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Review

NF-κB signaling pathway and free radical impact

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The activation of NF-κB transcription factor is critical for a wide range of processes such as immunity, inflammation, cell development, growth and survival. It is activated by a variety of stimuli including cytokines, ionizing radiation and oxidative stress. Redox modulations of NF-κB pathway have been widely demonstrated. Studies carried out during last years have advanced our knowledge about possible connections between NF-κB pathway and the impact of free radicals. This review is an endeavor to gather recent results focused on this issue, although an important question, whether oxidative stress plays a physiological role in NF-κB activation, seems to be still unanswered.

Keywords: NF-κB transcription factors, reactive oxygen species, nitrogen oxygen species

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INTRODUCTION

Since 1986, when Sen and Baltimore discovered the NF-κB transcription factors (Sen & Baltimore, 1986), there have been a lot of studies taking into consideration the role of NF-κB proteins and the possible ways or factors taking part in their regulation (Toledano & Leonard, 1991; Li & Karin, 1999; Gloire & Piete, 2009). Free radicals are well known as factors, which modulate the activity of many metabolic pathways. Activation of the NF-κB transcription factor is critical for a wide range of physiological processes, including immunity, inflammation, cell growth and survival, development and proliferation (Barket & Gilmore, 1999; Bonnizi & Karin, 2004; Shishodia & Aggarwal 2004). Even though during the recent years many laboratories have been trying to answer the question whether NF-κB is the sensor of oxidative stress, this issue remains unexplained, in spite of an extensive range of studies. This review is an attempt to gather current knowledge about the possible ways of the free radical impact on the NF-κB regulation.

NF-κB

Although NF-κB was initially identified in activated B cells, it rapidly appeared that this transcription factor exerts many biological functions in essential processes, such as both the innate and adaptive immunity, cell survival, and inflammation, also in other cells (Ghosh & Karin, 2002). The NF-κB transcription factor regulates expression of hundreds of genes that are involved in cell growth regulation, differentiation and development (Morgan & Liu, 2011), and can activate a great number of genes involved in stress responses, inflammation, and apoptosis (Wang *et al.*, 2002). Although much evidence strongly supports the anti-apoptotic function of NF-κB (Barkett & Gilmore, 1999; Kucharczak *et al.,* 2003; Lin & Karin 2003), there are a few reports suggesting that activation of this signaling pathway could lead to induction of pro-apoptotic molecules (Kasof *et al.*, 2001; Ravi *et al.*, 2001). This dimeric transcription factor is composed of different members of the Rel family, such as p65 (RelA), p50, p52, c-Rel and RelB. The mammalian NF-κB protein family contains five members: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel. These proteins share an evolutionary conserved domain called Rel-homology domain (RHD) or Rel-homology region (RHR). The RHD comprises domains for dimerization, DNA binding, and nuclear localization (Basak & Hoffmann, 2008; Gloire & Piette, 2009). NF-κB dimers bind to the promoters of a diversity of genes at sequences known as κB elements, whose consensus was defined as 5'GGGRNWYYCC3' (N: any base; R: purine; W: adenine or thymine; and Y: pyrimidine). Three Rel members of the family, RelA, RelB, and c-Rel have a C-terminal transcription activation domain (TAD) that serves to positively regulate gene expression. The two other mammalian NF-κB proteins are synthesized as larger p100 and p150 precursor proteins, which have C-terminal ankyrin repeats that inhibit DNA binding until processed to the smaller p50 and p52 products. These proteins are also characterized by the lack of TAD domains, and they repress transcription unless associated, as heterodimers, with other proteins from the Rel family (Morgan & Liu, 2011). Five related mammalian NF-

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Abbreviations: APE1/Ref, AP endonuclease1/redox factor 1; BAFF, B cell activating factor; COX-2, cyclooxygenase 2; CS, cigarette smoke; CuZn-SOD, Cu,Zn-superoxide dismutase; Cys, cysteine; ERK, extracellular-signal regulated kinase; FKHRL1, forkhead transcription factor FOXO3a; GSH, glutathione; HDAC, histone desacetylase; HIF, hypoxia-inducible factor; ICAM-1, intracellular adhesion molecule 1; IκB, inhibitor of κB; IKK, IκB-kinase; IL-1, interleukin-1; IL-6, interleukin 6; IL-8, interleukin 8; IFN-γ, interferon γ; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LTβ(R), lymphotoxin β (receptor); LPS, lipopolysaccharide; MAP, mitogen-activated protein; MAPKKK, MAPK kinase kinase; MCP-1, monocyte chemoattractant protein 1; MEKK1, mitogen-activated protein kinase kinase kinase 1; MHC, major histocompatibility complex; MIP-1α, macrophage inflammatory protein 1 α; MnSOD, manganese superoxide dismutases; MSK-1-2, mitogen and stress activated protein kinases 1-2; NAC, *N*-acetylcysteine; NAK, NF-κB activating kinase; NEMO, IκB kinase γ; NF-κB, nuclear factor-kappa b; NIK, NF-κB-inducing kinase; NLS, nuclear localization sequence; NO·, nitric oxide; NOS2, NO synthase 2; PH, Pleckstrin Homology; PHD1, PHD2, prolyl hydroxylases 1 and 2; PKAc, protein kinase A; PKD, protein kinase D; PEST, proline-, glutamic acid, serine-and threonine rich polypeptide sequences; RHD, Rel homology domain; RHR, Rel homology region; RNS, reactive nitrogen species; ROS, reactive oxygen species; SODs, superoxide dismutases; TAD, transcription activation domain; TAK1, TGFβ-activated kinase 1; TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; TRAFs, TNF receptor associated factors; TRX, thioredoxin; VCAM-1, vascular cell adhesion molecule 1.

