### Ceramide Analysis

Lipid of each tissue was extracted according to the method of Folch et al. [25]. Lipid in the liver and the adipose tissues was dissolved in chloroform to perform silica gel 60 TLC (Merck, Darmstadt, Germany). TLC separation was performed as previously described [26].

Quantitative measurement of ceramide species was made using a triple-quadrupole mass spectrometer (Finnigan MAT TSQ 7000). ESI-MS/MS was performed as previously described [11, 26]. HPLC was conducted with a $\mu$-Bondasphere column (5 $\mu$C18 100A Waters). Elution was performed at a flow rate of 0.2 ml/min with a mixture of 5 mM ammonium formate, methanol, and tetrahydrofuran at a volume ratio of 1:2:7. The mobile phase stream was connected to the ionspray interface of an ESI–MS/MS system. Standards and cellular ceramide extracts were stored at –20°C. Mass analysis was performed in the positive mode in a heated capillary tube at 250 °C with an electrospray potential of 4.5 kV, a sheath gas pressure of 70 psi, and a collision gas pressure of 1.6–2.0 mtorr. Under optimized conditions, monitoring ions were ceramide molecular species $(M+H)^+$ for the product ion at $m/z$ 264 of the sphingoid base. Standards and samples were injected with 5 μl of 5 pmol C8:0-ceramide as an internal standard for ESI–MS/MS. The quantity of each ceramide was calibrated from each ceramide/C8:0-ceramide ratio, assuming that the calibration curve of ceramides bearing C16–24 acyl chains was similar to that of C16:0-ceramide as previously described [11, 26]. Each sample was analyzed in duplicate.

### Western Blot Analysis

For electrophoresis, the sample was applied to 4% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblot analysis was performed, both as described previously [14, 15]. Proteins separated on the gel were electrophoretically transferred to PVDF membrane filters and immunoblotting analyses of apoB were performed as previously described [14, 15].

Anti-mouse apoB antiserum was prepared by immunizing mouse LDL to a rabbit. Chemiluminescence was analyzed with ATTO Densitograph Software Library (CS Analyzer Ver2.0).

### Statistical Analysis

The data were expressed as mean ± SE. Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD).

## Results

### Effect of Dietary Cholesterol on Body Weight and Lipids

The body weight of the apoE$^{-/-}$ control group was higher than those of the other groups (Table 1). Liver weight of apoE$^{-/-}$ mice was higher than that of the wild type control group. The weight of total white adipose tissue of the apoE$^{-/-}$ control group was higher than those of the wild control group and the apoE$^{-/-}$ cholesterol group. No differences were observed in daily food consumption among these four groups (data not shown).

Plasma cholesterol of apoE$^{-/-}$ mice fed a control diet was about 6.7 times higher than that of the wild type mice fed a control diet (Table 2). In the cholesterol group, plasma cholesterol of the apoE$^{-/-}$ mice was also about six times higher than that of the wild type mice. However, the liver cholesterol in the apoE$^{-/-}$ mice was not different from that of the wild type mice. Cholesterol levels of plasma and the liver in both the wild type and the apoE$^{-/-}$ mice fed cholesterol were higher than those in mice fed a diet without cholesterol. No difference was observed among all groups in plasma TG. However, the liver TG of the wild type control group was lower than that of the other groups.

**Table 1** Effect of dietary cholesterol on weights of body, liver, and white adipose tissue (WAT) of wild type and apoE$^{-/-}$ mice

