

Role of acid sphingomyelinase in the age-dependent dysregulation of sphingolipids turnover in the tissues of rats

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Abstract. Old age-associated pathologies usually coincide with altered sphingolipid metabolism. In the present article, the role of acid sphingomyelinase (ASMase) in the age-dependent changes of sphingomyelin (SM) and ceramide contents in the tissues has been investigated by means of ASMase inhibitors, imipramine and zoledronic acid. It has been determined that ceramide content and ceramide/SM ratio increased, while SM level decreased in the heart, liver, blood serum and skeletal muscles of 24-month old rats in contrast to 3-month old animals. Injections of imipramine or zoledronic acid to 24-month old rats resulted in significant downregulation of ASMase in the liver and skeletal and heart muscles. The both inhibitors decreased the ceramide content and ceramide/SM ratio and increased the SM content in all tissues studied, except the heart, of old rats to the levels close to those observed in the young animals. Long-term treatment of rats by inhibitors, which have different mechanisms of action on ASMase, exerts the similar, but not equal effects on enzyme activity and SM turnover. In summary, the data above strongly suggest that the age-dependent up-regulation of ASMase plays an important role in the modulation of ceramide and SM contents in rat tissues and that imipramine and zoledronic acid are useful tools for SM turnover manipulation at old age.

Key words: Imipramine — Zoledronic acid — Acid sphingomyelinase — Rat tissues — Aging

Introduction

Sphingolipids (SL) are essential cell membrane components. Moreover, sphingomyelin (SM) is a major membrane SL and its metabolites and precursors are responsible for membrane structure and signaling functions (Liu et al. 2006; Hannun and Obeid 2008; Pavoine and Pecker 2009). SM metabolite, ceramide, plays the central role not only in membrane raft clusterization and structurizing, but also is essential as a secondary messenger in signal pathways responsible for cell viability regulation (Liu et al. 2006). Ceramide-dependent pathways are essentially pro-apoptotic and antagonistic to such vital signal pathways, as phosphatidylinositol-3-kinase-dependent (Liu et al. 2006; Mullen et al. 2012). Ceramide accumulation under the cytostatic drugs action and at old age

is an important reason of cell death and tissues degradation (Algeciras-Schimnich et al. 2002; Tirodkar and Voelkel-Johnson 2012).

Ceramide accumulation in cells is closely related to the development of such age-associated pathologies, as atherosclerosis, Alzheimer's disease, metabolic syndrome and insulin resistance (Young et al. 2013). Ceramide is predominantly a minor cell membrane component, and the pool of ceramide messenger molecules can be formed through SM degradation by SMases (Jenkins et al. 2010). SMases activation under stress conditions is the important reason of ceramide overproduction in the different cells. Acid sphingomyelinase (ASMase) overexpression is associated with the development of age-dependent pathologies, such as cardiovascular disease and diabetes, hepatic cirrhosis, chronic hepatitis, and even progressive liver failure (Beckmann et al. 2014).

Elevated ASMase, as well as neutral SMase and ceramidase activity in the liver, kidney and brain at old age (Babenko and Shakhova 2006, 2014a; Sacket et al. 2009), is associated with inflammation and neurodegeneration. The

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SMase hyperactivity can promote the development of age-related asthenias in skeletal muscles (Loehr et al. 2014). However, the impact of SMase-dependent pathway of SL turnover in the ceramide accumulation and age-dependent tissues malfunction is not fully understood. It has been determined recently that treatment of hepatocytes, isolated from the liver of 24-month old rats, by inhibitor of ASMase, imipramine, reduced the elevated content of ceramide (Babenko et al. 2012) and increased the cell sensitivity to insulin action at old age (Babenko and Kharchenko 2015). It is noteworthy that action of imipramine, as well as other inhibitors of key enzymes of sphingolipid turnover, on the “old” hepatocytes, only partly improves ceramide content and cell response to insulin action, while the mixture of inhibitors of acid and neutral SMases and ceramide synthesis *de novo* nullifies the age-dependent malfunction of liver cell. These results clearly demonstrated the impact of multiple pathways of SL turnover on ceramide accumulation in the hepatocytes at old age. In contrast, in such brain structures, as hippocampus and neocortex, imipramine increased the SM level (Garkavenko et al. 2012), but did not change the ceramide content (Babenko and Shakhova 2014a). However, inhibition of neutral SMase by means of N-acetylcysteine or calorie-restricted diet nullified the age-induced ceramide accumulation in the brain (Babenko and Shakhova 2014a) and improved cognitive function of old rats (Babenko and Shakhova 2014b). On the other hand, the treatment of old rats with imipramine significantly reduced the ceramide content in such tissues, as kidney cortex, soleus muscle and blood serum (Babenko and Shakhova 2014a). Thus, it becomes evident that the tissue-specific changes of SL turnover can lead to the increase of ceramide content and thus finally to organs malfunction during aging. Taking together, these results reveal that specific inhibitors of SMases and other key enzymes of SL metabolism can be used to clarify the mechanism of ceramide-induced changes of cellular function during aging and to determine the exact targets for treatment to ameliorate these pathological conditions.

