Signaling from Toxic Metals to NF-κB and Beyond: Not Just a Matter of Reactive Oxygen Species

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The nuclear factor kappa B (NF-κB) family of transcription factors controls expression of a number of early response genes associated with inflammatory responses, cell growth, cell cycle progression, and neoplastic transformation. These genes include a multitude of cytokines, chemokines, adhesion molecules, immune receptors, stress proteins, apoptotic or anti-apoptotic regulators, and several oncogenes. Accumulating evidence indicates that a variety of toxic metals are able to affect the activation or activity of NF-κB, but the molecular mechanisms involved in this process remain largely unknown. The signaling pathways mediating cytokine- or microorganism-induced NF-κB activation have been well established recently. Whether the same signaling systems are involved in metal-induced NF-κB activation, however, is unclear. In the present review, we have attempted to evaluate and update the possible mechanisms of metal signals on the activation and function of NF-κB. *Key words:* kinase, metals, NF-κB, oxidative stress, signal transduction. *Environ Health Perspect* 110(suppl 5):807–811 (2002).
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Metal ions are essential life elements that regulate numerous biological and biochemical functions to every living cell (*1,2*). However, overwhelming exposure to heavy metals in a variety of environmental and occupational settings is highly toxic to eukaryotic cells (*3,4*). Epidemiologic studies have suggested that some metals and metal-containing compounds are possibly cancer inducers for human beings (*5*). These metals include chromium, arsenic, vanadium, nickel, and others. Unfortunately, traditional epidemiologic approaches have not been able to delineate the molecular mechanisms of human diseases caused by exposure to toxic metals.

The development of cancer involves multiple steps that promote the transformation of normal cells into highly malignant derivatives (*6*). In the case of toxic metal–induced carcinogenesis, it remains unclear which step or steps are effectively targeted by metals. For a given step known to be critically involved in the process of carcinogenic transformation of cells, such as nuclear factor kappa B (NF-κB) or cell growth control, how metals affect the signal transduction pathways leading to that step is also poorly understood. Because NF-κB is a critical transcription factor governing a number of cellular processes ranging from anti-apoptotic response to critical oncogene expression (*7,8*), in this brief review we focus our attention on the mechanisms linking NF-κB activation and possible carcinogenic transformation of cellular responses to toxic metals.

Kinase Pathways Leading to the Activation of NF-κB

The most classical form of NF-κB is a heterodimer of p50 and p65(RelA), which is sequestered in the cytoplasm in an inactive form through its association with one of several inhibitory molecules, including IκBα, IκBβ, IκBε, p105, and p100 (*8,9*). Diverse stimuli, which typically include cytokines, mitogens, environmental and occupational particles, toxic metals, intracellular stresses, viral or bacterial products, and ultraviolet light, induce the degradation of IκB or partial degradation of the C-termini of p105 and p100 precursors, allowing the translocation of NF-κB to the nucleus, where it induces transcription of a number of important genes. Many of the NF-κB–targeting genes are pivotal in mediating cell-to-cell interaction, intercellular communication, cell recruitment or transmigration, amplification or spreading of primary pathogenic signals, and initiation or acceleration of carcinogenesis (*10*). The consensus binding site of NF-κB on these target genes is composed of the GGGRNNYYCC sequence, where R is purine, Y is pyrimidine, and N is any base.

The kinases responsible for the signal-induced phosphorylation of IκB include IKKα/β and IKKi/ε (*9,11,12*). Several upstream kinases have been proposed to be the physiologically relevant IKK activators by direct phosphorylation of the IKK subunits. These kinases include MEKK1 [mitogen-activated protein (MAP) kinase kinase (MEK) K1] (*13*), (protein kinase B) PKB/Akt (*14*), NIK (NF-κB–inducing kinase) (*15*), NAK (NF-κB–activating kinase) (*12*), tumor growth factor β-activating kinase 1 (TAK1) (*16*), mixed lineage kinase 3 (MLK3) (*17*), and some atypical protein kinase C (PKC) isoforms (*18*). Under certain circumstances, overexpression of wild-type or a constitutively active form of these kinases stimulates IKK. In contrast, overexpression of dominant negative mutants of these kinases inactivates IKK as well as the NF-κB–dependent target gene transcription. In addition to phosphorylating or activating IKK, all of these kinases can also relay their upstream signals to several other non–NF-κB signaling molecules.