| | Wild control | Wild cholesterol | ApoE$^{-/-}$ control | ApoE$^{-/-}$ cholesterol |
|---|---|---|---|---|
| Body weight (g) | $32.3 \pm 0.9^{a}$ | $33.8 \pm 1.3^{a}$ | $38.1 \pm 1.3^{b}$ | $35.1 \pm 1.0^{a}$ |
| Liver weight (g) | $1.30 \pm 0.09^{a}$ | $1.67 \pm 0.12^{ac}$ | $1.84 \pm 0.11^{bc}$ | $1.84 \pm 0.18^{bc}$ |
| Epididymal WAT weight (g) | $0.68 \pm 0.11^{a}$ | $1.12 \pm 0.15^{b}$ | $1.33 \pm 0.13^{b}$ | $0.76 \pm 0.11^{a}$ |
| Perirenal WAT weight (g) | $0.39 \pm 0.07^{a}$ | $0.47 \pm 0.07^{ac}$ | $0.68 \pm 0.08^{b}$ | $0.38 \pm 0.08^{a}$ |
| Mesenteric WAT weight (g) | $0.38 \pm 0.06^{a}$ | $0.50 \pm 0.08^{ac}$ | $0.63 \pm 0.06^{bc}$ | $0.33 \pm 0.05^{a}$ |
| Total WAT weight (g) | $1.49 \pm 0.21^{a}$ | $2.08 \pm 0.30^{ac}$ | $2.64 \pm 0.27^{bc}$ | $1.44 \pm 0.22^{a}$ |

The values were mean ± SE for eight C57BL/6J and ten apoE$^{-/-}$ mice. Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD). Values with different superscript letters show significant difference at $P < 0.05$

**Table 2** Effect of dietary cholesterol on plasma and liver lipids of wild type and apoE$^{-/-}$mice

| | Wild control | Wild cholesterol | ApoE$^{-/-}$ control | ApoE$^{-/-}$ cholesterol |
|---|---|---|---|---|
| Plasma cholesterol (mg/dL) | 77.4 ± 12.7$^{a}$ | 140 ± 11$^{a}$ | 517 ± 46$^{b}$ | 873 ± 64$^{c}$ |
| Plasma triacylglycerol (mg/dL) | 30.4 ± 1.9 | 28.6 ± 2.0 | 42.4 ± 6.3 | 46.6 ± 13.4 |
| Liver cholesterol (mg/g) | 3.36 ± 0.44$^{a}$ | 14.3 ± 1.9$^{b}$ | 5.76 ± 0.48$^{a}$ | 15.2 ± 1.4$^{b}$ |
| Liver triacylglycerol (mg/g) | 44.2 ± 8.8$^{a}$ | 115 ± 30$^{b}$ | 179 ± 25$^{b}$ | 119 ± 21$^{b}$ |

The values were mean ± SE for eight C57BL/6J and ten apoE$^{-/-}$ mice. Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD). Values with different superscript letters show significant difference at $P < 0.05$

## Effect of Dietary Cholesterol on Ceramide

Table 3 shows the distribution of ceramide species in plasma. A major ceramide in plasma was C24:0 in both wild type and apoE$^{-/-}$ mice. The plasma level of total ceramide of the apoE$^{-/-}$ mice fed a control diet was about six times higher than that of the wild type mice fed a control diet. In the cholesterol group, the plasma level of total ceramide in the apoE$^{-/-}$ mice was also about 5.1 times higher than that of the wild type mice. In apoE$^{-/-}$ mice, the plasma level of total ceramide of the cholesterol group tended to be higher than that of the control group ($p = 0.08$), while C16:0, C24:1, and C24:2 of the cholesterol group were significantly higher than those of the control group. In the wild type mice, dietary cholesterol did not affect plasma levels of ceramide.

Table 4 shows the distribution of ceramide species in the liver. The major ceramide of the liver was also C24:0 in the wild type and the apoE$^{-/-}$ mice. In the liver, the total ceramide of the apoE$^{-/-}$ mice fed a control diet was about 1.5 times higher than that of the wild type mice fed a control diet. In the cholesterol group, the total ceramide of apoE$^{-/-}$ mice was not different from that of the wild type mice. Thus, the difference in total ceramide level between wild type and apoE$^{-/-}$ mice in the liver was less than that in plasma. In addition, dietary cholesterol did not affect ceramide levels of the liver in either the wild type or the apoE$^{-/-}$ mice.