In the present article we investigated the role of ASMase in the age-dependent changes of SM and ceramide contents in rat tissues by means of ASMase inhibitors, imipramine and zoledronic acid. Injections of imipramine or zoledronic acid to 24-month old rats resulted in significant downregulation of ASMase in the liver and skeletal and heart muscles. The both inhibitors decreased the ceramide content and ceramide/SM ratio and increased the SM content in the blood serum, liver and skeletal muscles of old rats to the levels close to those inherent in young animals, while in the heart the drugs induced more deep changes of SL contents. Long-term treatment of rats by inhibitors, which have different mechanism of action on ASMase, exerts the similar, but not equal effects on enzyme activity and SM turnover.

The data above strongly suggest that age-dependent up-regulation of lysosomal ASMase plays important role in the modulation of ceramide and SM contents in the rat tissues.

Materials and Methods

Materials

Imipramine hydrochloride (Melipramin Egis, Hungary), zoledronic acid (Zometa, Novartis Pharma Stein AG, Switzerland), [N-methyl-¹⁴C-phosphorylcholine]sphingomyelin (52 mCi/mmol) (PerkinElmer, USA), sodium chloride (0.9% NaCl injectable solution, Galich Farm, Ukraine), Sorbfil plates (Sorbopolimer, Russia) for thin-layer chromatography were used. Lipid standards (ceramide, SM) were obtained from Sigma (USA). Other chemicals used were of chemically pure grade.

Animal experimentation and drug administration

Experiments were carried out in accordance with the existing norms of the “European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes” (Strasbourg 1985) and of the national common ethical principles on the protection of animals from inhumane treatment in experiments (Decree of the 1st National Congress on Bioethics, Kyiv, 2001). The animals were fed with standard pellet diet and water *ad libitum*. All the animals were housed in polypropylene cages at a temperature 22–24°C and relative humidity of 50–60%. The animals were kept under alternate cycle of 12 hours of darkness and light. 3-month old (15 animals) and 24-month old (30 animals) male Wistar rats were used. 24-month old rats were divided into 4 groups: untreated rats, rats injected by 0.9% NaCl (control), rats injected by imipramine, intramuscularly, daily for 14 days (10 mg/kg body weight) and rats injected by zoledronic acid, intramuscularly (0.15 mg/kg body weight) for 10 days, one time in two days. Rats were euthanized 1 day after the last injection and tissues were used for the SL analysis, as described below.

Extraction and separation of lipids

The lipids were extracted according to the Bligh and Dyer protocol (Bligh and Dyer 1959). The chloroform phase was collected and dried in vacuum at 37°C. The lipids were redissolved in chloroform/methanol (1:2, v/v) and applied on the TLC plates. The individual lipids were separated by TLC on Sorbfil plates (Sorbpolymer, Russia) in solvent systems CH₃CH₂OCH₂CH₃ (system 1) and CHCl₃–CH₃OH–H₂O (40:10:1 v/v) (system 2). Lipid spots on chromatograms were

identified by comparison with the standards. SM content was determined by the Bartlett technique (Bartlett 1959). For quantitative estimation of the content of ceramides in the cells, lipid spots were transferred to test tubes and eluted by a mixture of chloroform and methanol (volumes 1:1) with subsequent elution by methanol. Combined eluates were subjected to vacuum evaporation and hydrolysis in HCl solution (0.5 mol/l) in methanol at 65°C for 15 h. Masses of ceramides were estimated by the release of long-chain compounds after hydrolysis of lipids (Babenko and Kharchenko 2012). The total protein amount was estimated by the Lowry technique (Lowry et al. 1951).

Determination of sphingomyelin turnover

The activities of ASMase were determined using the liver, heart and skeletal muscles lysates and [N-methyl-¹⁴C-phosphorylcholine]sphingomyelin (52 mCi/mmol), as enzyme substrate. The specific activity was adjusted by the addition of unlabeled SM. To determine activity of ASMase, samples of tissues were lysed in buffer containing 50 mmol/l CH₃COONa, pH 5.0, 0.65% Triton X-100. The reaction mixture contained 50 mmol/l CH₃COONa, pH 5.0, 0.65% Triton X-100, 1.5 mg protein and 38,000 dpm [methyl-¹⁴C] sphingomyelin in a final volume of 200 µl. 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 0.5 ml of chloroform and 0.5 ml of H₂O. The mixture was centrifuged for 5 min at 3,000 rpm. After phase separation, a portion of the upper, aqueous phase containing

[¹⁴C]phosphorylcholine, was removed and the radioactivity determined by liquid scintillation counting.

Statistical analysis

All mean values are presented with their standard deviation (mean ± S.D.). Data were analyzed using one-way analysis of variance (ANOVA) followed by Student's test. Differences were considered to be significant at a level of $p < 0.05$.

