REDOX-SENSITIVE SIGNALING FACTORS AND ANTIOXIDANTS

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Abstract

The redox state, like the pH or the osmotic pressure, represents chemical characteristics of the intracellular environment. The intracellular redox homeostasis can be disturbed by the installation of the "oxidative stress", which manifests itself through the dysregulation of the balance between the systems that produce oxidant agents and the antioxidant defense mechanisms (the redox balance). Essential cellular functions, such as gene expression, are influenced by the balance between pro- and antioxidant conditions. The mechanism by which the transcription of specific eukaryotic genes can be redox regulated, is complex, however, recent findings suggest that redox-sensitive transcription factors play an essential role in this process. This review is focused on the recent knowledge concerning some eukaryotic transcription factors, whose activation and DNA binding is controlled by the thiol redox status of the cell.

Rezumat

Starea redox, ca şi pH-ul sau presiunea osmotică, reprezintă caracteristici chimice ale mediului intracelular. Instalarea stresului oxidativ care se manifestă prin dereglarea balanței dintre sistemele care produc agenți oxidanți şi mecanismele de apărare antioxidantă (balanța redox) poate perturba homeostazia redox intracelulară. Echilibrul dintre condițiile pro- şi antioxidante influențează una dintre funcțiile celulare esențiale, cum ar fi exprimarea genelor. Mecanismul prin care are loc reglarea transcrierii genelor specifice la eucariote este un proces complex, în care factorii de transcriere redox-sensibili joacă un rol important. În acest articol este prezentat modul de acțiune al unor factori de transcriere eucariotici, a căror activitate şi legare de ADN este controlată de statusul redox tiolic al celulei.

Key words: oxidative stress, antioxidants, thioredoxin, gene expression, transcription factors, glutathione.

Introduction

Different forms of cellular stress constitute primary signals that are transduced *via* the plasma membrane into the cytoplasm and ultimately

stimulate the expression of specific genes in the cells nucleus. Oxidative stress may be defined as an imbalance between oxidant-producing systems and anti-oxidant mechanisms (redox balance), which results in excessive formation of reactive oxygen species (ROS) [1]. Oxidative stress has been implicated in a large number of human diseases including diabetes, cancer and aging [2].

In order to counteract oxidative stress and to maintain redox balance, enzymatic and non-enzymatic anti-oxidant systems have evolved. The products of genes that are induced in response to oxidative stress may confer protection against subsequent adversities or serve to signal stress to neighboring cells [50, 51].

Accumulating evidence suggests that ROS are not only injurious by-products of cellular metabolism but also essential participants, in cell signaling and regulation [3]. Nitric oxide (NO) functions as a signaling molecule mediating vasodilatation when produced in low concentrations by the constitutive isoform of nitric oxide synthase (NOS) in vascular endothelial cells and as a source of highly toxic oxidants used for microbicidal killing when produced in high concentrations by inducible NOS in macrophages [4].

ROS, as signalling molecules, regulate the expression of genes whose products serve important functions in the immune response, proliferation control, and differentiation processes during a general pathogenic response. Altered ROS balance may activate some signalling pathways and inhibit others leading to altered gene expression. As a consequence, these may lead to various seemingly unrelated pathologies, depending on the specific cell or tissue and the site of their production.

Some research studies have identified several transcription factors (NF-kB, AP-1, p53,) that are activated by fluctuations of the intracellular ROS level. In this paper, we will briefly review the biochemical basis of ROS formation and the induction of oxidative stress in the cells and will describe molecular mechanisms of the activation of some eukaryotic transcription factors controlled by redox-dependent processes.