Table 5 shows the distribution of ceramide species in the mesenteric white adipose tissue. Major ceramides of white adipose tissue were C24:0, C16:0, and C24:1. The ratio of C16:0 and C18:0 in adipose tissues was higher than that in plasma and the liver. In the wild type mice and the apoE$^{-/-}$ mice fed cholesterol, the content of C16:0 of the adipose tissue was similar to that of C24:0. In the adipose tissue, the total ceramide of the wild type was not different from that of the apoE$^{-/-}$ mice. In addition, dietary cholesterol did not affect ceramides of the adipose tissue in either wild type or apoE$^{-/-}$ mice.

## Effect of Dietary Cholesterol on Cross-Linked and Fragmented apoB

The band, which was larger than the band of apoB (512 kDa) was assumed to be a cross-linking product as previously reported [15] and the band, which was smaller than the band of apoB was assumed to be a fragmented product. However, neither cross-linking nor fragmentation

**Table 3** Effect of dietary cholesterol on ceramide concentration (nmol/ml) in the plasma

| | Wild control | Wild cholesterol | ApoE$^{-/-}$ control | ApoE$^{-/-}$ cholesterol |
|---|---|---|---|---|
| C16:0 | 0.75 ± 0.13$^{a}$ | 1.20 ± 0.18$^{a}$ | 4.11 ± 0.32$^{b}$ | 5.71 ± 0.42$^{c}$ |
| C18:0 | 0.07 ± 0.01$^{a}$ | 0.10 ± 0.02$^{a}$ | 0.75 ± 0.11$^{b}$ | 0.85 ± 0.1$^{b}$ |
| C22:0 | 3.05 ± 0.83$^{a}$ | 4.12 ± 0.80$^{a}$ | 25.5 ± 3.8$^{b}$ | 24.5 ± 3.3$^{b}$ |
| C24:0 | 6.16 ± 1.54$^{a}$ | 9.04 ± 1.46$^{a}$ | 34.3 ± 2.8$^{b}$ | 41.5 ± 4.6$^{b}$ |
| C24:1 | 3.36 ± 0.77$^{a}$ | 4.70 ± 0.98$^{a}$ | 15.3 ± 1.4$^{b}$ | 20.2 ± 2.2$^{c}$ |
| C24:2 | 0.14 ± 0.03$^{a}$ | 0.22 ± 0.04$^{a}$ | 2.03 ± 0.38$^{b}$ | 5.01 ± 0.67$^{c}$ |
| Total | 13.4 ± 3.3$^{a}$ | 19.1 ± 3.3$^{a}$ | 80.6 ± 6.8$^{b}$ | 97.8 ± 11.0$^{b}$ |

The values were mean ± SE for eight C57BL/6J and ten apoE$^{-/-}$ mice. Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD). Values with different superscript letters show significant difference at $P < 0.05$

**Table 4** Effect of dietary cholesterol on ceramide concentration (nmol/g tissue) in the liver

| | Wild control | Wild cholesterol | ApoE$^{-/-}$ control | ApoE$^{-/-}$ cholesterol |
|---|---|---|---|---|
| C16:0 | 19.6 ± 3.0$^{a}$ | 22.3 ± 2.6$^{ab}$ | 28.5 ± 2.4$^{b}$ | 27.5 ± 3.2$^{b}$ |
| C18:0 | 2.52 ± 0.5$^{a}$ | 2.16 ± 0.29$^{ab}$ | 3.90 ± 0.33$^{ac}$ | 4.36 ± 0.93$^{c}$ |
| C22:0 | 38.4 ± 4.1$^{a}$ | 47.1 ± 5.1$^{a}$ | 80.6 ± 6.3$^{b}$ | 50.0 ± 4.9$^{ac}$ |
| C24:0 | 75.0 ± 10.1$^{a}$ | 88.9 ± 6.5$^{ab}$ | 99.6 ± 7.4$^{b}$ | 97.7 ± 8.7$^{b}$ |
| C24:1 | 50.3 ± 8.2 | 61.8 ± 5.5 | 63.1 ± 5.9 | 71.6 ± 6.3 |
| C24:2 | 4.62 ± 0.84$^{a}$ | 5.57 ± 0.58$^{a}$ | 5.80 ± 0.74$^{a}$ | 9.59 ± 1.12$^{b}$ |
| Total | 190 ± 19$^{a}$ | 228 ± 17$^{ab}$ | 281 ± 19$^{b}$ | 261 ± 23$^{b}$ |

