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Review

Carcinogenic metals and NF-κB activation

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Abstract

Epidemiological and animal studies suggest that several metals and metal-containing compounds are potent mutagens and carcinogens. These metals include chromium, arsenic, vanadium, and nickel. During the last two decades, chemical and cellular studies have contributed enormously to our understanding of the mechanisms of metal-induced pathophysiological processes. Although each of these metals is unique in its mechanism of action, some common signaling molecules, such as reactive oxygen species (ROS), may be shared by many of these carcinogenic metals. New techniques are now available to reveal the mechanisms of carcinogenesis in precise molecular terms. In this review, we focused our attentions on metal-induced signal transduction pathways leading to the activation of NF-κB, a transcription factor governing the expression of most early response genes involved in a number of human diseases. (Mol Cell Biochem **222**: 159–171, 2001)

Key words: NF-κB, IKK, metals, signal transduction, ROS

Introduction

There is increasing evidence that most of the toxic metals or metal-containing particles from either environmental or occupational sources are human carcinogens [1–4]. However, a detailed molecular mechanism of metal-induced malignant transformation and cancer remains elusive. Emerging evidence indicates that cellular transformation and tumorigenesis in humans is a multistep process that requires both non-genetic and genetic alterations that promote the transformation of normal human cells into highly malignant tumors [5]. However, it is still not clear which step or steps are targeted by these metals. For example, how the metals affect critical carcinogenic transformation events, such as NF-κB or apoptosis, is poorly understood. It is generally believed that oxidative stress, resulting from metal-induced generation of reactive oxygen species (ROS), is a critical mediator for the malignant transformation. However, ROS-independent effects of metals on cellular signaling pathways and genomic stability may also play a significant role [6, 7].

This review will focus on pathways leading to the activation of the transcription factor, NF-κB, and its possible roles in cell apoptosis and cell cycle regulation induced by metals, especially arsenic, vanadium and chromium.

Signal transduction of NF-κB activation

A wide range of signals, which typically include cytokines, mitogens, environmental and occupational particles or metals, intracellular stresses, viral and bacterial products, and UV light, induce the expression of early response genes through the NF-κB family of transcription factors [8–12]. In resting cells, NF-κB is sequestered in the cytoplasm of most cells where it is bound to one of a number of inhibitory molecules, including IκBα, IκBβ, IκBε, p105, and p100. Activation of the NF-κB signaling cascade results in a complete degradation of IκB or partial degradation of the carboxy termini of p105 and p100 precursors, allowing nuclear translocation of the NF-κB complexes (Fig. 1). Activated NF-κB binds to specific DNA sequences in target genes, designated as κB-elements, and regulates transcription of genes mediating inflammation, carcinogenesis and anti-apoptotic reactions.

Three IκB proteins, IκBα, IκBβ and IκBε, have been identified, among which IκBα is the most abundant inhibitory protein for NF-κB [9]. All IκB proteins contain two conserved serine residues within their N-terminal domain. Phosphorylation of these conserved serine residues in response to inducers, leads to the immediate polyubiquitination of IκB proteins by the SCF-β-TrCP complex, a step which has been

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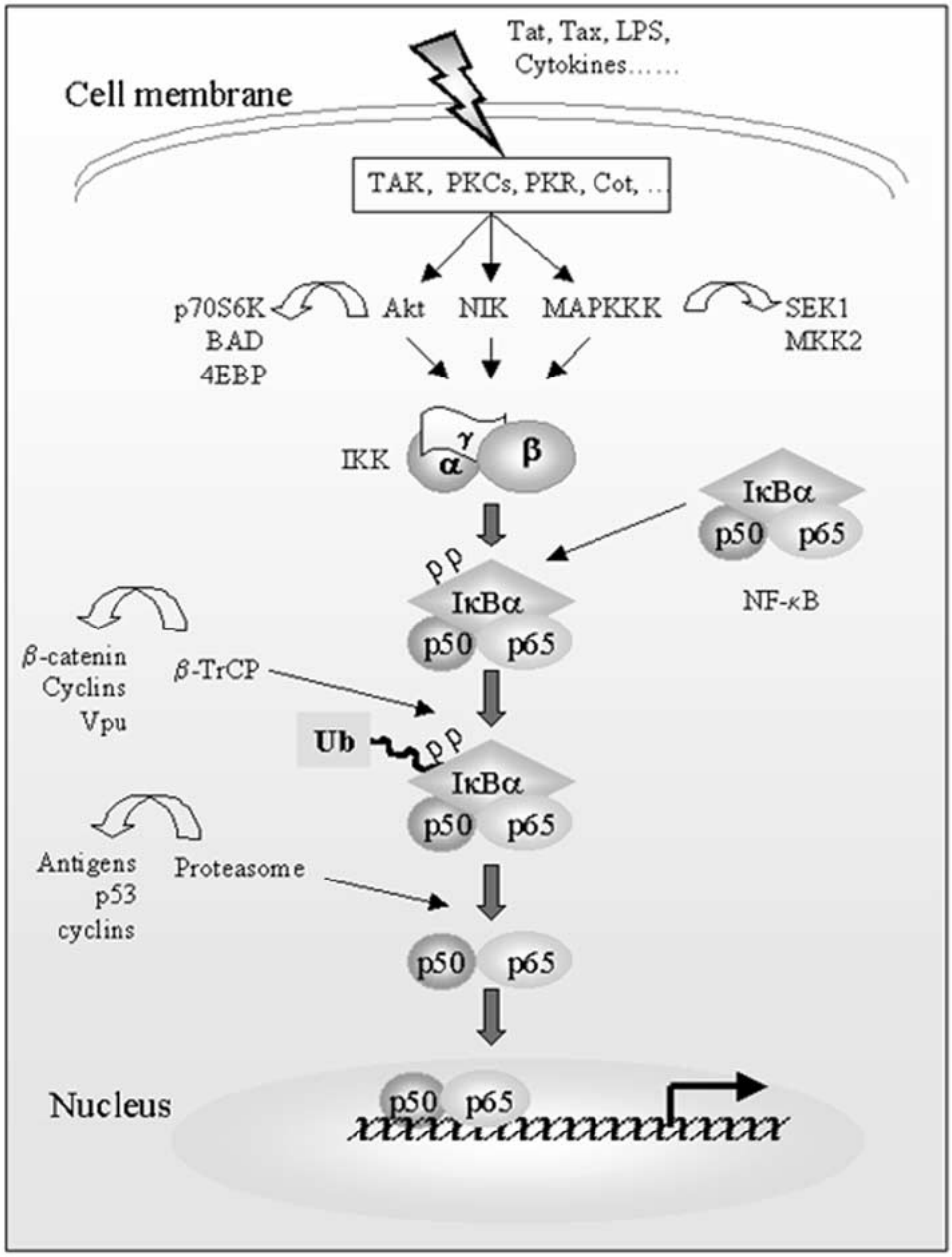

