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Full-length article

Discovery of sphingosine 1-*O*-methyltransferase in rat kidney and liver homogenates[1]

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Key words

sphingosine; *O*-methylation; methyltransferase; sphingosine kinase; lipid; sphingolipid

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Abstract

Aim: To characterize sphingosine methyltransferase in rat tissues. **Methods:** By using *S*-adenosyl-*L*-(methyl-^{3}H) methionine, enzymatic activity was measured in the rat liver and kidney homogenates. **Results:** The optimum pH and reaction time for the enzyme assay were pH 7.8 and 1 h. $ZnCl_2$ inhibited the activity, but not $MgCl_2$, $CaCl_2$, $CoCl_2$, or $NiCl_2$. In the kidney homogenate, enzymatic activity was detectable in the cytosol and all membrane fractions from the plasma membrane and other organelles; however, in the liver homogenate, enzymatic activity was detectable in all membrane fractions, but not in the cytosol. We also tested the enzymatic activity with structurally-modified sphingosine derivatives. **Conclusion:** We found sphingosine 1-*O*-methyltransferase activity in the rat liver and kidney homogenates.

Introduction

Sphingolipids are ubiquitous constituents of eukaryotic membranes. Their metabolism is a highly dynamic process that generates various secondary messengers, including ceramide, sphingosine, and sphingosine 1-phosphate (S1P)[1–3]. Ceramide is deacylated by ceramidases to generate sphingosines, which can be phosphorylated by sphingosine kinases to produce S1P[1]. It is now generally understood that ceramide/sphingosine and S1P have contrasting effects on the stress response[1–4]. Sphingolipids, including sphingosine, S1P, and ceramide, have been studied intensively over the last 2 decade[1,5]. *N*,*N*-dimethyl-*D*-erythro-sphingosine (DMS), a methylated derivative of sphingosine, is found naturally in cells, and the enzymes that convert sphingosine to DMS have been detected in several tissues[6–8]. DMS was first reported as a protein kinase C inhibitor along with *D*-erythro sphingosine[9–11]. Later, Spiegel *et al* reported its specific inhibitory action on the sphingosine kinase and excluded its inhibitory action on protein kinase C[12,13]. Thereafter, DMS has been used as a specific inhibitor of the sphingosine kinase. Its action on tumor cell migration and cancer cell growth have also been reported and are a fundamental basis for the development of chemotherapeutic agents[14–16]. However, sphingosine *N*-methyltransferase, the enzyme responsible for DMS production, has not been well characterized.

On a preliminary note, the enzymatic methylation of sphingosine in the mouse brain homogenate with *S*-adenosyl-*L*-methionine (SAM) as the methyl donor was reported[6]. The *N*-methyltransferase responsible for DMS synthesis was shown to be inde-pendent of the *N*-methyltransferase that converts phosphatidylethanolamine to phosphatidylmonomethyl-ethanolamine, phosphatidyldimethyl-ethanolamine, and phosphatidylcholine. The former was only detectable in the brain (not in the liver), while the latter was present in both the brain and liver, and probably in all cells[17,18]. In this study, we tried to identify this enzymatic activity; however, we found another methyltransferase activity for sphingosine in the rat kidney and liver homogenates that transfers a methyl group from SAM to the C1 OH group of sphingosine.

