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REVIEW

Reactive Oxygen Species and Regulation of Gene Expression

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Abstract—In eucaryotic cells reactive oxygen species (ROS) are produced in the reactions catalyzed by NAD(P)H oxidase and by some other specialized oxidases and also as an inevitable by-product of many redox reactions. Intracellular ROS synthesis is regulated by various hormones, cytokines, and growth factors. An increase in the ROS levels above a certain threshold (so-called oxidative stress) is accompanied by processes that are harmful for cell survival, such as lipid peroxidation and oxidative modification of proteins and nucleic acids. However, at low concentrations ROS act as secondary messengers responsible for a signal transduction from extracellular signaling molecules and their membrane receptors to the intracellular regulatory systems which control gene expression. Cellular transcriptional response to ROS is mediated mainly by activation of MAP protein kinases and submitted transcription factors AP-1, ATF, and NF-κB. A number of specific genes is also induced under hypoxia, i.e., under conditions opposite to oxidative stress. Cellular transcriptional response on hypoxia is mediated by activation of transcription factors HIF-1 and AP-1. Together with ROS, nitric oxide fulfills the role of a mobile and highly reactive redox-sensitive signaling molecule. Chemical reactions of NO with the superoxide anion and with other free radicals leads to production of highly reactive intermediates. Depending on the ratio of their intracellular concentrations, NO and ROS can either enhance or attenuate their reciprocal effects on cells.

Key words: reactive oxygen species, nitric oxide, protein kinases, gene regulation

In cells of aerobic organisms not less than 95% of the oxygen consumed is reduced by mitochondrial cytochrome oxidase, whereas the remaining part is reduced by various oxidases and aerobic dehydrogenases. For the complete reduction of an O_2 molecule, a simultaneous transfer of four electrons is required that produces two molecules of water or equivalent compounds. The reduction of oxygen by less than four electrons leads to generation of unstable metabolites that are called reactive oxygen species (ROS). Cellular ROS production can be augmented by the action of hormones, cytokines, and other physiological stimuli and also by external factors such as xenobiotics or UV-radiation. In high concentrations ROS are toxic for cells, especially due to membrane damage and irreversible DNA modifications. At a moderate level of synthesis, ROS act as specific signaling molecules involved in the control of immune processes, blood circulation, and regulation of the endocrine and some other physiological systems [1-5].

Abbreviations: ROS) reactive oxygen species; SOD) superoxide dismutase; GSNO) nitrosoglutathione; IL-1) interleukin 1; IL-8) interleukin 8; IFN-γ) interferon γ; MAP) mitogen-activated proteins; PKG) cGMP-dependent protein kinase; TGF-β) transforming growth factor β; TNF-α) tumor necrosis factor α.

SYNTHESIS AND CHEMICAL REACTIONS OF ROS IN CELLS

One-electron reduction of O_2 generates the superoxide anion ($O_2^{\cdot-}$). The nonenzymatic oxidation of semi-reduced ubiquinone ($UQ_{10}^{\cdot-}$) is one of the main sources of the mitochondrial synthesis of superoxide [6]:

$$UQ_{10}^{\cdot-} + O_2 \rightarrow O_2^{\cdot-} + UQ_{10}. \quad (1)$$

Superoxide generated also as an inevitable side product of the hydroxylation reaction catalyzed by cytochrome *P*-450 and through some other redox reactions. For instance, $O_2^{\cdot-}$ is generated as of one of the main products in reactions catalyzed by NAD(P)H oxidase, xanthine oxidase, and indoleamine dioxygenase [2]; $O_2^{\cdot-}$ is also generated in some nonenzymatic reactions (e.g., during catecholamine oxidation in the presence of iron ions, during the oxidation of semi-reduced flavins, semiquinones, and of heme iron by molecular oxygen). In the dismutase reaction with water, superoxide rather rapidly ($\tau_{1/2} \approx 10^{-6}$ sec, 37°C) generates hydrogen peroxide and O_2 (Fig. 1) [4].

Hydrogen peroxide (H_2O_2) is generated in two-electron reduction of molecular oxygen in reactions catalyzed

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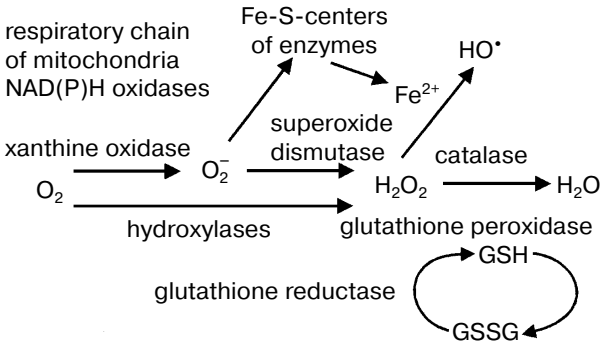

Fig. 1. Scheme of main pathways of generation and mutual transformations of ROS. Oxygen is reduced to $O_2^{\cdot-}$ by mitochondria in the reaction with ubisemiquinone ($UQ_{10}^{\cdot-}$) and also in reactions catalyzed by xanthine oxidase and specialized NAD(P)H oxidases. Hydrogen peroxide is generated from superoxide via the dismutase reaction and also during the two-electron reduction of oxygen by some hydroxylases. Superoxide oxidizes iron-sulfur centers of proteins that results in release of iron ions and in generation of hydroxyl radical in the reaction with H_2O_2.

by L-amino acid oxidase, aldehyde dehydrogenase, NO-synthase, monoamine oxidase, 5-lipoxygenase, superoxide dismutase, and some other enzymes [1, 4]. In contrast to $O_2^{\cdot-}$, hydrogen peroxide can penetrate through cell membranes and react with cellular components which are rather distant from the place of synthesis. Hydrogen peroxide is a rather stable compound in comparison with $O_2^{\cdot-}$, its reactivity is not very high. However, the accumulation of H_2O_2 is very dangerous for cells because by one-electron reduction of H_2O_2 in the presence of free Fe^{2+} or Cu^+ (the Fenton reaction) the highly reactive hydroxyl radical $OH^{\cdot}$ is generated:

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\cdot} + Fe^{3+}. \qquad (2)$$

This short-living free radical ($\tau_{1/2} \approx 10^{-9}$ sec, 37°C) can oxidize almost all organic compounds occurring near the place of its generation [4]. The toxic effects of $OH^{\cdot}$ are mainly consequences of irreversible nucleic acid and protein modifications and also due to initiation of chain reactions of unsaturated fatty acid peroxidation breaking the integrity of cell membranes.