κB proteins can potentially form 15 different homo- or heterodimeric complexes (Hoffmann & Baltimore, 2006). Almost all NF-κB proteins are capable of homodimerization or heterodimerization with the other NF-κB proteins, while the RelB protein is an exception and can only form heterodimers. Unlike most transcription factors, proteins of this family reside in the cytoplasm in a resting state through interaction with IκB inhibitory proteins (IκBs), and must be activated and translocated into the nucleus in order to function (Schoonbroodt & Piette, 2000). The IκB proteins contain multiple copies of the ankyrin repeats, which interact with the RHD of Rel/NF-κB proteins, thereby covering their nuclear localization sequence (NLS). Several members of the IκB family proteins have been identified, including IκB α, β, δ, ε and Bcl-3. The NF-κB/IκB interaction is disrupted by modification of IκB phosphorylation sites in response to stimulation. Phosphorylation of IκB results in its ubiquitination and degradation, while NF-κB is activated and translocated from the cytoplasm to the nucleus (Kabe *et al.*, 2005).

WAYS OF NF-κB ACTIVATION

NF-κB pathway may be activated via at least two distinct routes named the canonical and the noncanonical pathway, respectively (Oliver *et al.*, 2009). Some authors have distinguished another route, so that we can also consider three ways of NF-κB activation: the classical, atypical and alternative pathway (Gloire & Piette, 2009). The canonical (classical) pathway depends upon the activation of the IκB kinase (IKK) complex consisting of IKKα, IKKβ, and IKKγ (also known as NEMO), and usually results in nuclear localization of the p65/p50 dimers. IKKα and IKKβ have catalytic properties, while the third polypeptide, IKKγ, has a regulatory nature. This pathway relies on the phosphorylation of IκBα on serine 32 and 36, that leads to ubiquitination on a specific lysine residue. Ubiquitinated IκBs are directed to the proteasome for complete degradation, enabling the free p50/p65 heterodimers to enter the nucleus (Gloire & Piette, 2009). As it was shown, neither phosphorylation nor ubiquitination is sufficient to dissociate IκBs from the RelA/p50 heterodimers, and proteasomal degradation of IκBs is required for translocation of these dimers (DiDonato *et al.*, 1995). NF-κB activation is an example of feedback inhibition, due to genes encoding IκBα and IκBε, which are also NF-κB target genes. Therefore, NF-κB activation generates inhibition of the pathway (Basak & Hoffmann, 2008). The newly synthesized IκBα is able to enter the nucleus, remove NF-κB from its DNA-binding sites, and transport it back to the cytoplasm (Ghosh & Karin, 2002). These processes lead to the termination of the NF-κB-dependent gene transcription.

In most cases IκBα phosphorylation is dependent on IKKβ activity. The classical pathway is activated by a diverse set of stimuli including proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukins (IL-1 β, IL-6), bacterial lipopolysaccharide (LPS), DNA damaging agents (camptothecin, daunomycin), Toll-like receptor (TLR) agonists and**,** in some cell types**,** reactive oxygen species (Fig. 1). Classically activated NF-κB controls cell proliferation, differentiation, apoptosis, immunity and stress responses (Dejardin, 2006; Gloire & Piette, 2009).

In the alternative pathway the IKKα protein plays a pivotal role, and this type of activation is completely independent of IKKβ and IKKγ. This pathway is activated by a subset of tumor necrosis factor superfamily receptors (TNFSFRs) such as lymphotoxin β receptor (LTβR), B-cell activating factor receptor (BAFF) or CD40, and is dependent upon activation of IKKα homodimers by NF-κB-inducing kinase (NIK). Albeit all of these receptors belong to the TNFR family, it was shown that TNFR1 does not activate the alternative pathway (Bonizzi & Karin, 2004). Proteins of the TRAF family enable the recruitment of NIK. Although NIK is continuously synthesized, in resting cells it undergoes constitutive degradation via a TRAF3-dependent mechanism (Qing *et al.*, 2005). Ligand stimulation induces TRAF3 degradation and NIK stabilization, thus allowing NIK to phosphorylate and activate the IKKα. It was suggested that NIK might be able to induce the activation of both the noncanonical and the canonical IKK complex. Evidence provided by Zarnegar *et al.* have demonstrated that TRAF3 potently suppresses canonical NF-κB activation and gene expression *in vitro* and *in vivo* (Zarnegar *et al.*, 2008). Additionally, the authors revealed that in TRAF3 deficient cells accumulation of NIK was the source of deregulation of the canonical pathway. Activation of NF-κB through the alternative pathway leads to the processing of p100 to the mature p52 form and to nuclear localization of RelB/p52. This process is generally slower than the activation of the classical pathway but is important for secondary lymphoid organ development, B cell survival and homeostasis, adaptive immunity, and osteoclastogenesis (Dejardin, 2006).