The core subunits of IKK complex include two catalytic subunits, IKKα and IKKβ, and a structural component named IKKγ or NEMO/IKKAP (*9,11*). Sequence analysis revealed that at the amino acid level, the IKKα and IKKβ are highly homologous proteins with 51% sequence identity. Both IKKα and IKKβ contain a kinase domain at the NH2-terminus with a leucine zipper motif and a helix–loop–helix motif in the COOH-terminal region. In addition, both subunits contain a canonical MEK activation loop motif (S-X-X-X-S, where X is any amino acid) that appears to be essential for the activation of the kinase activity. It has been suggested that both IKKα and IKKβ are capable of phosphorylating S32/S36 of IκBα and S19/S23 of IκBβ (*9*). However, certain functional differences between IKKα and IKKβ have been demonstrated by *in vitro* and *ex vivo* experiments. IKKβ seems to be more responsible in mediating cytokine-, inflammation-, and/or MEKK1-induced NF-κB activation (*9,19*). On the other hand, IKKα is more important in mediating NIK signaling, p100 process, and keratinocyte differentiation (*20,21*). The IKKγ itself does not possess any kinase activity, but it is essential to relay upstream signals to IKK. Point mutations or genomic rearrangement resulting in partial deletion of IKKγ gene at the X-chromosome has been linked to the autosomal recessive diseases of hypohidrotic ectodermal dysplasia and incontinential pigmenti (*11*).

IKKι/ε, a newly identified protein with IKK kinase activity, has been suggested to be an independent serine/threonine kinase (*22–24*). Structurally, this new kinase has an

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overall topologic similarity to IKKα or IKKβ in the N-terminal kinase domain, C-terminal leucine zipperlike domain and helix-loop-helix region. The expression of IKKι/ε mRNA is in an inducible fashion, which is drastically different from that of IKKα or IKKβ. Using recombinant proteins and a peptide substrate, a recent study by Kishore et al. (*25*) demonstrated that the kinase activity of IKKι/ε is 50- to 100-fold higher than that of IKKβ. A yeast-two hybrid screening experiment suggested that the C-terminal portion of IKKι/ε could specifically associate with the N-terminal domain of TANK (TRAF-associated NF-κB activator) (*26*). The most recent biochemical evidence provided by Chariot et al. (*27*) demonstrated that physical interaction of IKKι/ε with TANK is sufficient to promote the association of TANK with IKKγ. Thus, it is possible that IKKι/ε may associate with a subset of classic IKK complex and act as an upstream kinase to activate IKKα or IKKβ. The association of IKKι/e with IKKα/β complex may serve to relay specific signals at special sites within cells.

NF-κB Activation Induced by Metals

Accumulating evidence suggests that many metals are able to affect the activation or activity of NF-κB transcription factor (*28*). To date, the results are not straightforward. Both activation and inhibition of NF-κB by metals have been reported (*29–31*). Several studies from different groups indicate that, at a noncytotoxic concentration, arsenic trioxide [As(III)] (*32*), chromium(VI) [Cr(VI)] (*28*), and vanadium(V) [V(V)] (*28*) are capable of activating NF-κB as monitored by either gel shift assay, reflecting the activation and nuclear translocation of NF-κB, or NF-κB–dependent reporter gene assay, an indicator of NF-κB activity. In contrast, it has been reported that Cr(VI), As(III), and other metals inhibit NF-κB activation through interfering with IKK NF-κB DNA binding, or the interactions with nuclear cofactor, cAMP-responsive element–binding protein (CREB)–binding protein (*30,31*). How can metals mediate both activation and inhibition of NF-κB? One possibility is that the final outcome of metals on NF-κB is either dose dependent or cell type dependent. Evidence to support this possibility comes from the studies by Hamilton et al. (*33*). Whereas NF-κB is clearly activated by both As(III) and Cr(VI) at lower concentrations in MDA epithelial-type cells, it is not activated by any of these metals at either lower (1 and 2 μM, respectively) or higher concentrations (20 and 100 μM, respectively) in H4IIE rat hepatoma cells.