Fig. 1. Signaling pathways activating NF-κB. Extracellular inducers, including cytokines, viral and bacterial products and ROS, activate IKK through upstream kinases directly or indirectly. Activated IKK phosphorylates N-terminal S32 and S36 residues of IκBα which is associated with the NF-κB p50 and p65 heterodimer. The β-TrCP complex recognizes phosphorylated IκBα and modifies IκBα with polyubiquitin chains. This is followed by proteasome-mediated degradation of IκBα. After degradation of IκBα, the activated NF-κB translocates into the nucleus and binds to the κB-sites of gene promoters or enhancers to up-regulate target gene expression. Line arrows and filled arrows denote the NF-κB signaling pathways; non-filled arrows denote the involvement in other signaling pathways.

shown recently to be inhibited by the nonpathogenic *Salmonella* bacteria in gut epithelial cells [13]. This modification subsequently targets IκB proteins for rapid degradation by the 26S proteasome [14]. The phosphorylation is accomplished by a specific IκB kinase (IKK) complex containing two catalytic subunits, IKKα and IKKβ, and a structural component named NEMO/IKKγ/IKKAP [15, 16]. An earlier report by Cohen *et al.* [17] suggested the presence of a scaffold protein named IKK complex-associated protein (IKAP) in the IKK complex, which could not be confirmed in other studies. A late study by the group, who originally identified IKAP, indicated that the observed association of IKAP with

IKK was due to a non-strict elusion condition of chromatographic extracts during the purification of IKK [18]. Recently, two groups independently reported the identification of a novel protein, CIKS/Act1, associated with the IKK complex and suggested that CIKS/Act1 functions as an anchoring protein in the assembly of the IKK complex and in providing a possible connection between IKK and JNK signaling [19, 20]. IKKα and IKKβ share 50% sequence similarity. Both proteins contain an amino terminal kinase domain, a carboxy terminal region with a leucine zipper, and a helix-loop-helix domain. *In vitro* or *in vivo* studies indicate that although both IKKα and IKKβ are capable of phosphorylating IκBα on ser32 and ser36, IKKβ is more potent in IκBα phosphorylation induced by proinflammatory stimuli. Several groups have recently indicated the existence of an additional IKK-like kinase complex in T cells, named IKKi/ε, which shares 27% homology with IKKα and IKKβ and possibly mediates NF-κB activating kinase (NAK) signaling and PMA/PKCε-induced S36 phosphorylation of IκBα and NF-κB activation [21, 22].

Diverse stimuli, with the possible exception of UV, activate NF-κB through activation of the kinase activity of IKK that phosphorylates IκBs, a critical step required for subsequent ubiquitination and degradation. UV-induced NF-κB activation may be through a mechanism that does not depend on IKK or N-terminal phosphorylation of IκB [23, 24]. It is known that the activity of IKK can be stimulated by TNFα, IL-1, LPS, Tax protein, ionizing radiation, vanadate, or double-stranded RNA. However, how these diverse stimuli activate IKK remains unresolved. A number of kinases, mostly based on transient overexpression in which wild-type forms activate IKK and catalytically inactive forms inhibit IKK, have been suggested to be able to phosphorylate and activate IKK. These kinases include MEKK1 [25], NIK [15], TBK1/NAK [22, 26], Akt [27, 28], PKC-θ [29, 30], PKCξ [31], Cot kinase [32], PKR [33], and MLK3 [34]. It is unclear whether each of these upstream kinases represents one of the many routes to IKK activation by specific stimuli, or represents a ubiquitous pathway leading to the activation of IKK induced by diverse stimuli.

