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Brain Regions with Reduced Amounts of Mevalonate Pyrophosphate Decarboxylase Correspond to Sites of Strokes in Stroke-Prone Spontaneously Hypertensive Rats

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Cholesterol deficiency is believed to result in fragile plasma membranes. It remains unclear whether a reduction in the amounts of both mevalonate pyrophosphate decarboxylase (MPD), which is involved in cholesterol biosynthesis, and cholesterol content occurs in the cerebrum and brain stem (diencephalon and midbrain) in or near the sites of strokes in stroke-prone spontaneously hypertensive rats (SHRSP). In this study, we investigated whether a reduction in both the amounts of MPD and cholesterol content corresponded to the sites of strokes in the SHRSP brain. The results obtained suggested that a reduction in the amount of MPD was involved in the decrease observed in cholesterol content, and was also important as a risk factor for stroke in SHRSP because the reductions in cholesterol content and MPD protein levels were associated with the sites of strokes. The mechanism responsible for reducing MPD protein levels in the brains of SHRSP differed with each region.

Key words mevalonate pyrophosphate decarboxylase, cholesterol, brain, stroke-prone spontaneously hypertensive rats

INTRODUCTION

Malignant neoplasms, heart disease, and stroke (cerebral hemorrhage) are the major causes of death in worldwide.[1] The major cause of stroke is hypertension, although an epidemiological study has identified a lower serum cholesterol level as another cause of stroke.[2] Cholesterol is a major constituent of cellular membranes; therefore, a reduction in cholesterol content in cells may lead to fragile plasma membranes.[3] Cholesterol is provided to vascular endothelial cells (VECs) in the brain via two pathways: the uptake of cholesterol from the serum, and the uptake of cholesterol biosynthesized in brain cells. Consequently, the reduced biosynthesis of cholesterol by the brain and lower cholesterol levels in the serum may induce angionecrosis in the brain.

The spontaneously hypertensive rat (SHR) is a widely used animal model of hypertension.[4] However, the incidence of hypertensive complications, such as stroke in SHR, was shown to be lower than that of other causes of death.[4] Stroke-prone spontaneously hypertensive rats (SHRSP) were reported to develop severe hypertension of approximately 240 mmHg and die due to stroke after a period of a few days to 24 weeks after the initial symptoms of stroke were noted.[4] Serum cholesterol levels in SHRSP were shown to be lower than those in normotensive age-matched Wistar Kyoto rats (WKY).[5] Therefore, we demonstrated that some factors other than hypertension (a reduction in serum cholesterol levels or the induction of oxidative stress) may be involved in the development of strokes in SHRSP as well as humans.[6,7] Sawamura *et al*. reported that in the livers of SHRSP, the activities of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, mevalonate kinase, and phosphomevalonate kinase, which play roles in the cholesterol biosynthetic pathway, were not significantly different from those in WKY.[6] We previously reported that in the livers of SHRSP, reductions in the activity and expression (protein and mRNA levels) of mevalonate pyrophosphate decarboxylase (MPD),[6,8,9] and hydroxysteroid 17-β dehydrogenase 7 (HSD17B7),[10] in expression of squalene epoxidase (SQE),[11] which is an enzyme involved in cholesterol biosynthesis, may be attributed to low serum cholesterol levels. Furthermore, when the tissue distribution of MPD was examined in WKY and SHRSP, we found that MPD levels were significantly lower in the brains of SHRSP.[12] The brain contains a quarter of all cholesterol in the body.[13] Cholesterol maintains membrane-rich structures such as axons, dendrites, and synapses and is a major constituent of the plasma membrane in VECs in the brain. Thus, reduced MPD levels in SHRSP may cause severe dysfunction in the nervous system and ultimately, stroke. We previously reported that MPD protein levels in the brains of SHRSP were reduced due to the accelerated degradation of proteins, or other regulation mechanisms, because MPD mRNA levels in SHRSP were similar to those in WKY whereas protein levels were lower.[8,9,14] Protein levels, or the regulation of HMG-CoA reductase, in the brains of rats have been shown to differ in each region (the hippocampus, cortex, cerebellum, and brain stem, containing the midbrain and diencephalon).[15] Differences have also been identified in the profiles of cholesterol biosynthetic enzymes between neurons and glial cells from postnatal rats.[16] Based on these findings, we reasoned the reduction in MPD protein levels and cholesterol content differs according to brain region in SHRSP. Lesions in the brains of SHRSP are commonly

