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Effects of Reduced and Oxidized Glutathione on Sphingomyelinase Activity and Contents of Sphingomyelin and Lipid Peroxidation Products in Murine Liver

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Abstract—Like the phosphatidyl inositol cycle, the sphingomyelin cycle produces a series of the secondary messengers transmitting extracellular signals from the cytoplasmic membrane into the nucleus. Sphingomyelin, ceramide, sphingosine, sphingomyelinase, and ceramidase are the main components of the sphingomyelin cycle. In spite of numerous data on the functional properties of sphingomyelin cycle products, the activation mechanism for the key enzyme of the sphingomyelin cycle, sphingomyelinase (SMase), is not well understood. We have discovered effects of both reduced (GSH) and oxidized (GSSG) glutathione on the activity of neutral SMase in animals. GSH administration (18 mg per mouse) inhibits this enzymatic activity in liver for 2 h and increases the sphingomyelin level exactly as occurs in cell culture. The levels of diene conjugates and ketodienes decrease simultaneously during the experiment, thus indicating the ability of GSH to suppress oxidative processes in the cell. GSSG administration (18 mg per mouse) has no effect on the SMase activity during the first 15 min, but increases it twofold after 1 h. A short-term decrease in this activity after 30 min may depend on the conversion of excess GSSG into its reduced form by glutathione reductase. Unlike GSH, GSSG has no effect on the level of ketodienes after 1 h, but it induces the accumulation of diene conjugates. A strong correlation exists between the changes in SMase activity and in the level of oxidation products caused by either GSH or GSSG. These data indicate a relationship between SMase activity and the level of peroxidation products and possibly a relation between two signaling systems: the sphingomyelin cycle and the oxidative system.

Key words: glutathione, sphingomyelinase, sphingomyelin, sphingomyelin cycle, lipid peroxidation

Like the phosphatidyl inositol cycle, the sphingomyelin cycle produces a series of the secondary messengers transmitting extracellular signals from the cytoplasmic membrane into the nucleus [1-4]. Sphingomyelin, ceramide, and sphingosine, as well as the enzymes sphingomyelinase and ceramidase, are the main components of sphingomyelin cycle. Ceramide and sphingosine appear to mimic the effects of cytokines [5-7], lipopolysaccharides [8], and other biologically active substances [9] on the final cell responses, such as differentiation [5], apoptosis, or cell cycle termination [10-12]. The mechanism of activation of sphingomyelinase, the main enzyme of the sphingomyelin cycle, is not well understood. Nevertheless, much data have accumulated on the functions of sphingomyelin cycle products.

Sphingomyelinase (sphingomyelin hydrolase, EC 3.1.4.12) cleaves sphingomyelin into ceramide and phosphocholine. Sphingomyelinase is found in nearly all cell types with its maximum level in brain (in myelin). The five known types of sphingomyelinase differ in intracellular localization, pH optimum, cation dependence, and role in mechanisms of cell regulation [13-16]. Two isoforms of the enzyme, acidic and Mg^{2+}-dependent neutral sphingomyelinases, are most important in proliferation and apoptosis. Study of intracellular sphingomyelinase distribution has revealed that the acidic form is mainly located in lysosomes [14]. The optimum activity of lysosomal sphingomyelinase lies in pH range 4.4-4.8. Neutral sphingomyelinase is a Mg^{2+}- and Mn^{2+}-dependent enzyme and is located in the plasma membrane [13, 15]. The signaling system of the sphingomyelin cycle interacts with other signaling systems, such as the system generat-

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ing reactive oxygen forms including NO [17-19]. Recently, arguments have appeared suggesting that sphingomyelinase activation depends on the level of oxidative processes in the cell, and the enzymatic activity is regulated by natural antioxidants, among which glutathione is most studied [20-22]. In these studies, glutathione was found to inhibit neutral but not acidic sphingomyelinase activity. These investigations were carried out on cell cultures or partially purified enzymes, but the antioxidative properties of glutathione are also well studied in animals *in vivo* [23, 24].

