Increase in Secretory Sphingomyelinase Activity and Specific Ceramides in the Aorta of Apolipoprotein E Knockout Mice during Aging

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Atherosclerosis is caused by many factors, one of which is oxidative stress. We recently demonstrated that systemic oxidative stress increased secretory sphingomyelinase (sSMase) activity and generated ceramides in the plasma of diabetic rats. In addition, we also showed that the total ceramide level in human plasma correlated with the level of oxidized low-density lipoprotein. To investigate the relationship between ceramide species and atherogenesis during aging, we compared age-related changes in ceramide metabolism in apolipoprotein E knock out mice (apoE$^{-/-}$) and wild type mice (WT). Although the total plasma ceramide level was higher in apoE$^{-/-}$ than that in WT at all ages, it decreased with increasing age. sSMase activity increased at 65 weeks (w) of age in both strains of mice. When apoE$^{-/-}$ developed atherosclerosis at 15 w of age, C18:0, C22:0, and C24:0 ceramide levels in the apoE$^{-/-}$ aorta significantly increased. Furthermore, at 65 w of age C16:0 and C24:1 ceramide levels were significantly higher than those in WT. These results suggested that elevation in levels of specific ceramide species due to sSMase activity contributed to atherogenesis during aging.

Key words aging; apolipoprotein E; atherosclerosis; ceramide; sphingomyelinase

Cardiovascular disease is associated with aging. Hyperlipidemia and long-term exposure to various stimuli such as oxidative stress lead to atherosclerosis. Despite several indications that aging causes atherosclerosis, a specific factor associated with atherosclerosis that increases or decreases with aging is yet to be entirely identified. One possible factor is elevation of bioactive lipid such as ceramide. Ceramide regulates cell cycle arrest, apoptosis, and cellular senescence,[1] and serves as an intracellular second messenger in these processes.[2] Oxidative stress inducers such as UV light, antineoplastic drugs, and radiation stimulate ceramide accumulation in the cell.[3–6] Ceramide belongs to the sphingolipid family and comprises a saturated or unsaturated fatty acid of C16–C26 chain length bound to the amino group of sphingosine.

Ceramide is generated by *de novo* synthetic pathway as well as from the hydrolysis of sphingomyelin (SM) by sphingomyelinase (SMase). Sphingomyelinases are classified into five types on the basis of their optimum pH, subcellular localization, and cation dependence.[5,7] Among these enzymes acid SMase (aSMase) (optimal pH=4.8) operates in the endosomal/lysosomal compartment or plasma membrane.[8] Of note, the aSMase gene (*smpd1*) gives rise to two different enzymes lysosomal SMase *i.e.*, aSMase, and secretory SMase (sSMase) *via* alternative trafficking of the same protein precusor.[9,10] The vascular endothelium and macrophages secrete sSMase, which is the only enzyme responsible for sphingolytic activity in plasma.[11]

Elevated ceramide levels were recently shown to correlate with atherogenetic processes such as low-density lipoprotein (LDL) aggregation,[12] and form cell migration.[13] In relation to atherogenesis, we previously reported that the ceramide level in human plasma was positively correlated with both total cholesterol and oxidized apolipoprotein B-100 (oxLDL) levels.[14] Deevska *et al.*[15] reported that LDL-SM content and sSMase activity in LDL receptor knockout mice, which were fed an atherogenic diet, were increased. Excessive cholesterol intake increased plasma ceramides in apolipoprotein E knockout mice (apoE$^{-/-}$), a typical animal model for atherosclerosis.[16] In addition, we previously reported that the increase in plasma ceramide level was caused by increased activity of sSMase in the streptozotocin-induced diabetic rats,[17] thus suggesting that the elevation of plasma ceramide was an important factor in atherogenesis. sSMase activity also increased in response to stimulation of macrophages obtained from patients with chronic heart failure[18] and type 2 diabetes.[19] These studies indicated that oxidative stress resulted in ceramide accumulation by increasing sSMase activity.