MEKK1

MEKK1 is a mammalian serine/threonine kinase in the mitogen-activated protein kinase kinase kinase (MAPKKK) group. It was found that MEKK1 was a far more important activator for JNK signaling than for ERK signaling as proposed originally [35, 36]. The first evidence, indicating the involvement of MEKK1 in signal-induced IKK activation, was provided by Lee *et al.* [25, 37]. In their studies, they reported that addition of the recombinant catalytic domain of MEKK1 (MEKK1Δ) to a partially enriched fraction of non-stimulated HeLa cells stimulated an IKK-like kinase activity that phosphorylated IκBα at serines 32 and 36 and induced subsequent ubiquitination and degradation of IκBα. It was subsequently demonstrated that an overexpression of MEKK1 stimulated NF-κB-dependent transcriptional reporter [38, 39]. The activation of NF-κB by HTLV Tax protein was shown to involve MEKK1 [40]. MEKK1 may also contribute to Toll- and IL-1 receptor-mediated IKK activation, since it was demonstrated that an adaptor protein named evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) could promote the proteolytic activation of MEKK1 and subsequent activation of NF-κB [41]. Further studies by Mercurio *et al.* [16] indicate the presence of a protein that is recognized by anti-MEKK1 anti-serum in the IKK complex. Both IKKα and IKKβ contain a MAPKK activation loop, 176/177 S-X-X-X-S 180/181, (where X is any amino acid), required for their activation. Substitution of these serines with alanine residues inactivates both kinases, while phosphomimetic glutamic acid substitutions at these positions result in constitutively active kinases. It remains to be confirmed whether MEKK1 is a physiologically important activator of IKK in cells in response to various stimuli. Indeed, a recent study by Xia *et al.* [42] demonstrated that inactivation of MEKK1 does not result in any impairment of NF-κB activation in response to TNFα, IL-1, LPS or dsRNA. In addition, several studies indicated that a modest expression of MEKK1, that was sufficient for JNK activation, failed to stimulate IKK [43]. Although some studies, using a dominant negative mutant of MEKK1, showed an inhibition of IKK, these results should be interpreted with caution since many dominant negatives can bind to their upstream partners upon overexpression and function as potent general inhibitors. The best approach to determine whether MEKK1 is a direct upstream kinase to phosphorylate and activate IKK is the use of MEKK1-deficient mice to examine whether cells or tissues from these mice display defects in activation of IKK or NF-κB in response to cytokines or other inflammatory stimuli.

NF-κB-inducing kinase (NIK)

NIK, a member of MAPKKK family, was originally identified as a TNFα receptor associating factor 2 (TRAF2)-interacting kinase whose overexpression results in potent NF-κB activation without any considerable effect on MAPKs [44, 45]. A study using yeast two-hybrid screens identified an interaction between NIK and IKK [15, 46], suggesting that NIK might be a direct upstream activator of IKK. Transient transfection of NIK into human embryonic kidney 293 cells indicated that IKKα was more responsive to NIK, whereas IKKβ was slightly more responsive to MEKK1 [47]. Most studies show that when the abilities of MEKK1 and NIK to

activate total IKK kinase activity are compared, NIK is a much stronger activator of the NF-κB transcriptional reporter than MEKK1 is [45, 48]. NIK can preferentially phosphorylate IKKα on ser176 in the activation loop, leading to the activation of IKKα kinase activity. In contrast, MEKK1 was found to preferentially phosphorylate the corresponding serine residue, ser177, in the activation loop of IKKβ. A dominant negative mutant of NIK blocked NF-κB activation by TNFα, IL-1, Fas [44], Toll-like receptors 2 and 4 [49, 50], LMP-1 [51], and CD3/CD28 stimulation [32]. Thus, NIK appears to be a general kinase mediating IKK activation induced by diverse stimuli. However, a recent analysis using a NIK-mutant mouse strain, *alymphoplasia (aly)*, contradicted this assumption. The *Alymohoplasia* mouse strain failed to develop lymphoid organs, such as lymph node, Peyer's patches, due to a point mutation in the NIK locus [52]. The mutation of NIK locus results in a disruption of interactions between NIK and IKKα or TRAF proteins. Further analysis indicated that the *aly* mutation did not affect TNFα-induced activation of NF-κB, but only blocked lymphotoxin-mediated activation of NF-κB. Similarly, studies using cells derived form NIK-deficient mice have indicated that NIK appears to be dispensable in IKK activation induced by TNFα or IL-1. It raises the possibility that NIK may be specifically involved in IKK activation induced by lymphotoxin but not other stimuli.

NF-κB activating kinase (NAK)

Three groups independently identified a novel serine/threonine kinase possibly activating IKK through direct phosphorylation in cells stimulated with PMA [22, 26, Tularik Inc., US patent 5,776,717 (1998)]. This novel kinase was named NAK, TANK-binding kinase 1 (TBK1) and T2K, respectively. Pomerantz and Baltimore [26] cloned NAK by a yeast two-hybrid screen using a N-terminal stimulatory domain of TANK 1-190 fused to GAL4 as bait and a human B-cell library fused to the GAL4 activation domain. The same kinase was also identified by PCR using degenerate primers based on sequences common to IKKα and IKKβ [22]. Amino acid sequence analysis indicated that NAK protein contains a kinase domain at its N-terminus that exhibits about 30% identity to the corresponding kinase domains of IKKα and IKKβ and more than 60% identity to the corresponding kinase domain of IKKε/i. Whereas the report by Pomerantz and Baltimore [26] showed that NAK might form a ternary complex with TANK and TRAF2, suggesting that NAK functions at distal upstream of signal cascade leading to IKK activation, *in vitro* kinase activation assay by Tojima *et al.* [22] demonstrated that NAK was a direct upstream kinase phosphorylating IKKβ. Intriguingly, activation of endogenous NAK resulted in only ser36, but not ser32, phosphorylation of IκBα, a phenomenon similarly observed in recombinant IKKε/i-mediated IκBα phosphorylation [53]. Since both IKKα and IKKβ are able to phosphorylate both ser32 and ser36 of IκBα protein, it is unclear whether IKKε/i or a novel IKK isozyme functions as a downstream kinase of NAK to induce ser36 phosphorylation of IκBα. Transient transfection studies showed that dominant negative NAK inhibited NF-κB transcriptional reporter activity induced by PMA, PKCε and PDGF, but not by TNFα, IL-1β, LPS, or ionizing radiation. These results, therefore, suggest that NAK is likely a downstream kinase of PKCε or related isozymes and an upstream kinase of IKK in the signaling pathway through which growth factors, such as PDGF, stimulate NF-κB activity.