The goal of the present work was to determine the effects of reduced and oxidized glutathione on neutral sphingomyelinase activity and sphingomyelin level in animal liver and to determine the relationship between the enzymatic activity and levels of lipid peroxidation (LPO) products such as diene conjugates and diene ketones. Our results elucidate the relationship between oxidative processes and the main enzyme of the sphingomyelin cycle in animals *in vivo*.

MATERIALS AND METHODS

The experiments on the effects of reduced and oxidized glutathione on LPO and sphingomyelin cycle levels were carried out on the *Balb/c* mouse strain.

Reduced or oxidized glutathione in Tris-HCl buffer (pH 7.0) was injected at dose 18 μg per mouse intraperitoneally as a single injection. Tris-HCl buffer was used as a control. The effect of glutathione was evaluated after 15 and 30 min and 1, 2, and 3 h. Murine liver homogenate was studied, three animals being used for each experimental point. Each experiment was done twice.

Mean values and standard deviations are given. The significance of differences between control values and those after oxidized or reduced glutathione administration was estimated using Student's *t*-test.

Sphingomyelinase activity was determined by the method of Hostetler [25] using 12 μM [N-methyl-^{14}C]-sphingomyelin (Amersham, England; specific activity 0.1 Ci/mmol) diluted with 50 μM "cold" sphingomyelin in buffer containing 50 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 1 mM EDTA, and 0.25% Triton X-100.

Lipids were extracted by the method of Bligh and Dyer [26]. Sphingomyelin was determined by HPTLC on silica gel followed by densitometry.

Protein was determined by the method of Lowry et al. [27].

LPO products (diene conjugates and diene ketones) were determined spectrophotometrically. Primary (diene conjugates) and secondary (diene ketones) LPO products have specific absorption maxima in their UV spectra at 233 and 270 nm, respectively. The lipids extracted from liver tissue were dissolved in methanol–hexane mixture (5 : 1 v/v) to final concentration 0.2-1.0 mg/ml. Absorption spectra in the range of 210-290 nm were recorded using a DU-6 spectrophotometer (Beckman, USA). LPO product quantities per mg lipid were calculated using the following molar extinction coefficients: 21,000 $M^{-1}\cdot cm^{-1}$ for diene conjugates; 23,000 $M^{-1}\cdot cm^{-1}$ for diene ketones [28].

RESULTS

Effect of reduced glutathione on sphingomyelinase activity and sphingomyelin level. The neutral sphingomyelinase isoform we tested is located in the plasma membrane and involved in the enzymatic degradation of sphingomyelin by triggering the sphingomyelin cycle of the cell.

Reduced glutathione (GSH), which exhibits antioxidative activity in interactions with free radicals [29], is a substrate for GSHS-transferase and GSH peroxidase [30].

Since GSH influences sphingomyelinase activity *in vitro*, it was of interest to elucidate whether GSH retains its effect on this activity *in vivo*, where its metabolic pathways appear to be more complicated. Figure 1 shows the changes in sphingomyelinase activity (curve *1*) and the level of sphingomyelin (an immediate substrate of this enzyme) (curve *2*) after GSH administration (18 mg per mouse in a single dose). The data are given in arbitrary units normalized to the corresponding controls (injection of the Tris-HCl buffer). The values of measured parameters per unit of protein or lipid are given in Table 1. Sphingomyelinase activity calculated