REGULATED SYNTHESIS OF ROS

Various enzymatic systems in different cell types augment the synthesis of ROS in response to extracellular signals [1]. The expression of the indoleamine dioxygenase gene is up-regulated in response to interferons and cytokines [7]. Activation of Ca^{2+}-dependent proteases leads to proteolytic transformation of xanthine dehydrogenase into $O_2^{\cdot-}$-producing xanthine oxidase [8]. ROS generation by mitochondria is increased in cells treated with IL-1 or TNF-α [6] and also under hypoxic conditions (see below). The mechanism of the cytokine-dependent stimulation of ROS synthesis by mitochondria is not sufficiently clear. It was suggested that this synthesis could be mediated by the activation of ceramide-producing sphingomyelinase and by other membrane-associated enzymes involved in phospholipid metabolism [9]. Besides mitochondria, NAD(P)H oxidase and 5-lipoxygenase located on the plasma membrane are also important sources of regulated ROS synthesis. The expression of NAD(P)H oxidase is typical for phagocytic blood cells (neutrophils, eosinophils, and macrophages), endothelial cells, chondrocytes, and astrocytes [10]. The expression of 5-lipoxygenase is typical for lymphocytes and astrocytes [11]. NAD(P)H oxidase catalyzes the one-electron reduction of O_2 accepting the reducing equivalent from NADH and NADPH. Certain constituents of NAD(P)H oxidase complex are present in several other cell types. NAD(P)H oxidase is activated by inflammatory cytokines (IFN-γ, TNF-α, TGF-β, IL-1) and by some growth factors. Under such conditions (the so-called "oxidative burst"), in neutrophils up to 90% of the consumed oxygen is reduced to $O_2^{\cdot-}$ and then to H_2O_2 [12]. The NAD(P)H oxidase complex includes cytochrome b_{558} which is permanently bound to the plasma membrane and consists of two protein subunits ($p91^{phox}$ and $p22^{phox}$). Besides, the binding of three initially cytoplasmic proteins ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) is necessary for the activation of this enzyme. Translocation of the cytoplasmic components of NAD(P)H oxidase is initiated by the binding of the cytokines with corresponding receptors and subsequent activation of a membrane G-protein Ras and cytoplasmic G-protein $p21^{rac}$. The latter in GTP-bound active form is translocated to the cytoplasmic membrane in a complex with $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$. Due to transmembrane location of cytochrome b_{558}, NAD(P)H oxidase produces $O_2^{\cdot-}$ into the extracellular medium [11, 13].

In addition to the tissue-specific NAD(P)H oxidase which is mainly expressed by the phagocytic blood cells, several homologous enzymes were described [14]. The active complexes of these oxidases includes the $p22^{phox}$ subunit of cytochrome b_{558} or other cytochromes of group *b*. For instance, recently a new oxidoreductase of the cytochrome *b* class was revealed that was designated as $b_5 + b_{5R}$. This superoxide-generating flavohemoprotein possessing NAD(P)H oxidase activity is expressed in cells of different types. The role of the $b_5 + b_{5R}$ oxidoreductase and the mechanism of its regulation were not studied yet. This enzyme was suggested to function as a cellular sensor for O_2 [15].

ROS are also generated through arachidonic acid oxidation in reactions catalyzed by cyclooxygenase and lipoxygenases. A substrate for these membrane-associated enzymes is free arachidonic acid. For its production an

activation of phospholipase A_2 (PLA_2) is required. This process is under control of some peptide hormones (angiotensin), cytokines (TNF-α), and growth factors (PDGF) [11, 16]. The enzymatic oxidation of arachidonic acid is accompanied with generation of peroxides and free radical eicosanoid derivatives that can generate ROS [17].

CELL PROTECTION FROM ROS AND INDUCTION OF REDOX-SENSITIVE GENES

The dismutase reaction of $O_2^{\cdot-}$ with water resulting in the generation of H_2O_2 occurs either spontaneously ($k = 5 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$) or mediated by superoxide dismutases (SOD). Three isoenzymes of SOD are known: the cytoplasmic (SOD1) and extracellular (SOD3) isoenzymes containing Cu^{2+} and Zn^{2+} in their heme groups, and the Mn^{2+}-containing mitochondrial isoenzyme (SOD2). The efficiency of superoxide dismutases is very high ($k_{cat} = 1.6 \cdot 10^9$ $M^{-1} \cdot sec^{-1}$) [1, 3]. Nitric oxide (NO) is a single cellular component that reacts with $O_2^{\cdot-}$ more efficiently than superoxide dismutases. When NO concentration in the cells is low, superoxide is usually degraded by superoxide dismutases before it can participate in other chemical reactions [6]. Therefore, the accumulation of H_2O_2 is the main consequence of $O_2^{\cdot-}$ synthesis in cells. Hydrogen peroxide is removed by various enzymes: catalases, glutathione peroxidase, and thioredoxin peroxidase. The oxidized thiol groups of glutathione and thioredoxin are reducing in reactions catalyzed by the specific NADPH-dependent reductases. In addition to H_2O_2, glutathione peroxidase is also able to reduce organic hydroperoxides (ROOH) [2, 4].

Protection of cells from the generation of $OH^{\cdot}$ requires the immobilization of iron ions and removal of labile heme groups. These processes are mediated by intracellular chelators, such as ferredoxin, metallothionein, and picolinic acid (a product of tryptophan degradation), and also heme oxygenase degrading the heme ring [4, 18]. The generation of free radicals during oxidation of semiquinones is prevented by quinone reductases. The peroxidation of lipids and of other cell components initiated by free radicals is prevented by ascorbic acid, lipophilic antioxidant α-tocopherol (vitamin E), and also by melatonin and bilirubin (the products of tryptophan and protoporphyrin catabolism, respectively). Acting as electron donors, low-molecular-weight antioxidants neutralize unpaired electrons of highly reactive thionyl, alcoxyl, and peroxyl radicals converting them to relatively more stable free radical compounds [4]. Under certain conditions (see below), nitric oxide can act as an antioxidant since it is soluble in the hydrophobic phase of cell membranes and is able to quench chain reactions of lipid peroxidation [19]. NO also generates nitrosyl complexes with iron ions that inhibit the hydroxyl radical production [20]. Another group of antioxidants is involved in the reduction of oxidized thiol groups. This function is fulfilled by glutathione and protein factors thioredoxin and Ref-1, which contain active SH-groups [2].