To investigate the effect of aging on ceramide metabolism, we compared changes in tissue ceramides and related enzymes in apoE$^{-/-}$ with wild-type mice (WT), particularly focusing on SMase activity, which increased because of oxidative stress.[17,20,21]

MATERIALS AND METHODS

Materials All the solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Authentic ceramides were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). Nitrobenzofurazan (NBD) C_6-SM was purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.).

Animals This study was approved by the Animal Care Committee of Nara Women's University. Female WT (SLC: C57BL/6J; 4 or 13 weeks of age) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan). Female apoE$^{-/-}$ were obtained from the Jackson Laboratory. The animals were housed in a room maintained at 24±2°C, with a 12 h/12 h

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light/dark cycle. Animals were fed commercial laboratory chow (CE-2, Oriental Yeast Co., Osaka, Japan) and water *ad libitum*. Mice were sacrificed at the age of 7, 15, and 65 weeks (w) of age.

Analytical Methods Mice were anesthetized with Nembutal, and blood samples were collected by left ventricular puncture using a syringe containing sodium heparin as an anticoagulant. After perfusion with saline, the liver and aorta were dissected out. Plasma was separated from whole blood sample by centrifugation.

SMase Activity Assay SMase activities were measured using NBD C_6-SM as a substrate, and the assay was principally performed based on previous studies.[17,22] For sSMase analysis, a 800 μL assay mixture consisted of 15 μL of plasma and 785 μL of an assay buffer (0.1 mM $ZnSO_4$, 1 nmol NBD C_6-SM, 0.1% NP-40/62, 0.1 M sodium acetate buffer at pH 5.0) was incubated for 2 h at 37°C. The reaction was stopped by adding 1 mL of methanol. For aortic aSMase, which depended on zinc ion, the aorta was dissected and cleaned of visible fat debris. The tissue was homogenized on ice in 400 μL of phosphate buffered saline (10 mM, pH 7.4). Protein concentrations were determined according to the method of Lowry *et al.*[23] Aortic aSMase activity was determined by suspending the homogenate (1–2 mg protein/mL) in 800 μL assay mixture comprising of 0.1 mM $ZnSO_4$, 1 nmol NBD C_6-SM, 0.1% NP-40/62, and 0.1 M sodium acetate (pH 5.0). The reaction was continued for 2 h at 37°C and stopped by the addition of 2 mL methanol. NBD C_6-SM and generated NBD C_6-Ceramide were subsequently extracted according to the method of Bligh and Dyer.[24] The extract was dissolved in 0.5 mL methanol and analyzed using HPLC as described below. The samples (20 μL) were directly analyzed by HPLC with a Nova Pak 4 μm C18 column (3.9×150 mm, Waters Corporation, Milford, MA, U.S.A.). Elution was performed 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. NBD fluorescence was determined using a fluorescence detector (Shimadzu, RF-10AXL, excitation at 466 nm and emission at 536 nm).

Data were expressed as mean±S.E.M., and analyzed by multiple comparison tests using Statcel software (OMS Publishing Inc., Tokyo, Japan). Using the Tukey–Kramer Procedure, the differences between group means were significant at $p<0.05$.

