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REVIEW

Interaction of the Nitric Oxide Signaling System with the Sphingomyelin Cycle and Peroxidation on Transmission of Toxic Signal of Tumor Necrosis Factor-α in Ischemia–Reperfusion

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Abstract—This review discusses the functional role of nitric oxide in ischemia–reperfusion injury and mechanisms of signal transduction of apoptosis, which accompanies ischemic damage to organs and tissues. On induction of apoptosis an interaction is observed of the nitric oxide signaling system with the sphingomyelin cycle, which is a source of a proapoptotic agent ceramide. Evidence is presented of an interaction of the sphingomyelin cycle enzymes and ceramide with nitric oxide and enzymes synthesizing nitric oxide. The role of a proinflammatory cytokine TNF-α in apoptosis and ischemia–reperfusion and mechanisms of its cytotoxic action, which involve nitric oxide, the sphingomyelin cycle, and lipid peroxidation are discussed. A comprehensive study of these signaling systems provides insight into the molecular mechanism of apoptosis during ischemia and allows us to consider new approaches for treatment of diseases associated with the activation of apoptosis.

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Key words: nitric oxide, sphingomyelin cycle, ceramide, lipid peroxidation, tumor necrosis factor-α, apoptosis, ischemia–reperfusion

Ischemia, or oxygen deficiency of tissues, is observed in myocardial infarction and in brain stroke and also during surgical operations on the liver and other organs. Ischemia and the subsequent reperfusion result in apoptosis mediated by signaling pathways, which can include secretion of the tumor necrosis factor-α (TNF-α) [1], nitric oxide (NO) [2], and initiation of lipid peroxidation (LPO) [3] and of the sphingomyelin cycle (SPM cycle) [4]. However, all these signaling systems in the ischemia zone were studied separately and mainly in the cells of heart [4] and brain [5].

Nitric oxide (NO) is involved in transmission of the TNF-α toxic signal and in induction of its expression. Intersection points of the signaling systems of the SPM cycle and NO have been found. Products of the SPM cycle, ceramide and sphingosine, can imitate cellular effects of cytokines, lipopolysaccharide, and other biologically active compounds in the final cellular response: differentiation, apoptosis, or arresting of the cell cycle [6]. Moreover, sphingosine is responsible for a synergic effect in combination with many preparations, in particular with TNF-α [7]. To understand the mechanism of ischemic damages on arresting the blood flow and also on its subsequent recovery, it is important to analyze changes in parameters of all signaling systems involved in these processes and the succession of their inclusion; finally, it

Abbreviations: BHA, butylhydroxyanisole; CAPK, ceramide-activated protein kinase; CAPP, ceramide-activated protein phosphatase; cGMP, cyclic guanidine monophosphate; DNIC, dinitrosyl iron complexes; DPI, diphenyl iodonium; eNOS, endothelial NO synthase; GSH, glutathione; GSNO, nitrosoglutathione; HNE, 4-hydroxy-2-nonenal; iNOS, inducible NO synthase; L-NAME-N^G, nitro-L-arginine methyl ester; LPO, lipid peroxidation; nNOS, neuronal NO synthase; NO, nitric oxide; 3-NP, 3-nitropropionic acid; PK, protein kinase; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SPM, sphingomyelin; SPMase, sphingomyelinase; SPM cycle, sphingomyelin cycle; TNF-α, tumor necrosis factor α; TNF-R1, TNF-R2, TNF-α-binding receptors.

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will be promising for more reasonable and effective therapy of organs in ischemia.

FEATURES OF NITRIC OXIDE AND ITS PHYSIOLOGIC EFFECTS

Nitric oxide (NO) is a diatomic molecule that attracts the attention of researchers due to its extreme diversity of biological functions. NO can act as a signaling molecule, a cytotoxic agent, a pro-oxidant, or an antioxidant. NO is involved in the signal transduction during vasodilation and neurotransmission, acts as a toxin in destruction of pathogens [8], etc. In mammals NO is synthesized under the influence of NO synthase. NO synthase (NOS, EC 1.14.13.19) catalyzes the generation of NO and L-citrulline from L-arginine and O_2 under the influence of the electron donor NADPH [8]. There are three isoforms of the enzyme: neutral (nNOS), endothelial (eNOS), and inducible (iNOS). The isoforms have some differences in their catalytic properties: Ca^{2+} is necessary for activities of eNOS and nNOS, whereas calmodulin is bound with iNOS so tightly that there is no need for addition of Ca^{2+} [9]. iNOS is expressed in various cells, including macrophages and hepatocytes. Stimulation with immunologic or proinflammatory agents results in production of a great amount of NO some hours later [10].

NO is required for functioning of the gastrointestinal tract and the urogenital system, for activities of secretory cells and tissues, in particular in producing insulin, for functioning of the respiratory organs and skin, and for the pain response. NO is involved as an inhibitory agent in the regulation of thrombogenesis and platelet adhesion on the blood vessel walls. The generation of NO in the vegetative nervous system endings is important for regulation of functioning of many internal organs. In the central nervous system NO is responsible for the release of neuromediators and is involved in the synaptic transmission and formation of long-acting connections between neurons, i.e. in the postsynaptic potentiation underlying memory and learning [11]. The regulatory functions of NO produced by the constitutive isoforms eNOS and nNOS are manifested at its concentration of some μmol per kg tissue. At the NO concentrations ≥100 μmol per kg tissue produced by iNOS when NO functions as an effector of the cell immunity system the cytotoxic or cytostatic effects of this agent are realized. The same level of NO generation detected under different pathological conditions in the smooth muscle of blood vessels, myocardium, nervous tissue, and in secretory organs can lead to nearly irreversible negative events [11].

