A Critical Role for IκB Kinase β in Metallothionein-1 Expression and Protection against Arsenic Toxicity*

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Arsenic is a widespread environmental toxic agent that has been shown to cause diverse tissue and cell damage and at the same time to be an effective anti-cancer therapeutic agent. The objective of this study is to explore the signaling mechanisms involved in arsenic toxicity. We show that the IκB kinase β (IκKB) plays a crucial role in protecting cells from arsenic toxicity. IκKBβ–/– mouse 3T3 fibroblasts have decreased expression of antioxidant genes, such as metallothionein 1 (Mt1). In contrast to wild type and IKKB-reconstituted IκKBβ–/– cells, IKKB-null cells display a marked increase in arsenic-induced reactive oxygen species (ROS) accumulation, which leads to activation of the MKK4-c-Jun NH2-terminal kinase (JNK) pathway, c-Jun phosphorylation, and apoptosis. Pretreatment with the antioxidant N-acetylcysteine (NAC) and expression of MT1 in the IκKBβ–/– cells prevented JNK activation; moreover, NAC pretreatment, MT1 expression, MKK4 ablation, and JNK inhibition all protected cells from death induced by arsenic. Our data show that two signaling pathways appear to be important for modulating arsenic toxicity. First, the IKK-NF-κB pathway is crucial for maintaining cellular metallothionein-1 levels to counteract ROS accumulation, and second, when this pathway fails, excessive ROS leads to activation of the M KK4-JNK pathway, resulting in apoptosis.

2 The abbreviations used are: ROS, reactive oxygen species; IKK, inhibitor of nuclear factor-κB kinase; MKK4, mitogen-activated protein kinase kinase 4; JNK, c-Jun NH2-terminal kinase; NAC, N-acetylcysteine; MAP, mitogen-activated protein; NF-κB, nuclear factor-κB; IκBα, inhibitor of NF-κB; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; DCFDA, 2′,7′-dichlorofluorescein-diacetate; CM-H2DCFDA, chloromethyl DCFDA; ES cells, embryonic stem cells; SOD, superoxide dismutase; TNF, tumor necrosis factor; MT, metallothionein; ERK, extracellular signal-regulated kinase; PARP, poly(ADP-ribose) polymerase; MEF, mouse embryonic fibroblast; DAPI, 4,6-diamidino-2-phenylindole; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; WT, wild type; As, arsenic.

* This work was supported in part by National Institutes of Health Grants ES11798 (to Y. X.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This issue is dedicated to the memory of the late Martin B. Seroff in honor of his contributions to the biological sciences.
IKKβ, which hinders recognition of the nuclear localization signal on NF-κB and prevents its nuclear translocation. Upon stimulation by various growth factors, cytokines and cellular stressors, the IKK complex binds to sequence-specific consensus DNA and activates gene transcription (33–35). NF-κB is a critical regulator of the expression of genes involved in adaptive anti-oxidative responses, including glutathione peroxidase, mitochondrial SOD2, and cytosolic SOD1. Cells and mice defective in NF-κB signaling are more susceptible to oxidative damage induced by TNFα, H2O2, and arsenic (36).

The IKK-NF-κB pathway has recently emerged as a master regulator for buffering cellular redox levels and suppressing ROS accumulation during inflammatory responses (37, 38). In the current studies we showed that IKKβ activity was essential for transcriptional activation of antioxidant genes, such as metallothionein 1, whose expression was markedly reduced in Ikkβ−/− cells. As a consequence, Ikkβ−/− cells had higher levels of ROS accumulation than wild type cells. Arsenic caused a ROS-dependent activation of the signaling cascade MKK4-JNK-c-Jun that led to apoptosis. The thiol-containing redox scavenger N-acetyl-L-cysteine (NAC), MKK4 ablation, SP600125, a JNK inhibitor, and reconstitution of IKKβ nonessential subunits are activated, which phosphorylate IκBα, which hinders recognition of the nuclear localization signal on NF-κB and prevents its nuclear translocation. The nuclear NF-κB complex binds to sequence-specific consensus DNA and activates gene transcription (33–35). NF-κB is a critical regulator of the expression of genes involved in adaptive anti-oxidative responses, including glutathione peroxidase, mitochondrial SOD2, and cytosolic SOD1. Cells and mice defective in NF-κB signaling are more susceptible to oxidative damage induced by TNFα, H2O2, and arsenic (36).

