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ARTICLE

Red Mold Rice Promotes Neuroprotective sAPPalpha Secretion Instead of Alzheimer's Risk Factors and Amyloid Beta Expression in Hyperlipidemic Aβ40-Infused Rats

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Amyloid beta (Aβ) peptide is closely related to the onset of Alzheimer's disease (AD). A high-cholesterol or high-energy diet was demonstrated to stimulate Aβ formation and deposition in the amyloid precursor protein (APP) pathway and, oppositely, downregulate the secretion of the neuroprotective soluble APP α-fragment (sAPPα). *Monascus*-fermented red mold rice (RMR) including multiple cholesterol-lowering agents, antioxidants, and anti-inflammatory agents has been proven to ameliorate Aβ40 infusion-induced memory deficit in our previous study. In this study, the ethanol extract of RMR (RE) and natural RMR were respectively tested for their effect on the mediation of the proteolytic process of APP in cholesterol-treated human neuroblastoma IMR32 cell, as well as their effect on memory and learning ability and the expression of AD risk factors in intracerebroventricular Aβ40-infused hyperlipidemic rats. In the results, RE suppressed cholesterol-raised β-secretase activity and further resulted in the increase of sAPPα secretion in the IMR32 cell. In the animal test, RMR potently reversed the memory deficit in the water maze and passive avoidance tasks. RMR administration could prevent against Aβ40 infusion plus the great damage caused by a high energy diet in hippocampus and cortex involved in the raise of thiobarbituric acid reactive substances and reactive oxygen species. The neuroprotection provided by RMR downregulates Aβ40 formation and deposition by suppressing the cholesterol-raised β-secretase activity and apolipoprotein E expression, as well as mediates the proteolytic process of APP toward neuroprotective sAPPα secretion in hippocampus.

KEYWORDS: Red mold rice; Alzheimer's disease; ApoE; β-secretase; amyloid; monacolin

INTRODUCTION

β-Amyloid (Aβ), a 40−43 amino acid peptide associated with Alzheimer's disease (AD), is derived from the integral membrane amyloid precursor protein (APP) by proteolytic cleavage of β- and γ-secretase (*1*). Oxidative stress and inflammatory response have been proven to be the important damage induced by Aβ deposition in the brains of AD patients (*2*, *3*). Cholesterol is found to increase the protein expression and activity of β-secretase (*4*). In addition, apolipoprotein E (ApoE), a serious risk factor of late-onset familial AD, is proven to stimulate the deposition of Aβ (*5*). A high-fat and -energy diet was found to raise the levels of AD risk factors, including ApoE, β-secretase, lipid peroxidation, and inflammatory response in the brain, and stimulate Aβ secretion and deposition. Therefore, more and more evidence has proved that cardiovascular disease is highly associated with AD by epidemiology (*6*). An Aβ40-infused rat fed with a high-cholesterol diet was proven as an animal model with more serious AD pathogenesis by a previous study (*7*).

Monascus-fermented rice, known as red mold rice (RMR) or red yeast rice, is a common food item found in China, used for many centuries to enhance the color and flavor of food, as well as a traditional medicine for digestive and vascular functions (*8*, *9*). Currently, RMR is regarded as a popular health food has also been developed as commercial capsules containing 0.5−1.0 g of red mold rice powder for hypolipidemic treatment in Asia and the United States. Monacolins, a group of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors with character identical with that of statins, have been proven to be the functional ingredients of RMR with hypolipidemic ability (*10*). RMR has been proven to ameliorate the impairment of memory and learning ability and repress Aβ accumulation in the hippocampus of Aβ-infused rat in our previous study (*11*). Furthermore, neuroprotection is contributed from the ability of antioxidative

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stress and anti-inflammatory response performed by monacolins and other *Monascus* metabolites (including dimerumic acid, tannin, phenol, sterol, monounsaturated fatty acid, azaphilones, and furanoisophthalides) (*12*).

RMR is proven to repress Aβ40 deposition in Aβ40-infused rats via antioxidation and anti-inflammatory response (*11*, *12*), but a more complex and serious AD model with high brain lipidemic level may frequently occur in AD pathogenesis. Brain cholesterol level is highly associated with Aβ secretion and deposition because of cholesterol-raised β-secretase activity and apolipoprotein E expression. On the basis of the neuroprotective and hypolipidemic ability of RMR, the ethanol extract of RMR (RE) is used to mediate the proteolytic process of APP in the cholesterol-treated IMR32 cell. Further, the ameliorative effects of the dietary administration of RMR on the expression of AD risk factor and memory deficit are confirmed in a hyperlipidemic AD rat model established with intracerebroventricular (icv) Aβ infusion (*11*) and high-energy (HE) diet (*13*) according to the previous studies. Memory and learning abilities are evaluated by water maze and passive avoidance tasks. AD risk factors, including β-secretase expression and activity, ApoE expression, cholesterol, and lipid levels in the hippocampus and cortex are monitored. In addition, neurotoxic Aβ accumulation and neuroprotective soluble amyloid precursor protein α-fragment (sAPPα) secretion were also investigated in this study.

MATERIALS AND METHODS

Materials. MEM medium and fecal bovine serum were purchased from Gibco BRL (Grand Island, NY) and Aβ40 from Tocris Bioscience (Ellisville, MO). Ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), nitro blue tetrazolium (NBT), superoxide dismutase (SOD), sodium dodecyl sulfate (SDS), ferricytochrome *c*, cholesterol, mevalonate, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), monoclonal sAPPα (6E10) antibody, apolipoprotein E antibody, and polyclonal β-secretase antibody were purchased from Sigma (St. Louis, MO). Cholesterol and triglyceride kits were purchased from Randox Laboratories Ltd. (Antrim, Belfast, U.K.). Polyclonal Aβ40 antibody was purchased from Novus Inc. (Littleton, CO).

Preparation of Ethanol Extract of Red Mold Rice. The long-grain rice was purchased from a local supermarket in Taiwan to be used for RMR production under solid-state cultivation. *Monascus purpureus* NTU 568, a mutant with high monacolin K productivity, was used to prepare RMR using the method proposed by our previous study (*14*). The RMR fermented by *M. purpureus* NTU 568 has been proven to include monaconlin K production at 9.5 mg/g and further perform a potent hypolipidemic effect in our previous study (*14*). After fermentation, the crushed and dried red mold rice (5 g) was further extracted by 50 mL of ethanol at 37 °C for 24 h and then dried under vacuum. The dried ethanol extract of *M. purpureus* NTU 568 fermented RMR (RE 568) was resolved to a final concentration of 1 mg/mL by distilled water.

