FULL PAPER

Laboratory Animal Science

Identification of changed proteins by retinoic acid in cerebral ischemic damage: a proteomic study

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ABSTRACT. Ischemic stroke is a severe neurodegenerative disease with a high mortality rate. Retinoic acid is a representative metabolite of vitamin A. It has many beneficial effects including anti-inflammatory, anti-apoptotic, and neuroprotective effects. The purpose of this study is to identify specific proteins that are regulated by retinoic acid in ischemic stroke. Middle cerebral artery occlusion (MCAO) was performed to induce focal cerebral ischemia. Retinoic acid (5 mg/kg) or vehicle was injected intraperitoneally into male rats for four days prior to MCAO operation. Neurobehavioral tests were performed 24 hr after MCAO and the cerebral cortex was collected for proteomic study. Retinoic acid alleviates neurobehavioral deficits and histopathological changes caused by MCAO. Furthermore, we identified various proteins that were altered by retinoic acid in MCAO damage. Among these identified proteins, adenosylhomocysteinase, isocitrate dehydrogenase [NAD$^+$] subunit α, glycerol-3-phosphate dehydrogenase, Rab GDP dissociation inhibitor β, and apolipoprotein A1 were down-regulated in MCAO animals with vehicle treatment, whereas retinoic acid treatment alleviated these reductions. However, heat shock protein 60 was up-regulated in MCAO animals with vehicle, while retinoic acid treatment attenuated this increase. The changes in these expressions were confirmed by reverse transcription-PCR. These proteins regulate cell metabolism and mediate stress responses. Our results demonstrated that retinoic acid attenuates the neuronal damage by MCAO and regulates the various protein expressions that are involved in the survival of cells. Thus, we can suggest that retinoic acid exerts neuroprotective effects on focal cerebral ischemia by modulation of specific proteins.

KEYWORDS: cerebral ischemia, neuroprotection, proteomics, retinoic acid

Stroke is a main cause of death and has a high mortality and morbidity [12]. Cerebral ischemic stroke is the most common type of stroke and is characterized by cerebral artery occlusion [43]. It causes oxygen deficiency and mitochondrial dysfunction due to reduction of blood flow [61]. It produces excessive reactive oxygen species (ROS) due to oxidative stress, and also causes serious changes such as membrane depolarization, glutamate excitotoxicity, and intracellular calcium imbalance [45]. Ischemic stroke destroys blood brain barriers that play an essential role in controlling homeostasis and defensive responses in the nervous system [16]. These changes cause irreparable nerve cell damage and cell death [28].

Retinoic acid is a major metabolite of vitamin A that plays an important role in neurodevelopment [44]. It has various biological effects, such as antioxidant, anticancer, and anti-inflammatory [7, 15, 48]. In addition, retinoic acid has neuroprotective effects by inhibiting oxidative stress and neuroinflammatory reactions in Alzheimer's disease, Parkinson's disease, and stroke [4, 8, 55]. Retinoic acid also promotes synaptic plasticity and behavioral recovery including learning and memory [26]. We recently reported the neuroprotective effect of retinoic acid in middle cerebral artery occlusion (MCAO) animal model [20]. Specifically, retinoic acid improves neurological behavioral disorders and infarction caused by MCAO damage and prevents the expression of apoptotic proteins in cerebral ischemia. Although many studies have been reported regarding the neuroprotective effect of retinoic acid in ischemic brain damage, various proteins regulated by retinoic acid in MCAO damage have not been clearly identified and further studies are still needed to elucidate the neuroprotective pathway of retinoic acid. We propose that retinoic acid exerts neuroprotective effects by controlling the expression of specific proteins in MCAO damage. The aim of this study was to detect various proteins that are modulated by retinoic acid in MCAO animal models. In this study, we investigated the proteins regulated by retinoic acid using proteomic study.

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MATERIALS AND METHODS

Experimental animals and drug treatment

Male Sprague-Dawley rats were purchased from Samtako Co. (Animal Breeding Center, Osan, Korea). All experimental protocols were performed according to the guidelines of the Institutional Animal Care and Use Committee of Gyeongsang National University (GNU-210302-R0023). Animals (200–220 g, n=60) were housed in controlled temperature (25°C) and light system (12 hr/12 hr light/dark cycle). Rats were freely supplied with feed and water, and randomly divided into the following groups: vehicle + MCAO, retinoic acid + MCAO, vehicle + sham, and retinoic acid + sham. Retinoic acid (Sigma Aldrich, St. Louis, MO, USA) was dissolved in solvent agent (polyethylene glycol, 0.9% NaCl, and ethanol; 70%/20%/10% by volume). Retinoic acid (5 mg/kg) or vehicle was intraperitoneally injected for four days before MCAO surgery by following previous study [23]. A solvent agent without retinoic acid was used as vehicle.

Middle cerebral artery occlusion surgery

MCAO was carried out as a previously described method [31]. Rats were anesthetized with Zoletil (50 mg/kg, Virbac, Carros, France) and a vertical midline incision was made in the neck. The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were consecutively exposed and dissected from adjacent tissues. CCA was temporally blocked with a microvascular clamp and ECA was ligated and cut. A 4–0 monofilament nylon suture with rounded end made by heating was inserted through the stump of ECA to ICA for the blocking origin of middle cerebral artery. The length of inserted nylon suture was almost 22–24 mm. ECA with inserted nylon was ligated and the skin was sutured. Sham-operated animals had same surgical operation without insertion of nylon suture. Rats were placed on heating pad to maintain body temperature during surgery and recovery. MCAO was maintained for 24 hr and neurological deficit test was performed. Animals were sacrificed immediately after the neurological deficit test, whole brains were isolated for further experiments.

Neurological deficit scoring test

Neurological function was evaluated according to a five-point scoring criterion [51]. The scoring was performed as follows: no neurological deficit (no deficit, 0), failure to extend the left forepaw (minor neurologic deficit, 1), circling to the ipsilateral side with normal posture (moderate neurologic deficit, 2), leaning toward the contralateral side and seizures (serious neurologic deficit) (3), no spontaneous locomotor activity and loss of consciousness (very serious deficit, 4).

