Vitamin E prevents the age-dependent and palmitate-induced disturbances of sphingolipid turnover in liver cells

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Abstract Sphingolipid turnover has been shown to be activated at old age and in response to various stress stimuli including oxidative stress. Reduction of vitamin E content in the liver under the pro-oxidant action is associated with enhanced sphingolipid turnover and ceramide accumulation in hepatocytes. In the present paper, the correction of sphingolipid metabolism in the liver cells of old rats and in the palmitate-treated young hepatocytes using α -tocopherol has been investigated. 3- and 24-month-old rats, [14 C] palmitic acid, [methyl- 14 C-choline]sphingomyelin (SM), and [14 C]serine were used. α -Tocopherol administration to old rats or addition to the culture medium of old liver slices or hepatocytes prevented age-dependent increase of ceramide synthesis and lipid accumulation, and increased SM content in liver tissue and cells. α -Tocopherol treatment of old cells decreased the neutral and acid sphingomyelinase (SMase) activities in hepatocytes and serine palmitoyl transferase activity in the liver cell microsomes. Effect of α - or γ -tocopherol, but not of δ -tocopherol, on the newly

synthesized ceramide content in old cells was correlated with the action of inhibitor of serine palmitoyl transferase (SPT) activity (myriocin) and SMase inhibitors (glutathione, imipramine). Addition of α -tocopherol as well as myriocin to the culture medium of young hepatocytes, treated by palmitate, abolished ceramide accumulation and synthesis. The data obtained demonstrate that α -tocopherol normalized elevated ceramide content in the old liver cells via inhibition of acid and neutral SMase activities and lipid synthesis de novo. α -Tocopherol, reducing ceramide synthesis, prevented palmitate-induced aging-like ceramide accumulation in young liver cells.

Keywords Old age · Liver cells · Vitamin E · α -Tocopherol · Palmitic acid · Ceramide · Sphingomyelinases

Introduction

Sphingolipids are components of biological membranes and important regulators of various stress responses and growth mechanisms. The factors that alter the metabolism of sphingolipids, including oxidative stress, increase risk, and progression of the pathogenesis of several age-related diseases: cancers, diabetes, atherosclerosis, and neuro-degenerative disorders (for review, see Cutler and Mattson 2001). Sphingolipid turnover in the liver (Kavok et al. 2003) and brain structures (striatum, hippocampus, and

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frontal cortex) (Crivello et al. 2005; Babenko and Semenova 2010) was found to be more active in the aged rats than in the adult ones. Ceramide accumulation in the aged liver and hepatocytes is accompanied by reduced sphingomyelin (SM) levels and increased acid sphingomyelinase (aSMase) and neutral sphingomyelinase (nSMase) activities (Lightle et al. 2000; Babenko and Shakhova 2006). The aging-associated oxidative stress in the liver (Nakata et al. 1996) was associated with ceramide elevation in the cells and could be mimicked by the dietetic saturated fatty acid supplementation to the adult animals (Nagle et al. 2009). Exposure of palmitic acid to liver cells led to the time-dependent reactive oxygen species (ROS) production (Strivastava and Chan 2007), ceramide accumulation (Wei et al. 2006), apoptotic hepatocyte death, and development of hepatic insulin resistance.

The oxidative stress-induced ceramide accumulation in the keratinocytes, neurons, and astrocytes can be blocked by a pretreatment of cells with the singlet oxygen quencher and antioxidant vitamin E (Grether-Beck et al. 2000; Mazière et al. 2001; Ayasolla et al. 2004; Yamagata et al. 2009). But the data on the mechanism of vitamin E action on sphingolipid turnover are controversial. Vitamin E prevents the solar ultraviolet (UVA)-mediated non-enzymatic pathway of ceramide formation in long-term cultured, normal human keratinocytes (Grether-Beck et al. 2000). However, under other experimental conditions, a direct cleavage of SM to ceramide by UVA was not observed in the human keratinocytes, while vitamin E pretreatment of the cells prevented UVA-induced ceramide accumulation (Mazière et al. 2001). Addition of a high dose of vitamin E to the culture media before reoxygenation and hypoxia of cortical neurons significantly reduced nSMase activity and increased the level of negative regulator of the nSMase, glutathione, in cells of stroke-prone spontaneously hypertensive and Wistar Kyoto rats (Yamagata et al. 2009). α -Tocopherol could inhibit *N*-acetylsphingosine (C2-ceramide) and SMase stimulation of ROS overproduction in the HEK293 and RAW264.7 cells, respectively (Bai et al. 2007; Hatanaka et al. 1998). CCl₄-induced oxidative stress reduced vitamin E content in the liver and kidney and activated the nSMase but not the aSMase activity in the tissues (Ichi et al. 2009). In other cell types, it was observed that generation of ceramide by aSMase depends on ROS (Denis et al. 2002; Scheel-Toellner et al. 2004; Sanvicens and Cotter 2006). The ROS-

induced activation of aSMase and consequent CD95 clustering in the lipid rafts are supposed to be a primary factor limiting the cell life span.