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**EXPERIMENTAL PROCEDURES**

Reagents, Antibodies, and cDNA Vectors—All cell culture reagents, including Dulbecco’s minimum essential medium (MEM) fetal bovine serum, l-glutamine, MEM nonessential amino acids, penicillin-streptomycin, and MEM vitamins were obtained from Invitrogen. The MAP kinase inhibitors SB203580, U0126, SP600125, and NF-κB activation inhibitor II (JSH23) were obtained from Calbiochem-Novabiochem. Antibodies for phospho-p38 and β-actin were from PharMingen, antibodies for phospho-ERK, phospho-JNK, phospho-MKK4, phospho-MKK7, phospho-c-Jun, and IKKβ were from Cell Signaling Technology, (Beverly, MA), and antibodies for poly-(ADP-ribose) polymerase (PARP), hemooxygenase-1, IκBα, and GADD45α and GADD45-β were from Santa Cruz Biotechnology, Inc. Sodium arsenite, NAC, butylated hydroxytoluene, vitamin E, and hydrogen peroxide were purchased from Sigma, and cytokine TNFα was obtained from Pepro Tech, Inc. (Rocky Hill, NJ). A mammalian expression vector containing full-length MT1 cDNA was kindly provided by Dr. Tim Dalton (University of Cincinnati, Cincinnati, OH), the vector for NF-κB-luciferase was described previously (39), and plasmids for β-galactosidase (Fisher) and green fluorescence protein (Promega, Madison, WI) were from commercial sources.

**Cell Lines and Arsenic Exposure**—Mouse embryonic fibroblasts (MEFs) derived from wild type and IKKβ gene knock-out (Ikkβ−/−) fetuses were provided by Dr. Michael Karin (University of California, San Diego, CA) (40). The cells were propagated using a 3T3 protocol and were developed to 3T3 fibroblasts. Genotyping detection of the Ikk-null allele was carried out as described (40). Stably reconstitution of IKKβ expression in Ikkβ−/− (Ikkβ−/−-R) cells were developed by Dr. Ebrahim Zandi (University of Southern California). The p65−/− fibroblasts were from Dr. Sankar Ghosh (Yale University). The AP-1-luciferase MEFs were isolated from E13.5 fetuses of transgenic mice harboring AP-1-driven luciferase gene. Cells were cultured in Dulbecco’s modified MEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin streptomycin, 1% MEM nonessential amino acids, 1% Eagle’s vitamins. The embryonic stem (ES) cells of wild type, Mkk4−/−, and Mkk7−/− were kindly provided by Dr. Nishina (Tokyo Women’s Medical University, Japan) and were maintained under conditions as described (41).

The Ikkβ adenovirus was a gift from Dr. Yinling Hu (University of Texas, MD Anderson Cancer Center, Houston, TX). Adenoviruses at 10⁷ plaque-forming units were used for infection of 90% confluent cells. Viral infection was carried out in serum-free Dulbecco’s modified Eagle’s medium for 2 h with gentle shaking and then washed with phosphate-buffered saline. After infection, the cells were incubated with growth medium at 37 °C and 5% CO₂ for 24 h. When the cells reached 60–80% confluence, they were exposed to various concentrations of sodium arsenite in fetal bovine serum-free Dulbecco’s modified Eagle’s medium for different lengths of time.

**Cell Toxicity Assays**—Cells cultured on cover-glass slides were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, washed, and treated with 0.25% Nonidet P-40 for 1 min. Cells were stained with 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 30 min, the staining was observed, and pictures were taken under a fluorescence microscope. Approximately 100–200 cells in 3 different fields/coverslip were photographed. The percentage of apoptotic cells with condensed or fragmented nuclei was calculated in comparison to the numbers of total cells number.