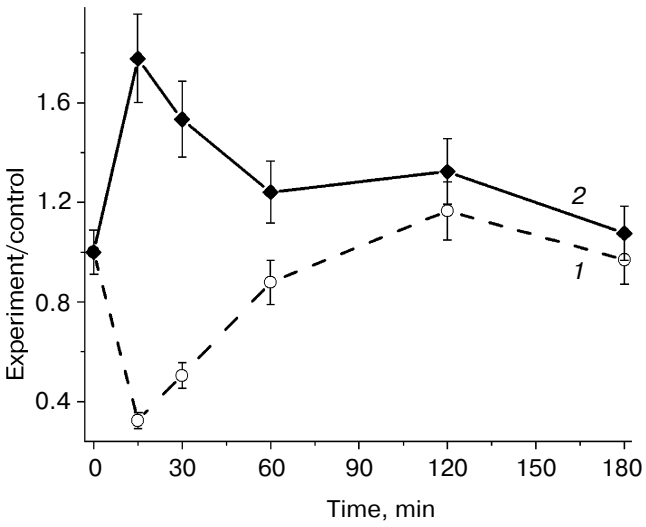

Fig. 1. Effect of reduced glutathione (18 mg per mouse) on neutral sphingomyelinase activity (cpm/mg protein) and sphingomyelin level (mg/mg total lipid) in mouse liver compared to control (injection of Tris-HCl buffer solution): *1*) sphingomyelinase activity; *2*) sphingomyelin content.

Table 1. Changes in sphingomyelin content and neutral sphingomyelinase activity in mouse liver after administration of reduced or oxidized glutathione

Time, min	Sphingomyelin, mg/mg protein	Sphingomyelin, mg/mg total lipid	Sphingomyelinase, cpm/mg protein
	Control (Tris-HCl buffer)		
0	0.0065 ± 0.0002	0.048 ± 0.001	63 ± 3
15	0.0068 ± 0.0002	0.043 ± 0.002	85 ± 2
30	0.0064 ± 0.0003	0.042 ± 0.002	117 ± 4
60	0.0065 ± 0.0002	0.048 ± 0.001	57 ± 5
120	0.0067 ± 0.0002	0.047 ± 0.002	54 ± 6
180	0.0066 ± 0.0003	0.049 ± 0.002	64 ± 6
	Experiment (GSH)		
0	0.0065 ± 0.0002	0.048 ± 0.005	63 ± 3
15	0.0114 ± 0.0011*	0.076 ± 0.008*	28 ± 3*
30	0.0090 ± 0.0009*	0.064 ± 0.006*	59 ± 6*
60	0.0084 ± 0.0008*	0.060 ± 0.006	50 ± 5
120	0.0096 ± 0.0009*	0.064 ± 0.006	63 ± 6
180	0.0070 ± 0.0007	0.052 ± 0.005	61 ± 6
	Experiment (GSSG)		
0	0.0065 ± 0.0002	0.048 ± 0.001	63 ± 3
15	0.0079 ± 0.0010	0.052 ± 0.005	86 ± 8
30	0.0082 ± 0.0009*	0.060 ± 0.003*	85 ± 9*
60	0.0074 ± 0.0008	0.050 ± 0.006	111 ± 5*
120	0.0071 ± 0.0005	0.043 ± 0.003	85 ± 4*
180	0.0074 ± 0.0005	0.041 ± 0.003	77 ± 5

Note: Mean values of six animals and corresponding standard deviations are given in Tables 1 and 2.
* Significant difference from control, $p < 0.05$.

per unit of protein was found to fall drastically 15 min after an injection of reduced glutathione and became three times lower than the control values. The level of the enzyme activity is drastically still decreased after 30 min and only after 1 h normalization was observed. The enzymatic activity is slightly higher than in control after 2 h, probably reflecting a compensatory reaction of the organism.

The change in sphingomyelin content in liver after administration of reduced glutathione is enigmatic. Sphingomyelin content calculated per unit protein and per unit total lipid increases 1.8-fold during a drastic decrease in sphingomyelinase activity, whereas the sphingomyelin level returns to the control as the enzyme activity normalizes. The data suggest two possibilities for the effect of glutathione on sphingomyelin metabolism. Reduced glutathione may induce an increase in sphingomyelin content either due to the acceleration of sphingomyelin synthesis or through the inhibition of sphingomyelinase activity. We think that sphingomyelin content is determined by the enzyme activity only because of lack of any evidence for a direct effect of glutathione on sphingolipid synthesis.

