Protective Roles of NF-κB for Chromium(VI)-Induced Cytotoxicity Is Revealed by Expression of IκB Kinase-β Mutant

Fei Chen¶§, Jacquelyn Bower, Stephen S. Leonard, Min Ding, Yongju Lu, Yon Rojanasakul†, Hsiang-fu Kung‡, Val Vallyathan, Vince Castranova, and Xianglin Shi§

The Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505, USA; †Department of Basic Pharmaceutical Sciences, West Virginia University, Morgantown, WV 26506, USA; and ‡Institute of Molecular Biology, University of Hong Kong, Hong Kong

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§ To whom requests for reprints should be addressed at:

Dr. Fei Chen/Xianglin Shi
PPRB of NIOSH
1095 Willowdale Road
Morgantown, WV 26505
Tel: (304)-285-6021/6158; Fax: (304) 285-5938; Email: lfd3@cdc.gov; xshi@cdc.gov

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Abstract

To delineate the molecular mechanisms of NF-κB-mediated regulation of chromium(VI)-induced cell death, the signaling pathway leading to the activation of NF-κB was interrupted by stable transfection of a kinase mutated form of IκB kinase β (IKKβ-KM). Here we demonstrate a novel role for the NF-κB transcription factor in inhibiting chromium(VI)-induced cell death. Inhibition of NF-κB by IKKβ-KM 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 anti-apoptotic proteins, cIAP1 and cIAP2, in the cells overexpression IKKβ-KM. Chromium(VI) treatment of these NF-κB-inhibited cells induced necrotic-like cell death. Such chromium(VI)-induced cell killing could be partially inhibited by expression of exogenous cIAP1, an inhibitor of caspases, indicating non-caspase cytotoxic mechanisms may be involved in chromium(VI)-induced cell death. Indeed, combination of cIAP1 and the antioxidant, N-acetylcysteine, resulted in a significant inhibition of chromium(VI)-induced cell death of NF-κB-inhibited cells. These results suggest that NF-κB is essential for inhibiting reactive oxygen species-dependent cytotoxicity. Such inhibition may involve up-regulation of the expression of anti-death proteins including cIAP1 that prevents spontaneous caspase activation and subsequent cleavage of Bcl-xl protein.
A wide range of signals, many of which are thought to be related to cellular stress, induce expression of early response genes through the nuclear factor-κB (NF-κB) family of transcription factors (1-4). In resting cells, NF-κB is retained in cytoplasm in its inactive form by interaction with 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 carboxyl terminal partial degradation of the p105 and p100 precursors, allowing nuclear translocation of the NF-κB complexes. Activated NF-κB binds to specific DNA sequences in target genes, designated as κB-elements, and regulates transcription of genes mediating inflammation, carcinogenesis and pro- or anti-apoptotic reactions. IκBα is the most abundant inhibitory protein for NF-κB (5). The mechanisms of signal-induced IκBα degradation involve phosphorylation of two serine residues, S32 and S36. This phosphorylation leads to polyubiquitination of two specific lysines on IκBα (K21 and K22) by an SCF-β-TrCP complex and its degradation by the 26S proteasome (6). 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 (3,5). IKKα and IKKβ share 50% sequence homology. Both proteins contain an amino terminal kinase domain, a carboxyl terminal region with a leucine zipper and a helix-loop-helix domain. In vitro and in vivo studies indicate that both IKKα and IKKβ are capable of phosphorylating IκBα on S32 and S36, but IKKβ is more potent in IκBα phosphorylation induced by proinflammatory stimuli. Recent studies by several groups indicate 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/PKCɛ-induced S36 phosphorylation of IκBα and thus NF-κB activation (7-11).

Increasing evidence indicates that NF-κB is either a pro- or anti-apoptotic transcription factor regulating a variety of apoptotic responses (12). NF-κB is activated in response to several pro-apoptotic stimuli including oxidative stress, cytotoxic drugs and ionizing radiations (13,14). Consistent with this notion, the gene encoding Fas ligand (FasL) has been shown to be transcriptionally regulated by NF-κB in response to T-cell activation signals and to chemotherapeutic agents (15,16). The evidence that NF-κB is also an anti-apoptotic transcription factor is mainly provided by gene knockout studies of NF-κB family members and IKK kinase subunits (17-19). Rel A (p65) deficient mice die during embryonic development through apoptosis of hepatocytes (17). IKKβ gene knockout mice die as embryos and show massive liver cell apoptosis, a response similar to that of NF-κB p65 gene knockout mice (19). Male mice with an inactivated X-linked gene encoding IKKγ/NEMO, an essential modulator of the IKK complex for NF-κB activation, die at mid-gestation due to a massive cortical and medulla lymphocyte apoptosis in the thymus in addition to degeneration of the liver (20,21). Thus, in certain situations, NF-κB is pro-apoptotic, but in alternative situations and cell types, NF-κB inhibits apoptosis and contributes to cell proliferation or transformation. Therefore, cell type and inducing stimuli appear to determine whether NF-κB is a causal or secondary event in apoptosis.