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located in the cortical or subcortical areas of the telencephalon (cerebrum), but have also been detected in the basal ganglia and intraventricular area (diencephalon and midbrain).[4] Sites exhibiting a reduction in MPD levels and cholesterol content in SHRSP have been associated with the development of cell damage of strokes in SHRSP, which suggests that the decrease in cholesterol levels due to a reduction in MPD levels may be an important factor of stroke. Therefore, reductions in MPD levels and cholesterol content need to be investigated across brain regions in WKY and SHRSP. In the present study, we determined whether a reduction in MPD levels and cholesterol content occurred in each region of the SHRSP brain, and also if brain regions with reduced cholesterol contents corresponded to the sites with decreased MPD protein levels in SHRSP. We also attempted to elucidate the mechanism responsible for reducing MPD protein levels in SHRSP.

MATERIALS AND METHODS

Animals Male (10-week-old) WKY (approximately 150 mmHg) and SHRSP (approximately 240 mmHg) were obtained from the Disease Model Co-operative Research Association, Japan. The experimental protocol was reviewed and approved by the Animal Care and Use Committee of Fukuyama University.

Cholesterol Levels in the Brain Brain tissues (100 mg) from each site (cerebrum, diencephalon, midbrain, cerebellum, pons, and medulla oblongata) were homogenized in 500 µL of homogenization buffer (50 mM Tris–HCl, pH 7.5 containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitors [1 mM leupeptin, 1 mM pepstatin A, 1 mM chymostatin, and 1mM antipain]) and centrifuged at 1000×g for 10 min. Forty microliters of postnuclear supernatant was mixed with 5 mL of Folch extract (chloroform–methanol, 2:1), and the mixture was incubated for 10 min at 37 °C. After the mixture was centrifuged at 3000×g for 10 min, 3 mL of the supernatant was evaporated to dryness by boiling at 100 °C and was then dissolved in 200 µL of isopropyl alcohol containing 1% Triton-X-100. Total cholesterol (containing free cholesterol and cholesterol esters) in the solution (20 µL) was determined using the Cholesterol E-test Wako (Wako, Osaka, Japan).

Protein Assay Protein levels were measured by the method of Lowry *et al*. using bovine serum albumin (BSA) as the standard.[17]

Immunoblot Analysis Sample preparation and immunoblot analysis were performed as described previously.[18] Each area (15 mg) of the brain was homogenized in 50 µL of homogenization buffer containing 0.1% Triton X-100 and centrifuged at 15,000×g for 10 min. The supernatants (sample) were subjected to SDS-PAGE followed by immunoblot analysis using anti-GAPDH IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-rat MPD antiserum raised against the 45 kDa MPD of rats, and goat anti-rabbit IgG conjugated to horseradish peroxidase (Invitrogen, Carlsbad, CA). SDS-PAGE was performed on 12% slab gels, and the blots were transferred to a nylon membrane by electrophoresis. The protein bands were visualized using the ECL Western blotting detection kit (Amersham Pharmacia, Amersham, UK) that contained a sensitive chemiluminescent substrate for horseradish peroxidase. Signals were measured by the Intelligent Quantifier (Bio Image). Relative protein levels of MPD were quantified using GAPDH as an internal control.

Quantitative Reverse Transcription-PCR (qRT-PCR) Rat brains (15 mg) were homogenized using a Biomasher (Nippi, Tokyo, Japan). The homogenate was dissolved using QuickGene RNA tissue kit SII (Fujifilm, Tokyo, Japan), and total RNA (60 µL) was isolated using the same kit and QuickGene-810 of Nucleic Acid Isolation System (Fujifilm). Total RNA (100 ng) from each tissue was subjected to reverse transcription (RT) using reverse transcriptase in a 50 µL reaction volume. Afterward, the cDNA template was amplified by using the SYBR Ex Script RT-PCR Kit (Takara Bio, Shiga, Japan). SYBR Green was used for the real-time PCR analysis of MPD (Fw-AGGACCGCATCTGGCTGAAC, Rv-TGACGCCACGTGCACCTTA; PCR products of 158 bp). Real-time PCR was performed using the ABI7500 system (Applied Biosystems Japan, Tokyo, Japan). The PCR conditions were 40 cycles of 96°C for 35 s, 64°C for 35 s, and 72°C for 35 s. Relative MPD mRNA levels were quantified using GAPDH (Fw-GGCACAGTCAAGGCTGAGAATG, Rv-ATGGTGGTGAAGACGCCAGTA; PCR products of 143 bp) as an internal control.