Vibrissae-evoked forelimb placing

The forelimb placing test was performed to evaluate the movement initiation abilities by stimulating the vibrissae [2]. The test was performed before and after MCAO. We caught the animal so that all four limbs can hang freely. The right or left vibrissae were brushed on the edge of the table. This triggered a forelimb placing response from the forelimb on the same side of stimulated vibrissae. Each experiment was performed for ten trials and the percentage of successful replacing response was recorded for each side.

Adhesive-removal somatosensory test

The adhesive-removal somatosensory test was performed to measure somatosensory deficiency as previously described [32]. The test was performed by measuring the time to remove the adhesive tapes from both forelimbs in the cages. Two adhesive tapes (12 mm in diameter) were attached to both hairless part of the forelimb to stimulate bilateral tactile sense and rats were transferred to a test cage. Rats removed the adhesive tapes and the time for tape removal was measured. The process was repeated five times. The experimental rats were trained for three days before surgery.

Hematoxylin and eosin staining

Tissues were fixed in 4% neutral buffered paraformaldehyde, washed with tap water for overnight, dehydrated from 70% to 100% gradient ethyl alcohol series, and cleaned with xylene. They were embedded with paraplast using paraffin embedding center (Leica, Wetzlar, Germany) and paraffin blocks were cut into 4 μm thickness using a rotary microtome (Leica). Paraffin sections were placed on slide glass, dried on slide warmer (Thermo Fisher Scientific, Waltham, MA, USA), and deparaffinized with xylene. They were rehydrated from 100% to 70% with a gradient ethyl alcohol series and washed with tap water. Sections were stained with Harris' hematoxylin solution (Sigma Aldrich) for 5 min and washed with tap water. They were briefly dipped in 1% HCl solution with ethyl alcohol, washed with water, and immersed 1% ammonia water. They were stained with Eosin Y solution (Sigma Aldrich) for 2 min, washed with water, and dehydrated with gradient ethyl alcohol series (from 70% to 100%). They were cleaned with xylene and mounted with mounting medium (Thermo Fisher Scientific). Tissues were observed using Olympus microscope (Olympus, Tokyo, Japan) and images were taken.

2-dimensional gel electrophoresis

A proteomic study was conducted according to the described method [21]. Right cerebral cortices were separated from whole brain and kept in −70°C. They were homogenized with lysis buffer (8 M urea, 4% CHAPS, ampholytes, and 40 mM Tris-HCl) and centrifuged at 15,000 g for 15 min at 4°C. The supernatants were collected and, precipitated with trichloroacetic acid, and centrifuged at 14,000 g for 15 min at 4°C. After centrifugation, the supernatants were discarded and the pellets were washed with acetone and were dissolved in lysis buffer. The protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, USA) with bovine

serum albumin as a standard. Isoelectric focusing (IEF) was performed using immobilized pH gradient (IPG) gel strip (pH 4–7, 17 cm, Bio-Rad). Protein samples (50 µg) were diluted in the rehydration buffer [8 Murea, 2% CHAPS, 20 mM dithiothreitol (DTT), 0.5% IPG buffer, bromophenol blue] and loaded into the IPG strip. IPG strips were incubated for 15 hr and first dimentional IEF using Ettan IPGphor 3 System (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with following conditions: 250 V for 15 min, 10,000 V for 3 hr, and then 10,000 to 50,000 V. Strips were incubated in equilibration buffer [6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 50 mM Tris- HCl, and bromophenol blue] with 1% DTT for 15 min and reacted with 2.5% iodoacetamide for 15 min. Equilibrated strips were loaded into gradient gels (7.5–17.5%), electrophoresed at 5 mA for 2 hr, and continuously followed at 10 mA at 15°C until the bromophenol blue dye reached the bottom of gel. Gels were fixed in fixation solution (12% acetic acid and 50% methanol) for 2 hr, washed with 50% ethyl alcohol for 20 min, and pretreated with 0.2% sodium thiosulfate for 1 min. They were washed with deionized water, stained with silver nitrate solution (0.2% silver nitrate and 0.75 mL/L formaldehyde) for 20 min, and washed with deionized water. They were developed in a developing solution (0.2% sodium carbonate and 0.5 mL/L formaldehyde) until protein spots were clearly apparent. Reaction was stopped by a stop solution (1% acetic acid). Obtained images were scanned by Agfa ARCUS 1200TM (Agfar Gevaert, Mortsel, Belgium) and the protein spots with difference in intensities of all groups were analyzed by PDQest 2-D analysis software (Bio-Rad). Targeted protein spots were cut from stained gel, destained, and digested by trypsin-containing buffer. Mass spectrometry was performed to analyze extracted peptides by Voyager System DE-STR MALDI-TOF mass spectrometer (Life Technologies, Carlsbad, CA, USA). Analyzed proteins were identified by MS-Fit and ProFound software and confirmed by SWISS-PROT and NCBI databases.

Reverse transcription-polymerase chain reaction amplification

Total RNA from right cerebral cortices was extracted with Trizol Reagent (Life Technologies) following the manufacturer's instructions. Single-stranded complementary DNA was synthesized from RNA samples (500 ng) with GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's manual. Target genes were amplified by polymerase chain reaction (PCR) with designed specific primers. Table 1 represents the primer sequence used in this study. PCR reaction was performed as the following conditions: initial denaturation step for 5 min at 94°C; 28–30 cycles of denaturation step at 94°C for 30 sec, annealing step at 54–56°C for 30 sec, and elongation step at 72°C for 1 min; and a final extension step for 5 min at 72°C. The amplified PCR products were loaded and electrophoresed in 1% agarose gel. The separated products were visualized under an ultraviolet light. The densities of PCR product were analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA) and expressed as a ratio of PCR product intensity to β-actin intensity.