The values were mean ± SE for eight C57BL/6J and ten apoE$^{-/-}$ mice. Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD). Values with different superscript letters show significant difference at $P < 0.05$

**Table 5** Effect of dietary cholesterol on ceramide concentration (nmol/g tissue) in the mesenteric white adipose tissues

| | Wild control | Wild cholesterol | ApoE$^{-/-}$ control | ApoE$^{-/-}$ cholesterol |
|---|---|---|---|---|
| C16:0 | 51.4 ± 4.7 | 52.7 ± 15.0 | 43.8 ± 10.0 | 55.9 ± 9.3 |
| C18:0 | 16.9 ± 3.5 | 19.7 ± 5.2 | 10.8 ± 1.8 | 19.9 ± 4.4 |
| C22:0 | 21.1 ± 2.9 | 17.3 ± 3.2 | 15.4 ± 2.8 | 16.9 ± 2.9 |
| C24:0 | 63.0 ± 10.4 | 53.3 ± 11.1 | 49.6 ± 11.1 | 56.5 ± 11.7 |
| C24:1 | 53.8 ± 9.0 | 47.6 ± 9.2 | 40.1 ± 8.4 | 49.6 ± 9.8 |
| C24:2 | 8.40 ± 1.41 | 7.68 ± 1.69 | 6.09 ± 1.05 | 10.2 ± 2.2 |
| Total | 215 ± 30 | 198 ± 45 | 159 ± 34 | 209 ± 38 |

The values were mean ± SE for eight C57BL/6J and ten apoE$^{-/-}$ mice. Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD). Values with different superscript letters show significant difference at $P < 0.05$

of apoB-48 (250 kDa) were detected (Fig. 1a). Western blot analysis of plasma revealed that apoB-100 with molecular weight of 512 kDa in the apoE$^{-/-}$ cholesterol group was lower than that in the apoE$^{-/-}$ control group (Fig. 1a, b). Though cross-linking of apoB-100 was not detected in apoE$^{-/-}$ mice, fragmentation of apoB-100 in the apoE$^{-/-}$ cholesterol group was 2.5 times higher than that in the apoE$^{-/-}$ control group (Fig. 1c). In the wild type mice, neither cross-linked nor fragmented apoB proteins were discernible (data not shown).

The amount of the aortic area covered with plaques was significantly greater in the apoE$^{-/-}$ mice fed cholesterol compared to the apoE$^{-/-}$ mice fed a control diet, as is well established (data not shown).

Effect of Dietary Cholesterol on Vitamin C

In plasma, vitamin C of apoE$^{-/-}$ mice was higher than that of wild type mice (Table 6). Vitamin C of both wild type and the apoE$^{-/-}$ mice fed a cholesterol diet was higher than for mice fed a control diet.

## Discussion

Sphingolipids such as ceramide and sphingosine-1-phosphate are bioactive lipid mediators [27]. The importance of sphingolipids as mediators in cardiovascular pathophysiology has recently been reported [28]. In addition, it was shown that SPT activity was higher in apoE$^{-/-}$ mice compared with C57BL/6J mice [9]. In this study, it was shown that the total ceramide level in plasma and the liver of the apoE$^{-/-}$ control group was higher than that of the wild control group. Therefore, decreasing ceramide levels in plasma and the liver may be beneficial for prevention of atherogenesis.