Arsenic

The first evidence indicating the activating effect of As(III) on NF-κB is provided by Barchowsky et al. (*32*), who demonstrated that lower concentrations of As(III) activated NF-κB possibly through oxidative stress in endothelial cells. Later studies by Hamilton et al. (*33*) suggested that activation of NF-κB by As(III) is dependent on cell types. Epithelial-like cells appear to be more responsible to As(III) on NF-κB activation. In airway epithelial cells, studies by Jaspers et al. (*34*) indicated that As(III) activated NF-κB through an alternative mechanism that did not require the inducible degradation of IκBα and the nuclear translocation of NF-κB proteins. In contrast to these studies, several reports suggest that As(III) inhibits NF-κB by either interfering with DNA binding of NF-κB or directly inactivating IKK (*35*). In HeLa cells and HEK293 cells, As(III) has been shown to be able to bind to cysteine 179 of IKKβ and inhibit IKK activity induced by tumor necrosis factor α (TNFα), interleukin (IL-1), and PMA (*35*). The controversial As(III) effects on NF-κB mostly result from dosages of As(III) used in each experimental system. It is certain that inhibition of NF-κB by As(III) will occur at nonphysiologic concentrations such as 100–500 μM used in the DNA binding studies (*36*). Using wild-type and *sek1* [stress-activated protein kinase (SAPK)/ERK kinase] gene knockout mouse embryo stem cells, our recent mechanistic studies suggest that As(III)-induced NF-κB is through a signaling pathway that involves SEK1 (MKK4)-JNK (*37*). Neither ERK nor p38 is required for As(III)-induced NF-κB activation. In the assay of As(III) effects on IKK activity, the inhibitory effect of As(III) on IKK was studied in the presence of TNFα, a cytokine that potentially activates both the NF-κB signaling pathway and the cell apoptosis pathway (*35*). It has been widely accepted that the simultaneous or asynchronous stimulatory events in any given cell type for a particular stimulation, for example, As(III), will alter the availability of As(III), intracellular redox status, and the accessibility of targeting molecules.

In the human bronchial epithelial cell line BEAS-2B, we observed that the activation of NF-κB by As(III) occurred in a very narrow dosage ranges (*38*). A 5- to 6-fold induction of NF-κB–dependent reporter gene activity was observed by As(III) at concentrations of 6–12 μM. In contrast, a substantial inhibition of NF-κB by As(III) was observed at concentrations higher than 25 μM. Obviously, at a physiologically relevant dose range, As(III) is not an inhibitor but rather an activator for NF-κB. To delineate the role of NF-κB in As(III)-induced cellular responses, we recently performed cDNA microarray analysis using mRNAs extracted from both normal and IKKβ-inhibited cells in response to 10 μM As(III). As depicted in Figure 1, blockage of the activation pathway of NF-κB by expression of dominant negative mutant of IKKβ potentiated the inducible expression of genes encoding heme oxygenase, heat shock protein chaperonin 10, and several proteasome subunits. As(III) is a potent inducer for the expression of several metallothionein proteins. However, the effect of NF-κB on the induction of these proteins by As(III) appears to be marginal.

Vanadate

An increasing concern has been raised in recent years regarding the release of vanadium into the atmosphere from anthropogenic sources (*39*). Vanadium is a major trace metal in particulate emissions resulting from combustion of fossil fuels and other industrial activities. The predominant forms of vanadium include V(IV) (vanadyl) and V(V) (vanadate). As an established toxic metal, vanadate exerts divergent biologic functions, from insulin-like effects to NF-κB activation, after entering cells (*40–42*). V(V) activates NF-κB in virtually all types of cells (*28*). The studies

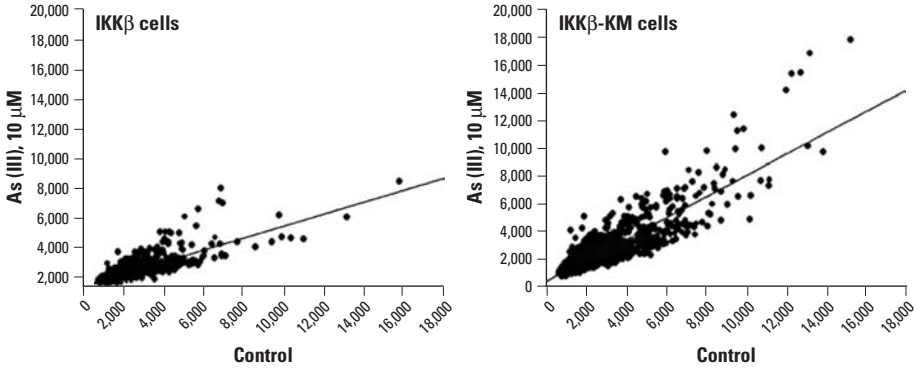

Figure 1. Scatter plots of gene expression for IKKβ cells and IKKβ-KM cells after 10 μM As(III) treatment for 12 hr. Gene induction in response to As(III) is visualized as a shift upward from the diagonal, whereas genes repressed are shifted downward.

by Schieven et al. (*43*) indicate that the activation of NF-κB by V(V) might be tyrosine kinase dependent. Studies by Imbert et al. (*44*) indicate that the activation of NF-κB by V(V) occurs independently of IκBα degradation. However, several recent studies suggest that V(V) does induce degradation of IκBα after the phosphorylation of serine or tyrosine (*45–47*). In RAW264.7 cells, V(V)-induced IκBα degradation occurred within 10–20 min, with peak degradation at 40 min (*42*). In human myeloid U937 cells or epithelial cells, V(V)-induced IκBα degradation occurred at 30 min and reached maximum at 240 min (*46*). A similar result was achieved in Jurkat E6.1 cells and the human B-cell lymphoma line Ramos (*34,45,47*). In contrast to an earlier report that no resynthesis of IκBα occurred after V(V) treatment (*44*), several studies have indicated that the resynthesis of IκBα indeed occurs at 80–180 min after V(V) treatment (*42,46*).

