Strong Inhibition of Secretory Sphingomyelinase by Catechins, Particularly by (–)-Epicatechin 3-O-Gallate and (–)-3′-O-Methylepigallocatechin 3-O-Gallate

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Summary 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 ($IC_{50} = 25.7 \mu M$). 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-methylepigallocatechin 3-O-gallate (EGCg-3′-O-Me), showed the strongest inhibitory effect ($IC_{50} = 1.7 \mu M$) on sSMase. This phenomenon was not observed for (–)-4′-O-methylepigallocatechin 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 sphingomyelinase, redox, catechin, antioxidant

Sphingomyelinases (SMases) are involved in the pathogenesis of many diseases such as atherosclerosis, diabetes mellitus, coronary heart disease, nonalcoholic fatty liver disease, Alzheimer's disease, and tumor metastasis (1–6). SMases hydrolyze sphingomyelin to generate ceramide, which is a well-known pro-apoptotic lipid (7) and serves as an intracellular second messenger (8). Oxidative stress caused, for example, by UV light, antineoplastic drugs, and radiation induces SMase activation and ceramide accumulation in the cell (9–11).

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.

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Abbreviations: aSMase, acid sphingomyelinase; Cg, (–)-catechin gallate; EC, (–)-epicatechin; ECg, (–)-epicatechin 3-O-gallate; ECG-3′-O-Me, (–)-3′-O-methylepicatechin 3-O-gallate; ECG-3″-O-Me, (–)-epicatechin 3-O-(3″-O-methyl)gallate; ECG-4′-O-Me, (–)-4′-O-methylepicatechin 3-O-gallate; ECG-4″-O-Me, (–)-epicatechin 3-O-(4″-O-methyl)gallate; EGC, (–)-epigallocatechin; EGC-3′-O-Me, (–)-3′-O-methylepigallocatechin; EGCg, (–)-epigallocatechin 3-O-gallate; EGCg-3′-O-Me, (–)-3′-O-methylepigallocatechin 3-O-gallate; EGCg-4′-O-Me, (–)-4′-O-methylepigallocatechin 3-O-gallate; GA, gallic acid; GSH, reduced glutathione; MG, methyl gallate; NBD, nitrobenzofurazan; PGG, penta-O-galloyl-β-D-glucose; SMase, sphingomyelinase; SMases, sphingomyelinases; SM, sphingomyelin; sSMase, secretory sphingomyelinase.

Fig. 1. Structures of catechins.

MATERIALS AND METHODS

Materials. All the solvents, catalase, and (–)-epicatechin 3-O-gallate (ECg) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (–)-Catechin 3-O-gallate (Cg) and penta-O-galloyl-β-D-glucose (PGG) hydrate were purchased from Sigma-Aldrich (St. Louis, MO). Seven methylated catechins were obtained from Nagara Science Co., Ltd. (Gifu, Japan); (–)-3'-O-methylepigallocatechin (EGC-3'-O-Me), (–)-3'-O-methylepigallocatechin 3-O-gallate (EGCg-3'-O-Me), (–)-4'-O-methylepigallocatechin 3-O-gallate (EGCg-4'-O-Me), (–)-3'-O-methylepigallocatechin 3-O-gallate (EGCg-3'-O-Me), (–)-4'-O-methylepigallocatechin 3-O-gallate (EGCg-4'-O-Me), (–)-epicatechin 3-O-(3''-O-methyl)gallate (ECg-3''-O-Me) and (–)-epicatechin 3-O-(4''-O-methyl)gallate (ECg-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 mM 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 mM 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,

Fig. 2. Changes in sSMase activity under oxidation by H₂O₂ (A) and reduction by GSH (B). sSMase was oxidized by 0, 10, 100, and 1,000 mM of H₂O₂. To prepare reduced sSMase, sSMase was incubated with 0, 4, 10, and 20 mM of GSH. Values are mean $\pm$ SEM for 4–12 runs. Different superscript letters indicate significant differences at $p < 0.01$ (Scheffe's method). Effect of redox cycling on sSMase activity (C). sSMase was reduced by 7, 10, 14, and 20 mM of GSH after the oxidation by 100 mM of H₂O₂. Different superscript letters indicate significant differences at $p < 0.01$ (Scheffe's method). Changes in sSMase activity by reoxidation (D). sSMase was oxidized by 100 mM of H₂O₂. After removing the residual H₂O₂ by catalase, GSH was added to a final concentration of 10 mM. Then the enzyme solution was reoxidized in the presence of 100 and 1,000 mM of H₂O₂. Values are mean $\pm$ SEM for 4–12 runs in each group. Different superscript letters indicate significant differences at $p < 0.01$ (Scheffe's method).