In the present paper, a significant decrease in the level of ceramide and increase of SM content has been observed in the liver of α -tocopherol-treated 24-month-old rats. Both the long- and short-term effects of α -tocopherol on sphingolipid turnover have been determined. α -Tocopherol could ameliorate palmitate-induced ceramide accumulation in the hepatocytes of young animals via the inhibition of sphingolipid synthesis. By using α -tocopherol, the intracellular accumulation of ceramides in aging can be prevented via a mechanism involving an inhibition of the acid and neutral SMases, as well as ceramide synthesis *de novo*.

Materials and methods

Materials

[¹⁴C]Palmitic acid (56 mCi/mmol; Amersham, GE Health Care UK), [methyl-¹⁴C-choline]sphingomyelin (52 mCi/mmol; PerkinElmer, USA), [¹⁴C]serine (25 mCi/mmol; BPO Isotop, Russia); silica gel plates for thin-layer chromatography from Sorbfil (Russia), α -tocopherol acetate from ZAT Technolog (Ukraine), δ -tocopherol, γ -tocopherol, myriocin, fumonisins B1, and *N*-acetylcysteine (NAC) from Sigma-Aldrich (UK), palmitoyl CoA from Sigma Chemical Inc., St. Louis, MO (USA), glutathione from Reanal, Budapest (Hungary), and imipramine from EGIS Pharmaceutical PLC, Budapest (Hungary). Palmitic acid, palmitoleic acid, and lipid standards (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), SM, and ceramide) were obtained from Sigma-Aldrich (UK).

Animals

The 3- and 24-month-old male Wistar rats were used in the experiments. They were kept at 24°C on a cycle of 12 h light/12 h darkness and had free access to a standard chow diet and drinking water *ad libitum*. Experimental procedures were approved by the Institutional Animal Care and Use committees at the Kharkov Karazin National University. The 24-month-old animals were divided into two groups. Animals were fed α -tocopherol (100 μ g/100 g body weight) or

corn oil intragastrically daily for 7 days. Rats were fasted overnight, anesthetized the next day with an injection of ketamine (75 mg/kg), and killed by decapitation. Their livers were isolated, homogenated, and used for lipid analysis as described below. The untreated 3- and 24-month-old rats which had free access to a standard chow diet and drinking water *ad libitum* were used for hepatocyte isolation.

Experiments with liver homogenates and slices

The livers of 3- and 24-month-old rats as well as α -tocopherol-treated and control 24-month-old rats were perfused with 0.9% NaCl, then removed and washed in Krebs–Henseleit buffer, pH 7.4, containing 2 mM CaCl_2 and 0.2% bovine serum albumin (BSA). For lipid extraction and separation, the liver homogenates prepared in the Krebs–Henseleit buffer, pH 7.4, were used. For sphingolipid synthesis, homogenates were prepared and incubated in Krebs–Ringer bicarbonate buffer, pH 7.4. Liver slices of 24-month-old rats were incubated in the Krebs–Ringer bicarbonate buffer, pH 7.4, in the presence of PC liposomes containing α -tocopherol (100 $\mu\text{g/ml}$) or PC liposomes (control) for 1.5 h at 37°C in the atmosphere of 5% CO_2 –95% O_2 . Preparation of liposomes containing vitamin E was done according Freisleben and Mentrup (1992). The lipids were extracted and analyzed as described below. Liver homogenates of α -tocopherol-treated or corn oil-treated 24-month-old rats were used for determination of total glutathione content by the method of Beutler et al. (1963).