ROS activate in cells the expression of redox-sensitive genes, of which many are required for cell defense from toxic effects of oxidative stress. These genes include the already mentioned glutathione peroxidase, quinone reductase, catalase, superoxide dismutase (SOD2), heme oxygenase-1, ferritin, glutathione reductase, thioredoxin, thioredoxin reductase, and metallothionein, and also cyclooxygenase-2 and γ-glutamylcysteine synthase. The antioxidant effect of cyclooxygenase seems to be a consequence of the decrease of the free arachidonic acid level caused by the activity of this enzyme. Glutamylcysteine synthase is involved in the synthesis of glutathione [1, 4]. Moreover, ROS activate the expression of genes that do not directly influence the increase the cell resistance to oxidative stress. The function of these genes is a participation in intercellular interactions. Thus, in epithelial cells and in the phagocytic blood cells, ROS induce some interleukins and chemokines such as IL-8 and MIP-1α [21]. In endothelial cells and in cartilaginous and connective tissues ROS activate the expression of stromelysin, collagenase 3, and of some other metallo-

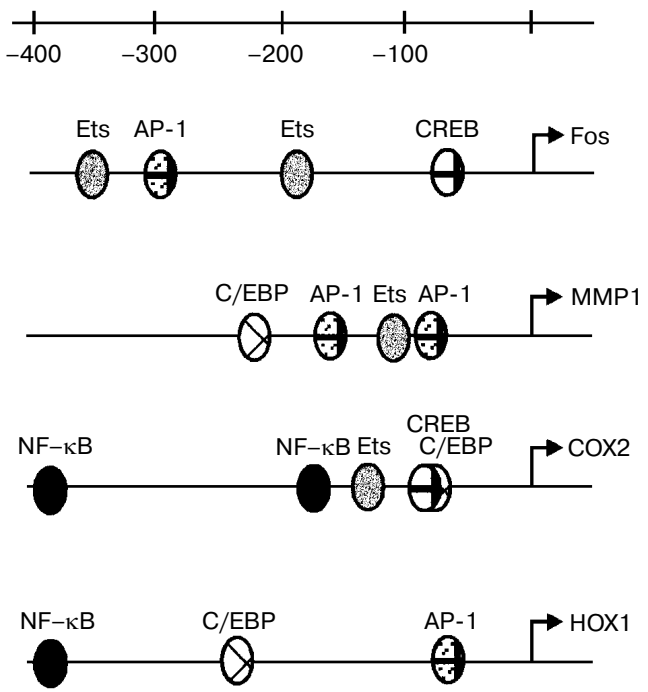

Fig. 2. Structure of promotor regions of some ROS-dependent human genes. The binding sites of the transcription factors and their coordinates (in nucleotides) are shown relatively to the starting point of the mRNA synthesis. Notations: Fos, c-*fos* gene; MMP1, collagenase (metalloprotease 1); COX2, cyclooxygenase-2; Hox1, heme oxygenase-1.

proteinases [22, 23]. Thus, ROS influence the state of the intercellular matrix.

The promotor regions of the ROS-inducible genes contain binding sites (operator sequences) of the transcription factors AP-1, ATF/CREB, Ets, C/EBP, and NF-κB (Fig. 2). In contrast to procaryotic transcription factors, which contain redox-sensitive iron-sulfur or thiol receptor groups (SoxR or OxyR, respectively) [1, 24], the activating effect of ROS on the transcription factors in eucaryotes is mediated by complex redox-sensitive systems. These systems are organized hierarchically consisting of multiple elements including regulatory GTPases, phospholipases, protein phosphatases, and also cGMP-dependent, phospholipid-dependent, and MAP protein kinases.

ACTIVATION OF MAP KINASES UNDER CONDITIONS OF OXIDATIVE STRESS

MAP (Mitogen-Activated Protein) kinases are one of the main regulatory systems mediating gene expression control by various extracellular stimuli [25-29]. The superfamily of MAP kinases consists of three main subgroups that are designated by including serine protein kinases ERK, p38, and JNK (synonym: SAPK), respectively. The activity of these protein kinases is controlled by a branched chain of double (threonine-tyrosine) protein kinases. These protein kinases are denoted as kinases of MAP protein kinases (MEK, MKK, MAPKK). The phosphorylation level and activity of kinases of MAP kinases is regulated also by a number of specific double protein phosphatases (MKP1, MKP2, PP2A, etc.). The expression of protein phosphatases MKP1 and MKP2 is up-regulated in response to the activation of MAP kinases. The activity of protein kinases of MAP kinases is under control of enzymes of the next regulatory level: double (serine-threonine) protein kinases of kinases of MAP kinases (MAP3K, MEKK, MAPKKK, etc.) (Fig. 3) [25, 27]. In general, ROS activate MAP kinases, however, the response of different members of MAP kinase family is a cell type depending process [30]. The relatively best elucidated mechanism of the redox-dependent MAP kinase activation is mediated by protein kinase ASK1 (Apoptosis Signal-Regulating Kinase-1). This protein kinase is a member of the MAP3K subfamily regulating MAP kinase cascades p38 and JNK. The ASK1 activity depends on thioredoxin, a multifunctional thiol-containing protein factor. In reduced form thioredoxin binds to ASK1 and inhibits this enzyme blocking the binding site of ASK1 with activating factor TRAF2 (TNF Receptor-Associated Factor-2). Oxidation of SH-groups of thioredoxin, which occurs, for instance, in thioredoxin peroxidase catalyzed reaction with H_2O_2 leads to thioredoxin dissociation from the complex with ASK1 [31]. ASK1 protein kinase phosphorylates and activates MAP kinases of the next regulatory level, such as MKK3, MKK6, MKK7, and SEK1, which in turn activate MAP kinases p38 and JNK (Fig. 4). Another pathway of JNK activation by ROS was revealed recently. This pathway is based on the reversible inactivation of JNK mediated by formation of its dimeric complex with redox-sensitive glutathione-S transferase (isoform π) [32]. Among all members of MAP kinase family, the JNK cascade is most sensitive to ROS, which induce a long-term (up to several hours) activation of JNK. The transcription factors c-Jun and ATF-2 (synonyms: CREB-2 and CREBP-1) are main substrates for JNK protein kinase [33]. In addition to its involvement in the regulation of transcription factors and of gene expression, JNK affects the cell regulatory systems that control apoptosis. JNK forms a complex with p53 that modifies the stability of this factor [34]. Moreover, JNK phosphorylates and activates caspase 3 (a latent protease which plays a key role in apoptosis) [35]. The role of MAP protein kinases and ROS in the regulation of apoptosis is reviewed in [36] in detail.

Mainly the activity of the MAP kinase cascade ERK (Extracellular Signal-Regulating Kinase) is under control of growth factors. ERK phosphorylates and activates transcription factors Elk-1 and c-Ets, which in turn induce the gene coding transcription factor c-Fos [33]. ERK is activated by H_2O_2 in cells of different types (lymphocytes, endothelial cells, fibroblasts). However, this activation continues for a shorter time (about 10-20 min) than the activation of JNK under the same conditions [37]. The mechanism of the ROS-dependent activation of ERK is not clear enough. The ROS-dependent activation of ERK is mediated by several proteins associated with the cell membrane and functionally located at the top level of MAP kinase cascades: G-proteins $p21^{Ras}$ and Rac1, tyrosine protein kinase Fyn (a member of the Src family), factor $p66^{Shc}$, and the double serine-serine protein kinase Raf [30]. MAP kinases and especially the ERK protein kinase are known to play a key role in the regulation of proliferation and realization of cellular effects of growth factors [25]. By regulation of MAP kinase activity, ROS influence cell proliferation controlling systems. Various transformed cells are characterized by decreased efficiency of antioxidant systems and by modifications in their sensitivity to ROS. Moreover, ROS were shown to be involved in the cell transformation stimulated by phorbol esters [38].