Cell Culture. IMR32 cells, the human neuroblastoma cell line with APP secretion and proteolytic ability, obtained from Bioresource Collection and Research Center (60014; BCRC, Hsinchu, Taiwan) were maintained in MEM medium supplemented with 10% fetal bovine serum, 50 U/mL of penicillin, and 50 mg/mL of streptomycin in a humified incubator aerated with 95% air and 5% CO_2 at 37 °C.

RE or Lovastatin Treatment for Cholesterol-Treated IMR32 Cells. IMR32 cells (3×10^6 cells) were cultured in the culture MEM medium for 24 h at 37 °C, with 5% CO_2. After pretreatment, the culture MEM medium was removed and changed to serum-free MEM medium. Cells were stimulated with cholesterol (1.6 mM) and then pretreated with either lovastatin or RE for 48 h. After treatment, cells were collected and lysed with lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 100 mM NaCl, 40 mM NaF, 0.2% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, and 1 mM Na_3VO_4) and brief sonication (10 s). Cell lysates were used for the determination of β-secretase activity and immunoblotting of sAPPα and β-scretase.

Animal Grouping and Experiment Schedule. Male Wistar rats (weighing 280−320 g) were obtained from the Laboratory Animal Center of National Taiwan University, College of Medicine. They were kept in a temperature-controlled room (23 °C) under a 12L:12D cycle (light on at 6:00) and were given free access to food and water. In the experiment, 49 rats were randomly divided into seven groups including (1) vehicle infused and feeding with normal chow diet (3.34 kcal/g) (V−N group), (2) vehicle infused and feeding with HE diet (4.85 kcal/g) consisting of 72.7% chow diet, 2.67% butter fat, and 1% cholesterol (V-HE group), (3) Aβ40 infused and feeding with normal chow diet (Aβ-N group), (4) Aβ40 infused and feeding with HE diet (Aβ-HE group), (5) Aβ40 infused, feeding with HE diet, and administration with lovastatin (1.43 mg/kg of rat per day) (LS-HE group), (6) Aβ40 infused, feeding with HE diet, and administration with one-fold dosage of RMR (151 mg/kg of rat per day) (RL-HE group), (7) Aβ40 infused, feeding with HE diet, and administration with 5-fold dosage of RMR (755 mg/kg of rat per day) (RH-HE group). The dosage of RMR is calculated in accordance with Boyd's formula of body surface area, as recommended by the FDA (Food and Drug Administration) (*15*, *16*). Feeding rat with RMR at a one-fold dosage per day corresponds to supplementing the daily diet with RMR at 2.0 g for an adult. In addition, the dosage of lovastatin for rat is used at 1.43 mg/kg per day because the concentration is the same as the monacolin K levels of a one-fold dosage of RMR.

The experiment schedule of the AD animal model was as follows. Aβ40 infusion on the zeroth day was continued for 28 days, and lovastatin or RMR in suspension was daily administrated to the rat from the 1st day to the 28th day. The behavioral test was started on the 19th day. The passive avoidance task was carried out from the 19th day to the 21st day. Subsequent reference memory tasks, probe tests, and working memory tasks were started on the 22nd day, the 24th day, and the 25th day. On the 28th day, rats were sacrificed and the brain tissues were collected for the measurement of AChE activity, inflammatory response, and oxidative stress.

Surgery for icv Aβ40 Infusion. Rats were anesthetized with sodium pentobarbital (50 mg/kg BW i.p.). The left skull was exposed and drilled (relative to the bregma; 0.8 mm posterior, 1.4 mm lateral) according to the atlas of Paxinos and Watson (*17*) using a stereotaxic frame (Narishige, Tokyo, Japan). Aβ40 was prepared in the vehicle solvent of 35% (v/v) acetonitrile plus 0.1% (v/v) trifluoroacetic acid (pH 2.0). The osmotic mini-pump (2004, Durect Co., Cupertino, CA) used to result in an animal model of AD with impaired memory was filled with Aβ40 solution or the vehicle solution. The outlet of infusion cannula was inserted 4.0 mm into the left ventricle and attached to the skull with dental cement, and then the mini-pump was quickly implanted into the backs of the rats. A 234 μL portion of Aβ solution contained in the osmotic pump was continuously infused into the left ventricle at a rate of 0.28 μL/h for 28 days. Finally, the total amount infused was approximately 4.9−5.5 nmol Aβ40 (*11*).

Passive Avoidance Task. The passive avoidance task was carried out from the 19th day to the 21st day according to our previous study (*11*).

Apparatus of Water Maze. The Morris water maze task was used to evaluate the memory and learning ability from the 22nd day to the 27th day (*11*). A black circular tank (diameter 140 cm, height 45 cm) was used as the apparatus of the water maze in which a movable escape platform (diameter 10 cm, height 25 cm) was located inside the tank. The tank was filled to a height of 27.5 cm with water at a temperature of approximately 23 °C; thus, the surface of the platform was 2.5 cm below the surface of the water. The circular tank was divided into four quadrants (I−IV), and a position with equal distance from center and edge in the middle of each quadrant was marked for the location of the platform. The water tank was located in a test room with many cues external to the maze. The room had adjustable indirect light, and the camera was set at the ceiling above the center of the water tank. The position of the cues remained unchanged throughout the water-maze task.

Morris Water-Maze Task. According to the procedure of our previous study (*11*), reference memory tasks were carried out from the 22nd day to the 24th day and included 4 continuous trials per day. A probe test was immediately carried out after the 12th training trial of the reference memory task on the 24th day. Working memory tasks were performed from the 25th day to the 27th day and consisted of 5 trials per day.

Preparation of Brains. After the behavioral studies were completed, the rats were anesthetized with sodium pentobarbital (65 mg/kg BW, i.p.)