qRT-PCR of microRNA Rat brains (15 mg) were homogenized with a Biomasher (Nippi). After the homogenate was dissolved with the appropriate reagents from the High Pure miRNA Isolation Kit (Roche, Mannheim, Germany), total RNA containing miRNA (*miR*) was isolated from the homogenate using the column of the same kit. Samples of total RNA (2 µg) from each group of tissues were subjected to RT using Taqman Micro RNA reverse transcriptase and Taqman Micro RNA primer (has-miRNA-214 or U6 snRNA) in a 20 µL reaction volume. Afterward, the cDNA template was amplified by using the Taqman Micro RNA Assay Kit (Life Technologies Japan, Tokyo, Japan). qRT-PCR analysis was performed using an ABI7500 system (Applied Biosystems Japan). Relative gene expression of *miR-214* was quantified using *U6 snRNA* as an internal control.

Statistical Analysis Statistical analysis was carried out using the Student's *t*-test. Data are presented as the mean ± SD.

RESULTS

Comparison of Cholesterol Contents in WKY and SHRSP To examine whether a reduction in cholesterol content occurred in each region of the SHRSP brain, and also whether brain regions with reduced cholesterol contents corresponded to the sites of strokes in SHRSP, we first evaluated the overall cholesterol content in rats. Statistical analysis was carried out using the Student's *t*-test (n=3). Cholesterol contents in the cerebrum, midbrain, diencephalon, and pons were lower, while contents in the cerebellum were higher in SHRSP than in WKY (Fig. 1). No significant differences were observed in cholesterol contents in the medulla oblongata between SHRSP and WKY. Total serum cholesterol levels were also low in SHRSP, as described previously (WKY: 117±9 mg/dL, SHRSP: 67±14 mg/dL; data not shown). These results indicated that the reductions in cholesterol contents in the brains of SHRSP differed with each region, and also that brain regions with reduced cholesterol contents corresponded to the sites (cerebrum [cortical or subcortical area of the telencephalon] and brain stem [diencephalon and midbrain containing the