Results

Age-related changes of sphingolipids content

Endogenous ceramide accumulates, while SM content reduces in the different rat tissues at old age in contrast to adult animals (Fig. 1–5). The most significant changes of ceramide content were found in the blood serum (Fig. 1) and liver (Fig. 2). The ceramide level enhanced in the liver and serum of 24-month old untreated rats by 96% and 89%, respectively, as compared with the 3-month old animals. The ceramide level enhanced in the liver and serum of 24-month old untreated rats by 96% and 89%, respectively, as compared with the 3-month old animals. However, muscle tissues have demonstrated lower rate of ceramide accumulation during aging, resulted in 20% rise of ceramide in heart muscle (Fig. 3), and 44% and 62% rise in soleus (Fig. 4) and gastrocnemius muscle (Fig. 5), respectively, in comparison with the 3-month old animals. On the other hand, the most significant

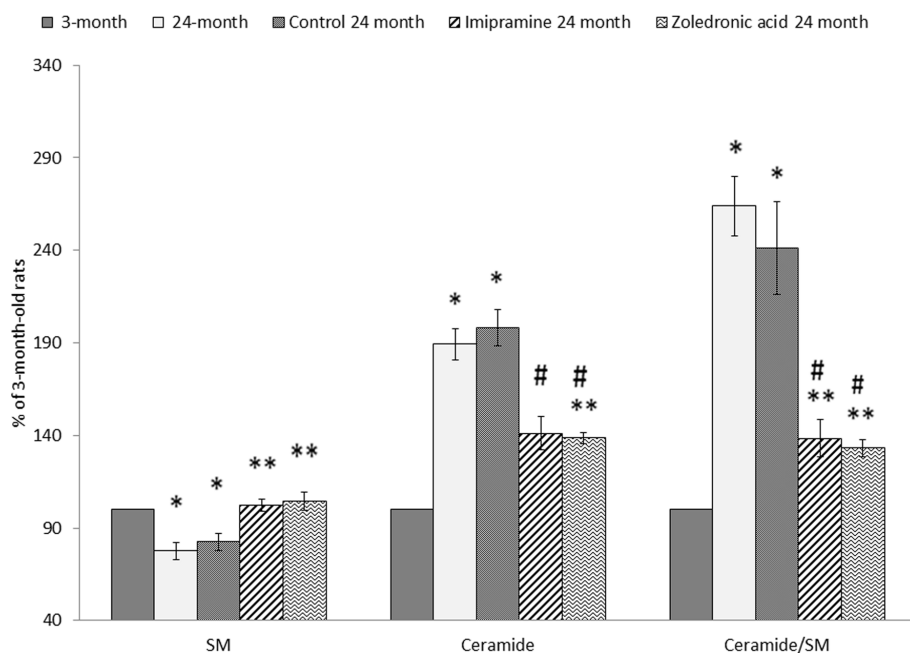


Figure 1. Effect of imipramine and zoledronic acid on sphingolipids content of old rats blood serum. Sphingomyelin (SM) content of the serum of 3-, 24-month old untreated and 24-month old control rats was 7.55 ± 0.53 , 5.86 ± 0.34 and 6.23 ± 0.34 nmol/mg protein, respectively. Ceramide content of the serum of 3-, 24-month old and 24-month old control rats was 6.35 ± 0.49 , 12.03 ± 0.54 and 12.57 ± 0.62 nmol/mg protein, respectively. Ceramide/SM ratio of the serum of 3-, 24-month old and 24-month old control rats was 0.841 ± 0.1 , 2.22 ± 0.13 and 2.029 ± 0.21 nmol/nmol, respectively. * $p < 0.05$ 24-month old vs. 3-month old rats; ** $p < 0.05$ drug-treated 24-month old rats vs. 24-month old control rats; # $p < 0.05$ drug-treated 24-month old rats vs. 3-month old rats.

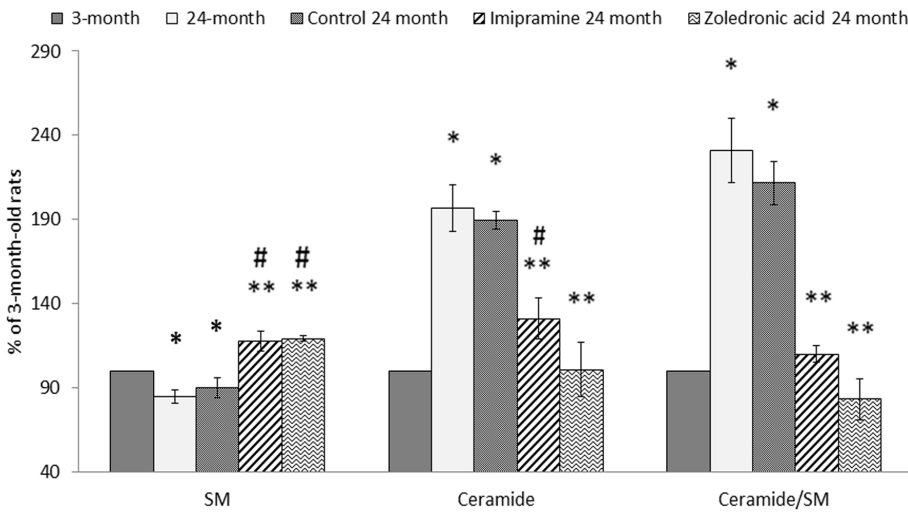


Figure 2. Effect of imipramine and zoledronic acid on sphingolipids content of old rats liver. Sphingomyelin (SM) content of the liver of 3-, 24-month old untreated and 24-month old control rats was 15.48 ± 0.54 , 11.78 ± 0.83 and 13.95 ± 0.89 nmol/mg protein, respectively. Ceramide content of the liver of 3-, 24-month old and 24-month old control rats was 5.99 ± 0.25 , 13.13 ± 0.59 and 11.36 ± 0.32 nmol/mg protein, respectively. Ceramide/SM ratio of the liver of 3-, 24-month old and 24-month old control rats was 0.39 ± 0.02 , 0.901 ± 0.07 and 0.830 ± 0.05 nmol/nmol, respectively. * $p < 0.05$ 24-month old vs. 3-month old rats; ** $p < 0.05$ drug-treated 24-month old rats vs. 24-month old control rats; # $p < 0.05$ drug-treated 24-month old rats vs. 3-month old rats.

