Sphingomyelinases (SMases) are key enzymes involved in many diseases which are caused by oxidative stress, such as atherosclerosis, diabetes mellitus, nonalcoholic fatty liver disease, and Alzheimer’s disease. SMases hydrolyze sphingomyelin to generate ceramide, a well-known pro-apoptotic lipid. SMases are classified into five types based on pH optimum, subcellular localization, and cation dependence. Previously, we demonstrated that elevation of secretory sphingomyelinase (sSMase) activity increased the plasma ceramide concentration under oxidative stress induced by diabetes and atherosclerosis in murine models. These results suggest that sSMase inhibitors can prevent the progress of these diseases. The present study demonstrated that sSMase activity was activated by oxidation and inhibited by reduction. Furthermore, we examined whether catechins inhibited the sSMase activity in a physiological plasma concentration. Among catechins, (−)-epicatechin 3-O-gallate (ECg) exhibited strong inhibitory effect on sSMase (IC50=25.7 μM). This effect was attenuated by methylation at the 3′- or 4′-position. On the other hand, (−)-epigallocatechin 3-O-gallate (EGCg) and (−)-catechin 3-O-gallate (Cg) exhibited weaker inhibitory activity than ECg, and (−)-epicatechin and (−)-epigallocatechin did not affect sSMase activity. Additionally, one synthetic catechin, (−)-3′-O-methylpygallocatechin 3-O-gallate (EGCg-3′-O-Me), showed the strongest inhibitory effect (IC50=1.7 μM) on sSMase. This phenomenon was not observed for (−)-4′-O-methylpygallocatechin 3-O-gallate. These results suggest that the reduction potential, the presence of the galloyl residue at the C-3 position, and the steric requirement to interact with sSMase protein are important for effective inhibition of sSMase.

**Key Words** sphingomyelinas, redox, catechin, antioxidant

SMases are classified into five types based on pH optimum, subcellular localization, and cation dependence (12). Among them, acid SMase (aSMase) with an optimal pH of 4.8 functions in the endosomal-lysosomal compartments or plasma membrane (13). The gene coding aSMase (smpd1) produces two different enzymes,—lysosomal SMase and secretory SMase (sSMase)—through alternative trafficking of the same protein precursor (14, 15). sSMase is secreted by the vascular endothelium and macrophages, and is the only enzyme responsible for sphingolytic activity in the plasma (16).

Previously, we demonstrated that increased sSMase activity resulted in an increase of the ceramide in the plasma of diabetic and atherosclerotic murine models which had been exposed to systemic oxidative stress (17, 18). These results suggest that inhibition of sSMase activity is effective in the prevention of these diseases.

Although many studies were conducted on the aSMase functional inhibitor, FIASMA (19), no report is available concerning sSMase. Because neutral SMase is inhibited by glutathione (GSH) (20), some chemicals with strong reducing power might affect sSMase activity.

In the present study, we investigated whether sSMase was affected by redox, as well as the inhibitory activity of catechins, well-known antioxidants, on sSMase to find effective sSMase inhibitors in food materials.
MATERIALS AND METHODS

Materials. All the solvents, catalase, and (−)-epicatechin 3-O-gallate (EC) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (−)-Epigallocatechin 3-O-gallate (EGC) and (−)-epicatechin 3-O-gallate (ECg) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (−)-Epicatechin 3-O-(3″-O-methyl)gallate (ECg-3″-O-Me) and (−)-Epicatechin 3-O-(4″-O-methyl)gallate (ECg-4″-O-Me) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents, (−)-epicatechin (EC), and (−)-epigallocatechin (EGC) were obtained from Sigma-Aldrich (St. Louis, MO). Seven methylated catechins were obtained from Nagara Science Co., Ltd. (Gifu, Japan); (−)-3′-O-methyl-epigallocatechin (EGC-3′-O-Me), (−)-3′-O-methylepicatechin 3-O-gallate (EGCg-3′-O-Me), (−)-4′-O-methyl-epigallocatechin 3-O-gallate (EGCg-4′-O-Me), (−)-3′-O-methyl-epigallocatechin 3-O-gallate (EGC-3′-O-Me), (−)-4′-O-methyl-epigallocatechin 3-O-gallate (EGCg-4′-O-Me), (−)-Epigallocatechin 3-O-gallate (EGCg) was a gift from Mitsui-Nourin Inc. (Tokyo, Japan). Gallic acid (GA) and methyl gallate (MG) were purchased from Fuji Chemical Industry Co., Ltd. (Wakayama, Japan). Procyanidin B1 was purchased from Extrasynthese S.A. (Lyon, France). Nitrobenzofurazan (NBD) C6-SM was purchased from Molecular Probes Inc. (Eugene, OR). All other reagents, (−)-epicatechin (EC), and (−)-epigallocatechin (EGC) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). The structures of these catechins are shown in Fig. 1.