Akt

The pro-survival function of Akt has been well documented. The kinase activity of Akt is activated via the phosphoinositide-3-OH kinase (PI3K) and PI3K-dependent kinase 1/2 (PDK1/2) signaling pathway [54]. Overexpression or constitutive activation of Akt has been associated with tumourigenesis in a number of studies. As a serine/threonine kinase, Akt is able to phosphorylate pro-apoptotic protein Bad, anti-apoptotic protein Bcl-x, apoptotic protease caspase-9, Forkhead transcription factors, and eNOS [54]. However, considerable controversies remain regarding the involvement of Akt in signal-induced IKK activation. Studies by Ozes *et al.* [27] and Xie *et al.* [55] indicate that Akt was required for TNFα- or G protein activator-induced NF-κB activation by directly phosphorylating and activating IKKα in 293, HeLa and ME-180 cells. A putative Atk phosphorylation site at amino acids 18–23 in both IKKα and IKKβ was identified. Akt induced Thr23 phosphorylation of IKKα both *in vitro* and *in vivo*. Mutation of Thr23 significantly decreased Akt-induced IKKα phosphorylation and TNFα-induced NF-κB activation in 293, HeLa and ME-180 cells [27]. In contrast, Romashkova *et al.* [28] demonstrated that Akt was involved in PDGF-mediated, but not in TNFα- or PMA-mediated, NF-κB activation in human or rat fibroblasts. In this study, the authors suggested that upon PDGF stimulation, Akt could transiently associate with IKK and induce IKK activation, especially IKKβ activation. Several other studies, however, suggested that the effects of Akt on NF-κB did not occur at the level of IKK activation in several cell types. Studies by Delhase *et al.* [56] indicate that Akt activation induced by IGF-1 failed to activate IKKα kinase activity, IκBα phosphorylation and degradation, and NF-κB DNA binding in HeLa cells, the same cell line used by Ozes *et al.* [27]. Similarly, several recent studies showed that Akt was not involved in TNFα-induced NF-κB activation in human vascular smooth muscle cells, skin fibroblasts, or endothelial cells [57, 58]. Rather, Akt might enhance the ability of

the p65 (RelA) transactivation to induce transcription [59, 60]. In Jurkat T-cells, Akt alone failed to activate NF-κB, but it was capable of potentiating NF-κB activation induced by PMA, partially by enhancing IκBβ degradation [61]. Thus, the questions remain to be answered are: (1) does Akt phosphorylate IKK in a cell context- and stimulation-dependent manner? and (2) do upstream kinases of Akt, such as PDK1 and PDK2, also activate IKK, since both IKKα and IKKβ contain a putative PDK1 phosphorylation site (S-F-X-G-T-X-X-Y-X-A-P-E) directly juxtaposed to MAPKKK phosphorylation site [62, 63]?

Mixed-lineage kinase 3 (MLK3)

MLK3, another member of MAPKKK family, contains a N-terminal SH3 domain, followed by the catalytic domain and two tandem leucine/isoleucine zippers, a basic region, a Cdc43/Rac binding motif, and a proline-rich C terminus [64]. Based on these structural characteristics, MLK3 may associate with a variety of protein modules. Studies by Hehner *et al.* [34] suggested that MLK3 could directly associate with IKK complex through its leucine zipper domain and phosphorylate Ser176 of IKKα and Ser177 and Ser181 of IKKβ. Transfection of Jurkat T cells with a kinase mutated form of MLK3 blocked the CD3-CD28 signal- and PMA-induced NF-κB transcriptional reporter. No significant influence of this mutated MLK3 was observed on either TNFα- or IL-1β-induced NF-κB activation. These results suggest that MLK3 may be important in mediating T cell costimulation-induced activation of IKK and consequent NF-κB-dependent transcription. MLK3 has also been shown to form a complex with a JNK scaffold protein, JIP, and stimulate JNK activation. Thus, MLK3 may function as integral molecule between the signaling pathways leading to the activation of NF-κB and JNK and provide a molecular explanation as to why many stimuli induce activation of NF-κB and JNK simultaneously under certain circumstances.

A variety of other kinases have been shown to function upstream of IKK. Because direct association of these kinases with IKK upon activation or specific phosphorylating site(s) of these kinases on IKK has not yet been demonstrated, whether these kinases are direct upstream kinases phosphorylating and activating IKK or far more distal kinases indirectly activating IKK remains unsolved. These kinases include Cot [32], PKCθ [29, 30], PKCα, PKCξ [31], TAK1 [65, 66], PKR [33], etc. Due to the fact that a variety of kinases either related or unrelated can activate IKK, it seems likely that different cell types and stimuli may utilize distinct upstream kinases for the activation of IKK. An example to support this notion is provided by the observation that PKCθ and Cot kinase participate in CD3-CD28 costimulation signal-induced but not TNFα-induced activation of NF-κB [30, 32].

Metals and NF-κB activation

A number of reports during the last few years indicate that some metals are able to affect the activation or activity of NF-κB transcription factors [67]. However, a detailed mechanistic understanding of metal-induced activation or inactivation of NF-κB is still lacking. In addition, certain results from different groups have been contradictory [68–71]. A possible explanation for this controversy is that previous studies have utilized different doses of metal ions and have examined the cellular response in different types of cells. These previous studies have not been conducted over a wide dose range and with the simultaneous evaluation of both bioactivity and cytotoxicity. Since it is possible that different doses of metals may either stimulate the activation of NF-κB or inhibit the activity of NF-κB as a result of their outright ability to initiate cell death, it will be important to examine the relationship between the dose level and the type of metal on the activation of NF-κB.