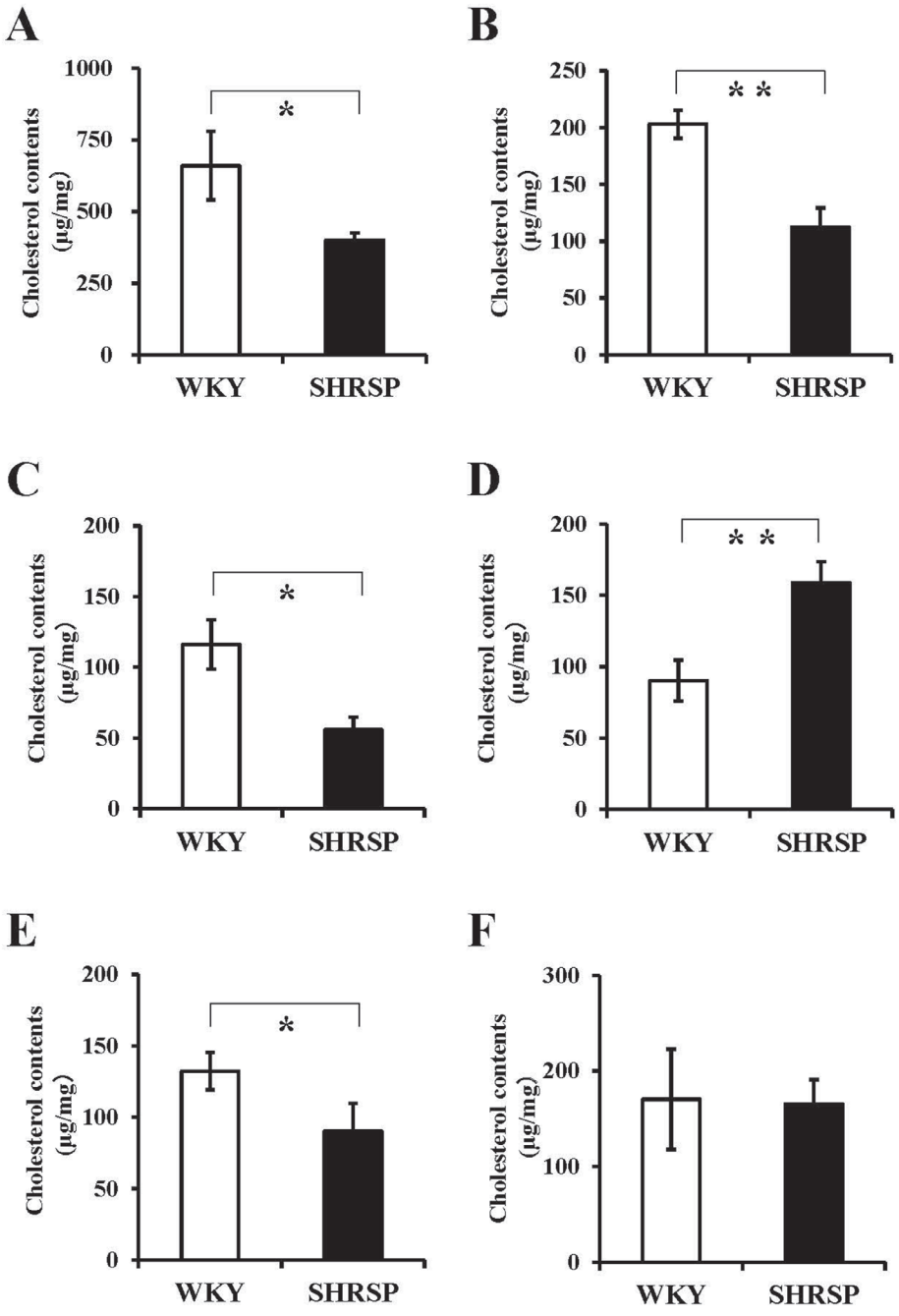

Fig. 1. Cholesterol Contents in the Brains of WKY and SHRSP

Cholesterol contents in the cerebrum (A), diencephalon (B), midbrain (C), cerebellum (D), pons (E), and medulla oblongata (F) of WKY and SHRSP, measured by a Cholesterol E-test Wako, as described in the Methods. Values are the means of three independent experiments. Significantly different: $*p < 0.05$, $**p < 0.005$

basal ganglia and intraventricular area]) of strokes in SHRSP.

Comparison of MPD Protein Levels in WKY and SHRSP In order to establish whether a reduction in MPD protein levels occurred in each region of the SHRSP brain, and if brain regions with reduced MPD protein levels corresponded to the sites of strokes or the sites of low cholesterol levels in SHRSP, we next investigated MPD protein levels. MPD protein levels in the cerebrum, diencephalon, midbrain, and pons, as measured by immunoblot analysis, were significantly lower in SHRSP than in WKY (Fig. 2). No significant difference was observed in MPD levels in the cerebellum or medulla oblongata between SHRSP and WKY. MPD protein levels at all sites in the SHRSP brain were not significantly different from those in the WKY brain. These results indicated that the reduction in MPD protein levels, as well as cholesterol contents in the brains of SHRSP, differed according to the site. Furthermore, sites in SHRSP brains that had reduced MPD levels corresponded to the sites with low cholesterol contents, as well as the sites of strokes.

Comparison of MPD mRNA Levels in WKY and SHRSP Lower mRNA levels of MPD were detected in the cerebrum, midbrain, cerebellum, and medulla oblongata of SHRSP than those in WKY, as measured by qRT-PCR (Fig. 3). MPD levels in the diencephalon and pons in SHRSP were similar to those in WKY. Based on these results, the mechanisms responsible for reducing MPD protein levels in the brains of SHRSP dif-

