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Effect of Bilirubin on Lipid Peroxidation, Sphingomyelinase Activity, and Apoptosis Induced by Sphingosine and UV Irradiation

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Abstract—The effect of bilirubin (BR) on sphingomyelin cycle activity, lipid peroxidation (LPO), and apoptosis induced by sphingosine and UV irradiation has been studied *in vivo*. Neutral Mg^{2+}-dependent sphingomyelinase (SMase) activity and LPO level were monitored in heart, kidney, and liver of mice after administration of BR. BR inhibited both LPO and SMase activities in heart and kidney. BR induced a mild increase in LPO level and moderate increase in lipid contents in liver, consistent with the functional role of liver in both BR and lipid metabolism. BR injected to mice causes simultaneous and unidirectional alterations in both LPO level and SMase activity with a significant ($p < 0.05$) positive linear correlation between these two parameters. Sphingosine administration results in increased lipid peroxidation in murine liver. Data on DNA fragmentation indicate that exogenous BR may effectively protect thymus cells against sphingosine- and UV-mediated apoptosis. These results have revealed a biochemical association between oxidative stress and BR on one hand and the sphingomyelin cycle and apoptotic cell death on the other hand. Our data show that BR as an antioxidant, due to its effect on the sphingomyelin cycle, can protect membrane lipids against peroxidation and cells against apoptosis induced by various factors.

Key words: bilirubin, sphingomyelinase, sphingosine, apoptosis, lipid peroxidation

Numerous publications indicate a close relationship between the level of oxidative processes and the physiological status of cells [1, 2]. It has been shown, for instance, that oxidative stress can induce programmed cell death, i.e., apoptosis [3, 4], whereas antioxidants such as N-acetylcysteine, ionol (2,6-di-*tert*-butyl-*p*-cresol), or coenzyme Q suppress the apoptosis [5-7].

A bile pigment, bilirubin, is a powerful antioxidant that plays a protective role in various pathologies such as ischemia/reperfusion, atherosclerosis, hemorrhagic insult, anaphylaxis, and chemical mutagenesis [8-12]. Since the bilirubin-producing enzyme, heme oxygenase, is highly inducible, bilirubin has been of special interest in recent years. A cell response to oxidative stimuli is a manifold increase in heme oxygenase activity, and the bilirubin–heme oxygenase system is presently assumed to be one of the important components of anti-peroxide defense, along with superoxide dismutase, catalase, glutathione peroxidase, and both water-soluble and fat-soluble antioxidants [13, 14]. In connection with its antioxidative properties, bilirubin is supposed to influence apoptosis.

The sphingomyelin cycle, including sphingomyelin, ceramide, sphingosine, and the enzymes sphingomyelinase and ceramidase as the main components, generates secondary messengers participating in the regulation of apoptosis [15]. The oxidative stress induced directly by pro-oxidants (UV irradiation or hydrogen peroxide) or indirectly (by cytokines, lipopolysaccharides, or ischemia/reperfusion) [16-21] results in the activation of the sphingomyelin cycle. The activation mechanism is not well understood; however, some evidence was published recently for the dependence of the main sphingomyelin cycle enzyme, sphingomyelinase, on the level of oxidative processes in the cell. Thus, glutathione, one of the main components of anti-peroxide defense, taken in physiological concentrations, almost completely inhibits sphingomyelinase activity *in vitro*. The experiments demonstrating the effect of glutathione on sphingomyelinase activity were conducted on cell cultures or partially purified enzymes. Both reduced and oxidized glutathione were found to possess inhibitory activity [22-25]. Although antioxidants are well known to inhibit apoptosis by acting as quenchers of active oxygen forms and peroxide radicals [5-7], we think a dependence of sphin-

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gomyelinase activity on oxidative processes is not so obvious. It is the oxidized-to-reduced glutathione ratio, not the overall glutathione level, that influences the intensity of oxidative processes in the cell. Moreover, correlation between glutathione contents and sphingomyelinase activity *in vivo*, as well as between glutathione contents and the level of cell oxidative processes may be far more complicated. The data on the effects of antioxidants other than glutathione on sphingomyelinase activity *in vitro* are contradictory [5, 7, 18, 24], and there is no data from experiments *in vivo*. Also, from the literature we found no correlation between sphingomyelinase activity and membrane lipid peroxidation (LPO) level *in vivo*.

Hence, the goal of the present work was to determine the nature of the bilirubin effect on neutral sphingomyelinase activity in various organs, to reveal the relationship between the level of enzyme activity and contents of LPO products (conjugated dienes and diene ketones), and to study the effect of bilirubin on apoptosis induced by one of the sphingomyelin cycle products (sphingosine) or UV irradiation.

MATERIALS AND METHODS

The experiments were carried out on strain *Balb/c* mice. The following chemicals were used: bilirubin (Fluka, Switzerland); sphingosine (Sigma, USA); [N-methyl-^{14}C]sphingomyelin (Amersham, UK); HEPES, RPMI 1640 medium, cattle serum, proteinase K, and RNAse A (Sigma, USA); agarose 33258 (Hoechst, Germany).