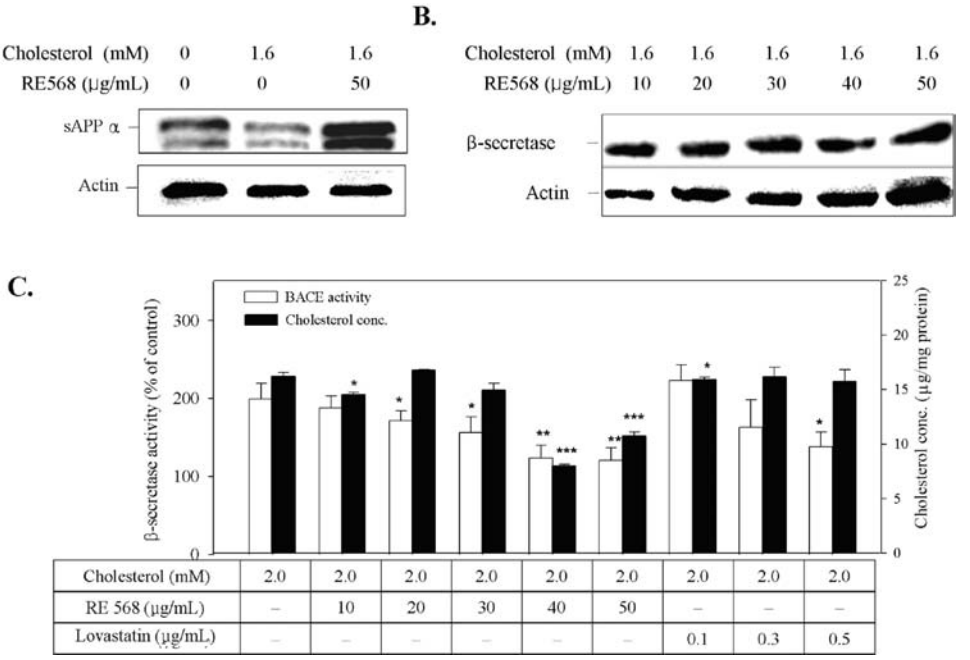

Figure 1. Effects of RE on the sAPPα secretion (**A**) and β-secretase expression (**B**) and activity (**C**) in cholesterol-treated IMR32 cells. Cells (3×10^6 cells) were treated with cholesterol (1.6 mM) and then treated with lovastatin or RE for either 48 h. Data are presented as means ± SD ($n = 3$). Legend: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus control group).

and the blood was collected; the cerebral cortex and hippocampus were separated from the whole brain on ice, blotted gently with filter paper to remove blood and extraneous tissue fragments, and then flash-frozen with liquid nitrogen and stored at −80 °C until use. Hippocampus and cortex tissues (100 mg) were crushed with an amalgam mixer (UT-1600, Sharp, Osaka, Japan) and suspended in 1.0 mL of ice-cold Tris saline (50 mM Tris-HCl, pH, 7.6, 0.15 M NaCl) buffer containing 1% (v/v) Triton X-100 and protease inhibitor cocktail and then sonicated for 30 s. The homogenate was centrifuged at 100 000*g* for 30 min, and the supernatant was used for thiobarbituric acid reactive substances (TBARS) and reactive oxygen species (ROS) assay. With regard to the protein extraction for immunoblotting, the tissue (100 mg) was homogenated in 1.0 mL of lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 100 mM NaCl, 40 mM NaF, 0.2% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, and 1 mM Na_3VO_4) and brief sonication (10 s). The homogenate was centrifuged at 100 000*g* for 30 min, and the supernatant was used for immunoblotting assays.

Determination of TBARS and ROS Levels in the Hippocampus and Cortex. Hippocampus and cortex homogenates were centrifuged at 4000*g* for 15 min, and the supernatant was used for neurochemical assays. The TBARS level was determined by the method of thiobarbituric acid (TBA) colorimetric analysis, and the optical density (OD) value was measured at 532 nm (*18*). In the measurement of ROS, homogenates were added to 96-well plates, and NBT reduction was measured by absorbance at 550 nm in triplicate (*11*).

Brain Cholesterol Measurements. Cholesterol in the hippocampus and cortex was extracted by the method of Folch et al (*19*). A 10 μL amount of supernatant was evaporated to dryness, and the residue was dissolved in 10 μL of DMSO. Then, the cholesterol content was determined using a cholesterol analysis kit (Randox Laboratories Ltd.).

Measurement of β-Secretase. β-Secretase activity in the hippocampus, cortex, or cell lysate was determined using a commercial analysis kit (FP002, R&D System, Minneapolis, MN).

Immunoblotting of ApoE, β-Secretase, and sAPPα. Protein concentrations were determined by the bicinchoninic acid (BCA) method. The samples were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. After blocking in a gelatin-NET solution, blots were incubated with polyclonal Aβ40 antibody (1:1000), monoclonal sAPPα antibody (1:1000), or polyclonal ApoE antibody (1:1000) at room temperature for 1 h. Then, bands were incubated with specific horse radish peroxidase (HRP) conjugated secondary antibodies (1:200 000) at room temperature for 1 h and visualized by enhanced chemiluminescence (ECL) substrate with UVP AutoChemi Image system (UVP Inc., Upland, CA). Protein loading was evaluated by antiactin antibody (1:3000). Band intensities were quantified using UVP LabWork 4.5 software (UVP Inc.).

Immunohistochemical Stain of sAPPα and Aβ40 in the Hippocampus. The brain tissue was fixed in 10% formalin at pH 7.4. Brains were blocked, and serial 35 μm thick frozen sections cut on a sledge microtome were collected sequentially without interruption into wells. Sections from regions containing the hippocampus were processed for immunohistochemical stains according to our previous study (*11*).

Statistics. Data are expressed as the mean ± SD. The statistical significance in the behavioral and biochemical effects was determined by one-way analysis of variance (ANOVA), followed by ANOVA with Duncan's multiple test.

RESULTS

sAPPα and β-Secretase Expression and β-Secretase Activity in IMR32 Cells. The in vitro result of the APP process by RE treatment was shown in **Figure 1**. sAPPα expression was repressed by cholesterol supplements, but the reduced level was significantly enhanced by RMR treatment (**Figure 1A**). This study further focused the target on the expression and activity of β-secretase affected by RE or lovastatin treatments. RE treatment has a significant effect on suppressing β-secretase activity (**Figure 1C**) but not β-secretase expression (**Figure 1B**). The suppressing effect performed by RE was as potent as that of lovastatin. The in vitro results proved that RMR was able to mediate the proteolytic process of APP toward the sAPPα pathway via suppressing β-secretase activity. This study further investigated whether the neuroprotection provided from RMR was able to apply to in vivo therapy or not.

