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Role of Tumor Necrosis Factor Alpha and Sphingomyelin Cycle Activation in the Induction of Apoptosis by Ischemia/Reperfusion of the Liver

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Abstract—The signal transduction pathways triggering apoptotic mechanisms after ischemia/reperfusion may involve TNF-α secretion, ceramide generation, and initiation of lipid peroxidation. In the present study involvement of the TNF-α, sphingomyelin cycle, and lipid peroxidation in the initiation of apoptosis induced in liver cells by ischemia and reperfusion was investigated. Wistar rats were subjected to total liver ischemia (for 15, 30 min, and 1 h) followed by subsequent reperfusion. Ischemia caused sharp decrease of neutral sphingomyelinase activity. Activity of acidic sphingomyelinase initially decreased (during 15-30 min ischemia) but then increased (after 1 h of ischemic injury). Reperfusion of the ischemic lobe of the liver caused increase in neutral sphingomyelinase activity and decrease in acidic sphingomyelinase activity. A small amount of TNF-α detected by immunoblotting analysis was accumulated in the ischemic area of liver rapidly and the content of this cytokine dramatically increased after the reperfusion. TNF-α is known to induce free radical production. We found that the accumulation of TNF and increase of sphingomyelinase activity during the development of ischemic/reperfusion injury coincided with increase in content of lipid peroxidation products (conjugated dienes) and DNA degradation detected by gel electrophoresis. Recently it was shown that superoxide radicals are used as signaling molecules within the sphingomyelin pathway. This suggests the existence of cross-talk between the oxidation system and the sphingomyelin cycle in cells, which may have important implications for the initial phase and subsequent development of post-ischemic injury.

Key words: ischemia/reperfusion, tumor necrosis factor alpha, sphingomyelinase, sphingomyelin, sphingomyelin cycle, lipid peroxidation, apoptosis

Ischemia (or oxygen deficiency) of tissues is observed in myocardial infarction, stroke, and during surgical operations on the liver and other organs. Tissue damage occurs due to apoptotic cell death. Signaling pathways responsible for triggering apoptotic mechanisms after ischemia/reperfusion may involve secretion of tumor necrosis factor-α (TNF-α) [1], stimulation of lipid peroxidation [2], and the sphingomyelin cycle [3, 4]. However, in the ischemic zone these signaling systems are usually studied separately and these studies have been mainly focused on myocardial [4], brain [5], and kidney cells [3]. There are no indications on the activation of the sphingomyelin cycle and the expression of TNF-α in the liver subjected to ischemia/reperfusion. Lack of such information (and particular interest in the liver as a research object) may be explained by weak induction of apoptosis in liver cells by TNF-α [6]. (It should be noted that Kupffer cells can synthesize this cytokine [7].) Induction of apoptosis in liver requires not only TNF-α, but also inhibitors of RNA or protein synthesis [6]. However, in the damaged liver unable for regeneration (protein synthesis suppressed after 80% hepatectomy) cell death involves both apoptosis and necrosis. The remaining part of the liver is characterized by significant accumulation of TNF-α [8]. Co-administration of TNF-α and sphingosine, a product of sphingomyelin hydrolysis, induces apoptosis in liver cells [9, 10]. Taking into consideration these data we suggest that ischemia/reperfusion of the liver may cause accumulation of TNF-α and activate the sphingomyelin cycle. The key event of this cycle consists in enzymatic cleavage of sphingomyelin and formation of ceramide and phosphocholine. Ceramide can be further cleaved by ceramidase with sphingosine formation. The sphingomyelin cycle together

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with the phosphatidylinositide cycle generate the second messengers responsible for signal transduction from plasma membrane into nucleus [11, 12]. Ceramide and sphingosine may mimic effects of cytokines, lipopolysaccharide, and other biologically active molecules on the terminal cell response: differentiation, apoptosis, or arrest of the cell cycle [11]. It was also shown that sphingosine exerts a synergic effect in combination with various preparations including TNF-α [13, 14].

Sphingomyelinase (sphingomyelin hydrolase, EC 3.1.4.12) is the main enzyme of sphingomyelin cycle catalyzing sphingomyelin cleavage to ceramide and phosphocholine. This enzyme has been found in all cells; however, the highest amounts of sphingomyelinase are found in brain cells (in myelin) [15]. There are eight types of sphingomyelinase which differ in intracellular localization, pH optimum, cation requirement, and the role in the cell regulation [16]. The sphingomyelin cycle signaling system interacts with other signaling systems including reactive oxygen species and NO [17]. Such interaction may potentiate toxic effects of cytokines on liver cells. (Oxidative stress induced by TNF-α is the key event of the cytotoxic effect of this cytokine [18].) Certain evidence exists that sphingomyelinase activation depends on the intensity of oxidative processes in the cell [19, 20], with regulation of this enzyme by natural antioxidants (glutathione and bilirubin) [21, 22]. So, "triple" study of TNF-α, lipid peroxidation, and the sphingomyelin cycle would allow better characterization of mechanism(s) inducing apoptosis in liver cells subjected to ischemia/reperfusion injury.

In the present study we have investigated the interrelationship between expression of TNF-α with activation of sphingomyelin cycle and lipid peroxidation in liver after ischemia/reperfusion injury.