Effect of reduced glutathione on the contents of diene conjugates and diene ketones. Reduced glutathione has antioxidative properties causing a decrease in the levels of LPO products (Fig. 2), diene conjugates (curve *1*) and diene ketones (curve *2*). The product levels in Fig. 2 are given in arbitrary units normalized to the control values. The magnitudes of LPO product contents calculated per unit protein or lipid in mouse liver are given in Table 2. The levels of diene conjugates or diene ketones vary synchronously and are below the control during the entire period monitored, i.e., reduced glutathione inhibits LPO intensity in normal hepatic cells. The data are in complete agreement with the data from cell culture experiments [22].

Effect of oxidized glutathione on the sphingomyelinase activity and sphingomyelin level. Oxidized glutathione induces a short-term decrease in sphingomyelinase activity by 30% 30 min after the injection of oxidized glutathione at dose 18 mg per mouse. Unlike the reduced form, oxidized glutathione does not influence the enzyme

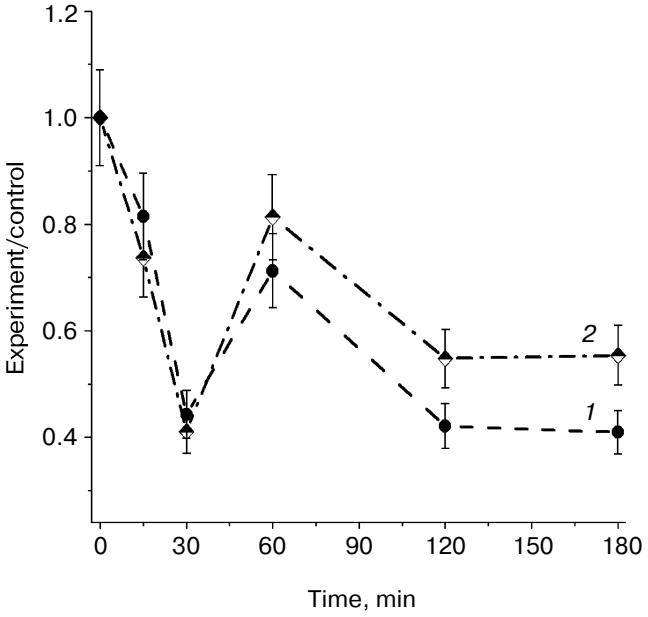

Fig. 2. Effect of reduced glutathione administration (18 mg per mouse) on lipid peroxidation product level (nmol/mg lipid) in mouse liver compared to control values: *1*) diene conjugates; *2*) diene ketones.

activity during the first 15 min, but activates it about twofold after 1 h and 1.6-fold after 2 h (Fig. 3, curve *1*). In these experiments, reduced glutathione was found to decrease sphingomyelinase activity after 30 min (Fig. 1, curve *1*).

Sphingomyelin level changes in accordance with changes in the sphingomyelinase activity under the influence of oxidized glutathione, i.e., it increases when the enzyme activity decreases (after 30 min) and decreases while the enzyme activity increases (after 1 and 2 h) (Fig. 3, curve *2*). Magnitudes of sphingomyelin levels calculated per protein or lipid are given in Table 1. Thus, sphingomyelin level in liver correlates with sphingomyelinase activity changes after the injection of reduced or oxidized glutathione into animals.

Effect of oxidized glutathione on the levels of diene conjugates and diene ketones. Oxidized glutathione administration (18 mg per mouse as a single dose) induces a decrease in LPO product levels after 30 min only (Fig. 4), i.e., at the moment when sphingomyelinase activity decreases. It is then that the reduced glutathione can be accumulated by conversion from oxidized glutathione in animal's body, as mentioned above.