Apoptosis is a process in which cell death is initiated and completed in an orderly fashion through the activation of various apoptotic pathways (22,23). However, in cases of severe injury, cells may instead undergo necrosis, a passive death resulting in cellular lysis (23). Most apoptotic cells are characterized by unique morphological features, such as membrane blebbing,
cell shrinking, cytosolic and nuclear condensation, and breakdown of chromosomal DNA. In contrast, cells dying by necrosis are characterized by cellular edema and loss of cell membrane integrity. Depending on the involvement of caspases or reactive oxygen species, cell death can be either apoptotic, necrotic, or both (24). In fact, under many circumstances, different death pathways can co-exist in the same cell and are switched on by specific stimuli. A number of studies have revealed that when a cell dies by a typical apoptotic process, usually a late-phase necrosis also occurs (25-29).

Chromium(VI) [Cr(VI)] compounds, widely used in industry, have been shown to have serious toxic and carcinogenic effects on humans. Although the biochemical features of the signals that associate Cr(VI) with NF-κB activation and cell death have so far remained unclear, both reactive oxygen species (ROS)-dependent and ROS-independent mechanisms have been proposed (30-32). The importance of NF-κB as an anti-apoptotic factor is evident mainly from the studies of gene knockout mice and the apoptotic pathways of TNFα signaling (17-19,33). Much less is known concerning the role of NF-κB in Cr(VI)-induced cell death. The objective of the present investigation was to clarify the involvement of NF-κB in Cr(VI)-induced cell death and to determine if NF-κB plays a protective or promoting role in cell death triggered by Cr(VI).
Materials and Methods

Cells and Reagents – The human bronchial epithelial cell line, BEAS-2B, from American Type Culture Collection (ATCC, Rockville, MD) was cultured in keratinocyte basal medium (Sigma Chemical Company, St. Louis, MO) supplemented with 30 µg/ml of bovine pituitary extract and 5 ng/ml of human epidermal growth factor. Mouse embryo fibroblasts (MEF) derived from wild-type mice and IKKβ gene knockout mice were a gift from Dr. Michael Karin (University of California, San Diego, La Jolla, CA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS). Cr(VI) was purchased from Aldrich Chem. Co (Milwaukee, WI). The luciferase assay kit was from Promega (Madison, WI). All antibodies against NF-κB family members, IKKβ, pro-caspase-3, Bcl-xl, and Myc-tag were from Santa Cruz Biotechnology (Santa Cruz, CA) or Upstate Biotechnology (Lake Placid, NY). Anti-flag monoclonal antibody was from Sigma Chemical Company (St. Louis, MO). ECL Western blotting detection reagents were from Amersham Life Science (Buckingham, England).

Cell Transfection – pCR-Flag-IKKβ and pCR-Flag-IKKβ-KM (K44A) were gifts from Dr. Hiroyasu Nakano (Juntendo University, Japan). pcDNA3-myc-IAP1 was provided by Dr. John C. Reed (The Burnham Institute, Lajolla, CA). pEGFPLuc vector was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). BEAS-2B cells were plated in 6-well tissue culture plates at 5 × 10^5 cells/well for two days. The cells were transfected with a control vector (pCR3) or indicated expression vectors along with a 2 × κB-dependent luciferase reporter construct using lipofectamine (Life Technologies, Rockville, MD) as previously described (34). Single clones of BEAS-2B cells, stably transfected with the control vector (pCR3), wild-type IKKβ, or IKKβ-KM, and luciferase reporter genes, were isolated in 700 µg/ml of G418 for three weeks and
tested by Western blotting and luciferase activity assay for expression of the transfected genes. Stably transfected cells were maintained in regular culture media supplemented with 200 µg/ml of G418. To minimize possible clone variations during the course of selection, several independently derived cell lines expressing each transfected vector with similar expression levels were pooled together for the experiments described below.

**Electrophoretic Mobility Shift Assay (EMSA)** – For nuclear protein extraction, cells were harvested and resuspended in hypotonic buffer A (10 mM HEPES [pH 7.6], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) as previously described (35). Briefly, cells were incubated in buffer A for 10 min on ice, then vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12,000 × g for 20 sec and were resuspended in buffer C (20 mM HEPES [pH 7.6], 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 30 min on ice. The supernatants containing nuclear proteins were collected after centrifugation at 12,000 × g for 2 min and stored at -70 ºC. For EMSA, 4 µg of nuclear extract were mixed with the 32P-labeled double-stranded oligonucleotide containing a κB sequence (5'-CAACGGCAGGGGAAATTCCCTCTCCT-3') in a reaction mixture containing 100 µCi [32P]dCTP plus 5 mM dATP, dGTP and dTTP. The reaction solution was incubated at room temperature for 30 min and electrophoresed on a native 5% polyacrylamide gel in 0.25 × TBE buffer for 2 to 3 h. The DNA binding proteins were visualized by autoradiography.