It is not clear why V(V) is able to induce degradation of IκBα in some types of cells but not in others. The explanation for this may be the use of different forms of V(V). It was noted that some studies used sodium vanadate whereas others used peroxovanadate. The latter form is a reactive product of V(V) in the presence of H_2O_2. There is evidence indicating that sodium vanadate and peroxovanadate exhibit different effects on the induction of cell apoptosis, inactivation of protein phosphatase, and generation of reactive oxygen species (ROS) (*48*). An additional explanation for varying effects of V(V) on IκBα degradation is the types of cells used in each experiment. It is well known that cells originating from different tissues exhibit different capacities for the degradation of cellular proteins, generation of ROS, and response to metal or exogenous ROS stimulation. Finally, the dosage of V(V) used in each experiment may affect the degradation of IκBα protein. For example, the degradation of IκBα could be induced by 5–80 μM V(V) but not by 100–1,000 μM V(V).

Chromium(VI)

It has been well known that the hexavalent state of chromium [Cr(VI)] is the strongest oxidizing form and most carcinogenic form of chromium (*49,50*). Cr(VI) is able to activate NF-κB at lower concentrations (<50 μM) in T cells (*51*), macrophages (*52*), bronchial epithelial cells (*53*), and human breast cancer cells (*29*). The final concentration of Cr(VI) is critical for this metal to induce or inhibit NF-κB. The inhibitory effect of Cr(VI) at higher concentrations (>50 mM) on NF-κB may be due to the cytotoxic effect on the cells or interference with the DNA binding activity of NF-κB (*31*). We recently demonstrated that Cr(VI) activated NF-κB at 5–10 μM in human bronchial epithelial cells cultured at a relatively higher cell density, possibly through activating IKK. We further showed that activation of NF-κB is a protective response for the cells from Cr(VI)-induced cytotoxicity. Inhibition of NF-κB by expression of a dominant negative mutant of IKKβ or IKKβ gene deficiency resulted in a spontaneous cleavage of Bcl-xl anti-apoptotic protein due to the elevated caspase-3 activity. DNA microarray assay suggested a decreased expression of genes encoding the anti-apoptotic proteins cIAP1 and cIAP2, in the cells overexpressing kinase-mutated IKKβ (IKKβ-KM). Cr(VI) treatment of these NF-κB–inhibited cells induced necrotic-like cell death. Such Cr(VI)-induced cell death could be partially inhibited by expression of exogenous cIAP1, an inhibitor of caspases, indicating noncaspase cytotoxic mechanisms may be involved in Cr(VI)-induced cell death. Indeed, combination of cIAP1 and the antioxidant *N*-acetylcysteine resulted in a significant inhibition of Cr(VI)-induced cell death of NF-κB–inhibited cells (*53*). These results suggest that NF-κB is essential for inhibiting ROS-dependent cytotoxicity. Such inhibition may involve up-regulation of anti-death proteins, including cIAP1, which prevents spontaneous caspase activation and subsequent cleavage of Bcl-xl protein.

Questions of ROS Effects on Metal-Induced NF-κB

A number of reports suggest that NF-κB can be activated by a variety of ROS that cause oxidative stress (*54,55*). It has been realized for decades that oxidative stress is the major effect of toxic metals on cellular events (*56*). It appears logical, therefore, to assume that the activation of NF-κB by toxic metals is through the induction of ROS. Nevertheless, several obstacles are still unsolved (*57–60*). If oxidative stress is a common mechanism for toxic metal-induced cellular response, one will speculate that all of the metals should have the same or similar effects on NF-κB. However, it is not true in reality. One example is the activation of NF-kB induced by As(III) and Cr(VI). Whereas Cr(VI) is stronger than As(III) in the induction of ROS generation, Cr(VI) is much weaker than As(III) in the induction of the NF-κB reporter gene activity (*28*). Even if oxidative stress is the true reason for the metal-induced activation of NF-κB, several questions remain to be answer. Are these ROS essential mediators for the activation of NF-κB or bystanders during the activation of NF-κB? When antioxidants were used in experimental system to support the claims of ROS-dependent activation of NF-κB by metals, did these antioxidants solely attenuate the oxidative stress without other cellular effects (*61,62*)? How do we reconcile the activation of NF-κB by ROS with the fact that oxidation of NF-κB proteins decreases the DNA-binding activity of this transcription factor (*63–67*)? Does direct interaction occur between metal ions and signaling proteins for the activation of NF-κB? If this is the case, the binding of metals with signaling proteins will certainly alter the functions of these proteins without the ROS effect.