Table 2. Changes in lipid peroxidation products in mouse liver after administration of reduced or oxidized glutathione

Duration, min	Diene conjugates, nmol/mg lipid	Diene conjugates, nmol/mg protein	Diene ketones, nmol/mg lipid	Diene ketones, nmol/mg protein
		Control (Tris-HCl buffer)		
0	44.3 ± 2.6	6.18 ± 0.66	21.9 ± 1.3	2.67 ± 0.19
15	41.1 ± 3.4	6.14 ± 0.63	21.4 ± 0.7	3.25 ± 0.43
30	73.4 ± 1.0	11.21 ± 1.41	37.8 ± 2.6	5.94 ± 1.34
60	45.0 ± 2.2	6.53 ± 0.41	21.6 ± 1.9	3.11 ± 0.23
120	44.9 ± 2.7	6.28 ± 0.62	21.9 ± 1.3	2.67 ± 0.19
180	44.6 ± 2.8	6.21 ± 0.69	21.9 ± 1.3	2.67 ± 0.19
		Experiment (GSH)		
0	44.3 ± 4.6	6.18 ± 0.66	21.9 ± 2.0	2.67 ± 0.19
15	33.5 ± 3.3*	4.68 ± 0.47*	15.8 ± 1.6*	2.21 ± 0.22*
30	32.5 ± 3.3*	4.55 ± 0.46*	15.5 ± 1.6*	2.17 ± 0.22*
60	32.1 ± 3.2*	4.49 ± 0.45*	17.6 ± 1.8	2.46 ± 0.25
120	18.6 ± 1.9*	2.61 ± 0.26*	12.0 ± 1.2*	1.68 ± 0.17*
180	18.1 ± 1.8*	2.54 ± 0.25*	12.1 ± 1.2*	1.69 ± 0.17*
		Experiment (GSSG)		
0	44.3 ± 2.6	6.18 ± 0.66	21.9 ± 1.3	2.67 ± 0.19
15	65.2 ± 0.7*	9.20 ± 1.16*	21.7 ± 1.2	3.07 ± 0.52
30	61.7 ± 2.0*	8.30 ± 0.84*	24.4 ± 1.0*	3.30 ± 0.22*
60	61.6 ± 4.3*	9.81 ± 0.71*	20.9 ± 2.3	3.33 ± 0.38
120	61.5 ± 1.8*	9.52 ± 0.07*	19.8 ± 0.1	3.07 ± 0.05
180	55.6 ± 1.5*	8.39 ± 0.87*	21.7 ± 0.5	3.29 ± 0.50

* Significant difference from control, $p < 0.05$.

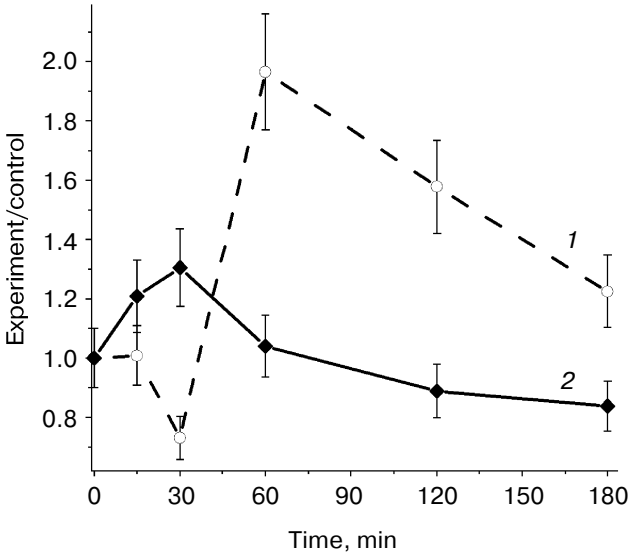

Fig. 3. Effect of single-dose intraperitoneal injection of oxidized glutathione (18 mg per mouse) on the sphingomyelin content (mg/mg total lipid) and sphingomyelinase activity (cpm/mg protein) versus control in mouse liver: *1*) sphingomyelinase activity; *2*) sphingomyelin content.