Biological effect of NO can be significantly reduced by its elimination in the reaction with superoxide anions or by its binding with other compounds, first of all with iron-containing complexes, e.g. hemoglobin. Therefore, to protect NO against such interactions and increase its efficiency, Nature uses the ability of this molecule to incorporate into different compounds. This promotes the transfer of NO both inside cells and between cells and tissues. Such compounds as S-nitrosothiols (RS-NO) and dinitrosyl complexes of nonheme iron ($(RS)_2Fe(NO)_2$) are shown to be directly generated in living cells and tissues [12-14]. The inclusion of NO as nitrosonium ions NO^+ into low molecular weight compounds (RS-NO) or $(RS)_2Fe(NO)_2$ significantly increases the number of intracellular biomolecules capable of reacting with NO. First of all, it concerns various proteins with thiol groups available for nitrosonium ions transferred by low molecular weight RS-NO species with formation of protein S-nitrosothiols. This conversion sharply increases the reactivity of thiol groups of proteins. Because thiol groups play an important role in functioning of many enzymes, S-nitrosylation of proteins is one of the major pathways used by NO for activation or suppression of various biochemical and physiological processes providing for the vital activity of cells and tissues. Thus, S-nitrosylation of proteins in calcium channels of heart cells increases the conductivity of calcium ions. Low molecular weight S-nitrosothiols activate transcription processes in *Escherichia coli* cells that results in expression of genes for synthesis of proteins responsible for antioxidant protection. S-Nitrosothiols also stimulate synthesis of stress proteins, which are necessary for supporting the integrity of cells and tissues under stress conditions, e.g. in hyperthermia or in the presence of various toxic agents. S-Nitrosylation inhibits caspases, i.e. proteins initiating programmed cell death, or apoptosis [11, 13].

Dual Effect of NO in Apoptosis

Dualism of NO manifests itself in apoptosis. The NO source and concentration are critical determinants of its either pro- or antiapoptotic role. However, the cell type is determinative. Thus, in macrophages, enterocytes, and muscle cells NO promotes apoptosis, and it inhibits it in hepatocytes and endothelial cells [10, 15, 16].

Apoptotic features of NO. In most cases, NO-induced apoptosis is mediated by caspases, DNA damage, and activation of p53 protein accompanied by subsequent disorders in the permeability of mitochondria and by their depolarization. One of the immunological functions of NO is induction of cytotoxicity directed against tumor cells and the surrounding tissues [17]. At high concentrations of NO and its subsequent conversion into peroxynitrite in the reaction with $O_2^{\cdot-}$, apoptosis is triggered in some cells, including macrophages [18], thymocytes [19], cells of the pancreas insulae [20], some neurons [21], and tumor cells [22]. Some proapoptotic effects of NO in these cells are mediated by cyclic guanidine monophosphate (cGMP). Factors influencing the cell-

specific sensitivity to the NO-caused apoptosis can be associated with the redox-state of the cell and formation of complexes with transition metals inside the cell [23] and also with the expression of antiapoptotic genes [24]. NO activates a group of mitogen-activated protein kinases (MAPKs) JNK/SAPK, which is involved in cell death. NO generated either by donors or by NOS induces apoptosis and activation of JNK/SAPK and p38 MAPK and activation of caspase-3, whereas their suppression protects RAW264.7 cells against apoptosis induced by the NO donor SNP [10].

Influence of NO on functions of mitochondria. The involvement of NO in apoptosis is manifested by its influence on functions of mitochondria. NO can directly cause the release of cytochrome *c* (CytC) that results in the loss of the mitochondrial transmembrane potential. NO binds with CytC-oxidase (complex IV) in the mitochondrial chain of electron transfer [25]. The $O_2^{\cdot-}$ generated by mitochondria under these conditions interacts with NO with production of peroxynitrite, which causes dysfunction of mitochondria, release of CytC, and apoptosis of thymocytes [26], neurons [27], and tumor cells [28]. Although physiological concentrations of peroxynitrite induce apoptosis in the cell lines HL-60 and U-937, they do not affect normal human monocytes and endothelial cells [28]. This indicates that the transformed cells are more sensitive to the peroxynitrite-induced cytotoxicity than normal healthy cells. It seems that the effect of NO on the CytC release depends on the intracellular redox potential and the number of target molecules, such as GSH and $O_2^{\cdot-}$.

Antiapoptotic features of NO. As it has been noted, NO can cause apoptosis. However, under certain conditions NO can display antiapoptotic features in some cells, including hepatocytes [16, 24, 29]. NO protects cells against apoptosis induced by different stimuli, including TNF-α [30], oxidative stress [31], and a shortage of Ca^{2+} or glucose [32]. The biochemical mechanism underlying the NO-dependent antiapoptotic effects is also cell-specific and is mediated by a multiplicity of signaling pathways.

Inhibition of apoptosis by the cGMP-dependent pathway. NO prevents the development of apoptosis by the cGMP-dependent pathway in hepatocytes [29], PC-12 nerve cells [32], embryonic motor neurons [33], B-lymphocytes [34], eosinophils [35], and oocytes [36]. However, some works have shown that the antiapoptotic effect of NO is not prevented in other cell types on inhibition of soluble guanylate cyclase (sGC), and stable cGMP analogs capable of penetrating across the membrane, e.g. 8-Br-cGMP, do not influence the protective effects of NO [37]. This indicates that the antiapoptotic mechanisms of NO action can be subdivided into cGMP-dependent and cGMP-independent ones that are specific for different type cells. In hepatocytes, in the PC-12 and U-937 cell lines the antiapoptotic effect of NO is associated with production of cGMP, which suppresses the release of mitochondrial CytC [30, 31], generation of ceramide [38], and activation of caspases [28], and this suppression is weakened by an inhibitor of protein kinase G [30]. Moreover, both NO and cGMP protect B-lymphocytes of the spleen against apoptosis due to increase in Bcl-2 expression [34] and activation of Akt/PKB, which induces phosphorylation of Bad and procaspase-9 along with expression of cytoprotective genes through NF-κB activation [10, 39].

Inhibition of caspase activities by S-nitrosylation. Caspases play an important role in the apoptotic signaling cascade. All caspases contain a single cysteine molecule in the catalytic center of the enzyme. This thiol-containing amino acid residue is very suitable for redox modifications and can be effectively S-nitrosylated by the ionized NO form, the nitrosonium ion NO^+. Seven kinds of recombinant caspases can be reversibly inhibited by NO^+ [40]. S-Nitrosylation of caspase-3 and caspase-1 was also shown *in vivo*. Inhibiting the caspase activities, NO prevents apoptosis in hepatocytes [23, 29, 32] and endothelial cells [30].