MATERIALS AND METHODS

Liver ischemia/reperfusion modeling in rats. Wister rats (250-300 g) fasted overnight were used in experiments. Animals were anesthetized with intravenous injection of briethal (50 mg per kg). After median laparotomy liver ischemia (~40% of the organ) was achieved by application of microclip on the basis of left lateral lobe with transparenchymatous clenching of the vascular stem. During the period of ischemia the wound was covered with aseptic dressing. After the period of ischemia (which varied from 15 to 60 min) the microclip was removed and reperfusion restored (for 5-60 min). After the reperfusion rats were decapitated and their livers were perfused, dissected, and homogenized. Three animals were used for each experimental point and corresponding control. Experiments were repeated twice. Livers of sham operated rats subjected to the same treatments (anesthetic administration, laparotomy, manipulations on left lateral lobe) except microclip application were used as controls.

Detection of TNF-α in the liver was carried out by ECL immunoblotting using polyclonal antibodies (Santa Cruz, USA) that specifically bind mouse and rat TNF-α.

Proteins of liver homogenate were separated by electrophoresis in 13% polyacrylamide gel [23] and transferred onto nitrocellulose filters. Recombinant mouse TNF-α obtained from the laboratory of V. G. Korobko (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences) was used as control. Nonspecific sorption of protein was prevented by preincubation of membranes in TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 1% BSA. After such pretreatment nitrocellulose strips were incubated with antibodies against TNF-α, washed with 0.1% Tween-20 in TBS and then incubated with horseradish peroxidase conjugated with goat anti-rabbit immunoglobulins. Detection of antibody binding to TNF-α was carried out using non-radioactive ECL reagent (Amersham Life Sciences, England).

Determination of sphingomyelinase activity. The activity of neutral and acidic sphingomyelinases was determined in the liver after ischemia/reperfusion injury as described by Hosteler et al. [24] using [N-methyl-^{14}C]sphingomyelin (specific activity 58 mCi/mmol). The reaction mixture (final volume 0.8 ml) for neutral sphingomyelinase contained 50 mM Tris-HCl buffer, pH 7.2, 10 mM $MgCl_2$, 1 mM EDTA, 0.25% Triton X-100, 50 μM labeled sphingomyelin (the ratio labeled/non-labeled sphingomyelin was 1 : 10), and 2-3 mg of liver homogenate protein. In the case of acidic sphingomyelinase the reaction mixture contained the same reagents but citrate buffer (pH 5.0) was used instead of Tris buffer.

Sphingomyelinase activity was evaluated by accumulation of water soluble reaction product, [^{14}C]phosphocholine, in the water–methanol phase. Radioactivity was counted using an aliquot of upper, water–methanol phase and a Delta counter (The Netherlands). Specific activity was expressed per mg protein.

Lipids were extracted by the method of Bligh and Dyer [25] and their content was determined gravimetrically.

Protein content was determined by the Lowry method [26].

Determination of products of lipid peroxidation (LPO)—conjugated dienes and ketodienes. Primary (conjugated dienes) and secondary (ketodienes) LPO products are characterized by absorbance maxima at 233 and 270 nm, respectively. Extracted hepatic lipids were dissolved in a mixture methanol–hexane (5 : 1 v/v); their concentration varied from 0.2 to 1.0 mg per ml. Electronic spectra of these solutions were registered using a Beckman (USA) spectrophotometer in the range of wavelength from 210 to 290 nm. The amount of LPO products (per mg lipids) was calculated using molar absorbance coefficients 21,000 and 23,000 $M^{-1}\cdot cm^{-1}$ for conjugated dienes and ketodienes, respectively [27].

Detection of apoptosis in the liver after ischemia/reperfusion. The fraction of low molecular weight DNA was isolated as described in [28]. Cells were lysed in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.2% Triton X-100, and centrifuged at 13,000*g*. The supernatant was mixed with ethanol and sodium acetate (final concentrations of these reagents were 67% and 0.3 M, respectively) and this mixture was incubated overnight at −18°C. The resultant mixture was centrifuged at 13,000*g*; the sediment was dried under aerobic conditions and the residue was resuspended in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, and mixed with buffer (10 : 1 v/v) containing 25% Ficoll 400, 5% SDS, 0.25% bromophenol blue, and RNase A (final concentration 0.1 mg/ml). The resultant mixture was incubated at 37°C for 1 h, mixed with proteinase K (final concentration 0.1 mg/ml) and incubated again at 37°C for 1 h.

The isolated low molecular weight DNA was analyzed by horizontal electrophoresis in 1.5% agarose gel. The latter was prepared using 0.5 M TBE buffer (0.045 M Tris, pH 8.0, 0.045 M boric acid, 1 mM EDTA). Electrophoresis was carried out in the same buffer at 4°C for 9 h using a GNA-200 (Pharmacia, Sweden) and voltage of 2 V/cm.

Sphingomyelin was determined by thin layer chromatography using silica gel coated plates Merck (Germany) and subsequent densitometry.

The results were treated by methods of variation statistics. Differences were considered as statistically significant at $p \leq 0.05$. Data presented on figures represent means (±SEM) of parameters of experimental and control animals. Sham operation corresponding to the duration of ischemia/reperfusion treatment did not influence the parameters studied which were in the range of values obtained for intact liver.

RESULTS

Effect of ischemia/reperfusion of the liver on the activity of sphingomyelinase. Ischemia is characterized by reduced or totally absent blood flow. In our experiments we have investigated the activity of neutral and acidic sphingomyelinase in ischemic lobe of liver and the effect of subsequent reperfusion.