Using mouse JB6 fibroblast cells stably expressing both the NF-κB-dependent chloramphenicol acetyltransferase (CAT) reporter gene and AP-1-dependent luciferase reporter gene, we recently investigated the potency of individual metals and some metal-containing particles, such as silica and residual oil fly ashes (ROFA), to activate NF-κB. All of these metals produced an optimal dose-dependent induction of NF-κB-dependent and AP-1-dependent reporter gene activity (Fig. 2). Under the optimal dose range, cobalt and copper showed the strongest induction of NF-κB reporter gene activity. Arsenic (As (III) and As (V)) and vanadium (V) (V (V)) exhibited a similar mild induction of NF-κB, whereas chromium (VI) (Cr (VI)) and nickel were relatively weak in their ability to induce NF-κB reporter gene activity. It is interesting to note that very low doses of Cr (VI) as compared to other metals, i.e., from 4–100 ng/ml, are sufficient for the induction of NF-κB. Higher doses of Cr (VI), (> 200 ng/ml), showed considerable cytotoxicity on this type of cells. In human bronchial epithelial cell line, the optimal dose range of Cr (VI) required for stimulating NF-κB activity is between 0.125–2 μg/ml (data not shown). Thus, NF-κB induction by metals appears to be both dose-dependent and cell type-dependent.

Figure 2 also showed that arsenite, arsenate, copper, vanadate, and silica induced NF-κB and AP-1 with a similar dose-response curve, whereas both cobalt and zinc preferentially induce NF-κB but not AP-1. Although both the original ROFA and the washing buffer of ROFA exhibited a similar dose-response induction of both NF-κB and AP-1, the washed ROFA could only induce NF-κB, indicating that the AP-1 induction by ROFA was dependent upon water soluble components of this particle.

Previous studies by several groups indicate that, at a non-cytotoxic dose range, Cr (VI), As (III) or V (V) is capable of

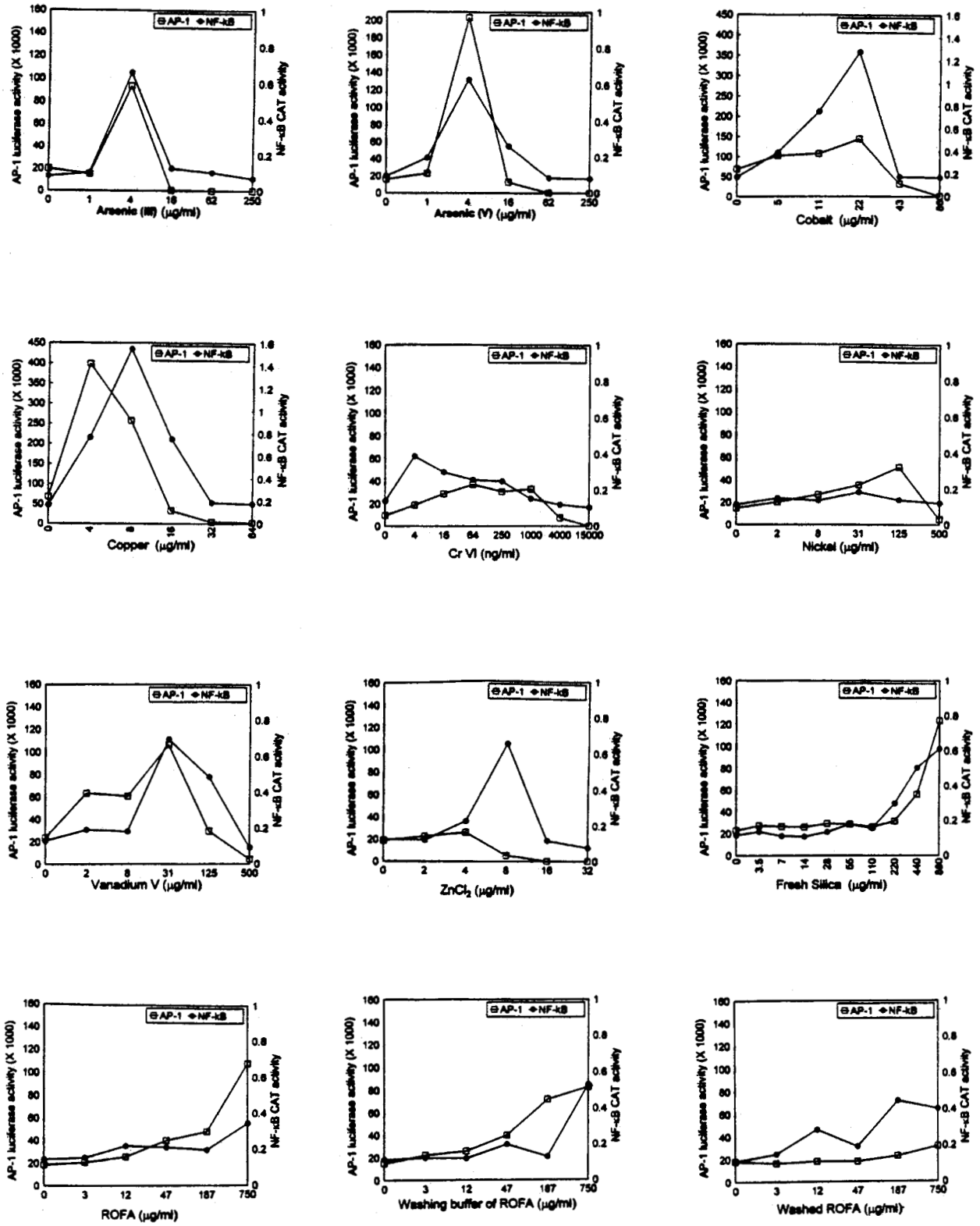

Fig. 2. Metals or metal-containing particles induce NF-κB-dependent CAT activity and AP-1 dependent luciferase activity. JB6 mouse epidermal cells stably transfected with NF-κB- and AP-1-dependent reporter genes were cultured with 0.1% FBS for 24 h in 24-well tissue culture plates. Cells were then treated with various doses of metals or metal-containing particles as indicated for an additional 12 h. Total cell lysates were prepared and subjected to CAT and luciferase activity assay, respectively.

activating NF-κB as determined by either a gel shift assay, which reflects the activation and nuclear translocation of NF-κB, or NF-κB-dependent reporter gene assay, an indicator of NF-κB activity. In contrast, Cr (VI), As (III), and other metals have been reported to inhibit NF-κB activation through interfering with IKK kinase, DNA binding, or the interactions with nuclear co-factor, cAMP-responsive element-binding protein (CREB)-binding protein (CBP) [70, 71]. These results raise the mechanistic question of how metals affect NF-κB activation and activity. Although some metals showed similar capabilities on either the activation or inhibition of NF-κB, different metals may use different signal transduction pathways to affect NF-κB.