Statistical analysis

All experimental data are represented as means ± standard error of mean (S.E.M). The intensity analysis was performed using SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, USA). The results of each group were compared by two-way analysis of variance (ANOVA) followed by *post-hoc* Scheffe's test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Figure 1 shows the results of a neurological deficit scoring test, vibrissae-evoked forelimb placing test, and adhesive-removal somatosensory test. MCAO injury induced neurobehavioral disorders, such as involuntary circling and loss of movement and retinoic acid treatment migrated MCAO-induced deficits. These neurological deficits were not observed in sham operated animals. Neurological scores were 3.40 ± 0.19 in vehicle + MCAO animals and 2.07 ± 0.18 in retinoic acid + MCAO animals (Fig. 1A). Moreover, the

Table 1. Sequence of the primers used for PCR amplification

| Gene	Primer sequences (F, forward; R, reverse)	Product (bp)
Adenosylhomocysteinase	F: 5′-AAGCTGCCATGGAGGGCTAC3′ R: 5′-GATGGCAGCTGGAAGGTGAA-3′	583
Isocitrate dehydrogenase [NAD$^+$] subunit α	F: 5′-AAAAATCCATGGCGGTTCTGTG-3′ R: 5′-GGTCCCCATAGGCGTGTCG-3′	404
Glycerol-3-phosphate dehydrogenase	F: 5′-GGCCCTTTCACAGACTCCGT-3′ R: 5′-TCCATGTTCTCGGGGCTGT-3′	117
Rab GDP dissociation inhibitor β	F: 5′-ACCAAGTCAACCGCAAGTCA-3′ R: 5′-AGGTCGCTGATGCTGACAAA-3′	187
Apolipoprotein A-I	F: 5′-GGGAGTTCTGGCAGCAAGAT-3′ R: 5′-GCTGTTTGCCCAAAGTGGAG-3′	138
60 kDa heat shock protein	F: 5′-AGGCATGAAGTTTGATAGAG-3′ R: 5′-TTGGCAATTTCAAGAGCAGG-3′	150
β-actin	F: 5′-TACAACCTTCTTGCAGCTCCTC-3′ R: 5′-CCTTCTGACCCATACCCACC-3′	205
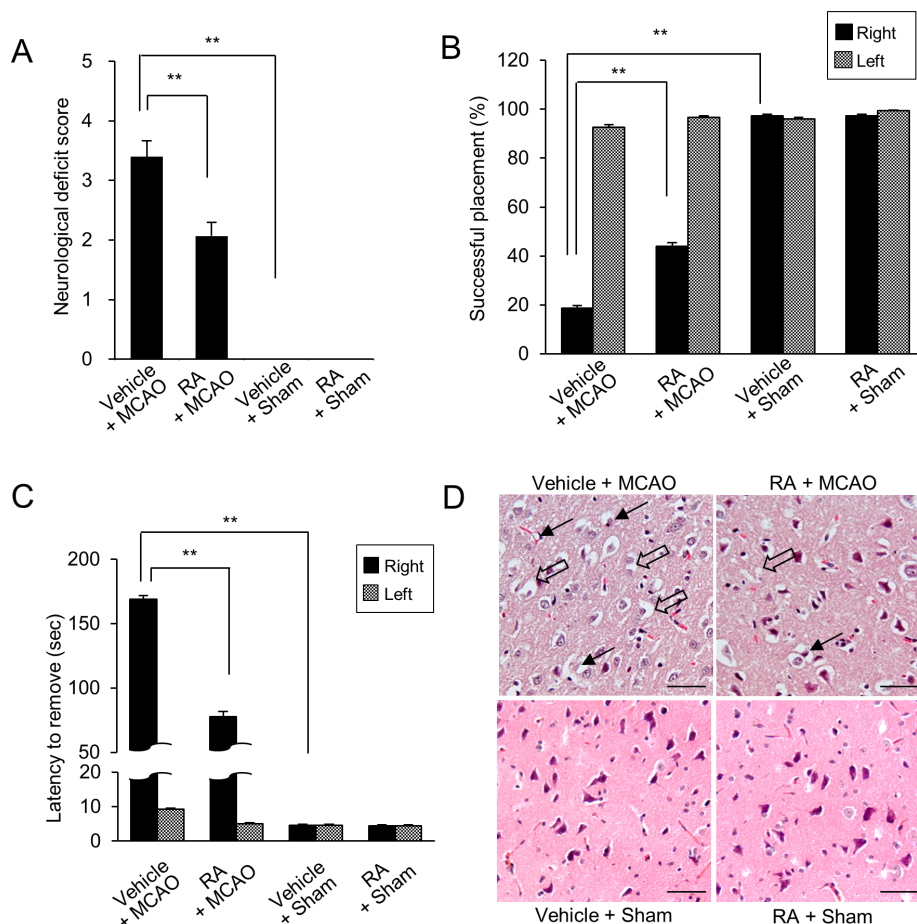
Fig. 1. Neurological deficit scoring test (**A**), vibrissae-evoked forelimb placing test (**B**), adhesive-removal somatosensory test (**C**), and hematoxylin and eosin staining (**D**) in vehicle + middle cerebral artery occlusion (MCAO), retinoic acid (RA) + MCAO, vehicle + sham, and RA + sham animals. RA improved neurological behavioral defects caused by MCAO and alleviated histopathological changes. Arrows indicate shrunken nuclei and open arrows indicate vacuoles cytoplasm. Scale bar=100 μm. Data (*n*=15 per group) are represented as the mean ± S.E.M. **P<0.01 vs. vehicle + MCAO animals.

forelimb placing test showed that MCAO damage induces decreased tactile and sensory functions in right vibrissae stimulation, and retinoic acid improves the dysfunction caused by MCAO (Fig. 1B). There were no significant changes on left stimulation. The percentages of successful placement of the right forelimb were $18.7 \pm 0.99\%$ and $44.0 \pm 1.41\%$ in vehicle + MCAO and retinoic acid + MCAO animals, respectively. Adhesive-removal test showed the change of sensorimotor function in left paw of MCAO animals (Fig. 1C). MCAO damage delayed the time to remove the adhesive tapes on the left paw and retinoic acid treatment restored these changes. There was no significant change in the time to remove the tape on the right paw in all animals, regardless of MCAO damage. The removal time was 168.9 ± 2.91 sec and 77.5 ± 4.29 sec in vehicle + MCAO animals and retinoic acid + MCAO animals, respectively. We observed the histopathological changes in the cerebral cortex of MCAO animals (Fig. 1D). Sham animals showed typical normal shapes and had pyramidal cells with large round nuclei and well-developed dendrites. However, MCAO damage induced morphological changes including nuclei condensation, cytoplasmic vacuoles, and dendrites shrinkage. Retinoic acid treatment alleviated these changes by MCAO. We observed reductions in cytoplasmic vacuole and nuclear condensation in retinoic acid-treated animals compared to the vehicle-treated animals.