SM content decrease has been observed in skeletal muscles: 58% and 47% reduction in the soleus (Fig. 4) and gastrocnemius (Fig. 5) muscles, respectively, related to 3-month old animals. The SM content showed 15% and 12% decrease in the liver and heart of 24-month old rats, respectively, as compared with adult ones. These results indicate that the age-dependent ceramide accumulation and SM content drop occur in a tissue-specific manner. Considering that SM

is the substrate for SMases and that ceramide/SM ratio can demonstrate the activation of these enzymes in the cells, we have studied the age-dependent fluctuations of ceramide/SM ratio in rat tissues. The ceramide/SM ratio decreased in all studied tissues of 24-month old rats, with respect to 3-month old animals (Fig. 1–5). The most significant ceramide/SM ratio increase has been observed in the soleus (Fig. 4) and gastrocnemius (Fig. 5) muscles and blood serum (Fig. 1)

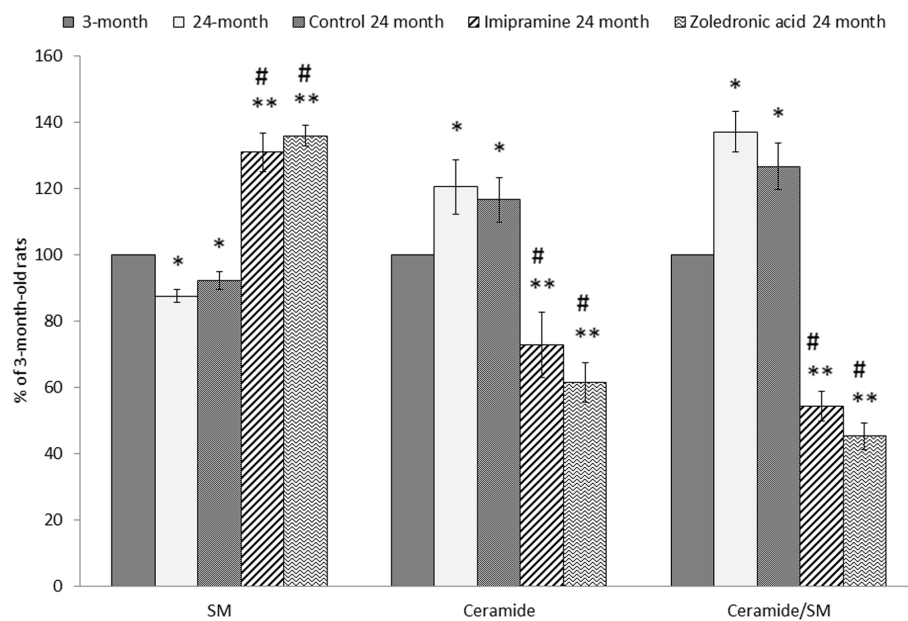


Figure 3. Effect of imipramine and zoledronic acid on sphingolipids content of old rats heart. Sphingomyelin (SM) content of the heart of 3-, 24-month old untreated and 24-month old control rats was 29.49 ± 2.47 , 25.85 ± 0.60 and 27.18 ± 0.80 nmol/mg protein, respectively. Ceramide content of the heart of 3-, 24-month old and 24-month old control rats was 10.73 ± 0.45 , 12.93 ± 0.89 and 12.52 ± 0.73 nmol/mg protein, respectively. Ceramide/SM ratio of the heart of 3-, 24-month old and 24-month old control rats was 0.364 ± 0.02 , 0.499 ± 0.02 and 0.46 ± 0.03 nmol/nmol, respectively. * $p < 0.05$ 24-month old vs. 3-month old rats; ** $p < 0.05$ drug-treated 24-month old rats vs. 24-month old control rats; # $p < 0.05$ drug-treated 24-month old rats vs. 3-month old rats.

3-month old rats; ** $p < 0.05$ drug-treated 24-month old rats vs. 24-month old control rats; # $p < 0.05$ drug-treated 24-month old rats vs. 3-month old rats.