Reactive species and oxidative stress

Molecular ground-state oxygen can be activated to a ROS by means of energy transfer, forming singlet oxygen ($^1O_2$), or by electron transfer, forming "incomplete" reduction products, i.e., the superoxide anion radical ($O_2^{\cdot-}$). Small amounts of oxygen (between 0.4 and 4% of all oxygen consumed) are reduced to $O_2^{\cdot-}$ by the mitochondrial electron transport chain during the course of normal oxidative phosphorylation, which is essential for generating ATP. Subsequently, $O_2^{\cdot-}$ can be converted into other ROS and reactive nitrogen species (RNS) (Fig.1) [5]. Under normal conditions, $O_2^{\cdot-}$

molecules are quickly converted to H_2O_2 by the key mitochondrial enzyme, manganese superoxide dismutase (Mn-SOD) within the mitochondria and by copper and zinc (CuZn-SOD) in the cytosol [6].

H_2O_2 is then either detoxified to H_2O and O_2 by glutathione peroxidase (in the mitochondria) in conjunction with glutathione reductase, or diffuses into the cytosol and is detoxified by catalase in peroxisomes. The peroxiredoxin (Prdx) family of antioxidant enzymes uses redox-active cysteines to reduce peroxides, lipid hydroperoxides, and peroxynitrites [7]. H_2O_2 can also be converted to the highly reactive hydroxyl radical ($HO^{\cdot}$) in the presence of reduced transition metals such as Cu or Fe (Fenton reaction) [8].

Production of one ROS may lead to the production of others through radical chain reactions. As summarized in (Fig. 1) $O_2^{\cdot-}$ is produced by one electron reduction of oxygen by several different oxidases including NAD(P)H oxidase, xanthine oxidase, cyclooxygenase and even endothelial nitric oxide synthase (eNOS), under certain conditions [8, 9].

RNS include free radicals like $NO^{\cdot}$ and nitrogen dioxide ($NO_2^{\cdot}$), as well as non-radicals such as peroxynitrite ($ONOO^-$). $NO^{\cdot}$, also known as endothelium-derived relaxing factor (EDRF), produced from L-arginine by eNOS in the vasculature is considered a vasculoprotective molecule [10]. Some of the physiological effects may be mediated through the intermediate formation of *S*-nitroso-cysteine or *S*-nitroso-glutathione. However, $NO^{\cdot}$ easly reacts with $O_2^{\cdot-}$, generating the highly reactive molecule $ONOO^-$. Thus, variation in the production of $NO^{\cdot}$ and $O_2^{\cdot-}$ by endothelium, might provide one mechanism for the regulation of vascular tone and hence blood pressure.

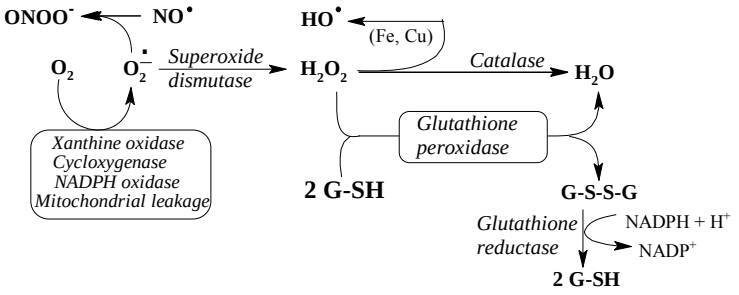

Figure 1 (figure adapted from [8])
Endogenous stimuli leading to ROS and RNS generation. The endogenous antioxidant enzymes: superoxide dismutase, glutathione peroxidase, and catalase function to maintain redox equilibrium (NADPH= nicotinamide adenine dinucleotide phosphate)
G-SH= reduced glutathione; G-S-S-G= oxidized glutathione

Although in the organism the oxygen pressure is reduced to the metabolic comfort limit and the free Fe^{2+} and Cu^{+} ions concentrations are rigorously controlled, the conditions for ROS formation can not be fully eliminated. Their uncontrolled formation in cells, i.e., the generation of a ROS load exceeding the antioxidant capacity of the cell, results in damage and oxidation of lipids, proteins, and nucleic acids, as well as various transcriptional factors [52].