Ischemia for 15 min had minor effect on the activity of neutral sphingomyelinase. Reduced enzyme activity was found after 30 min of ischemia and remained at this level for up to 60 min of ischemia (Fig. 1, curve *1*). Reperfusion for 30 min (after 30 min ischemia) normalized the enzyme activity (Fig. 2, curve *1*). In the case of longer ischemia (for 60 min) reperfusion caused significant 1.5-fold activation of sphingomyelinase (compared with the enzyme in ischemic liver) which even exceeded control values after 10 min of reperfusion (Fig. 2, curve *2*).

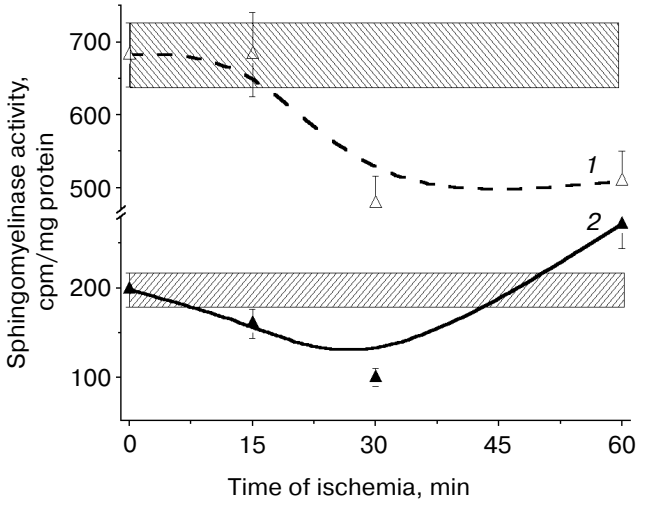

Fig. 1. Effect of ischemia on the activity of hepatic neutral (*1*) and acidic (*2*) sphingomyelinase. Here and in subsequent figures hatched regions indicate the range of parameters studied in control (sham operated) animals.

Thus, ischemia caused decrease of neutral sphingomyelinase activity, whereas reperfusion activated the enzyme.

During ischemia the activity of acidic sphingomyelinase demonstrated biphasic behavior; the enzyme activity decreased after ischemia for 30 min but longer ischemia (for 60 min) produced an opposite effect (Fig. 1, curve *2*). This suggests that prolonged ischemia results in activation of the lysosomal form of this enzyme which cleaves intracellular sphingomyelin. However, intracellular sphingomyelin content represents not more than 10%

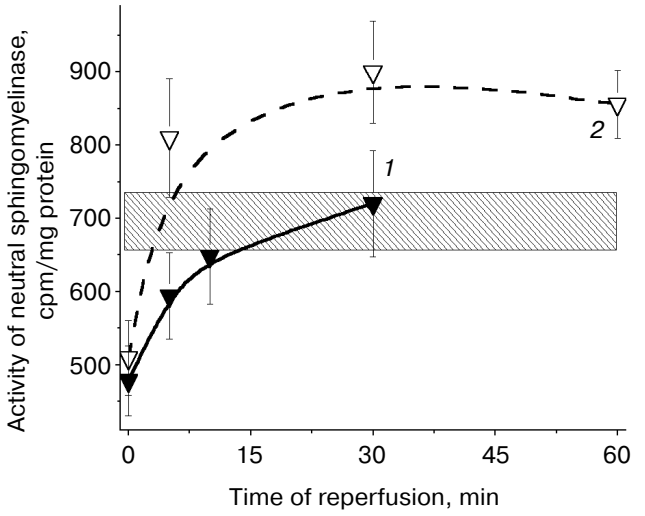

Fig. 2. Effect of reperfusion on the activity of neutral sphingomyelinase after hepatic ischemia for 30 (*1*) and 60 (*2*) min.

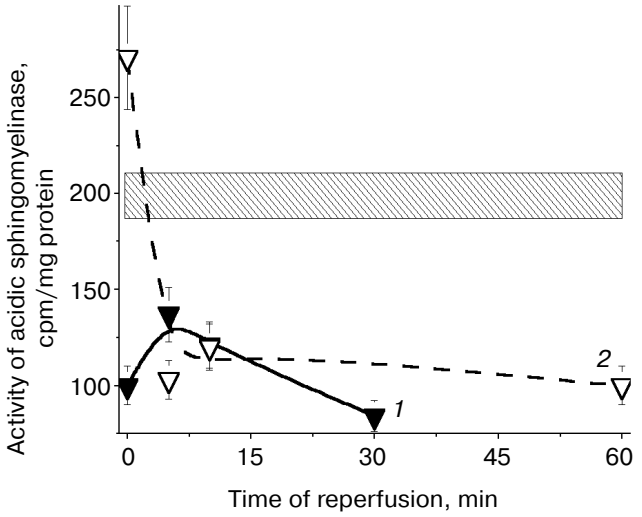

Fig. 3. Effect of reperfusion on the activity of acidic sphingomyelinase after hepatic ischemia for 30 (*1*) and 60 (*2*) min.

of total cellular sphingomyelin (the major proportion of sphingomyelin is localized in the plasma membrane). Reperfusion after 30 min ischemia did not normalize sphingomyelinase activity (Fig. 3, curve *1*), whereas reperfusion of the liver after longer ischemia (for 60 min) was accompanied by a decrease in sphingomyelinase activity which was noted after 10 min of reperfusion (Fig. 3, curve *2*).