As a measure of total cell death, lactate dehydrogenase (LDH) activity was determined in a colorimetric assay following the manufacturer’s instructions (Pierce). Briefly, 50 μl of cell culture supernatant were mixed with 200 μl of reaction mixture and incubated for 30 min at 37 °C in the dark. The remaining cells were lysed with water for 30 min and processed as described above. Absorbance was measured in a microplate reader at 490 nm. Total LDH = LDH in supernatant + LDH in water-treated cells. LDH release (%) = (supernatant LDH/total LDH) × 100.

Fluorescence based terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed using the labeling protocol provided by the manufacturer (Roche Applied Science).

**ROS Measurement**—Direct detection of intracellular steady-state levels of ROS was carried out on living cells using 2’,7’-dichlorofluorescein-diacetate (CM-H₂DCFDA). Cells cultured
on glass coverslips were incubated with 10 μM CM-H$_2$DCFDA (Molecular Probes, Inc., OR) in fetal bovine serum-free Dulbecco’s modified Eagle’s medium in the dark for 30 min. The cells were washed with phosphate-buffered saline and stained with DAPI. ROS generation, resulting in the oxidative production of dichlorofluorescein (excitation, 488 nm; emission, 515–540 nm), was detected under fluorescence microscopy.

Alternatively, cells cultured on 10-cm$^2$ plates were trypsinized and centrifuged, the cell pellets were incubated in fetal bovine serum-free culture medium with 5 μM H$_2$DCFDA for 30 min at 37°C. Cells were washed and suspended at 1 × 10$^6$ cells/ml in phosphate-buffered saline and analyzed by FACSCalibur (BD Biosciences Immuno cytometry Systems), which was equipped with a 488 argon laser for measurements of intracellular fluorescence. Logarithmic detectors were used for the FL-1 fluorescence channel necessary for DCF detection. Mean log fluorescence intensity values were obtained by the CELLQUEST software program (BD Biosciences).

**Cell Lysates and Western Blotting**—Whole cell extracts were prepared using radioimmunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μM leupeptin) at 4°C for 60 min. Samples were boiled and analyzed by SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane, and Western blotting was performed using antibodies as indicated.

**Reverse Transcription and Quantitative PCR**—Total RNA was extracted by using RNeasy mini kit (Qiagen Sciences), and RNA (10 μg) was reverse-transcribed to cDNA using random hexamer primers and the Stratascript enzyme following the instructions from the company (Invitrogen). Quantitative PCR was performed using an MX3000p thermal cycler system and SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). The conditions for the amplification were optimized for specific PCR reactions. At the end of the PCR the samples were subjected to melting curve analysis. All reactions were performed at least in triplicate. Oligonucleotides used as the specific primers to amplify mouse antioxidant genes cDNA are as follows: Mt1, 5’-CAGGACCTCTCAAGCTCC and 5’-CAGCCAGGAGCGAGCA-GCTCTTCTTGCA; Mt2, 5’-GCTCTAGAATCTTTCCAAAC and 5’-CAGCCAGGAGCGAGCA-GCTCTTCTTGCA; Gapdh, 5’-CCATGGGAGGCTGGG and 5’-CAGAGTT-
Transfection and Luciferase Assay—Cells were plated in 12-well tissue culture plates at 2 × 10⁴ cells/well the evening before transfection. The cells were transfected with 1 μg of NF-κB-dependent luciferase reporter plasmid along with 1 μg of β-galactosidase plasmid or with expression vectors for MT1 and green fluorescence protein (GFP) following the manufacturer’s instructions (Invitrogen). Twenty-four hours after transfection, cells were treated by arsenic and followed by staining by CM-H₂DCFDA, anti-p-c-Jun, and DAPI.

For the luciferase assay, cells were starved overnight and treated with 20 ng/ml TNFα for 16 h. Cells were lysed, and luciferase activity and β-galactosidase activity of cell lysates were determined using the luciferase reporter kit and β-galactosidase reporter kit (Promega) according to the manufacturer’s protocol.