Ceramide Measurement Lipids were extracted according to the method of Folch *et al.*[25] Lipids were dissolved in chloroform and subjected to chromatography on Silica gel 60 thin-layer chromatography (TLC) plates (Merck, Darmstradt, Germany). Separation was performed using silica gel 60 TLC plates (Merck, Germany) as previous studies.[6,26] In brief, the first elution was made with a mixture of *n*-butanol–acetic acid–water (30 : 10 : 10, v/v/v) to the one third of the plate and the second elution was made to the top of the plate with a mixture of diethyl ether–*n*-hexane–acetic acid (90 : 10 : 1, v/v/v). The ceramide spot was visualized under the UV by staining with primulin spray. The ceramide spot was scratched from the TLC plates and collected into a glass tube. Extraction was made with 2 mL of a mixture of H_2O–CH_3OH–$CHCl_3$ (20 : 30 : 50, v/v/v) under shaking for 30 min. After centrifugation, the lower layer was collected. To the upper phase was added 1.5 mL of $CHCl_3$, and extraction was made an additional two times. The collected $CHCl_3$ solution was evaporated and resuspended in a mixture of 10 mL (liver) or 2 mL (aorta and plasma) of $CHCl_3$–CH_3OH (1 : 9, v/v). Standards and tissue ceramide extracts were stored at −20°C. The quantities of major ceramide species were measured by LC-MS/MS using a triple-quadrupole mass spectrometer [the ACQUITY TQD mass spectrometer (Waters Corporation, Milford, MA, U.S.A.)] equipped with the ACQUITY ultra performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA, U.S.A.). The ceramide species were separated using a column, [ACQUITY UPLC BEH, 1.7 μm, 2.1×50 mm, C18 (Waters Corporation, Milford, MA, U.S.A.)] at 50°C. UPLC gradient elution was applied beginning with 15% of mobile phase A (water containing 0.2% formic acid and 5 mM ammonium acetate) and 85% of mobile phase B (methanol containing 0.2% formic acid and 5 mM ammonium acetate) at a flow rate of 0.4 mL/min. The initial solvent condition was maintained for 3 min, and the percentage of the B solution was gradually increased with a linear gradient to 99% for 6 min. The column was equilibrated for 4 min with 85% mobile phase B prior to next injection. The total run time per injection was 13 min. The mass spectrometry settings were as follows: electrospray ionization (ESI) positive ion mode, capillary voltage, 3.0 kV; cone voltage, 20 V; source temperature, 120°C; and desolvation temperature, 350°C. The flow rates of nitrogen gas in the cone and desolvation gas were 50 and 600 L/h, respectively. Argon gas was used for collision-induced dissociation and maintenance of collision cell pressure at 10^{-4} mbar. Results were analyzed using multiple reaction monitoring (MRM). Mass spectra (*m/z*) for an internal standard (IS) and six major ceramides were set up at 426.4→264.2 for C8:0 ceramide (IS), and 538.4→264.2, 566.5→264.2, 622.5→264.3, 650.6→264.3, 648.6→264.3, 646.6→264.3 for C16:0, C18:0, C22:0, C24:0, C24:1, and C24:2 ceramides, respectively. Data acquisition was carried out by MassLynx software (version 4.1, Waters Corporation). Ceramide species were quantified using standard curves and ratios of the integrated peak areas of each ceramide species and C8:0 ceramide, which was used as an internal standard for quantification of the other ceramide species. Total ceramide content was calculated by addition of the amount of C16:0, C18:0, C22:0, C24:0, C24:1, and C24:2 ceramides. Data were expressed as an mean±S.E.M.

RESULTS

Change in SMase Activity Plasma sSMase activity was significantly elevated at 65 w of age in both apoE$^{-/-}$ and WT compared with level observed at 7 w of age (Table 1). At 7 w

Table 1. Changes in sSMase Activity in Plasma (pmol/mL/min) and Aortic aSMase Activity (pmol/mg protein/min) of Wild-Type and ApoE$^{-/-}$ Mice

	7 w WT	15 w WT	65 w WT	7 w apoE$^{-/-}$	15 w apoE$^{-/-}$	65 w apoE$^{-/-}$
sSMase	1599.6±171.6^{ab}	1666.7±83.6^{ab}	2688.7±175.1^{c}	657.3±86.9^{d}	1187.2± 121.7^{ad}	1990.2±207.1^{b}
Aortic aSMase	79.2±9.4^{a}	130.8±12.5^{bc}	271.5±14.3^{d}	113.8±13.5^{ab}	167.1±8.3^{c}	66.4±5.0^{a}

Values are presented as mean±S.E.M. for 4 or 5 animals in each group. Different superscript letters indicate significant differences at $p<0.05$ (Tukey–Kramer *post hoc* test).

of age, plasma sSMase activity was lower in apoE$^{-/-}$ than that in WT.

