wako Junyaku, Osaka, Japan) dissolved in the same mixture was used. The analyte and matrix were consecutively spotted in a 1:1 ratio on a stainless steel target and dried under ambient conditions. All spectra acquired by MALDI-TOF MS were externally calibrated with peptide calibration standard II (Bruker Daltonics, Germany). An MS condition of 2500 shots per spectrum was used. Automatic monoisotopic precursor selection for MS/MS was performed using an interpretation method based on the 12 most intense peaks per spot with an MS/MS mode condition
of 4000 laser shots per spectrum. The minimum peak width was one fraction and mass tolerance was 80 ppm. Adduct tolerance was (m/z)±0.003. MS/MS was performed with a gas pressure of 1×10⁻⁶ bar in the collision cell. Ambient air was used as the collision gas. Data analyses were performed with Data Explorer version 4.9 (Applied Biosystems) software, and proteins were identified with the Mascot search engine (www.matrixscience.com; Matrix Science, Boston, MA, U.S.A.) (peptide mass tolerance: 60 ppm; MS/MS tolerance: 0.3 Da; maximum missed cleavages: 1) using the NCBI nr protein database. Proteins identified by MALDI-TOF MS with a score of 79 or higher were considered significant (p<0.05). Single peptides identified by MALDI-TOF/TOF MS/MS with individual ions scores greater than 47 indicated identity or extensive similarity (p<0.05).

**Western Blot Analysis** Western blotting was performed as previously described but with some modifications.[31] Briefly, the spinal cord and sciatic nerve were homogenized in homogenization buffer and diluted with an equal volume of 2× SDS sample buffer (0.5 M Tris–HCl [pH 6.8], 10% SDS, 12% β-mercaptoethanol, 20% glycerol, 1% bromophenol blue). Each sample was heated for 3 min at 97°C and protein samples (20 µg) were separated via electrophoresis on 12% (w/v) (spinal cord) or 15% (w/v) (sciatic nerve) SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes (BioRad, Hercules, CA, U.S.A.) at 15 V for 50 min. Membranes were blocked (60 min at room temperature) in Tris-buffered saline (TBS) (pH 7.6) with 0.1% Tween 20, and either 5% bovine serum albumin (BSA) (Sigma-Aldrich) for dihydroxyimidine-related protein 2 (CRMP-2), Nδ, N⁴-dimethylarginine dimethylaminohydrolase 1 (DDAH1), dihydroxyimidine-related protein 3 (CRMP-4), myosin regulatory light chain 2 (MLC2), or 5% skim milk (GE Healthcare, Tokyo, Japan or Wako Pure Chemical Industries, Ltd., Osaka, Japan) for myelin basic protein (MBP), and glyceraldehyde 3 phosphate dehydrogenase (GAPDH; loading control). Membranes were incubated with the primary antibodies (in their corresponding blocking solution, overnight at 4°C): rabbit anti-CRMP-2 (1:1000, Cell Signaling, MA, U.S.A.), rabbit anti-DDAH1 (1:1000, Abcam, Tokyo, Japan), rabbit anti-CRMP-4 (1:500, Abcam), rabbit anti-MLC2 (1:500, Cell Signaling), mouse anti-MBP (1:1000, Abcam), and mouse anti-GAPDH (1:20000, Chemicon, CA, U.S.A.). Blots were then incubated (for 1 h at room temperature (r.t.)) in horseradish peroxidase (HRP)-conjugated secondary antibodies: anti-rabbit immunoglobulin G (IgG) (1:1000, KPL, Guildford, U.K.) for CRMP-2, DDAH1, CRMP-4, MLC2, and anti-mouse IgG (KPL) for MBP (1:1000) and GAPDH (1:10000). All visualization of immunoreactive bands were visualized with enhanced chemiluminescence Western immunoblotting substrate (Pierce; Thermo Scientific, Rockford, IL, U.S.A.) followed by a Light-Capture Instrument (AE-6981; ATTO, Tokyo, Japan). The signal intensity of immunoreactive bands was analyzed using a Cs-Analyzer (Ver. 3.0; ATTO).