Immunofluorescent Staining for Phospho-c-Jun—Cells grown on cover glasses were fixed with 4% formaldehyde and permeabilized with 0.25% Nonidet P-40. Cells were blocked with 5% bovine serum albumin (BSA) for 1 h and incubated overnight with rabbit polyclonal anti-phospho-c-Jun antibody diluted 1:500 in 1% BSA followed by rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin-G antibody (Molecule Probes) diluted 1:500 in 1% BSA for 30 min. Slides were analyzed using a fluorescent light microscope.

Statistical Analysis—Statistical comparisons were performed with Student’s two-tailed paired t test and analysis of variance. Values of p < 0.01 were considered statistically significant.

RESULTS

IKKβ Protects Cells from Arsenic Toxicity—The identity of the cells used for these studies was confirmed by genotypic analyses, which detected the presences of the Ikkβ-null alleles (40) in Ikkβ−/− and Ikkβ−/−-R but not in wild type cells (Fig. 1A). Western blot analyses showed that expression of IKKβ proteins, apparent in wild type, Ikkβ−/−-R, and Ikkβ−/−-Ad, was completely absent in Ikkβ−/− (Fig. 1B).

To evaluate the role of IKKβ in arsenic toxicity, we exposed wild type and Ikkβ-deficient cells to different concentrations (0–200 μM) of sodium arsenite for various length of time, as indicated (Fig. 1, C–F). Under normal growth condition, IKK status had not much influence on cell survival; when exposed to sodium arsenite, the Ikkβ−/− cells were more sensitive to toxicity than wild type cells, as indicated by the higher levels of LDH release and apoptosis. There was a clear association of arsenic dose and exposure time with increased death of all cells, but in each treatment condition toxicity was more severe in Ikkβ−/− than in wild type cells. Specifically, although 50 μM arsenite exposure for 6 h did not cause much toxicity of wild type cells, it induced 20% condensed nuclei, indicative of apo-
Cross-talk of IKKβ and JNK Pathways in Arsenic Toxicity

FIGURE 3. Inactivation of NF-κB in Ikkβ−/− cells is responsible for arsenic toxicity. A, WT and Ikkβ−/− cells were co-transfected with a NF-κB-driven luciferase reporter plasmid (NF-κB-Luc) together with a β-galactosidase expression vector driven by an actin promoter. After 36 h cells were treated with 20 ng/ml TNFα for 16 h, and luciferase activity was determined and normalized for β-galactosidase expression. Induction of luciferase activity by TNFα versus untreated cells is shown. B, WT and Ikkβ−/− cells were exposed to 50 μM arsenite, 20 ng/ml TNFα, and 100 μM hydrogen peroxide (H2O2) for the indicated times. Cell lysates were immunoblotted with anti-IκBα and anti-actin. C and D, WT cells were pretreated with a NF-κB inhibitor II at 30 μM for 2 h followed by a 6-h exposure to 50 μM sodium arsenite. C, cells were subjected to CM-H2DCFDA labeling and the DCF-positive cells were identified by fluorescence microscopy. D, condensed nuclei, used as markers of apoptosis, were identified by fluorescence microscopy, and the relative apoptotic cell numbers were quantified in three different fields. Results are presented as the mean ± S.D. from three independent experiments. The asterisks indicate significant difference (*, p < 0.01) compared with wild type cells (A) under the same experimental conditions or compared with untreated cells (D). E, WT and p65−/− cells were exposed to 50 μM arsenite for the indicated times. Cell lysates were subjected to Western blotting using anti-PARP. Cleaved PARP (cPARP) was apparently induced in the p65−/− cells exposed to arsenite for 6 h.

Ikkβ−/− cells are defective in maintaining redox levels after arsenic exposure. Aromatic is known to elicit intracellular ROS, which may be the cause of massive death of Ikkβ−/− cells. To test this possibility, wild type, Ikkβ−/−, Ikkβ−/−-R, and Ikkβ−/−-Ad cells were labeled with CM-H2DCFDA after arsenic exposure, and ROS-activated fluorescence positive cells were identified by flow cytometry and fluorescence microscopy. There was a slight increase in fluorescence levels in all arsenic-exposed cells; however, the intensity was significantly higher in Ikkβ−/− than in wild type, Ikkβ−/−-R, and high levels of ROS and apoptosis induced by arsenic. The Ikkβ−/− cells were also extremely sensitive to exogenous H2O2, suggesting that these cells might lack ROS scavengers (Fig. 2E).