Molecular oxygen and transition metal ions, such as iron and copper, which can *in vivo* react with NO are known electron acceptors. S-Nitrosylated groups can also arise during the interaction of NO with iron–sulfur complexes [41]. Consequently, the ability of NO to S-nitrosylate caspases will depend on the presence of these molecules and other thiols such as GSH and Cys. The product of NO reaction with iron-sulfur complexes, dinitrosyl-iron complexes, are shown to S-nitrosylate caspases through production of NO^+ [23]. S-Nitrosylation of caspases in iron-enriched hepatocytes [22] occurs more effectively than in the iron-impoverished MCF-7 and RAW264.7 cells, until these cells were treated with iron [23, 42]. Thus, regulation of NO production and of the level of intracellular iron level allows us either to prevent an undesirable apoptosis, or to develop new therapeutic strategies for elimination of tumor cells during apoptosis [10]. Antiapoptotic features of NO also include its ability to reversibly inhibit the permeability of mitochondrial pores and the CytC release from mitochondria into the cytoplasm, as well as to induce expression of cytoprotective genes, in particular, of heat shock proteins, cyclooxygenase-2, hemoxygenase-1, and metallothionein [43, 44].

Pro- and Antioxidant Features of NO

NO endogenously produced by iNOS is well known as a mediator involved in inflammation, vasorelaxation, hypotension, and organ damage. The worsening of state of iNOS-deficient mice under conditions of TNF-α-induced shock associated with the NOS inhibition suggests that iNOS is responsible for additional important

protective functions in transmission of the TNF-α toxic signal [45, 46]. Molecular mechanisms of this protective effect can determine antioxidant and antiapoptotic functions of NO. The toxic effect of TNF-α is realized through induction of oxidative stress and accumulation of NO [20, 47]. This model of oxidative stress was used to study antioxidant features of NO. As a TNF-α-induced marker of LPO, 4-hydroxy-2-nonenal (HNE) was used [47], and its metabolite generated in the presence of LPO products was recorded immunohistochemically. The NO content on TNF-α-caused induction was lowered by injecting the NO synthase inhibitor L-NAME. These manipulations increased the content of the HNE metabolite that indicated antioxidant functions of NO [46]. In equimolar concentrations NO and $O_2^{\cdot-}$ produce peroxynitrite, which is a very strong oxidant [48, 49]. Under physiological conditions the production of peroxynitrite is minimal, and possible negative results of its influence are under the control of the endogenous system of antioxidant protection [50]. However, even a slight increase in the concurrent generation of NO and superoxide results in production of a significant amount of peroxynitrite: the tenfold increase in the generation of NO and superoxide results in a 100-fold increase in the peroxynitrite production. This situation can be observed in various pathologies [51]. The generation of peroxynitrite is recorded in diabetic, cardiological, vascular, neurodegenerative, and oncological diseases. Nevertheless, peroxynitrite can also display protective features. Thus, in the ischemia–reperfusion model in rat liver the injection of peroxynitrite (2 μmol/kg) 0, 60, and 120 min after the ischemia significantly weakened infiltration of neutrophils and liver damage [52]. The development of all these diseases can be accompanied by cell death through apoptosis. The involvement of peroxynitrite in apoptosis has been shown on HL-60 cells [28], PC-12 [53], fibroblasts [54], the neuroblastoma SN-SY5Y cells [55], neurons [33], astrocytes [56], oligodendrocytes [57], endothelial cells [58], β-cells of pancreatic islands [59], neutrophils [60], chondrocytes [61], cardiomyocytes [62], and cells of renal tubules [63, 64]. The peroxynitrite cytotoxicity is mainly characterized by the ability to induce peroxidation in membranes [65], liposomes, and lipoproteins that results in generation of lipid hydroperoxides, diene conjugates, and aldehydes [66]. These radicals in turn attack unsaturated fatty acids generating additional radicals and promoting the development of a chain of free radical reactions and degradation of membrane lipids [65, 67] by increasing their permeability and fluidity [68]. Peroxynitrite plays a critical role in inflammatory diseases of the nervous system due to inducing LPO of myelin with subsequent demyelinization [69]. Peroxynitrite is a strong oxidizer of low-density lipoproteins (LDL) [70]. Peroxynitrite-modified LDL with a high affinity for receptors causes accumulation of oxidized cholesterol esters and formation of foam cells that is a key marker of early atherogenesis [71]. The interaction of peroxynitrite with membrane lipids also results in generation of some lipid nitro-derivatives (mediators in transmission of biological signals under both physiological conditions and during the progress of some pathologies) or of intermediate products, such as isoprostanes and HNE, which can induce secondary oxidative processes [65]. Nevertheless, NO can also protect against oxidative stress [72]: it neutralizes $O_2^{\cdot-}$ and lipoperoxyl radicals, regulates the functioning of superoxide dismutase (SOD) [73], and inhibits the activity of NADPH-oxidase [74] and of cytochrome P450 [75]. Consequently, in $O_2^{\cdot-}$ excess in the presence of NO peroxidation develops, whereas at high concentrations of NO the effect of $O_2^{\cdot-}$ is neutralized and peroxidation is inhibited [72]. Thus, NO prevents oxidative stress and membrane *in vivo* oxidation due to inhibition of enzymatic sources of superoxide and owing to a direct reaction with superoxide and lipid radicals. It has been shown *in vitro* that NO is a stronger inhibitor of peroxidation than vitamin E and can protect even vitamin E against oxidation [64, 76].

NO and SPM Cycle

At present there is no an unambiguous concept about mutual influence of the SPM cycle and NO. There are data on just opposite influences of NO-generating compounds on the SPM cycle components [77]. High doses of NO induce apoptosis and generation of ceramide and also activate Mg^{2+}-dependent neutral and acidic SPMases [78], whereas physiological concentrations of NO inhibit apoptosis and the SPM cycle [79, 80]. Ceramide, in its turn, can increase the level of NOS expression, activate NOS, and promote accumulation of nitrites and nitrates in the intracellular medium [81], or, on the contrary, inhibit NOS [82].