Arsenic

The pioneer study of arsenic-induced NF-κB activation was carried out by Barchowsky *et al.* [72]. This study suggested that lower concentrations of As (III) activated NF-κB through increasing intracellular oxidant levels in vascular endothelial cells. Later studies by Hamilton *et al.* [6] indicated that the induction of NF-κB by As (III) is cell-type dependent. As (III) is able to induce NF-κB in MDA epithelial-like cells, whereas As is unable to induce NF-κB in H4IIE rat hepatoma cells. Mechanistic studies by Jaspers *et al.* [73] indicated a possible non-classic signaling pathway involved in mediating arsenite-induced NF-κB activation in airway epithelial cells. In contrast to other NF-κB inducers such as vanadyl sulfate, inducible IκBα degradation or p65 nuclear translocation did not occur in As (III)-induced NF-κB reporter gene activity. At higher concentrations, As (III) appears to exhibit an inhibitory effect on NF-κB by interfering with the DNA binding of NF-κB, possibly through a direct modification of As (III) on the sulfhydryls of NF-κB proteins [74]. If that is the case, there are a number of intracellular proteins, either within NF-κB signaling pathways or other pathways, which contain exterior sulfhydryls and would be targeted by As (III). Indeed, a direct inhibition of IKK kinase activity by As (III) has been demonstrated recently [70, 75]. 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 TNFα, IL-1 and PMA [75]. These latest findings provided a new insight into the role of As (III) on NF-κB signaling, but still did not solve the dilemma of the stimulation of NF-κB by As (III) at lower concentrations. One may speculate that the activation of NF-κB by lower concentrations of As (III) is possibly through the regulation of the redox status of cells as suggested by the earlier studies [72], while the inhibition of NF-κB by lower or higher concentrations of As (III) is through direct binding of As (III) to either IKK or NF-κB proteins. However, a more difficult question to answer is how redox changes participate in the signaling pathways leading to the activation of NF-κB. Obviously, IKK kinase is unlikely to be activated directly by changes in cellular redox status, since, similar to the modification by As (III), the oxidation of cysteine 179 in the activation loop of IKKβ will inactivate the activity of this kinase.

Vanadium

Vanadium is a transitional element that exists in oxidation states ranging from –1 to +5. The most common forms of vanadium include V (IV) and V (V). The first evidence indicating the induction of NF-κB by V (V) was provided by Schieven *et al.* [76] who demonstrated that V (V) potentiated ROS-induced NF-κB activation in a tyrosine kinase-dependent fashion [76]. Further studies consistently showed that V (V) or V (IV) could activate NF-κB in almost all types of cells tested, such as B-lymphocyte precursors, T-lymphocytes [77], monocytes [78], macrophages [79], epidermal cells [68] and epithelial cells [73]. In human myeloid ML-1a cells, V (V) itself exhibited a mild induction of NF-κB, but it attenuated the activation of NF-κB induced by TNFα [80]. The mechanism involved in vanadium- or vanadium compound-induced NF-κB activation is a matter of debate. Early study by Imbert *et al.* [77] indicated that the activation of NF-κB by V (V) occurred independently of IκBα degradation. However, this observation could not be reproduced by several recent studies, suggesting that V (V) did induce degradation of IκBα following the phosphorylation of serine or tyrosine [79, 81]. In mouse macrophage RAW264.7 cells, V (V)-induced IκBα degradation occurred within 10–20 min with a peak degradation at 40 min [79]. In human myeloid U937 cells or epithelial cells, V (V)-induced IκBα degradation occurred at 30 min and reached a maximum at 240 min [81]. A similar result was obtained in Jurkat E6.1 cells, Ramos, a human B cell lymphoma line, and normal human bronchial epithelial (NHBE) cells [73, 82, 83]. In contrast to the earlier report that no resynthesis of IκBα occurred after V (V) treatment [77], several subsequent studies have indicated that the resynthesis of IκBα indeed occurs at 80–180 min after V (V) treatment [79, 81]. Phosphorylation on either tyrosine 42 or serine 32/36 sites of IκBα has been demonstrated in cells treated with V (V). The phosphorylation of IκBα on these sites may contribute to the subsequent degradation of this protein. At present, there are limited data available regarding which kinase or how many kinases contribute to the phosphorylation of IκBα. Since IKK is required for LPS- and inflammatory cytokine-induced IκBα phosphorylation and NF-κB activation, IKK may also contribute to vanadium-induced NF-κB activation. Indeed, in mouse macrophage RAW264.7 cells, the activation of IKKβ kinase by V (V) is both dose- and time-dependent [79]. In comparison to cytokine- or LPS-induced IKKβ kinase activation, V (V)

induces IKKβ activation in a relatively persistent manner. In addition, blockage of the JNK signaling pathway resulted in a partial inhibition of IKK kinase activity induced by V (V). In the case of V (IV)-induced NF-κB activation, studies by Jaspers *et al.* [84] suggest that the p38 MAPK pathway may contribute to the NF-κB activation induced by V (IV). The phosphorylation of Tyr42 of IκBα might be through a passive enhancement of tyrosine kinase activity due to the inhibition of tyrosine kinase phosphatases by V (V). A new model has been proposed for the V (V)-induced, tyrosine phosphorylation-dependent NF-κB activation [85]. In this model, p85α, the regulatory subunit of PI3-kinase, had been suggested as a sequester of IκBα through specific association of its Src homology 2 domains with tyrosine-phosphorylated IκBα *in vitro* and *in vivo* after stimulation of T cells with pervanadate.