Experiments with isolated hepatocytes

Hepatocytes were isolated from the 3- and 24-month-old untreated rats by the method described in Babenko and Shakhova (2006). The preparation of hepatocytes started between 9:00 and 10:00 AM. The cells ($10^6/\text{ml}$) were cultured on the 60-mm Petri dishes coated with rat tail collagen in Eagle medium containing 10% fetal calf serum, 100 units/liter streptomycin, and 100 units/liter penicillin in a humidified atmosphere of 5% CO_2 in a tissue culture incubator at 37°C. Isolated hepatocytes of untreated 24-month-old rats were incubated in the presence of PC liposomes containing α -tocopherol (100 $\mu\text{g/ml}$) or PC liposomes (control) for 24 h at 37°C. Old hepatocytes ($2 \times 10^7/\text{ml}$) were incubated in the presence

of 1 $\mu\text{Ci/ml}$ [^{14}C]palmitic acid and α -tocopherol or δ -tocopherol liposomes (100 $\mu\text{g/ml}$), inhibitors of nSMase (3 mM glutathione), aSMase (50 μM imipramin), SPT (5 μM myriocin), or ceramide synthase (1 μM fumonisins B1) inhibitors for 2 h. Control cells were incubated in the presence of PC liposomes or ethanol for 2 h. Isolated hepatocytes of 3-month-old rats were cultured in the presence of palmitic acid or palmitoleic acid (0.5 mM), bound to fatty acid-free bovine serum albumin (1:1, molar ratio), or palmitic acid (0.5 mM) and PC liposomes containing α -tocopherol (100 $\mu\text{g/ml}$) or palmitic acid+myriocin (5 μM) for 24 h at 37°C. Control cells were incubated in the presence of fatty acid-free bovine serum albumin and PC liposomes or ethanol for 24 h at 37°C. The cells were released from the dishes by a mild trypsin treatment. Hepatocytes were allowed to settle, and the pellet was resuspended in the Krebs–Henseleit buffer, pH 7.4, containing 2 mM CaCl_2 , 25 mM HEPES, and 0.1% BSA. To determine the cell viability, the trypan blue exclusion into hepatocytes has been studied. The lipids were extracted and analyzed as described below. Liver cells were pre-incubated in the presence of α -tocopherol liposomes (100 $\mu\text{g/ml}$) or PC liposomes (control), inhibitors of nSMase (3 mM glutathione), aSMase (50 μM imipramine), or SPT (5 μM myriocin) for 2 h and used for determination of SMases activities and isolation of microsomes and determination of SPT activity as described below.

Determination of sphingolipids turnover

The liver homogenates (1.5 mg protein/ml) were incubated for 90 min in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 20 $\mu\text{Ci/ml}$ [^{14}C]palmitic acid (56 mCi/mmol) bound to fatty acid-free bovine serum albumin (1:1, molar ratio), and the reaction was terminated by the addition of chloroform/methanol (1:2, v/v). Isolated hepatocytes ($2 \times 10^7/\text{ml}$) were incubated in the presence of 1 $\mu\text{Ci/ml}$ [^{14}C]palmitic acid (56 mCi/mmol) bound to fatty acid-free bovine serum albumin (1:1, molar ratio), or 2.5 $\mu\text{Ci/ml}$ [^{14}C]serine (25 mCi/mmol) for 2 h and 24 h, and the reaction was terminated by the addition of chloroform/methanol (1:2, v/v). Lipids were extracted, and the newly synthesized SM and ceramide were analyzed as described below. Activities of the neutral and acid SMases were determined using the liver cell homogenates and [methyl- ^{14}C -choline] sphingomyelin

(52 mCi/mmol). For nSMase activity determination, the reaction mixture was used which contained 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM magnesium chloride, 0.65% Triton X-100, 1.5 mg protein, and 38,000 dpm [14 C]sphingomyelin in a final volume of 200 μ l. For aSMase activity determination, the reaction mixture contained 50 mM sodium acetate, pH 5.0, 0.65% Triton X-100, 1.5 mg protein, and 38,000 dpm [14 C]sphingomyelin in a final volume of 200 μ l. The reaction proceeded up to 1 h at 37°C and then was terminated by the addition of 1.5 ml of chloroform/methanol (1:2, v/v), followed by 1 ml of chloroform and 1 ml of H₂O. The mixture was centrifuged for 5 min at 3,000 rpm. After the phase separation, a portion of the upper aqueous phase, containing [14 C]phosphorylcholine, was removed, and the radioactivity was determined by liquid scintillation counting. Isolated liver cell microsomes and [14 C]serine were used for the determination of SPT activity (Merrill et al. 1985). The reaction volume of 0.1 ml contains 100 mM HEPES (pH 8.3), 5 mM dithiothreitol, 2.5 mM EDTA (pH 7.0), 50 μ M pyridoxal phosphate, and microsomes containing 100 μ g protein. The reaction was initiated by the addition of 200 μ M palmitoyl CoA and 1 mM [14 C]serine (25 mCi/mmol). Reactions were incubated for 10 min at 37°C. The reaction was terminated by addition of 1.5 ml chloroform/methanol (1:2, v/v), and the 3-ketosphinganine was isolated by phase separation and quantified by a standard scintillation procedure.