Materials and methods

Materials Cold SAM was purchased from Sigma (St

Louis, MO, USA). *D*-erythro-sphingosine (synthetic), DMS, *N*-methyl-*D*-erythro-sphingosine (MMS), and *N*,*N*,*N*-trimethyl-*D*-erythro-sphingosine (TMS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). *S*-adenosyl-*L*-(methyl-^{3}H) methionine was procured from Amersham (GE Healthcare UK, Buckinghamshire, UK), while E^3 enhancer was purchased from PerkinElmer Life & Analytical Sciences (Boston, MA, USA). Silica gel high performance thin layer chromatography (HPTLC) (60 F_{254}, 20×20) was obtained from Merck (Darmstadt, Germany), and Kodak Medical X-ray film was purchased from Eastman Kodak (Rochester, NY, USA). *D*-threo-sphingosine, *L*-threo-sphingosine, and *L*-erythro-sphingosine were from Matreya (Pleasant Gap, PA, USA). C3-deoxy-sphinganine was kindly provided by SK CHUNG at Postech (Pohang, Gyungbuk, Korea)[19]. C1-deoxy-sphinganine was synthesized by WK LEE at Sogang University (Seoul Korea). All other materials were purchased from Sigma–Aldrich Korea (St Louis, MO, USA).

Preparation of tissue homogenates Rat tissue homogenates were prepared as described[6] with some modifications. Spleen, lung, thymus, heart, brain, and liver tissues were removed from Sprague-Dawley rats (n=2–3) and separately washed in cold phosphate-buffered saline. The tissues were placed in 10 mL cold 20 mmol/L Tris-HCl (pH 7.5) and 2 mmol/L EDTA solution, and homogenized with a Dounce homogenizer at 4 °C. The homogenate was centrifuged at 500×g for 10 min to remove unbroken cell debris, and the supernatant was used as an enzyme source for sphingosine methyltransferase.

Assay method for sphingosine methyltransferase We employed the same assay conditions as Igarashi and Hakomori to observe the enzymatic activity of sphingosine methyltransferase[6]. The complete reaction mixture included 40 mmol/L Tris-HCl (pH 7.8), 3 mmol/L $MgCl_2$, 10 μmol/L ^{3}H-SAM (1 mCi/mmol), 300 μmol/L sphingosine, and tissue homogenates (~5 mg of protein) in a final volume of 1 mL. Protein amounts were determined by the Micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Sphingosine was added to the reaction mixture as an exogenous substrate (300 μmol/L) and incubated for 1 h at 25 °C. The reaction was started by the addition of the tissue homogenate (~5 mg of protein) to the preheated reaction solution. After 1 h, the incubation was terminated by adding 100 μL of 10 mol/L HCl. To extract the metabolites, 5 mL chloroform:methanol mixture (2:1) was added and the tubes were capped and shaken.

The organic extracts of the reaction mixture were washed twice with the upper phase. The organic phase from each tube was then transferred and dried under nitrogen gas. The samples were reconstituted in 50 μL of chloroform:methanol mixture and spotted on 20×20 HPTLC silica gel plates for TLC. The plates were developed in chloroform:methanol:ammonium hydroxide (80:20:2) at room temperature and were allowed to air dry. Spots were visualized with an E^3 enhancer spray, and autoradiography was determined with Kodak medical X-ray film (3 d exposure at –80 °C).

Results

Sphingosine methyltransferase activity in kidney and liver Sphingosine methyltransferase activity was measured in rat tissue homogenates from the spleen, lung, thymus, kidney, heart, brain, and liver. As shown in Figure 1, enzymatic activity was detected in the kidney and liver homogenates. Standard sphingosine and methylated sphingosines moved to the position indicated by the arrow. In the liver homogenate, methylated lipids were also observed between methylated sphingosine and the origin of the HPTLC, implying further metabolism of the methylated sphingosine to sphingolipid metabolites, such as ceramide or sphingomyelin. In other homogenates, including the brain, enzymatic activity was undetectable, in contrast to the previous report in which sphingosine *N*-methyltransferase activity was detected in the mouse brain, but not in the liver[6]. The enzymatic activity in the kidney homogenate was measured under various pH conditions to find the optimum pH. The enzymatic activity was greatest at pH 7.8 (data not shown). To optimize the conditions for enzymatic activity, we measured the activity at 5, 15, 30, 60, and 120 min. The 60 min incubation was found to be the best (data not shown). Thus, subsequent experiments were conducted at pH 7.8 for 60 min.