Bilirubin was dissolved in several drops of NaOH (0.1 M) and diluted with 0.1 M Tris-HCl buffer, pH 7.4, to neutral pH; sphingosine was dissolved in ethanol and then in saline to final ethanol concentration in the solution below 1%. Bilirubin and sphingosine were injected intraperitoneally, 1.25 mg (50 mg/kg) and 20 or 100 μg per mouse (0.8 or 4 mg/kg), respectively; Tris-HCl buffer or saline was injected into the control mice. Liver, kidney, heart, and thymus were investigated, three animals being taken for each experimental point, and each experiment was repeated twice.

Sphingomyelinase activity was determined in liver, heart, and kidney homogenates by the method of Hostetler [26] using 12 μM [N-methyl-^{14}C]sphingomyelin, specific activity 0.1 Ci/mmol, diluted with 50 μM "cold" sphingomyelin in 50 mM Tris-HCl buffer, pH 7.2, containing 10 mM $MgCl_2$, 1 mM EDTA, and 0.25% Triton X-100.

Lipids were extracted by the method of Bligh and Dyer [27]; lipid contents were determined by weighing.

Protein was determined quantitatively by the method of Lowry et al. [28].

Spectrophotometric determination of LPO products (conjugated dienes and diene ketones). Primary (conjugated dienes) and secondary (diene ketones) products of LPO have characteristic absorption maxima in the UV spectral region at 233 and 270 nm, respectively. Lipids extracted from the liver tissue were dissolved to 0.2-1.0 mg/ml in methanol–hexane mixture (5:1 v/v). Spectra of the lipid solutions were recorded using a Beckman spectrophotometer (USA) in the range 210-290 nm. LPO products were quantified in 1 mg of lipids studied using the molar absorption coefficients 21,000 $M^{-1}\cdot cm^{-1}$ for conjugated dienes and 23,000 $M^{-1}\cdot cm^{-1}$ for diene ketones [29].

Effect of bilirubin on apoptosis. The effect of bilirubin on the apoptosis induced *in vitro* by sphingosine or UV irradiation was studied by mouse thymus DNA fragmentation evaluated by gel electrophoresis [30]. Samples were irradiated with a DRK-120 ultraviolet arc lamp (200 J/m^2). The resulting bilirubin concentration was 100 μM and sphingosine concentration was 50 μM.

Extracted organs were homogenized in a homogenizer with loosely fitting glass pestle for cell suspension preparation. To remove erythrocytes, the cell suspension was washed with 0.83% NH_4Cl. Cells were incubated with the substances under study in RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 25 μM HEPES, and 5% serum.

For the detection of apoptosis, the low molecular weight DNA fraction was isolated. Cell lysate was prepared in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.2% Triton X-100; lysate was centrifuged at 13,000*g*, ethanol was added to the supernatant to final concentration 67% and sodium acetate to 0.3 M, and then the mixture was incubated overnight at −18°C. The prepared mixture was centrifuged at 13,000*g*, and the pellet was dried in air. The pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.5, with 1 mM EDTA, and then sample application buffer (1/10 volume) was added (25% Ficoll 400, 5% SDS, and 0.25% Bromophenol Blue), RNAse A was added to 0.1 mg/ml, and the sample was incubated for 1 h at 37°C. Then proteinase K was added to 0.1 mg/ml, and the sample was incubated for 1 h at 37°C.

Low polymer DNA isolated was analyzed by horizontal electrophoresis in 1.5% agarose gel prepared in 0.5 M TBE buffer (0.045 M Tris, pH 8.0, 0.45 M borate, and 1 mM EDTA). Electrophoresis was carried out in the same buffer at 4°C and intensity of 2 V/cm for 9 h using GNA-200 Techware (Pharmacia, Sweden).

The resulting data were processed using variation statistics; the differences were taken as significant when $p \le 0.05$. Mean values are given on the figures and in the table. Experimental errors are given as average dispersions.

RESULTS

Effect of bilirubin on the sphingomyelinase activity and contents of LPO products in organs of experimental

animals. Sphingomyelinase (EC 3.1.4.12; sphingomyelin hydrolase) splits sphingomyelin to ceramide and phosphocholine. Five types of sphingomyelinases are known; two of them, acidic and Mg^{2+}-dependent neutral, are most important in proliferation and apoptosis. The acidic form of the enzyme is located in plasmatic membrane [15]. Glutathione was shown to inhibit neutral sphingomyelinase but not acidic sphingomyelinase activity *in vitro* [22-25]. Thus, activity changes of the neutral enzyme isoform that were studied on injection of antioxidant (bilirubin) into experimental animals to elucidate the relationship between the sphingomyelinase activity and LPO processes *in vivo*.

Figure 1 shows the changes in sphingomyelinase activity in heart (a), kidney (b), and liver (c) after the injection of bilirubin into mice in a single dose of 1.25 mg per animal. The data are given in arbitrary units normalized to the corresponding controls (injection of Tris-HCl buffer, pH 7.4). Magnitudes of the measured indices are given in the table.