and 62 mM sodium acetate (pH 5.0). After incubation, 4 nmol of NBD C₆-SM in 10 μ L 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 μ L of methanol. Twenty μ L of the solution was directly applied to a HPLC system (Waters, 1525 Binary HPLC Pump) using a Nova Pak 4 μ m C18 column (3.9 $\times$ 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 μ M 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 $\pm$ 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

Fig. 3. Changes in sSMase activity under incubation by catechins. sSMase was incubated by five types of catechins: EC, EGC, ECg, EGCg, and Cg (50 μ M as final concentration). Values are mean $\pm$ 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).

Fig. 4. Inhibition reversal of sSMase activity by catechins by reoxidation. sSMase was oxidized by 100 mM of H₂O₂. After removing the residual H₂O₂ by catalase, 50 μ M of ECg was added. Then, the enzyme solution was reoxidized with 1,000 mM of H₂O₂. Values are mean $\pm$ SEM for 4 runs in each group. Different superscript letters indicate significant differences at $p < 0.01$ (Scheffe's method).

under oxidative and reductive conditions. sSMase was activated by H₂O₂ in a dose-dependent manner (Fig. 2A). H₂O₂ at 100 mM (final concentration) significantly activated sSMase compared with the control without H₂O₂. H₂O₂ at 1,000 mM activated sSMase significantly more than H₂O₂ at 100 mM (Fig. 2A).

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 decomposi-

Fig. 5. Inhibition of sSMase activity by galloyl chemicals and catechin dimer. sSMase was incubated with GA, MG, PGG, and procyanidin B1 (final concentration at 50 μ M). Values are mean $\pm$ SEM for 4–9 runs in each group. Different superscript letters indicate significant differences at $p < 0.01$ (Scheffe's method).

tion 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 μ M.

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 μ M ECg for 15 min at 37°C. The activity of the resulting sSMase was reduced significantly to 17% of the oxidized enzyme with H₂O₂ (Fig. 4). Then the reduced enzyme was significantly reactivated by 1,000 mM of H₂O₂ (Fig. 4).

sSMase inhibition by galloyl compounds and catechin dimer

The inhibition of sSMase by various catechins indicated that the galloyl group was essential. We investigated the effect of some galloyl compounds on the sSMase activity (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-(4 β -8)-catechin), had no effect on sSMase.

Fig. 6. Inhibition of sSMase activity by methylated ECg molecules (A, B). Values are mean $\pm$ SEM for 4 runs in each group. Different superscript letters indicate significant differences at $p < 0.01$ (Scheffé's method). Inhibition of sSMase activity by methylated EGCg (C). Values are mean $\pm$ SEM for 4 runs in each group. Different superscript letters indicate significant differences at $p < 0.01$ (Scheffé's method). Inhibition of sSMase activity by methylated EGC (D). Values are mean $\pm$ SEM for 4 runs in each group. Different superscript letters indicate significant differences at $p < 0.01$ (Scheffé's method).

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 ($IC_{50} = 1.7 \mu\text{M}$) 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 EGCg. 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 $5 \mu\text{M}$ concentrations of EGCg-3'-O-Me and fitted to the Michaelis-Menten model. The V_{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

Table 1. The change of K_m values and V_{max} values when sSMase was inhibited by EGCg-3'-O-Me.

	EGCg-3'-O-Me concentration		
	0 μM	2.5 μM	5 μM
V_{max} (pmol/mL/min)	455.10	343.02	261.64
K_m (μM)	19.31	15.27	15.03

Data were fitted to the Michaelis-Menten model by using the R.

sSMase by EGCg-3'-O-Me did not follow the competitive inhibition mechanism.

DISCUSSION

Although aSMase 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 ECg-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 ($IC_{50}=1.7\ \mu M$). 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 H_2O_2 similar to the case of GSH, reduction of the cysteine group(s) may be

Fig. 7. Double reciprocal plot of sSMase activity versus NBD C₆-SM concentrations in the presence of EGCg-3'-O-Me.

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, EGC, 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.

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