**Immunofluorescence** Immunofluorescence was performed as described previously, with some modifications.[22] Mice were transcardially perfused with saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) on day 1 after BCAO. The sciatic nerve was incubated and fixed in ice-cold PBS containing 4% PFA at 4°C for 3 h, then dehydrated in 10% sucrose for 3 h, followed by 20% overnight at 4°C. Tissues were then embedded and frozen in the Tissue-Tek optimal cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and stored at −80°C until future use. Frozen blocks were cut into 20-µm-thick sections with a cryostat (Leica CM1850; Microsystems GmbH, Wetzlar, Germany) and mounted on a MAS-coated glass slide (Matsunami Glass Ind., Ltd., Osaka, Japan). Sections were post-fixed in 10% formaldehyde at r.t. for 15 min and then washed with PBS containing 0.1% Tween 20 (PBS-T) 3 times at 5-min intervals. Sections were exposed to 3% BSA in PBS +0.3% Triton-X for 1 h at r.t. and then incubated (at 4°C for 24 h) with the primary antibodies (in PBS +0.3% Triton-X with 1% BSA): mouse anti-myelin basic protein (MBP) (1:200, Abcam), rabbit anti-myosin regulatory light chain 2 (1:200, Cell Signaling, MA, U.S.A.). Sections were incubated (r.t., 2 h) with the secondary antibody: Alexa Fluor 488 goat polyclonal anti-mouse (1:200, Life Technologies Inc., Carlsbad, CA, U.S.A.). Alexa Fluor 594 goat polyclonal anti-rabbit (1:200, Life Technologies Inc.). After washing, cover slips were applied to the sections with Fluoromount/Plus (Thermo Shandon Inc., Pittsburgh, PA, U.S.A.). Immunoreactive signals were detected with a confocal laser scanning microscope (FV1000, OLYMPUS, Tokyo, Japan). Quantitatively the immunofluorescent intensity were analyzed using image analysis software (ImageJ, NIH, Bethesda, MD, U.S.A.).

**Statistical Analysis** Data are expressed as mean±standard error of the mean (S.E.M.). Statistical significance was assessed using the Tukey test or one-way ANOVA. Unpaired Student’s t-test and ANOVA were performed at the 95% significance level to identify proteins differentially expressed in sham and BCAO mice.

**RESULTS**

**Development of Mechanical Allodynia on Day 1 after Global Cerebral Ischemia** The number of escape behaviors in response to the stimulation applied by the von Frey filament was significantly increased on day 1 after BCAO as compared with sham group (p<0.01 vs. sham) (Fig. 1). Escape behaviors in pre were not different between the sham and BCAO group. An increase in escape behaviors after BCAO was observed for both hind paws.

**Representative 2-D Gel for Proteins on Day 1 after Global Cerebral Ischemia** The levels of proteins in the spinal cord and sciatic nerve of BCAO mice were compared to those of sham mice. The altered levels of proteins were quantified and identified in 2-DE gels using Prodigy SameSpots software for MALDI-MS/MS. We show representative 2-DE gels stained with SYPRO Ruby for the spinal cord and sciatic nerve of sham and BCAO mice (Figs. 2, 3). There were approximately 300 spots in each SYPRO Ruby-stained 2-DE gel from the spinal cord and sciatic nerve of the sham and BCAO mice (Figs. 2, 3).