A decrease in sphingomyelinase specific activity in heart (1.3-fold) was observed 15 min after bilirubin injection in comparison to control (injection of Tris-HCl buffer). The level of enzyme activity further decreased by a factor of 1.6 during the next 30 min, and 1 h later a tendency toward normalization was observed (Fig. 1a), however, the values did not reach the initial level.

A drastic drop of LPO products (conjugated dienes and diene ketones) in heart, respectively, 2.8- and 1.6-fold in 15 min and 3.1- and 2.5-fold 30 min after the beginning of the experiment, was observed in the experimental animals versus controls (Fig. 2a), in complete agreement with the antioxidant properties of bilirubin. The quantities of both LPO products varied synchronously and reached the control level 1 h after the beginning of experiment. The changes in absolute values of the studied parameters (see table) showed a drastic increase in LPO product levels and to a lesser extent in sphingomyelinase activity due to the stress of the injection into the control animals in the early stage of the experiment.

Injection of bilirubin not only prevented the changes caused by stress but also decreased significantly the LPO and sphingomyelinase activity indices compared to the intact organ, that is, bilirubin decreased the LPO that occurs in normal cardiac cells. These results are consistent with data in the literature. Some authors suggest that it is the myocardium where bilirubin as a product of heme oxygenase plays a particularly important role in cell protection [31]. This is related to the relatively low antioxidant level in myocardium in comparison with other organs, e.g., liver [31].

Similar but less prominent changes compared to heart were observed in kidney (Figs. 1b and 2b and the table). The maximal decrease in LPO intensity (1.5-fold versus control) occurred 30 min after the beginning of the experiment; a small apparent decrease in sphingomyelinase activity was not significant (1.25-fold). Elevation of LPO and sphingomyelinase activity indices in the kidney of control animals under stress conditions was less prominent than in heart, and it was completely prevented by bilirubin. All the studied indices returned to the initial level 1 h after the injection of bilirubin or Tris-HCl buffer.

The nature of the changes observed in the liver of experimental animals was quite different. Both sphingomyelinase activity and LPO product levels tended to increase versus control animals 15 min after bilirubin injection, and relative contents of LPO products

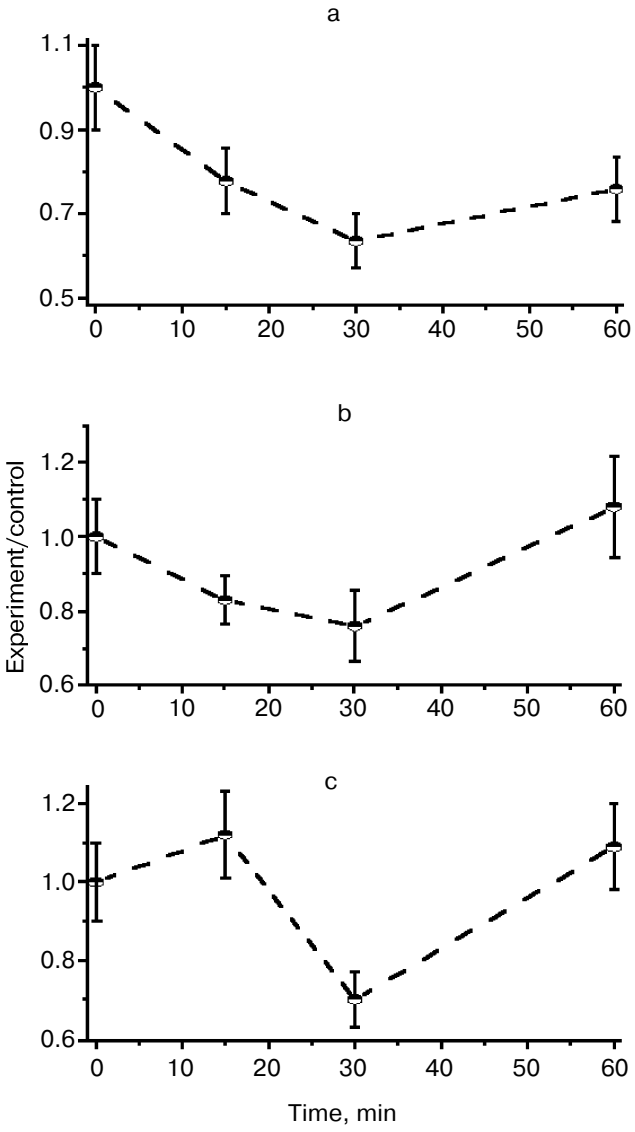

Fig. 1. Effect of intraperitoneally injected bilirubin (1.25 mg per mouse) on neutral sphingomyelinase activity in mouse heart (a), kidney (b), and liver (c). The data on sphingomyelinase activity changes (cpm per mg protein) are given in arbitrary units normalized to the corresponding controls (injection of Tris-HCl buffer).