sSMase enzyme from rats. This study was approved by the Animal Care Committee of Nara Women’s University. We used the plasma of ten-week-old male rats (SLC: Sprague-Dawley strain), which were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The animals were housed in a room at 24±2°C, with a 12 h/12 h light–dark cycle. Animals were fed commercial laboratory chow (CE-2, Oriental Yeast Co., Ltd., Osaka, Japan) and water ad libitum. The rats were anesthetized with pentobarbital, and sacrificed by collecting the blood from the inferior vena cava using a syringe containing sodium heparin as an anticoagulant. The plasma was separated from the blood sample by centrifugation and used as an enzyme solution.

Change in sSMase activity by the redox state. sSMase activities were measured using NBD C6-SM as a substrate, as previously described (17, 18). To examine the effect of oxidation on sSMase activity, 100 μL of rat plasma was incubated with 500 μL of H2O2 solution at 0, 10, 100, and 1,000 μM final concentrations for 15 min at 37°C in 20 mM Tris-HCl buffer (pH 7.5) (the oxidized sample). To examine the effect of reduction, 100 μL of plasma was incubated with 500 μL of reduced glutathione (GSH) at 0, 4, 10, and 20 mM final concentrations for 15 min at 37°C in 20 mM Tris-HCl buffer (pH 7.5) (the reduced sample). To examine the effect of redox cycling, sSMase in 100 μL of plasma was treated with H2O2 at 100 μM final concentration for 15 min at 37°C, then H2O2 was removed by 920 units of catalase (Wako Pure Chemicals Industries, Ltd., 9,200 units per mg), and the resulting enzyme solution was incubated with GSH at 0, 7, 14, and 20 mM final concentrations for 15 min at 37°C (the redox cycling sample). To prepare the reoxidized sample, H2O2 was added to the sample reduced by 10 mM GSH at 1,000 mM final concentration (the reoxidized sample).

Inhibition of sSMase by catechins and other compounds. Five catechins, seven methylated catechins, and galloyl compounds (50 μM final concentration) were incubated with sSMase in 15 μL of plasma for 15 min at 37°C. The total volume of the incubation mixture was 800 μL containing 0.1 mM ZnSO4, 0.1% Nonidet P-40,
Sphingomyelinase Inhibition by Catechins

and 62 mM sodium acetate (pH 5.0). After incubation, 4 nmol of NBD C₆-SM in 10 μL of methanol was added as a substrate. The enzymatic reaction was performed for 2 h at 37°C, and was stopped by adding 2 mL of chloroform and methanol. After vortexing and centrifugation, the chloroform layer was collected, evaporated, and dissolved in 500 μL of methanol. Twenty μL of the solution was directly applied to a HPLC system (Waters, 1525 Binary HPLC Pump) using a Nova Pak 4 μm C18 column (3.9×150 mm, Waters Corp. Milford, MA). NBD C₆-ceramide generated from NBD C₆-SM was eluted at a flow rate of 1 mL/min with a mixture of water, acetonitrile, and phosphoric acid at a volume ratio of 35:65:0.2. The fluorescence of NBD of these compounds was recorded with a fluorescence detector (Waters, type 2479, excitation at 466 nm and emission at 536 nm).

Kinetic data analysis. To determine the inhibition pattern, sSMase was reacted in the presence of 0, 2.5, and 5 μM concentrations of EGCg-3′-O-Me and the contents of NBD C₆-SM were changed in a range from 1 to 8 nmol. Duplicated experiments were performed to determine $K_m$ and $V_{max}$. The kinetic data for $K_m$ and $V_{max}$ were analyzed by using the R (Free Software under the terms of the Free Software Foundation's GNU General Public License). The velocities were fitted to the Michaelis-Menten model by using package “drc” and its function “drm”. The Figure was drawn as double reciprocal plot with Excel.

Statistical analyses. Data were expressed as mean±SEM and analyzed through a multiple comparison test using the Statcel software (OMS Publishing Inc., Tokyo, Japan). Differences between group means were considered significant at $p<0.01$ using the Scheffe’s method generated by this program.

RESULTS

Change in sSMase activity by the redox state

To examine whether sSMase is affected by the redox state, we determined the change in sSMase activity
Kobayashi K et al. 