The atypical NF-κB activation occurs in response to phosphorylation of IκBα on Tyr 42 or on serine residues in the IκBα PEST do-

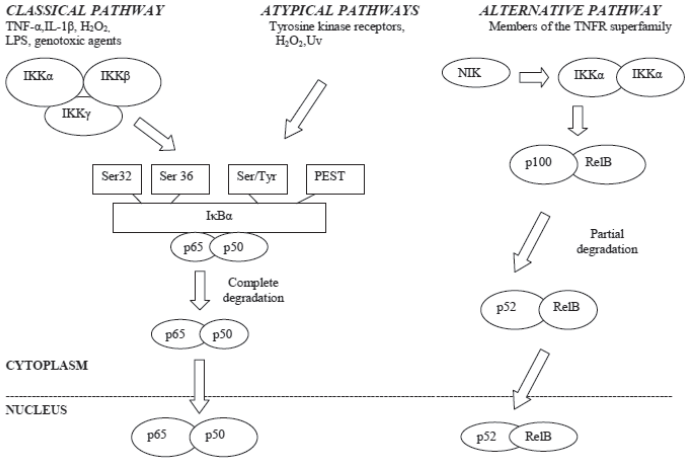

Figure 1. Pathways of NF-κB activation.
The most known, classical way of activation involves activity of IKK, subsequent phosphorylation of IκBα on Ser 32 and Ser 36, IκBα ubiquitination and degradation. Classically activated NF-κB controls cell proliferation, differentiation, apoptosis and immune and stress responses. The atypical NF-κB pathway relies on IκBα Tyr 42 or Ser and Thr phosphorylation in the PEST region, without IKK activation. This pathway could be caused by hypoxia/reoxygenation or stimulation of a tyrosine kinase receptor, pervanadate, UV irradiation, and in some cell lines, by H_2O_2. In the alternative pathway the activation occurs *via* NIK and IKKα and leads to p100 phosphorylation, resulting in p52 translocation into the nucleus in a complex with RelB. This pathway is activated by LTβ, BAFF or CD40 (members of the TNFR superfamily). Activation through this pathway is important for secondary lymphoid organ development, homeostasis and adaptive immunity.

main (Gloire & Piette, 2009). In this way the atypical activation is IKK-independent and could be caused by hypoxia/reoxygenation or stimulation of tyrosine kinase receptor, pervanadate, UV irradiation and, in some cell lines, by H_2O_2 (Gloire *et al.*, 2006; Gallagher *et al.*, 2007). Tyrosine phosphorylation causes IκBα degradation and dissociation from NF-κB.

Because NF-κB can be activated in many cells by a diverse set of stimulating agents with redox regulation properties, reactive oxygen species have been proposed to be involved in the activation of the NF-κB pathway (Schreck *et al.*, 1991). This model was found not to be universal, since a relation between NF-κB activation and generation of intracellular reactive oxygen species was only detected in certain cell lines (Schoonbroodt & Piette, 2000) and it seems to be highly cell type-dependent. Although Hayakawa *et al.* provided evidence that ROS did not mediate NF-κB activation (Hayakawa *et al.*, 2003), reactive oxygen species are still considered as second messengers that are implicated in NF-κB pathway modulation (Kamata *et al.*, 2002; Storz & Toker 2004; Gloire *et al.,* 2006; Jamaluddin *et al.*2007). NF-κB activation has a dual and opposite dependence on oxidative events, which seems to be connected with cellular localization of the affected steps as wells as with the duration of the exposure to the oxidative factor (Byun *et al.*, 2002).

OXIDATIVE STRESS ROS/RNS

Molecular oxygen is indispensable for aerobic organisms in the respiration process. In spite of its necessity for living, the respiration process could be harmful due to the formation of reactive oxygen species (ROS). Reactive oxygen/nitrogen species are produced in living cells not only by normal metabolism, but they also arise from pathophysiological processes and extracellular sources, such as UV and γ-irradiation. The group of moieties termed reactive oxygen species include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and their molecular by-products (e.g. hydrogen peroxide H_2O_2). Reactive nitrogen species (RNS) comprise nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$). To maintain redox homeostasis cells have developed a series of mechanisms, which include nonenzymatic and enzymatic antioxidants (Gloire & Piette, 2009). Several types of intracellular antioxidant molecules, such as glutathione (GSH), catalase, superoxide dismutases (SODs), thioredoxin (TRX) and thioredoxin reductase exist to protect cells from oxidative damage (Kabe *et al.*, 2005).

Moderate levels of certain ROS produced as a consequence of electron transfer reactions in mitochondria, peroxisomes and cytosol can be scavenged by cellular defense systems or may regulate normal physiological pathways and cellular functions. High levels of ROS are toxic for cells and lead to apoptosis and/or necrosis. When ROS production overwhelms the antioxidative defense, the state referred to as oxidative stress occurs (Sies & Cadenas, 1985). An appropriate cellular response to oxidative stress is critical in order to prevent further oxidative damage and to maintain cell survival. It is generally accepted that reactive oxygen species generated during oxidative stress are important mediators in the expression of genes involved in the antioxidant defense and inflammatory processes. Reactive oxygen species are able to trigger both the apoptotic and necrotic cell death depending on the severity of the oxidative stress (Saito *et al.*, 2006). One of the possible factors which are responsible for the maintenance of the balance between cell life and death is the activity of the NF-κB pathway. Although the expression of NF-κB target genes in most events promotes cell survival, nevertheless there are some exceptions when NF-κB activation may lead to cell death. Therefore ROS could modulate NF-κB response and NF-κB target gene products could attenuate ROS to promote survival (Morgan & Liu, 2011). In multicellular organisms, activation of the apoptotic program in response to oxidative stress is considered as a mechanism preventing seriously damaged cells from accumulating DNA mutations that could lead to cancer or other diseases. The fragile balance between life and death in that case could depend on a proper activation of the NF-κB pathway. The antiapoptotic activity of signaling proteins is crucial in numerous human diseases (Bubici *et al.*, 2004).