Diene conjugate levels are 1.5-fold elevated versus the control at 15 min, 1 h, and 2 h after injection of oxidized glutathione (Fig. 4, curve *1*). Diene ketone levels do not vary significantly from the control in this time range (Fig. 4, curve *2*). Relative values of oxidized products are given in Fig. 4, and the magnitudes calculated per mg protein and per mg lipid are given in Table 2. Thus, in contrast to reduced glutathione that inhibits oxidative processes for a long time, oxidized glutathione inhibits them for a short time and does not influence the diene ketone levels 1 h after the injection, and diene conjugates accumulate in this time range.

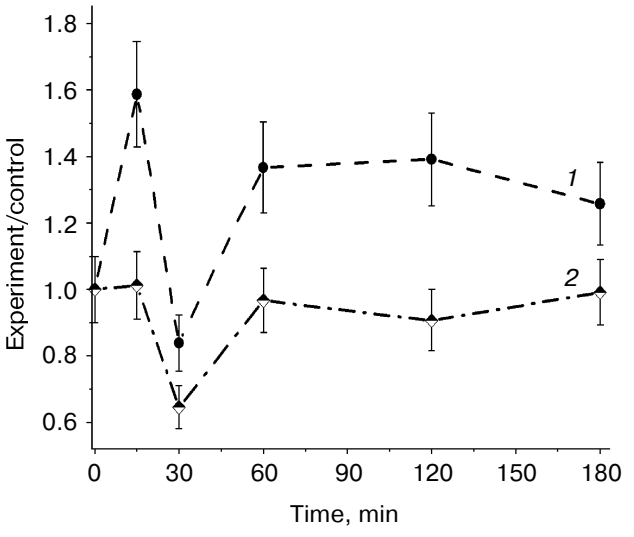

Fig. 4. Effect of oxidized glutathione administration (18 mg per mouse) on lipid peroxidation product level (nmol/mg lipid) versus control in mouse liver: *1*) diene conjugates; 2) diene ketones.

DISCUSSION

The sphingomyelin cycle is important for cell life, and thus there is significant interest in investigations of the regulation of the key enzymes of this signaling system. In spite of studies on sphingomyelinases, their properties, and various factors influencing their activity over a long period, the fact that the activity of some sphingomyelinase isoforms is controlled by the oxidative system in the cell was established only in the last three years [20-22]. A few studies performed on the cellular level or on isolated enzymes demonstrated that the activity of neutral sphingomyelinase depends on glutathione present in the cell in reduced and oxidized forms [23].

The level of intracellular glutathione, its physiological concentration being 1-20 mM, is so important for the functioning of the organism that the ratio of the reduced to oxidized (GSSG) forms is considered as a regulator of cell development [31]. GSH level in various cell types is 100-500 times higher than that of GSSG [30]. Changes in the GSH–GSSG system can be reversible or irreversible. Reversible changes in reduced glutathione to glutathione disulfide ratio are generally due to the reactions with GSH-peroxidase (enzymatic GSH oxidation), diazenes (nonenzymatic GSH oxidation), and various disulfides. Under certain conditions, these changes can result from the impairment of reduced glutathione formation from its oxidized form by GSSG reductase [31]. Irreversible changes in GSH to GSSG ratio in cells can result from the inhibition of glutathione synthesis [31]. A proper ratio of reduced glutathione to glutathione disulfide is required for normal functioning of both cell and whole organism. Substances inhibiting glutathione synthesis activate LPO in cells.