Figure 2 shows images of proteins that were regulated by retinoic acid treatment in MCAO damage. These proteins were screened using two-dimensional gel electrophoresis. Nearly 537 protein spots were detected in each image. Twenty-nine protein spots were selected that had more than a two-fold difference in intensity between vehicle- and retinoic acid-treated animals with MCAO damage and no significant difference in intensity between sham group (Table 2). There were no other protein spots that are suitable in these conditions. Among these protein spots, 26 proteins were identified by MALDI-TOF analysis and the sequence coverage of these proteins was 17–61%. However, three protein spots were not matched and named as unknown proteins. We focused on adenosylhomocysteinase, isocitrate dehydrogenase [NAD$^+$] subunit α, glycerol-3-phosphate dehydrogenase, Rab GDP dissociation inhibitor β, apolipoprotein A1, and heat shock protein 60 that are related with cellular metabolism and stress. The expressions of adenosylhomocysteinase, isocitrate dehydrogenase [NAD$^+$] subunit α, glycerol-3-phosphate dehydrogenase, Rab GDP dissociation inhibitor β, and apolipoprotein A1 were decreased in the MCAO animals with vehicle, and retinoic acid treatment migrated these decreases. However, MCAO damage
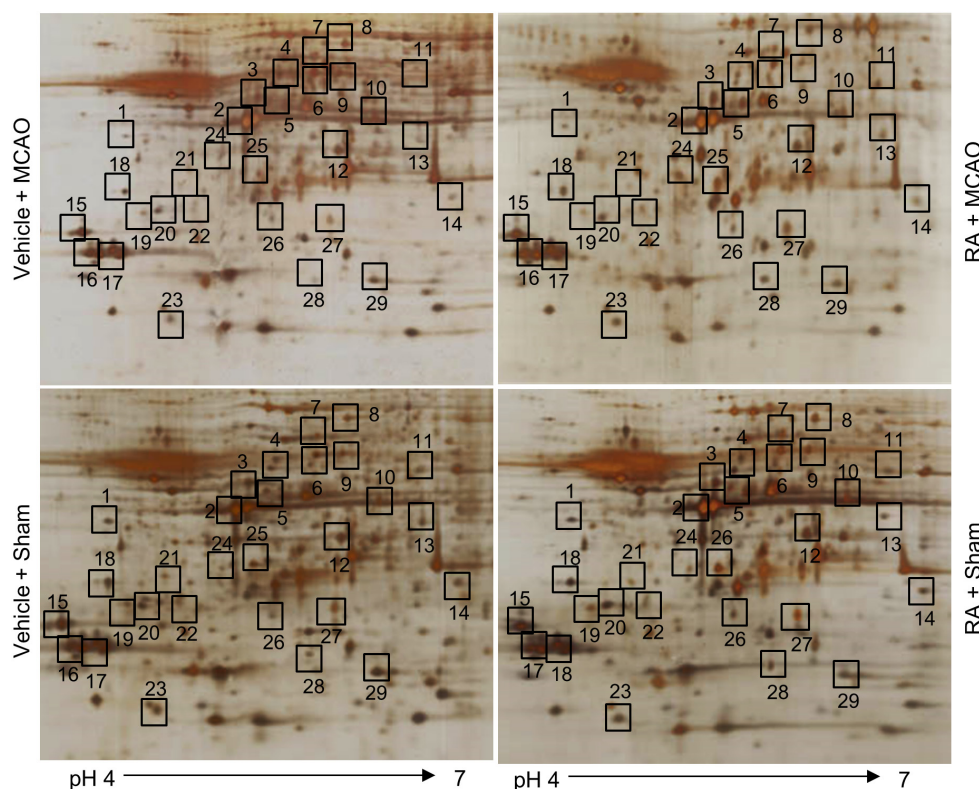
Fig. 2. Image of two-dimensional electrophoresis analysis in vehicle + middle cerebral artery occlusion (MCAO), retinoic acid (RA) + MCAO, vehicle + sham, and RA + sham animals (*n*=5 per group). Isoelectric focusing was performed at pH 4–7 IPG strips and electrophoresed on 7.5–17.5% gradient SDS gels. Squares indicate the significantly changed protein spots between vehicle + MCAO and RA + MCAO animals.

elevated heat shock protein 60 expression and this increase was attenuated by retinoic acid treatment. Regulated proteins by MCAO damage remained at the same level in sham-operated animals, regardless of vehicle or retinoic acid treatment.

Figure 3 shows the magnified pictures of these focused proteins. Expressions of adenosylhomocysteinase, isocitrate dehydrogenase [NAD$^+$] subunit α, glycerol-3-phosphate dehydrogenase, Rab GDP dissociation inhibitor β, and apolipoprotein A1 were decreased and heat shock protein 60 expression was increased in MCAO animals with vehicle. Retinoic acid treatment alleviated the MCAO-induced changes of these proteins. Moreover, expression levels of these proteins were similar in sham operated animals. The results of reverse transcription-PCR analysis showed similar changes to the results of proteomic study (Fig. 4). Adenosylhomocysteinase mRNA levels were 0.61 ± 0.05 and 1.28 ± 0.06 in the vehicle + MCAO animals and retinoic acid + MCAO animals, respectively. Isocitrate dehydrogenase [NAD$^+$] subunit α mRNA levels were 0.34 ± 0.02 in the vehicle + MCAO animals and 1.36 ± 0.05 in the retinoic acid + MCAO animals. Expression levels of glycerol-3-phosphate dehydrogenase mRNA were 0.30 ± 0.01 in the vehicle + MCAO and 0.91 ± 0.02 in the retinoic acid + MCAO animals. Rab GDP dissociation inhibitor β mRNA levels were 0.52 ± 0.02 in the vehicle + MCAO animals and 1.02 ± 0.02 in the retinoic acid + MCAO animals. Apolipoprotein A1 mRNA levels were 0.37 ± 0.02 in the vehicle + MCAO animals and 1.13 ± 0.02 in the retinoic acid + MCAO animals. Expression levels of heat shock protein 60 mRNA were 1.89 ± 0.05 in the vehicle + MCAO and 0.95 ± 0.05 in the retinoic acid + MCAO animals.