ROS/RNS are highly reactive and can easily react with biological macromolecules, resulting in lipid peroxidation, oxidation of amino acid side chains (especially cysteine), formation of protein-protein cross-links and DNA damage (Li & Karin, 1999). Although there is a plethora of works devoted to these issues, the matter of how the redox events regulate the NF-κB pathway is still unclear. Among the enormous variety of changes, which can be caused by ROS/RNS, their effect on the phosphorylation/dephosphorylation and oxidation of thiol residues appears to play a critical role in ROS-induced modification of the NF-κB signaling pathway. Phosphorylation and dephosphorylation processes are crucial in the activation of preexisting NF-κB and IκB proteins, as well as in the activation of factors responsible for de novo transcription of the NF-κB and IκB genes. Phosphatases play an important role in NF-κB regulation by influencing kinases or directly dephosphorylating IκBs (Wang, Zhang, & Li, 2002), whereas oxidation of protein thiols has structural, conformational, and direct catalytic consequences. Cysteine residues can be modified in many different ways such as: (i) formation of inter -or intramolecular disulfide bonds, (ii) oxidation of the cysteine sulfhydryl to sulfenic acid (R-SOH) or to higher order sulfinic ($R\text{-}SO_2H$) or sulfonic ($R\text{-}SO_3H$) acids, (iii) formation of mixed disulfides with small intracellular thiols (e.g. glutathione, GSH) through a process known as glutathionylation, glutathiolation or S-thiolation; (iv) modification by reactive nitrogen species: S-nitrosylation (Cross & Templeton, 2004b).

REDOX REGULATION OF NF-κB PATHWAY

According to numerous data there are still many inconsistencies concerning the influence of oxidative stress on NF-κB activity. ROS can modulate NF-κB activity both positively and negatively. Reduction/oxidation control of both the cytoplasmic and nuclear steps of NF-κB activation involves IκBs degradation, NF-κB DNA binding, NF-κB transcriptional activity, and chromatin remodeling (Gloire & Piette, 2009). Because many stages of the aforementioned processes have been shown to be prone to the ROS impact, it is worthy to point out their main elements which can be regulated by ROS.

IκB KINASE (IKK)/IκBS PATHWAY

The IκBs (inhibitors of NF-κB) are the central elements of the canonical (classical) pathway. In unstimulated cells NF-κB is locked in cytoplasm by binding with IκBs. Upon stimulation, iterative intracellular path-

ways are activated, leading to changes in the IκBs kinase (IKK) complex, and finally to IκB phosphorylation at specific amino acid residues by IKK. The major function of IKK is to connect proinflammatory stimuli and signals generated by pathogen-associated molecular patterns to the activation of the NF-κB transcription factor. IKKβ is the major kinase that leads to phosphorylation-induced NF-IκBs degradation. IKKα and IKKβ are structurally related but functionally distinct polypeptides that contain a serine/threonine kinase domain at their N-terminus and protein-protein interaction motifs including a leucine zipper (LZ) and a helix-loop-helix (HLH) at their C-terminal part (Rothwarf & Karin, 1999). IKKγ contains several coiled-coil motifs that mediate its oligomerization.

A vast majority of studies on oxidant-induced NF-κB activation have used H_2O_2 as the main source of ROS (Oliveira-Marques *et al.*,2009; Dudek *et al.*, 2001). The direct evidence that ROS can influence the NF-κB pathway was provided by using H_2O_2 and cultured cells (Schreck *et al.*, 1991, Byun *et al.*, 2002). Studies concerning ROS influence on NF-κB activity have shown that H_2O_2 can act as an activator of IKKs or can inactivate these proteins, probably depending on the cell-type (Kamata *et al.*, 2002; Korn *et al.*, 2001). According to some results, H_2O_2 can be a factor that potentiates dimerization of IKKγ/NEMO by inducing the formation of disulfide bonds between Cys54 and Cys347 (Herscovitch *et al.*, 2008). Although the authors showed that H_2O_2 could modulate the NEMO monomer vs. dimer ratio, implicating that NEMO was positively regulated by ROS, it is most likely that H_2O_2 inhibits IKK activation by directly affecting IKKβ. This report was the first demonstration that NEMO also contains redox-sensitive cysteine residues. Another study has shown that H_2O_2 is able to potentiate phosphorylation of serine residues in the activation loops of IKK in HeLa cells (Kamata *et al.*, 2002). Activation of NF-κB in HeLa cells through IKK was also shown in another study (Storz *et al.*, 2004). The authors emphasized the role of IKKβ in response to oxidative stress. In this case the role of the serine/threonine kinase protein kinase Dδ (PKD) seems to be the most important. NF-κB can be regulated e.g. via tyrosine phosphorylation of IκBα or *via* the canonical IκB kinase (IKK) complex. The serine/threonine kinase protein kinase D (PKD) promotes NF-κB activation leading to protection of HeLa cells from oxidative stress-induced death. The authors showed that, following the oxidative stress, phosphorylation of protein kinase D (PKD) at Y463 in the Pleckstrin Homology (PH) domain was mediated by the Src and Abl tyrosine kinase signaling pathway. Full activation of the kinase was achieved by additional phosphorylation of PKD activation-loop residues S738/742 in the catalytic domain. PKD activates NF-κB through the IKK complex, mainly IKKβ, which leads to IκBα degradation (Storz & Toker, 2003). Additionally, tyrosine (Tyr42) phosphorylation of IκBα has been shown to mediate NF-κB activation following hypoxia/reoxygenation (H/R) or pervanadate treatment in Jurkat T cells (Imbert *et al.*, 1996; Livolsi *et al.*, 2001). This pathway is different from the canonical proinflammatory pathways, which mediate NF-κB activation through serine phosphorylation of IκBα by the IKK complex. Results of those works demonstrated that pervanadate or H/R treatment led to tyrosine phosphorylation of IκBα and that activation of NF-κB was IKK-independent. The influence of reactive oxygen species on NF-κB activation was also demonstrated in pyropheophorbide-mediated photosensitization of endothelial cells. The authors have shown that singlet oxygen was important in NF-κB activation, and during this process IKKs were not activated by photosensitization but required an intact tyrosine residue at position 42 on IκBα (Volanti *et al.*, 2002).