Comparison of the intensities of protein spots from the sham and BCAO in the spinal cord and sciatic nerve showed that the levels of 10 (spinal cord, Table 1) or 7 (sciatic nerve, Table 2) protein spots (ANOVA <0.05) were altered. Protein levels in spinal cord were significantly increased for DDAH1, 6-phosphogluconolactonase isoform 1, and precursor apoprotein A-I and decreased for CRMP-2, enolase 1B, rab GDP dissociation inhibitor beta, septin-2 isoform a, isocitrate de-
hydrogenase subunit alpha, cytosolic malate dehydrogenase, and ATP synthase (Table 1). The protein levels in sciatic nerve were significantly increased for mimecan precursor, myosin light chain 1/3, and MLC2 and decreased for CRMP-4, protein disulfide-isomerase A3 precursor, 3-hydroxy-3-methylglutarylcoenzyme A synthase 1, and B-type creatine kinase (Table 2).

Changes in the Expression Levels of Some Proteins on Day 1 after Global Cerebral Ischemia

The protein levels in sciatic nerve were significantly increased for mimecan precursor, myosin light chain 1/3, and MLC2 and decreased for CRMP-4, protein disulfide-isomerase A3 precursor, 3-hydroxy-3-methylglutarylcoenzyme A synthase 1, and B-type creatine kinase (Table 2).
sion levels of CRMP-2 in the spinal cord and CRMP-4 in the sciatic nerve tended to be increased/decreased in the BCAO group than in the sham group on day 1 after BCAO (Figs. 4A, C). DDAH1 in spinal cord and MLC2 in sciatic nerve protein levels were tend to increase in the BCAO group than in the sham group on day 1 after BCAO (Figs. 4B, D).

**DISCUSSION**

Pain signaling is initiated by the detection of noxious stimuli through specialized primary nociceptors located in peripheral endings within the skin and in internal organs. The spinal cord and sciatic nerve are important regions for explaining the transmission of pain signaling. Thus, to search for factors regulating the development of CPSP, we performed a compre-

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>GI accession-number</th>
<th>Identified protein name</th>
<th>Theoretical Mass/PI</th>
<th>Score</th>
<th>Sequence coverage (%)</th>
<th>Number of peptides</th>
<th>Fold Increase/Decrease</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>40254595</td>
<td>Dihydropyrimidinase-related protein 2 (CRMP-2)</td>
<td>62638/5.95</td>
<td>309</td>
<td>23</td>
<td>8</td>
<td>0.59</td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>70794816</td>
<td>Enolase 1B</td>
<td>47453/6.37</td>
<td>79</td>
<td>6</td>
<td>2</td>
<td>0.56</td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>40254781</td>
<td>Rab GDP dissociation inhibitor beta</td>
<td>51018/5.93</td>
<td>108</td>
<td>13</td>
<td>4</td>
<td>0.77</td>
</tr>
<tr>
<td>4</td>
<td>gi</td>
<td>6754816</td>
<td>Septin-2 isoform a</td>
<td>41727/6.10</td>
<td>136</td>
<td>14</td>
<td>4</td>
<td>0.77</td>
</tr>
<tr>
<td>5</td>
<td>gi</td>
<td>38371755</td>
<td>(N^\alpha,N^\beta)-Dimethylarginine dimethylaminohydrolase 1</td>
<td>31760/5.64</td>
<td>128</td>
<td>17</td>
<td>4</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>gi</td>
<td>18250284</td>
<td>Isocitrate dehydrogenase [NAD] subunit alpha</td>
<td>40069/6.27</td>
<td>205</td>
<td>24</td>
<td>7</td>
<td>0.83</td>
</tr>
<tr>
<td>7</td>
<td>gi</td>
<td>387129</td>
<td>Cytosolic malate dehydrogenase</td>
<td>36625/6.16</td>
<td>140</td>
<td>8</td>
<td>3</td>
<td>0.83</td>
</tr>
<tr>
<td>8</td>
<td>gi</td>
<td>13384778</td>
<td>6-Phosphogluconolactonase isoform 1</td>
<td>27465/5.55</td>
<td>70</td>
<td>17</td>
<td>3</td>
<td>1.4</td>
</tr>
<tr>
<td>9</td>
<td>gi</td>
<td>16741459</td>
<td>ATP synthase</td>
<td>18809/5.52</td>
<td>98</td>
<td>23</td>
<td>3</td>
<td>0.83</td>
</tr>
<tr>
<td>10</td>
<td>gi</td>
<td>50015</td>
<td>Precursor Apoprotein A-I</td>
<td>30569/5.64</td>
<td>210</td>
<td>32</td>
<td>9</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Proteins of mouse spinal cord were separated by 2-DE and identified by MALDI-TOF MS/MS, following in-gel digestion with trypsin. The spots representing the identified proteins are indicated in Fig. 2 and are designated with their gene accession numbers (GI) of SwissProt database. Scores of the probability assignment, theoretical molecular weight, and pl, and sequence coverage (SC) values, are given. Score and sequence coverage were calculated by the MASCOT search engine (http://www.matrixscience.com). Data were analyzed by ANOVA.