DISCUSSION

Cerebral ischemic damage causes neurological deficits and dysfunction, including cognitive impairment and memory loss [52]. Our previous study showed that retinoic acid alleviates neuronal disorder and infarction, and exerts neuroprotective effects against cerebral ischemia following MCAO [20]. Previous studies have shown that administration of retinoic acid before and after MCAO surgery has neuroprotective effects [22, 23, 47, 59]. We administered retinoic acid before MCAO surgery to demonstrate the preventive effect of retinoic acid on ischemic damage induced by MCAO. We confirmed the neuroprotective effects of retinoic acid against MCAO-induced cerebral ischemic damage through the evaluation of neurological behavioral tests. Cerebral ischemia induces severe neurological deficits and cognitive impairments, while retinoic acid prevents these dysfunctions. Retinoic acid also alleviates histopathological changes caused by MCAO damage. In addition, this study identified various proteins that are modulated by retinoic acid in MCAO damage. Among these identified proteins, the changes in adenosylhomocysteinase, isocitrate dehydrogenase [NAD$^+$] subunit α, apolipoprotein A1, glycerol-3-phosphate dehydrogenase, Rab GDP dissociation inhibitor β, and heat shock protein 60 protein were

Table 2. List of identified proteins which significantly differentially expressed in vehicle- and retinoic acid (RA)-treated animals with middle cerebral artery occlusion (MCAO)

Spot no.	Protein name	Accession no	Mw (kDa)	pI	Mass matched	Sequence Coverage (%)	Value of vehicle + MCAO vs. RA + MCAO
1	γ-enolase	P07323	47.14	5.00	14/70	34	*
2	Prolactin-8A5 isoform XI	P33579	27.26	5.47	7/97	22	*
3	Eukaryotic initiation factor 4A-II	Q5RKI1	46.73	5.33	16/82	40	***
4	Rab, GTPase-GDP dissociation stimulation stimulator 1	P52306	66.31	5.20	20/156	53	**
5	Eukaryotic initiation factor 4A-II	Q5RKI1	46.73	5.33	16/82	40	**
6	Dehydropyrimidinase-related protein 2	P47942	62.24	5.95	8/47	29	*
7	60 kDa heat shock protein	P63038	60.96	5.91	19/45	41	**
8	Unknown						**
9	Unknown						**
10	Unknown						**
11	Adenosylhomocysteinase	P10760	47.53	6.07	15/132	33	***
12	Isocitrate dehydrogenase [NAD$^+$] subunit α	Q99NA5	39.58	6.47	8/93	31	**
13	Rab GDP dissociation inhibitor β	P50399	50.50	5.90	9/150	23	***
14	Glycerol-3-phosphate dehydrogenase	O35077	37.43	6.16	8/105	25	**
15	14-3-3 Gamma	P61983	28.28	4.80	8/77	35	*
16	14-3-3 Zeta/delta	P63102	27.77	4.70	8/125	36	*
17	14-3-3 Beta/alpha	P35213	28.05	4.80	11/110	43	*
18	Ubiquitin carboxy-terminal hydrolase L1	Q7TQI3	27.61	4.85	11/66	58	***
19	Ubiquitin thiolesterase OTUB1	B2RYG6	31.27	4.80	14/39	61	*
20	Thioredoxin	Q920J4	32.23	4.84	8/87	42	*
21	Thioredoxin	Q920J4	32.23	4.84	8/87	42	**
22	Ubiquitin carboxy-terminal hydrolase L1	Q66HC4	27.61	5.60	2/126	17	**
23	Peroxiredoxin 2	Q61171	21.64	5.30	9/51	46	***
24	Mu-crystallin	Q9QYU4	33.55	5.34	9/86	24	***
25	Mu-crystallin	Q9QYU4	33.55	5.30	6/114	21	***
26	Unknown						**
27	Proteasome subunit alpha type3	P18422	28.40	5.30	7/112	27	***
28	Apolipoprotein A-I	P04693	30.04	5.25	16/116	50	***
29	PeroxiredoxIn-6	O35244	24.80	5.64	11/64	61	**

Protein names and accession numbers are listed according to the SWISS-PROT database. MW, molecular weight; pI, isoelectric point. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

further investigated. These proteins are associated with cell metabolism and stress response. The regulation of these proteins by retinoic acid in MCAO damage has been discussed.

Adenosylhomocysteinase is an enzyme that reversibly catalyzes the catabolism of adenosylhomocysteine to homocysteine and adenosine [39]. It is abundantly present in the hippocampus, cerebral cortex, cerebellum, and neocortex [42]. It is an inhibitor of methyltransferases and regulates intracellular adenosylhomocysteine concentration. Adenosylhomocysteinase plays an important role for transmethylation reactions [39]. Moreover, it regulates adenosylhomocysteine in streptozotocin-induced diabetes [37]. It also acts as a neuromodulator in brain tissues and exerts a neuroprotective effect against brain ischemic damage [39, 42]. This study showed that MCAO damage reduces adenosylhomocysteinase expression and retinoic acid attenuates this decrease. The reduction of adenosylhomocysteinase induces the accumulation of adenosylhomocysteine and decrease of methylation capacity, and it eventually leads to a decrease in adenosine level [39, 42]. Down-regulation of adenosylhomocysteine also indicates a decrease in adenosine activity. The maintenance of adenosylhomocysteine preserves adenosine levels and is involved in neuroprotection in ischemic damage. Deficiency of adenosylhomocysteinase is involved in DNA hypomethylation that induces pathological changes in ischemic stroke [56, 62]. Therefore, preventing adenosylhomocysteinase is an important role in protecting neurons. Regulation of adenosylhomocysteine expression by retinoic acid is considered an important event for protecting neurons. Therefore, it can be shown that retinoic acid contributes to neuroprotective effects in ischemic damage by controlling adenosylhomocysteinease expression and regulating adenosylhomocysteine levels.