Oxidant – Antioxidant (OA) balance

To prevent the deleterious effects of free radicals produced by normal metabolism, cells are equipped with an antioxidant system composed of enzymes such as: superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase and nonenzymatic substances such as: glutathione (GSH), thioredoxin (Trx), lipoic acid, ubiquinol, albumin, uric acid, copper and iron chelators like the proteins ceruloplasmin and transferrin, flavonoids, vitamins A, E and C [5, 11].

The role of antioxidants seems to be to sacrifice them in order to protect the essential cellular structures and molecules against ROS. In biological systems, OA are associated mainly with a lipophilic phase (e.g., membranes or lipoproteins such as vitamin E and ubiquinols) or can operate in aqueous phase such as ascorbate, GSH and Trx. Uniquely, lipoic acid as dihydrolipoate is able to interact with both phases and is a bridge between them. When present in sufficient concentrations, alpha lipoic acid acts as the anchor of the antioxidant network. In addition, these OA may act by coupling to protect both the membrane and cytoplasm.

Together with the glutathione system, Redox factor 1 (Ref-1) protein and Trx maintain, in the nucleus, the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. Oxidised glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of the oxidative stress of an organism. Reduced glutathione is regenerated most efficiently from oxidised form GSSG by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione reductase, glyoxalases and enzymes involved in leucotriene synthesis [12]. Glutathione is highly abundant in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) and is the major soluble antioxidant in these cell compartments. In the cell, there are many other redox couples: examples include NAD^{+}/NADH, ascorbate/dehydroascorbate, $NADP^{+}$/NADPH and alpha-lipoic acid (LA)/Dihidrolipoic acid (DHLA). DHLA is the driving force for maintaining high concentrations of the reduced antioxidant forms

of the major cellular antioxidants such as glutathione, Trx and ascorbic acid in the aqueous phase, and vitamin E and ubiquinol in membranes or lipoproteins. The amount of alpha lipoic acid naturally present in the body may not be adequate for obtaining the antioxidant benefits. Increasing the amount of alpha lipoic acid through dietary supplementation can improve this vital function [12, 13, 50].

Antioxidants scavenge free radicals, especially ROS, inhibit generation of ROS, inhibit metabolic activation of carcinogens, and alter the intracellular redox potential [14]. Redox state, in turn, regulates the activity of many transcription factors.

Redox and structural changes of transcription factors

The synthesis of new proteins is most frequently regulated at the transcriptional level by transcription factors binding to regulatory DNA sequences within target genes. Transcription factors are composed of two essential functional regions: a DNA-binding domain and an activator domain. The DNA-binding domain consists of amino acids that recognize specific DNA bases near the start of transcription. Transcription factors are typically classified according to the structure of its DNA-binding domain, which are of one of the following types: zinc fingers, helix-turn-helix, leucine zipper, helix-loop-helix, and high mobility groups. The interaction of transcription factors with the components of the transcriptional apparatus (RNA polymerase) and with other regulatory proteins, can be activated by physiological, therapeutical and pathological stimuli. Their sensibility to certain stimulus is variable and depends on protein structure and its capacity to be modified through phosphorilation, glicozilation or oxidation (depending on the intracelular redox state) [15].

Several transcription factors (nuclear factor kB (NF-kB), activator protein 1 (AP-1), protein 53 (p53), nuclear receptors (such as glucocorticoid receptor and estrogen receptor) contain conserved cysteine residues in their structure (sensitive to redox variation due to thiols groups) that ensure their interaction with DNA specific binding regions. These interactions occur through hydrogen bonds formation between free thiols of transcription factors and DNA azotic bases, intra- or intermoleculare disulfide bonds (which ensure favorable conformation for DNA binding of transcriptional factors), cations coordination (specially zinc, that determine zinc-finger motifs formation in the structure of transcription factors, favoring its interaction with DNA) (Fig. 2) [16].