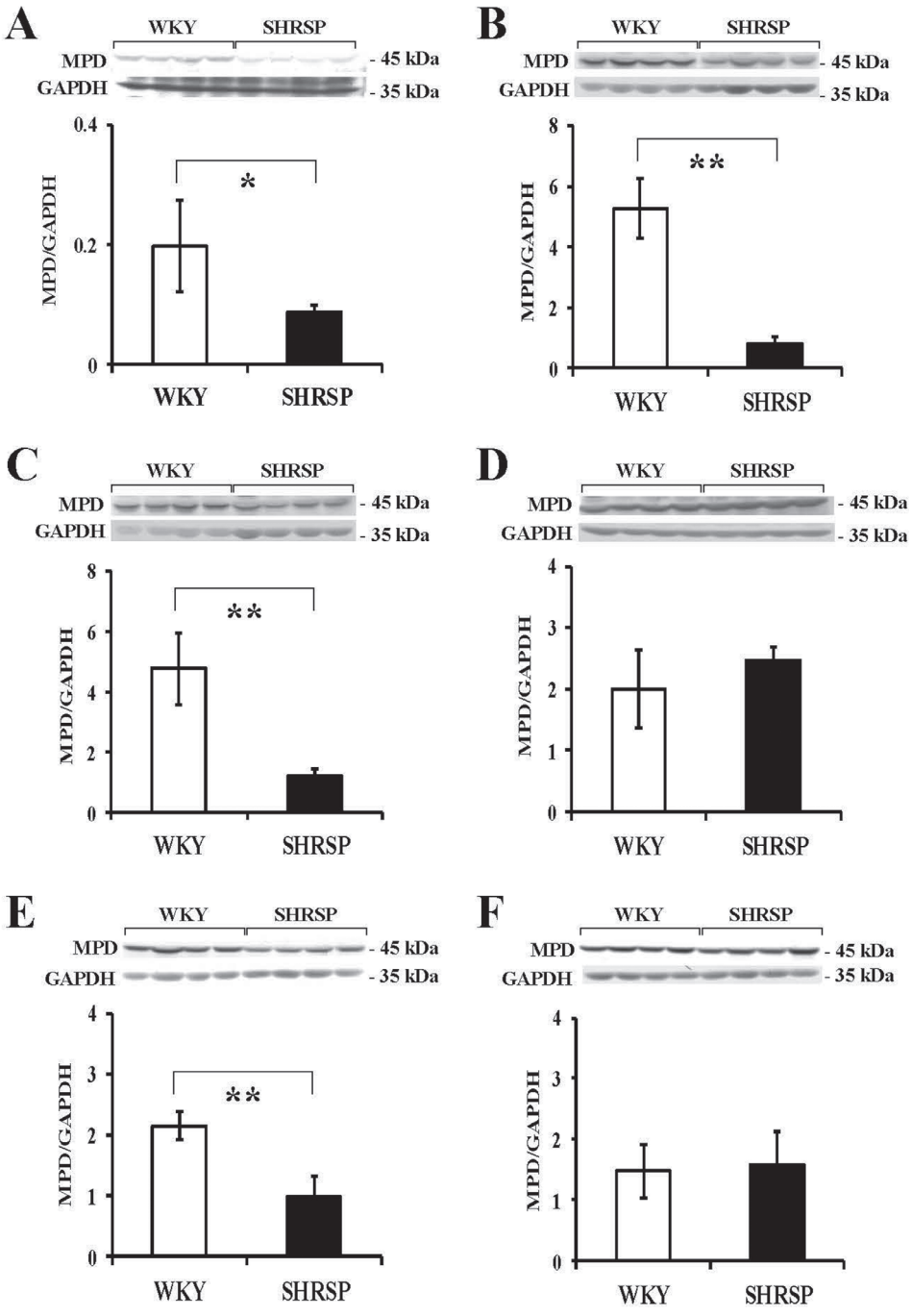

Fig. 2. MPD Protein Levels in the Brains of WKY and SHRSP

Samples (15 μg) from the cerebrum (A), diencephalon (B), midbrain (C), cerebellum (D), pons (E), and medulla oblongata (F) of WKY and SHRSP were subjected to SDS-PAGE followed by immunoblot analysis. MPD bands at each site of the brain were quantified using the Intelligent Quantifier. Relative protein levels of MPD were quantified using GAPDH as an internal control. Values are the means of four independent experiments. Significantly different: $*p < 0.05$, $**p < 0.005$

fered with each region. The results of the present study indicated that MPD mRNA levels in the brain regions of SHRSP differed according to the collection site. We previously reported that mRNA levels were similar in the brains of SHRSP and WKY[9]; however, MPD mRNA levels might have been measured in the diencephalon rather than in the cerebrum at that time.