Effect of metal ions on sphingosine methyltransferase The effects of $MgCl_2$, $CaCl_2$, $CoCl_2$, $NiCl_2$, and $ZnCl_2$ on enzymatic activity were tested. $ZnCl_2$ strongly inhibited enzymatic activity, but other metal ions did not influence the enzymatic activity in the kidney and liver homogenates (Figure 2A and 2C). The inhibitory effect of $ZnCl_2$ was concentration dependent (Figure 2B and 2D).

Fractionation of enzymatic activity by centrifugation The kidney and liver homogenates were fractionated by sequential centrifugation at 1000×g for 10 min, 3000×g for 10 min, 10000×g for 20 min, and 100000×g for 40 min. After centrifugation, we obtained 4 pellets (P1, P2, P3, and P4) and 1 supernatant (S4). Fraction P1 contained

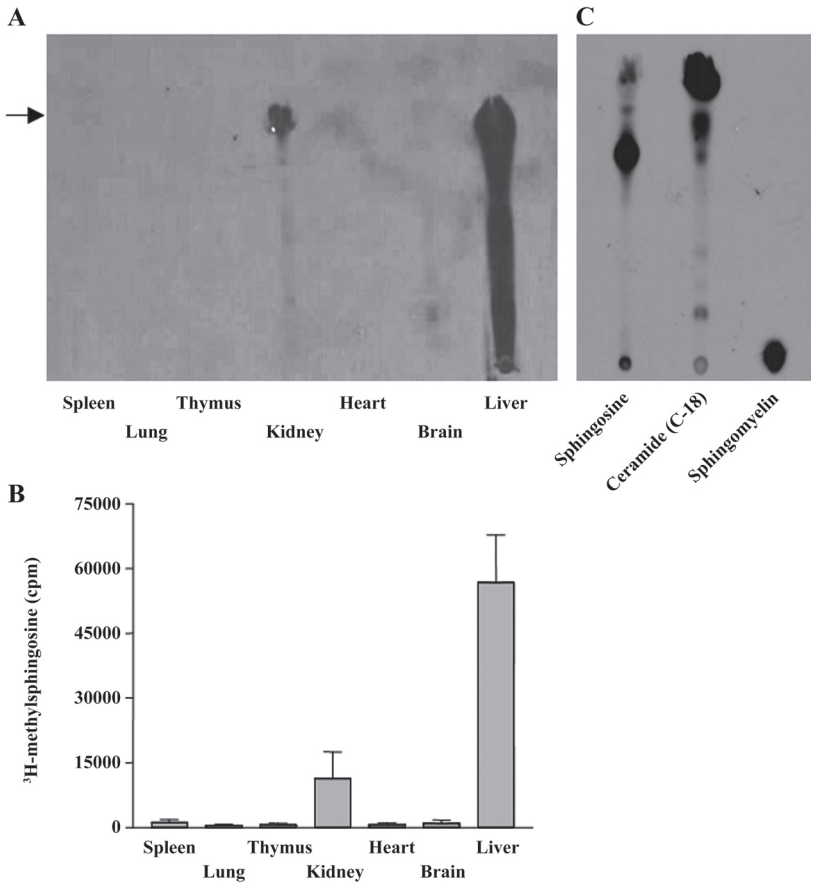

Figure 1. Tissue specificity of sphingosine methyltransferase activity in rat tissues. (A) rat tissue homogenates from Sprague–Dawley rats were incubated in the presence of exogenous *D*-erythro-sphingosine (300 μmol/L) with ^{3}H-SAM (250 μmol/L, 2 mCi/mmol) for 60 min at 25 °C. Lipids extracted with $CHCl_3$:MeOH were separated by TLC developed in $CHCl_3$:MeOH:NH_4OH (80:20:2). Autoradiography was performed for 3 d at –80 °C with Kodak X-OMAR film. Arrow indicates the position of the methylated sphingosine. (B) each area of methylated sphingosine from TLC was scrapped out, and radioactivity was measured with a liquid scintillation counter. Histogram shows the results from 2 independent experiments. (C) audioradiograph of TLC development of 3 standard sphingolipids in the same developing condition. Standards were *D*-erythro-(3-^{3}H)sphingosine, (palmitoyl-1-^{14}C) *N*-palmitoyl-*D*-sphingosine (C-18 ceramide), and (*N*-methyl-^{14}C)sphingomyelin.