Aortic aSMase activity in WT was significantly elevated at 15 w of age compared with the level observed at 7 w of age, the level increased further at 65 w of age (Table 1). In contrast, aortic aSMase activity in apoE$^{-/-}$ was unchanged at 15 w of age compared with that at 7 w of age and declined significantly at 65 w of age. Liver aSMase and neutral SMase activities were unchanged during aging in both apoE$^{-/-}$ and WT (data not shown).

Changes in Tissue Ceramide Levels The total aortic ceramide level in WT at 65 w of age increased significantly compared with that observed at 7 w of age (2.6-fold increase) (Table 2A). In the apoE$^{-/-}$ aorta, the total ceramide level was increased at 15 w and 65 w of age compared with the level observed at 7 w of age (2.0, 1.9-fold, respectively). The total aortic ceramide concentration of apoE$^{-/-}$ was similar to that of WT at 7 w of age (Table 2A); however, it was significantly higher than that of WT (2.7-fold) at 15 w of age. Table 2A demonstrates the six major ceramide levels. In the WT aorta, C24:0, C24:1, and C24:2 ceramide levels at 65 w of age were significantly higher than those at 7 w of age (2.6, 2.9, 6.0-fold, respectively). In contrast, C18:0, C22:0, and C24:0 ceramide levels in apoE$^{-/-}$ aorta increased at 15 w of age compared with those observed at 7 w of age (2.7, 2.4, 2.2-fold, respectively). The C16:0, C24:1, and C24:2 ceramide levels in apoE$^{-/-}$ aorta at 65 w of age increased compared with those at 7 w of age (2.5, 2.3, 2.6-fold, respectively) (Table 2A).

Although the levels of almost all ceramide species changed in a similar manner to the total level, some ceramides showed a different behavior (Table 2A). The levels of C16:0, C18:0, C22:0, and C24:0 ceramides in the apoE$^{-/-}$ aorta were significantly higher than those of WT (2.4, 4.1, 3.5, 3.5-fold, respectively) at 15 w of age.

The total plasma ceramide level in apoE$^{-/-}$ was significantly higher (4.4–5.9-fold) than that observed in WT at all ages

Table 2A. Levels of Ceramides in the Aorta of Wild-Type and ApoE$^{-/-}$ Mice

(A) Aorta ceramide levels (nmol/mg protein)						
Aorta	7 w WT	15 w WT	65 w WT	7 w apoE$^{-/-}$	15 w apoE$^{-/-}$	65 w apoE$^{-/-}$
C16:0	1.08±0.18^{a}	1.53±0.15^{a}	2.48±0.32^{ac}	2.03±0.46^{ac}	3.70±0.52^{bc}	4.98±0.53^{b}
C18:0	0.38±0.04^{a}	0.38±0.03^{a}	0.74±0.07^{a}	0.58±0.14^{a}	1.53±0.14^{b}	0.47±0.04^{a}
C22:0	0.45±0.06^{ab}	0.44±0.05^{b}	0.95±0.13^{a}	0.66±0.14^{ab}	1.56±0.18^{bc}	0.94±0.08 ^{a}
C24:0	1.36±0.21^{ac}	1.33±0.10^{a}	3.52±0.46^{bd}	2.10±0.39^{ad}	4.69±0.55^{b}	3.03±0.29^{cd}
C24:1	0.58±0.12^{a}	1.03±0.12^{ab}	1.70±0.15^{b}	1.22±0.24^{ab}	1.79±0.25^{b}	2.82±0.31^{c}
C24:2	0.17±0.03^{a}	0.39±0.06^{a}	1.02±0.13^{b}	0.45±0.08^{a}	0.50±0.10^{a}	1.15±0.13^{b}
Total	4.02±0.58^{a}	5.09±0.48^{a}	10.40±1.17^{bc}	7.03±1.42^{ab}	13.77±1.73^{cd}	13.39±1.23^{cd}

Values are presented as mean±S.E.M. for 4 or 5 animals in each group. Total values are sum of six ceramide species. Different superscript letters indicate significant differences at $p<0.05$ (Tukey–Kramer *post hoc* test).