The ischemia for 30 min was accompanied by marked increase in sphingomyelin proportion in phospholipid fractions (Fig. 4a). However, the amount of total phospholipids in the ischemic liver was lower than in control (Fig. 4b): after ischemia for 60 min the amount of phospholipids was 1.5-fold less than in control. Reduction of total phospholipid content is often seen in ischemic organs; this reduction is attributed to activation of phospholipase A_2 [29]. In contrast to other phospholipids, the absolute content of sphingomyelin (per g protein) remained unchanged during ischemia. This is consistent with ischemia-induced inhibition of the sphingomyelin degrading enzyme, sphingomyelinase, and reduction of synthetic potential of cells due to energy deficit under ischemic conditions.

Effect of ischemia/reperfusion on TNF-α content in liver. We failed to detect TNF-α in the liver of sham operated rats. However, after short-term ischemia (for 15 min) the content of TNF-α was 2.54 μg per g of total protein. Prolonged ischemia (for 60 min) caused almost double increase in TNF-α content (Fig. 5). Such rapid accumulation of TNF-α in the ischemic lobe of the liver may be attributed to lack of TNF-α transportation to other organs due to interrupted blood flow. However, reperfusion for 5, 15, and 30 min after ischemia (for 30 min) caused further increase in TNF-α in the ischemic zone up to maximal values. The level of TNF-α after reperfusion for 15 min was more than two times higher than in the ischemic lobe. It is possible that restored blood circulation brings a new portion of TNF-α to the reperfused liver lobe and TNF-α produced by this lobe is associated with its receptors and cannot be transported from the liver. It is also possible that ischemic liver is characterized by increased amount of TNF-α receptors. In this case the new portion of TNF-α may interact with these receptors. Reperfusion of the hepatic lobe subjected to prolonged ischemia for 60 min did not influence high content of TNF-α which insignificantly differed from the values observed in the ischemic lobe before reperfusion. Long-term ischemia (for 60 min) caused profound changes of blood vessels of the ischemic lobe (microscopic observation) and reperfusion was less effective than that after

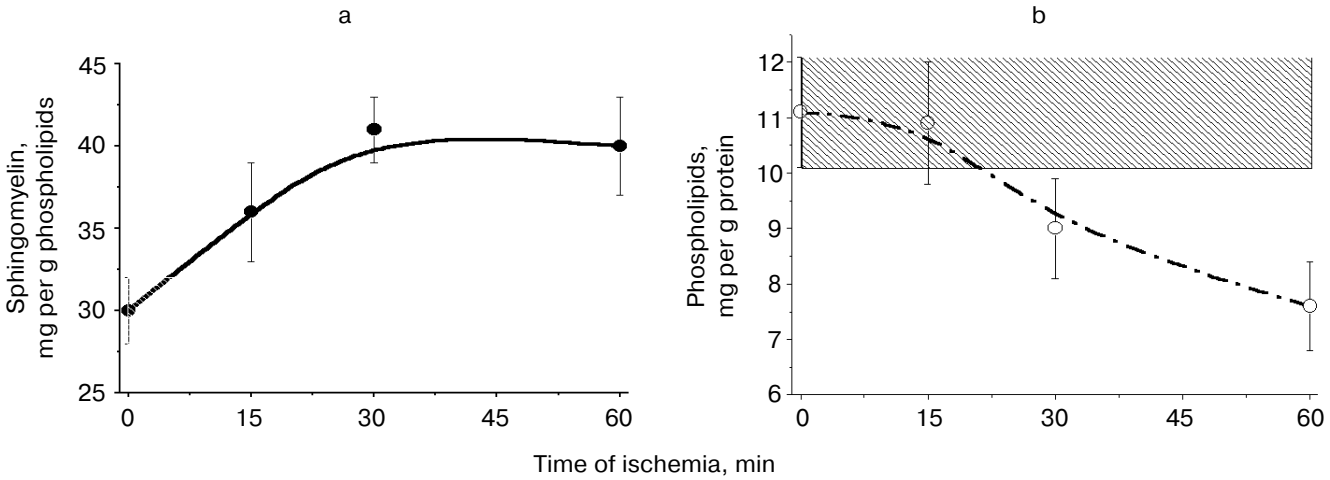

Fig. 4. Effect of ischemia on hepatic content of sphingomyelin (a) and total phospholipids (b).

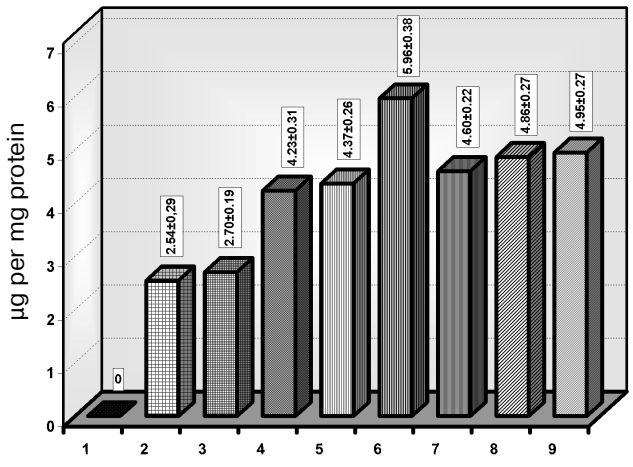

Fig. 5. Effect of ischemia/reperfusion of the liver on the content of TNF-α: *1*) sham operated control; *2-4*) ischemia for 15 (*2*), 30 (*3*), and 60 min (*4*); *5-9*) reperfusion for 5 min after ischemia for 30 min (*5*), for 15 min after ischemia for 30 min (*6*), for 30 min after ischemia for 30 min (*7*), for 30 min after ischemia for 60 min (*8*), and for 60 min after ischemia for 60 min (*9*).

shorter ischemia (for 30 min). This may explain lack of changes in TNF-α after long-term ischemia followed by reperfusion.