Learning-Related Behavior. *Passive Avoidance Tasks.* Passive avoidance tasks are used to evaluate the memory and learning ability in animal models. Step-through latency from a light chamber to a dark chamber is used as the marker to evaluate the memory and learning ability in passive avoidance tasks. As shown in **Figure 2A**, both the V−N group and V-HE group

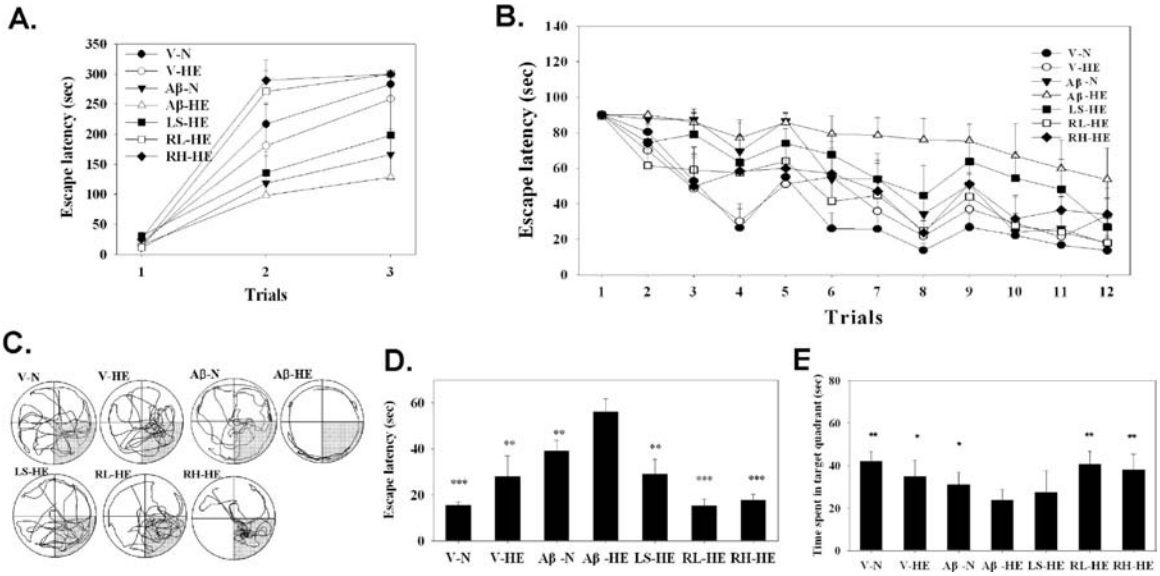

Figure 2. Effect of RMR on memory and learning ability in Aβ40-infused hyperlipidemic rats: (**A**) Sstep-through latency of multiple-trial passive-avoidance tasks; (**B**) escape latency in reference memory tasks. (**C**) time spent in probe trial; (**D**) swimming pathway in probe trial; (**E**) escape latency in working memory tasks. Passive-avoidance tasks were carried out per 24 h from the 19th day to the 21st day. Reference memory tasks were carried out from the 22nd day to the 24th day. After reference memory tasks, the probe trial was accomplished immediately on the 24th day. The working memory task was carried out from the 25th day to the 27th day. the V−N group and Aβ-N group fed with a normal diet were icv infused vehicle solution and Aβ40 solution, respectively. The other rats were fed with an HE diet as well as icv infused vehicle solution (V-HE group) or Aβ40 solution (Aβ-HE group). The test groups were treated with Aβ infusion and an HE diet as well as administrated with lovastatin (1.43 mg/kg/day) (LS-HE group), 1-fold dosage RMR (151 mg/kg/day including 1.43 mg monacolin K) (RL-HE group), or 5-fold dosage RMR (755 mg/kg/day including 7.15 mg monacolin K) (RH-HE group). Data are presented as means ± SD (n = 7). Legend: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus Aβ-HE group).

without Aβ40 infusion performed similarly in passive avoidance tasks. However, Aβ40-infused rats (Aβ-N and Aβ-HE group) had shorter step-through latency. However, an HE diet aggravated the memory deficit, so that the Aβ-HE group showed poorer learning ability as compared to the Aβ-N group. Administration with a low dose or high dose of RMR gave longer step-through latency than for the Aβ-HE group, as well as the LS-HE group.

Reference Memory Tasks. **Figure 2B** indicated thata high-energy diet would not lead to significant differences in reference memory tasks between the V−N and V-HE groups but between the Aβ-N and Aβ-HE groups, especially in the 6th to the 12th tests. The poorest learning ability was exhibited by the Aβ-HE group because of the treatment with Aβ infusion and an HE diet. RMR treatment (RL-HE group and RH-HE group) was proven to assist Aβ-infused rats in reducing escape latency from the 2nd to the 12th test. The LS-HE group treated with lovastatin had an escape latency as short as that of the Aβ-HE group.

Probe Tests. The escape platform was removed from the pool, and probe tests were immediately started after the end of reference memory tasks. The rats with good spatial memory and learning ability would spend much time in the target quadrant, in which the platform of reference memory tasks was located. As shown in **Figure 2C**, The Aβ-HE group had the poorest results at 23.83 on 90 s of spatial probe test among each group. The Aβ-HE group searched the target quadrant with directionless escape and around the wall of pools (**Figure 2D**). In addition, an HE diet slightly caused the V-HE (34.83 s) and Aβ-HE groups (23.83 s) to have poorer spatial memory and learning ability than the V−N (42.08 s) and Aβ-N groups (31.02 s), respectively. According to the escape pathway results , RL-HE and RH-HE groups would focus the pathway on the target quadrant, which also results in the fact that the times spent by the RL-HE (40.66 s) and RH-HE groups (38.00 s) were both longer than that spent by the Aβ-HE group. Therefore, the results proved that RMR certainly has the function to ameliorate Aβ40 and HE diet-induced memory deficits. However, lovastatin treatment still had less of an effect on probe tests than RMR treatment, even though the lovastatin concentration was equal to the total monacolin K concentration of RMR.

Working Memory Tasks. The escape latency in the working memory tasks of short-term learning ability is shown in **Figure 2E**. An HE diet was proven to weaken working memory ability in rats without Aβ infusion according to comparison between the V-N (15.59 s) and V-HE groups (28.00 s). The Aβ-HE group exhibited the poorest learning ability (56.17 s of escape latency) in working memory tasks among each group. However, RL-HE, RH-HE, and LS-HE groups had shorter escape latencies of 15.27 s ($p < 0.001$), 17.74 s ($p < 0.001$), and 29.11 s ($p < 0.01$), proving that RMR treatment was able to ameliorate Aβ40 infusion plus an HE diet-induced memory deficit and had a greater effect than lovastatin treatment.

Cholesterol Level in the Hippocampus and Cortex. Cholesterol level in the brain is highly associated with the formation of AD risk factors (*20*). The results (**Table 1**) indicated that an HE diet increased hippocampus and cortex cholesterol levels of V-HE and Aβ-HE groups as compared with the V-N group, respectively. The increased cholesterol levels significantly decreased with dose response. The lowering effects in the cortex and hippocampus respectively increased to 26.79% ($p < 0.05$) and 37.86% ($p < 0.01$) by treatment with a high dose of RMR.