The Role of IKKβ in ROS Modulation Is Likely Mediated through NF-κB—Lack of ROS scavenging capacity in Ikkβ−/− cells may be due to defective activation of NF-κB, a redox-sensitive transcription factor known to play an important role in antioxidant gene expression. Indeed, TNFα, a known IKK-NF-κB pathway activator, caused an apparent induction of IκBα degradation and NF-κB promoter-driven luciferase activity in wild type but not in Ikkβ−/− cells (Figs. 3, A and B). On the other hand, neither arsenic nor H2O2 induced IκBα degradation in wild type or Ikkβ−/− cells, suggesting that these agents did not cause an apparent activation of NF-κB through immediate induction of the IKKβ-IκBα pathway (Fig. 3B).

To determine whether NF-κB has a role in arsenic toxicity, we pretreated wild type cells with a NF-κB inhibitor for 2 h before they were exposed to sodium arsenite. NF-κB inhibitor-treated cells behaved like Ikkβ−/− cells, exhibiting increased ROS accumulation and apoptosis after arsenic exposure (Fig. 3, C and D). Moreover, cells deficient in the p65 component exhibited an obvious induction of PARP cleavage upon arsenic exposure (Fig. 3E). Thus, there is the existence of an IKKβ-NF-κB pathway that helps to maintain cellular redox levels and prevent apoptosis in response to arsenic.
Cross-talk of IKKβ and JNK Pathways in Arsenic Toxicity

A Role for the IKKβ-NF-κB Pathway in Antioxidant Gene Expression—Based on the above observations, we hypothesized that IKKβ ablation might prevent NF-κB activation by cytokines under normal growth conditions, leading to defective antioxidant gene expression in \( \text{Ikkb}^{-/-} \) cells (36, 38). This idea was tested by examination of the basal transcription levels of key antioxidant genes in wild type and \( \text{Ikkb}^{-/-} \) cells using real-time PCR (Fig. 4A). There was less mRNA accumulation for \( \text{Mrp1}, \text{Mt1}, \text{Mt2}, \text{mitochondrial Sod2}, \text{cytosolic Sod1} \), and glutathione peroxidase in \( \text{Ikkb}^{-/-} \) than in wild type cells; conversely, the expression of catalase was slightly increased in IKKβ-null cells. Reconstitution of IKKβ expression in \( \text{Ikkb}^{-/-} \) cells could partially restore the expression of \( 
\text{Mrp1}, \text{Mt1}, \text{Sod1}, \text{and Sod2} \), whereas it did not rescue the expression of glutathione peroxidase and catalase.

Among the IKKβ-dependent genes, the most strikingly changed was \( 
\text{Mrp1} \), as its expression levels were high in wild type cells but were suppressed by as much as 4000-fold in the \( \text{Ikkb}^{-/-} \) cells (Figs. 4, A and B). Although arsenic, like TNFα, slightly enhanced \( 
\text{Mrp1} \) transcription in wild type and IKKβ-reconstituted \( \text{Ikkb}^{-/-} \) cells, it failed to activate \( 
\text{Mrp1} \) expression in \( \text{Ikkb}^{-/-} \) cells (Fig. 4B). To determine whether the basal \( 
\text{Mrp1} \) expression was under the control of NF-κB, we pretreated wild type cells with NF-κB-I for 2 h, which resulted in a 4-fold suppression of \( 
\text{Mrp1} \) expression (Fig. 4C). Taken together, our results suggest a crucial role for IKKβ in \( 
\text{Mrp1} \) expression, which may be mediated through NF-κB.

If lacking MT1 expression were the reason for the arsenic sensitivity of \( 
\text{Ikkb}^{-/-} \) cells, re-introducing \( 
\text{Mrp1} \) expression should rescue the \( 
\text{Ikkb}^{-/-} \) cells from apoptosis after arsenic exposure. To test this hypothesis, expression vectors for \( 
\text{Mrp1} \) and a GFP were used to transfect \( 
\text{Ikkb}^{-/-} \) cells. Although the \( 
\text{Mrp1} \)-positive cells in \( 
\text{Mrp1} \) and GFP co-expression displayed marked resistance to arsenic toxicity, those in GFP expression alone were similar to the parental \( 
\text{Ikkb}^{-/-} \) cells, displaying high sensitivity to arsenic-induced apoptosis (Fig. 4D).