Thus, results of these experiments demonstrate increase in hepatic TNF-α content which depended on the duration of the ischemic period. Reperfusion did not decrease its content in the ischemic zone.

Effects of ischemia/reperfusion on LPO products. Changes in the content of primary (conjugated dienes) and secondary (ketodienes) LPO products reflect altered balance between pro- and antioxidant systems in the ischemic organ. Ischemia/reperfusion injury is known to be characterized by significant changes in the content of lipids, proteins, and nucleic acids. Long-term ischemia (for 60 min) caused 1.5-fold reduction of the ratio total lipids: protein (Fig. 6a). Reperfusion influenced this parameter, and the effect depended on duration of ischemia and reperfusion. Short-term ischemia (up to 30 min) insignificantly influenced lipid/protein ratio and after reperfusion for 30 min this value was within the control range (Fig. 6b, curve *1*). However, reperfusion of hepatic lobe subjected to long-term ischemia (60 min) was accompanied by stable increase in lipid content in the ischemic zone (Fig. 6b, curve *2*).

We have calculated the content of conjugated dienes and ketodienes per mg protein and per mg lipids. Figure 7 shows the effect of ischemia (from 15 to 60 min) on the content of conjugated dienes (curve *1*) and ketodienes (curve *2*) in the ischemic lobe. The results expressed per mg lipids clearly demonstrate increase in accumulation LPO products observed in all periods of ischemia (Fig. 7a). However, when the amount of LPO products was expressed per mg protein the content of conjugated dienes remained unchanged over all periods of ischemia (Fig. 7b, curve *1*), whereas an increase in ketodienes was noted only after short-term ischemia (15-30 min) and the content of ketodienes returned to the control value after long-term ischemia (for 60 min) (Fig. 7b, curve *2*).

These data suggest activation of LPO during ischemia. More pronounced accumulation of ketodienes than conjugated dienes may be attributed to further decay of conjugated dienes or their subsequent involvement into oxidative processes. However, irrespectively to the mode of calculation the present study revealed accumulation of ketodienes during short-term (within 30 min) ischemia and "normalization" of this parameter after long-term ischemia. Reduction of LPO products seen in lipids of the ischemic lobe may be explained by various reasons such as

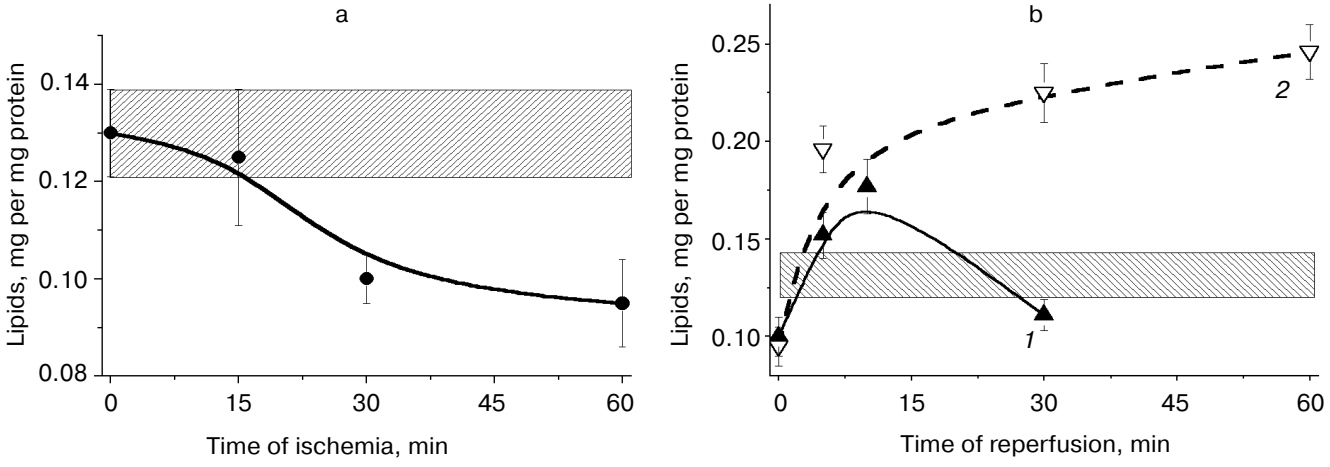

Fig. 6. Effect of ischemia (a) and reperfusion after 30-min (*1*) and 60-min (*2*) ischemia (b) on lipid content.