SPM cycle. Sphingolipids perform a great number of biological functions. They are involved in cell transformation, differentiation, and proliferation, and many sphingolipids are described as secondary messengers and regulators of cell activity. Sphingomyelin (SPM) is one of the lipids producing biologically active metabolites. SPM residues are located mainly on the external side of the plasma membrane and form hydrophobic bonds with cholesterol. Traditionally, SPM is considered as a fundamental structural component of membranes of mammalian plasma cells. However, some agonists including TNF-α, 1-25-dihydroxyvitamin D_3, endotoxin, interferon, interleukins, the nerve growth factor, ionizing radiation, chemotherapeutical agents, and heating induce SPM hydrolysis to ceramide and phosphocholine [83-85]. In 1989 a signaling pathway was identified which was called the sphingomyelin–ceramide pathway or sphingomyelin cycle. This cycle is responsible for SPM cleavage with a phospholipase C-like enzyme SPMase

(ceramide phosphocholine phosphodiesterase, EC 3.1.4.12) with production of a secondary messenger ceramide [86]. Moreover, on hydrolyzing SPM, SPMase triggers a cascade of secondary lipid messengers (ceramide, sphingosine, sphyngosine-1-phosphate, ceramide-1-phosphate) and some other active molecules involved in the transmission of extracellular signals [85, 87, 88]. This process starts some minutes or hours after the cell is subjected to an exposure and can continue for different time resulting in hydrolysis of about 40% of the intracellular SPM. Then the amount of SPM is usually recovered to the initial level, and the SPM cycle is terminated.

Types of sphingomyelinases. At least seven types of SPMases have been identified in cells, tissues, and biological fluids of mammals [89]. Maximal activities are displayed by Mg^{2+}-dependent isoforms: the neutral isoform, the membrane-bound SPMase (which is often called N-SPMase), which was first isolated from the nervous tissue [90], and the acidic lysosomal SPMase (A-SPMase) [91].

Mg^{2+}-dependent N-SPMase. In 1998 Mg^{2+}-dependent human and mouse SPMases were cloned and sequenced [92]. The mouse and human enzymes consist, respectively, of 419 and 423 amino acid residues, and their molecular weights are 47.6 and 47.3 kDa. At the C-terminus of SPMase two membrane-bound hydrophobic domains are located, the catalytic domain being faced to the cytosol, and only its minor part is faced to the extracellular space. As shown by analysis of extracts from rat tissues, the expression level of N-SPMase is high in intestine, kidney, brain, liver, and tongue, but is low in spleen. Note that the expression level not always correlates with the enzyme activity. This suggests a possibility of an additional posttranslational regulation of N-SPMase activity [92].

At present, some N-SPMase isoforms have been identified, one of which is redox-sensitive, and just this isoform is involved in transmission of extracellular signals [93]. Activation of this N-SPMase by NO is a key link involved in apoptosis in different types of cells.

Role of ceramide in regulation of cell death. The product of SPM hydrolysis, ceramide, plays an important role in the regulation of proliferation, growth, development, and death of cells [94-96], and this role depends on the place of ceramide generation and on its subsequent metabolism. Many recent data suggest the involvement of ceramide in apoptosis [95, 97, 98]. Thus, ceramide regulates apoptosis induced by TNF-α or FAS-L [84]. On addition into the medium, exogenous ceramide and its analogs induce apoptosis of neurons, astrocytes, and oligodendrocytes [99-101]. However, depending on the cell type and the dose, ceramide can either inhibit or activate apoptosis [96, 102, 103]. Thus, depending on the concentration and place of generation, and, possibly, also on the age and differentiation of the cells, ceramide can manifest opposite effects, but in the majority of works it is considered to be a toxic agent.

In connection with such diversity of functions, ceramide is supposed to have various molecular targets. These targets can be presented by stress-activated kinases of the MAPK family, protein kinases (PK) B and C, NF-κB, Bcl-2, the Ras suppressor kinase, caspases, and ceramide-activated protein phosphatases (CAPP) and kinases (CAPK) [88, 104, 105]. The CAPP-mediated dephosphorylation of PKC can be a mechanism of SAPK activation and ERK inactivation by ceramide [104]. MAP-kinases regulated by peptide factors such as TNF-α and nerve growth factor (NGF) can be activated by ceramide, most likely, under the influence of p21 Ras, as it has been shown in cerebellum granular cells on the receptor $p75^{NGF}$ stimulation or on treatment with C_2-ceramide. Both JNK and p38 MAPK are involved in the process of cell death, whereas ERK is involved in cell proliferation and differentiation. In primary cortical neurons ceramide inhibits ERK (possibly due to activation of the ERK negative regulators, such as protein phosphatase) and stimulates JNK and p38 MAPK-cascades leading to cell death.

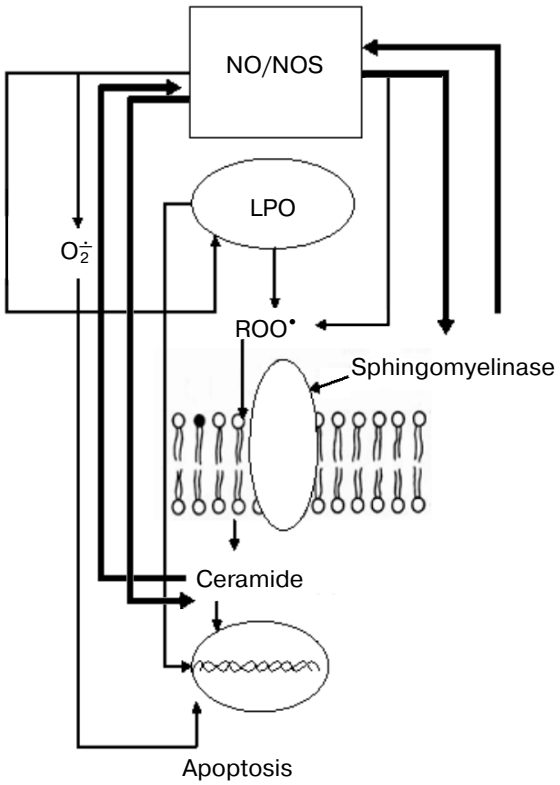
Some researchers record a role of oxidative stress and activation of NF-κB in ceramide-induced apoptosis [106, 107]. An increase in the NF-κB content in ceramide-induced apoptosis was observed in cells of spinal marrow ganglia roots, in midbrain neurons, and in PC-12-cells [107] that indicated the involvement of NF-κB in the apoptotic effect of ceramide. Increased content of ceramide can arrest cell growth due to inhibition of the signaling pathway that involves phospholipase D, whereas the mitogenic effect of sphingosine-1-phosphate can be caused by stimulation of this enzyme [108].