Emerging data suggest that oxidation of protein-SH (formation of sulfenic −SOH, sulfinic $-SO_2H$, and sulfonic acids$-SO_3H$) as in AP-1 transcription factor leads to changing the protein ability to bind to DNA

specific sequences [17]. Also, unfavorable intra- or intermolecular disulfide bonds formation as for glucocorticoids receptors (GR) can prevent transcription factor dimerization or change its conformation influencing the interaction with DNA (Fig.2).

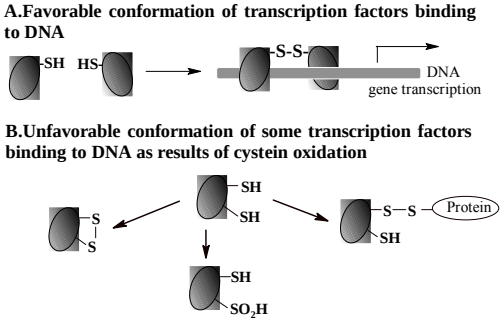

Figure 2 (figure adapted from [17])
Redox regulation. Oxidation may activate or inactivate protein functions

The Nuclear Factor kB (NF-kB), and p53 are among the most studied transcription factor families. They have been identified as important components of signal transduction pathways leading to pathological outcomes such as inflammation and tumorogenesis.

NF-kB

The heterodimeric protein NF-kB, is a ubiquitous redox-sensitive transcription factor involved in the control of a large number of normal cellular processes, such as immune and inflammatory responses, cellular growth, and apoptosis [18]. Dysregulated NF-kB activity occurs in a number of chronic inflammatory diseases and certain types of cancers making NF-kB signaling an attractive target for the development of anti-inflammatory and anti-cancer drugs. Several studies have identified NF-kB as a critical component to bridge inflammation and cancer. The role of NF-kB in carcinogenesis was further corroborated with the fact that NF-kB antagonizes the function of the tumor suppressor protein p53 [19].

The DNA binding, nuclear form of NF-kB is a protein heterodimer made up of p-65 and p-50 subunit, but other hetero- and homodimeric species are found [20, 21]. In non-stimulated cells, NF-kB exists as an inactive cytoplasmic complex with inhibitory kB (IkB) proteins. The transcriptional activation of NF-kB has two distinct steps with respect to the cellular compartment. One step is IkB degradation in the cytoplasm and following nuclear translocation of the complex. Another step is DNA binding of the complex and transactivation in the nucleus [22].

Extracellular stimuli such as pro-inflammatory cytokines (TNFα and IL-1β), ionizing radiation or ROS and mitogens lead to activation of the IkB kinase complex, IKK, which phosphorilates IkB on two serine residues, and this, targets IkB for ubiquitination and subsequent degradation by the proteasomes (Fig.3). Dissociation of IkB from NF-kB-IkB complex allows the activated free dimmer p50/p65 to translocate to the nucleus, where it binds to NF-kB element located in the promoter or enhancer regions of target genes, controlling their expression [23]. NF-kB may be also activated independently of IkB_α degradation by phosphorylation of IkB_α at tyrosine residues rendering this protein resistant to proteasomal degradation but rather facilitating its dissociation from NF-kB [24]. ROS appear to control IkB phosphorylation which may explain why NF-kB activation is blocked by a host of antioxidants.

The redox control of NF-kB DNA-binding

NF-kB was the first transcription factor recognized to be redox-regulated in eukaryotic cells [25]. It has been shown that, whereas oxidants enhance NF-kB nuclear translocation, oxidation of NF-kB decreases its DNA binding activity. In the nuclear compartment, for NF-kB to bind to DNA, a cysteine residue in the DNA binding region of the p50 subunit (cysteine 62) must be in a reduced state [26]. P50 Cys62 is highly oxidized in the cytoplasm, but is rapidly reduced once NF-kB has migrated into the nucleus. This cysteine has been shown to participate in intermolecular disulfide formation [27].