Table 2B. Levels of Ceramides in the Plasma of Wild-Type and ApoE$^{-/-}$ Mice

(B) Plasma ceramide levels (nmol/mL)						
Plasma	7 w WT	15 w WT	65 w WT	7 w apoE$^{-/-}$	15 w apoE$^{-/-}$	65 w apoE$^{-/-}$
C16:0	0.53±0.04^{a}	0.50±0.09^{a}	0.47±0.05^{a}	5.63±0.42^{b}	4.33±0.26^{c}	3.34±0.39^{c}
C18:0	0.14±0.03^{a}	0.20±0.03^{a}	0.16±0.02^{a}	0.82±0.10^{b}	0.85±0.05^{b}	0.47±0.07^{c}
C22:0	1.06±0.12^{a}	0.98±0.15^{a}	0.79±0.06^{a}	8.50±1.12^{b}	6.61±0.44^{b}	3.77±0.26^{c}
C24:0	4.86±0.48^{a}	3.89±0.68^{a}	2.96±0.38^{a}	25.76±3.44^{b}	16.81±0.82^{c}	12.08±0.64^{c}
C24:1	2.82±0.46^{a}	3.20±0.55^{a}	2.33±0.24^{a}	14.84±1.90^{b}	12.93±0.58^{bc}	9.43±0.59^{c}
C24:2	0.20±0.05^{a}	0.22±0.04^{a}	0.21±0.02^{a}	1.05±0.05^{a}	1.01±0.07^{ab}	1.37±0.44^{b}
Total	9.62±1.14^{a}	8.99±1.48^{a}	6.92±0.72^{a}	56.60±6.54^{b}	42.53±1.81^{c}	30.46±1.74^{c}

Values are presented as mean±S.E.M. for 4 or 5 animals in each group. Total values are sum of six ceramide species. Different superscript letters indicate significant differences at $p<0.05$ (Tukey–Kramer *post hoc* test).

Table 2C. Levels of Ceramides in the Liver of Wild-Type and ApoE$^{-/-}$ Mice

(C) Liver ceramide levels (nmol/g tissue)						
Liver	7 w WT	15 w WT	65 w WT	7 w apoE$^{-/-}$	15 w apoE$^{-/-}$	65 w apoE$^{-/-}$
C16:0	47.03±4.12^{a}	72.29±5.66^{b}	60.20±5.22^{ab}	52.47±4.87^{ab}	56.07±2.02^{ab}	55.09±3.71^{ab}
C18:0	8.03±0.85^{a}	16.48±2.15^{b}	13.10±0.29^{ab}	10.19±1.54^{a}	12.02±1.35^{ab}	8.07±0.98^{a}
C22:0	44.14±1.96^{a}	65.93±3.69^{b}	67.06±3.28^{b}	66.16±7.25^{b}	65.48±4.23^{b}	38.75±2.36^{a}
C24:0	128.87±5.43^{ab}	169.37±10.78^{a}	156.39±16.47^{ab}	178.40±19.69^{a}	153.40±6.30^{ab}	105.94±4.78^{b}
C24:1	201.38±11.52^{a}	283.16±13.98^{b}	239.44±12.91^{ab}	200.30±8.90^{a}	247.01±11.85 ^{ab}	208.74±10.94^{a}
C24:2	16.67±0.97	22.33±1.70	20.40±3.24	21.30±1.83	24.23±1.11	24.11±3.87
Total	446.12±22.95^{ac}	629.55±29.14^{b}	556.58±26.09^{ab}	528.82±35.68^{abc}	558.22±15.30^{ab}	440.69±19.06^{c}

Values are presented as mean±S.E.M. for 4 or 5 animals in each group. Total values are sum of six ceramide species. Different superscript letters indicate significant differences at $p<0.05$ (Tukey–Kramer *post hoc* test).

(Table 2B). Almost all plasma ceramide species in apoE$^{-/-}$ decreased with age with the exception of C24:2 ceramide (Table 2B).