**Kinase Activity Assay –** The IκB kinase (IKK) activity assay was performed by the method reported by Geleziunas et al. (36) with minor modifications. Briefly, transfected BEAS-2B cells, seeded at a concentration of 5 × 10⁶ cells/ml and cultured for two days, were treated with indicated agents and lysed in a lysis buffer containing 1 % Nonidet P-40, 250 mM NaCl, 50 mM HEPES (pH 7.4), 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10
µg/ml aprotinin and 10 µg/ml leupeptin. After centrifugation of the lysate at 16,000 × g for 20 min at 4°C, the supernatant was incubated with anti-IKKβ antibody H-470 or anti-Flag antibody with rotation for 4 h at 4°C, followed by the addition of 20 µl of Protein A-Agarose and incubation at 4°C for an additional 2 h. The immunoprecipitate was collected by centrifugation at 2,000 × g and washed three times with lysis buffer and two times with kinase buffer containing 20 mM HEPES (pH7.4), 20 mM β-glycerophosphate, 1 mM MnCl₂, 5 mM MgCl₂, 2 mM NaF, and 1 mM DTT. To monitor the kinase reaction, the immunoprecipitate was incubated in 20 µl kinase buffer supplemented with 5 µCi of [γ-32P]ATP and 1 µg of GST-IκBα (1-54) (Clontech, Palo Alto, CA) for 30 min at 30°C. The reaction was stopped by addition of SDS sample buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which was then transferred onto a nitrocellulose membrane and subjected to autoradiography.

Clonogenic Survival Assay and Cell Death Assay – Logarithmically growing cells stably transfected with indicated expressing vectors were harvested by typsinization. Cell suspensions were seeded into 6-well tissue culture plates with a concentration of 10³ cells/well. After allowing cells to adhere for 12 h, the cells were treated with various concentrations of Cr(VI) for an additional 12 h. After the treatment, cells were washed and incubated for one week in tissue culture medium containing 5% FBS. At the end of culture, the cell colonies were washed and fixed by the addition of water:methanol (1:1, vol:vol) containing crystal violet (1 mg/ml) and counted under microscope. Clonogenic survival rate was calculated based on the number of colonies that grew and the number of cells plated into each well. For the analysis of cell death, stably co-transfected cells with the indicated vectors were cultured in six-well tissue culture plates for 48 h before the experiments. Percentage of green cells was determined by
fluorescence microscopy. Five independent counts in each experiment were used to determine a mean and standard deviation.

*Genefilter Microarray and RT-PCR* – The genefilter membrane (gf2l1) from Research Genetics (Huntsville, AL), which covers 3,965 genes, was used for mRNA expression profiling following the manufacture's instructions. Briefly, 1 µg of total RNA extracted from transfected cells was incubated with 2 µg of oligo dT, 1.5 µl of reverse transcriptase, 20 mM of dATP, dGTP and dTTP, and 100 µCi 32P-dCTP in 30 µl of DEPC-treated water for 90 min at 37 °C. After purification through a Bio-Spin 6 Chromatography Column, labeled probe was mixed with prehybridization solution and incubated with Genefilter membranes overnight at 42 °C. To minimize possible variations among individual membranes, the same membrane was stripped and re-hybridized with a second probe after the first round of hybridization. To verify the microarray data, some of the differentially regulated genes in the transfected cells, wild type- or IKKβ-/--MEF were analyzed by RT-PCR. The primers used for RT-PCR were designed by using Primer3 software [http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and indicated in Table 1.

*Western Blotting* – Whole cell extracts were mixed with 3 × SDS-PAGE sample buffer and then subjected to SDS-PAGE in 10 or 16% gels. The resolved proteins were transferred to a nitrocellulose membrane. Western blotting was performed using antibodies against IKKβ, Flag, Myc-tag, Bcl-xl, caspase-3, and anti-rabbit IgG-horseradish peroxidase conjugates.
Results

Inhibition of IKKβ blocks NF-κB activation – IKKβ has been considered as the major IkBα kinase in response to a variety of stimuli (3,5). To determine whether overexpression of a kinase-mutated form of IKKβ (IKKβ-KM) can lead to inhibition of NF-κB, we characterized BEAS-2B cell clones stably expressing either wild-type IKKβ or IKKβ-KM in combination of a NF-κB-dependent luciferase reporter construct. BEAS-2B cells transfected with the empty vector pCR3 were employed as a control. To exclude the potential problem associated with overexpression, we selected clones with a range of expression of the exogenous proteins relative to the endogenous IKKβ and identified clones with comparable levels of expression of wild-type IKKβ and IKKβ-KM. We first confirmed the previously observed inhibition of NF-κB in the cells expressing IKKβ-KM (37). The nuclear proteins were prepared from the transfected clones in the absence or presence of various doses of Cr(VI) for 1 h and subjected to EMSA. Figure 1A shows that NF-κB DNA binding activity in the cells transfected with a control vector or wild-type IKKβ could be induced by Cr(VI) in a dose-dependent manner. In contrast, no or very marginal induction of NF-κB DNA binding activity by Cr(VI) could be observed in the cells transfected with IKKβ-KM (Fig. 1A, lanes 6 through 10, upper panel). The same nuclear extracts were also analyzed for the Sp-1 DNA binding activity. As shown in Figure 1A, overexpression of IKKβ-KM did not alter the Sp-1 DNA binding activity in the cellular response to Cr(VI) (Fig. 1A, bottom panel).