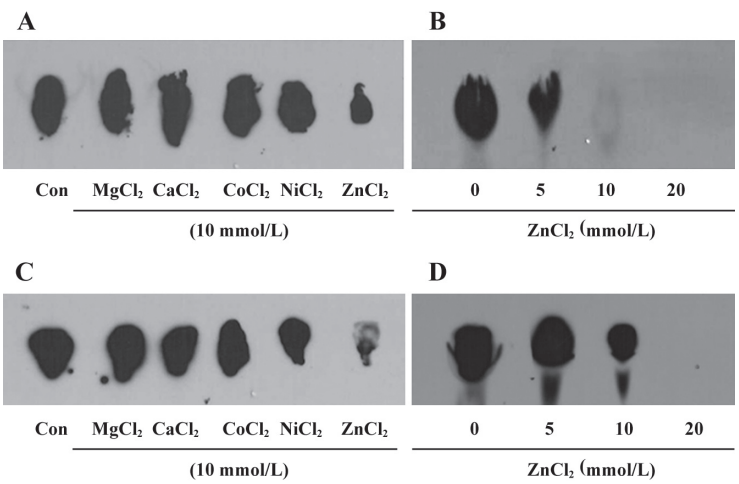

Figure 2. Inhibitory effect of Zn^{2+} on sphingosine methyltransferase activity. (A) rat kidney homogenate from SD rats was incubated in the presence of exogenous *D*-erythro-sphingosine (300 μmol/L) with ^{3}H-SAM (250 μmol/L, 2 mCi/mmol) at pH 7.8 for 60 min at 25 °C with 10 mmol/L of the indicated divalent cations. (B) enzymatic reaction of the kidney homogenate was conducted in the absence or presence of the indicated concentration of $ZnCl_2$. Lipid extraction, TLC, and autoradiography were carried out as described in materials and methods. (C) rat liver homogenate from SD rats was incubated in the presence of exogenous *D*-erythro-sphingosine (300 μmol/L) with ^{3}H-SAM (250 μmol/L, 2 mCi/mmol) at pH 7.8 for 60 min at 25 °C with 10 mmol/L of the indicated divalent cations. (D) enzymatic reaction of the liver homogenate was conducted in the absence or presence of the indicated concentration of $ZnCl_2$. Lipid extraction, TLC, and autoradiography were carried out as described in materials and methods.

nuclei, plasma membrane sheets, heavy mitochondria, and unbroken cells. Fraction P2 generally contained heavy mitochondria and plasma membrane fragments. Fraction P3 consisted of mitochondria, lysosomes, peroxisomes, Golgi membranes, and some rough endoplasmic reticulum, while fraction P4 contained membrane vesicles (microsomes) from smooth and rough endoplasmic reticulum. Fraction S4 contained all of the soluble

components from the cytoplasm.

In the kidney homogenate, the centrifugation fractions, pellets P1, P2, P3, and P4, exhibited enzymatic activity along with the supernatant (S4; Figure 3A). In the liver homogenate, fractions P1, P2, P3, and P4 showed stronger enzymatic activity than fractions from the kidney homogenate; there was no activity in the liver supernatant (S4; Figure 3B).