ROS AND ACTIVATION OF THE TRANSCRIPTION FACTORS AP-1 AND ATF

The transcription factor AP-1 (Activator Protein 1) binds to specific palindromic sequences with similar structure, namely TGACTCA and TGACGTCA. These two homologous sequences are designated as regulatory

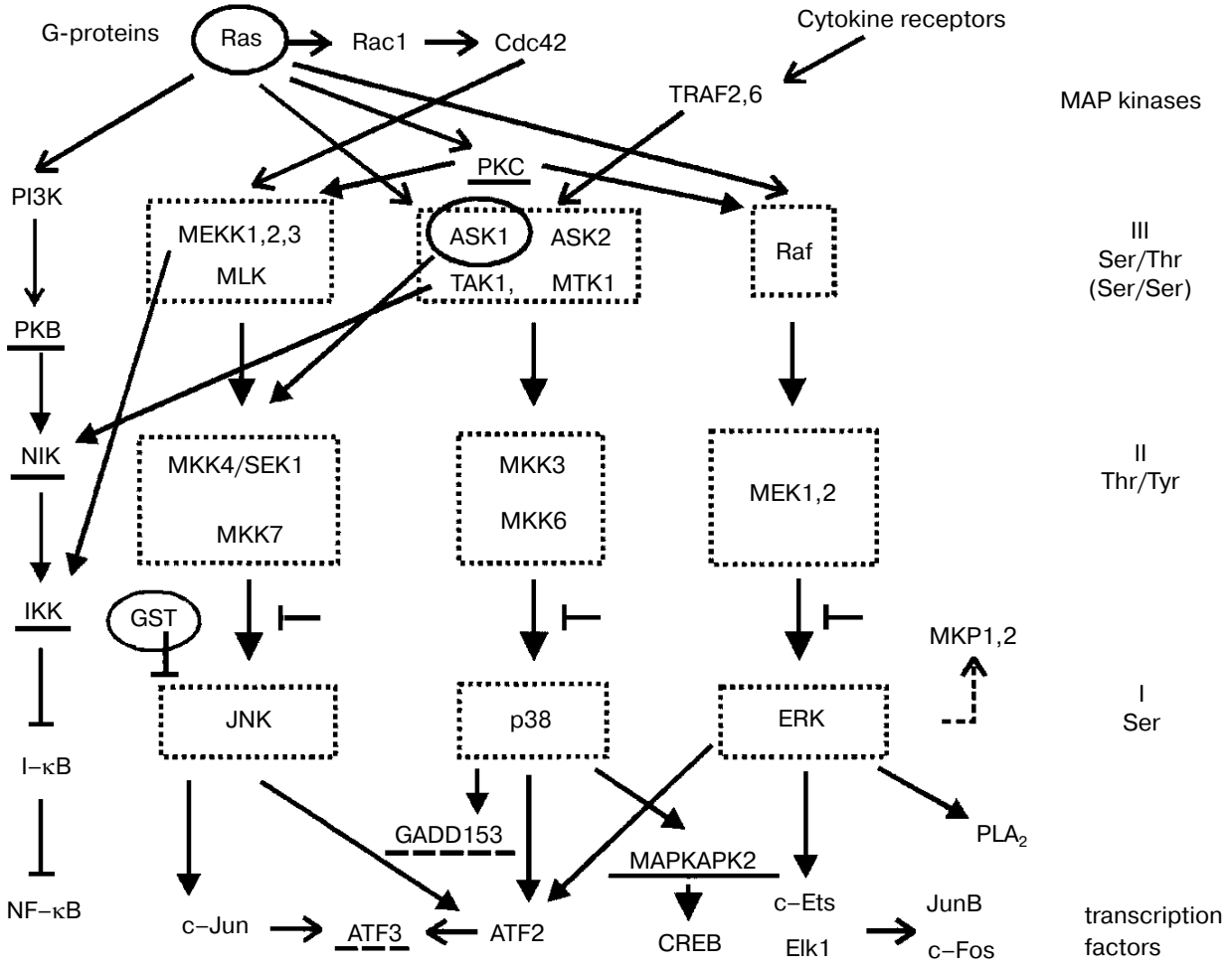

Fig. 3. Kinase systems involved in the redox-dependent regulation of transcription factors AP-1 and NF-κB. Activities of MAP kinases and of kinases that control NF-κB depend on membrane G-proteins and on receptors of cytokines. The system of MAP kinases consists of three cascades (JNK, p38, and ERK), each including three groups of enzymes: serine, threonine-tyrosine, and serine-threonine (or serine-serine) kinases. The activation of MAP kinases results in the induction of tyrosine-threonine phosphatases of MAP kinases (MKP1 and MKP2). MAP kinases are placed inside dotted rectangular frames. Notations of other proteinases are underlined. ROS-sensitive elements of regulatory systems are encircled. Regulatory interactions caused by phosphorylation of proteins are shown by arrows with hatched ends. The transcription factors with inhibiting effects are underlined with the broken line. Notations: GST, glutathione-S transferase π; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C_α; PLA_2, phospholipase A_2.

elements TPE (tetradecanoyl phorbol-acetate-responsive element) and CRE (cAMP-responsive element). The factor AP-1 consists of two subunits. At least one of them is a member of families of DNA-binding proteins Jun or Fos (c-Jun, JunB, JunD, c-Fos, FosB, Fra1, Fra2) [39]. The second subunit of AP-1 can be a member of the ATF (Activating Transcription Factor) family [40]. The subunit composition determines the AP-1 affinity to the operator DNA sequence and its interaction with other transcription factors assembling the promotor complex. Thus, the homodimer c-Jun–c-Jun has a higher affinity to the TPE sequence, whereas the heterodimer c-Jun–ATF-2 has a relatively higher affinity to the CRE

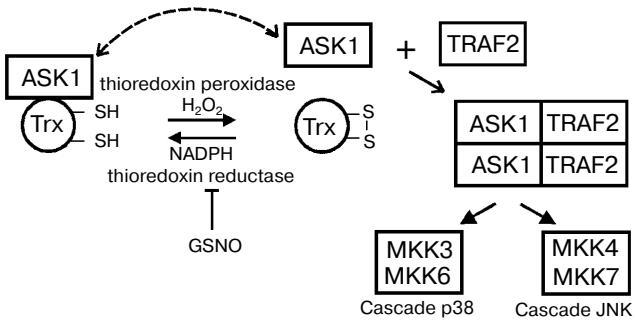

Fig. 4. Scheme of activation of MAP kinase ASK1 under the influence of ROS. Notations: Trx, thioredoxin; TRAF2, adaptor proteinaceous factor associated with the receptor TNF-α.

sequence. Dimers JunB–c-Fos and c-Jun–c-Fos bind to both operator sequences with a similar efficiency [39]. The binding of c-Jun and ATF-2 with subunits Fra1, Fra2, and ATF-3 suppresses the functional activity of these transcription factors [39]. AP-1 is activated by various effectors: growth factors, cytokines, hormones, heavy metals, xenobiotics, ultraviolet radiation, mechanical and osmotic stress, membrane depolarization, oxidative stress, and hypoxia. The responses of certain AP-1-dependent genes to each of these stimuli are not the same. These variations are determined partly by effector-specific activation of AP-1 of different subunit composition [41].