Oxidative Stress and Inflammatory Response. Aβ deposition-induced oxidative stress and inflammatory response are proven to result in neuron damage and memory deficit (*7*). A high-fat or HE diet is highly associated with lipid accumulation and serious lipid peroxidation (*7*, *21*). As shown in **Figure 3**, the TBARS and ROS levels in the cortex and hippocampus were increased by feeding an HE diet or Aβ40 infusion, but more serious damage was found

Table 1. Effect of RMR on Levels of Cholesterol in the Cortex and Hippocampus of AD Rats Fed with a High-Energy Diet[a]

	cholesterol (mg/g of protein)	
group	cortex	hippocampus
V−N	31.6 ± 6.8**	28.8 ± 4.9*
V-HE	47.9 ± 5.8	40.0 ± 11.4
Aβ-N	30.7 ± 5.4**	29.5 ± 7.1*
Aβ-HE	52.3 ± 6.5	37.7 ± 8.0
LS-HE	44.3 ± 5.3*	35.2 ± 3.8
RL-HE	39.6 ± 6.7*	30.2 ± 3.0*
RH-HE	32.5 ± 8.0**	27.6 ± 2.3*

[a] V-N and Aβ-N groups fed with a normal diet were icv infused vehicle solution and Aβ40 solution, respectively. The other rats were fed with an HE diet as well as icv infused vehicle solution (V-HE group) or Aβ40 solution (Aβ-HE group). The test groups were treated with Aβ infusion and HE diet as well as administrated with lovastatin (1.43 mg/kg/day; LS-HE group), l-fold dosage RMR (151 mg/kg/day including 1.43 mg monacolin K; RL-HE group), or 5-fold dosage RMR (755 mg/kg/day including 7.15 mg monacolin K; RH-HE group). Data are presented as means ± SD ($n = 7$): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus Aβ-HE group).

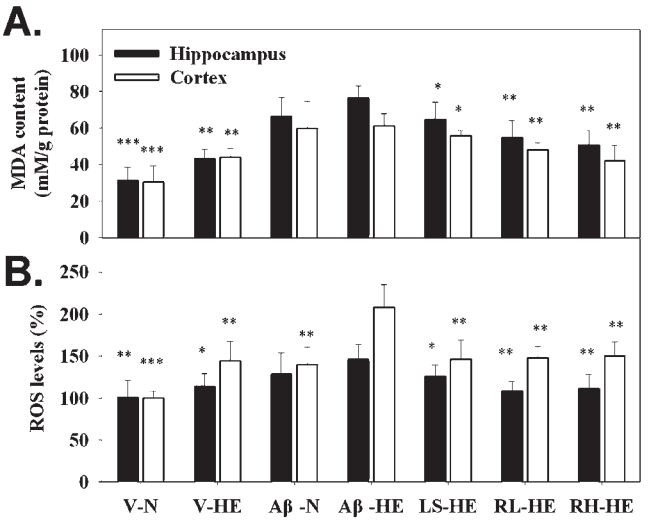

Figure 3. Effect of RMR on the TBARS and ROS levels in the hippocampus and cortex of Aβ40-infused rats with or without hyperlipidemia. V-N and Aβ-N groups fed with a normal diet were icv infused vehicle solution and Aβ40 solution, respectively. The other rats were fed with an HE diet as well as icv infused vehicle solution (V-HE group) or Aβ40 solution (Aβ-HE group). The test groups were treated with Aβ infusion and an HE diet as well as administrated with lovastatin (1.43 mg/kg/day; LS-HE group), l-fold dosage RMR (151 mg/kg/day including 1.43 mg monacolin K; RL-HE group), or 5-fold dosage RMR (755 mg/kg/day including 7.15 mg monacolin K; RH-HE group). Data are presented as means ± SD ($n = 7$). Legend: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus Aβ-HE group).

in the treatment with Aβ40 infusion plus feeding HE diet. TBARS in the cortex and hippocampus was able to be significantly lowered to 48.08 and 54.64 mM/g protein by treatment with a low dose of RMR and further lowered to 42.05 and 50.63 mM/g protein by treatment with a high dose of RMR. With regard to ROS levels, ROS levels in hippocampus and cortex of RL-HE and RH-HE groups were less than that of the Aβ-HE group. Although the ROS level could be repressed by lovastatin treatment, the effect in the hippocampus was proven to be poorer than that of RMR treatment.

Protein Expression and Activity of β-Secretase. The protein expression and activity of β-secretase in the brain are proven to be associated with Aβ formation (*1*). Therefore, repressing β-secretase expression or activity is considered as the target of drug development for AD prevention (*22*). β-Secretase expression in the cortex and hippocampus were found to significantly increase by Aβ40 infusion plus feeding HE diet as compared with V-N ($p < 0.01$), and V-HE groups ($p < 0.05$) (**Figure 4A**). The increased β-secretase expression was significantly reduced by administration with a low dose ($p < 0.01$) and high dose ($p < 0.01$) of RMR, and further, the inhibition caused by RMR treatment was more remarkable in the hippocampus than in the cortex. Lovastatin treatment was found to reduce both cortex and hippocampus β-secretase expression.