Substrate specificity Substrate specificity was next tested. MMS, DMS, and TMS were substrates for the enzyme (Figure 4A, 4D). Because TMS could not be further methylated on the C2 ammonium moiety, the enzymatic activity does not involve *N*-methylation on the C2 NH_2 group of sphingosines. Therefore, we tested whether 1-deoxy-sphinganine and 3-deoxy-sphinganine were substrates for the enzyme. As shown in Figure 4B, 3-deoxy-sphinganine was methylated, which indicated that *O*-methylation on C3 OH did not occur. Finally, we synthesized 1-deoxy-sphinganine and tested it as a substrate. 1-Deoxy-sphinganine was not methylated, suggesting that methylation occurs on the C1 OH group of sphingosines (Figure 4B, 4E). As shown in Figure 4C and 4F, no methylation was observed also in the liver homogenates with 1-deoxy-sphinganine, conclusively indicating that the enzyme is sphingosine 1-*O*-methyltransferase (Figure 5).

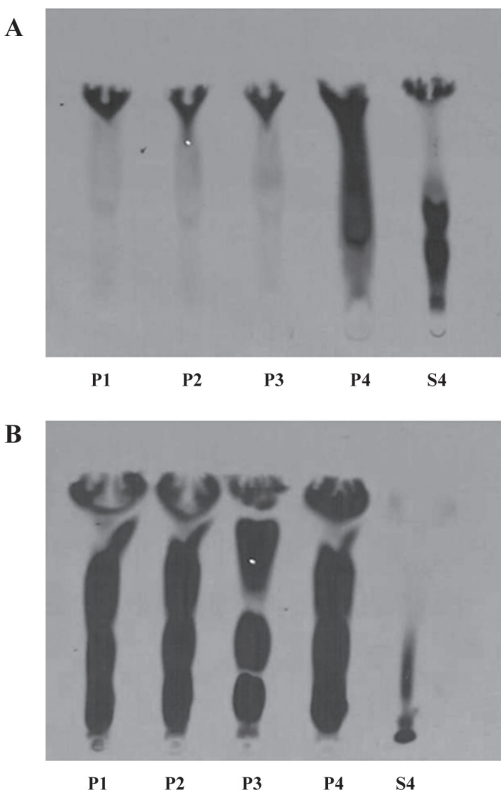

Figure 3. Fractionation of sphingosine methyltransferase activity from kidney and liver homogenates. (A) rat kidney homogenate from Sprague–Dawley rats was fractionated by centrifugation as described in Materials and methods. Each pellet and supernatant was used to assay the enzymatic activity in the presence of exogenous *D*-erythro-sphingosine (300 μmol/L) with ^{3}H-SAM (250 μmol/L, 2 mCi/mmol) at pH 7.8 for 60 min at 25 ^{o}C. (B) Rat liver homogenate from Sprague–Dawley rats was fractionated by centrifugation, and each pellet and supernatant was used to assay the enzymatic activity in the presence of exogenous *D*-erythro-sphingosine (300 μmol/L) with ^{3}H-SAM (250 μmol/L, 2 mCi/mmol) at pH 7.8 for 60 min at 25 ^{o}C.

Discussion

Initially, we attempted to detect sphingosine *N*-methyltransferase activity by converting sphingosine to DMS. However, after substrate-specific experiments, we concluded that the enzymatic activity that we had isolated was sphingosine *O*-methyltransferase on C1 OH (Figure 5). We found enzymatic activity in the liver and kidney homogenates, but not in the brain, contrary to the previous report in which sphingosine *N*-methyltransferase was detected in the mouse brain under the same experimental conditions[6].