Several enzymes have been reported to control the reduction of nuclear p50 Cys62. Thioredoxin (Trx), a small, ubiquitous protein with two redox-active half-cysteine residues in its catalytic active center, mainly localized in the cytoplasm, translocates into the nucleus upon cellular stimulation by TNFα, where it reduces p50 Cys62 in order to promote NF-kB DNA binding [28]. Recently it has been shown that APE1/Ref-1 ratio (where APE1 means apurinic endonuclease 1) can regulate the DNA binding activity of NF-kB through promoting the reduction of p50 Cys62 by antioxidant proteins, such as GSH and Trx [29]. Nitric oxide (NO) can also modify either p50 C62 or p65 Cys38 by S-nitrosylation thereby inhibiting NF-kB DNA binding activity [30]. It is though that p50 C62 S-nitrosylation may represent a negative regulation mechanism of NF-kB activation since NOS, which produces $NO^{\cdot}$, is NF-kB-dependent gene. It is suggested that $NO^{\cdot}$ produced by NOS represents a negative feed-back mechanism to avoid excessive inflammation [31].

NF-kB is highly activated at the sites of inflammation in different diseases and induces the expression of mRNA of a variety of pro-

inflammatory mediators including tumor necrosis factor (TNF-α), interleukins (2 and 8), adhesion molecules (ICAM-1 and VCAM-1) and enzymes (like cyclooxygenase 2 and inducible nitric oxide synthase (iNOS) [32].

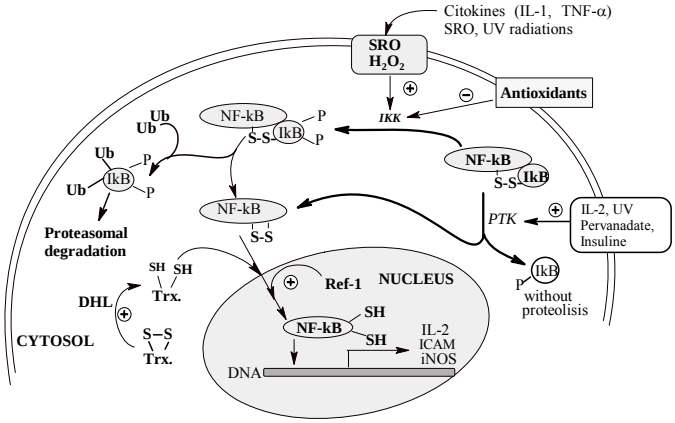

Figure 3 (figure adapted from [22])

NF-kB mediated signaling pathway. Exposure of cells to oxidative and proinflammatory stimuli causes activation of a series of upstream kinases such as IKK which then activate NF-kB by phosphorylation-mediated degradation of IkBα. Activated upstream tyrosine kinases (PTK) may also phosphorylate IkBα at tyrosine residues rendering this protein resistant to proteasomal degradation but rather facilitating its dissociation from NF-kB. Free activated NF-kB, in the form of p 65-p 50 heterodimer, translocates to the nucleus, where it binde to kB sequences located in the promoter of the target genes. Thioredoxin (Trx) reduced in cytosol by dihidrolipoic acid (DHL) and redox factor 1 (Ref-1) function to regulate the redox status of NF-kB in the nucleus. Thioredoxin translocates to the nucleus concomitant with NF-kB activation and may physically interact with the p50 subunit prior to DNA binding; Ub= ubiquitin

NF-kB cysteine residues are the target of several anti-inflammatory and anti cancer agents. Eriocalyxin B (Eri-B) isolated from *Isodon eriocalyx*, an asiatic herbal plants used in traditional medicine, was reported to inhibit the NF-kB activation by blocking the binding of NF-kB to its response element possibly through the reversible interaction with the two NF-kB subunits, p65 and p50, at an allosteric site. It suppressed the transcription of NF-kB downstream gene products including cyclooxygenase-2 and inducible nitric-oxide synthase induced by tumor necrosis factor-α or lipopolysaccharide in macrophages and hepatocarcinoma cells [33].