Isocitrate dehydrogenase [NAD$^+$] subunit α is a key enzyme that regulates glucose metabolism. It catalyzes oxidative decarboxylation and transfers isocitrate to α-ketoglutarate in the tricarboxylic acid cycle [35]. It also inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidation [13]. This procedure generates NADPH and α-ketoglutarate, reduces oxidative stress, and alleviates oxidative damage [29]. NADPH oxidase is associated with ROS generation in the brain after cerebral ischemic damage [17]. Excessive production of ROS after ischemia injury is associated with increased NADPH oxidase [9]. Isocitrate dehydrogenase [NAD$^+$] subunit α is one of the most important producers of NADPH in the brain. Retinoic acid also has the ability to reduce oxidative stress and
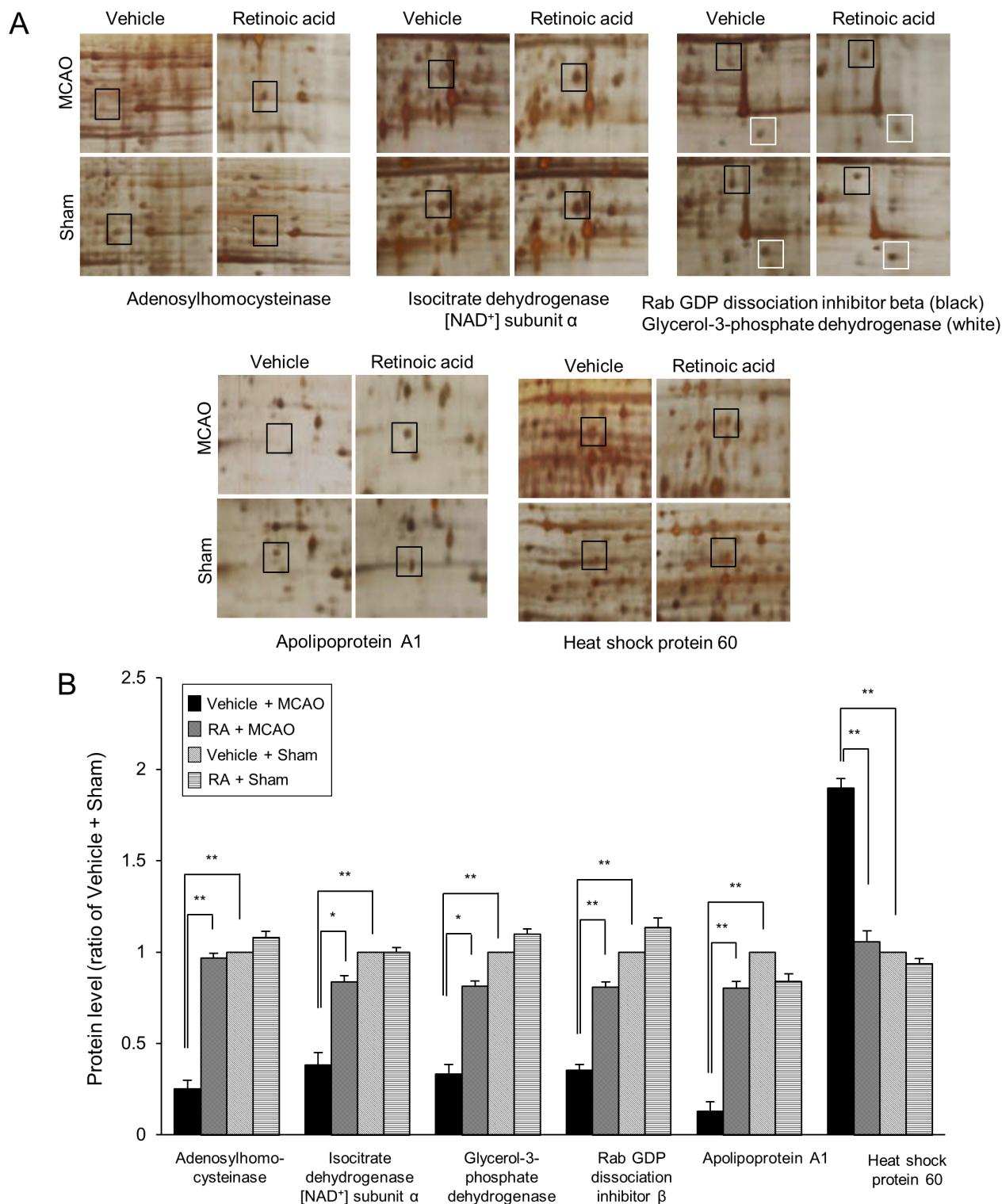
Fig. 3. Magnified protein spots (**A**) of adenosylhomocysteinase, isocitrate dehydrogenase [NAD$^+$] subunit α, cytosolic glycerol-3-phosphate dehydrogenase, Rab GDP dissociation inhibitor β, apolipoprotein A1, and heat shock protein 60 in the cerebral cortex from vehicle + middle cerebral artery occlusion (MCAO), retinoic acid (RA) + MCAO, vehicle + sham, and RA + sham animals. Each square indicates the protein spots. The band intensity of protein spot (**B**) is expressed as a ratio of that of vehicle + sham. Data (*n*=5 per group) are represented as the mean ± S.E.M. *P<0.05, **P<0.01 vs. vehicle + MCAO animals.