To verify that the inhibition of NF-κB was a result of the functional disruption of IKK in cells expressing IKKβ-KM, we examined IKK kinase activity in these cells in the absence or
presence of Cr(VI). Cell extracts prepared at a 40 min time point after treatment with Cr(VI) were immunoprecipitated using IKKβ antiserum and subjected to an immune-complex kinase assay using GST-κBα (amino acids 1 to 54) as the substrate. As depicted in Fig.1B, Cr(VI) stimulated IKK kinase activity in the cells transfected with a control vector and wild-type IKKβ (Fig. 1B, lanes 1 through 3 and lanes 7 through 9, top panel). Only marginal IKK kinase activity was induced by Cr(VI) in the cells stably expressing IKKβ-KM (Fig. 1B, lanes 4 through 6, top panel). Essentially equal amounts of IKKβ proteins were present in the extracts from the cells transfected with vector, IKKβ-KM or IKKβ as verified by immunoblot using anti-Flag and anti-IKKβ antibodies (Fig. 1B, the middle and bottom panels). Since the transfected IKKβ and IKKβ-KM were consistently of the expected size in the immunoblot using anti-Flag antibody (Fig. 1C, the third panel), it seemed unlikely that the IKKβ-KM coding region had undergone mutation or rearrangement during plasmid amplification or integration into genomic DNA. Thus, these results suggest that the IKK kinase activity is indeed inhibited in the cells expressing a kinase mutated form of IKKβ, IKKβ-KM.

IKKβ inhibition enhances cell death – Evidence that cells lacking NF-κB activity undergo apoptosis suggests that NF-κB activation provides protection against apoptotic signals (17). The above data show that NF-κB activation in response to Cr(VI) is defective in the cells expressing IKKβ-KM. We next determined whether NF-κB inhibition by expression of IKKβ-KM sensitized cells to apoptosis in response to Cr(VI). To our surprise, Cr(VI) (5 µM) treatment for 12 h induced a necrotic-like, rather then apoptotic, cell death of IKKβ-KM cells. Morphologic analysis of phase contrast images of cells indicates that only a few control vector-transfected cells or wild-type IKKβ-expressing cells exhibited partial cell shrinkage and
condensation after the treatment with Cr(VI) (Figs. 2F and 2G). In contrast, after the same treatment, IKKβ-KM expressing cells manifested cell blebbing, swelling and loss of membrane integrity, characteristics similar to those seen in cells undergoing necrosis (Fig. 2H).

The role of IKKβ and NF-κB in controlling Cr(VI)-induced cytotoxicity was further investigated genetically using knockout mouse embryo fibroblast (MEF) cell line lacking IKKβ subunits. A dramatic loss of cell viability in response to Cr(VI) was observed in IKKβ−/− MEF (Fig. 2J), but not in wild-type MEF (Fig. 2I). Thus, these results excluded the potential artifacts associated with the use of dominant negative IKKβ kinase mutant in overexpression experiments (Fig. 2H).

To further assess the cytotoxic effect of Cr(VI) on the cells in which NF-κB was inhibited due to overexpression of IKKβ-KM or deficiency of IKKβ gene, IKKβ-expressing cells, IKKβ-KM-expressing cells, wild-type MEF, and IKKβ−/− MEF were treated with increasing concentrations of Cr(VI). Cytotoxicity was determined by both LDH release analysis and clonogenic survival assay. As indicated in Fig. 3A and 3B, compared to their wild-type counterparts, a substantial increase of LDH release was observed in IKKβ-KM cells (Fig. 3A) and in IKKβ−/− cells (Fig. 3B) in response to various doses of Cr(VI). Consistent with this observation, the clonogenic survival assay indicated that exposure to increasing amounts of Cr(VI) inhibited clonogenic survival in IKKβ-KM cells and IKKβ−/− MEF more effectively than in the cells expressing wild-type IKKβ or wild-type MEF (Fig. 3C). Figure 3D depicts a representative clonogenic survival experiment.

Spontaneous cleavage of Bcl-xl in IKKβ-KM cells or IKKβ−/− fibroblasts – It has been demonstrated that the bcl-x gene is a transcriptional target of NF-κB in both mouse and human cells (35,38). Enhanced NF-κB activity has been correlated with the upregulated expression of
Bcl-xl, an important anti-apoptotic protein that can stabilize mitochondrial membranes and prevent the release of cytochrome c and apoptosis inducing factor (AIF) (39-41). A possible explanation for the increased vulnerability of IKKβ-KM expressing cells in response to Cr(VI) is that these cells may lack sufficient anti-death proteins, such as Bcl-xl, due to the impairment of NF-κB signaling. Decreased expression of Bcl-xl can cause either apoptosis due to the increase of mitochondrial membrane permeability or necrosis due to the collapse of fragile mitochondria (42). However, gene expression profiling showed no difference of bcl-xl gene expression between IKKβ and IKKβ-KM expressing cells (data not shown). Unexpectedly, spontaneous cleavage of Bcl-xl protein was observed in IKKβ-KM expressing cells but not in control vector- or wild-type IKKβ-transfected cells (Fig. 4A, left panel). A 17 kD fragment occurred concomitantly with a disappearance of the 30 kD intact Bcl-xl protein band in non-stimulated or Cr(VI)-stimulated IKKβ-KM expressing cells. There are two potential cleavage sites of caspase-3 (HLAD61/S and SSLS76/A) that are located in the loop region between the BH4 and BH3 domains of the Bcl-xl protein (43,44). Cleavage of these sites by activated caspases releases a C-terminal product that lacks the BH4 domain, an anti-apoptotic domain of Bcl-xl protein. The spontaneous cleavage of Bcl-xl in IKKβ-KM cells indicated possible activation of caspases in these cells. Indeed, immunoblotting shows a basal activation of caspase-3 as judged by the cleavage of the 32 kD precursor caspase-3 with the appearance of a 12 kD activated caspase-3 fragment (Fig. 4A, right panel). Cr(VI) treatment did not further alter the cleavage of Bcl-xl and activation of caspase-3, indicating Cr(VI) itself has no effect on proteases responsible for the cleavage of Bcl-xl or the activation of caspase-3.