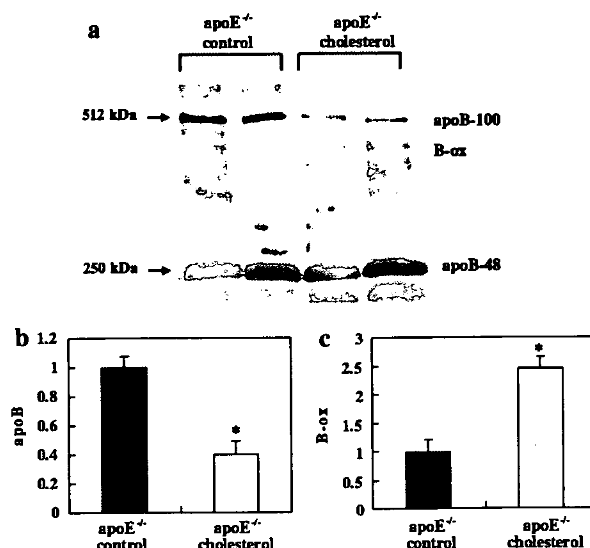


**Fig. 1** ApoB and fragmented apoB-100 proteins in plasma of apoE$^{-/-}$ mice fed a standard diet and supplemented with 1% cholesterol. Plasma was loaded on 4% SDS-PAGE gel and Western blot analysis was performed (**a**). Densitometry of apoB (**b**) and fragmented apoB (**c**) in plasma of apoE$^{-/-}$ mice. The values were means ± SE for 4–5 apoE$^{-/-}$ mice and *asterisks* indicated significant differences from the corresponding the apoE$^{-/-}$ control group

**Table 6** Effect of dietary cholesterol on plasma Vitamin C concentration (nmol/mL) of wild type and apoE$^{-/-}$ mice

| | Wild control | Wild cholesterol | ApoE$^{-/-}$ control | ApoE$^{-/-}$ cholesterol |
|---|---|---|---|---|
| Vitamin C | 69.6 ± 8.5$^{a}$ | 96.3 ± 3.5$^{b}$ | 115 ± 6$^{b}$ | 146 ± 5$^{c}$ |

The values were mean ± SE for five C57BL/6J and six apoE$^{-/-}$ mice. Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD). Values with different superscript letters show significant differences at $P < 0.05$

In the present study, we investigated the effects of dietary cholesterol on ceramide and oxidative products of apoB in apoE$^{-/-}$ mice, of which the plasma and aorta responded sufficiently to cholesterol-enriched diets. Our recent study demonstrated that the correlation coefficient between plasma cholesterol and total ceramide in human subjects was particularly high among lipid markers associated with atherosclerosis [11]. Treatment with myriocin, which is a potent and specific SPT inhibitor and is known to have an immunosuppressive activity [29], significantly lowered plasma cholesterol levels of apoE$^{-/-}$ mice in a dose-dependent manner [30]. In this study, we demonstrated that a cholesterol-enriched diet did not affect ceramide levels of the tissues in the wild type mice. However, in apoE$^{-/-}$ mice, the plasma levels of several ceramides in the cholesterol group were significantly

higher than those in the control group and the plasma level of total ceramides in the cholesterol group also tended to be higher than for mice fed a control diet. This result indicated a correlation between increased cholesterol intake and the elevation of the plasma levels of ceramide.

In the liver, the total ceramides of apoE$^{-/-}$ mice were higher than those of the wild control group. However, the difference of ceramides levels between the wild type and the apoE$^{-/-}$ mice in the liver was not so large as that in plasma. In plasma, the difference of total ceramides between the wild type and the apoE$^{-/-}$ mice coincided with the change of cholesterol. Furthermore, the cholesterol-enriched diet did not cause a significant increase in liver ceramide levels. Therefore, a relationship between cholesterol accumulation and ceramide metabolism change in the liver was not supported.

It is well known that a fat-enriched diet is an important factor in the development of atherosclerosis. Overnutrition leads to hypertrophy of adipocytes, and high-fat diets promote obesity [31]. The islet obese fa/fa Zucker diabetic fatty rats exhibit an increase in de novo synthesis of [$^3$H]-ceramide from [$^3$H]-palmitate [32]. Based on these reports, a correlation between ceramide and deposition of visceral fat is suggested. In this study, although the weight of total white adipose tissues in the apoE$^{-/-}$ control group was higher than in the other groups, ceramide levels in the white adipose tissue of the apoE$^{-/-}$ control group were not higher than those of other groups. Therefore, it is suggested that fat accumulation in the white adipose tissue did not increase ceramide content.