Arsenic-induced ROS Triggers the MKK4-JNK Apoptotic Pathway—To search for the molecular events downstream of ROS that might be responsible for arsenic toxicity, we looked at MAP kinase activation, known to be induced by cellular stress. A strong induction of JNK and ERK phosphorylation but a weak induction of p38 phosphorylation was observed in arsenic-treated \( 
\text{Ikkb}^{-/-} \) but not in wild type cells (Fig. 5A). To evaluate the contributions of MAP kinases to arsenic toxicity, wild type and \( 
\text{Ikkb}^{-/-} \) cells were pretreated with specific MAP kinase inhibitors: SP600125 for JNK, SB202190 for p38, and PD 98059 for ERK. Although the JNK inhibitor significantly reduced arsenic induced apoptosis of the \( 
\text{Ikkb}^{-/-} \), the p38 inhibitor had less and the ERK inhibitor had no effect on arsenic toxicity (Fig. 5B). Based on these results, we propose that JNK activation is mainly responsible for \( 
\text{Ikkb}^{-/-} \) cell toxicity in response to arsenite.

The onset and level of MAP kinase phosphorylation correlated very well with the ROS status, as induction was strong in \( 
\text{Ikkb}^{-/-} \) but weak if at all in wild type cells (Fig. 5A). The onset of ROS formation was first detectable at 2 h of exposure, and its level increased with time (data not shown); correspondingly, weak JNK phosphorylation was detected at 2 h, became stron-
ger at 6 h, and persisted for at least 24 h post-exposure. Moreover, pretreatment of cells with NAC completely blocked JNK phosphorylation and reduced hemeoxygenase-1 expression over, pretreatment of cells with NAC completely blocked JNK activation during the delayed phase of exposure.

To investigate whether an upstream signaling pathway was involved in JNK activation, we examined the phosphorylation of MKK4 and MKK7, the direct kinases for JNK. Arsenite exposure did not cause strong induction of MKK4 and MKK7 phosphorylation in wild type cells, but it induced phospho-MKK4 in Ikkβ−/− cells in a exposure- and time-dependent manner, similar to the induction pattern of phospho-JNK (Fig. 5D). On the contrary, phospho-MKK7 levels were higher in Ikkβ−/− than in wild type cells, but its level was not further increased after arsenite exposure.

To further evaluate the roles of MKK4 and MKK7 in JNK signaling, we examined phospho-JNK levels in wild type, Mkk4−/−, and Mkk7−/− mouse ES cells exposed to various concentrations of sodium arsenite for the indicated times (Fig. 5E). Again, there was an arsenic concentration- and exposure time-dependent increase of phospho-JNK, which was detected only in wild type and Mkk7−/− but not in Mkk4−/− ES cells. In agreement with the notion that JNK activity was essential for arsenic to induce apoptosis, the Mkk4−/− cells were largely resistant to arsenic toxicity, in contrast to wild type and Mkk7−/− ES cells that underwent significant apoptosis after arsenic exposure (Fig. 5F).

NF-κB-mediated MT1 Expression Connects the Ikkβ Pathway and the JNK-c-Jun Pathway—To evaluate the role of NF-κB in arsenic-induced JNK activation, wild type cells were pretreated with NF-κB-1, which itself caused a slight induction of JNK phosphorylation (Fig. 6A). Exposure to arsenic led to a weak JNK phosphorylation in wild type cells but a much stronger JNK phosphorylation in NF-κB-1-treated cells. Hence, similar to Ikkβ ablation, inhibition of NF-κB potentiates JNK activation in response to arsenite.