Chromium

Chromium occurs primarily in the trivalent state (III), which is the most stable form, or in the hexavalent state (VI), which is a strong oxidizing agent and carcinogen [86, 87]. NF-κB activation by Cr (VI) was observed in T cells [88], macrophages [67], bronchial epithelial cells (Chen *et al.*, unpublished), and human breast cancer cells [69]. The ability of Cr (VI) to activate NF-κB is also dose-dependent. It has been frequently observed that at higher concentrations, Cr (VI) exhibits an inhibitory effect on NF-κB activation or activity due to its cytotoxicity, interference with NF-κB DNA binding, and/or disruption of the interaction between NF-κB p65 protein and CEBP-binding protein [71, 74]. Our recent observations suggest that the stimulation or inhibition of Cr (VI) with a given dose-range on NF-κB is also cell density-dependent. The activation of NF-κB by Cr (VI) could only be detected at a relatively higher cell density in the presence of 1 to 5% of serum. We are currently testing the possibility that Cr (VI)-induced NF-κB activation is through a secondary cell-damaging effect in which damaged cells induced by Cr (VI) elicit an activation process for NF-κB in the adjacent undamaged cells. It has been suggested that Cr (VI) is one of the most potent metals which can elicit an oxidative stress response in cells through the generation of ROS, including superoxide anion, hydroxy radical, nitric oxide, and the by-product of superoxide, H_2O_2 [89–92]. Thus, under certain circumstances, this Cr (VI)-induced ROS generation may be accountable for the activation of NF-κB.

ROS generation in metal-induced NF-κB

A number of excellent review articles suggesting a central role of ROS in signal-induced NF-κB activation have been published and well cited during the last few years [93–99]. A high amount of ROS generated from chronic and acute inflammatory responses or environmental stresses is cytotoxic. It is known that limited production of ROS, as a consequence of electron transfer reaction in cytosol, peroxisomes and mitochondria, is buffered or scavenged by both enzymatic and nonenzymatic antioxidant systems inside the cells [99]. The mechanisms of ROS generation by metals under biologically relevant conditions may involve Fenton/Haber–Weiss chemistry and autoxidation [92]. In macrophages or other phagocytes, metal-induced ROS generation may also involve the activation of hypochlorous acid, lipid peroxides and nucleoside hydroperoxides [100]. Studies using of ROS scavengers or antioxidants have suggested the involvement of cellular redox regulation with certain kinases, leading to the activation of NF-κB or other transcription factors [97]. In an early model, ROS-induced oxidative stress was proposed as a universal mechanism for NF-κB activation by diverse agents [101]. An unanswered and difficult question is which point in the activation pathway of NF-κB is targeted by ROS. The signal transduction pathways, such as the upstream and proximal kinases (e.g., IKK, for NF-κB activation induced by TNF, LPS or CD28) have recently been identified [8, 21]. However, no evidence has been presented to indicate that these kinase cascades would be oxidant-responsive or redox-regulated. Evidence provided by Li and Karin [102] demonstrated that the ROS scavenger, N-acetyl-L-cysteine (NAC), reduced TNFα-induced IκBα degradation and NF-κB DNA-binding activity, but failed to affect TNFα-induced IKK kinase activity in HeLa cells. These results raise the possibility that ROS may not target the activation pathway of IKK, but rather may interfere with the steps of ubiquination and degradation of IκB. The model proposed by Roederer *et al.* [103] suggests that NF-κB activation is controlled by intracellular thiol levels, and that NF-κB inducers somehow potentiate oxidative stress by depleting glutathione levels. However, this model contradicts several later observations, which showed that a glutathione-oxidizing agent could inhibit NF-κB through interference with its DNA binding [104–106]. Several studies indicate that cysteine 62 of the p50 subunit of NF-κB is essential for NF-κB DNA binding and that oxidation of cysteine 62 inhibits NF-κB DNA binding activity. Thioredoxin, a ubiquitous dithiol-reducing enzyme, can reduce cysteine 62 and restore DNA-binding activity of NF-κB [104]. It thus appears that the observed NF-κB activation by metal-induced ROS may depend on some alternative pathways, such as MAP kinases, Ras and Rac1, with the potential to stimulate the IKKs or affect the transcriptional activity of NF-κB. Indeed, JNK inhibition by a transient transfection of macrophages with a dominant negative SEK1, an upstream kinase of JNK, decreased V (V)-induced IκBα degradation [79].

ROS and some transition metals, including V (V) and As (III), are potent inhibitors of protein tyrosine phosphatases

(PTP) [107, 108]. All PTPs contain a signature active site characterized by the sequence of His-Cys-X-X-Gly-X-X-Arg-Ser/Thr, where X is any amino acid [109]. Oxidation of the cysteine residue in this signature motif that is essential for the achievement of phosphatase activity will inactivate PTPs. Protein serine/threonine phosphatases, such as PP1, PP2A, PP2B and PP2C, may also be subject to redox regulation through the oxidative formation of a disulfide bond between a conserved pair of cysteine residues [110]. Because the extent of protein phosphorylation, such as IκBα phosphorylation, reflects the balance between the opposing actions of protein kinases and phosphatases, changes in either can consequently shift the extent of phosphorylation (Fig. 3). It has been suggested that hypoxia and reoxygenation induce NF-