Role of NO generation in ceramide-induced cell death. A functional relationship has been observed between the intracellular contents of ceramide and NO during activation of apoptosis and necrosis in some normal and transformed cells [77, 109, 110]. SPM and enzymes regulating its metabolism are located in caveolae and rafts similarly to the constitutive NOS isoform [111, 112]. These membrane microdomains are supposed to be generation sites of NO and ceramide that trigger signaling cascades of apoptosis and necrosis. The NO and ceramide accumulation in caveolae and rafts is thought to result in damage to cell membranes in necrosis and apoptosis [113].

Although molecular mechanisms involved in the relationship between ceramide and NO are poorly studied, it is reasonable to suppose that some signaling interactions should exist. The activation of iNOS leads to accumulation of ceramide under the influence of A- and N-SPMases [78] and also to inhibition of neutral [114] and acidic [115] ceramidases (ceramide-metabolizing enzymes) during apoptosis and necrosis [78, 116]. However, ceramide can inhibit activities of NO-synthases and induction of cyclooxygenase-2 [117]. Some data

confirm that ceramide and SPMase can stimulate iNOS expression [118]. Moreover, cytotoxic effects caused by ceramide accumulation in cells can be caused by the presence of different pharmacological sources of NO, such as spermine-NO, sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), in normal renal, mesangial, and glomerular endothelial cells and also in transformed cells of HL-60 promyelocytic carcinoma [78]. Thus, upon 4 h of incubation of HL-60 cells with 1 mM of NO donor SNP, the ceramide content in the cells increased 1.6-fold and the activity of N-SPMase increased about 1.5-fold. In endothelial and mesangial cells A-SPMase was also activated along with inhibition of acidic and neutral ceramidases.

Ceramide can also mediate cell death under conditions of nitrosative and oxidative stress induced by NO donors and by superoxide-generating compounds [119]. Moreover, NO and its derivatives generated in caveolae and rafts can act as mediators responsible for transmission of external influences induced by ceramide on the cell membrane surface into the intracellular medium. A high ability for diffusion and hydrophobicity of NO allow it to react with various molecules and enzymes, such as lipoxygenases, peroxidases, and prostaglandin synthases [120]. Note that the activation of eNOS by C_2-ceramide can be detected using an immunofluorescence approach by its translocation from the external surface of the endothelial cell membrane onto its internal surface [81]. Moreover, NO and its derivatives induce damage to mitochondrial membranes and initiate caspase cascades and apoptosis in various types of cells [116].



Interaction between the NO/NOS signaling system and the SPM cycle in the induction of apoptosis

NO and inhibition of ceramide generation. Exogenous NO prevents death of human monocytes due to inhibition of TNF-α-caused binding of TRADD, activation of caspase-8, and generation of ceramide [38]. The NO donors SNAP and DETA/NO in doses lower than 100 μM inhibit the caspase-3 activity and prevent apoptosis in neurons, but higher doses of these compounds decrease the cell lifespan [121]. Similarly, NO prevents the $p75^{NTR}$-induced apoptosis in the neuroblastoma cells, but on increase in the ceramide generation by higher doses of NO signs of apoptosis are observed in these cells [122]. In dendrite cells NO prevents inhibition of endocytosis induced by accumulation of intracellular TNF-α-induced ceramide, as well as of exogenous ceramide [123]. This finding confirms that NO can act both before and after the generation of ceramide [77].

Inhibitory effects of NO donor compounds SIN1 and SNP on the activity of Mg^{2+}-dependent N-SPMase were also shown *in vitro*. In work [79] incubation of 5 μM NSM1 with the NO donor SIN1 at the concentration of 125-250 μM for 10 min at 37°C inhibited about 90% of the enzyme activity [79].

Ceramide and NO in transmission of apoptosis signal. NO is both an inducer and mediator during apoptosis in different cell systems [19, 117, 124]. Ceramide and NO are supposed to be interrelated in the transmission of the cell death signal. The treatment of cells with donors of NO leads to a dose-dependent increase in the ceramide content and to activation of A- and N-SPMases [78, 80, 125, 126], whereas the acidic and neutral ceramidases are inhibited by NO [125].

However, in some works exogenous NO was shown to protect monocytes against apoptosis due to inhibition of caspase-8 and production of ceramide [38, 80]. NO also prevented the inhibition of endocytosis on ceramide accumulation under the influence of TNF-α or the incubation of cells with a short-chain ceramide [123].

Thus, NO modulates (positively or negatively) the production of ceramide. In turn, ceramide increases the effect of TNF-α on NO generation and expression of inducible NO-synthase in cells [127]. Moreover, the generation of NO was activated in smooth muscle cells treated with exogenous SPMase [128]. Antioxidants such as N-acetylcysteine can inhibit the induction of genes and NO generation caused by sphingolipids in mouse macrophages [129] or rat astrocytes [118], and this suggests a role of the redox state of the cell in this process. Thus, NO and the SPM cycle are interrelated in the transmission of apoptosis signal (figure). However, the molecular mechanism of interaction between the NO/NOS signaling system and the SPM cycle needs to be studied in more detail.