To rule out the possibility that above observations are artifacts due to overexpression of IKKβ-KM, we next examined the status of Bcl-xl proteins and caspase-3 in MEF cells derived
from both wild-type mice and IKKβ gene knockout mice. As depicted in Figure 4B, IKKβ protein is absent in IKKβ-deficient MEF (IKKβ−/−, Fig. 4B, top panel). However, these cells express comparable levels of IKKα as observed in wild-type cells (Fig. 4B, the second panel). The spontaneous cleavage of Bcl-xl protein and activation of caspase-3 are evident in IKKβ−/− cells (Fig. 4B, the third and bottom panels, respectively).

**Decreased cIAP expression in IKKβ-KM cells** – The spontaneous activation of caspase-3 in IKKβ-KM cells implied an impaired anti-apoptotic function in these cells. It is known that NF-κB may regulate the expression of several anti-apoptotic genes, such as cIAP1 and cIAP2. The failure of IAP antibody to detect IAP proteins in our system prompts us to analyze the basal gene expression profile of both wild-type IKKβ and IKKβ-KM expressing cells by DNA microarray. Both wild-type IKKβ and IKKβ-KM expressing cells were cultured in medium for 12 h. cDNA probes were generated from the RNAs of both cell lines and used for sequential hybridization with the human GeneFilter gf2l1, which contains 3,965 sequence-verified known human genes. The majority of these genes were expressed at similar levels in cells stably expressing either wild-type IKKβ or IKKβ-KM. In IKKβ-KM cells, several genes encoding proteins involved in the P450 function/cellular redox regulation, protein degradation, cell cycle, and transforming growth factor-β signaling were up-regulated by more than 2.5 fold in comparison to IKKβ cells (Fig. 5). Thus, these data indicate that NF-κB may negatively regulate the expression of these genes. At least two recent reports also demonstrated that NF-κB suppressed the expression of the P4501A1 (cyp1a1) gene (45) and a proteasome C3 subunit gene (46). Under the basal condition, many of the documented NF-κB target genes, such as cytokines and chemokines, were not changed (data not shown). However, we did note a decreased expression of both cIAP1 and cIAP2 genes in IKKβ-KM cells. Both cIAP1 and cIAP2 have
been originally identified as direct inhibitors for caspases, especially for caspase-3, caspase-7 and caspase-9 (47). In addition, the expression of genes encoding transcription factor E2F5, keratin 18, and an antioxidant protein PDIR is decreased in the IKKβ-KM cells. Therefore, the observed spontaneous activation of caspase-3 in IKKβ-KM cells may be explained as the lack of sufficient endogenous caspase inhibitors, such as cIAP1 and cIAP2.

To verify the difference of gene expression observed by microarray analysis between IKKβ and IKKβ-KM expressing cells, we next performed RT-PCR using equal amount of total RNAs from IKKβ expressing cells, IKKβ-KM expressing cells, wild-type MEF, or IKKβ−/− MEF. The results of the RT-PCR analysis confirmed decreased expressions of cIAP1 and cIAP2 and increased expression of eNOS and POH1 in IKKβ-KM cells (Fig. 5B). In fact, the cIAP2 expression appears to be undetectable in the cells stably expressing IKKβ-KM in this RT-PCR analysis (Fig. 5B, lane 4 of cIAP2 panel). In addition, we also compared the expression levels of eNOS, POH1, cIAP1, and cIAP2 between wild-type MEF and IKKβ−/− MEF. Similar to the BEAS-2B cells stably expressing IKKβ-KM, the IKKβ−/− MEF exhibited an increased expression of POH1 and decreased expression cIAP1 (Fig. 5B, lane 6). We failed to detect the expression of eNOS and cIAP2 in both wild-type and IKKβ−/− MEF. For unknown reasons, we also failed to detect the expression of XDH gene in both BEAS-2B cells transfected with different vector and MEF with different genetic backgrounds in several RT-PCR analyses (data not shown).

**Cr(VI)-induced cell death can be partially inhibited by exogenous cIAP1** – To determine whether Cr(VI)-induced necrotic-like cell death of IKKβ-KM cells was in fact due to the reduced expression of cIAP1 genes, we tested whether overexpression of cIAP1 was capable of reducing Cr(VI)-induced cell death. The IKKβ-KM cells were further transfected with a control vector, pcDNA, or a vector expressing Myc-tagged cIAP1, and cultured for 48 h. Cells were then left
untreated or treated with various concentrations of Cr(VI). After an additional 12 h, the caspase-3 activation, Bcl-xl cleavage and LDH release were determined. As depicted in Figure 6A, IKKβ-KM cells transfected with the control vector exhibited spontaneous activation of caspase and Bcl-xl cleavage as judged by the disappearance of procaspase-3 bands and intact Bcl-xl bands (Fig. 6A, lanes 4 through 6, top and middle panels). In contrast, transfection of Myc-tagged cIAP1 significantly blocked caspase-3 activation and Bcl-xl cleavage under either basal or Cr(VI)-treated conditions (Fig. 6A, lanes 1 through 3, top and middle panels).