Fig. 4. Changes in the Expression Levels of Some Proteins on Day 1 after Global Cerebral Ischemia

Representative Western immunoblots and quantification (ratio to GAPDH) as % of sham group on day 1 after BCAO: (A) CRMP2 and (B) DDAH1 in spinal cord, (C) CRMP4 and (D) MLC2 in sciatic nerve. Student’s t-test, Results are presented as the mean±S.E.M. (n=8).
hensive proteome analysis of the spinal cord and sciatic nerve.

DDAH1 modulates nitric oxide (NO) signaling by catalyzing the hydrolysis of asymmetric dimethylarginine, an endogenous inhibitor of all three isoforms of NO synthase.33) NO is associated with the development of neuropathic pain.34) The increase of DDAH1 may be involved in the development of CPSP via enhancement of nitric oxide signaling.

6-Phosphogluconolactonase converts 6-phosphogluconolactone to 6-phosphogluconate, is an intermediate in the pentose phosphate pathway.35) The pentose phosphate pathway was involved in a broad range of oxidation–reduction reactions through the enhancement of nicotinamide adenine dinucleotide phosphate and glutathione synthesis.36) In previous study, the pentose phosphate pathway is increased after traumatic brain injury.37) These suggested that increase of 6-phosphogluconolactonase may contribute to enhance of anti-oxidative stress after cerebral ischemia. Apoprotein A-I, which the major component antiatherogenic properties of high density lipoprotein (HDL) are mainly related to reverse cholesterol transport process (i.e., removing excess cholesterol from the arterial wall's foam macrophages to the liver for metabolism). Physiologically, the efficiency of reverse cholesterol transport depends on the activity of lecithin:cholesterol acyltransferase (LCAT), and apoprotein A-I activates LCAT to facilitate reverse cholesterol transport and HDL maturation.38–40) Thus, the function of apoprotein A-I and LCAT is essential for maintaining body cholesterol homeostasis. However, there are no reports regarding the development of pain signaling of 6-phosphogluconolactonase and apoprotein A-I.

It is noteworthy that four of them (enolase, isocitrate dehydrogenase, creatine kinase, and apoprotein A-I) are increased after traumatic brain injury. These proteins were identified in the sciatic nerve by proteomic analysis. The results of the proteomic analysis are summarized in Table 2.