The mechanism of the ROS-dependent activation of JNK protein kinase was described above. JNK phosphorylates and activates c-Jun and ATF-2 constituents of the AP-1 and promotes the induction of the transcription factor ATF-3 [42, 43]. Because a number of AP-1 binding sites are located in the promotor region of the *c-jun* gene, the phosphorylation and the subsequent activation of the c-Jun factor result in the induction of its own gene [39]. Such regulation of the c-Jun activity leads to long-term (for several hours) activation of the transcription factor AP-1 and for a steady increase of the expression levels of the submitted genes under oxidative stress conditions [44]. Protein kinase p38 phosphorylates and activates transcription factors ATF-2 [45] and GADD153 (Growth Arrest and DNA Damage-Inducible Protein 153) [46]. The GADD153 factor is a member of a family of DNA-binding proteins C/EBP (CCAAT Enhancer Binding Protein). GADD153 is able to produce dimers with other transcription factors of its own family and with members of ATF/CREB family. This dimerization leads to suppression of the activity of C/EBP partners [47]. Besides the enlisted substrates, protein kinase p38 phosphorylates and activates the serine protein kinase MAPKAPK-2 (MAP Kinase-Activated Protein Kinase-2), which is located in the cell nucleus and, in turn, phosphorylates the transcription factor CREB and a heat shock protein HSP27 [48].

The ROS-dependent activation of protein kinase ERK leads to phosphorylation and activation of transcription factors c-Ets and Elk-1 [33]. These two homologous factors recognize the DNA sequence CCGGAAG. In turn, c-Ets and Elk-1 activate the expression of genes encoding the transcription factors JunB and c-Fos, which are constituents of AP-1 [49]. Unlike c-Jun, oxidative stress causes a short-term activation of JunB and c-Fos because of a low stability of their mRNAs [39] together with the already mentioned time-limited ROS-dependent ERK activation [37, 50, 51]. The factor JunB (unlike c-Jun) is able to activate the transcription of AP-1-dependent genes under the condition that their promotor regions contain several copies of the CRE sequence [52].

A group of transcriptional factors involved in cell protection from oxidative stress includes factors of the Nrf (Nuclear Respiratory Factor) family. Factors Nrf1 and Nrf2 control expression of the antioxidant-sensitive genes such as NAD(P)H:quinone oxidoreductase, glutathione-S transferase, UDP-glucuronosyl transferase, and epoxide hydrolase [53]. Products of these genes are implicated in the metabolism of xenobiotics and prevent their involvement in ROS-generating intracellular reactions. The binding site of Nrf family factors to DNA is RTGACNNNGCA (R, purine nucleotide) and this sequence is denoted as ARE (Antioxidant-Responsive Element). ARE is composed of two incomplete TPE-like sites that enable the cooperative binding of the Nrf and AP-1 transcription factors to the ARE region [54]. The mechanism of redox-dependent activation of Nrf factors remains unclear.

ROS AND ACTIVATION OF TRANSCRIPTION FACTOR NF-κB

The transcription factor NF-κB is activated by numerous extracellular agents such as inflammatory cytokines, xenobiotics, bacterial endotoxins, and viral double-stranded RNA [55-57]. ROS activate NF-κB in cells of many types (but not all), whereas antioxidant action attenuates its activity. NF-κB up-regulate the expression of many genes that maintain cellular resistance to stress, participate in apoptosis control, and regulation of cell immunity. The sequence of NF-κB binding site on DNA is GGANYYYCC (Y is a pyrimidine nucleotide). Similarly to AP-1, the dimeric factor NF-κB occurs in several isoforms with different subunit composition. The most representative isoform of NF-κB consists of the Rel (synonyms: RelA and p65) and p50 subunits. The minor NF-κB isoforms contain structurally related proteins c-Rel and RelB instead of RelA and either protein p52 or any protein of the Rel family instead of p50. In inactive form NF-κB factor is bound to an inhibitor I-κB (seven isoforms were identified) preventing its nuclear translocation from the cytoplasm. Most of the signals causing the NF-κB activation (with exception of UV radiation and double-stranded RNA) activate finally the IKK protein kinase (I-κB Kinase) [55]. The activity of IKK is directly or indirectly regulated by protein kinases NIK, IRAK, MEKK1, PKR, PKB (Akt), PKC_{ζ}, and PKC_{0} [58]. The phosphorylation of I-κB on serine residues by IKK leads to dissociation of this factor from the complex with NF-κB and its subsequent proteolytic degradation [59]. Besides, NF-κB factor activates the transcription of the gene encoding I-κBα and promotes the recovery of the initial level of its own inhibitor [55].

In ROS-dependent activation of NF-κB the various cellular signaling systems are implicated. One of the regulatory pathways is based on the redox-dependent activation of the G-protein $p21^{Ras}$ and subsequent activation of membrane phosphatidylinositol-3 kinase (PIP3K) and of

phosphatidylinositol-3,4,5-triphosphate-dependent protein kinases: PKB (Protein Kinase B) as well as serine protein kinases PKC_ζ and PKC_θ [60, 61]. In NF-κB regulation are implicated also MEKK1, TAK1, and MKK6 protein kinases, which are members of the MAP kinase cascades JNK and p38 (Fig. 3) [62]. Increase of cellular level of oxidized thioredoxin leads to NF-κB activation. Similarly to the mechanism of AP-1 activation, this process is mediated by ASK1 protein kinase [31]. The redox-dependent regulation of NF-κB is probably mediated by cytoplasmic thioredoxin peroxidase (isoform AOE372), which oxidizes thioredoxin in reaction with H_2O_2 [63]. On the contrary, an increase of the intracellular level of reduced thioredoxin (in experiments with transfection with a thioredoxin-expressing plasmid) leads to the inhibition of NF-κB [64].