The possible protective role of cIAP1 on Cr(VI)-induced cytotoxicity was also determined by cell viability analysis of IKKβ-KM cells co-transfected with pEGFPLuc and Myc-tagged cIAP1 or pcDNA control vector (Fig. 6B). While 5 µM Cr(VI) substantially decreased the percentage of green cells of IKKβ-KM cells co-transfected with pEGFPLuc and control vector, less effect of Cr(VI) on the loss of percentage of green cells was observed in IKKβ-KM cells co-transfected with pEGFPLuc and Myc-tagged cIAP1.
Discussion

The results presented here provide evidence for a novel function of NF-κB in inhibiting Cr(VI)-induced necrotic-like cell death. In the cells stably expressing IKKβ-KM, an essential component of NF-κB signaling, IKKβ, is defective (Fig. 1B). EMSA indicates a pronounced decrease of NF-κB DNA binding activity in these IKKβ-KM expression cells in response to Cr(VI) (Fig. 1A). Cell morphologic analysis demonstrates that treatment of the cells expressing IKKβ-KM with Cr(VI) induced a necrotic-like cell death (Fig. 2). Analysis of the protein expression levels for both Bcl-xl and caspase-3 shows that IKKβ-KM expressing cells or IKKβ gene knockout MEF exhibited spontaneous cleavage of Bcl-xl protein and activation of caspase-3 (Fig. 4A and Fig. 4B). The globe gene expression profiling analysis shows that inhibition of IKKβ to block NF-κB signaling decreased the expression of two important anti-apoptotic genes, cIAP1 and cIAP2. Transfection of the cells expressing IKKβ-KM with cIAP1 partially prevents caspase-3 activation, Bcl-xl cleavage (Fig. 6A), and protects the cells from Cr(VI)-induced cytotoxicity (Fig. 6B).

While the mechanism by which NF-κB protects cells from death signals remains to be further investigated, it may be related to its transcriptional regulation on several anti-apoptotic genes (33). The observations presented in this paper support the notion that NF-κB plays a pivotal role in the expression of both cIAP1 and cIAP2 genes. These data also support a model for the consequent effects of NF-κB inhibition on Cr(VI)-induced cell death (Fig. 7). The levels of cIAPs and Bcl-xl may determine whether necrotic cell death or apoptosis ensues in cellular response to Cr(VI). In NF-κB inhibited cells, such as the expression of IKKβ-KM and IKKβ gene knockout, caspase-3 was activated due to the reduced expression of cIAP1 and cIAP2.
Activated caspase-3 cleaves Bcl-xL, which not only weakens the protective mechanism of Bcl-xL on the mitochondrial outer membrane, but also converts this anti-apoptotic protein to a killer molecule (40,41). Under this pre-disposed condition, Cr(VI) treatment may result in necrosis rather than apoptosis due to severe damage of mitochondria. Severely damaged mitochondria release an excessive amount of cytochrome c that interrupts electron transport in the inner membrane, causing ATP depletion and consequently switching the cells from apoptosis to necrosis. However, if the levels of cIAP1 and cIAP2 are maintained by a normal NF-κB activation response, the cleavage of Bcl-xL will be prevented by IAP-mediated inhibition of caspases (Fig. 6).

The protective effect of cIAP1 on Cr(VI)-induced death of IKKβ-KM cells is distinct from the previous reports indicating that peptidyl caspase inhibitors potentiate TNFα- or double-stranded RNA-induced cytotoxicity (48,49). It should be noted that there are several substantial differences between cIAPs and peptidyl caspase inhibitors. In addition to their function as endogenous inhibitors for caspases, cIAP1 and cIAP2 have recently been shown to regulate several signal transduction pathways leading to the activation of NF-κB and JNK (50,51), and act as ubiquitin ligases modulating protein degradation (52,53). Thus, the observed protection of cIAP1 from Cr(VI)-induced killing of IKKβ-KM cells might not only be the result of inhibition of caspases, but also the result of regulation of intracellular signal transduction.

It has been proposed that Cr(VI)-induced cellular responses are both ROS-dependent and ROS-independent. A limited amount of ROS can be buffered in cells by glutathione and thioredoxin (54,55). This raises the possibility that the increased vulnerability of IKKβ-KM expressing cells to Cr(VI) may be partially due to a reduced generation of oxidative buffering molecules. Indeed, the gene expression profiling study showed that the lowest expressed gene in
IKKβ-KM cells, compared with that in IKKβ cells, is the gene encoding protein disulfide isomerase-related protein (PDIR) (Fig. 5), an important member of the thioredoxin superfamily participating in redox regulation (55). Lowered oxidative buffering could lead to oxidative stress. Under this circumstance, the mitochondrial respiratory chain would be easily disrupted. The cells would undergo necrosis rather than apoptosis due to the depressed activation of caspases by Cr(VI) or ROS. It has been demonstrated that activation of caspases requires ATP and reduction of cysteine in the essential active center of caspases (24). To support this, combined treatment of cells with cIAP1 and NAC to elevate intracellular thio-containing molecules, such as GSH, partially protected IKKβ-KM cells from Cr(VI)-induced killing (Chen et al, unpublished observation).