The total ceramide level in WT liver increased at 15 w at age (Table 2C) and the levels of C16:0, C18:0, C22:0, and C24:1 ceramides were highest at 15 w of age (Table 2C). In apoE$^{-/-}$ liver, C22:0 ceramide level at 7 w of age was significantly higher than that observed in WT, whereas C22:0 ceramide level and total ceramide level at 65 w of age were significantly lower in apoE$^{-/-}$ than those in WT.

DISCUSSION

The total plasma ceramide level in apoE$^{-/-}$ was comparable to that reported for human atherosclerotic subjects,[14] and was significantly higher than the level observed in WT at all ages, indicating that the aorta of apoE$^{-/-}$ was exposed to a higher plasma ceramide level throughout aging. The difference in the plasma ceramide levels between WT and apoE$^{-/-}$ may be attributed to the LDL level in plasma because LDL is the major ceramide carrier.[27,28] The plasma ceramide level correlated with the LDL cholesterol and oxLDL levels in human samples.[14] The plasma ceramide level in apoE$^{-/-}$ decreased during aging. As a similar phenomemon was reported for oxLDL by Itabe and colleagues[29]: the level of oxLDL increased at 20 w of age and decreased toward the basal level at 40 w of age in apoE$^{-/-}$. These authors suggested that oxLDL appeared before the development of atherosclerotic lesions. Moreover, Schissel *et al.*[30] indicated that ceramides, generated by sSMase from the lesional LDL, but not from the plasma LDL, participated in LDL aggregation. Combining these reports with the results from this study, in which aortic ceramide levels increased at 15 w of age in apoE$^{-/-}$, it is suggested that an elevation in ceramide level at the beginning of atherogenesis is involved in the pathogenesis of atherosclerosis.

Although the aortic ceramide level increased significantly in both WT and apoE$^{-/-}$, the distribution pattern of ceramide species in WT and apoE$^{-/-}$ was different. At 15 w of age, when atherogenesis was assumed to initiate in apoE$^{-/-}$,[31,32] the levels of C16:0, C18:0, C22:0, and C24:0 ceramides in the aorta increased significantly, whereas only C16:0 and C24:1 ceramide levels increased at 65 w of age. The aortic levels of C18:0, C22:0, and C24:0 ceramides caused a similar change in aortic SMase activity. On the basis of these results, we suggest that the increase in C18-24 ceramide levels and the elevation of aortic aSMase activity may play a role in the initiation of atherogenesis. In addition, this is the first study showing elevation of aortic ceramide level during aging in apoE$^{-/-}$ as well as in WT.

In contrast to C24 ceramide, C16:0 ceramide, a major ceramide in the aorta of apoE$^{-/-}$ that increased at 15 and 65 w of age, seemed to have a different function. Ceramide chain length affects the physicochemical properties of lipid membranes;[33] thus, C16:0 ceramide easily mixes with cholesterol in contrast to C24 ceramide.[34] Mesicek *et al.*[35] reported that the overexpression of ceramide synthase (CerS) 5 lead to generation of C16:0 ceramide and an increase in apoptosis, whereas overexpression of CerS2 yielded C24 ceramide and provoked pro-survival signals. Furthermore, in the liver of CerS2 null mice, elevated ROS levels are associated with an increase in C16:0 ceramide and a decrease in mitochondria complex IV activity.[36] In this regard, cellular homeostasis appears to be maintained by a balance between C16:0 and C24:0 ceramides. Although the present study showed that C16:0 ceramide behaved in a manner similar to the very long, unsaturated C24:1 ceramide, the relationship between atherogenesis and apoptosis caused by ceramides, particularly C16:0 and C24 ceramides, requires further examination.

Liver ceramide levels were unchanged with aging in either apoE$^{-/-}$ or WT. Similar results were obtained in diabetic rats.[17] These observations suggested that aging did not affect hepatic ceramide metabolism.

In conclusion, this study suggests that elevation of ceramide species such as C16:0 in the aorta as a result of sSMase activity in the plasma contributes to atherogenesis during aging.

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