INHIBITION OF TRANSCRIPTION FACTORS UNDER CONDITIONS OF OXIDATIVE STRESS

Factors c-Jun and c-Fos and protein p50 (a subunit of NF-κB) contain in their DNA-binding domains highly sensitive to ROS cysteine residues (Cys252, Cys154, and Cys61, respectively). Oxidation of their SH-groups reversibly inactivates AP-1 and NF-κB [2]. The back reduction of these groups is mediated by the active thiol-containing protein factor Ref-1 (Redox Factor-1), which is then reduced without enzymes in the thiol disulfide exchange reaction with thioredoxin (Fig. 5) [65]. In addition to its involvement in the redox-dependent regulation of transcription factors, Ref-1 also fulfills some other functions in the cell. Due to its DNase activity, Ref-1 is involved in the reparation of DNA, and in this role this factor is designated as endonuclease APE (Apurinic/Apyrimidinic Endonuclease) [2]. Moreover, Ref-1 activates the p53 factor that controls apoptosis [66].

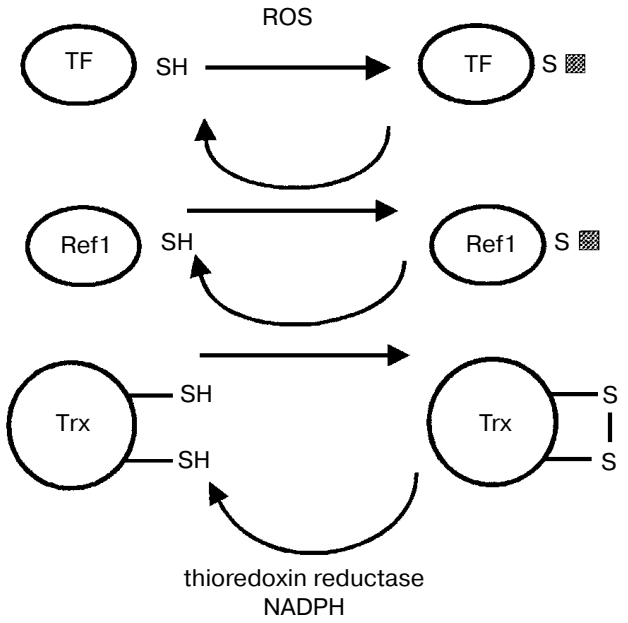

Fig. 5. Scheme of the reducing reactivation of transcription factors in the reaction with Ref-1. Notations: TF, redox-sensitive transcription factors; Trx, thioredoxin; squares, oxidized thiol groups (disulfides R-SS-R and sulfenoids R-SOH).

Oxidative inactivation of AP-1 and NF-κB transcription factors occurs at higher ROS concentrations compared to ROS levels required for MAP kinase activation. Intense oxidative stress is accompanied by a decrease in the activities of AP-1 and NF-κB and attenuation of transcription of the submitted genes. Products of many of these genes are involved in the cell protective anti-stress systems. This suggests that the mobilization of the cell protective systems will be attenuated after a certain threshold level of ROS, whereas the intensity of apoptosis stimulation will increase. ROS are also involved in the oxidation of active SH groups and in the inhibition of constitutive transcription factors NF-1, Sp1, USF, and MyoD. As a result, the expression of a number of genes is suppressed. For instance, the oxidation of NF-1 to decreased transcription of several isoforms of cytochrome *P*-450 [2]. ROS also inhibit tyrosine protein phosphatases by oxidation of cysteine residues within catalytic centers of these enzymes [67].

ACTIVATION OF TRANSCRIPTION FACTORS UNDER CONDITIONS OF HYPOXIA

The activity of specific transcription factors is modulated and the subsequent expression of specific genes is modulated not only under oxidative stress, but also under decreased oxygen pressure (hypoxia and anoxia). The progression of hypoxia in the organism is usually associated with ischemia progression and some other pathophysiological situations [68]. It is worth noting that AP-1 is activated equally under hypoxia and oxidative stress conditions [69]. This occurs because hypoxia promotes the accumulation of semiquinones and reduced flavins that cause the increase of ROS generation in mitochondria and a subsequent activation of the MAP kinase system JNK [70]. However, various AP-1 constituents differ in their sensitivity to hypoxia and to oxidative stress. Under the normal oxygen concentration in the cell environment (normoxia) ROS mainly activate c-Jun, ATF-2, and c-Fos [37, 51], whereas JunB and c-Fos factors are more sensitive to ROS generated under conditions of hypoxia [71]. Under the influence of antioxidants, the JNK-mediated induction of ATF-3 occurs that leads to suppression of the binding of the functionally active dimeric form (c-Jun:ATF-2) of the factor AP-1 to the operator sequence CRE [42, 72]. Under conditions of hypoxia, JunB and c-Fos activation is mediated by the

MAP kinase cascade ERK (Fig. 3). ERK protein kinase phosphorylates and activates the transcription factors Elk-1 and ATF-2 that leads to subsequent induction of submitted c-Fos and JunB factors [33]. Unlike AP-1, the redox-sensitive transcription factor NF-κB is up-regulated only by oxidative stress, while hypoxia suppresses its activity [69]. It is worth mentioning that under conditions of hypoxia (and also of oxidative stress), the activation of expression of several redox-sensitive genes can be mediated by a selective stabilization of their mRNAs. The expression of ferritin and of some other genes is regulated by Fe-S cluster-containing RNA-binding proteins IRP1 and IRP2 [73]. For many other genes, the mechanism of ROS-dependent control of the mRNA degradation remains unclear [74].

Unlike AP-1, the redox-dependent activation of the transcription factor HIF-1 (Hypoxia-Induced Factor-1) is activated only under conditions of decreased oxygen tension. Under the normal oxygen concentration, HIF-1 is also activated, but by other than ROS factors, such as inflammatory cytokines, peptide hormones, and NO [75]. In response to NO, HIF-1 is activated only under normoxia. On the contrary, under conditions of hypoxia, NO inhibits this transcription factor [76]. In cooperation with AP-1 (the c-Fos-JunB subunit composition) and p300 factor, HIF-1 activates the transcription of the submitted genes [76, 77]. HIF-1-sensitive genes are involved in the control of proliferation of smooth muscle cells of epithelium, such as vascular endothelium growth factor (VEGF), collagenase 4 (MMP-9), endothelin-1. HIF-1 also stimulates the expression of erythropoietin (in kidneys), NADPH:quinone oxidoreductase, platelet growth factor (PDGF-B), and several glycolytic enzymes [75].