IKK activity depends on its phosphorylation, and is inhibited by protein phosphatase 2A (DiDonato *et al.*, 1997). Due to the essential role of IKKs in NF-κB activation these peptides are also subject to negative regulation in order to prevent activation of NF-κB. Indeed, in some cells H_2O_2 has been shown to inactivate IKK. This inhibitory effect may be mediated by ROS-mediated oxidation of IKKβ on cysteine 179, which in turn leads to inactivation of its kinase activity and reduction of NF-κB signaling (Reynaert *et al.*, 2006). Additionally, it has been found that oxidation of Cys-179 occurs upon the action of anti-inflammatory prostaglandins PGA and 15-PGJ_2, providing a proof for oxidation under physiological circumstances (Rossi *et al.*, 2000).

Korn *et al.* (2001) have revealed that exposure of mouse alveolar epithelial cells to H_2O_2 was not sufficient to activate IKK, degrade IκBα, or activate NF-κB. Additionally, in the presence of H_2O_2, the ability of TNFα to induce IKK activity was significantly decreased thus precluding IκBα degradation and NF-κB activation. As the authors have explained, the observed reduction in IKKβ activity was due to oxidation of cysteine residues in the IKK complex.

More than 20 transcription factors have been described to be to some degree sensitive to oxygen species, of which the hypoxia-inducible factor (HIF) is the best studied example (Oliver *et al.,* 2009). Recent studies have indicated that the same hydroxylases which confer oxygen sensitivity to the HIF pathway may also play a role in oxygen sensing in the NF-κB pathway. Specifically, two prolyl hydroxylases PHD1 and PHD2 seem to act as repressors of the canonical NF-κB pathway through mechanisms, which could include direct hydroxylation of IKKβ (Cummins *et al.*, 2006).

Duration of the exposure to the oxidative agents is of much importance. As it was shown in the article by Wu *et al.* (2009), in human lens, epithelial cells sustained exposure to physiologically relevant levels of H_2O_2 and did not cause degradation of IκB and activation of the NF-κB pathway. These conditions have resulted in attenuation of the TNFα-induced IκB degradation and activation of NF-κB. Additionally, it was demonstrated that the impairment of NF-κB activation by sustained oxidative stress was due to the inactivation of proteasome (Wu *et al.*, 2009). These results also emphasize that, whereas a sustained oxidative stress may impair NF-κB activation, transient exposure to such conditions leads to activation of this pathway. According to this work a low level of oxidative stress can stimulate NF-κB activation, while higher levels may lead to the inhibition of activation.

MAP KINASE PATHWAY/JNK

Regulation of NF-κB activity has been shown to occur through the activation of upstream protein kinases including NIK (NF-κB-inducing kinase), MEKK1 (mitogen-activated protein kinase kinase kinase 1), NAK (NF-κB activating kinase), and IKKε/I, all of which mediate NF-κB activation by converging on the IKK (IκB-kinase) complex (Peters & Maniatis, 2001). NF-κB regulates transcription of a large number of genes that are important in cell survival, while regulation of cell death and survival is controlled in part by the mitogen-activated protein kinase (MAPK) cascades, which are activated by

various cellular stresses and are also involved in diverse biological phenomena such as differentiation, proliferation, and cytokine production (Sakon *et al.*, 2003). The term mitogen-activated protein kinase signaling pathways generally refers to a family of signaling cascades, which consist of the extracellular signal regulated kinase (ERK 1/2 and ERK 3/4), Jun N-terminal kinase (JNK), p38 kinase, and big mitogen–activated protein kinase 1 (BMK1) pathways (Zhou *et al.*, 2009). In general, oxidative stress is correlated with activation of JNK, as well as p38 and ERK kinases (Cross & Templeton, 2004a). Each MAPK is activated by sequential phosphorylation through a MAPK module. For instance MAPK kinase kinase (MAPKKK) phosphorylates MAPK kinase, which in turn phosphorylates MAPK (Nakano *et al.*, 2006). In the case of the JNK cascade, the MAPKKKs include apoptosis-signal regulating kinase (ASK)1, MAP/ERK kinase kinase (MEKK)s, MEKK4 (also known as MKT1), and TGFβ-activated kinase (TAK)1. Activated JNK regulates many cellular functions such as apoptosis, cellular proliferation, embryonic morphogenesis and tumorigenesis (Davis, 2000).