RELATIONSHIP BETWEEN SIGNALING SYSTEMS OF THE SPM CYCLE AND OXIDATIVE STRESS

Common activators and inhibitors. Some factors capable of inducing apoptosis cause oxidative stress and activate the SPM cycle. Such proinflammatory cytokines as TNF-α promote the generation of reactive oxygen species (ROS) in cells [130] and also induce generation of SPM cycle products. Studies on the relationship between ROS production and generation of ceramide and sphingosine during apoptosis were mostly performed using antioxidants. Thus, N-acetylcysteine and pyrrolidin dithiocarbamate inhibited the TNF-α-mediated hydrolysis of sphingomyelin associated with production of ceramide and apoptosis of carcinoma cells. These antioxidants also inhibited N-SPMase and apoptosis induced by daunorubicin or by a nucleoside analog 1-β-*D*-arabinofuranosyl cytosine in human leukemia cells [89]. The interrelationship between oxidative stress, the production of ceramide, and apoptosis was demonstrated in the case of photodynamic therapy on mouse embryonic fibroblasts [131], human lymphoblasts [132], and by treatment of neuroblastoma cells with a synthetic retinoid *N*-(4-hydroxyphenyl)-retinamide, which inhibits carcinogenesis in some tumor models in animals [133]. Generation of ROS and activation of the SPM cycle were observed during the cell death induced by ionizing or ultraviolet (UV) radiation. Thus, the action of UV on keratinocytes resulted in an increase in the ROS content and in production of ceramide [134, 135]. However, vitamin E prevented the effect of UV, whereas the prooxidant L-buthionine-(S,R)-sulfoximine, on the contrary, significantly increased the UV-induced production of ceramide [134]. Induction of apoptosis by ionizing radiation, which generates hydroxyl radicals, was also accompanied by an increase in the ceramide content [136]. In our experiments on animals it was shown for the first time that there is an intersection of signaling pathways of NO, the SPM cycle, and LPO that can play a crucial role in the development of apoptosis. Addition of glutathione to the cells *in vitro* [137] and its injection into animals [138] inhibited SPMases. This was associated with a decrease in the amount of peroxides, which confirmed the ability of glutathione to inhibit oxidative processes acting as an antioxidant. The synchronicity of these events indicates the dependence of SPMase on the LPO intensity and, as a result, on contents of the LPO products.

Injection into animals of NO donors (S-nitrosoglutathione, dinitrosyl iron complexes, and $NaNO_2$) caused a dose-dependent change in the SPMase activity and accumulation of LPO products (conjugated dienes and diene ketones) [139]. The enzyme activity was inhibited during the first 30 min of the experiment and was normalized by 4 h. Changes in the contents of LPO products were reflected as mirror changes in the enzyme activity during the experiment [139]. These results can be easily explained by the dependence of SPMase on the oxidative potential of the cell. However, the enzyme activity could be inhibited by peroxynitrite, which interacts with the thiol groups of SPMase [93]. But the exact molecular mechanism of regulation of the SPMase activity by pro- and antioxidants is still unclear. Understanding of this mechanism would allow us to purposefully influence cell death and possibly aging. The main endogenous source of ROS in the cells is the electron transport in the mitochondrial electron chain, which results in the generation of a superoxide anion-radical [140]. A ceramide analog capable of penetrating into the cell was shown to directly influence mitochondria, inhibit the electron transport chain, and activate the ROS generation that resulted in changes in the mitochondrial permeability [113, 141]. Thus, many studies have shown that mitochondria, and in particular, the respiratory electron chain, are targets for ceramide, and this indicates an immediate relationship between ceramide and oxidative stress in mitochondria.

INTERACTION OF NO, TNF-α, AND SPM-CYCLE DURING APOPTOSIS

Functional properties of TNF-α. TNF-α is a polypeptide that is mainly produced by macrophages and monocytes in response to some external influences. This cytokine is expressed as a transmembrane integral 26-kDa precursor protein, which due to proteolysis is converted into a mature 17-kDa protein. The functionally active protein forms a trimer, which can be either in a membrane-bound or in a free form. Synthesis of TNF-α is regulated by many factors: lipopolysaccharides (LPS), fungi, mycoplasma, yeast, RNA- and DNA-containing viruses, cytokines, mitogens, PKC activators, and ionizing radiation. TNF-α is involved in the immune response, influences cell growth and differentiation, and performs many other biological functions [142]. A specific role of TNF-α in various systemic processes is essentially determined by the totality of biochemical and biophysical factors acting in parallel with TNF-α.

Role of lipids in transmission of TNF-α signal. Lipid peroxidation. The cytotoxic effects of TNF-α are largely due to processes associated with changes in the lipid component of cell membranes, including LPO. In work [143] it was first shown that TNF-α disturbed the functioning of mitochondria and promoted the appearance of free radicals and lipid peroxides, which was indicated by the generation of malonic dialdehyde. After this work disorders in the respiratory chain and of TNF-α-induced generation of ROS leading to oxidative stress were confirmed directly [144, 145]. In work [145] the role of mitochondria in TNF-α-mediated cytotoxicity was confirmed. The clone L929 of cells genetically deprived of

functioning mitochondria was shown to be resistant to cytotoxic effect of TNF-α. Moreover, these cells lost the ability for the TNF-α-induced synthesis of interleukin-6, and this at least partially confirmed the intersection between the ROS generation pathways and gene expression. This was also supported by the protective action of antioxidants preventing the cytotoxic effect of TNF-α on a cell culture [146].

TNF-α and the SPM cycle. The role of the SPM cycle products in the transmission of the TNF-α signal was first noted in works [147, 148]. Activation of SPM hydrolysis was shown on HL-60 cells in response to 15-min treatment with TNF-α, and a peak was recorded 30-60 min later. The SPM content acquired its initial level after 2-3 h. No changes were found in the content of phosphatidylcholine. The role of ceramide was also determined in the TNF-α-caused effects on HL-60 cells, such as induction of differentiation and decrease in the expression level of the gene *c-myc* of early response [147, 148]. The TNF-R1 receptor domain containing the amino acid sequence from 205 to 344 determines activation of N-SPMase, proline kinase, and phospholipase A_2 [149]. In Kronke's laboratory, the protein FAN was detected of the protein family with WD-repeats, which interacted with the TNF-R1-receptor of TNF-α selectively activating N-SPMase [150].