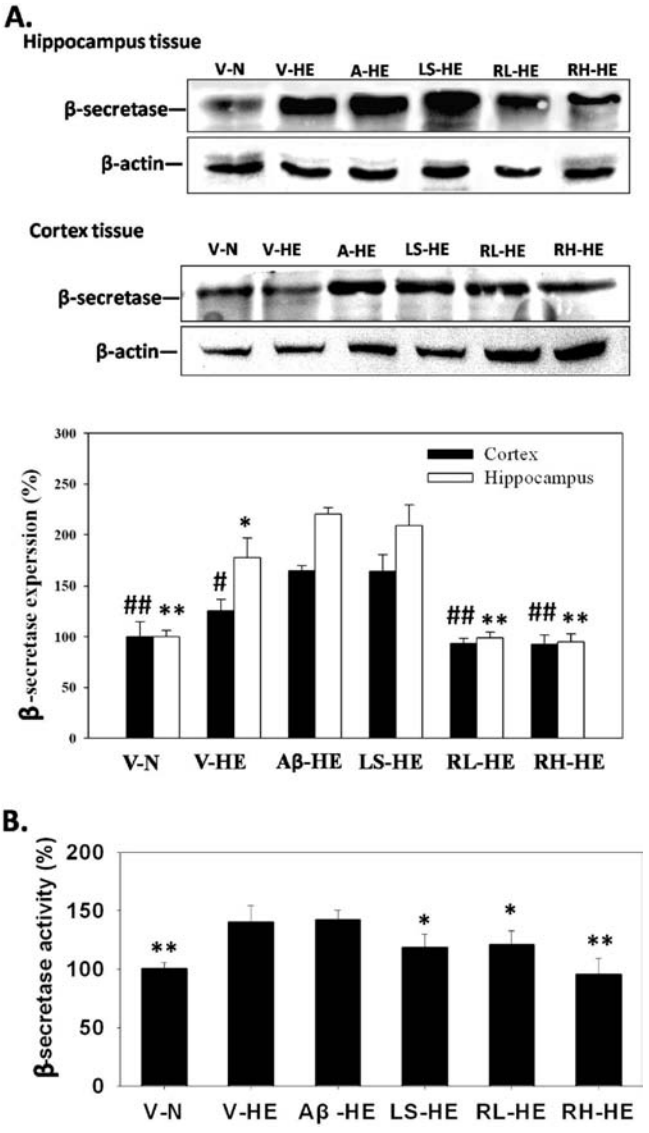

Figure 4. Effect of RMR on β-secretase expression (A) and activity (B) in the hippocampus and cortex of Aβ40-infused rats with or without hyperlipidemia. β-Secretase expression was visualized using immunoblotting and quantified using Image J software. V-N and Aβ-N groups fed with a normal diet were icv infused vehicle solution and Aβ40 solution, respectively. The other rats were fed with an HE diet as well as icv infused vehicle solution (V-HE group) or Aβ40 solution (Aβ-HE group). The test groups were treated with Aβ infusion and HE diet as well as administrated with lovastatin (1.43 mg/kg/day; LS-HE group), l-fold dosage RMR (151 mg/kg/day including 1.43 mg monacolin K; RL-HE group), or 5-fold dosage RMR (755 mg/kg/day including 7.15 mg monacolin K; RH-HE group). Data are presented as means ± SD ($n = 7$). Legend: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus the hippocampus sample of Aβ-HE group); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ (versus the cortex sample of the Aβ-HE group).

The result of brain β-secretase activity was similar to that of in vitro results (**Figure 1C**). After treatment with an HE diet, hippocampus β-secretase activities of the V-HE and Aβ-HE groups significantly increased by 39.99% and 40.73%, as compared to the V-N group (**Figure 4B**). However, β-secretase activities of a hyperlipidemic Aβ-infused rat were suppressed by 14.68% and 32.70% by treatment with low and high doses of RMR, respectively. Importantly, RMR has a greater suppression effect on β-secretase activity than does lovastatin.

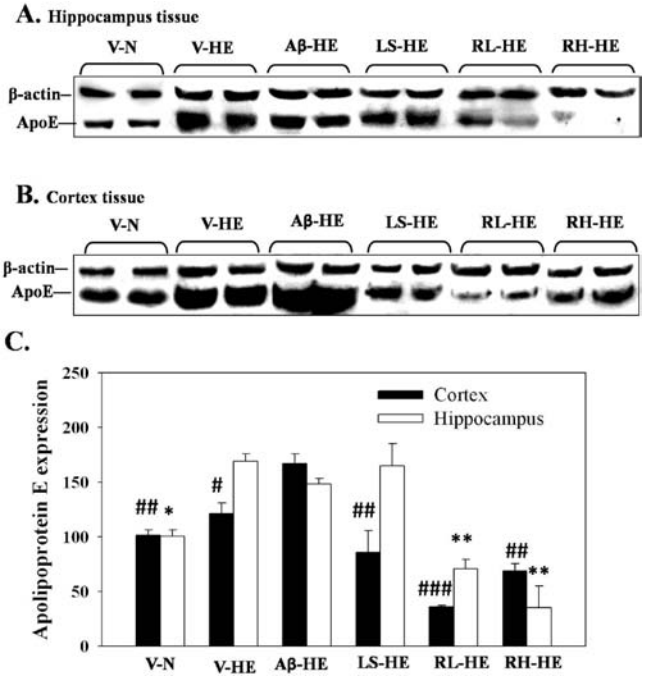

Figure 5. Effect of RMR on ApoE expression in the hippocampus and cortex of Aβ40-infused rats with or without hyperlipidemia. ApoE expression was visualized using immunoblotting (**A**) and quantified using Image J software (**B**). V-N group fed a normal diet were icv infused vehicle solution. The other rats were fed with an HE diet as well as icv infused vehicle solution (V-HE group) or Aβ40 solution (Aβ-HE group). The test groups were treated with Aβ infusion and an HE diet as well as administrated with lovastatin (1.43 mg/kg/day; LS-HE group), 1-fold dosage RMR (151 mg/kg/day including 1.43 mg monacolin K; RL-HE group), or 5-fold dosage RMR (755 mg/kg/day including 7.15 mg monacolin K; RH-HE group). Legend: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus the hippocampus sample of the Aβ-HE group); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ (versus the cortex sample of the Aβ-HE group).

ApoE Expression. ApoE has been proven to combine with Aβ so that more Aβ deposition and tangle fibrils are formed to aggravate neuron damage and memory deficit. A high-energy or high-fat diet is highly associated with ApoE expression of the brain (*5*). RMR and lovastatin with hypolipidemic ability should have a greater effect on repressing ApoE expression. As shown in **Figure 5**, the ApoE expression in the hippocampus and cortex of RL-HE and RH-HE groups would be less than for V-HE and Aβ-HE groups. The results also indicated that ApoE expression in the cortex but not in the hippocampus could be repressed by lovastatin treatment, suggesting that lovastatin had a weaker ability than RMR even though they contain equal monacolin K levels.