Comparison of *miR-214* Expression Levels in SHRSP and WKY microRNA was recently shown to cause a reduction in protein levels by inhibiting its synthesis from mRNA or accelerating the degradation of mRNA.[19,20] We previously reported the upregulation of *miR-214* and downregulation of MPD transcription in the liver of SHRSP rats, and that *miR-214* was directly affected by binding to 3′-untranslated region (UTR) of MPD mRNA.[21] Thus, we investigated whether *miR-214* was involved in reducing MPD mRNA levels in the brain. *miR-214* levels in the cerebrum, midbrain, diencephalon, pons, and medulla oblongata of SHRSP were similar to those in WKY, whereas lower *miR-214* levels were detected in the cerebellum (Table 1). These results indicated that *miR-214* in the

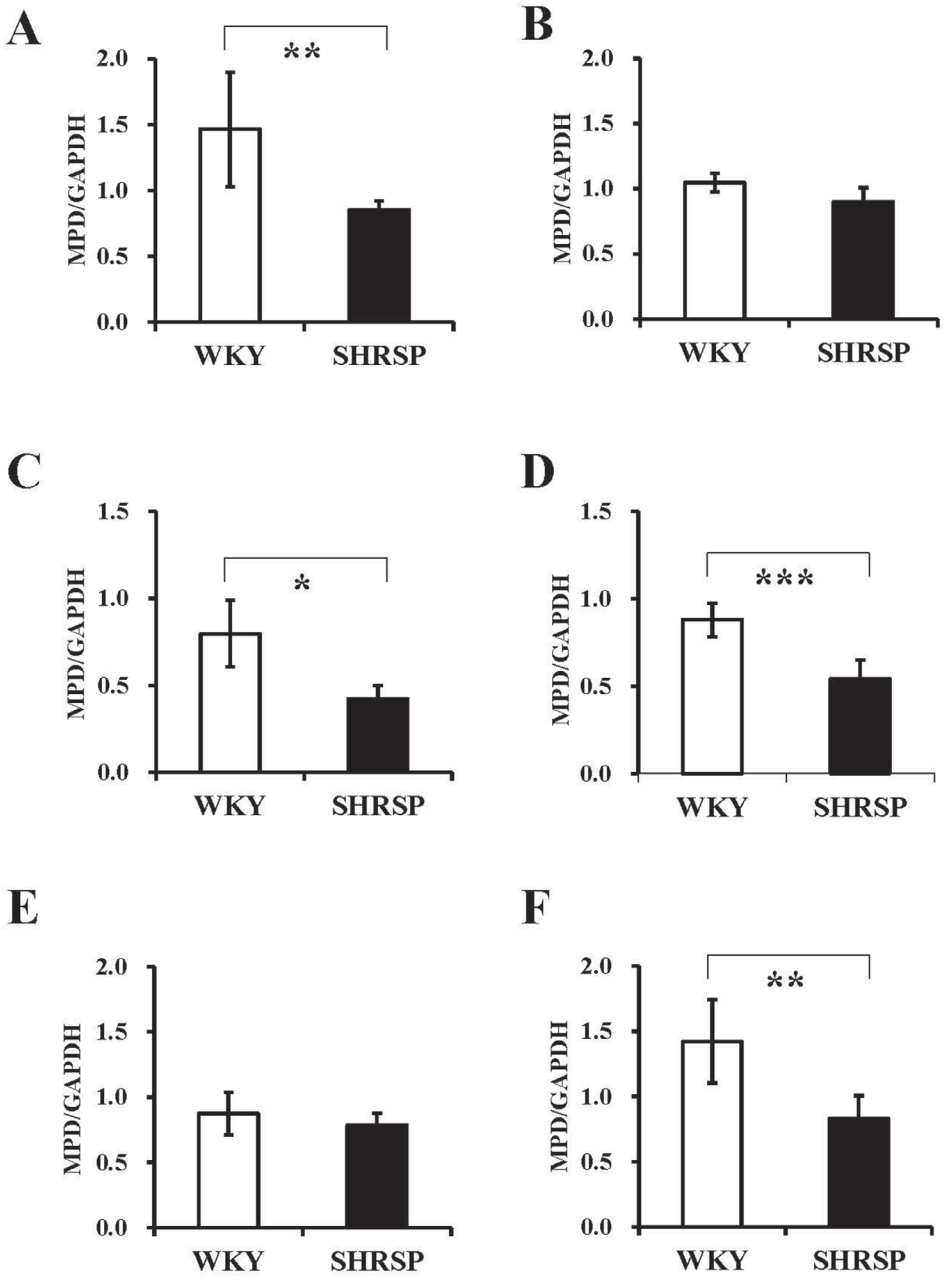

Fig. 3. MPD mRNA Levels in the Brains of WKY and SHRSP

Real-time PCR was performed using primer pairs for MPD or GAPDH from total RNA in the cerebrum (A), diencephalon (B), midbrain (C), cerebellum (D), pons (E), and medulla oblongata (F) of WKY and SHRSP. Relative protein levels of MPD were quantified using GAPDH as an internal control. Values are the means of three or four independent experiments. Significantly different: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$