The factor HIF-1 consists of two subunits: HIF-1α and HIF-1β. The subunit HIF-1α which is a transcription factor of the helix-loop-helix (HLH) family. This subunit is responsible for the binding to DNA and for the interaction with RNA polymerase. The factor HIF-1α binds to the operator sequence TACGTGCT. The HIF-1α subunit contains non-heme iron (two atoms of Fe(II) per protein molecule) and an active cysteine residue. The HIF-1β subunit is designated also as factor Arnt (Aryl Hydrocarbon Receptor Nuclear Translocator) [78, 79] since besides HIF-1α, this protein is able to promote nuclear translocation of xenobiotics-sensitive AhR transcription factor [75]. The stability of HIF-1α depends from concentrations of O_2 and ROS. The mechanism of redox-dependent activation of HIF-1α is not finally determined. According to many data, the iron-sulfur cluster in HIF-1α plays a role of the receptor of ROS [80]. As it has been mentioned above, under conditions of hypoxia the ROS generation in mitochondria is increased and this initiates the oxidation and following dissociation of iron ions bound to HIF-1α [70]. This modification suppresses the proteolytic degradation of HIF-1α and stimulates its translocation into the nucleus. Unlike the ROS synthesis in mitochondria which increases under conditions of hypoxia, cells also contain enzymatic systems which increase the production of ROS on an response to increase of the O_2 concentration in the extracellular medium. These enzymes ("oxygen sensors") are NAD(P)H oxidase family members which produce superoxide anion [15]. In contrast to HIF-1α activation by ROS of mitochondrial origin, ROS generated by NAD(P)H oxidases inhibit this transcription factor [81, 82].

OXIDATIVE STRESS AND NO

There is a similarity between cell sensitivity and their transcriptional response to the influence of NO and ROS because these simplest signaling molecules operate with common intracellular targets, such as proteins containing the reactive heme, thiol, and iron-sulfur (Fe-S) groups [24, 83-86]. An example of parallel effects of NO and ROS is induction of heme oxygenase-1 and cyclooxygenase-2 genes under conditions of oxidative stress and by NO. The transcriptional activation of both genes is mediated by MAP kinases [87, 88]. Nevertheless, the transcriptional responses of some other genes to NO and ROS are different. Thus, superoxide dismutase (SOD2) is induced by ROS and not by NO, whereas NO activates an expression of the gene of the vascular endothelium growth factor (VEGF) which transcription is suppressed under conditions of oxidative stress [76, 89]. Most pathways regulating NO and ROS syntheses are the same. In the phagocytic cells the expression of the inducible NO synthase and the activity of the membrane NAD(P)H oxidase are increased after the treatment with inflammatory cytokines, interferons, peptide hormones, and bacterial endotoxins. Under conditions of hypoxia, the mitochondrial synthesis of ROS and the NO synthase-induced production of NO are increasing simultaneously in cells of different types [10, 90].

The oxidation of NO with molecular oxygen leads to production of short-living active nitrogen derivatives: nitrosonium ion NO^+, nitrous acid anhydride N_2O_3, and radical $NO_2^{\cdot}$ (Fig. 6). Unlike a rather chemically stable NO, these compounds can nitrosylate cysteine and nitrosate tyrosine residues of proteins [20]. In the lipid phase of membranes the rate of NO reaction with oxygen is ~300-fold higher than in water. One can suggest that membrane proteins are more sensitive to NO-dependent modifications than the proteins located in the cytoplasm [91]. NO was shown to modulate the activity of G-protein $p21^{Ras}$, cyclooxygenase-2, and lipoxygenases [92-94]. The Ras activation by NO is mediated by modification of the Cys118 residue of this protein. The activation of Ras causes the subsequent activation of phosphatidylinositol-3′ kinase, phos-

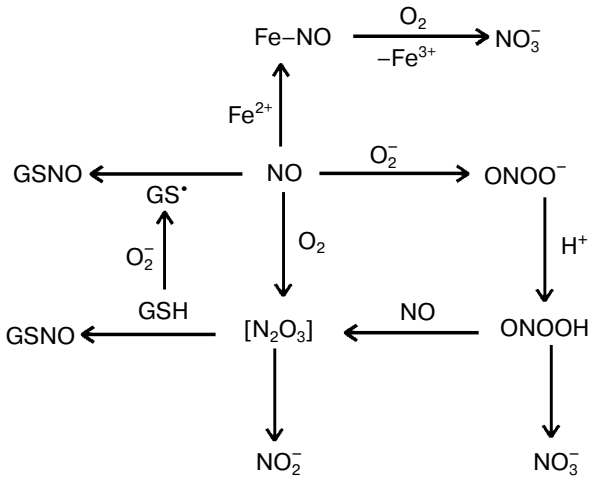

Fig. 6. A scheme of NO reactions with oxygen, superoxide, and glutathione. To nitrosylate glutathione (GSH), a glutathionyl radical (GS$^\bullet$) or active derivatives of NO are to be previously produced in the reaction with ROS and molecular oxygen. NO forms iron-nitrosyl complexes with Fe(II)-containing compounds.

phatidylinositol-dependent protein kinases, and the PKB protein kinase-mediated activation of the NF-κB factor [44, 92, 95]. The effect of NO on NF-κB is a highly concentration-depending process. Low doses of NO activate this transcription factor, whereas high doses of NO inhibit it [96]. The inhibiting effect of NO is caused by nitrosylation of active cysteine residue within p50 subunit preventing the binding of NF-κB to DNA [97]. Some findings suggest that the NO-dependent activation of the transcription factor HIF-1 is also mediated by PKB protein kinase [75].

Under conditions of intense synthesis of NO and in the presence of ROS, glutathione, which is the most represented cellular thiol compound, is nitrosylated, and S-nitrosoglutathione (GSNO) is accumulated. GSNO is synthesized in the cells through an intermediate generation of glutathionyl (GS$^\bullet$) and other free radicals. GSNO participates the reaction of transnitrosylation with accessible SH-groups of proteins [98]:

$$\text{R-SH} + \text{GSNO} \leftrightarrow \text{R-SNO} + \text{GSH}. \quad (3)$$

The transnitrosylation reaction is catalyzed by glutathione peroxidase but can occur also without enzymes [99]. Nitrosylation of thioredoxin reductase in the reaction with GSNO is associated with an inhibition (IC_{50} = 10^{-5} M) of this enzyme [100]. The inhibition of thioredoxin reductase is suggested to cause an accumulation of the oxidized thioredoxin and subsequent activation of ASK1 and of ASK1-dependent MAP kinases. Among MAP kinase cascades, the system of JNK is the most sensitive to the activating effect of NO [101].