Extraction and separation of lipids

The lipids were extracted according to the Bligh and Dyer (1959) protocol. The chloroform phase was collected and dried under N₂ at 37°C. The lipids were re-dissolved in chloroform/methanol (1:2, v/v) and applied on TLC plates. We used chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, v/v) for PC, PE, PI, and PS separation. For analyses of sphingolipids, we incubated dried lipids in the chloroform/methanol (1:1, v/v) containing 0.1 M KOH for 60 min for acylglycerolipid hydrolysis. Lipids were re-extracted (Bligh and Dyer 1959) and dried. To separate SM and ceramide, the plates with loaded lipid extract were developed with chloroform/ethylacetate/isopropylalcohol/methanol/0.25% KCl (25:25:25:10:9, v/v). The appropriate standards were

applied on each plate for quantification. The R_f values for ceramide and SM were 0.90 and 0.13, respectively. The contents of PC, PE, PI, PS, and SM in chromatographic fractions were determined by the method of Bartlett (1959). The regions migrating with the standard ceramide were scraped from the plate and ceramide eluted from the silica with 1 ml chloroform/methanol (1:1, v/v) followed by 1 ml of methanol. The combined eluates were dried in vacuo, and ceramide mass was quantified by long-chain bases released after an acid hydrolysis in 0.5 M HCl in methanol at 65°C for 15 h (Sathishkumar et al. 2005). Free long-chain bases were analyzed as described by Lauter and Trams (1962). Content of protein in the samples was determined according to Lowry et al. (1951). The gel spots containing [14 C]lipids were scraped and transferred to scintillation vials. Radioactivity was measured by a scintillation counter.

Statistical analysis

Data were analyzed by one-way analysis of variance followed by post hoc Fisher's protected least significant difference (Fisher PLSD) test. Results shown represent the means $\pm$ standard error of the mean and deemed statistically significant when $p < 0.05$.

Results

Our results show that administration of α -tocopherol to the 24-month-old rats increased the mass of endogenous SM in the liver (Fig. 1). However, 7-day-long treatment of the old rats with α -tocopherol had no effect on PC, PE, PI, and PS contents in the liver. Treatment of the old rats with α -tocopherol significantly decreased ceramide mass in the liver to the same level as in 3-month-old animals (Fig. 2a). Addition of α -tocopherol to the culture medium of liver slices (Fig. 2b) or isolated hepatocytes (Fig. 2c) nullified ceramide accumulation at old age. An administration of α -tocopherol to the 24-month-old rats significantly increased the content of newly synthesized [14 C]SM and decreased the content of [14 C]ceramide in the liver with respect to control animals (Fig. 3a). Culturing of hepatocytes isolated from livers of 24-month-old rats in the presence of α -tocopherol or γ -tocopherol and [14 C]palmitic acid increased [14 C]SM and decreased

Fig. 1 Effect of α -tocopherol on the phospholipids contents in the liver of 24-month-old rats. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserin (PS), sphingomyelin (SM). Animals were fed corn oil (control rats; white bars) or α -tocopherol (100 μ g/100 g body weight; black bars) daily for 7 days. The animals were starved overnight prior to the experiments. Liver homogenates were used for the determination of phospholipids contents. Lipid extraction and separation were determined as described in the “Materials and methods”. Results are mean $\pm$ SE of six experiments performed in duplicate. Significant differences are shown. * $P < 0.05$ (vitamin E-treated vs. control)

[14 C]ceramide contents as compared with the control cells (Fig. 3b, Table 1). However, treatment of the old hepatocytes by δ -tocopherol had no significant effect on [14 C]ceramide levels in the liver cells (Table 1). α -Tocopherol effect on the newly synthesized ceramide content in old cells correlated with the action of inhibitor of SPT activity (myriocin) on the hepatocytes (Table 1). It has been demonstrated that myriocin

addition to the culture medium of old hepatocytes significantly decreased [14 C]ceramide content with respect to control cells. In contrast with the myriocin, inhibitor of ceramide synthesis (fumonisins B1), did not significantly reduce the level of newly synthesized [14 C]ceramide in the hepatocytes of 24-month-old rats (Table 1).