display a protective effect [1]. This study showed that MCAO damage reduces isocitrate dehydrogenase [NAD$^+$] subunit α expression, and retinoic acid attenuates MCAO-induced reduction in this protein and mRNA expressions. Thus, we suggest that regulation of isocitrate dehydrogenase [NAD$^+$] subunit α expression by retinoic acid in ischemia is associated with the neuroprotective mechanism
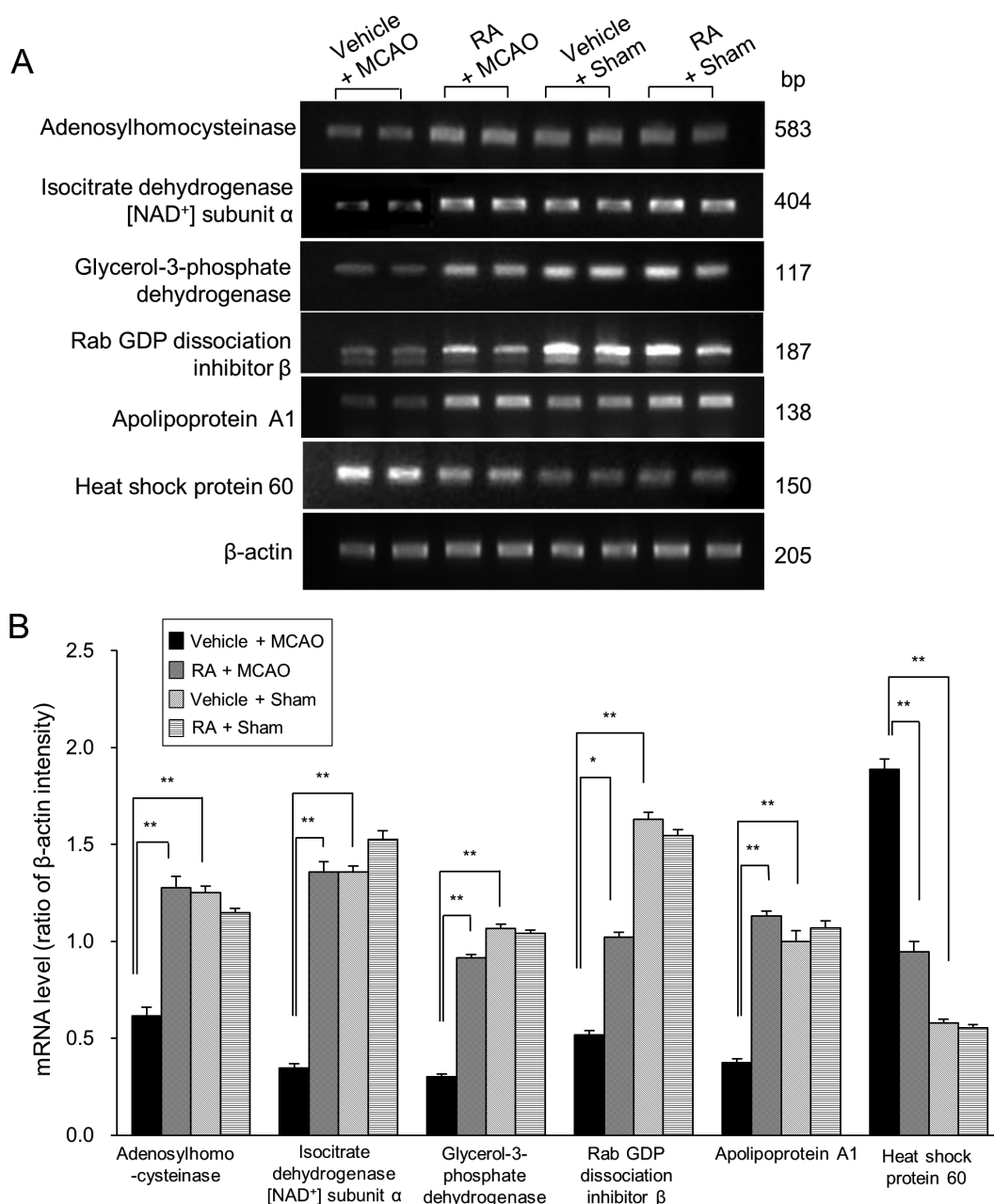
Fig. 4. Reverse transcription-PCR products (**A**) of adenosylhomocysteinase, isocitrate dehydrogenase [NAD$^+$] subunit α, cytosolic glycerol-3-phosphate dehydrogenase, Rab GDP dissociation inhibitor β, apolipoprotein A1, and heat shock protein 60 in the cerebral cortex from vehicle + middle cerebral artery occlusion (MCAO), retinoic acid (RA) + MCAO, vehicle + sham, and RA + sham animals. The band intensity of PCR product (**B**) is expressed as a ratio of β-actin product intensity. Data (n=5 per group) are represented as the mean ± S.E.M. *P<0.05, **P<0.01 vs. vehicle + MCAO animals.

of retinoic acid. Although further studies are needed to investigate the relationship between retinoic acid and isocitrate dehydrogenase [NAD$^+$] subunit α, it is evident that alleviation of isocitrate dehydrogenase [NAD$^+$] subunit α reduction by retinoic acid treatment during cerebral ischemia contributes to the neuroprotective effect of retinoic acid.

Glycerol-3-phosphate dehydrogenase acts as a major link between carbohydrate and lipid metabolism, and is highly expressed in neurons and astrocytes [40, 60]. It modulates lipid biosynthesis, regulates the redox potential in the inner mitochondrial membrane, and also contributes to the electron transfer chain of mitochondria [60]. It exists as a mitochondrial and cytosolic type. Mitochondrial glycerol-3-phosphate dehydrogenase catalyzes the glycerol-3-phosphate to dihydroxyacetone phosphate. Cytosolic glycerol-3-phosphate dehydrogenase reverses dihydroxyacetone phosphate to glycerol-3-phosphate [40]. It oxidizes NADH and produces NAD$^+$ during catalytic reactions and is recognized as a major regulatory factor in learning and memory [33]. Cerebral ischemic damage accelerates hyperglycemia, reduces glycerol-3-phosphate expression, and causes excessive production of dihydroxyacetone phosphate

[19]. The deficiency of cytosolic glycerol-3-phosphate dehydrogenase increases lipid oxidation [46]. Furthermore, hyperglycemia worsens brain damage by reducing NAD^+ and overproducing ROS [19]. Thus, glycerol-3-phosphate and NAD^+ level maintenance contributes to neuroprotective effects against ischemic brain damage [30]. The neuroprotective effects of retinoic acid have been identified through a variety of experimental approaches, including neurobehavioral tests and brain edema and infarction measurements [20]. Retinoic acid performs neuroprotective functions against MCAO damage by controlling apoptosis-related proteins. Moreover, retinoic acid decreases hyperglycemia and induces nerve regeneration in diabetic neuropathic animal models [11]. The proteomic approach showed that MCAO damage reduces cytosolic glycerol-3-phosphate dehydrogenase expression, and retinoic acid treatment attenuates this decrease. These results were confirmed by reverse transcription-PCR analysis. We can demonstrate that the maintenance of cytosolic glycerol-3-phosphate dehydrogenase by retinoic acid contributes to the neuroprotective effect of retinoic acid against MCAO damage can be demonstrated. Therefore, our findings suggest that retinoic acid exerts neuroprotective effects by regulating cytosolic glycerol-3-phosphate dehydrogenase expression against MCAO damage.