Oxidative modification of LDL and its recognition by macrophages have been suggested as being an initial event of atherosclerosis [17]. In this study, we analyzed the oxidation profile of apoB, namely the sum of fragmented and conjugated apoB proteins determined by an immunoblot assay. We reported that these oxidation products of apoB-100, termed B-ox are a reliable indicator of atherosclerosis [15]. In human plasma, the conjugated apoB-100 was higher than the fragmented apoB-100. However, conjugated apoB-100 was not detected clearly in apoE$^{-/-}$ mice. In addition, in plasma of apoE$^{-/-}$ mice it was reported that the major apoB is apoB-48 and the minor apoB is apoB-100 [33]. In this study, conjugated and fragmented apoB-48 were not detected in apoE$^{-/-}$ mice. It is necessary to examine the difference of apoB-100 oxidation between human and mice and the difference of oxidation between apoB-100 and apoB-48.

Our previous studies demonstrated that the reactivity of apoB toward radicals is extremely high and even comparable to vitamin E [14]. Hence degraded apoB fragments were present in normal human plasma and tended to increase with aging [15]. In this study, the apoE$^{-/-}$ cholesterol group, which demonstrated a significant increase in the size of lesions as is well established [33], also exhibited decreased apoB and increased fragmented apoB proteins compared to the control group. These results demonstrated that the cholesterol diet increased the development of atherosclerotic lesions and promoted oxidation of apoB in the apoE$^{-/-}$ mice. We reported that cross-linked and fragmented apoB-100 is a reliable index of atherosclerosis and oxidative stress [15]. Therefore, it is suggested that fragmentation of apoB is also a reliable indicator of atherosclerosis in apoE$^{-/-}$ mice as well as humans.

OxLDL have been shown to induce apoptosis of culture cells [34, 35]. Ceramides also have been shown to cause apoptosis in a variety of cells. Apoptosis of endothelial cells is widely implicated in the early stage of atherosclerosis. It was reported that oxLDL was involved in the formation of various sphingolipid mediators [36] and activated the generation of ceramide in endothelial cells [37]. Our results also demonstrated that apoE$^{-/-}$ mice, which showed increased size of lesions, exhibited higher ceramide levels and fragmented apoB in plasma. Based on these results, a correlation between increased oxLDL and ceramide is suggested. However, oxLDL-induced activation of the SMase-ceramide pathway has not yet been fully studied and is still controversial. Further studies are needed to clarify the relationship between LDL oxidation and ceramide metabolism.

Vitamin C is a potent water-soluble antioxidant that scavenges reactive oxygen species [38, 39]. Sublethal lipopolysaccharide, which is associated with oxidative stress, temporarily increased liver vitamin C in the mouse [40]. In addition, the deficiency of glutathione, which plays various important roles in the protection against oxidant stress [41], increased hepatic ascorbate synthesis in mice [42]. These studies indicate that vitamin C synthesis is enhanced by oxidative stress in mice. In this study, plasma vitamin C in apoE$^{-/-}$ mice was higher than that in the wild type mice. It is suggested that apoE$^{-/-}$ mice at this young age increased vitamin C production compared to the wild type mice to prevent increased oxidative stress. In addition, plasma vitamin C in the wild type and apoE$^{-/-}$ mice fed a cholesterol diet was higher than in those fed a control diet. The increase of B-ox in the apoE$^{-/-}$ mice fed a cholesterol diet suggested enhanced stress, which resulted in the elevation of plasma vitamin C just like in the mice under enhanced oxidative stress as described above [40, 42].

In conclusion, this study demonstrated that dietary cholesterol increased ceramide levels and products of oxidized LDL in plasma of the apoE$^{-/-}$ mice. In addition, we propose that ceramides, the toxicities of which are much higher than that of cholesterol, are a new risk factor for atherosclerosis.

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