Aβ40 Accumulation in the Hippocampus. According to the above results, feeding Aβ40-infused rats with an HE diet including 1% cholesterol increased brain cholesterol, as well as aggravated Aβ40-induced oxidative stress and inflammatory response. In addition, the AD risk factor, ApoE expression and β-secretase activity, increased with hyperlipidemic lesion. These damages have been demonstrated to be associated with Aβ deposition and Aβ-induced memory deficit (*20*). However, **Figure 6** indicates that a greater Aβ accumulation could be found in teh Aβ-HE group than in the V-HE or V-N groups. Although lovastatin treatment was able to suppress Aβ accumulation, its effect is inferior to that performed by RMR treatment. Infused Aβ40 accumulated in the hippocampus of the RH-HE group, suggesting that RMR should be a functional material for the prevention of Aβ40 accumulation in the hippocampus.

sAPPα Expression. sAPPα, a fragment of APP cleaved by α-secretase, is proven to have potent neurotrophic and neuroprotective activities against excitotoxic and oxidative insults in various cellular models (*23*). As shown in the results of **Figure 7**, vehicle-induced rats with or without hyperlipidemia would have

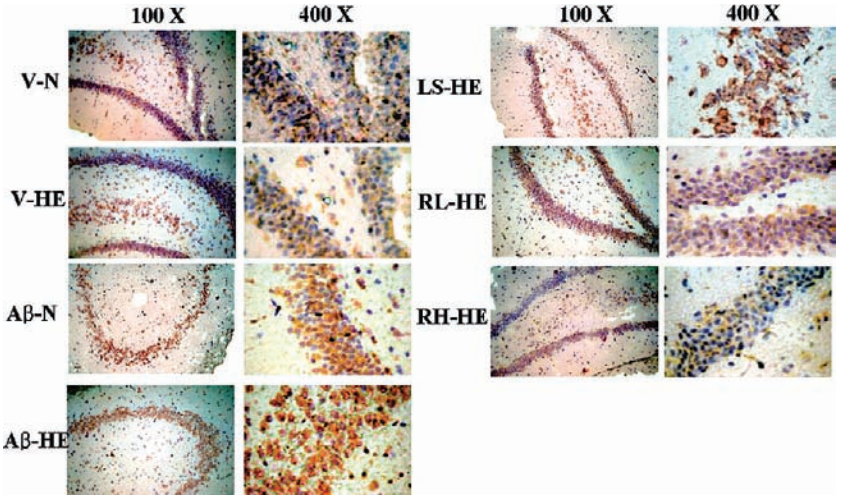

Figure 6. Effect of RMR on Aβ40 accumulation in the hippocampus of Aβ40-infused rats. Immunohistochemical staining was carried out using the nonbiotin hydrogen peroxidase kit. The Aβ40 accumulation in the hippocampus was monitored by microscopic examination (100$\times$ and 400$\times$) and presented as the brown dye in the graph. V-N and Aβ-N groups fed with a normal diet were icv infused vehicle solution and Aβ40 solution, respectively. The other rats were fed with an HE diet as well as icv infused vehicle solution (V-HE group) or Aβ40 solution (Aβ-HE group). The test groups were treated with Aβ infusion and HE diet as well as administrated with lovastatin (1.43 mg/kg/day; LS-HE group), 1-fold dosage RMR (151 mg/kg/day including 1.43 mg monacolin K; RL-HE group), or 5-fold dosage RMR (755 mg/kg/day including 7.15 mg monacolin K; RH-HE group).

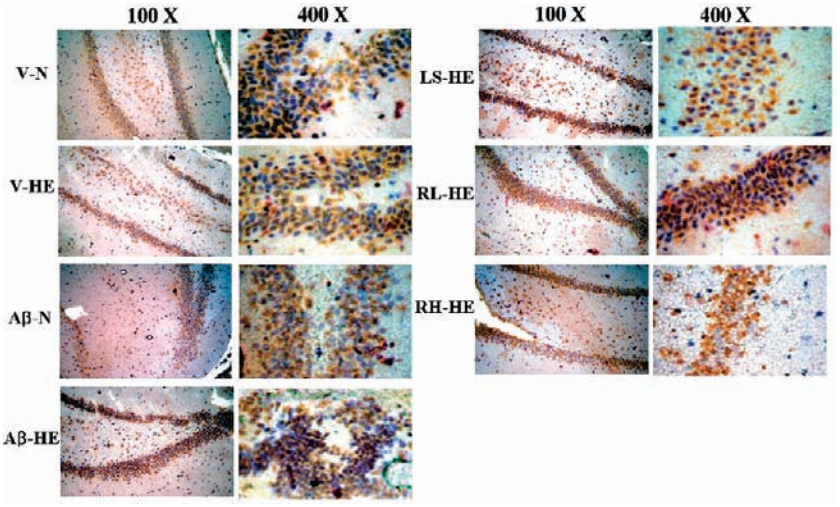

Figure 7. Effect of RMR on the sAPPα secretion in the hippocampus of Aβ40-infused rats. Immunohistochemical staining was carried out using the nonbiotin hydrogen peroxidase kit. The sAPPα secretion in the hippocampus was monitored by microscopic examination (100× and 400×) and presented as the brown dye in the graph. V-N group Aβ-N groups fed with a normal diet were icv infused vehicle solution and Aβ40 solution, respectively. The other rats were fed with an HE diet as well as icv infused vehicle solution (V-HE group) or Aβ40 solution (Aβ-HE group). The test groups were treated with Aβ infusion and HE diet as well as administrated with lovastatin (1.43 mg/kg/day; LS-HE group), 1-fold dosage RMR (151 mg/kg/day including 1.43 mg monacolin K; RL-HE group), or 5-fold dosage RMR (755 mg/kg/day including 7.15 mg monacolin K; RH-HE group).

large sAPPα expression in the hippocampus. However, the treated groups, LS-HE, RL-HE, and RH-HE groups, would repress Aβ-infusion-decreased sAPPα expression. However, lovastatin treatment does not have a more potent effect than RMR treatment.

DISCUSSION

Our previous studies have proven that RMR ameliorated Aβ-induced impairment of memory and learning ability via repressing Aβ40 accumulation in the hippocampus (*11*), because RMR included antioxidants (dimerumic acid, tannin, phenol, and sterols) and anti-inflammatory agents (monacolins, the six azaphilones monascin, ankaflavin, rubropunctatin, monascorburin, rubropunctamine, and monascorburamine, the two furanoisophthalides xanthomonasin A and xanthomonasin B, and the two amino acids (+)-monascumic acid and (−)-monascumic acid) (*24*−*27*). Although RMR is able to resolve Aβ40 deposition in Aβ40-infused rats via antioxidation and anti-inflammatory response, a more complex and serious AD model with high brain lipidemic level may frequently occur in AD pathogenesis. Current studies have found that hyperlipidemia and cardiovascular disease are highly associated with AD development (*6*). In addition, Hashimoto et al. fed Aβ40-infused rats with a high-cholesterol diet in order to increase the hypercholesterolemia-induced AD risk factor. The results indicated that increasing brain cholesterol increasde Aβ accumulation and aggravated memory and learning ability (*7*). Cholesterol has been proven to stimulate neuron damage of radical and ROS (*2*), as well as increase ApoE expression and β-secretase activity in the brain (*4*). However, this study found that many AD risk factors in the brain, including cholesterol, TBARS, ROS, ApoE, and β-secretase activity, increased, and the neuroprotective sAPPα expression was found to be lowered by Aβ40 infusion combined with feeding a high-energy diet plus 1% cholesterol. This and previous studies all proved that AD risk factors and Aβ-induced memory deficits are stimulated and aggravated by hyperlipidemia (*4*, *20*). Lowering the brain cholesterol level has been suggested as a beneficial action to ameliorate Aβ-induced impairment of memory and learning ability by an increasing number of AD-related studies (*4*, *5*).