Rab GDP dissociation inhibitors are proteins that react with Rab proteins to regulate GDP/GTP exchange reactions [36]. They transport Rab proteins to target membranes and recover Rab proteins after a completed catalytic cycle [36]. They have the ability to control Rab3A, which regulates calcium exocytosis and synaptic vesicles release [41]. Rab GDP dissociation inhibitors are mainly expressed in brain tissue and have two isoforms; Rab GDP dissociation inhibitor α and β [38]. Rab GDP dissociation inhibitor β is expressed ubiquitously and regulates vesicular trafficking in various types of cells [38]. It regulates GTPases access to GTPase activating protein and regulatory guanine nucleotide exchange factors [36]. In brain damage, oxidative stress reduces Rab GDP dissociation inhibitors expression, increases apoptosis, and diminishes synaptic function [18]. Rab GDP dissociation inhibitor β also induces apoptosis in various tumor cells [24, 34]. Furthermore, regulation of Rab GDP dissociation inhibitors expression exerts a neuroprotective effect against ischemic damage by inhibiting excitotoxicity [5]. The decrease of Rab GDP dissociation inhibitor β increases neuronal excitotoxicity in the ischemic cerebral cortex. The proteomic approach determined a decrease in Rab GDP dissociation inhibitor β expression in MCAO-induced cerebral cortex damage, and retinoic acid treatment mitigated this decrease. Moreover, reverse transcription-PCR analysis confirmed the regulation of Rab GDP dissociation inhibitor β expression by retinoic acid in MCAO damage. Retinoic acid alleviated reduction of Rab GDP dissociation inhibitor β expression due to MCAO damage and maintained its normal condition. In ischemic condition, decrease of Rab GDP dissociation inhibitor β induces excitotoxicity and synaptic dysfunction, and leads to apoptotic cell death. Therefore, it can be thought that the regulation of Rab GDP dissociation inhibitor β expression plays an important role in ischemic brain damage. This study shows that retinoic acid attenuates the decrease in Rab GDP dissociation inhibitor β in brain injury. Thus, these findings suggest that preserving Rab GDP dissociation inhibitor β expression by retinoic acid treatment alleviates brain damage and protects brain tissues from ischemic damage.

Apolipoprotein A1 is a major protein component of high-density lipoprotein (HDL) and is abundant in cerebrospinal fluid [10]. It is a cholesterol carrier in the central nervous system and has neuroprotective effects by inhibiting inflammatory reactions and controlling the polymerization of actin filaments after neuronal damage [49]. Moreover, apolipoprotein A1 regulates brain lipid homeostasis because it can enter the choroid plexus [53]. HDL and apolipoprotein A1 have various properties including anti-inflammatory and antioxidant effects [54]. They attenuate mitochondrial damage through inhibition of ROS formation [58]. The deficiency of apolipoprotein A1 expression exacerbates cognitive deficiency in an Alzheimer's disease model and increases cerebral amyloid angiopathy [25]. Ischemic stroke reduces the apolipoprotein A1-specific peptide that can be used as a diagnostic biomarker for acute ischemic stroke [63]. The decrease in apolipoprotein A1 increases subcortical infarction and is associated with a high risk of ischemic stroke [3]. Apolipoprotein A1 expression activates the extracellular signal-regulated kinase pathway and contributes to actin polymerization, and decreases neuronal injury [49]. It also mitigates morphologic changes and reduces infarct size in heart ischemic models [58]. Furthermore, retinoic acid treatment increases the expression of apolipoprotein A1, upregulates cellular retinol binding protein II, and leads to intestinal vitamin A absorption [27]. The proteomic approach showed reduced expression of apolipoprotein A1 after MCAO-induced injury through a proteomic approach and reverse transcription-PCR and retinoic acid treatment alleviates this decrease. The study also confirmed the regulation of apolipoprotein A1 mRNA expression by retinoic acid. Thus, it clearly demonstrated that retinoic acid regulates apolipoprotein A1 expression and contributes to neuroprotection in cerebral ischemic damage.

Heat shock protein 60 is a family of heat shock proteins that has 60 kDa molecular weight. Heat shock protein 60 is a stress-induced mitochondrial protein and is known as chaperonins. It plays an important role in eliminating misfolded proteins and folded protein aggregation in harmful conditions, such as heat and stress. Moreover, heat shock protein 60 expression is significantly raised in focal cerebral ischemia [14, 57]. Heat shock protein 60 is considered as a marker of damage caused by stress. Ischemic brain damage induces accumulation of abnormal proteins and leads to neuronal cell damage. It has been reported that ischemic brain damage significantly increases the expression of heat shock protein 60. An increase in heat shock protein 60 expression in focal cerebral ischemia was previously identified [50]. Preventing heat shock protein 60 overexpression regulates the inflammatory pathway, modulates the activation of microglial cells, and exerts neuroprotective effects microglia activation, and exerts neuroprotective effect [6]. This study showed the alleviation of MCAO-induced up-regulated heat shock protein 60 expression by retinoic acid treatment. Attenuation of heat shock protein 60 increased by retinoic acid represents a mitigation of damage from ischemic injury and a protective effect of retinoic acid. This study demonstrated that retinoic acid modulates specific proteins and protects cortical neurons from cerebral ischemic damage caused by MCAO. Retinoic acid alleviates the expression of adenosylhomocysteinase, isocitrate dehydrogenase [NAD^+] subunit α, cytosolic glycerol-3-phosphate dehydrogenase, Rab GDP dissociation inhibitor β, and apolipoprotein A1 decreased by MCAO. Conversely, MCAO damage increases heat shock protein 60 expression in the cerebral cortex and retinoic acid attenuates this increase. Adenosylhomocysteinase, isocitrate dehydrogenase [NAD^+] subunit α, and cytosolic glycerol-3-phosphate dehydrogenase are metabolic enzymes involved in energy and glucose metabolisms. Rab GDP dissociation inhibitor β is a protein that regulates GTPase

and performs various functions. Apolipoprotein A1 is a cholesterol carrier that regulates lipid homeostasis and protects nerve cells from damage. Furthermore, heat shock protein 60 is accepted as a representative stress protein. The described proteins in this study are associated with energy and glucose metabolisms, and stress response for the maintenance and survival of cells. These findings showed that retinoic acid regulates various proteins that mediates cell metabolism and function and exerts neuroprotective effects against cerebral ischemia. In conclusion, we suggest that retinoic acid performs neuroprotective function by controlling specific proteins.

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