Under oxidative and reductive conditions. SSMASe was incubated by five types of catechins: EC, EGC, ECg, EGCg, and Cg (50 μM as final concentration). Values are mean ± SEM for 4–8 runs in each catechin group and 8 runs in the control. Different superscript letters indicate significant differences at p<0.01 (Scheffe's method).

On the other hand, addition of GSH decreased the SSMASe activity in a dose-dependent manner. GSH at 10 mM significantly inhibited the SSMASe activity, as compared with the control without GSH, and GSH at 20 mM further inhibited SSMASe (Fig. 2B). Additionally, dithiothreitol showed a stronger inhibition effect than GSH (data not shown).

Next, we examined whether the effect of the redox state on SSMASe was reversible. The SSMASe activity was activated by 100 mM H2O2 followed by H2O2 decomposition by catalase. This activation was suppressed by treatment with 7 mM and higher GSH concentrations (Fig. 2C). Moreover, SSMASe, which was activated by 100 mM H2O2 and then inhibited by 10 mM of GSH, was activated again by 1,000 mM of H2O2 (Fig. 2D). However, 100 mM of H2O2 did not activate the SSMASe reduced by GSH, probably because H2O2 was promptly decomposed by GSH.

Since SSMASe was easily activated under aerobic conditions, we presented the SSMASe activities as relative values versus the corresponding control values. Inhibition of SSMASe by five catechins

Catechins are well-known antioxidants with a strong reducing power. We examined the inhibitory effect of five catechins contained in green tea on SSMASe (Fig. 3). ECg significantly inhibited SSMASe. Other gallate-type catechins such as EGCg and Cg also significantly inhibited SSMASe; however, these catechins had a weaker inhibitory effect than ECg. By contrast, EC and EGC did not affect SSMASe activity. Among these catechins, ECg showed the strongest inhibitory effect on SSMASe. The IC50 value of ECg was 25.7 μM.

SSMASe inhibition by ECg and its reversal by H2O2

The SSMASe activated by 100 mM H2O2 that is followed by decomposition of H2O2 with catalase was incubated with 50 mM ECg for 15 min at 37˚C. The activity of the resulting SSMASe was significantly higher than that by ECg. By contrast, EC and EGC did not affect SSMASe activity. Among these catechins, ECg showed the strongest inhibitory effect on SSMASe. The IC50 value of ECg was 25.7 μM.

SSMASe inhibition by galloyl chemicals and catechin dimer

The inhibition of SSMASe by various galloyl compounds and catechin dimer (Fig. 5). Gallic acid (GA) and methyl gallate (MG) did not show any inhibitory effect. PGG with five galloyl residues exhibited a significant inhibitory effect, which was weaker than that by ECg. In addition, procyanidin B1, which is a dimer of catechin (epicatechin-(4β-8)-catechin), had no effect on SSMASe.
sSMase inhibition by seven methylated catechins

To determine the necessary hydroxyl group(s) for the inhibitory activity of catechins, the inhibitions of sSMase by four methylated ECgs were compared. The inhibitory activity of ECg was significantly attenuated by methylation at the 3′-, 4′-, 3″-, or 4″-position of the galloyl residue (Fig. 6A, and B). The inhibitory activity of ECg-3′-O-Me was significantly stronger than that of ECg-4′-O-Me (p<0.01).

Furthermore, the inhibitory activity of two methylated EGCgs (Fig. 6C) was examined. EGCg-3′-O-Me showed the strongest inhibitory effect on sSMase (IC$_{50}$=1.7 μM) among the catechins examined in the present study. It was much stronger than that of ECg. The inhibitory activity of EGCg-4′-O-Me was significantly weaker than that of ECg, EGC, and EGC-3′-O-Me did not inhibit sSMase (Fig. 6D).

To determine the inhibitory pattern of EGCg-3′-O-Me, sSMase was reacted with 0, 2.5, and 5 μM concentrations of EGCg-3′-O-Me and fitted to the Michaelis-Menten model. The $V_{\text{max}}$ and $K_m$ values were different under each reaction condition (Table 1). In addition, the three plots in the double reciprocal plot did not intersect at one point. These results indicated that the inhibition of sSMase by EGCg-3′-O-Me did not follow the competitive inhibition mechanism.

### DISCUSSION

Although sSMase was activated by copper-promoted oxidation (21), this is the first study demonstrating that sSMase in plasma is also activated by oxidation. Furthermore, sSMase is inhibited by reductants such as GSH and dithiothreitol, and the redox regulation of the sSMase activity is found to be reversible. Therefore it is expected that the sSMase activity reflects the oxidative stress in plasma and it is a useful marker of diseases.
such as diabetes (17) and atherosclerosis (18), because oxidative stress is involved in their pathogenicity.