Fig. 2 Ceramide content in the liver of rats of different age and old α -tocopherol-treated animals. Three-month-old rats (light gray bar), 24-month-old rats (rising left hatched). Twenty-four-month-old animals were fed corn oil (control rats; white bars) or α -tocopherol (100 μ g/100 g body weight; black bars) daily for 7 days. The animals were starved overnight prior to the experiments. Liver homogenates **a** of corn oil or α -tocopherol-treated rats were used for the determination of ceramide contents. Liver slices **b** of untreated 24-month-old rats were incubated in the presence of PC liposomes (control; white bars) or α -tocopherol liposomes (100 μ g/ml; black bars) for 1.5 h. Hepatocytes **c** of untreated 24-month-old rats were incubated in the presence of PC liposomes (control; white bars) or α -tocopherol liposomes (100 μ g/ml; black bars) for 24 h. α -Tocopherol-containing liposomes were prepared, and lipid extraction and separation were determined as described in the “Materials and methods”. Results are mean $\pm$ SE of six experiments performed in duplicate. Significant differences are shown. * $P < 0.05$ (24-month-old vs. 3-month-old rats). ** $P < 0.05$ (α -tocopherol-treated vs. control)

Fig. 3 Effect of α -tocopherol on the ceramide synthesis in the liver and hepatocytes of 24-month-old rats. **a** Animals were fed corn oil (control rats; white bars) or α -tocopherol (100 μ g/100 g body weight; black bars) daily for 7 days. The animals were starved overnight prior to the experiments. Liver homogenates were used for the determination of sphingolipids synthesis in the presence of [14 C]palmitic acid. **b** Hepatocytes

were incubated in the presence of [14 C]palmitic acid and PC liposomes (control; white bars) or α -tocopherol liposomes (100 μ g/ml; black bars) for 2 h. Sphingolipid synthesis, lipid extraction, and separation were determined as described in the “Materials and methods”. Results are mean $\pm$ SE of six experiments performed in duplicate. Significant differences are shown. * $P < 0.05$ (α -tocopherol-treated vs. control)

To demonstrate that α -tocopherol decreases the synthesis of ceramide de novo, the key enzyme of sphingolipid synthesis was investigated in the isolated

Table 1 Effects of tocopherols and inhibitors of sphingolipid turnover and synthesis on ceramide content in the liver cells of 24-month-old rats

Tocopherols/inhibitors	Ceramide (% of untreated cells)
Untreated	100
Control	100 $\pm$ 7.3
Δ -Tocopherol	95.6 $\pm$ 4.5
α -Tocopherol	61.6 $\pm$ 3.6*
γ -Tocopherol	73.3 $\pm$ 4.8*
Glutathione	68.0 $\pm$ 3.7*
<i>N</i> -acetylcysteine	75.3 $\pm$ 4.6*
Imipramine	42.2 $\pm$ 4.7*
Myriocin	48.5 $\pm$ 6.0*
Fumonisin B1	85.9 $\pm$ 6.6

Hepatocytes were incubated in the presence of [14 C]palmitic acid and α -tocopherol, δ -tocopherol, γ -tocopherol liposomes (100 μ g/ml), *N*-acetylcysteine (3 mM), inhibitors of nSMase (3 mM glutathione), aSMase (50 μ M imipramine), SPT (5 μ M myriocin), or ceramide synthase (1 μ M fumonisins B1) for 2 h. Control cells were incubated in the presence of PC liposomes or ethanol. Tocopherol-containing liposomes were prepared, and [14 C]ceramide formation was determined as described in the “Materials and methods”. Results are mean $\pm$ SE of six experiments performed in duplicate

* $P < 0.05$, α -tocopherol or inhibitor-treated vs. control

liver microsomes. Using the [14 C]serine, it has been demonstrated that treatment of old liver cells by α -tocopherol or myriocin reduced SPT activity in the liver microsomes (Fig. 4).