Soy isoflavones, a component a soybeans, decrease the amount of APE1/Ref-1 available to reduce NF-kB in prostate cancer cells, which enhances the therapeutic effect of radiotherapy in prostate cancer [34].

Methylglyoxal (MG) is a cytotoxic metabolite produced *in vivo* mainly from glycolysis. Increased production of methylglyoxal can be induced by TNFα and occurs in a number of pathological conditions, including diabetes and neurodegenerative disorders. MG suppresses NF-kB activation by targeting p65 Cys38 [35]. This may represent a therapeutically relevant feedback mechanism decreasing NF-kB activation.

P53

The p53 protein is an ubiquitous multifunction, zinc-binding transcription factor that is activated in response to multiple forms of stress (ionizing radiation, oxidative base modification by ROS, nucleotide deprivation, etc.) [36]. The interest in p53 is engendered not by an essential role for p53 during normal growth and development, but by the contribution of p53 to tumor suppression [37]. P53 exerts its tumor suppressor function mainly through transcriptional induction of target genes involved in several processes, including cell cycle checkpoints, apoptosis, and regulation of cell redox status. The best described mechanism is its ability to modulate gene expression [38]. P53 binds directly and specifically as a tetramer [39] to target sequences of DNA (p53-responsive elements) and promotes transcription of a large number of target genes including expression of some well-known antioxidant genes, glutathione peroxidase, mitochondrial SOD2, and mammalian sestrin homologs SESN1 and SESN2, which are involved in regeneration of overoxidized peroxiredoxins [40, 41].

In response to stress signals, p53 activity is modulated by post-translational modifications, including phosphorylation, acetylation, methylation and ubiquitination that affect its stability, interaction with the negative regulator Mdm2 protein (where Mdm2 means the murine double minute) and other proteins, and its ability to interact with DNA [42, 43]. The strength of p53 binding to a specific DNA sequence is influenced by the redox state of the protein which modulate its conformation [44]. The protein exists in a latent conformation (inactive for DNA binding) and in an active conformation, where it binds to DNA. There are several cysteine residues in the central domain of the p53 protein. Some of these residues are crucial for the p53 protein binding to the specific DNA sequence. A reduced state is required for these cysteine residues to ensure that p53 protein would bind to specific consensus DNA and transactivate target genes [45]. *In vivo,* oxidative conditions decrease the specific affinity of p53 for certain promoters.

Three of these cysteine residues are involved in zinc coordination [46]. Therefore, p53 has a double redox sensibility. Most p53 mutations found in human tumors are located in the DNA-binding domain and involve

amino acid residues that contact DNA or are required for proper folding [47]. Links between ROS and p53 activity have previously been reported [48]. Activated p53 increases cellular ROS by enhancing the transcription of proapoptotic genes. Moreover, scavenging of ROS by antioxidant therapy, decreases apoptosis induced by p53. It has been shown that the role of p53 is contextually controlled: low amounts of p53 suppress ROS and protect against oxidative DNA damage and genomic instability under normal physiological conditions but high p53 expression promotes ROS accumulation in conditions of profound pathological stress [49].

Conclusions

The regulation of gene expression by redox state has promising therapeutic implications. The binding sites of the redox-regulated transcription factors NF-kappa B (NF-kB) and p53 are located in the promoter region of a large variety of genes that are directly involved in the pathogenesis of diseases, e.g. cancer, atherosclerosis, AIDS and diabetic complications. The role of ROS as signal transducing molecules has been supported by many observations. Biochemical and clinical studies have indicated that antioxidant therapy may be useful in the treatment of many diseases. Many basic events of cell regulation such as protein phosphorylation and binding of transcription factors to consensus sites on DNA are driven by physiological oxidant-antioxidant homeostasis, especially by the thiol-disulfide balance. Endogenous glutathione and thioredoxin systems may be considered to be effective regulators of redox-sensitive gene expression. The modulation of afore mentioned transcription factors by antioxidative and anti-inflammatory phytochemicals would provide important opportunities for chemoprevention based on molecular targeting.

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