The NF-κB activation pathways by TNF, IL-1, Toll, LPS, and CD28 have been clearly identified. However, no direct evidence is available to suggest the responsiveness of signaling molecules in these pathways to ROS (*68–71*). The evidence to implicate ROS as stimulators of IKK is based on the elevated IKK activity in human epithelial cells or mouse fibroblast cells caused by the H_2O_2 treatment (*72,73*). In our own studies, we found a modest induction of IKK activity in cellular response to Cr(VI), a potent intracellular H_2O_2 inducer (*53*). However, H_2O_2 itself neither stimulates IKK activity nor induces NF-κB reporter gene activity at a wide dose range, suggesting that other mechanisms, rather than oxidative stress, may be responsible for the Cr(VI)-induced NF-κB activation. Similarly, Korn et al. (*71*) found that H_2O_2 itself failed to stimulate IKK but rather inhibited TNFα-induced IKK activity. It is likely that H_2O_2 inactivates IKK through direct oxidation of a conserved cysteine 179 in the kinase domain of IKKβ, a mechanism similar to the inactivation of IKKβ by 15d-prostoglandin J2 and a high concentration of arsenic (Figure 2) (*35,74*). In comparison with several other kinases, including JNK, p38, PDK1, CKII, and MEKK1, only IKKβ and IKKα contain a cysteine residue in its kinase activation domain (Figure 2). This structural characteristic indicates that IKK but not kinases for the MAP kinase signaling is susceptible to oxidative inactivation. Thus, if ROS are truly capable of inducing

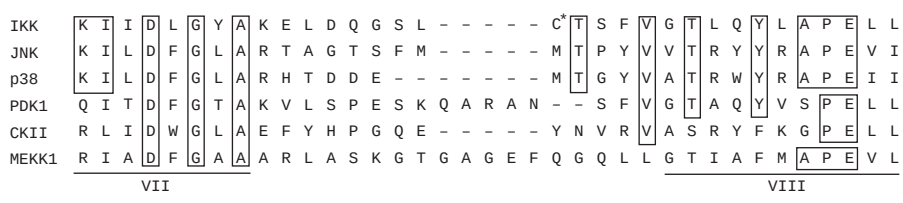

Figure 2. Alignment of the activation domain of IKKβ with the corresponding domains of other kinases. Conserved residues are boxed. Kinase domains VII and VIII are underlined. The unique cysteine 179 residue in IKKβ is marked with an asterisk.

NF-κB, they are most likely to do so through the regulations of other kinases or protein phosphatases.

Summary

Human beings are continuously exposed to diverse environmental stimuli. It is of great importance that these stimuli are correctly interpreted by the cell, a basic unit of our body, to avoid deteriorating cellular responses such as carcinogenic transformation. A number of cellular proteins play pivotal roles in this process. By associating with specific partners, these proteins are able to integrate these external stimuli with internal signal transduction pathways, contributing to the ability of the cell to respond correctly to its environment. However, a sustained exposure to these stimuli will result in the disturbance of normal cellular functions and consequently malignant transformation during tumor development.

What is so important about the NF-κB signaling pathway in metal-induced cellular responses? First, NF-κB is a transcription factor highly conserved in virtually all types of cells, from macrophage cells to epithelial cells, a sign of its importance. Second, the involvement of NF-κB in cellular response to metals provides insights into the regulatory circuitry that controls the biochemical responses of the cells, an essential process that, if overreacted, is harmful to the cell. The dramatic cell death observed when NF-κB is inhibited in epithelial cells further emphasizes the need to keep a precise balance of pro- and anti-apoptosis molecules throughout the cell growth cycle. The next challenge is to understand where the metals or their ROS derivatives interact with cellular signaling molecules. This issue is puzzling because metals and their ROS derivatives appear to have numerous targets intracellularly. Pinpointing the exact mechanisms of metal-induced activation of NF-κB will be crucial for the development of novel preventive measures and therapeutic strategies for diseases related to toxic metal exposure.

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