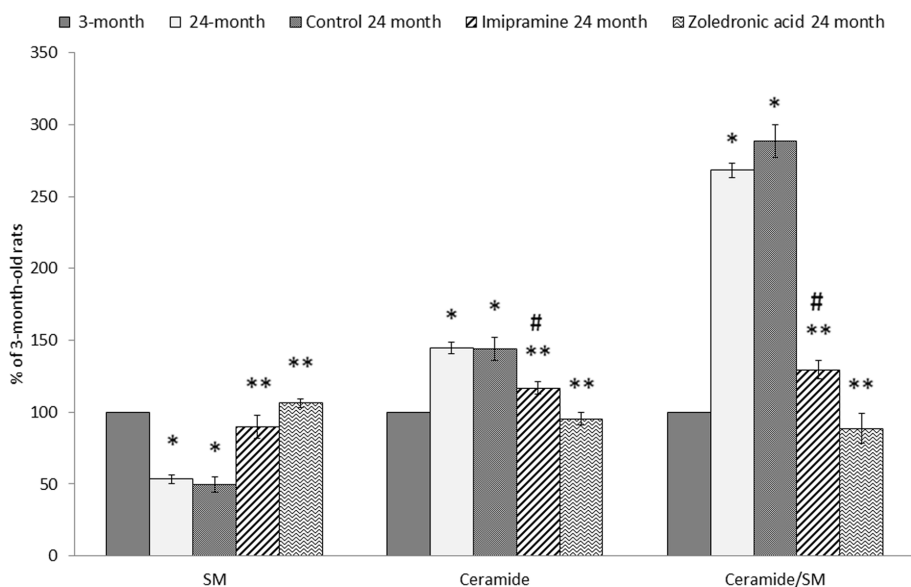


Figure 4. Effect of imipramine and zoledronic acid on sphingolipids content of old rats soleus muscle. Sphingomyelin (SM) content of the soleus muscle of 3-, 24-month old untreated and 24-month old control rats was 1.23 ± 0.07 , 0.65 ± 0.03 and 0.54 ± 0.04 nmol/mg protein, respectively. Ceramide content of the soleus muscle of 3-, 24-month old and 24-month old control rats was 0.71 ± 0.06 , 0.95 ± 0.08 and 0.95 ± 0.09 nmol/mg protein, respectively. Ceramide/SM ratio of the soleus muscle of 3-, 24-month old and 24-month old control rats was 0.59 ± 0.05 , 1.49 ± 0.15 and 1.83 ± 0.13 nmol/nmol, respectively.

* $p < 0.05$ 24-month old vs. 3-month old rats; ** $p < 0.05$ drug-treated 24-month old rats vs. 24-month old control rats; # $p < 0.05$ drug-treated 24-month old rats vs. 3-month old rats.

of aged animals: by 197%, 168% and 164%, respectively, as compared to 3-month old animals. Thus, taking together these results and data obtained previously (Babenko and Shakhova 2006; Sacket et al. 2009; Garkavenko et al. 2012) it is evident that at old age SMase activity increases in the rat tissues.

Imipramine and zoledronic acid effects on the body and organs weight

In humans chronic administration of imipramine could lead to body weight gain and even to metabolic syndrome development (Shahsavand Ananloo et al. 2013). On the other

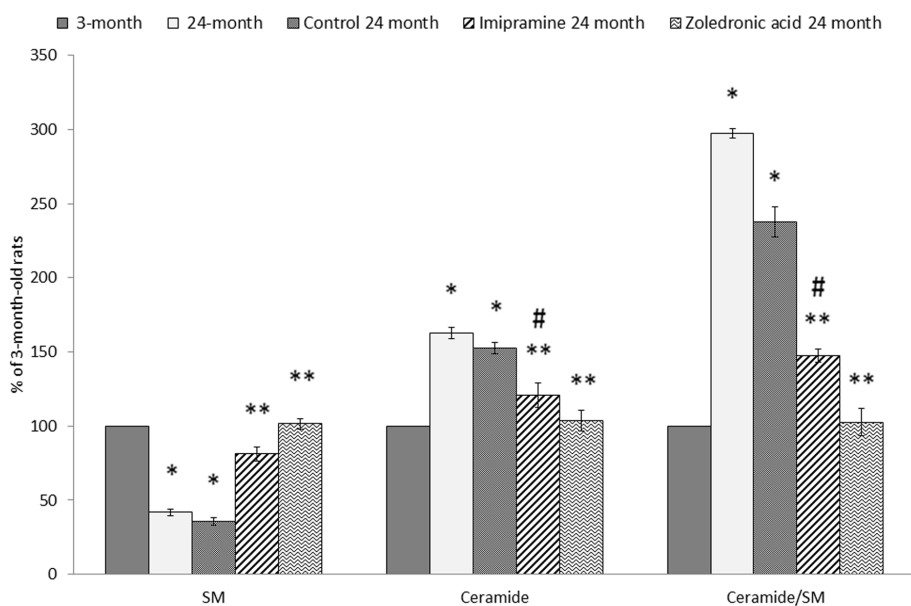


Figure 5. Effect of imipramine and zoledronic acid on sphingolipids content of old rats gastrocnemius muscle. Sphingomyelin (SM) content of the gastrocnemius muscle of 3-, 24-month old untreated and 24-month old control rats was 2.07 ± 0.09 , 0.82 ± 0.03 and 0.74 ± 0.02 nmol/mg protein, respectively. Ceramide content of the gastrocnemius muscle of 3-, 24-month old and 24-month old control rats was 0.82 ± 0.09 , 1.06 ± 0.06 and 1.19 ± 0.05 nmol/mg protein, respectively. Ceramide/SM ratio of the gastrocnemius muscle of 3-, 24-month old and 24-month old control rats was 0.41 ± 0.05 , 1.30 ± 0.08 and 1.63 ± 0.09 nmol/nmol, respectively.

* $p < 0.05$ 24-month old vs. 3-month old rats; ** $p < 0.05$ drug-treated 24-month old rats vs. 24-month old control rats; # $p < 0.05$ drug-treated 24-month old rats vs. 3-month old rats.