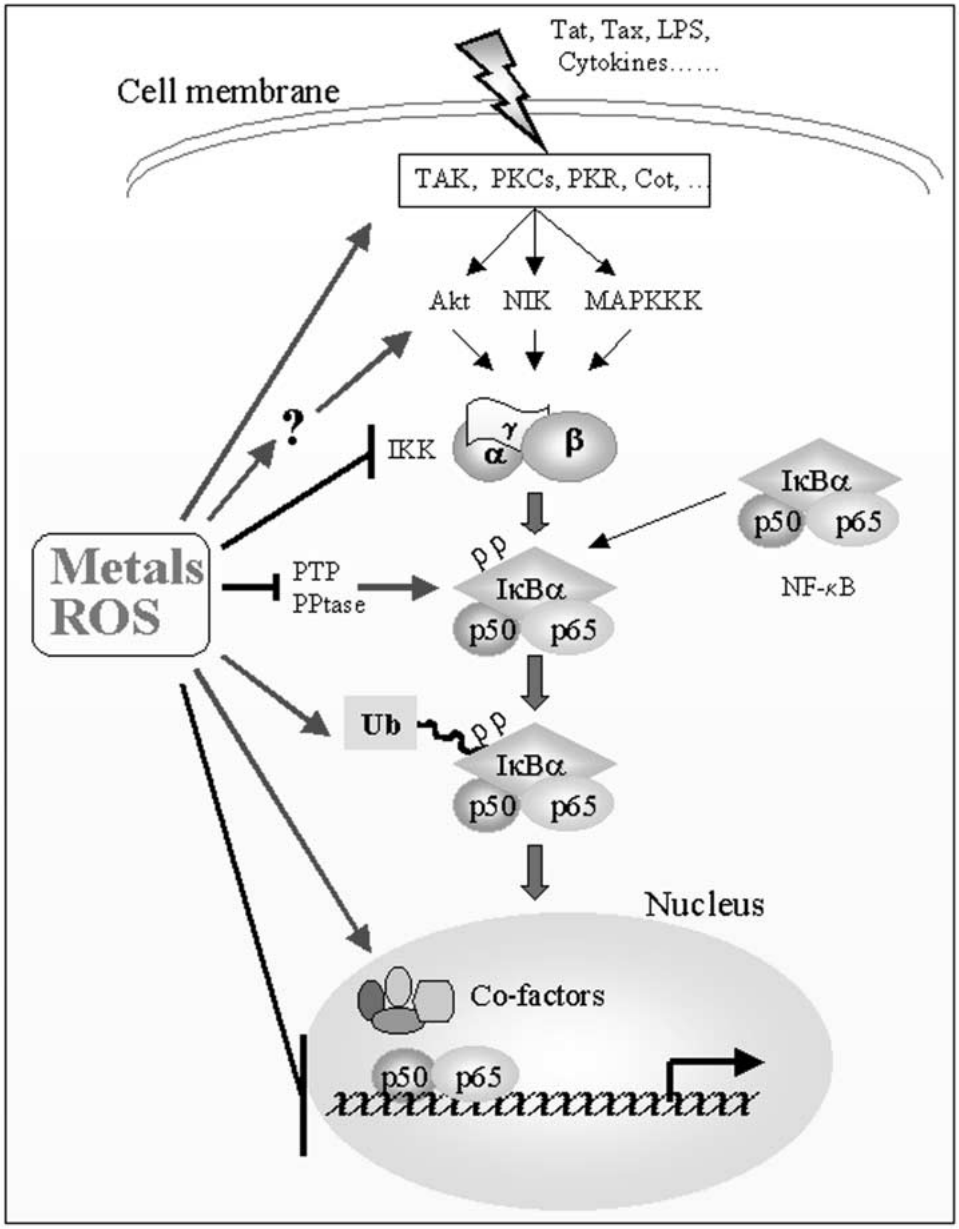

Fig. 3. Conflicting effects of metals or their ROS derivates on NF-κB signaling. Metals or ROS can possibly activate or potentiate the activation of up-stream kinases leading to the activation of IKK kinase complex. Meanwhile, metals or ROS can also inhibit IKK kinase activity through a direct modification of cysteine residues in the activation loop of IKK kinases. The phosphorylation of IκBα may be potentiated by metals or ROS due to the direct inhibition of tyrosine phosphatase (PTP) or serine/threonine phosphatases (PPtase) by metals or ROS. In the nucleus, metals or ROS may activate co-factors required for NF-κB-mediated transcriptional regulation on target genes, whereas they may also inhibit DNA binding activity of NF-κB by direct oxidation of critical cysteine residues in the DNA binding domain of NF-κB p50 or p65 proteins. →: activation; ⊣: inhibition.

κB activation through the induction of phosphorylation of tyrosine at position 42 on IκBα. Although this notion remains controversial, it will be important to determine whether tyrosine kinases are required for metal-induced NF-κB activation.

Summary

One of the major challenges in understanding the mechanisms of metal-induced NF-κB activation is to elucidate how signal transduction pathways are activated and how signaling cross-talk and specificity are achieved when several signaling pathways eliciting different cellular responses are activated at the same time by metals. For instance, why does activation of the NF-κB coincide with obvious MAPK activation in cells treated with metals under many circumstances, whereas only activation of NF-κB or activation of MAPK occurs under other circumstances? Since metals or their ROS derivatives are highly reactive but nonspecific molecules, an activation of only one specific signaling molecule, for example, an up-stream kinase, is hard to achieve in the cells in response to metals. It is a common scenario that metals or ROS can in principle induce conflicting signals by interfering with signaling molecules at different levels (Fig. 3). A good example is that oxidative stress amplifies or potentiates NF-κB activation, whereas at the same time oxidation of NF-κB proteins inhibits its NF-κB function. Translating the knowledge gained by studying the signaling pathways leading to the activation of NF-κB in the cellular response to metals may aid in developing novel preventive measures and therapies for diseases related to environmental and occupational exposures to metals.

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