Methylation by SAM-dependent *O*-methyltransferases (OMT) is a common alkylation reaction that is observed in the metabolism of mammals and in natural product biosynthesis. Catechol *O*-methyltransferase (COMT) is a well-known enzyme that catalyzes the transfer of the methyl group of SAM to the phenolic group of catechol compounds, including norepinephrine and dopamine[20]. In mammals, COMT is distributed throughout various organs. In plant biosynthesis[21], plant class I OMT are a group of low molecular weight (23–27 kDa), Mg^{2+}-dependent enzymes that were initially characterized as caffeoyl CoA OMT and shown to play an important role in the methylation of guaiacyl residue precursors of lignin, like caffeoyl or 5-hydroxyferuloyl CoA[22]. The substitution of Mg^{2+} with Ca^{2+} or Zn^{2+} in the caffeoyl CoA OMT assays marginally affected the substrate turnover rate and the substrate preference[23]. *O*-methylation by an inducible chlorophenol *O*-methyltransferase was almost completely inhibited by several metal ions, including Cu^{2+}, Hg^{2+}, Zn^{2+}, and Ag^{+}, whereas the inhibitory effects of Ca^{2+}, Cs^{+}, Fe^{2+}, and NH_4^{+} were weak. In our experiments, sphingosine 1-*O*-methyltransferase activity was inhibited by $ZnCl_2$, but not by $MgCl_2$, $CaCl_2$, $CoCl_2$, or $NiCl_2$, suggesting variable susceptibility to metal ions among OMT.

Igarashi and Hakomori found sphingosine *N*-methyltransferase activity in the mouse brain homogenate[6]. They used mouse brain and liver tissue homogenates to establish