Figure 6. Imipramine and zoledronic acid inhibit the acid sphingomyelinase activity in the tissues of old rats: skeletal muscle (A) and liver (B). To determine the enzyme activity, [methyl- ^{14}C -phosphorylcholine]sphingomyelin was used. [^{14}C]phosphorylcholine release was determined as described in the “Materials and Methods”. * $p < 0.05$ drug-treated vs. control.

hand, in rats, chronic imipramine treatment can inhibit body weight gain (Okiyama et al. 1986). At the same time, zoledronic acid chronic administration has been reported not to influence on body weight gain in rats, but in humans it can lead to either weight increase or weight loss (Khajuria et al. 2015). Our previous works showed that optimal administration period for imipramine to inhibit effectively ASMase activity in 24-month old rats was 14 days (Garkavenko et al. 2012). Effective period of zoledronic acid administration is known to be about 10 days (Yamashita et al. 2011). In these

periods chronic effects of both imipramine and zoledronic acid on body weight have not been manifested. Considering results obtained in the present experiment, it can be assumed that imipramine and zoledronic acid injections did not affect the body and organs weights of experimental animals (Table 1).

Imipramine and zoledronic acid effects on acid sphingomyelinase activity

From significant ceramide/SM ratio increase in the old rats’ tissues, observed in the present paper, one can suppose the impact of SMase in the age-associated violation of SL turnover. To clarify this point, we used the inhibitors of ASMase activity, imipramine (Arenz 2010; Kornhuber et al. 2010) and zoledronic acid (Roth et al. 2009). Using the [methyl- ^{14}C -choline]sphingomyelin, it has been determined that imipramine administration to old rats reduced the ASMase activity in the liver (Fig. 6A), skeletal soleus muscle (Fig. 6B) and heart of the 24-month old rats *versus* the control animals of the same age. Imipramine reduced ASMase activity in the liver and soleus muscle by 40% and 47%, respectively (Fig. 6A, B). Imipramine reduced the ASMase activity in the heart muscle of 24-month old rats by 59% (0.65 ± 0.03 nmol/mg protein *per* hour, $p < 0.05$) in comparison to control animals (0.83 ± 0.05 nmol/mg protein *per* hour). Taking into consideration that imipramine is not absolutely specific for ASMase, but can, as other tricyclic antidepressants, inhibit acid ceramidase (Zeidan et al. 2006) and lysosomal phospholipases (Pappu and Hostetler 1984), we used, additionally to imipramine, the specific inhibitor of ASMase, the zoledronic acid. Zoledronic acid administration to the 24-month old rats reduced ASMase activity in the liver (Fig. 6A) and soleus muscle (Fig. 6B) by 45% and 86%, respectively.

Effect of imipramine on sphingolipids content

Injections of imipramine to 24-month old rats resulted in significant decrease of the ceramide content and ceramide/SM ratio and in increase of the SM content in all studied tissues, except the heart, of old rats to the levels close to

Table 1. Imipramine and zoledronic acid effects on the body and organs weight of old rats

Group	Body weight (g)		Organ weight (g)			
	Before experiment	After experiment	Heart	Liver	Gastrocnemius muscle	Soleus muscle
Control	353 ± 4.41	401 ± 4.41*	1.1 ± 0.10	12.33 ± 0.46	1.3 ± 0.12	0.14 ± 0.03
Imipramine	351 ± 1.67	386 ± 6.67*	1.08 ± 0.02	11.43 ± 0.58	1.7 ± 0.20	0.16 ± 0.05
Zoledronate	348 ± 7.26	388 ± 6.01*	1.03 ± 0.09	11.08 ± 0.38	1.45 ± 0.16	0.13 ± 0.02

* $p < 0.05$ vs. before experiment.

those in the young animals (Fig. 1–5). Upon the imipramine administration, ceramide level and ceramide/SM ratio in the heart decreased to the level lower than that in the 3-month old rats, while the SM content increased up to the level higher than that in the heart tissue of adult animals (Fig. 3). These results demonstrated that ASMase up-regulation can play important role in the age-dependent disturbances of SL turnover in all studied rat tissues.

Effect of zoledronic acid on sphingolipids content

Zoledronic acid administration to the 24-month old rats decreased significantly the ceramide content and ceramide/SM ratio and increased the SM level in the blood serum (Fig. 1), liver (Fig. 2), heart (Fig. 3) and skeletal muscles (Fig. 4, 5) with respect to control rats of the same age. However, both the zoledronic acid and imipramine had more pronounced effect on SLs contents in the heart in contrast to other studied tissues. Treatment of old rats by zoledronic acid reduced the ceramide content and ceramide/SM ratio in the heart to the levels less than in the tissue of 3-month old rats (Fig. 3). The SM content in the heart tissue of zoledronic acid-treated rats was by 36% higher, while ceramide content was by 38% lower than that in the 3-month old animals.