The NO effect on cells depends on the ratio of intracellular concentrations of NO and ROS. Depending on the cell type, NO can either stimulate or inhibit apoptosis and other cytotoxic effects associated with oxidative stress [44, 93]. An example of cooperation between NO and ROS are reactions between NO, oxygen, and superoxide with the resulting generation of peroxynitrite ($ONOO^-$) and other short-living intermediates (Fig. 6) [98, 102, 103]. When concentrations of NO and O_2^- are comparable, these two free radicals reciprocally neutralize each other. Because the rate of the bimolecular reaction of O_2^- with NO is three times higher than the rate of SOD-mediated dismutation reaction, NO competes with SOD for superoxide. The intracellular concentrations of NO and SOD are comparable and correspond to nanomolar levels. It seems that until a certain threshold, the generation of $ONOO^-$ is not dangerous for the cell because the damaging effect of $ONOO^-$ is lower than toxic effects of acting separately either O_2^- or high-dose NO [93]. During spontaneous transmutation, peroxynitrite is rather rapidly ($\tau_{1/2} \approx 10^{-6}$ sec) converted to nitrate [20]:

$$ONOO^- + H^+ \rightarrow OH^\bullet + NO_2^- \rightarrow NO_3^- + H^+. \quad (4)$$

Under conditions of a rather intense synthesis of $ONOO^-$, peroxynitrite oxidizes SH-groups, Fe-S-centers of proteins and other intracellular compounds [104]. The oxidation of iron-sulfur centers is accompanied by releasing of Fe^{2+} that promotes the generation of a short-lived nitrosonium ion, the synthesis of nitroso compounds, and reactions of transnitrosylation [24, 105]. If the concentration of NO is higher than the concentration of superoxide, NO reacts with $ONOO^-$ and facilitate the generation of active intermediates and the subsequent synthesis of nitrosothiols (Fig. 6) [103]. This suggests that at the low concentration O_2^- can promote NO to nitrosylation of glutathione and other compounds containing active SH-groups.

The ratio of the intracellular concentrations of NO and ROS determines the effect of these compounds on processes involving in the regulation of apoptosis. Under condition favorable for accumulation of peroxynitrite, NO stimulates apoptosis that is associated, first of all, with the activation of protein kinase JNK, factors p53 and Bax, releasing of cytochrome *c* from mitochondria, and subsequent activation of caspases. On the contrary, at the low level of O_2^-, nitric oxide causes the cGMP-dependent induction of apoptosis suppressors, such as HSP32, HSP70, and Bcl-2 proteins. Under conditions promoting the nitrosylation of proteins, NO inactivates caspase 3 which is one of key elements of the system responsible for apoptosis [106].

REACTIVE OXYGEN SPECIES, NITRIC OXIDE, AND cGMP

Guanylate cyclase is the most sensitive cellular receptor of NO. Whereas MAP kinases are up-regulated by products of NO oxidation, guanylate cyclase (a cytoplasmic isoenzyme) is directly activated by NO itself [107, 108]. Guanylate cyclase contains a heme prosthetic group with a high affinity for NO ($K_s \leq 250$ nM) [109]. The binding of NO leads to enzyme activation and cGMP synthesis. A cGMP-dependent serine-threonine protein kinase (PKG) phosphorylates and activates many intracellular targets including ion channels, transcription factors, and components of MAP kinase systems. The effect of PKG on MAP kinase cascades depends on the cell type. The activation of PKG in neuroendocrine cells treated by membrane-penetrating analogs of cGMP induces a strong activation of the MAP protein kinase JNK system and subsequent up-regulation of the transcription factors AP-1 and ATF-2 [110]. The activation of guanylate cyclase in smooth muscular cells and embryonic fibroblasts by NO and cGMP analogs leads to cGMP-mediated activation of protein kinase ERK and submitted transcription factors c-Fos and JunB [111, 112]. NO is also able to stimulate the cGMP-dependent activation of MAP kinase p38 that is typical for HeLa cells [87]. On the contrary, in BHK cells the activation of PKG suppresses the stimulating effect of growth factors on the activity of ERK, and this event is associated with the inhibition of the ERK cascade-controlling protein kinase c-Raf through phosphorylation of the Ser43 residue together with induction of the double threonine-tyrosine protein phosphatase MKP-1 [113]. Transcriptional activation of cGMP-dependent genes is mediated not only by MAP kinases but also by some other signal systems. Thus, PKG, similarly to the cAMP-dependent protein kinase PKA, provides the phosphorylation and activation of the transcription factor CREB [114, 115].

Besides NO, the activity of guanylate cyclase depends on the redox status of the cells. Guanylate cyclase contains active SH-groups, and their oxidative modification inhibits the enzyme. Guanylate cyclase functions as an NO-dependent O_2 sensor since molecular oxygen is able to oxidize heme nitrosyl complex Fe(II)–NO to NO_2^- and Fe(III) that reversibly inactivates the enzyme [116]. The reverse transformation of the heme iron of guanylate cyclase into the initial Fe(II) form is catalyzed by a NADPH-dependent oxidoreductase, which is homologous to methemoglobin reductase of erythrocytes [117]. Various ROS differently affect the activity of guanylate cyclase. Whereas superoxide is a potent inhibitor of guanylate cyclase, hydrogen peroxide increases its NO-dependent activation [118, 119].

At present, the most completely studied are the cellular signal systems that are based on phosphorylation and dephosphorylation of proteins by specific protein kinases and phosphatases. The protein kinase systems form branched chains with a complex organization that promotes amplification of the initial stimuli and fulfill coordination between numerous influences on the cell. In addition to protein kinases, other regulatory systems based on biochemical reactions other than protein phosphorylation are also known. The effect of these systems is based on the control on turnover of phospholipids and nucleotides, proteolytic degradation of proteins, changes in the ion gradients and membrane permeability, and at last is mediated by the mean of oxidative modification of redox-sensitive proteins and low-molecular-weight compounds. ROS play a role of signaling molecules produced by specialized cellular enzymatic systems under normal physiological conditions. An example of the ROS-dependent signal transmission is the coupled system of thioredoxin, Ref-1 factor, thioredoxin-dependent ASK1 protein kinase and submitted transcription factors AP-1 and NF-κB. Another example of ROS-dependent signaling is transcription factor HIF-1α.

NO is a redox-sensitive signaling molecule with a similar to ROS function. Like ROS (H_2O_2) , NO can penetrate through the cell membranes acting not only as intracellular signaling molecules but also as paracrine factors. In a differ to secondary messengers which are organic compounds, ROS and NO participate in chemical reactions with their specific receptors as well as with many other cellular components. Chemical reactions between NO and ROS generate active intermediates, and their effects on the cells differ from the effect of the initial compounds [93, 120]. Compared to other signal transduction systems, the signaling mediated by ROS and NO might be characterized by a higher reactivity and plasticity that is achieved by relative reduction of the specificity and selectivity of their effects. At the same time, equally ROS and NO are able to affect simultaneously a number of signal transduction pathways, such as MAP kinases, G-proteins, cGMP system, and transcription factors.

Apparently, many components of redox-sensitive regulatory systems have not yet been revealed. Some biochemical mechanisms underlying the ROS and NO signaling effects, such as the role of the amino acid environment on the sensitivity of cysteine residues to ROS and NO, mechanism of cysteine residues re-oxidation under physiological conditions, as well as compartmentalization and local concentrations of ROS and NO inside the cells and in extracellular medium adjacent to the plasma membrane are yet not elucidated in detail.

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