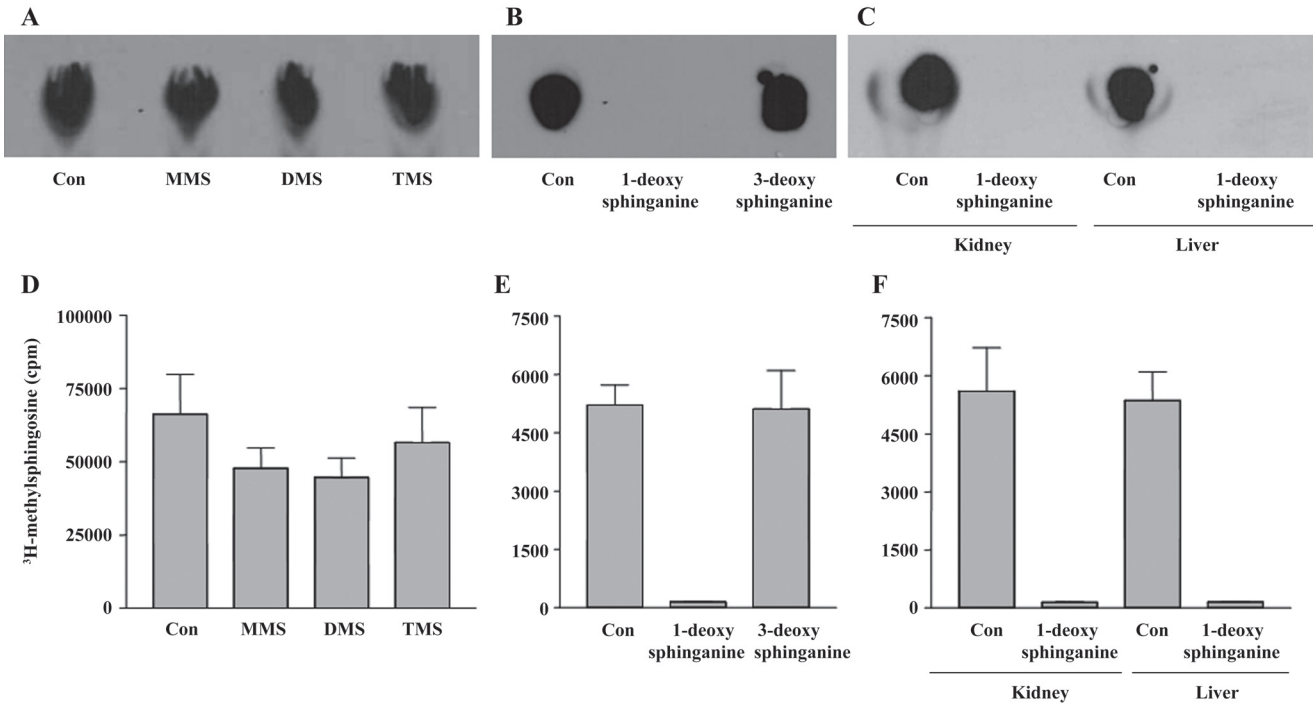

Figure 4. Substrate specificity of sphingosine methyltransferase activity in rat kidney homogenate. Rat kidney or liver homogenates from Sprague–Dawley rats were incubated in the presence of 300 μmol/L exogenous *D*-erythro-sphingosine (control), MMP, DMS, TMS, 3-deoxy-sphinganine, or 1-deoxy-sphinganine. Lipid extraction, TLC, and autoradiography were carried out as described in materials and methods. (A) representative results from the rat kidney homogenate with sphingosine (control), MMS, DMS, and TMS. (B) representative results from the rat kidney homogenate with sphingosine (control), 1-deoxy-sphinganine, and 3-deoxy-sphinganine. (C) representative results from the rat kidney and liver homogenates with sphingosine (control) and 1-deoxy-sphinganine. (D, E, F) Each area of methylated sphingosine from TLC was scrapped out, and radioactivity was measured with a liquid scintillation counter. Histogram shows the results from 2 independent experiments.

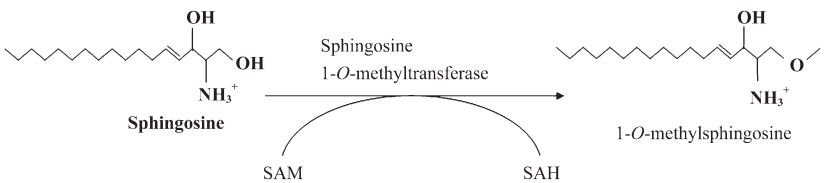

Figure 5. Proposed biochemical reaction. Sphingosine 1-*O*-methyltransferase transfers the methyl group of *S*-adenosyl-*L*-methionine to C1-oxygen of sphingosine to produce 1-*O*-methylsphingosine. SAH, *S*-adenosyl-*L*-homocysteine.

the *N*-methyltransferase enzymatic activity. However, we could not find *N*-methyltransferase activity in the rat brain homogenate. In contrast, we found sphingosine *O*-methylation on the C1 OH group of sphingosines in the rat kidney and liver homogenates. Currently, we can not explain why our results differ from the previous report, because we were not able to detect sphingosine *N*-methyltransferase activity in the mouse brain and liver homogenates also (data not shown).

We found the activity in the cytosol and membrane fractions of the kidney homogenate. This might imply that 2 isoforms of sphingosine 1-*O*-methyltransferase are present in the kidney. One is an integral protein in membrane fractions and the other is a cytosolic protein in the cytosol. This could be 2 separate gene products, or the same gene could be transcriptionally modified, such as splicing variants or at the post-translation stage, such as palmitoylation for the enzyme in the membrane fractions. Further investigation to identify the gene would elucidate truth. In the liver, we only detected the activity in membrane fractions, but not in the cytosol. This suggests that only 1 gene is expressed in the liver or the modification in the kidney does not happen in the liver.

In summary, we found sphingosine 1-*O*-methyltransferase activity in the rat liver and kidney homogenates is also responsible for the conversion of sphingosine to

methylated sphingolipids. Further investigation is necessary to determine whether 1-*O*-methylsphingosine is present in the liver and kidney to elucidate the pathophysiological significance of 1-*O*-methyl-sphingosine and to identify the enzyme at the molecular level for further studies of its regulation and relationship with certain diseases.

Author contribution

Dong-soon IM designed research; Santosh J SACKET performed research; Santosh J SACKET and Dong-soon IM wrote the paper.

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