In conclusion, we have demonstrated a novel function of NF-κB in inhibiting Cr(VI)-induced cell death. The levels of cIAPs that are transcriptionally regulated by NF-κB are critical in determining the activation and activity of caspases and the integrity of the Bcl-xl protein. Investigations are currently underway to address whether other oxidative stress inducers, such as H2O2, and nitric oxide, exhibit a similar effect on the cells where NF-κB was specifically inhibited by different approaches, for example, gene knockout for IKKβ or p65, transfection of degradation resistant IκBα, or delivery of peptidyl inhibitors for the IKK complex.
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Footnotes

1 Abbreviations used are: NF-κB, nuclear factor-κB; IKK, IκB kinases; Cr(VI), chromium(VI); ROS, reactive oxygen species; cIAP, cellular inhibitor of apoptosis.
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**Figure legends**

Fig. 1. Inhibited activation of NF-κB in the cells transfected with IKKβ-KM.  

**A,** Cells were transfected with indicated vectors and treated with different doses of Cr(VI) for 1 h. NF-κB (top panel) or Sp1 (bottom panel) DNA binding activity was determined by EMSA. N.S.: non-specific binding.  

**B,** Transfected cells treated with 5 or 10 µM Cr(VI) for 40 min. In vitro IKK kinase activity analysis and immunoblotting using anti-flag antibody and anti-IKKβ antibody were performed as described in "Materials and Methods".

Fig. 2. Cr(VI)-induces necrotic-like cell death in NF-κB-inhibited cells. Phase contrast morphologic analysis of the cells transfected with indicated vectors or MEF derived from wild-type mice (WT) or IKKβ gene knockout mice (IKKβ−/−) in the absence (**A** to **E**) or presence of 5 µM Cr(VI) (**F** to **J**) for 12 h.

Fig. 3. Cr(VI) increases LDH release from and inhibits clonogenic survival of IKKβ-KM cells and IKKβ−/− cells.  

**A,** Cells transfected with indicated vectors were treated with various doses of Cr(VI) for 12 h. LDH release was determined as described in the “Materials and Methods”. Values are means ± SD of 5 determinations.  

**B,** Mouse embryo fibroblast (MEF) cells derived from wild-type or IKKβ−/− mice were treated with Cr(VI) and analyzed for LDH release as in A.  

**C,** The effect of Cr(VI) on clonogenic survival was determined in the cells transfected with indicated vectors or the cells with indicated genetic background. Data indicates survival as a percentage of untreated cells. Values are means ± SD of 3 determinations.  

**D,** typical clonogenic survival assay of cells expressing IKKβ or IKKβ-KM after the treatment of Cr(VI) as described in the “Materials and Methods”.
Fig. 4. Cleavage of Bcl-xl protein and activation of caspase-3 in IKKβ-KM cells or IKKβ−/− MEF. A, Total cellular proteins extracted from transfected cells with indicated vectors and treated with 5 μM Cr(VI) for 12 h were subjected to immunoblotting using antiserum against C-terminal Bcl-xl (left panel) or caspase-3 (right panel). The intact 30 kD Bcl-xl protein band and the 32 kD pro-caspase-3 are indicated by arrows. The arrow heads indicate the cleaved C-terminal 17 kD Bcl-xl fragment and activated 12 kD caspase-3, respectively. The relative molecular weights are indicated as kD on the right side of each panel. N.S.: non-specific bands. B, Wild-type and IKKβ−/− MEF cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 5 μM Cr(VI) for 12 h. Total cellular proteins were extracted and subjected to immunoblot using antibodies against IKKβ, IKKα, Bcl-xl, and caspase-3.

Fig. 5. Inhibition of IKKβ decreases the expression of anti-apoptotic genes encoding cIAP1 and cIAP2. A, cDNA microarray analysis of gene expression was performed by using genefilter membrane (gf211) and 32P-dCTP-labelled cDNA probe synthesized from poly(A)+ mRNA that was extracted from the IKKβ-KM-expressing cells and IKKβ-expressing cells. The magnitude of the changes reported was computed as fold changes of the average values over the 2 sets of comparisons. Only those genes with a more than 2.5-fold change were shown. Filled bars indicate those genes encoding products that participate in the P450 function or cellular redox regulation; bars filled with dots indicate the genes involved in ubiquitin-proteasome degradation pathways; hatched bars indicate those genes encoding proteins participating in cell cycle regulation; open bars indicate
TGFβ family genes; cross hatched bars toward left indicate the genes with a decreased expression in IKKβ-KM cells. **B.** Representative RT-PCR analysis confirming some of the genes showing altered expression by the microarray in **A.** The primers and RT-PCR conditions are shown in Table 1. The bottom panel shows the RT-PCR product of 7S RNA to document an equal amount of RNAs used in this assay.