Table 1. *miR-214* Levels in the Brains of WKY and SHRSP

Brain regions	WKY	SHRSP
Cerebrum	1.06 ± 0.13	1.04 ± 0.27
Diencephalon	0.92 ± 0.24	0.69 ± 0.20
Midbrain	1.04 ± 0.66	1.72 ± 1.00
Pons	1.69 ± 1.07	1.65 ± 0.22
Cerebellum	1.25 ± 0.26	0.59 ± 0.10*
Medulla oblongata	1.55 ± 0.93	1.61 ± 1.17

*n= 3~4 *P < 0.005*

cerebrum, midbrain, diencephalon, pons, and medulla oblongata of SHRSP was not involved in reducing MPD mRNA levels.

DISCUSSION

In the present study, we demonstrated that the reductions in cholesterol contents and MPD protein levels were associated with the cell damage elements (cerebrum, midbrain, and diencephalon) of strokes in SHRSP (Fig. 1, 2). The decrease observed in cholesterol contents may have been caused by a reduction in MPD protein levels. The decrease in cholesterol levels due to the reduction in MPD is considered to have

an influence on VECs in or near the cerebrum (cortical layer) and brain stem (basal ganglia and intraventricular area) of SHRSP. We previously reported that the increase in oxidative stress in the brain of SHRSP is intrinsic (a direct effect of oxidative stress in the brain itself), although the reason for such high levels remains unclear.[7,22] Thus, it is not only damaged VECs in or near the cerebrum and brain stem of SHRSP that may contribute to hypertension, but also the high levels of oxidative stress and decrease in cholesterol levels (the reduction in cholesterol in the serum and in the brain cell itself) due to a reduction in MPD.

Furthermore, we indicated that the mechanism responsible for reducing MPD protein levels in the brains of SHRSP differed with each region. We suggested that the reduction in mRNA levels may be an important cause of the decrease in MPD protein levels in the cerebrum and midbrain of SHRSP. We previously reported that MPD protein levels in the brains of SHRSP were reduced from as early as 2 weeks of age.[12] Therefore, the reduction in MPD mRNA levels in the cerebrum and midbrain of SHRSP may occur from 2 weeks of age. We also hypothesized that the lower MPD mRNA levels in the cerebrum and midbrain of SHRSP were caused by an increase in repressor binding to the silencer element in the promoter sequence of MPD, or a decrease in activator binding to the enhancer element in the promoter region of MPD from 2 weeks of age, if the reduction in MPD mRNA levels that occurred due to changes in transcriptional factors is considered. However, the promoter element containing the silencer and enhancer of MPD was not examined. Therefore, the promoter region and transcriptional factors of MPD need to be identified in order to clearly understand the mechanism responsible for reducing MPD mRNA levels in the SHRSP brain. It is also possible that the reduction in MPD protein levels in the cerebrum and midbrain of SHRSP occurred due to the accelerated degradation of proteins or another regulation mechanism. A large number of miRNAs were detected when the miRNAs that combined with the 3′-UTR of the cholesterol biosynthetic enzyme were searched using miRBASE.[23] For example, the numbers of miRNAs that combined with the 3′-UTR of HMG-CoA reductase and Squalene synthase were 27 and 13, respectively.[21] However, the only miRNA that combined with the 3′-UTR of MPD was *miR-214*. We previously reported that *miR-214* exerted a direct effect by binding to 3′-UTR of MPD mRNA.[21] Therefore, the lower MPD mRNA levels in the cerebrum and midbrain of SHRSP may have been caused by an increase in a novel miRNA combining with the 3′-UTR of MPD, if the reduction in MPD mRNA levels that occurred due to the novel miRNA is considered.