Two members of the MAP kinase family, NIK and MEKK1, have been shown to directly interact with IKK, and activate the kinase subunits (Nemoto *et al.*, 1998; Lin *et al.*, 1999) (Fig. 2). Since IKK was also identified as a NIK-interacting protein, it was suggested that NIK might be an upstream kinase for IKK. TNF-α and IL-1 have been shown to activate NIK and MEKK1 coordinately and synergistically. Two other members of the MEKK family (MEKK2 and MEKK3) have been reported to induce IKK activation and site-specific IκBα phosphorylation (Schoonbroodt & Piette, 2000). On the basis of numerous studies it seems reasonable to consider MAP3K and IKK as the core elements of the signaling cascade. According to studies focusing on the role of ROS in MAPK activation ROS directly activate various kinases, including ASK1, MEKK1, EGFR, PDGFR, c-src, which in turn can activate the MAPK cascade (Droge, 2002). ROS have appeared as bridging factors, which can mediate the crosstalk between NF-κB and JNK (Nakano *et al.*, 2006). Additionally, it was demonstrated that NF-κB downregulates JNK activation by suppressing TNFα-induced ROS accumulation (Sakon *et al.*, 2003; Ventura *et al.*, 2004). Inhibition of ROS accumulation is a crucial mechanism by which NF-κB blocks TNFα-induced programmed cell death. In accordance with the literature data, ROS cytotoxicity is mediated in part by the JNK pathway, because ROS are required for permanent JNK induction by TNFRs. This induction depends on the TRAF2-binding MAPKKK, ASK1/MEKK5 (Matsuzawa *et al.*, 2002). TNF-activated TRAF-2 interacts with several MAP kinase kinase kinases (MAP3Ks or MEKKs) leading to activation of JNK or p38 (Lin, 2003)(Fig. 2). It is important to emphasize that according to some data TNFα induces early and transitional JNK activation in wild-type cells, while TNFα induces ROS accumulation leading to sustained JNK activation in NF-κB activation-deficient cells (Nakano *et al.,* 2006). Although JNK does not directly phosphorylate the NF-κB subunit, it can coordinately activate IKK by inducing phosphorylation of IκB serine residues in response to TNF (Lee *et al.*, 1997). JNK may also interact with the c-Rel subunit, which results in NF-κB transactivation. Moreover, MEKK1 and NIK can directly activate IKKα and β subunits within the IKK complex, but it is still questionable, whether these two pathways operate independently or overlap each other in the regulation of NF-κB.

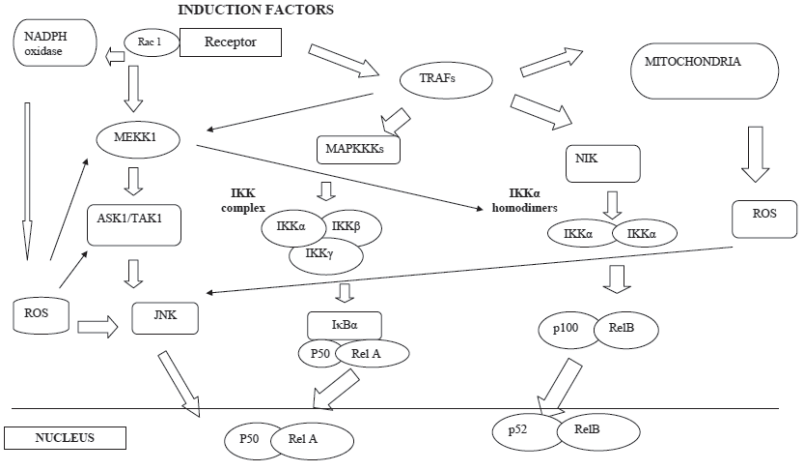

Figure 2. Crosstalk between ROS, NF-κB, JNK and NIK
ROS appear as bridging factors, which can mediate the crosstalk between NF-κB, JNK and NIK. They may activate JNK directly or by activation of various kinases such as ASK1, MEKK1. They are also required for permanent JNK induction. JNK, through interaction with the c-Rel subunit, leads to NF-κB transactivation, but without phosphorylation of NF-κB subunits. MEKK1 and NIK can directly activate IKKα within the IKK complex. Elevated level of ROS may also up-regulate the JNK-mediated activation of NF-κB.

ANTIOXIDANTS

The main mechanism, by which NF-κB activity can impact the level of ROS, is *via* increased expression of antioxidant proteins. On the other hand, a number of antioxidants such as *N*-acetylcysteine (NAC), vitamin E and its derivatives have been shown to block NF-κB activation (Wang *et al.*, 2002). This was the main reason why ROS have been previously considered as possible factors able to influence NF-κB activity. Additionally, it was shown that phosphorylation of IκB at Ser 32 and Ser36 could be inhibited by antioxidant dithiocarbamates, which provided the basis to suggest that ROS can activate IκB (Traenckner *et al.*, 1995).

Superoxide dismutases (SODs) are at the first line of defense in the detoxication of products resulting from oxidative stress (Johnson & Giulivi, 2005). They are a family of enzymes that very efficiently catalyze dismutation of the superoxide radical anion to hydrogen peroxide and molecular oxygen. CuZn-SOD (SOD1), located in the cytosol, is responsible for the majority of total SOD activity. CuZn-SOD (*SOD1, Sod1*) transgenic and mutant mice have been widely used to study the role of ROS in different experimental systems (Huang *et al.*, 2002). According to ample data, a deficiency in various forms of SOD promotes oxidative damage in a wide range of organisms (Fridovich, 1997). Increased levels of CuZn-SOD usually confer resistance to oxidative impact, whereas decreased expression renders mutant mice more susceptible (Elchuri *et al.*, 2005). A CuZn-SOD–deficient mouse is an effective model to study the influence of oxidative stress on the NF-κB pathway *in vivo*. In our

recent study we have demonstrated a statistically significant increase in the DNA-binding activity of NF-κB1 (p50) protein in CuZn-SOD-deficient mice without any changes in the activity of the RelA/p65 subunit. Moreover, the aforementioned increase was restricted to kidney, while no statistically significant changes were found in liver or brain (Siomek *et al.*, 2010).