Furthermore, to clarify if α -tocopherol could reduce the free fatty acid-induced ceramide accumulation in the liver cells of young animals, effects of palmitic acid as well as palmitoleic acid on sphingolipid synthesis and mass were investigated in the isolated hepatocytes. Pretreatment of hepatocytes, isolated from the liver of 3-month-old rats, with palmitic acid but not by palmitoleic acid caused loss of cell viability. The contents of the blue cells in the control and palmitate-treated cells were 3 ± 0.5 and $17 \pm 3\%$, respectively. Trypan blue staining indicated that α -tocopherol addition to the culture medium prevented the palmitate-induced hepatocyte damage. Addition of palmitic acid to the culture medium of young hepatocytes significantly increased the content (Fig. 5a) and synthesis (Fig. 5b) of ceramide in the cells. Pretreatment of hepatocytes with α -tocopherol or SPT inhibitor (myriocin) abolished palmitic acid-induced ceramide accumulation and synthesis. Treatment of hepatocytes of 3-month-old rats with palmitoleic acid had no significant effect on ceramide accumulation (Fig. 5a) and synthesis (Fig. 5b).

Next, we investigated effects of SMases inhibitors on ceramide accumulation in the hepatocytes of old rats. Culturing of hepatocytes in the presence of α -

Fig. 4 Effect of α -tocopherol and inhibitors of sphingolipid turnover and synthesis on sphingomyelinase and serine palmitoyl transferase activities in the liver cells of 24-month-old rats. **a** Liver cells were pre-incubated in the presence of PC liposomes (control; white bars) or α -tocopherol liposomes (100 μ g/ml; black bars) or myriocin (5 μ M; horizontally hatched) for 2 h and used for serine palmitoyl transferase activity (SPT) determination as described in the “Materials and methods”. **b** Liver cells were pre-incubated in the presence of PC liposomes (control; white bars) or α -tocopherol liposomes (100 μ g/ml; black bars) or glutathione (3 mM; dark gray bars), or imipramine (50 μ M; light gray bars) for 2 h and used for neutral sphingomyelinase (nSMase) and acid sphingomyelinase (aSMase) activities determination as described in the “Materials and methods”. Results are mean $\pm$ SE of six experiments performed in duplicate. Significant differences are shown. * P <0.05 (α -tocopherol-treated vs. control), ** P <0.05 (inhibitor-treated vs. control)

tocopherol or aSMase inhibitor (imipramine) as well as in the presence of nSMase inhibitor (glutathione) as well as NAC decreased [14 C]ceramide content in the old cells (Table 1). Moreover, it has been demonstrated that administration of α -tocopherol to

Fig. 5 Effect of α -tocopherol and myriocin on ceramide content and synthesis in the palmitate-treated hepatocytes of 3-month-old rats. Palmitoleic acid (POA), palmitic acid (PA), α -tocopherol (α -toc), myriocin (Myr). Control cells (white bars) were incubated in the presence of fatty acid-free bovine serum albumin and PC liposomes for 24 h at 37°C. Isolated hepatocytes of 3-month-old rats were cultured in the presence of palmitoleic acid (0.5 mM) (rising left hatched) or palmitic acid (0.5 mM; dark gray bars), bound to fatty acid-free bovine serum albumin (1:1, molar ratio) or palmitic acid (0.5 mM) and PC liposomes containing α -tocopherol (100 μ g/ml; black bars) or myriocin (5 μ M; horizontally hatched) for 24 h at 37°C. Ceramide content **a** in the hepatocytes was determined as described in the “Materials and methods”. To study the ceramide synthesis **b** in the hepatocytes, the [14 C]serine was added to the culture medium. Lipid extraction and separation were determined as described in the “Materials and methods”. Results are mean $\pm$ SE of six experiments performed in duplicate. Significant differences are shown. * P <0.05 (palmitate-treated vs. control), ** P <0.05 (α -tocopherol+palmitate-treated vs. palmitate-treated), *** P <0.05 (myriocin+palmitate-treated vs. palmitate-treated)

the 24-month-old rats increased the endogenous nSMase inhibitor glutathione content in the liver.

The glutathione contents in the liver of 24-month-old control and α -tocopherol-treated rats were 10.8 ± 0.7 and 15.7 ± 0.6 $\mu\text{g}/\text{mg}$ protein ($P < 0.05$), respectively. To demonstrate that α -tocopherol indeed affect SMases, activities of enzymes were studied in α -tocopherol-treated and control old hepatocytes. Using the [methyl- ^{14}C -choline]sphingomyelin, it has been determined that administration of α -tocopherol to 24-month-old liver cells reduced the neutral and acid SMases activities (Fig. 4).