Changes in lipid and LPO product contents and neutral sphingomyelinase activity in mouse organs after bilirubin injection

Exposure duration, min	Total lipids, mg per g mouse tissue	Conjugated dienes, nmol per mg lipids	Diene ketones, nmol per mg lipids	Sphingomyelinase, cpm per mg protein
		Heart		
Experiment				
0	52.4 ± 5.2	35.8 ± 3.6	23.1 ± 2.3	157 ± 16
15	50.4 ± 5.0	32.2 ± 3.2*	19.5 ± 1.9*	130 ± 13*
30	59.8 ± 6.0	23.0 ± 2.3*, **	13.4 ± 1.3*, **	109 ± 11*, **
60	59.2 ± 5.9	30.1 ± 3.0*	18.3 ± 1.8*	111 ± 11*, **
Control				
15	53.5 ± 5.4	98.5 ± 9.8 **	31.4 ± 3.1**	167 ± 17
30	56.2 ± 5.6	73.9 ± 7.4 **	24.6 ± 2.5	172 ± 17
60	53.8 ± 5.4	39.2 ± 3.9 **	24.6 ± 2.5 **	146 ± 15
		Kidney		
Experiment				
0	54.3 ± 3.6	20.4 ± 1.2	13.2 ± 0.8	51 ± 4
15	47.6 ± 3.9	29.0 ± 0.5	17.5 ± 0.7**	47 ± 3*
30	54.0 ± 0.2	23.1 ± 1.3*	17.5 ± 3.0	36 ± 2*, **
60	60.5 ± 1.2	21.9 ± 0.1	13.7 ± 0.3	53 ± 4
Control				
15	51.4 ± 4.7	30.7 ± 1.2**	17.3 ± 2.5**	58 ± 6
30	52.2 ± 0.1	29.2 ± 0.8**	19.2 ± 0.2**	47 ± 1
60	62.3 ± 2.7	20.6 ± 0.2	12.8 ± 1.0	45 ± 5
		Liver		
Experiment				
0	44.1 ± 3.2	43.7 ± 2.6	22.6 ± 1.3	63 ± 3
15	50.9 ± 2.8	51.0 ± 3.5**	31.5 ± 0.4*	99 ± 6**
30	59.3 ± 2.8*, **	79.5 ± 0.8*, **	46.8 ± 1.2*, **	82 ± 3*, **
60	53.1 ± 2.9*, **	44.2 ± 1.3	23.4 ± 2.6	63 ± 2
Control				
15	46.9 ± 3.1	44.1 ± 3.4	29.4 ± 0.7**	88 ± 8**
30	45.8 ± 2.1	63.4 ± 1.0**	37.8 ± 2.6**	118 ± 4**
60	44.1 ± 2.8	45.0 ± 2.2	21.6 ± 1.9	58 ± 5

Note: Mean values from six animals and corresponding standard deviations are given in the table.
* Significant difference from control ($p < 0.05$).
** Significant difference from the intact organ ($p < 0.05$).

increased significantly in liver (unlike heart and kidney) 30 min after the injection. Sphingomyelinase activity at this time was substantially decreased (by a factor of 1.4). This elevation of LPO activity in liver of control mice may be caused by stress; however, the more prominent intensification of oxidative reactions in liver after bilirubin injection seems to conflict with the antioxidant properties of this bile pigment. This fact may result from the direct involvement of hepatic cells in bilirubin excretion. When the level of serum bilirubin is normal, the liver converts it by glucuronization into a water-soluble form that then goes to the duodenum together with bile. Excess bilirubin may be oxidized by cytochrome P-450. As shown in work [32] devoted to the microsomal oxidation and conjugation of bilirubin, this pigment is successfully oxidized by the NADPH-dependent LPO system. The authors suggested microsomal oxidation to be the main metabolic pathway for bilirubin in pathologies accompanied by glucuronization defect, such as Gilbert's disease or Crigler–Najjar syndrome. In short, intensification of LPO under conditions of excess bilirubin in liver is supposed to be a response directed toward reducing the bilirubin level.

Unlike heart, liver has a very strong antioxidative defense that is first seen in high levels of glutathione and both fat- and water-soluble antioxidants (tocopherol, carotenoids, and ascorbate) as well as enzymes participating in the detoxification of active oxygen forms. The