Since the activity of sSMase is attenuated by reduction, an antioxidant may affect the sSMase activity. This study demonstrated that catechins, a typical food-derived antioxidant and reductant, effectively inhibited sSMase. Four catechins—EC, EGC, ECg, and EGCg—are the main components of green tea. Among them, ECg showed a strong inhibitory effect on sSMase. EGCg also exerted an inhibitory effect, although it was weaker than ECg. However, the reducing ability of EGCg was stronger than that of ECg (22), suggesting that the steric interaction of the sSMase protein with ECg is more favorable than that with EGCg.

The results concerning gallate type catechins (Fig. 3) indicate that the presence of the galloyl group at the C-3 position is important for the inhibitory effect on sSMase. The same phenomenon is observed in procyanidin B1 with no galloyl residue (Fig. 5). In addition, the results of ECg-3′-O-Me and EGCg-4′-O-Me (Fig. 6A) indicated that the galloyl structure was important. Meanwhile, the inhibitory activity of PGG with five galloyl residues was not stronger than that of ECg (Fig. 5). It is suggested that both the alcohol part and the flavanol structure of ECg are necessary to exhibit the inhibitory activity on sSMase. This idea is consistent with the results for methyl gallate, which had no inhibitory effect (Fig. 5).

It is worthwhile to note that the catechol structure of the B-ring is important for the inhibition of sSMase. On the other hand, Cg, which is the epimer of ECg and has a catechol structure similar to that of ECg, showed weaker inhibitory activity on sSMase than that of ECg. Since the reduction potential of Cg may approximate that of ECg, the difference between these epimers at the C-2 position may be ascribed to the steric discrimination by the sSMase protein.

There are some green teas that contain methylated catechins, such as Benifuuki. Methylated catechins have an anti-allergic effect (23). Among seven types of methylated catechins, EGCg-3′-O-Me showed the strongest inhibitory effect on sSMase (IC50 = 1.7 μM). Matsuura et al. (21) reported that the pattern of electric potential of EGCg-3′-O-Me resembled that of ECg, indicating that the redox character of the pyrogallol moiety of EGCg-3′-O-Me was changed to that of catechol by methylation of the 3′-position, which also caused stabilization of EGCg. On the other hand, the redox pattern of EGCg-4′-O-Me resembled that of MG, showing that methylation of the 4′-position of the B-ring of EGCg abolished the redox character of its B-ring (21). From these results, it is suggested that the ortho-diphenol structure at the B-ring was necessary to inhibit sSMase.

Gallate-type catechins have been reported to easily form a conjugated bond to the cysteine of many proteins such as albumin, and GAPDH (24, 25). Such an addition reaction could occur between the cysteine residue at the C-terminal domain of sSMase and catechins. However, considering the result that the inhibition by ECg was reversed by oxidation with H2O2 similar to the case of GSH, reduction of the cysteine group(s) may be a preferred mechanism for the inhibition of sSMase by catechins.

It is worthwhile to note that catechins have metal ion chelating activity. As sSMase needs a zinc ion, the inhibitory effect of catechins might be due to their chelating activity. However, the zinc-chelating activities of EC, ECg, EGCg, and procyanidin B1 were almost similar as detected by the decrease of the fluorescence emission of zinc-Zinquin complexes (26). Therefore, it is concluded that the zinc-chelating activity does not primarily contribute to the sSMase inhibition.

The results of the Michaelis-Menten model and the double reciprocal plot indicate that EGCg-3′-O-Me mainly affects sSMase activity through a reduction reaction and does not compete with SM for the binding site of the enzyme (Fig. 7, Table 1).

Oxidative stress is implicated in the pathogenesis of diabetes and atherosclerosis. Moreover, sSMase activity, which is activated by oxidation, is increased in these diseases, resulting in increased plasma ceramide (17, 18). Ceramide is suggested to be a risk factor for these diseases (27, 28, 29). Therefore, it is possible that the inhibition of sSMase effectively prevents these diseases. The beneficial effects of catechins in preventing these diseases are well documented (30), and the present study suggests that one of these effects of catechins can be ascribed to their ability to inhibit sSMase.

In conclusion, EGCg-3′-O-Me and ECg have a strong inhibitory effect on sSMase in a physiological plasma concentration, i.e., micromolar order. This inhibition may be due to their specific structures such as methoxycatechol and galloyl residue, and it depends on the reducing power as well as the steric interaction with the sSMase protein.