Discussion

It is well documented that increase of sphingolipid turnover in liver is associated with aging and that age-dependent ceramide accumulation in liver cells could be linked to the increased state of oxidative stress (Lightle et al. 2000; Kavok et al. 2003; Babenko and Shakhova 2006; Rutkute et al. 2007). In the previous investigations, it has been found that ceramide mass is significantly higher and SM content lower in the liver and isolated hepatocytes of 24-month-old rats as compared with the 3-month-old animals (Babenko and Shakhova 2006). Our results show that administration of α -tocopherol to old rats increased SM content and had no effect on other phospholipids contents in the liver. α -Tocopherol-induced increase of SM content was associated with ceramide content drop in the liver tissue of 24-month-old animals up to the level in 3-month-old rats. The similar results were obtained after addition of α -tocopherol as well as γ -tocopherol to the culture medium of old liver slices or isolated old hepatocytes. In contrast with α - or γ -tocopherol action on old liver hepatocytes, δ -tocopherol, which is less active as antioxidant than α - and γ -tocopherol, did not significantly alter ceramide content in the cells. α -Tocopherol imitates other antioxidants actions on SM and ceramide contents in the liver of old animals (Babenko and Shakhova 2006) and pro-oxidant-treated young rats (Babenko and Shakhova 2008). Administration of plant polyphenols, which are reported to exhibit antioxidant properties, to old rats nullified age-dependent ceramide accumulation and increased SM content in the liver of old rats. Taking together, the above results indicate that α -tocopherol could abolish ceramide accumulation in the liver and hepatocytes of old rats. These effects are specific

and could be dependent on α -tocopherol antioxidant activity.

Formation of ceramides via SM hydrolysis or from de novo pathways was observed in response to different inducers of stress and at old age. Ceramide accumulation in the liver cells of old rats was associated with the activation of the neutral and acid SMase activities (Babenko and Shakhova 2006; Rutkute et al. 2007). It has been determined that glutathione depletion is responsible for the age-related upregulation of nSMase activity in rat hepatocytes and NAC or glutathione addition to the culture media significantly decreased the activity of nSMase in aged cells to a level similar to that of young ones (Rutkute et al. 2007). It was well documented that the decreased cellular level of glutathione is the feature of the aging-associated oxidative stress in the liver (Nakata et al. 1996). The glutathione has been found to be a powerful negative regulator of the nSMase activity in different cells (Liu and Hannun 1997). Different antioxidants (melatonin, *N*-acetyl serotonin, and vitamin E) inhibited the intracellular accumulation of peroxide, stabilized glutathione content, reduced the nSMase activity, and prevented the oxidant-induced death of mouse hippocampal HT22 cells and cortical neurons (Post et al. 1998; Yamagata et al. 2009). Elevated at old age, neutral and acid SMases activities could be significantly decreased by the plant polyphenolic antioxidants (Babenko and Shakhova 2006), which are known could increase the level of glutathione depleted by oxidative stress in the liver (Su et al. 2003). In the present work, it has been demonstrated that glutathione or its precursor NAC as well as inhibitor of aSMase activity imipramine or α -tocopherol addition to the culture media of old hepatocytes decreased the content of ceramide in [^{14}C]palmitate-labeled cells. α -Tocopherol increased glutathione content and decreased neutral and acid SMases activities in the liver of old rats. The results obtained are in line with other experiments on α -tocopherol action on glutathione content in the liver (Bansal et al. 2005) and activity of nSMase in liver cells (Martín et al. 2001; Rutkute et al. 2007).

The increased nSMase activity in the hepatocytes of old rats is dependent on the glutathione depletion, while the increased aSMase activity in the liver cells can deplete the glutathione stores (Garcia-Ruiz et al. 2000). However, it has been recently shown that

generation of reactive oxygen species an important step for aSMase activation in cells (Dumitru and Gulbins 2006; Charruyer et al. 2005). The aSMase could be activated by the protein kinase C δ (PKC δ), which is overexpressed under oxidative stress (Zeidan and Hannun 2007) and a premature aging disorder—Werner syndrome (Massip et al. 2010). Based on these results, reasonable assumption can be made that α -tocopherol prevents ceramide accumulation in the liver of old rats, at least in part, via the glutathione-dependent nSMase and PKC δ -dependent aSMase inhibition.