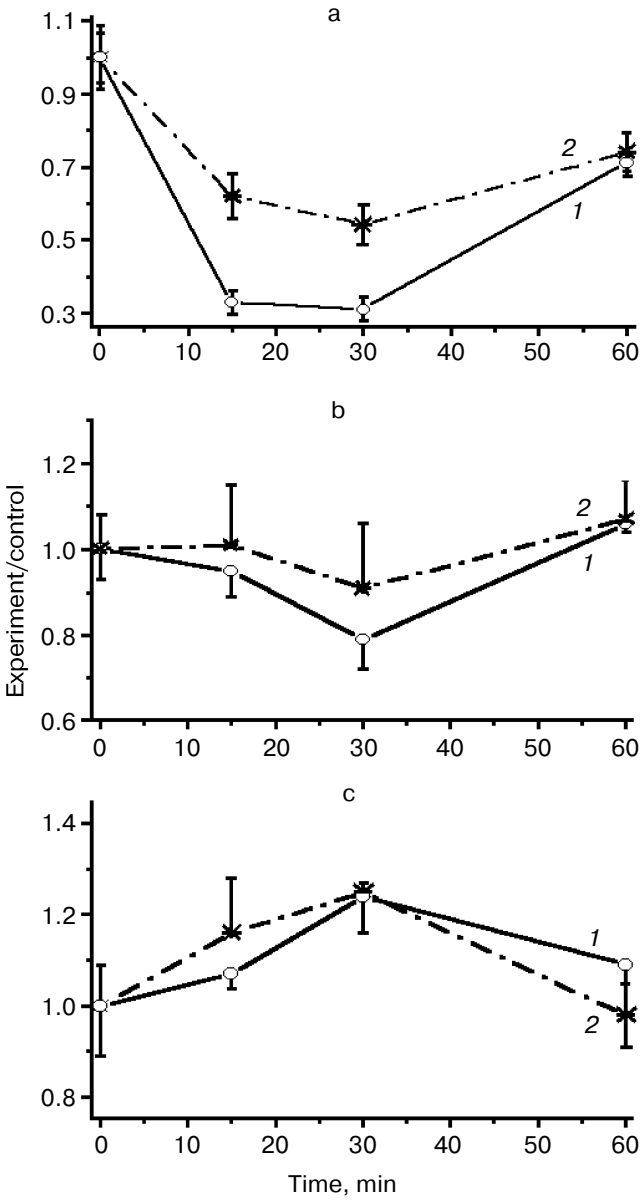

Fig. 2. Effect of intraperitoneally injected bilirubin (1.25 mg per mouse) on the contents of lipid peroxidation products (nmol per mg lipid) in mouse heart (a), kidney (b), and liver (c): *1*) diene conjugates; *2*) diene ketones. The data are given in arbitrary units normalized to the control.

decrease in sphingomyelinase activity versus controls 30 min after bilirubin injection may be due to the accumulation of glutathione, which inhibits sphingomyelinase [22-25]. Elevation of glutathione content in liver under the influence of bilirubin may result from the competition between the organic ion conjugates of glutathione and the large amount of bilirubin for excretion into bile.

Note that substantial (30-35%) elevation of lipid level occurred in liver, but not in heart or kidney, of experimental animals (Fig. 3 and the table). This increase became visible from the 30th min of the experiment, and the lipid level remained elevated after 1 h, when other parameters returned to the norm. Lipid level returned to the norm only 2-3 h after injection of bilirubin. Elevation of hepatic lipid level under the influence of bilirubin is a result of functional features of the liver, which plays a key role in lipid and bilirubin metabolism in the body. Lipids and bilirubin are excreted by common pathways, through the bile, and competitive interactions between these compounds are obvious.

Effect of sphingosine on LPO intensity in mouse liver. Sphingosine, a product of the sphingomyelin cycle, can simulate is cells effects of cytokines, lipopolysaccharides, and other biologically active compounds during cell proliferation and apoptosis [33, 34]. Although there is a considerable amount of data on the functional properties of sphingomyelin cycle products in the literature, the mechanism of action of sphingosine is not well understood. Particularly, in spite of the obvious connection between apoptosis and oxidative reactions, we did not find any evidence in the literature concerning the influence of sphingosine on LPO processes in experimental animals.

Figure 4 shows changes in LPO intensity in mouse liver after injection of sphingosine in two doses: 20 (curves *1* and *2*) and 100 μg per mouse (curves *3* and *4*). Both doses induced the accumulation of LPO products. The higher dose intensified LPO within 2 h after the experiment was started. The LPO product level became still higher in 4 h and reached a value exceeding the initial level by a factor of 1.7 for diene conjugates and 2.1 for diene ketones. The LPO parameters remained markedly elevated to the end of the experimental observation. The lower dose of sphingosine induced similar changes, but the LPO product level returned to normal 6 h after the injection. The mechanism of LPO enhancement by

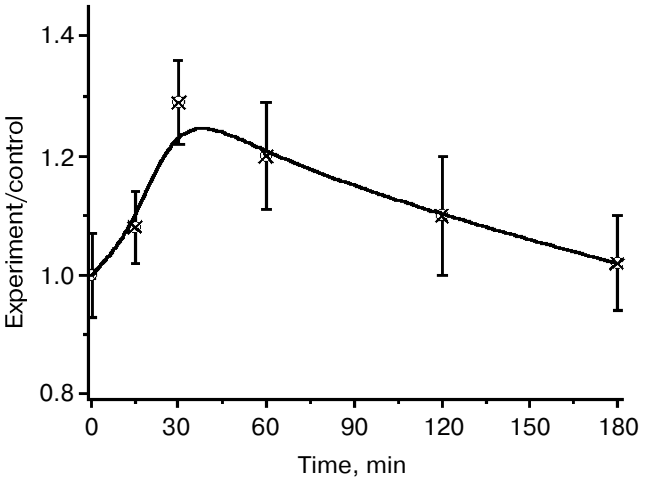

Fig. 3. Effect of intraperitoneally injected bilirubin (1.25 mg per mouse) on lipid content (mg per g tissue) in mouse liver. The data are given in arbitrary units normalized to control.