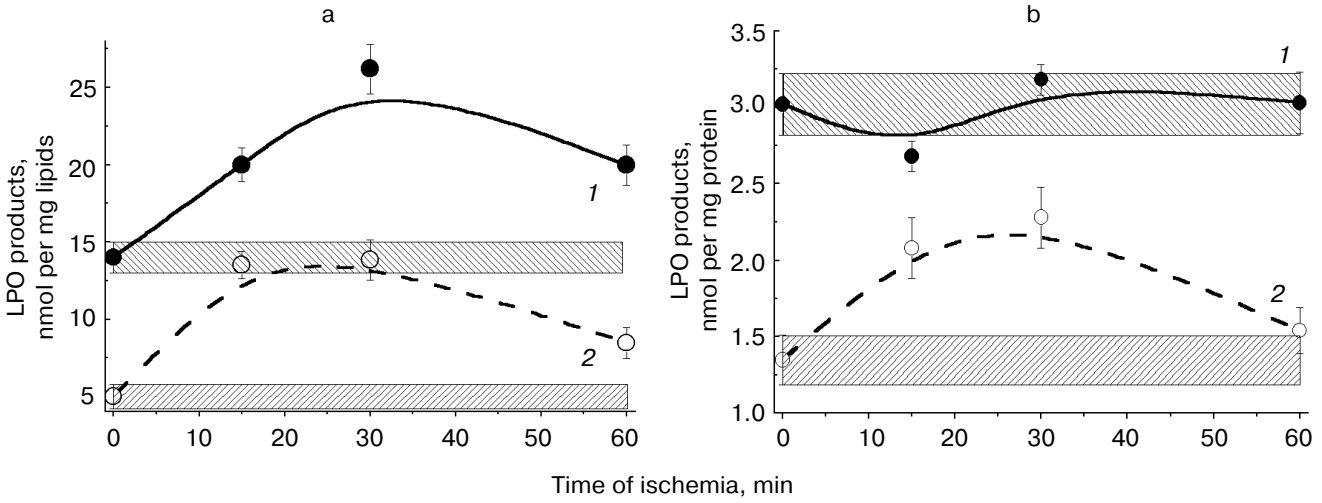

Fig. 7. Effect of ischemia on the content of LPO products expressed in nmol per mg lipids (a) and nmol per mg protein (b): *1*) conjugated dienes; *2*) ketodienes.

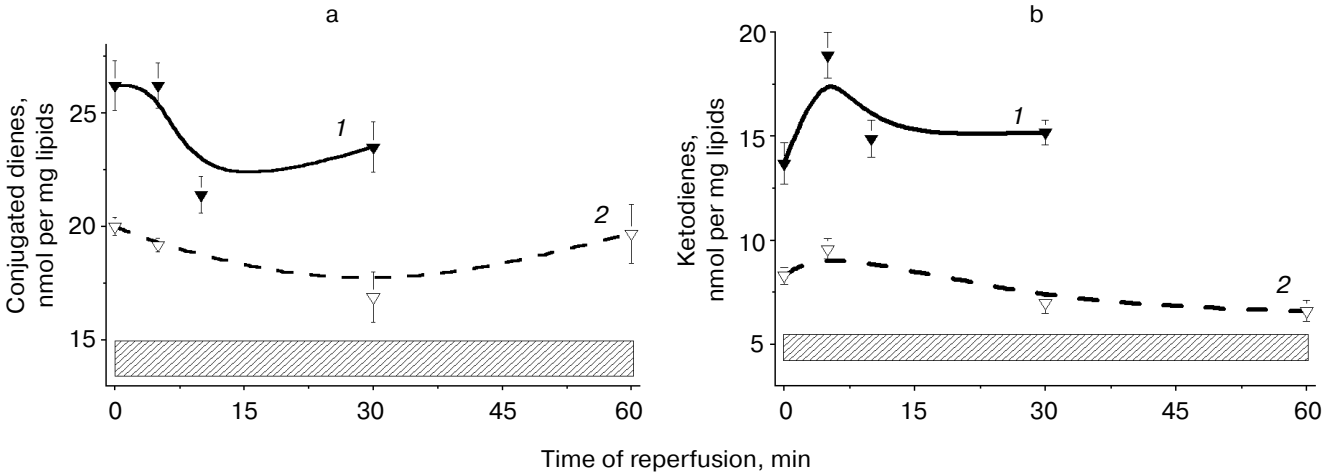

Fig. 8. Effect of hepatic reperfusion after ischemia for 30 min (*1*) and 60 min (*2*) on the content of conjugated dienes (a) and ketodienes (b) expressed in nmol per mg lipids.

depletion of the oxidative substrates (polyunsaturated fatty acids) and oxygen deficit. Unchanged amount of conjugated dienes in the ischemic lobe (expressed per mg protein) may be attributed to significant reduction of lipid content and more stable protein content. So correct comparison of literature data on the amount of LPO products in an injured organ requires analysis of methods employed for calculations.

Early periods of liver reperfusion after ischemia for 30 and 60 min were characterized by some decrease in the content of conjugated dienes (expressed per mg lipids) (Fig. 8a) and increase in ketodienes in the ischemic zone. Subsequently, the content of conjugated dienes in lipids increased whereas ketodienes decreased. However, in all periods of reperfusion both parameters significantly exceeded control levels. Relatively small changes in the content of LPO products in the ischemic lobe after the restoration of blood flow may reflect an increase in lipid content (see Fig. 6).