Discussion

The ASMase is an endo-lysosomal protein, which catalyzes the hydrolysis of SM to phosphorylcholine and ceramide at acidic condition (pH 5.0). Two forms of ASMase have been described: a lysosomal and a secretory one. The lysosomal ASMase can be activated by various stimuli *via* different mechanisms: phosphorylation with protein kinase C delta, resulting in ASMase translocation to the plasma membrane (Zeidan and Hannun 2007; Zeidan et al. 2008); the proteolytic cleavage of ASMase by caspase-7 (Edelmann et al. 2011); the modification of a free, C-terminal cysteine (Qiu et al. 2003); and due to translocation of the enzyme to the plasma membrane upon CD95 and action of numerous other stimuli on the cells (Beckmann et al. 2014). Although little is known about the precise mechanism of a secretory ASMase secretion and activation, the sustained activation of secretory ASMase was observed at age-dependent pathologies. Elevation of secretory ASMase in the blood serum was demonstrated in acute systemic inflammation (Haimovitz-Friedman et al. 1997; Garcia-Barros et al. 2003), in patients with type II diabetes (Gorska et al. 2003) and cachectic heart failure (Doehner et al. 2007). Increase in the secretory ASMase secretion from the human coronary artery endothelial cells under the interferon-gamma and interleukin-1beta action is related with a decrease in lysosomal ASMase (Marathe et al. 1998; Tabas 1999). Thus,

it cannot be excluded that cytokine induces the transfer of common ASMase precursor from lysosomes into the Golgi secretory pathway.

The enhancement of lysosomal ASMase activity was determined at old age in the hepatocytes (Lightle et al. 2000; Babenko and Shakhova 2014a), liver and kidney (Babenko and Shakhova 2006; Sacket et al. 2009) in contrast to young or adult rats. Our data indicate that ceramide content and ceramide/SM ratio increase, while SM level decreases in the liver and muscle tissues and blood serum of old rats in respect to adult animals. It is well known that ceramide and SM are associated with lipoproteins and that SM of the atherogenic lipoprotein particles is the substrate for secretory ASMase in blood serum. Moreover, ceramide and SM can be synthesized in the liver and after that can be transferred to the blood. The results obtained in the present work suggest the important role of the secretory ASMase in the ceramide accumulation in the blood serum at old age, while activation of the lysosomal ASMase leads to the changes of SLs contents in the liver and muscles. To clarify this point, the functional inhibitor of ASMase, imipramine, was used.

Imipramine, desipramine and other tricyclic-based drugs are widely used in clinics as antidepressants, blocking adrenergic neuromediator reuptake in central nervous system synapses. Besides this main effect, the tricyclic antidepressants are able to inhibit irreversibly ASMase activity. Moreover, the antidepressants effect on the neuronal proliferation and survival is mediated by the ASMase (Gulbins et al. 2013). Some researchers showed that ASMase inhibition contributes to imipramine antidepressant action on organism (Zhao et al. 2009). Tricyclic drugs cause ASMase degradation (Canals et al. 2011) by disturbing its linkage with negatively charged lipids of lysosomal membranes and inducing the enzyme proteolysis (Arenz 2010; Kornhuber et al. 2010). The facts that tricyclic antidepressants treatments can not completely downregulate ASMase in the cells and never lead to the development of the SM storage phenotype make these drugs the useful tool for determining the ASMase role in the cell physiology.

Upon imipramine administration, the ceramide content and ceramide/SM ratio decreased, while the SM level increased in all studied tissues. However, the imipramine treatment of old rats did not change the ceramide content and ceramide/SM ratio in blood serum, liver and heart up to the levels observed in the adult animals. In contrast, imipramine reduced the ceramide content and increased the SM content in gastrocnemius and soleus muscles of old rats to the levels close to those in the adult rats. Taken together, the above data make possible to suggest the important role of ASMase in the age-dependent dysregulation of SM turnover in the all studied tissues. In addition, the results obtained demonstrated that age-dependent accumulation of ceramide in blood serum depends on lysosomal ASMase in liver, but

not on secretory ASMase upregulation, as imipramine can inhibit the lysosomal, but not secretory ASMase. Moreover, desipramine can induce proteolysis of mature acid ceramidase and inhibit its activity in the human prostate cancer cell line DU145, bladder cancer cell line 5637, and HeLa cervical cancer cells (Canals et al. 2011) and thus reduce the ceramide content. As not only ASMase, but other catabolic enzymes of SL metabolism activated in the different cells at old age (Babenko and Shakhova 2006; Sacket et al. 2009) and tricyclic antidepressants were not completely specific for ASMase (Zeidan et al. 2006), we used other ASMase inhibitor, zoledronic acid, to clarify the role of ASMase in the age-dependent disturbances of ceramide and SM contents in rat tissues.

Biphosphonate-based drugs, such as zoledronic acid, as well as tricyclic antidepressants, can inhibit ASMase activity (Roth et al. 2009). Zoledronic acid is used in osteoporosis, bone cancer, and several other bone diseases treatments (Russell 2006). Drugs selectivity for ASMase is more than 5000 times greater than that for the neutral SMase. Biphosphonate-based drugs are more potent inhibitors of ASMase as compared with the most potent known inhibitor, phosphatidylinositol-3,5-bisphosphate (Kölzer et al. 2003). These drugs are successfully used in experimental selective ASMase suppression in cell cultures *in vitro* (Roth et al. 2009; Jenkins et al. 2010).

It can be seen from our data, that both the zoledronic acid and imipramine significantly inhibit the ASMase activity in the liver and muscle tissues of old rats. Zoledronic acid treatment causes the changes of ceramide and SM contents similar to those observed with imipramine treatment in the tissues studied. However, more pronounced effect of zoledronic acid on SL contents in comparison with imipramine was found in muscle and liver, but not in blood serum and heart. Taking into consideration that tricyclic antidepressants can inhibit ceramidase in some cells (Canals et al. 2011) it is possible to suggest that in the liver and skeletal muscle tissues imipramine inhibits the both ceramide production and degradation. The ceramide content in the liver and muscle of the imipramine-treated rats did not reach the level in the young animals, while zoledronic acid nullified age-dependent changes of ceramide content.