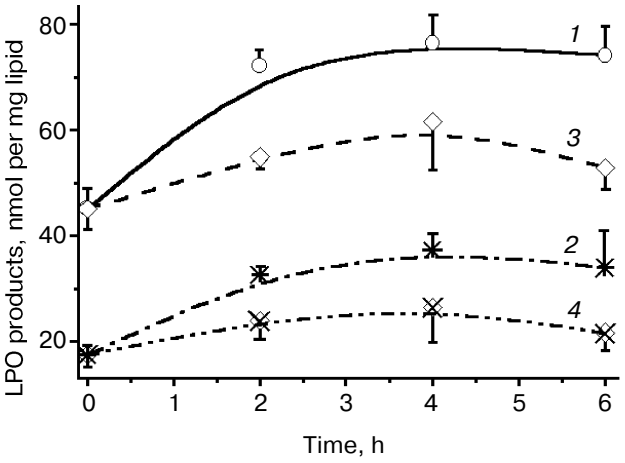

Fig. 4. Influence of two different doses of sphingosine on the contents of lipid peroxidation products in mouse liver: *1* and *2*) diene conjugates and diene ketones (nmol per mg lipid) (sphingosine dose, 20 μg per mouse, intraperitoneally); *3* and *4*) diene conjugates and diene ketones (nmol per mg lipid) (sphingosine dose, 100 μg per mouse, intraperitoneally).

sphingosine is not clear since its molecular structure gives no evidence of possible oxidative properties. However, there are data showing a toxic effect of sphingosine on isolated mitochondria. As this occurs, a steep rise in hydrogen peroxide production is observed [35], and this may enhance lipid peroxidation.

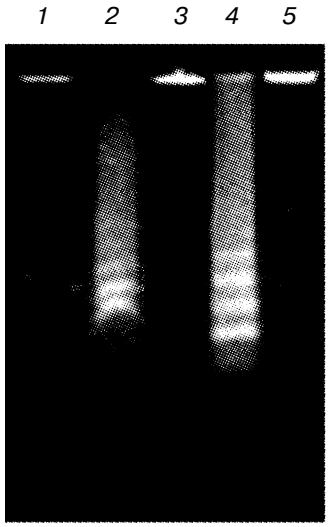

Fig. 5. Effect of bilirubin (100 μM) on apoptosis induced in murine thymocytes *in vitro* by sphingosine (50 μM) or UV irradiation (200 J/m^2) (DNA fragmentation was monitored by gel electrophoresis). Lanes: *1*) control; *2*) sphingosine; *3*) sphingosine + bilirubin; *4*) UV irradiation; *5*) bilirubin added after UV irradiation.

We showed earlier that sphingosine injected in a single dose of 20 μg per mouse induced reversible DNA degradation to low molecular weight fragments in liver, spleen, and thymus [36]. Sphingosine administration (100 μg per mouse) caused more noticeable DNA fragmentation and the distribution shifted to smaller fragments. DNA degradation was most prominent in thymus. Maximal DNA fragmentation was observed in liver 4 h after administration with both sphingosine doses. Thus, time and concentration dependencies of LPO intensification in mouse liver after sphingosine administration coincide with those observed in apoptosis induction by this compound.

Effect of bilirubin on apoptosis induced by sphingosine or UV irradiation. It is now know that oxidative stress plays a role in apoptosis; active forms of oxygen are able not only to trigger the process, but also can be generated during apoptosis induced by other, non-oxidative, stimuli. Antioxidants that protect cells against oxidative stress inhibit apoptosis effectively [3-7]. As indicated above, the activity of neutral sphingomyelinase that plays a key role in apoptosis decreases in various animal organs under the influence of bilirubin. Bilirubin as an antioxidant and inhibitor of sphingomyelinase may influence apoptosis too. This hypothesis was verified using two models, apoptosis induction by direct oxidative action (UV irradiation) and apoptosis induction by the sphingomyelin cycle product sphingosine, whose chemical structure does not suggest pro-oxidant activity.

The experiments were conducted using isolated murine thymocytes. Data on DNA fragmentation from gel electrophoresis are shown in Fig. 5. The simultaneous administration to thymocytes of bilirubin with sphingosine protects the cells adequately against sphingosine-induced apoptosis. Bilirubin added to cells after UV irradiation also inhibited internucleosomic DNA degradation. The pronounced antiapoptotic effect of bilirubin is apparently due to both its antioxidant properties and its influence on sphingomyelinase activity. Our results are consistent with data on a pro-oxidant effect of sphingosine (see above) and with data in the literature on a protective effect of bilirubin against damage caused by UV irradiation [37].

DISCUSSION

The role of bilirubin as one of the most important antioxidants in extracellular liquids such as blood serum is unquestioned. However, the role of this compound in the intracellular system of defense against oxidative damage is debated. Bilirubin content among cell membrane lipids is still unknown. Bilirubin formed in the body passes into the liver, where it conjugates with glucuronic acid and is excreted with bile into the duodenum. This elimination pathway for the initial reduced form is unknown

for any other antioxidant. There is widespread opinion among clinicians that bilirubin is not utilized in the body, and in fact may have a toxic effect, for instance, it may cause central nervous system damage in newborns. Nevertheless, indirect evidence for a possible important role of bilirubin in defense against LPO as an intracellular antioxidant has appeared in recent years. These studies [12, 13, 37] are mainly devoted to the induction of microsomal heme oxygenase, the key enzyme responsible for bilirubin formation, under oxidative stress.