### Table 2. Identification of Altered Proteins on Day 1 after Global Cerebral Ischemia in the Sciatic Nerve

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>GI accession-number</th>
<th>Identified protein name</th>
<th>Theoretical Mass/PI</th>
<th>Score</th>
<th>Sequence coverage (%)</th>
<th>Number of peptides</th>
<th>Fold Increase/Decrease</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>6681219</td>
<td>Dihydropyrimidinase-related protein 3 (CRMP-4)</td>
<td>62296/6.04</td>
<td>84</td>
<td>7</td>
<td>3</td>
<td>0.48</td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>112293264</td>
<td>Protein disulfide-isomerase A3 precursor</td>
<td>57099/5.88</td>
<td>313</td>
<td>21</td>
<td>8</td>
<td>0.59</td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>21618633</td>
<td>3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1</td>
<td>58144/5.65</td>
<td>159</td>
<td>12</td>
<td>7</td>
<td>0.56</td>
</tr>
<tr>
<td>4</td>
<td>gi</td>
<td>10946574</td>
<td>Creatine kinase B-type</td>
<td>42971/5.40</td>
<td>45</td>
<td>5</td>
<td>2</td>
<td>0.77</td>
</tr>
<tr>
<td>5</td>
<td>gi</td>
<td>10946574</td>
<td>Mimecan precursor</td>
<td>34333/5.52</td>
<td>177</td>
<td>27</td>
<td>6</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>gi</td>
<td>29789016</td>
<td>Myosin light chain 1/3</td>
<td>20695/4.98</td>
<td>326</td>
<td>44</td>
<td>7</td>
<td>1.8</td>
</tr>
<tr>
<td>7</td>
<td>gi</td>
<td>7949078</td>
<td>Myosin regulatory light chain 2</td>
<td>19057/4.83</td>
<td>166</td>
<td>27</td>
<td>4</td>
<td>3.9</td>
</tr>
</tbody>
</table>

The proteins of the mouse sciatic nerve were separated by 2-DE and identified by MALDI-TOF MS/MS, following in-gel digestion with trypsin. The spots representing the identified proteins are indicated in Fig. 3 and are designated with their gene ID accession numbers (GI acc. No.) of the Swiss Prot database. Scores of the probability assignment, theoretical molecular weight, and pl, and sequence coverage (SC) values, are given. The score and sequence coverage were calculated by the MASCOT search engine (http://www.matrixscience.com). Data were analyzed by ANOVA.

Fig. 5. Changes in MBP and MLC2 in the Sciatic Nerve on Day 1 after Global Cerebral Ischemia

Tissues samples prepared on day 1 after BCAO. (A) Expression levels of MBP. Results are presented as the mean±S.E.M. (n=8). (B–H) Results of immunofluorescence stain of MBP and MLC2. (B, D, F) Sham group, (C, E, G) BCAO group, (B, C) MBP, (D, E) MLC2, (F, G) Merge. Scale bar: 50 µm. (H, I) Quantitatively the immunofluorescent intensity were analyzed using ImageJ. (H) MBP, (I) MLC2. *p<0.05, Student's t-test. Results are presented as the mean±S.E.M. (n=8).
hydrogenase, malate dehydrogenase and ATP synthase) are cellular enzymes, and their levels are all decreased in spinal cord.\textsuperscript{31,42} In addition, septin-2 make up the cytoskeleton of basal epithelial cells or acts as a scaffold to anchor the acto-myosin ring to the plasma membrane during furrowing.\textsuperscript{43,44} Rab guanosine 5'-triphosphatases (GTPases), one of the Ras superfamily members of monomeric GTPases, are small G proteins and involved in membrane trafficking.\textsuperscript{45,46} The Rab GTase controls a fast endocytic recycling pathway and must be activated for septin cytoskeleton localization at the intercellular bridge, and thus for completion of cytokinesis.\textsuperscript{47} Above these reasons, it is possible that it may be caused cytoskeleton disfunction mediated by global cerebral ischemia. However, it is not unclear whether these changes in spinal cord represent primary causes or consequences of the development of CPSP.

Mimecan (osteoglycin), a member of the small leucine-rich proteoglycan gene family, was initially isolated in a truncated form from bovine bone and subsequently characterized as one of the 3 major keratan sulfate-containing proteoglycans in the cornea, along with lumican and keratan.\textsuperscript{48,49} This protein may play a role in angiogenesis, inflammation, and cellular growth control, as inferred from the ability of growth factors and cytokines to modulate its mRNA expression in corneal keratocytes and vascular smooth muscle cells.\textsuperscript{50–52} Mimecan was increased by glucocorticoid hormones, which regulate stress response.\textsuperscript{53} Thus, the elevated mimecan may be involved in the ischemia-induced stress response.