In addition, we have found a statistically significant, about 60%, increase of 8-oxo-7,8-dihydro-2'-deoxyguanosine (a reliable biomarker of oxidative stress) level in DNA isolated from livers of mice deficient in SOD1 in comparison with age-matched controls. Our results demonstrate that this level was also significantly elevated in kidneys of the *SOD1*$^{-/-}$ mice, albeit the change was not as distinct as that in liver. Our observation and earlier data (Elchuri *et al.,* 2005) showed that CuZn-SOD-deficient animals developed organ-specific, widespread oxidative stress and oxidative damage. Since NF-κB is recognized to be redox-regulated, it is possible that in some organs of *SOD1*$^{-/-}$ animals (e.g. in liver) this pathway is constitutively activated, which may be an additional factor responsible for organ-specific cancer development. The results presented in our work suggest that the increased DNA-binding activity of the NF-κB p50 protein in kidney may be linked to the inhibition of proinflammatory activity of the canonical NF-κB pathway, which in turn may protect this organ from cancer development in CuZn-SOD-deficient animals.

The manganese-containing superoxide dismutase (Mn-SOD) is the primary member of the mitochondrial enzymatic defense mechanism that converts the superoxide anion radical to hydrogen peroxide, which is then further degraded by catalase, peroxiredoxins and gluthatione peroxidases. It is the most recognized/renowned of NF-κB targets with antioxidant capacity (Morgan & Liu, 2011). The *SOD2* gene promoter is under the control of several transcription factors, mainly the nuclear factor κB (NF-κB) and the Forkhead transcription factor FOXO3a (FKHRL1) (Storz *et al.*, 2005). In both cases extracellular H_2O_2 has been shown as a regulatory agent (Nemoto & Finkel, 2002; Storz & Toker, 2003). A specific signaling pathway leading to the induction of *SOD2* was described. These authors have found that protein kinase D (PKD) plays an important role in the regulation of oxidative stress responses, and that under exposure to both exogenous and mitochondrial ROS this protein is potently phosphorylated and activated. Subsequently, PKD may dissociate from mitochondria and, through phosphorylation, can activate the IKK complex. They also indicate that, upon increased mitochondrial ROS release, PKD activates NF-κB, which in turn induces *SOD2*, resulting in increased Mn-SOD expression (Storz, Doppler, & Toker, 2005).

OXIDATIVE STRESS IN THE NUCLEUS

ROS can directly interact with the components of the NF-κB pathway leading to its up- or down-regulation in the cytoplasm. In spite of this, the appearance of ROS in the nucleus might lead exclusively to the reduction of NF-κB binding to DNA (Kabe *et al.*, 2005). There are a few well known ways in which ROS can influence the DNA binding activity of NF-κB. NF-κB is subject to a series of post-translational modifications that are required for the complex activation of NF-κB-dependent genes (Gloire & Piette, 2009). DNA is packaged with histones and non-histone proteins into chromatin. Chromatin relaxation and remodeling are necessary for DNA accessibility to NF-κB. These processes are in large part regulated by acetylation/deacetylation of lysine residues in histone N-terminal tails. The NF-κB-dependent transcription requires different co-activators such as p300/CBP, P/CAF, and SRC-1/NcoA1, which possess histone acetyltransferase (HAT) activity (Sheppard *et al.*, 1999). There is a strong link between acetylation events and NF-κB -mediated gene transcription. It was demonstrated that acetylation of the p50/p65 heterodimers at multiple lysine residues could change their transcriptional function, DNA-binding affinity, and IκBα binding affinity (Chen & Greene, 2003). On the other, hand it was observed that in human fibroblasts TNFα stimulation led to increased p50/p65 binding via p50 acetylation, and that NF-κB bound to its specific sequence recruited additional p300 leading to amplified transcriptional activation (Deng *et al.*, 2003).

Optimal NF-κB -mediated transcription is dependent on both chromatin remodeling and direct modifications of p65 through the activity of protein kinases and histone acetyltransferases and deacetylases (Gloire & Piette, 2009). It is well known that cigarette smoke (CS) contains an estimated 10^{17} free radicals and many ROS-producing chemical agents per puff. CS-mediated oxidative stress can not only activate the IKK complex, but it is also an important factor of chromatin modification that enhances the transcription of proinflammatory NF-κB-dependent genes. The regulation of NF-κB-mediated transcription is operated by a HDACs-containing repressor complex (histone deacetylases) (Calao *et al.*, 2008). It was shown that cigarette smoke extract (CSE)-derived oxidants could change the expression level and the activity of HDAC1-3 in macrophages (Yang *et al.*, 2006). Furthermore, the expression and activity of HDAC2 is decreased in smokers as well as in chronic obstructive pulmonary disease and asthma patients.

The post-translational modification of p65 is a well known process. After IκBα degradation phosphorylation of p65 Ser276 occurs and promotes the interaction of p65 with CBP (CREB binding protein) and p300, two transcriptional coactivators (Gloire & Piette, 2009). This phosphorylation is achieved by the catalytic subunit of protein kinase A (PKAc) or MSK-1 and -2 (Zhong *et al.*, 1998; Vermeulen *et al.*, 2003). Phosphorylation of p65 Ser276 by PKAc plays the most important role in the control of NF-κB-mediated transcription (Gloire & Piette, 2009). According to a lot of evidence, this PKAc-mediated Ser276 phosphorylation is redox-regulated, as the antioxidant treatment inhibits Ser276 phosphorylation and CBP/p300 binding (Jamaluddin *et al.*, 2007). On the other hand, N-acetylcysteine (NAC), widely used as an antioxidant, was shown to be an inducer of Ser536 phosphorylation of RelA, and this event was strongly connected with RelA DNA-binding activity (Liu *et al.*, 2008).