Sphingomyelinases are now known to influence the cellular glutathione level. When added to hepatocyte culture, *Bacillus cereus* neutral sphingomyelinase elevates intracellular GSH level, whereas the human placenta acidic sphingomyelinase hydrolyzing sphingomyelin to the same extent as neutral sphingomyelinase does, decreases the GSH depot [20]. Interestingly, in spite of the difference between the effects of these forms on GSH content, both the sphingomyelinases increased the level of γ-glutamyl-cysteine synthetase heavy chain mRNA. Furthermore, acidic sphingomyelinase was shown to induce mitochondrial depolarization and caspase 3 acti-

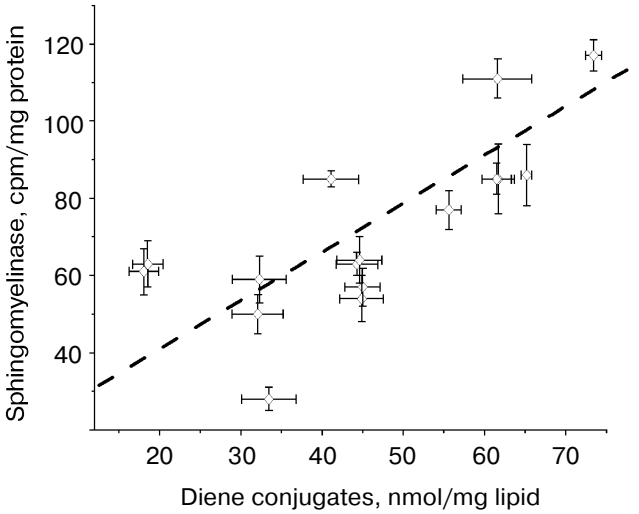

Fig. 5. Relationship between changes in LPO product level (diene conjugates, nmol/mg lipid) and neutral sphingomyelinase activity (cpm/mg protein) in mouse liver after intraperitoneal injection of reduced or oxidized glutathione (18 mg per mouse) ($R = 0.70$, $p = 0.002$).

vation, which may result in oxidative stress and the development of apoptosis [20].

There is a distinct relationship between glutathione content and sphingomyelinase activity. Neutral sphingomyelinase isolated from plasma membrane is inhibited by reduced glutathione [22]. However, a membrane-bound sphingomyelinase isoform from endoplasmic reticulum is inhibited by oxidized glutathione and by the products of free-radical oxidation [32].

The influence of glutathione, its analogs, and individual fragments on the activity of various sphingomyelinase isoforms has been studied elsewhere [22, 32, 33]. An inhibitory effect of GSH on the neutral Mg^{2+}-dependent sphingomyelinase was shown, the γ-glutamyl-group of glutathione being responsible for the effect. Most experimental data indicate reversible inhibition of the enzyme, where GSH is not metabolized and there is no irreversible change in the sphingomyelinase.

The effects of GSH and GSSG on the neutral sphingomyelinase activity in mice were demonstrated in the present work. Like on the cellular level, GSH inhibits the enzyme activity causing sphingomyelin accumulation. Diene conjugate and diene ketone levels decreased synchronously during all the experiments, confirming the ability of GSH to inhibit oxidative processes in the cell due to its antioxidative properties. A somewhat different pattern was observed with GSSG, which did not influence the enzymatic activity for 15 min and activated it about twofold after 1 h and 1.6-fold after 2 h (Fig. 3, curve *1*). Short-term decrease of enzymatic activity after 30 min may result from conversion of excess oxidized glutathione injected into the animal to the reduced form by glutathione reductase. Unlike reduced glutathione that inhibited oxidative processes for a long time, oxidized glutathione inhibited them for only a short time (for 30 min after injection) and did not influence the level of diene ketones after 1 h or result in accumulation of diene conjugates in this time range.

Note that the changes in LPO product levels and in neutral sphingomyelinase activity are similar under the influence of either GSH or GSSG. The relationship between these two parameters with a significant correlation coefficient of +0.50 ($p < 0.05$) is shown in the Fig. 5. This relationship suggests the dependence of sphingomyelinase activity on the LPO intensity and LPO product level as an intervention. It is not clear what stage of LPO process has the crucial effect on the enzyme activity. It will be elucidated in future experiments whether free oxygen radicals or lipid derivatives or products of more complete oxidation of various types are responsible for the effect.

The revealed influence of LPO components on the sphingomyelin cycle signaling system should elucidate and lead to more successfully correction of pathologies involving this system.

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