Among the proteins identified in the present study, we focused closely on MLC2. The phosphorylation of MLC2 following RhoA/Rho kinase activation regulates the activity of myosin II, which generates the force for cytoskeletal contractility.\textsuperscript{54} Activated myosin II generates cortical actin filaments and leads to inhibition of neuritogenesis.\textsuperscript{55,56} Myosin II is a hexameric complex composed of 2 myosin heavy chains, 2 MLCs, and 2 essential light chains.\textsuperscript{57} Levels of non-muscle myosin II decrease as a function of oligodendrocyte differentiation and inhibition of myosin activity increases branching and myelination,\textsuperscript{58,59} although their expression levels correlate negatively with myelination.\textsuperscript{59,60} The demyelination observed in neuropathic pain due to nerve lysoosphatidic acid-induced injury occurs on Schwann cells.\textsuperscript{59} By immunofluorescence staining, we observed that MLC2 was increased by global cerebral ischemia in the sciatic nerve. In addition, MLC2 was co-localized with MBP, one of schwann cells marker, and MBP were decreased by BCAO. We hypothesize that global ischemia-induced elevated MLC2 induces demye- lination in the sciatic nerve, resulting in the development of CPSP.

The dihydropyrimidinase related protein (DRP) family, also known as CRMPs, consists of 5 members, DRP1–5. Members of this family have been proposed to be part of the semaphorin signal transduction pathway involved in neurite outgrowth and in neuronal differentiation and death. CRMP-2 was first identified in a screen for proteins that mediate semaphorin 3A activity.\textsuperscript{62} Semaphorin 3A is a potent inhibitor of nerve growth factor (NGF)-responsive nociceptive C-fiber growth during development, as well as an inhibitor of sprouting into the mature spinal cord.\textsuperscript{63–65} Additionally, CRMP-2 and NGF have function of the neurite outgrowth.\textsuperscript{66} In some reports, neurite outgrowth induced the development of neuropathic pain.\textsuperscript{67,68} Some studies have shown that administration of antibodies directed against NGF or TrkA-Ig chimera greatly reduced NGF-induced pain responses and also reduced both sprouting and neuropeptide expression.\textsuperscript{69,70} We hypothesize that enhancement of pain behavior is mediated by a decrease in spinal CRMP-2-induced dysfunction of semaphorin 3A or of neurite extension. Dihydropyrimidinase-related protein 3, also known as CRMP-4 or ULIP1, regulates F-actin bundling, which may be relevant for growth cone collapse.\textsuperscript{71} CRMP-4 regulates neurite outgrowth, with a critical role in neurite outgrowth inhibition, and associates with vesicles in the growth cone.\textsuperscript{72,73} One report suggested that CRMP-4 was involved in the development of neuropathic pain.\textsuperscript{74} Thus, it is possible that CPSP is regulated by CRMP-4 in the sciatic nerve via modulation of neurite outgrowth.

Three of them (protein disulfide isomerase A3 precursor, 3-hydroxy-3-methylglutaryl-CoA synthase 1 and creatine kinase) other than CRMP4 are cellular enzymes, and their levels are all decreased in sciatic nerve.\textsuperscript{75–77} In particular, creatin kinase regulates ATP regeneration and energy homeostasis by catalyzing the reversible transfer of high-energy phosphate from phosphocreatine to ADP.\textsuperscript{77,78} Tissues such as the brain, skeletal and cardiac muscles, retinas, and spermatozoa express large amounts of creatin kinase to produce adequate energy stores for dynamic energy requirements.\textsuperscript{79,80} Thus, it is suggested that energy balance of sciatic nerve may be not able to regulate by global cerebral ischemia, but it is not unclear the detail mechanisms.

In conclusion, these findings suggest that proteome analysis is useful for identifying factors in the development of CPSP. Some proteins may be involved in the regulation of CPSP.

**Acknowledgment** This study was supported by Grants-in-Aid and by Special Coordination Funds from Grants-in-Aid for Scientific Research (C) (25462458) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