Metabolism of cellular sphingolipids is well studied due to the existence of some genetic diseases associated with disorders in sphingolipid metabolism [151]. The role of the SPM cycle in transmission of the TNF-α signal is not clear. On one hand, activation of the NF-κB transcription factor in Jurkat cells is associated with ceramide [152], but on the other hand induction of HL-60 cell differentiation, activation of nerve growth factor (NGF) receptors through the ceramide-activated protein kinase, and clusterization of some receptors are realized due to N-SPMase [147, 153, 154]. Today it is obvious that the activation of SPMase is a crucial moment in the transmission of not only of the TNF-α signal, but also of many other cytokines realizing their effects through the plasma membrane.

Ischemic damage is usually accompanied by an increase in TNF-α content [155]. Our studies on changes in the SPMase activities in the ischemic liver and upon its reperfusion revealed a sharp decrease in the N-SPMase activity throughout the duration of ischemia and a sharp increase in the activity during the reperfusion [156]. At the initiation of ischemia (15 and 30 min) A-SPMase was inhibited, and upon 1 h of ischemia the enzyme activity increased and became 1.8-fold higher than initially. The subsequent reperfusion did not cause a further increase in the enzyme activity. In work [157] molecular types of sphingomyelins and ceramides were studied by mass spectrometry during the reperfusion of ischemic liver. The total amount of ceramides and sphingomyelins, including other sphingolipids, was significantly increased after 1 h of the reperfusion. After 6 h of the reperfusion the contents of ceramide and SPM were the most increased as compared to the control. The increase in the content of sphingolipids was determined by appearance of new molecular forms 6 h after the reperfusion. The authors explained this effect by a possible synthesis *de novo* of new forms of sphingolipids, whereas the increase in the ceramide contents on the early stages of reperfusion was likely to depend on TNF-α, which was noticeably accumulated in the re-perfused organ after ischemia. Thus, changes in the amounts and profiles of molecular types of sphingolipids, mainly of SPM and ceramide, can be a determinative link during ischemia–reperfusion.

Role of NO in transmission of TNF-α signal. Cytokines including TNF-α can display a toxic effect via inducing NO-synthases that finally results in nitrosylation of heme and nonheme proteins [13]. However, NO can protect cells against the toxic action of TNF-α [15].

To study the protective role of NO under the toxic action of TNF-α, a model was developed in mice that included the injection of TNF-α combined with the NOS inhibitor L-NAME. This combined injection strengthened sensitivity to the TNF-α cytotoxicity and resulted in a significantly earlier death of the mice [46]. These experiments indicate that NO can protect animals against the toxic action of TNF-α. This was confirmed in experiments when wild type mice were compared with eNOS- and iNOS-deficient mice. It was established that iNOS could serve as a source of NO and be responsible for the protective effect in the TNF-α-caused oxidative shock. The iNOS-deficient animals are much more sensitive to TNF-α (the dose not lethal for the wild type and eNOS-deficient mice was 100% lethal for the iNOS-deficient mice); the addition of L-NAME over a wide range of concentrations did not influence this sensitivity. Thus, as differentiated from the conventional opinion, iNOS not only displays a damaging effect, but is also a source of NO, which is necessary for survival under conditions of TNF-α toxicity [158].

To confirm the protective effect of endogenous NO as an antioxidant under conditions of TNF-α-induced shock, mice were treated with antioxidants after the combined injection of TNF-α and the NOS inhibitor L-NAME, or the animals were injected with inhibitors of superoxide-generating systems. The lipophilic antioxidant butylhydroxyanisole (BHA) and SOD analogs less penetrating into the cell effectively prevented the increase in liver cell sensitivity to TNF-α, which was due to L-NAME-caused decrease in the NO content [46]. On the contrary, when the oxidation process is reduced on inhibition of xanthine oxidases or in the absence of gp91 NADPH-oxidase enzyme (Nox2) the effect of L-NAME was not weakened. In most cases, respiratory mitochondrial chains are the main source of ROS inside cells. Inhibition of complexes I and II protects cells against the TNF-α toxicity [145]. However, inhibition in mice of

complex I and/or II by rotenone, diphenyliodonium (DPI), and 3-nitropropionic acid (3-NP) gave a very weak protective effect. An inhibitor of phospholipase A2, aristolochic acid, significantly reduced the effect of the NOS inhibitor L-NAME on manifestations of the TNF-α toxic effect [46].

The best protection against the increased toxic action of TNF-α caused by injection of L-NAME was displayed by BHA and aristolochic acid. The protective properties of lipophilic BHA seemed to indicate that ROS produced during the TNF-α action were generated and/or acted in a lipophilic, hydrophobic medium, such as cell membranes and/or membranes of intracellular organelles. However, along with the ability to react with free radicals, BHA effectively inhibited mitochondrial respiration [159] and suppressed the TNF-R1-induced release of arachidonic acid [160].

NO in ischemia–reperfusion. Normally NO is expressed in the liver in eNOS sinusoidal endothelial cells [161]. In ischemia–reperfusion Kupffer cells are activated and produce proinflammatory cytokines, free radicals, oxidants, and significant amounts of NO generated by iNOS. This results in activation of neutrophils, endothelial cells, and hepatocytes, which release toxic amounts of ROS and NO generated by iNOS [162]. During ischemia–reperfusion of the liver both the protective and damaging effects of NO are observed [163-167]. Both the constitutive and inducible forms of NOS are expressed in the liver cells. Some works confirmed protective effects of nonspecific inhibitors of NOS in the liver, whereas in other works more pronounced cell damage under ischemia–reperfusion conditions were observed in eNOS- and iNOS-deficient rats. It was also shown that in ischemia–reperfusion eNOS could be a source of a cytoprotective NO, as in the eNOS-deficient mice the damage was more pronounced than in the wild type mice [163], and iNOS acted as a damaging agent. Upon 1 h of ischemia expression of TNF-α and IL-12 increases in eNOS-deficient but not in iNOS-deficient animals [164]. Using selective inhibitors of iNOS [165] and iNOS-knockout mice [166] revealed the damaging properties of iNOS during liver ischemia–reperfusion.

Moreover, donor compounds of NO had a protective effect and caused a decrease in iNOS expression. In other works iNOS inhibitors improved conditions of the liver subjected to ischemia–reperfusion after LPS-induced endotoxemia. Some authors reported that NO generated by iNOS protected liver cells. Thus, the Kupffer cells protected the liver by an iNOS-dependent mechanism. In fibrosis, inhibition of iNOS led to apoptosis and necrosis: iNOS-deficient mice are more sensitive to ischemia–reperfusion [167]. These contradictions attract attention to the complicated functions of NO and its relationship with other functionally important systems during the development of various pathologies or functional states of the cell.