**Fig. 6.** Exogenous cIAP1 inhibits spontaneous activation of caspase-3 and cleavage of Bcl-xl. **A,** Cells stably expressing IKKβ-KM were transiently transfected with a Myc-tagged cIAP1 (lanes 1 through 3) or a control vector, pcDNA (lanes 4 through 6). Approximately 48 h posttransfection, cells were treated with various doses of Cr(VI) as indicated for an additional 12 h. Thereafter, extracts were prepared and analyzed for caspase-3 activation (top panel) and Bcl-xl cleavage (middle panel). The expression of transfected Myc-tagged cIAP1 was verified in the same extracts by immunoblotting using anti-Myc antibody (bottom panel). **B,** IKKβ-KM cells were co-transfected with pEGFPLuc (GFP) and Myc-tagged cIAP1 (IAP1) or a control vector (pcDNA) and subjected to cell viability analysis following 5 µM Cr(VI) treatment. Values are means ± SD of 5 determinations.

**Fig. 7.** Possible mechanisms of Cr(VI)-induced necrosis in NF-κB-inhibited cells. Overexpression of a kinase-mutated IKKβ (IKKβ-KM) leads to the inhibition of basal and subsequent inducible NF-κB activation, resulting in decreased expression of cIAP1 and cIAP2. Caspase-3 is spontaneously activated under this circumstance, which causes cleavage of Bcl-xl protein. Bcl-xl cleavage not only weakens the protective mechanism of Bcl-xl on mitochondrial outer membrane, but also converts this anti-apoptotic protein to killer molecules of mitochondria. Necrosis, rather then apoptosis, will occur upon
persistent insults, such as Cr(VI) or overwhelming ROS. Small up and down line arrows indicate increased and decreased activities, respectively.
### Table 1: Sequences of PCR primers used for the RT-PCR experiments*

| mRNA  | GenBank ID | Primers (left/right)** | Region     | Product size |
|-------|------------|-------------------------|------------|--------------|
| XDH   | H: XM_054071<br>M: NM_011723 | 5'-CCGCACAGATATTGTCATGG-3'<br>5'-CTGAAGTCAATGGAGATGCT-3' | H: 3606-3803<br>M: 3556-3753 | 198bp |
| eNOS  | H: XM_054647<br>M: NM_008713 | 5'-GCCCTCACGCTACAACAT-3'<br>5'-GCTCATTCTCCAGGTGCTTC-3' | H: 1105-1303<br>M: 1109-1307 | 199bp |
| POH1  | H: XM_002532<br>M: NM_021526 | 5'-TTTGCTATGCCACAGTCAGG-3'<br>5'-CAAGGCTTCAAAGGTCTCGT-3' | H: 416-607<br>M: 450-641 | 192bp |
| cIAP1 | H: U45878<br>M: U88908 | 5'-CAATTGGGAACCGAAGGATA-3'<br>5'-ACTTGCAAGCTGCTCAGGAT-3' | H: 1087-1282 | 196bp |
| cIAP1 | M: U88908 | 5'-TTGCTTACACCTGTGAAC-3'<br>5'-GCAAAGCAGGCCACTCTATC-3' | M: 718-916 | 199bp |
| cIAP2 | H: U45879<br>M: U88909 | 5'-CCGACACAGATATTGTCATGG-3'<br>5'-CTGAAGTCAATGGAGATGCT-3' | H: 3606-3803<br>M: 3556-3753 | 198bp |
| 7S RNA | H: V00477<br>M: X04211 | 5'-CTCCTCCTAGGGAACCGAAGG-3'<br>5'-CCTCCTTCAAGCTGCTCGT-3' | H: 31-171<br>M: 15-159 | 141bp |

* The temperatures for reverse transcription are: 50 °C for 30 min and 94 °C for 2 min.

The temperatures for the 35 cycles of PCR are: 94 °C for 20s, 54 °C for 30s, 68 °C for 40s. At the end of PCR, the reactions were incubated at 68 °C for 10 min.

** The maximum mismatch numbers of homo (H) vs. mus (M) primers is 2 nucleotides.
### A

|       | Vector | IKKβ-KM | IKKβ  |
|-------|--------|---------|-------|
| 0     | 1.25   | 2.5     | 5     | 10    |
| [Cr(VI), µM] |        |         |       |

**Fig. 1A & B**

### B

|       | Vector | IKKβ-KM | IKKβ  |
|-------|--------|---------|-------|
| 0     | 1.25   | 2.5     | 5     | 10    |
| [Cr(VI), µM] |        |         |       |

**NF-κB**

**N.S.**

**Sp-1**

**N.S.**

**Flag**

**GST-IκBα**

**Flag**

**IKKβ**

**Fei Chen et al.**

**Anti-Flag**

**Anti-IKKβ**
Fig. 2A-J
Fig. 3A & B
Fei Chen et al.

Fig. 3C & D
Fig. 4A & B
Fei Chen et al.

Fig. 5
Fig. 6A & B
Fig. 7
Protective roles of NF-kB for chromium(VI)-induced cytotoxicity is revealed by expression of IκB kinase-b mutant

Fei Chen, Jacquelyn Bower, Stephen S. Leonard, Min Ding, Yongju Lu, You Rojanasakul, Hsiang-fu Kung, Val Vallyathan, Vince Castranova and Xianglin Shi

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