Next, we asked whether JNK activation signals were further transmitted to the nucleus. We examined the phosphorylation of c-Jun, a well-defined nuclear phosphorylation target of JNK. In arsenic-exposed Ikkβ−/− cells, there was a strong induction of c-Jun phosphorylation correlated to elevated ROS accumulation; in contrast, in wild type cells there was no detectable ROS and c-Jun phosphorylation (Fig. 6B). Pretreatment of the AP-1-luc cells with NF-κB-I also resulted in nearly 2-fold more induction of luciferase activity by arsenic, supporting the notion that inhibition of Ikkβ-NF-κB pathway led to enhanced c-Jun and AP-1 activation in the nucleus.

Interestingly, transient overexpression of MT1 in Ikkβ−/− cells completely prevented arsenite from inducing ROS and activating c-Jun, underlying the predominant role of MT1 in modulating the Ikk-JNK axis in arsenic toxicity (Fig. 6B). Taken together, our results establish a critical role for the Ikkβ pathway in MT1 expression and redox homeostasis, uncovering the mechanisms by which Ikkβ prevents activation of the apoptotic JNK pathway during arsenic toxicity.

DISCUSSION

Our results clearly support a role for Ikkβ in offering protection against arsenic toxicity (Fig. 6D). Under normal growth conditions, Ikkβ is required for NF-κB activation and antioxidant gene expression, which constitutes the redox modulating machinery that counteracts the oxidative stress caused by arsenite. Inactivation of this pathway in Ikkβ−/− cells results in high sensitivity to arsenic-induced ROS accumulation and cell death, whereas reconstituting Ikkβ expression either transiently by adenovirus-mediated gene expression or stably by introducing Ikkβ cDNA reverses the arsenic sensitivity of Ikkβ−/− cells.

The results of our work are consistent with several previous studies. A dominant-inactive IkB has been shown to prevent NF-κB activation, thereby rendering cells sensitive to arsenic (43). Moreover, Ikkβ-null MEFs are highly sensitive to arsenic
Cross-talk of IKKβ and JNK Pathways in Arsenic Toxicity

Several lines of evidence provide novel mechanistic insight of the role of IKKβ in arsenic toxicity. First, we have identified MT1 as one of the major transcription targets of the IKKβ pathway. NF-κB has been previously shown to regulate the expression of antioxidant defense enzymes, including mitochondrial SOD2 and cytosolic SOD1, glutathione peroxidase, and catalase (36). These enzymes metabolize and detoxify superoxide anions and H₂O₂ to H₂O and O₂. In addition, IKKβ has been shown to negatively regulate Cyp1b1, whose expression at least partly is responsible for sensitivity to arsenic toxicity (44). We find only a slightly reduction in the expression of detoxification enzymes SOD1 and SOD2 in the Ikkβ¹/⁻ cells, whereas the expression of glutathione peroxidase and catalase is not strictly dependent on IKKβ. The most strikingly affected gene by IKKβ ablation is Mti, whose expression is markedly decreased in the Ikkβ¹/⁻ cells and restored by reintroducing IKKβ expression in these cells.

Second, we have established a crucial role for MT1 in counteracting arsenic-induced ROS and apoptosis. MTs are low molecular weight cysteine-rich metal-binding proteins that have multiple functions, such as detoxification of nonessential metals and protection of cells against oxidative damage (47, 48). Introducing MT1 expression into the Ikkβ¹/⁻ cells protects against ROS accumulation, JNK pathway activation, and apoptosis induced by arsenic. Hence, MT1 appears to act as a mediator between IKK and JNK pathways, and high levels of MT1 expression are sufficient for protection against arsenic-induced apoptosis (49–51).

Third, we have shown that ROS activate the MKK4-JNK pathway, leading to apoptosis; however, JNK activation is not the consequence of a JNK phosphatase inactivation as shown previously (38). ROS act as second messengers to modulate many signal transduction pathways through oxidation of critical target molecules such as protein kinase C and protein-tyrosine phosphatases. Previous studies show that TNFα-mediated ROS in NF-κB-deficient cells cause sustained JNK activation through inactivation of a JNK-specific protein-tyrosine phosphatase (38). In the Ikkβ¹/⁻ cells, arsenite-induced ROS accumulation also leads to prolonged JNK activation, which however, is mediated through activation of a specific JNK upstream kinase MKK4 but not MKK7. The activation of MKK4 may in turn result from the activation of members of toxicity, displaying excessive H₂O₂ accumulation (44) and higher levels of GADD45α expression after exposure (45). However, a recent study using IKKβ-null MEFs originated from precisely the same source has reached a completely opposite conclusion. In their studies, Song et al. (46) report that IKKβ ablation protects cells against apoptosis in response to arsenic and that the IKKβ-null MEFs have reduced levels of GADD45α.