However, when the amount of LPO product was expressed per mg protein we found that reperfusion of liver after short-term ischemia (for 30 min) normalized the parameters 30 min after the restoration of blood flow. However, the time course of reperfusion after long-term ischemia (for 60 min) was accompanied by stable increase in both LPO products studied (Fig. 9). The highest (1.8-

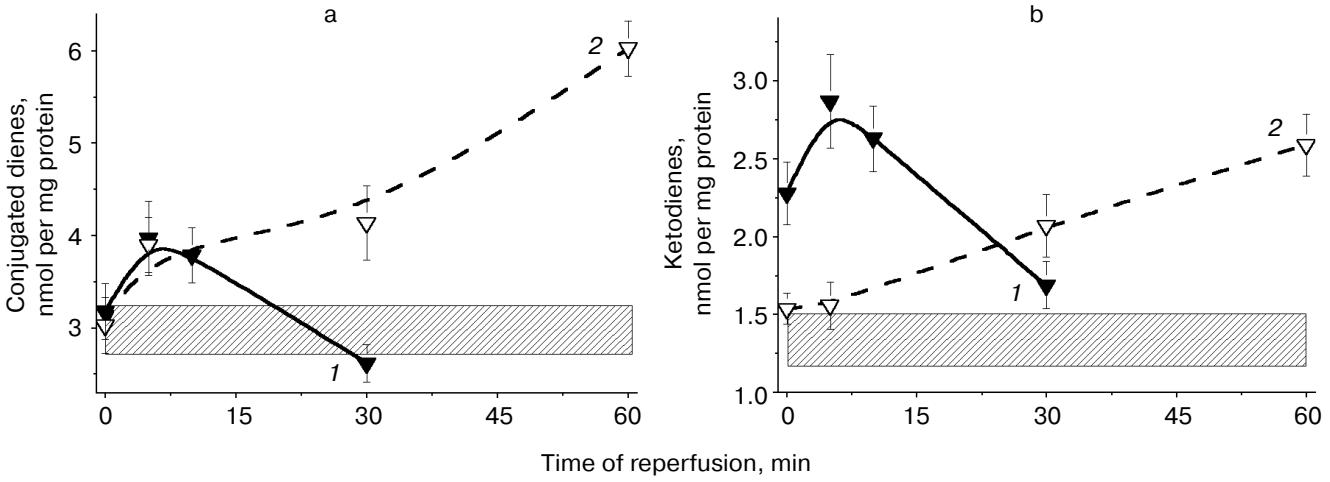

Fig. 9. Effect of hepatic reperfusion after ischemia for 30 min (*1*) and 60 min (*2*) on the content of conjugated dienes (a) and ketodienes (b) expressed in nmol per mg protein.

2-fold) increase in LPO products was observed 60 min after restoration of blood flow in the hepatic lobe subjected to prolonged ischemia (for 60 min). It should be noted that such long-term ischemia is characterized by high lethality of animals (up to 80%) [30]. It is possible that the increase in LPO products during reperfusion after long-term ischemia reflects the development of fatally irreversible processes leading to lethal intoxication.

Effect of ischemia/reperfusion on DNA degradation. It is well recognized now that ischemia/reperfusion injury is characterized by apoptosis, the programmed cell death. Internucleosomal degradation of DNA is one of the most typical signs of apoptosis. Analysis of low molecular weight DNA isolated from the hepatic lobe subjected to long-term ischemia followed by subsequent reperfusion (for 15-60 min) revealed that during the ischemic period DNA is quite stable and its fragmentation begins after onset of reperfusion (Fig. 10). Prolonged reperfusion (for 60 min) caused the appearance of DNA fragments representing the typical apoptotic "ladder". Oxidative stress induced by restoration of blood flow in the ischemic organ is accompanied by apoptotic events. It is thought that such process may be useful for the ischemic organ because it helps to eliminate damaged cells and supply surviving cells with useful building blocks released from apoptotic cells.

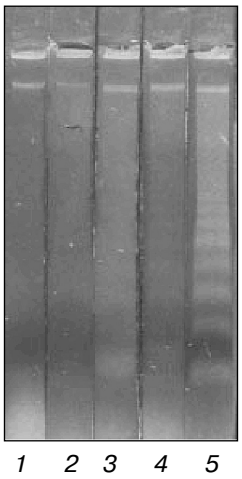

Fig. 10. Effect of ischemia/reperfusion of the liver on the induction of apoptosis (DNA fragmentation): *1*) sham operated control; *2*) ischemia for 60 min; *3*) reperfusion for 15 min after ischemia for 60 min; *4*) reperfusion for 30 min after ischemia for 60 min; *5*) reperfusion for 60 min after ischemia for 60 min.

DISCUSSION

Ischemia is a typical kind of hepatic damage seen in various poisonings and surgical operations (including liver transplantation) and therefore knowledge of precise biochemical mechanisms responsible for cell death would allow finding new strategies for prevention or attenuation of ischemic damage.

Certain evidence exists in the literature that impairment of hepatic microcirculation influences production of various cytokines including TNF-α [1]. In the intact liver Kupffer cells (macrophages fixed in the liver) synthesize TNF-α, which is released into blood circulation and transported to other organs and tissues [7]. We have found that accumulation of TNF-α in the ischemic zone is the first significant event after onset of ischemia; reperfusion of the ischemic lobe significantly increased its con-

tent. Rapid accumulation of TNF-α seen after 15 min of ischemia may be attributed to impossibility of its transportation from Kupffer cells due to termination of blood circulation. Sharp increase of TNF-α during reperfusion may be explained by increased delivery of TNF-α from other organs synthesizing this cytokine and possible increase in number or affinity of its receptors in the ischemic zone.

Cytotoxic effects of TNF-α are realized by oxidative stress (i.e., imbalance between formation of reactive oxygen species and antioxidant defense systems) and stimulation of the sphingomyelin cycle. The latter involves activation of sphingomyelinase, the enzyme catalyzing sphingomyelin cleavage, which is accompanied by accumulation of reaction product, ceramide.