Acknowledgments
This work was supported by Nara Women’s University Intramural Grant for Project Research (2013).

REFERENCES
1) Marathe S, Kuriakose G, Williams KJ, Tabas I. 1999. Sphingomyelinase, an enzyme implicated in atherogen-
Sphingomyelinase Inhibition by Catechins

Yamada Y, Kajiwara K, Yano M, Kishida E, Masuzawa Y, Schuchman EH. 2010. Acid sphingomyelinase, cell membranes and human disease: lessons from Niemann-Pick disease. *FEBS Lett* **531**: 38–46.

Fujita Y, Hidaka A, Kutsukake M, Tabas I. 1999. Secretory sphingomyelinase. *Chem Phys Lipids* **102**: 123–130.

Kobayashi K, Ichii I, Nakagawa T, Kamikawa C, Kitamura Y, Koga E, Washino Y, Hoshinaga Y, Kojo S. 2011. Increase in plasma ceramide levels via secretory sphingomyelinase activity in streptozotocin-induced diabetic rats. *Med Chem Commun* **2**: 536–541.

Kobayashi K, Nagata E, Sasaki K, Harada-Shiba M, Kojo S, Kikuzaki H. 2013. Increase in secretory sphingomyelinase activity and specific ceramides in the aorta of apolipoprotein E knockout mice during aging. *Biol Pharm Bull* **36**: 1192–1196.

Kornhuber J, Tripal P, Reichel M, Mühl C, Rhein C, Muehlbacher M, Groemer TW, Gulbins E. 2010. Functional inhibitors of acid sphingomyelinase (FIASMAs): a novel pharmacological group of drugs with broad clinical applications. *Cell Physiol Biochem* **26**: 9–20.

Liu B, Hannun YA. 1997. Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. *J Biol Chem* **272**: 16281–16287.

Qi H, Edmunds T, Baker-Malcolm J, Karye KP, Estes S, Schwarz C, Hughes H, Van Patten SM. 2003. Activation of human acid sphingomyelinase through modification or deletion of C-terminal cysteine. *J Biol Chem* **278**: 32744–32752.

Matsuura K, Usui Y, Kan T, Ishii T, Nakayama T. 2014. Structural specificity of electric potentials in the coulo- metric-array analysis of catechins and theaflavins. *J Clin Biochem Nutr* **55**: 103–109.

Maeda-Yamamoto M, Inagaki N, Kitaura J, Chikumoto T, Kawahara H, Kawakami Y, Sano M, Miyase T, Tachibana H, Nagai H, Kawakami T. 2004. O-methylated catechins from tea leaves inhibit multiple protein kinases in mast cells. *J Immunol* **172**: 4486–4492.

Ishii T, Mori T, Tanaka T, Mizuno D, Yamaji R, Kumazawa S, Nakayama T, Akagawa M. 2008. Covalent modification of proteins by green tea polyphenol (+-)epigallocatechin-3-gallate through antioxidation. *Free Radic Biol Med* **45**: 1384–1394.

Li M, Hagerman AE. 2014. Role of the flavan-3-ol and galloyl moieties in the interaction of (+)-epigallocatechin gallate with serum albumin. *J Agric Food Chem* **62**: 3768–3775.

Quesada IM, Bustos M, Blay M, Pujadas G, Ardevol A, Salvadó MJ, Bláez C, Arola L, Fernández-Larrera J. 2011. Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells. *J Nutr Biochem* **22**: 153–163.

Ichii I, Nakahara K, Kiso K, Kojo S. 2007. The effect of dietary cholesterol and high fat on ceramide concentrations in rat tissues. *Nutrition* **23**: 570–574.

Ichii I, Takashima Y, Adachi N, Nakahara K, Kamikawa C, Harada-Shiba M, Kojo S. 2007. Effects of dietary cholesterol on tissue ceramides and oxidation products of apolipoprotein B-100 in apoE deficient mice. *Lipids* **42**: 893–900.

Ichii I, Nakahara K, Miyashita Y, Hidaka A, Kutsukake S, Inoue K, Maruyama T, Miwa Y, Harada-Shiba M, Tsushima M, Kojo S, Kisei Cohort Study Group. 2006. Association of ceramides in human plasma with risk factors of atherosclerosis. *Lipids* **41**: 859–863.

Crespy V, Williamson G. 2004. A review of the health effects of green tea catechins in vivo and animal models. *J Nutr* **134**: 3431S–34408.