An increase in the brain cholesterol level was regarded as the risk action for AD prevention because it stimulates ApoE expression and β-secretase activity (*4*, *5*). ApoE expression in the hippocampus and cortex of Aβ40-infused rats was inhibited by daily RMR administration. Apolipoproteins are lipid carrier molecules that transport cholesterol and fatty acids around the circulatory system and brain, and ApoE is a major apolipoprotein in the central nervous system (CNS), where it is thought to redistribute lipoprotein cholesterol among neurons and to maintain cholesterol homeostasis (*28*). ApoE has been shown to bind Aβ and tau protein in an isoform-dependent manner, which would promote Aβ deposition in the brain (*5*). In addition, recent studies also suggest that ApoE enhances Aβ production. Therefore, ApoE has been suggested as a potential therapeutic target for AD. Lovastatin has been used to lower ApoE expression in glia cell (*29*). However, RMR treatment had greater effect on ApoE repression than lovastatin treatment in hyperlipidemic Aβ-infused rat. In addition, HE-diet-raised β-secretase activity may enhance the difficulty in ameliorating the memory deficit. β- and γ-secretase act as key enzymes for the proteolytic process from APP to Aβ. Therefore, increasing β-secretase expression or activity would increase in vivo Aβ formation from APP. Many studies have suggested that lowering brain cholesterol suppresses the activity of β-secretase (*4*, *20*). Recently, stains have been proven to suppress in vitro or in vivo Aβ formation through repressing cholesterol-mediated β-secretase activity (*20*, *30*). Although the potent inhibition of β-secretase activity performed by lovastatin treatment was also verified by this study, RMR treatment was found to have a greater effect than lovastatin treatment. More and more studies have evidenced that RMR has a greater cholesterol-lowering effect in serum than lovastatin, even though they include equal levels of monacolin K or lovastatin (*31*, *32*). A comparison of cholesterol-lowering effects in the brain between RMR and lovastatin has never been reported. This study has found that potent suppression of β-secretase activity and cholesterol level in the hippocampus and cortex is performed by RMR treatment but not by lovastatin treatment.

The most important effect of RMR on the prevention of AD pathogenesis is to repress Aβ40 accumulation. The neuroprotection

was suggested to highly associate with the amelioration of Aβ-induced memory deficit. Inhibiting the deposition and accumulation of Aβ has been suggested as the therapeutic target by many AD studies. Regarding a related study, docosahexaenoic acid (DHA) was frequently reported to ameliorate the Aβ-infusion-induced memory deficit because of the clearance ability for Aβ deposition (*7*). A greater level of Aβ accumulation is found in the brain of hyperlipidemic Aβ-infused rat than in the brain of Aβ-infused rat without hyperlipidemic rat (*7*). The greater Aβ accumulation in the brain of hyperlipidemic Aβ-infused rat was probably caused by Aβ40 infusion and cholesterol-raised Aβ formation. In addition, both Aβ-induced oxidative stress and cholesterol-raised ApoE expression would aggravate Aβ deposition (*4*, *29*). However, the results evidenced that the vicious circle leading to Aβ40 accumulation was able to be suppressed by RMR treatment, which should be carried out through multifunctional treatment. RMR had a potent effect on lowering brain cholesterol level via hypolipidemic agents, which would further prevent the aggravation of lipid peroxidation and oxidative stress as well as increase the ApoE expression and β-secretase activity. The infused Aβ40 was also the cause of a vicious circle between Aβ-induced oxidative stress and oxidative stress induced Aβ deposition because the RMR treatment includes potent antioxidants and anti-inflammatory agents. However, lovastatin treatment had a significant effect on lowering hippocampus cholesterol and TBARS levels as compared with the Aβ-HE group, showing that a lack of protection probably decreased the ability to repress Aβ40 accumulation.

sAPPα produced by the nonamyloidogenic pathway has potent neurotrophic and neuroprotective activities against excitotoxic and oxidative insults (*33*). Furthermore, sAPPα is proven to promote neurite outgrowth (*34*), regulate synaptogenesis (*35*), and exert trophic effects on cerebral neurons in culture (*36*) and stabilizes neuronal calcium homeostasis (*37*). Therefore, it can be suggested that sAPPα can serve as a neuroprotective agent against the toxic activity of Aβ. This study indicated that sAPPα secretion decreased with an increase of Aβ40 accumulation and various AD risk factors. However, the reduced sAPPα secretion in the hippocampus of hyperlipidemic Aβ-infused rat increased by RMR treatment. The proteolytic process of APP was mediated toward neurotoxic Aβ40 secretion by β-secretase or toward neuroprotective sAPPα secretion by α-secretase (*1*). This study has demonstrated that RMR treatment was able to suppresse cholesterol-mediated β-secretase activity. Many studies have suggested that suppressing Aβ formation via decreasing β-secretase activity would promote sAPPα secretion (*30*). Therefore, RMR was proven to suppress β-secretase activity and Aβ accumulation, leading to neuroprotective sAPPα formation.

Aβ secretion and deposition is the commonly accepted major risk factor in the development of AD; however, the occurrence of Aβ deposition is suggested to result from multiple chain reactions involved in cholesterol and isoprenoid biosynthesis, oxidative stress, inflammatory response, ApoE expression, β-secretase activity, etc, by many studies (*2*, *4*, *5*, *20*). Our previous study has proven that the neuroprotective ability against Aβ deposition performed by RMR should result from the anti-inflammatory agent and antioxidants in Aβ-infused rat with normal brain lipidemic level. In this study, a complex AD model involved in Aβ-infusion and high brain cholesterol would perform serious expression of AD risk factors associated with more Aβ formation and rapid deposition. However, the amelioration effect of RMR on memory deficit and Aβ40 accumulation in Aβ-infusion hyperlipidemic rat should result from the downregulation of β-secretase activity and ApoE expression via the suppression of brain cholesterol and lipid formation. Importantly, the Aβ formation and deposition pathway is first found to be replaced by an upregulation of the neuroprotective sAPPα secretion pathway in the APP proteolytic pathway of Aβ infusion hyperlipidemic rat treated with RMR.

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