Cell death seen during ischemia/reperfusion injury occurs via apoptosis [1]. However, mechanisms of apoptosis triggering in ischemic organs are not completely understood. Some authors suggest involvement of products of the sphingomyelin cycle, ceramide and sphingosine, as proapoptotic agents which influence activity of key enzymes of apoptosis (caspases, kinases, etc.) [31, 32]. However, in these *in vitro* studies heart [33], brain [5] or kidney [3, 34] cells were used. The role of TNF-α and the sphingomyelin cycle is the best studied in ischemia/reperfusion injury of the heart [4, 35-40]. In agreement with our results, it was shown that ischemia/reperfusion is accompanied by activation of sphingomyelinase, and accumulation of the reaction products of sphingomyelin degradation (ceramide and sphingosine) [4, 35]. It is generally accepted that TNF-α plays the key role in these processes [36-40].

We have also found that accumulation of TNF-α precedes activation of both neutral and acidic sphingomyelinases in the ischemic zone. This suggests that TNF-α is responsible for activation of the sphingomyelin cycle during ischemia/reperfusion. A possibility of involvement of TNF-α and products of the sphingomyelin cycle in the development of apoptosis in liver after ischemia/reperfusion injury has not been widely investigated. Lack of much interest in this problem can be explained resistance of hepatic cells to apoptotic effect of TNF-α and therefore many researchers do not consider TNF-α as the critical agent responsible for induction of apoptosis in the ischemic liver. The actual reason for potentiation of toxic effect of endogenous TNF-α in the liver remains unknown. Results of the present report suggest that activation of the sphingomyelin cycle followed by accumulation of ceramide and sphingosine is one of the key events involved in transduction of apoptotic signal induced by TNF-α in other cells. Two isoforms of the key enzyme of the sphingomyelin cycle, acidic sphingomyelinase and Mg^{2+}-dependent neutral sphingomyelinase, play an important role in proliferation and apoptosis [11, 15]. Study of subcellular distribution of sphingomyelinases revealed that acidic sphingomyelinase is located in lysosomes. This enzyme has optimum pH at 4.4-4.8. Neutral sphingomyelinase is a Mg^{2+},Mn^{2+}-dependent enzyme of plasma membrane. The development of ischemia caused sharp decrease in neutral sphingomyelinase activity, whereas reperfusion was accompanied by increase in this parameter. Early periods of ischemia (15-30 min) were characterized by inhibition of acidic sphingomyelinase which was replaced by significant activation (by 1.8-fold compared with control level) after prolonged ischemia (for 60 min). Subsequent reperfusion did not cause further increase in the enzyme activity. Thus, long-term ischemia (for 60 min) resulted in activation of acidic sphingomyelinase, whereas reperfusion caused activation of neutral sphingomyelinase.

Effect of TNF-α on various cells is accompanied by sharp LPO activation [18]. We have demonstrated that accumulation of TNF-α in the ischemic zone preceded activation of LPO (as well as activation of the sphingomyelin cycle). Stable increase in conjugated dienes (Fig. 8a) and ketodienes (Fig. 8b) expressed per mg protein was observed in all periods of reperfusion after prolonged ischemia (for 60 min). When the amount of LPO products was expressed per mg lipids no increase in LPO products was observed, possibly due to reperfusion-induced increase in lipid content in the ischemic zone.

Significant increase in LPO products was found 60 min after restoration of blood circulation in the hepatic lobe subjected to long-term ischemia (for 60 min). During this reperfusion period we found internucleosome ladder, the characteristic sign of apoptotic DNA fragmentation.

Thus, accumulation of TNF-α occurs both during ischemia and after reperfusion; however, DNA fragmentation is observed only after activation of neutral sphingomyelinase resulting in accumulation of proapoptotic agents of the sphingomyelin cycle and LPO products. This is consistent with our previous results that only co-administration of TNF-α and sphingosine induce apoptosis in the liver [10] whereas single administration of TNF-α to animals resulted only in large-scale DNA degradation [9, 10]. The interrelationship between sphingomyelinase activity and antioxidant status of cells [19, 20] suggests that LPO products generated in the presence of TNF-α may mediate the enzyme activation. Previously we demonstrated direct dependence of hepatic neutral sphingomyelinase activity on changes of conjugated dienes and ketodienes during administration of natural antioxidants (glutathione and bilirubin) to animals [21, 22].

The data of the present study suggest that accumulation of TNF-α preceding activation of sphingomyelin cycle and LPO is the main signaling event which accompanies hepatic ischemia/reperfusion injury. The TNF-α effect results in sphingomyelinase activation followed by subsequent accumulation of second messengers (ceramide and sphingosine) involved in apoptotic signal

transduction, whereas accumulation of LPO products provokes cell death.

This proposed mechanism of apoptosis induction in the liver subjected to ischemia/reperfusion injury allows the introduction of new schemes for prevention of ischemic injuries during liver transplantation and surgical operations on this organ. We do believe that sphingomyelinase inhibitors should be included into the list of preparations employed for prevention of ischemia/reperfusion injury. Results of our present and previous studies on the inhibition of sphingomyelinase activity *in vivo* by natural antioxidants suggest that known anti-ischemic effect of antioxidants may be now attributed not only to a decrease of LPO processes but also to their effect on the sphingomyelin cycle. Blockade of the sphingomyelin cycle can prevent the development of apoptosis even in the presence of high level of TNF-α, which cannot independently induce apoptosis in the liver. At the same time ceramide and sphingosine can induce apoptosis in various cell types even in the absence of TNF-α. So, in the ischemic organ it is very important to inhibit sphingomyelinase activity involved in the generation of apoptosis.

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