The effect of directly injected bilirubin on LPO activity in animal organs is shown in our experiments. Bilirubin inhibited LPO on the cellular level in heart and kidney of experimental animals as it did in blood serum [8, 9]. This effect was quite short-term due to the fast elimination of excess bilirubin by liver cells. However, in liver bilirubin did not induce any decrease in LPO, but in fact increased LPO intensity. This fact, along with elevation of lipid level, is connected with the characteristics of bilirubin metabolism in this organ, one of the main functions of which is excretion of various substances, including bilirubin and lipids, from the body with bile.

Since bilirubin has antioxidant properties, and oxidative reactions play a substantial role both in induction of heme oxygenase (the enzyme responsible for bilirubin formation) and induction of apoptosis, a relationship between bilirubin metabolism and the systems responsible for apoptosis can be assumed. In spite of the long history of studies on the properties of sphingomyelinases and the influence of various factors on their activities, the fact that the activities of some sphingomyelinases are connected with the cell oxidative system has been established only in the last three years. It has been shown in a few investigations made on the cell level or on the isolated enzyme that neutral sphingomyelinase activity depends on glutathione, the most important intracellular antioxidant [22-25]. Both reduced and oxidized glutathione were found to inhibit neutral Mg^{2+}-dependent sphingomyelinase with γ-glutamyl-residue of glutathione responsible for the effect. That is, the inhibitory effect of glutathione on sphingomyelinase is probably not due to its antioxidant properties. The inhibition of this enzyme by glutathione was reversible and did not result from any irreversible changes in sphingomyelinase. Cell treatment with inhibitors of glutathione synthesis was accompanied by enhanced sphingomyelin hydrolysis and ceramide production.

There are various contradictory data in the literature related to the influence of other antioxidants on the activity of the enzyme. Particularly, simultaneous inhibition of LPO, neutral sphingomyelinase, and apoptosis by ascorbate, tocopherol, or coenzyme Q was found during induction of apoptosis in cells deprived of serum [7]. The synthetic antioxidant ionol and superoxide dismutase were shown to prevent apoptosis induced by sphingomyelinase activation caused by oxidized low-density lipoproteins [18]. SH-bearing antioxidants, such as N-acetyl-cysteine and pyrrolidine dithiocarbamate, also inhibit both apoptosis and neutral sphingomyelinase [5]. However, the absence of any effect of antioxidants other than glutathione was shown in a study on apoptosis induced by hypoxia [24]. All the works cited were performed on cell cultures.

The changes in LPO product contents mimicked the changes in neutral sphingomyelinase activity in all the organs studied in both experimental and control animals.

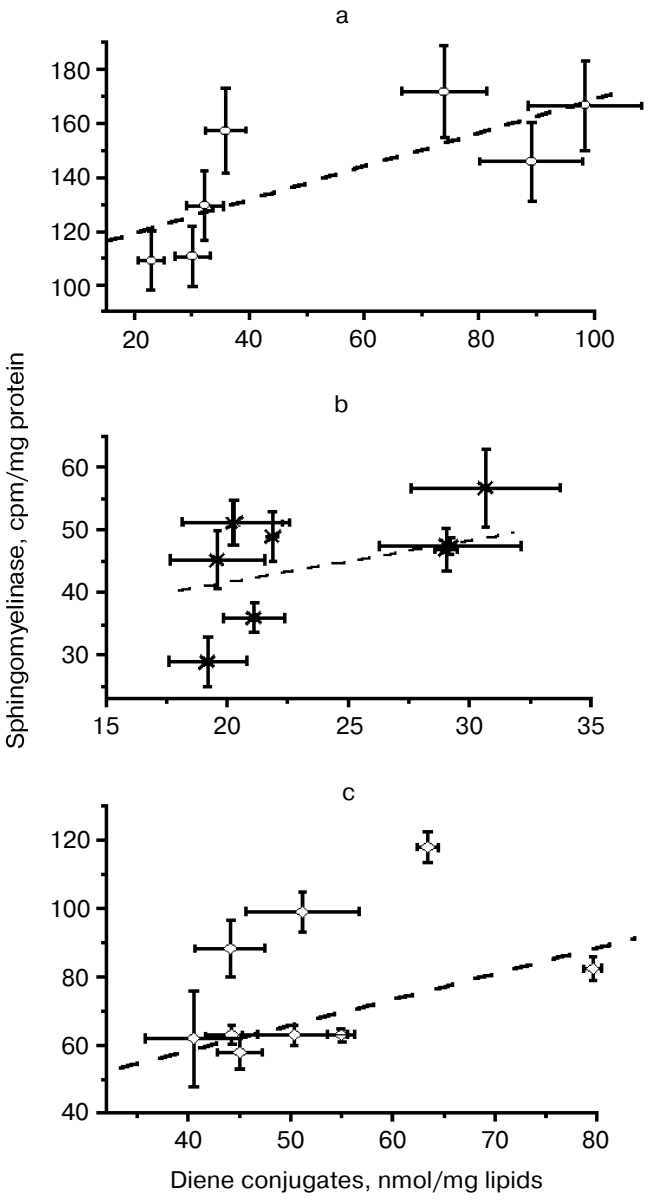

Fig. 6. Relationship between changes in LPO product contents (diene conjugates, nmol per mg lipid) and neutral sphingomyelinase activity (cpm per mg protein) in mouse heart (a), kidney (b), and liver (c) after intraperitoneal injection of bilirubin at dose 1.25 mg per mouse ($R = 0.70$ (a), 0.49 (b), and 0.58 (c); $p < 0.05$ (a, c), $p < 0.09$ (b)).