Although MPD protein levels were reduced in the diencephalon and pons of SHRSP, mRNA levels were similar to those in WKY (Fig. 2, 3). It was previously reported that the protein level of insulin receptors was decreased in ataxin-2 knock-out mice, although the mRNA levels increased.[24] Namely, it was indicated that the reduction in the protein level of insulin receptors occurred as a post-transcriptional event. The low MPD protein levels in the diencephalon and pons of SHRSP from 2 weeks of age may be caused by a post-transcriptional effect (accelerated protein degradation or another regulation mechanism) other than the reduction in mRNA levels.

MPD protein levels in the medulla oblongata of the SHRSP brain were similar to those in WKY, although mRNA levels were lower (Fig. 2, 3). These results suggested that MPD protein levels in the medulla oblongata of the SHRSP brain, due to the inhibited degradation of proteins or another mechanism, were the same as those in WKY.

MPD protein levels in the cerebellum of the SHRSP brain were similar to those in WKY, whereas cholesterol contents were higher (Fig. 1, 2). The high levels of cholesterol in the cerebellum of the SHRSP brain may be caused by an increased amount of the cholesterol biosynthetic enzyme other than MPD protein levels. No significant differences were observed in MPD protein levels in the cerebellum between SHRSP and WKY, although the mRNA levels of SHRSP were lower (Fig. 2, 3). Therefore, MPD protein levels in the cerebellum of the SHRSP brain may be the same as those in WKY through the inhibited degradation of proteins, or another mechanism. Although *miR-214* levels in the cerebellum of the SHRSP brain were lower than those in WKY, MPD mRNA levels were not higher (Fig. 3, Table 1). Thus, coupling *miR-214* with the 3′-UTR of MPD mRNA is considered to have no influence on the reduction observed in mRNA levels in the cerebellum of SHRSP. The reduction in mRNA levels in the cerebellum of SHRSP may have occurred due to an increase in novel miRNA combined with the 3′-UTR of MPD mRNA or the inhibition of transcription.

The stability of cell membranes is ascribed to free cholesterol. The decline in cholesterol content in the SHRSP brain may be due to the following factors: 1) Decrease in cholesterol biosynthesis by low levels of MPD in the brain, 2) Reduction in uptake due to a lower serum cholesterol caused by decreased expression of MPD, SQE, and HSD17b7 in the liver,[8,10,11] and 3) Effect of cholesterol esterification due to increased acyl-CoA cholesterol acyltransferase (ACAT) in the brain. We previously reported that MPD activity in testicles, brains, and livers was proportional to its protein level.[8] The specific activity of the purified protein in SHRSP was also similar to that of WKY.[7] Furthermore, post-translational modification of MPD like HMG-CoA reductase (activity regulation by phosphorylation and dephosphorylation) has not been reported.[25] From these data, it was suggested that the protein level of MPD corresponds to its activity. Therefore, the reduction in cholesterol content in each part of the SHRSP brain may be the reason for low MPD protein levels.

The cerebellum participates in the regulation of movement. Ataxia has been attributed to a decrease in cholesterol in the cerebellum because a cholesterol deficiency is considered to lead to fragile plasma membranes and abnormalities in axons, dendrites, and synapses. A previous study reported that SHRSP could be characterized not only by hypertension, but also hyperactivity.[26] Ataxin-2-deficient mice also exhibited locomotor hyperactivity, and cholesterol contents in the cerebellums of ataxin-2 deficient mice were the same as those in wild-type mice.[24] Attention deficient hyperactivity disorder has been suggested to be related to low levels of serum cholesterol.[27] In the present study, we found a high cholesterol content in the cerebellum and low cholesterol levels in the cerebrum and serum of SHRSP. Dyskinesia may be caused by abnormal nerve conduction due to low cholesterol content in the cerebrum; however, SHRSP are mobile because of high cholesterol content in the cerebellum. This reveals that the hyperactivity disorder in SHRSP was caused by low and high cholesterol levels in the cerebrum and cerebellum, respectively.

In conclusion, the results of the present study suggest that

the reduction in MPD levels, which is involved in decreasing cholesterol contents in the serum, cerebrum, and brain stem, is an important risk factor for stroke. We also showed that the mechanism responsible for reducing MPD protein levels in the brains of SHRSP differed with each region. Further studies are required to elucidate the mechanism responsible for reducing MPD protein levels in the SHRSP brain.

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Conflict of interest The authors declare no conflict of interest.

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