For our knowledge, this is the first report of zoledronic acid effects on ASMase activity and SLs contents in the different tissues of old rats. These results clearly demonstrated that activation of SM turnover under ASMase action plays an important role in the age-dependent changes of ceramide and SM contents in the rat tissues. It should be mentioned, that although both drugs inhibit ASMase in the liver and muscle tissues, nevertheless the zoledronic acid nullifies the age-induced changes of ceramide contents and ceramide/SM ratio in the skeletal muscles and in the liver, but not in the blood serum and heart. Upon the imipramine or zoledronic

acid administration, the ceramide level and ceramide/SM ratio in the heart decreased to the level lower than that in the 3-month old rats, while the SM content increased up to the level higher than that in the heart tissue of adult animals. It looks like that the heart tissue is extremely sensitive to ASMase inhibition and downregulation of ASMase-dependent ceramide production can cause the failure of membrane structure and function.

It is known, that upon certain stimuli, ASMase translocates from the lysosomes to the outer leaflet of the plasma membrane, then hydrolyzes the main SL of plasma membrane, SM, to ceramide molecules, finally resulting in the formation of ceramide-enriched membrane platforms, rafts. These platforms selectively trap or exclude such specific proteins as receptors and signaling molecules and thus facilitate or amplify signaling processes, involved in the apoptosis, autophagy, inflammation, and senescence (Gulbins and Kolesnick 2003; Gulbins and Li 2006). Using tricyclic antidepressant, desipramine, the role of ASMase/ceramide-dependent reorganization of lipid rafts in the myocardial ischemic reperfusion injury was determined (Der et al. 2006). Intravenous administration of the SMase inhibitor, D609, as well as desipramine, prior to the induction of ischemia, can abolish the heart tissue ceramide accumulation and ischemia-induced decrease in the left ventricular pressure, aortic flow and reduce the infarct size, expression of the antiapoptotic protein Bcl-2 and cardiomyocytes death (Argaud et al. 2004; Cui et al. 2004; Der et al. 2006). Although the ASMase-mediated accumulation of ceramide in the ischaemic heart is related to apoptosis and cardiac dysfunction, the desipramine treatment can promote apoptosis after the ischaemia/reperfusion and prevent an increase in antiapoptotic sphingosine-1-P content in the preconditioned heart (Cui et al. 2004).

Deficiency of ASMase and SM accumulation in the cells of patients with Niemann–Pick disease (NPD) is associated with the atherogenic lipid profile and the presence of early atherosclerotic plaques in the most affected patients (McGovern et al. 2004). Moreover, among clinical manifestations of NPD is cardiac disease (McGovern et al. 2013). High efficiency of ASMase downregulation and ceramide and SM contents modulation in heart muscle with imipramine and zoledronic acid, shown in our work, can contribute in the development of side effects of these drugs on myocardium. Imipramine can potentially cause lethal cardiotoxic side effects including hypotension, ventricular tachycardia, and decreased cardiac output (Lee et al. 2010). Imipramine-induced cardiac depression in rats is suggested to be able to reversibly inhibit heart rate, left ventricular developed pressure, and velocity of pressure change, accompanied by the total magnesium efflux (Lee et al. 2010). Furthermore, imipramine can induce ERK 1/2 activation and increase Mg^{2+} influx in cardiomyocytes. At the same time,

the antidepressants inhibit different membrane receptors and ionic channels, including the L-type calcium channel, *via* specific interaction at a receptor site (Zahradnik et al. 2008). Osteoporosis treatment with zoledronate can also be related with a high risk of atrial fibrillation and arrhythmias, as a result of altered intracellular electrolyte homeostasis and proinflammatory, profibrotic, and antiangiogenic effects (Pazianas et al. 2010; Yazici et al. 2013; Cipriani et al. 2015). In spite of that, the role of ASMase downregulation in imipramine- and zoledronic acid-induced side effects was not yet determined, it is evident, that both the upregulation and deficiency of ASMase can be harmful for organism.

In conclusion, the results obtained clearly demonstrated that the age-related SM turnover dysregulation is closely related to ASMase hyperactivity. Tricyclic antidepressant, imipramine, as well as biphospanate-based drug, zoledronic acid, are effective ASMase inhibitors in the tissues of old rats. Using inhibitors of ASMase activity, the enzyme role in the age-dependent ceramide accumulation and SM level drop in the liver, blood serum, heart and skeletal muscles was determined. Long-term treatment of old rats by inhibitors, which have different action mechanisms on ASMase, exerts the similar, but not equal effects on SM turnover. Moreover, effects of the both imipramine and zoledronic acid on the SLs contents were tissue-specific. Old rat myocardium was the most sensitive to the action of the both drugs. These drugs changed the SM turnover in the liver, blood serum and skeletal muscle to the level close to that of adult animals, but did not nullify the age-dependent differences. Thus, the impact of the other, than the ASMase, enzymes of SL metabolism in the age-dependent dysregulation of SL contents can not be excluded. Taking into account that tricyclic antidepressant and zoledronic acid are both licensed for use in humans, they can be used in future, for treatment of age-dependent pathologies, associated with the ASMase dysregulation.

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