To elucidate the further mechanisms of α -tocopherol action on ceramide content in the old liver cells, the SM and ceramide synthesis in the presence of precursor of sphingolipid synthesis de novo have been investigated. Long-term feeding of the old rats with α -tocopherol or addition of the antioxidant to the culture media of hepatocytes significantly increased the level of the newly synthesized SM and reduced the newly synthesized ceramide content in the liver cells. Previous studies have demonstrated that the production of ceramide in [14 C]palmitate-labeled human keratinocytes was parallel to the level of reactive oxygen species, and the free radical scavenger vitamin E prevented the pro-oxidant (UVA radiation) effect on sphingolipid accumulation (Mazière et al. 2001; Grether-Beck et al. 2005). Moreover, UVA-induced ceramide accumulation could be prevented by inhibitors of key enzymes of sphingolipid synthesis de novo (myriocin) and ceramide synthesis (fumonisins B1), but not by glucosylceramide inhibitor (*D,L*-threo-PDMP; Grether-Beck et al. 2005). The observed increase in ceramide content by UVA treatment was parallel to increase of the SPT activity and expression in keratinocytes. To clarify if α -tocopherol effect on ceramide synthesis could be correlated with known inhibitors of sphingolipid synthesis in the isolated old hepatocytes, myriocin and fumonisins B1 action on the newly synthesized ceramide content was investigated. Results obtained demonstrated that myriocin as well as α -tocopherol significantly decreased the newly synthesized ceramide content, while fumonisins B1 was less potent in the old liver cells. This might reflect impact of de novo pathway of sphingolipid synthesis in ceramide accumulation in old cells. There is growing evidence implicating SPT as the enzyme controlling de novo ceramide synthesis (Perry et al. 2000; Watson et al. 2009). Taking into account that α -tocopherol

reduced activity of a key enzyme in the de novo synthesis of ceramide from palmitoyl-CoA and serine and that this effect was imitated by myriocin, it could be suggested that SPT is an important target for antioxidant action in the old cells.

Aging-like increase of ceramide production and accumulation could be induced in different cells by oversupply of sphingolipid precursor palmitic acid. Infusion of saturated fatty acids to the mice significantly increased ceramide content in the liver and soleus muscles, while myriocin, an inhibitor of SPT activity, injection to the animals blocked ceramide production in tissues (Holland et al. 2007). Palmitic acid addition to the culture media of hepatocytes induced significantly the intracellular accumulation of ceramide (Wei et al. 2006; Nakamura et al. 2009). Palmitate-induced ceramide accumulation in the liver cells and astrocytes was prevented by the inhibitors of SPT (L-cycloserine) or ceramide synthase (fumonisins B1), respectively, but not by the aSMase inhibitor (disipramine) (Wei et al. 2006; Blázquez et al. 2000). Data from the present study demonstrated that palmitic acid, but not palmitoleic acid, increased the both content of newly synthesized ceramide and sphingolipid mass in the isolated hepatocytes of 3-month-old rats.

Trypan blue staining indicated that there was disruption of the palmitate-treated hepatocyte membranes. The results obtained are in line with other investigations which demonstrated increase in the cell death, ceramide synthesis, and content in the liver and other cell types under the oversupply of saturated free fatty acids in the culture media (Merrill et al. 1995; Wei et al. 2006; Listenberger et al. 2003; Blázquez et al. 2000). Culturing of hepatocytes in the presence of α -tocopherol prevented the palmitate-induced cell damage. α -Tocopherol treatment resulted in initial drop of trypan blue inclusion into the palmitate-treated cells up to the level in the control young hepatocytes. An addition of α -tocopherol or myriocin, an inhibitor of SPT activity, to the culture medium of hepatocytes prior to palmitate prevented ceramide accumulation and decreased the lipid synthesis de novo in the liver cells. Thus, these findings provide definitive evidence that α -tocopherol could restore ceramide content in the palmitate-treated young liver cells reducing lipid synthesis de novo.

In conclusion, our study has demonstrated that α -tocopherol is a powerful modulator of sphingolipid

turnover in the liver cells. Abnormalities of sphingolipid turnover in the livers of old animals could be ameliorated by the long- and short-term administration of α -tocopherol. Effect of α -tocopherol on ceramide content is specific, dependent on its antioxidant properties, and similar to that of γ -tocopherol, NAC, and known inhibitors of sphingolipid metabolism (glutathione, imipramine, myriocin). The in vivo as well as in vitro effects of α -tocopherol on ceramide and SM contents and metabolism in the liver cells were determined. Treatment of old rats with α -tocopherol decreased content and synthesis of ceramide, increased content and synthesis of SM, and did not influence the other glycerol phospholipid contents in the liver tissue. α -Tocopherol could prevent the palmitate-induced aging-like disturbances in ceramide metabolism in liver cells of young animals via suppression of ceramide synthesis de novo. The aging-induced accumulation of ceramide could be normalized by α -tocopherol in liver cells via inhibition of acid and neutral SMases and lipid synthesis de novo.

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