Role of TNF-α in ischemia–reperfusion. The proinflammatory cytokine TNF-α is produced in liver, heart, brain, and other organs in acute ischemia and subsequent reperfusion [155, 168, 169]. In the above-mentioned publications there is no unambiguous answer to the question of the functional role of TNF-α during the development of ischemia in the experiment. Thus, TNF-α was shown to reduce the sensitivity of some organs to ischemia and, as a result, to protect them. And in experimental myocardial infarction in mice deprived of TNF-α receptors the size of infarction was greater than in wild type mice. The absence of the TNF-α signaling system increased cell death in response to a prolonged ischemic insult [170].

However, in other works the infarct size in mice without receptors to TNF-α was shown to decrease [171], and the authors believed that this was associated with the absence of inflammation provoked by TNF-α. Data on the damaging effect of TNF-α in ischemia–reperfusion were also supported by a decrease in the infarct size in mice injected with antibodies to TNF-α [171]. The most studied mechanism of the TNF-α induction of apoptosis in hepatocytes is activation of caspases, which play a key role in the development of apoptosis. However, TNF-α-induced apoptosis can develop by a mechanism independent of caspases. The best effect can be achieved on neutralization of ROS upon stimulation of TNF-α expression [172]. To find agreement with these data, it is reasonable to suppose that TNF-α can trigger the cell survival program in ischemia, and that this protective function of the cytokine can be abolished by the proinflammatory cascade induced by TNF-α during reperfusion. This difference in the TNF-α effects in ischemia–reperfusion can also depend on the duration of ischemia and on the absolute content of TNF-α at different times of ischemia–reperfusion.

Liver ischemia for 5-15 min in rats, the so-called ischemic preconditioning previous to prolonged ischemia–reperfusion, had a protective effect on the liver. This was associated with a significantly reduced zone of necrosis of hepatocytes and with a decrease in the activity of alanine aminotransferase and other enzymes – markers of necrosis of hepatocytes [173, 174]. Similar data concerning ischemia of organs were obtained in experiments with TNF-α-containing drugs inducing oxidative stress [168]. The protective mechanisms of TNF-α include mobilization of adenosine and of the second type adenosine receptors, NO generation by NO synthases, and acceleration of the entrance of hepatocytes into the cell cycle. The protective properties of TNF-α are thought to be associated with activation of nuclear factor NF-κB [175].

TNF-α caused functional and morphologic damage in ischemia–reperfusion acting through TNF-R1 and TNF-R2 receptors [176, 177]. Inhibition of TNF-α in ischemia and immediately afterward saves functions of the eye retina [176]. However, the treatment with a

recombinant TNF-α 48 h after the ischemia–reperfusion significantly strengthened the retinal damage as compared with its treatment upon the 2-h reperfusion [177]. Activation of the NF-κB transcription factor of TNF-α also can be an explanation of the opposite effects of TNF-α in ischemia–reperfusion. Thus, authors of work [177] found that both TNF-R1 and TNF-R2 receptors activated the NF-κB-dependent pathway but with different kinetics, and that a long-term activation of NF-κB induced by TNF-R2 was necessary for survival of neurons. These data suggested that TNF-α could play a protective or a damaging role depending on the duration of ischemia–reperfusion. Thus, endogenous NO can cause not only a hemodynamic collapse and/or display a cytotoxic effect but can also limit the development of TNF-α-induced oxidative stress and LPO *in vivo*, which is significantly contributed to by the SPM cycle and phospholipase A2-dependent signaling systems [46]. Although interactions of the signaling systems have been studied in some detail, the problem of multiple intersections of these interactions with NO has not been considered up to now. Moreover, this process is virtually unstudied for ischemia–reperfusion models in animals.

We were the first to show the relationship between the signaling systems of NO, SPM cycle, LPO, and TNF-α expression on a liver ischemia–reperfusion model [139]. We found that during ischemia for 15-60 min TNF-α was insignificantly accumulated in the zone of ischemia and that the content of this cytokine sharply increased on reperfusion of the ischemic liver lobe. The action of TNF-α on various cells caused a sharp activation of LPO [142]. In our experiments after a 1-h ischemia, throughout the duration of reperfusion a stable increase was observed in the amount of diene conjugates and diene ketones calculated per mg protein. The increase in the amount of LPO products was especially sharp 1 h after the recovery of blood flow in the liver lobe subjected to ischemia for 1 h. And just at this moment of reperfusion of the liver lobe after 1-h ischemia DNA degradation specific for apoptosis was recorded – the so-called "internucleosomal ladder". Activities of A- and N-SPMases were sharply changing during the ischemia–reperfusion. In the ischemic and reperfused liver the NO content increased even at the start of ischemia and sharply grew during the reperfusion. Thus, we have shown that all signaling systems involved in apoptosis are sharply induced on the reperfusion of the ischemic organ when the signs of apoptosis are detectable. It seems that activation of the SPM cycle induced by TNF-α and NO is a crucial event, which results in accumulation of apoptotic agents such as ceramide and sphingosine [139].

The mechanism of induction of apoptosis after ischemia–reperfusion of liver proposed by us allows to consider new preparations for preventing ischemic damage associated with transplantation of a donor's liver with surgical operations on the liver. Inhibitors of SPMases can be used as such preparations. Based on the presented data and on our previous findings on the *in vivo* inhibition of SPMase by natural antioxidants [138], it is supposed that the well-known anti-ischemic effect of various antioxidants can be explained not only by a decrease in the LPO intensity but also by their inhibitory effect on the SPM cycle. Inhibition of the SPM cycle will prevent the development of apoptosis even in the presence of a high content of TNF-α, which is unable to cause apoptosis of liver cells without assistance. However, ceramide and sphingosine can induce apoptosis in various cells even in the absence of TNF-α. Therefore, it is first of all necessary to inhibit in an ischemic organ the activities of SPMases that are involved in the generation of inducers of apoptosis.

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