One possible explanation for the apparent contradicting results is that our knock-out cells are mistaken for wild type cells and vice versa. We performed genotyping analyses and detected the mutant Ikkβ allele only in knock-out but not in wild type cells. The genotypes together with IKKβ protein expression patterns, confirmed the correct identity of cells used in our studies. When IKKβ is re-expressed, as shown in Ikkβ¹/⁻-R and Ikkβ¹/⁻-Ad, it is clearly sufficient for protecting the Ikkβ¹/⁻ cells from arsenic-induced ROS accumulation, JNK activation, and apoptosis. Moreover, ablation of the NF-κB p65 subunit also renders the p65¹/⁻ cells highly sensitive to arsenic-induced cell apoptosis. All these results together support a role of the IKK-NF-κB pathway in protecting cells from arsenic toxicity.

![Image](https://example.com/image.png)
MAP kinase kinase kinase (MAP3K), as recent findings by Yan et al. (52) show that arsenite activates Ask1, a redox-sensitive MAP3K. Hence, arsenic-elicited ROS likely activate a signaling cascade of MAP3K-MKK4 that leads to JNK activation.

The ROS effect may not be very specific, because we find strong induction of JNK and ERK and to a lesser extent p38 phosphorylation in IkκB−/− cells. Interestingly, only JNK activity is required for arsenite toxicity, as a JNK inhibitor or MKK4 ablation markedly decreased apoptosis, whereas inhibition of ERK does not affect toxicity induced by arsenite in the IkκB−/− cells. The ERK pathway is commonly associated with cell proliferation and cell cycle progression; hence, its activation may contribute to other toxic outcomes of arsenite exposure, such as tumor promotion.

One caveat is the role of NF-κB in the regulation of MT1 expression. Several transcription factors, such as SP1, AP-1, and MTF1, have been shown implicated in the regulation of MT, but a role for the IKK or NF-κB in MT regulation has never been previously established. Because the MT1 gene promoter contains NF-κB binding sites, it is possible that the IKKa-containing NF-κB activation transcriptionally up-regulates MT1 expression, which would take place only in wild type and IkκB−/− cells but not in IkκB−/− cells. However, NF-κB inhibitor treatment of wild type cells results in a 4-fold reduction of MT1 expression, way below the reduction caused by IKKα ablation. One possibility is that the IkκB−/− cells represent a state of chronic IKK-NF-κB pathway inactivation, having molecular alterations that cannot be mimicked by a transient NF-κB inhibition by the chemical compounds. The NF-κB inhibitor II exerts cytotoxicity when used for long-term treatment (data not shown). An alternative possibility is that the IKKa may control MT1 expression through other downstream effectors in addition to or independently of NF-κB. These interesting prospects require further investigation.

Arsenic exerts diverse toxic and therapeutic effects that may be attributed to ROS, which in turn act as critical signaling molecules to modulate biological systems, such as carcinogenesis and apoptosis. In this regard, modulating NF-κB activity may be used to directly affect arsenic toxicity. Activation of the IKK-NF-κB pathway increases ROS scavenging capacity that may depress tumor growth, whereas inhibition of this pathway may result in excess ROS that would trigger cancer cell apoptosis. Detailed molecular studies suggest additional approaches may exist, such as changing the levels of MT1 expression and JNK activity, which may lead to more specific and convenient means to modulate arsenic toxicity.

Acknowledgments—We thank Drs. Michael Karin for IkκB−/− cells, Sankar Ghosh for p65−/− cells, Hiroshi Nishina for providing MKK4- and MKK7-deficient ES cells, Yining Hu for providing IKKa adenovirus, and Tim Dalton for MT1 expression vector.

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