Figure 6 shows the relationship between these two parameters with high linear correlation coefficients of +0.70 in heart, +0.49 in kidney, and +0.58 in liver ($p < 0.05$). The revealed phenomenon is itself necessary but insufficient to conclude that neutral sphingomyelinase activity depends on antioxidant levels in cells. For instance, the possibility of a specific interaction between bilirubin and sphingomyelinase (as in the case of glutathione) cannot be completely excluded. Further studies are needed to determine the effects of other antioxidants on sphingomyelinase and also to investigate relations between LPO and sphingomyelinase activity in pathologies differing in the intensity of oxidative processes. However, to summarize the data of the literature and the results of the present paper, a dependence of sphingomyelinase activity on LPO intensity and, as a consequence, on LPO product contents, seems very probable.

In spite of an apparent effect of reduced glutathione on the contents of other antioxidants and LPO intensity in cells, inhibition of neutral sphingomyelinase by glutathione has specific features that are not related to its antioxidant properties. This is evident from both the effect of glutathione on the purified enzyme and the fact that the γ-glutamyl-, but not the SH-group, of glutathione is active. Other antioxidants, particularly bilirubin, may influence the enzyme activity through the reduction of a free-radical oxygen form or oxidation product levels and also through changes in lipid composition or other physicochemical membrane properties, but not through the modulation of glutathione level in the cell.

The data from the literature concerning the influence of sphingomyelinase and products of the sphingomyelin cycle on LPO processes and glutathione level are very conflicting. Various authors studying the effect of exogenous sphingomyelinase on various cell cultures have obtained contrasting results. Exogenous bacterial neutral sphingomyelinase was shown to increase intracellular glutathione content in cultivated hepatocytes, whereas the acidic form of the enzyme from human placenta, which hydrolyzes sphingomyelin to the same extent as the neutral enzyme, reduced glutathione content [38]. Other authors [6] revealed a sharp elevation of active oxygen forms and NO under the influence of bacterial sphingomyelinase. Antioxidants, such as tocopherol and N-acetylcysteine, prevented the NO accumulation induced by sphingomyelinase. The treatment of cells with bacterial sphingomyelinase or ceramides does decrease the reduced glutathione level [25], which in turn does not protect cells from apoptosis induced by ceramides. The seemingly contradictory data are probably due to the variety and specificity of cell cultures and sphingomyelinases studied.

Our present data and the data of [36] demonstrate that apoptosis induced by a single injection of sphingosine (a product of the sphingosine cycle) into experimental animals is accompanied by prominent intensification of LPO reactions in mouse liver. There are numerous confirmations in the literature of the protective role of antioxidants against apoptosis; however, we could not find any data on the protective function of bilirubin against apoptosis. The inhibition of either UV irradiation- or sphingosine-induced apoptotic DNA degradation by bilirubin shown in our study may be useful in tactical developments for the treatment of diseases accompanied by jaundice. Particularly, hyperbilirubinemia may seriously affect the effectiveness of cancer therapy in patients with tumors in the hepatic-pancreatic-duodenal region. The treatment of such patients (radio-, chemo-, or photodynamic therapy) is induction of oxidative stress that in turn leads to apoptotic tumor cell death. Antiapoptotic and antioxidant properties of bilirubin may decrease the effectiveness of treatment. However, jaundice in viral hepatitis may be considered as a favorable response toward protection of the body from intoxication and the stabilization of hepatocytes [8]. It is theorized also that jaundice in newborns is a manifestation of a fetal adaptation to hyperbaric stress that takes place under the changing of partially anaerobic conditions in the uterus to the conditions of atmospheric oxygen [39]. Induction of heme oxygenase activity resulting in fast degradation of erythrocyte heme from the mother's blood to bilirubin, which in turn begins to fulfill the role of endogenous quencher of active oxygen forms generated during hyperbaric stress, is a protective biochemical reaction directed against the stress.

In spite of substantially elevated interest in bilirubin in the last 15 years and a large number of studies, the mechanism of the effect of bilirubin on cells is not well understood. Our data demonstrate that bilirubin probably plays a role in sphingomyelinase inhibition and so influences apoptotic cell death apart from the direct antioxidative effect in free radical oxidation and membrane lipid protection. The phenomena revealed here, such as a dependence of sphingomyelinase activity on LPO intensity and the effect of bilirubin both on LPO and the signaling system of the sphingomyelin cycle and apoptosis may be useful for more complete understanding of important processes such as cell proliferation and death.

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