Another molecular change that can occur is tyrosine nitration of the p65 protein at Tyr 66 and Tyr152 located within RHD domain, caused by reactive nitrogen species (RNS) (Gloire & Piette, 2009). A source of this modification is peroxynitrite generated from $NO^{\bullet}$ and $O_2^{\bullet-}$. Due to nitration NF-κB is abruptly inactivated by replacement of the p65/p50 with the p50/p50 complex, or by association of p65 with IκBα followed by relocation to the cytoplasm. RelA (p65) also could be S-nitrosylated on Cys38 due to NOS2 expression upon cytokine stimulation of cells. Nuclear SNO-p65 levels have shown inverse correlation with NF-κB DNA binding and transcriptional activity, which could suggest a negative feed-

back mechanism that prevents inordinate inflammation (Gloire & Piette, 2009).

Oxidation of NF-κB in the nucleus decreases or inhibits its DNA binding activity (Toledano & Leonard, 1991). Cys62 of p50 is especially sensitive to oxidation. It is localized in the RHD domain and therefore its oxidation inhibits DNA binding activity. This is a critical cysteine residue that needs to be reduced for efficient DNA binding. P50 Cys62 is mostly oxidized in the cytoplasm, but is rapidly reduced after NF-κB migration to the nucleus (Nishi *et al.*, 2002). There are many enzymes involved in the control of the reduction of nuclear p50 Cys62 such as thioredoxin or AP endonuclease/redox factor 1(APE1/Ref-1). It has been shown that APE1/Ref-1 can act by directly reducing proteins or by promoting the reduction of p50 Cys62 by other antioxidant proteins such as GSH or TRX (Walker *et al.*, 1993; Ando *et al.*, 2008). Nitric oxide ($NO^{\bullet}$) can also inhibit NF-κB-binding to DNA through the process of S-nitrosylation of the Cys 62 residue of p50. $NO^{\bullet}$ is produced by the iNOS protein that is up-regulated as an NF-κB target (Kelleher *et al.*, 2007). In that way, S-nitrosylation is a clear example of a negative regulation mechanism of NF-κB activation associated with ROS/RNS influence.

TARGET GENES

Some ROS, like $O_2^{\bullet-}$ and H_2O_2, can regulate physiological pathways, cell function, as well as transcriptional/posttranslational control of gene expression. NF-κB target genes mainly encode regulators of the immune/inflammatory response, such as cytokines (TNFα, IL-1, IL-6), chemokines (MCP-1, IL-8, MIP-1α), adhesion molecules (ICAM-1, VCAM-1), enzymes (COX-2, iNOS), and immune receptors (MHC, IL-2 receptor, IFN-γ receptor) (Wang *et al.*, 2002). NF-κB also enhances transcription of anti-apoptotic proteins and antioxidant enzymes. After many years of research one still knows very little about the target genes that are transcriptionally activated by moderate levels of ROS. A precise knowledge about the molecular processes induced by oxidative stress on that level could be very useful for new anticancer therapies.

In our recent research (Brzoska *et al.*, 2011) we compared the expression of 84 genes related to NF-κB signaling between CuZn-SOD-deficient and wild type animals with regard to liver and kidney. Even though we observed a higher level of oxidative DNA damage in both organs, elevated NF-κB activity was observed only in kidney (Siomek *et al.,* 2010). We decided to analyze expression of genes as a function of CuZn-SOD deficiency as well as NF-κB redox-sensitivity — an approach, which could be helpful in answering the questions concerning the organ-specific response to oxidative stress.

We have found that in the case of kidney four genes were up-regulated in CuZnSOD- deficient animals in comparison with wild-type mice, *Egr1,Fos, Il1b,Tnfrsf10b.* Three genes were down-regulated, *Card10,Ikbk and Tgfbr2.* In the liver eleven genes showed statistically significant changes. Six of them were up-regulated: *Fos, Il1b, Il1r1, Jun, Tlr7, Tnfrsf10b*, whereas five down-regulated: *Casp8, Ikbke, Irak1, Nfkb1, Raf1.* According to our findings only three of them, *Fos, IL1b*, and *Tnfrsf10b,* were regulated in the same manner in both analyzed organs (Brzoska *et al.,* 2011). Among the analyzed genes, the expression of eight was decreased in liver or kidney of CuZn-SOD-deficient mice. Five of them, *Ikbkb, Ikbke, Tgfbr2, Irak1*and *Raf1,* encode proteins, which have kinase activity and are involved in NF-κB signaling pathway regulation. These findings can be a confirmation showing that oxidative stress caused by CuZn-SOD deficiency influence the NF-κB signaling pathway. This, in turn, could be the next proof of the relation between oxidative stress and the NF-κB transcription factor.

CONCLUSIONS

During recent years the role of oxidative stress in the pathogenesis of a wide range of diseases has been a target of intensive experiments and trials. Because the activity of the NF-κB pathway is important in many processes such as cell survival, immunity and development, the elucidation of its possible modulation by the redox state could provide a new insight into the role of oxidative conditions in physiological and pathological conditions. Like many other transcription factors, NF-κB manifests a dual response to oxidative stress. It is definitely a cell-dependent and/or stimulus-modified phenomenon.

Although there still are many unanswered questions concerning the role of oxidative stress in the regulation of the NF-κB pathway, results of numerous works have demonstrated that the NF-κB activation and/or transcriptional activity is mediated by reactive oxygen and/or nitrogen species. Moreover, elucidation of the precise mechanisms of modulation of the NF-κB pathway may provide additional insight into molecular basis of numerous pathologies and be helpful in the development of potential drugs and therapies.

Oxidative stress can influence cytoplasmic and nuclear steps of NF-κB activation, and according to the results presented in this review this impact may be diverse depending on the localization of the affected elements, source of oxidative stress and cell type.

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