Zinc Inhibits Nuclear Factor-κB Activation and Sensitizes Prostate Cancer Cells to Cytotoxic Agents

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ABSTRACT

Prostate carcinogenesis involves transformation of zinc-accumulating normal epithelial cells to malignant cells, which do not accumulate zinc. In this study, we demonstrate by immunoblotting and immunohistochemistry that physiological levels of zinc inhibit activation of nuclear factor (NF)-κB transcription factor in PC-3 and DU-145 human prostate cancer cells, reduce expression of NF-κB-controlled antiapoptotic protein c-IAP2, and activate c-Jun NH_2-terminal kinases. Preincubation of PC-3 cells with physiological concentrations of zinc sensitized tumor cells to tumor necrosis factor (TNF)-α, and paclitaxel mediated cell death as defined by terminal deoxynucleotidyl transferase-mediated nick end labeling assay. These results suggest one possible mechanism for the inhibitory effect of zinc on the development and progression of prostate malignancy and might have important consequences for the prevention and treatment of prostate cancer.

INTRODUCTION

The Rel/NF-κB[2] family of eukaryotic transcription factors is composed of several structurally related proteins that form homo- and heterodimers. The most common Rel/NF-κB dimer in mammals contains p50-RelA subunits and is specifically called NF-κB. The activity of NF-κB is regulated by interaction with inhibitory IκB proteins, which block the ability of NF-κB to enter the nucleus and bind to DNA (1). Early response genes controlled by NF-κB contribute to malignant transformation (2) and progression of breast (3) and prostate (4) cancer to hormone-independent growth and are key elements of drug resistance in a wide variety of solid (5) and hematological (6) malignancies. Suppression of NF-κB activity in human prostate cancer cells sensitizes them to TNF-α mediated apoptosis (7) and inhibits their tumorigenic and metastatic properties *in vivo* by suppressing angiogenesis and invasion via down-regulation of vascular endothelial growth factor, interleukin-8, and matrix metalloproteinase-9 molecules (8).

Normal human prostate accumulates the highest levels of zinc of any soft tissue in the body. This unique capability is retained in benign prostatic hyperplasia. In contrast, the zinc level in prostate adenocarcinoma is markedly decreased (9, 10). Although special functions associated with the high zinc level in the normal prostate have not been resolved, substantial information exists that implicates the changes in zinc accumulation in the development and progression of prostate malignancy (11–13).

The present report reveals that physiological levels of zinc inhibit NF-κB activity in androgen-independent PC-3 and DU-145 prostate cancer cells, reduce constitutive expression of the antiapoptotic protein c-IAP2, activate JNK, and sensitize malignant cells to apoptosis induced by cytotoxic agents.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Androgen-independent human prostate cancer cell lines PC-3 and DU-145 were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), gentamicin (50 mg/l), sodium pyruvate (1 mM), and nonessential amino acids (0.1 mM) under conditions indicated in the figure legends.

Antibodies and Reagents. Antibodies to RelA, IκBα, and c-IAP2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to JNK was obtained from Promega Corp. (Madison, WI). Secondary horseradish peroxidase-conjugated donkey antirabbit antibodies were purchased from Amersham (Arlington Heights, IL). Antibody to poly(ADP-ribose) polymerase was obtained from BD Transduction Laboratories (Lexington, KY). FITC-conjugated secondary antibody was purchased from BD PharMingen (San Diego, CA). Antibody to actin, $ZnSO_4$, pyrithione, and TNF-α were obtained from Sigma Chemical Co. (St. Louis, MO). Caspase inhibitor Z-VAD-fmk was purchased from Biomol (Plymouth Meeting, PA). An APO-BRDU kit was obtained from The Phoenix Flow Systems, Inc. (San Diego, CA).

Western Blot Analysis. Nuclear and cytoplasmic extracts and whole-cell lysates were prepared as described previously (14). Protein concentrations were measured with BCA protein assay reagents (Pierce, Rockford, IL). Equivalent

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[2] The abbreviations used are: NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; JNK, c-Jun NH_2-terminal kinase; Z-VAD, fmk, z-Val-Ala-Asp-fluoromethyl ketone; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

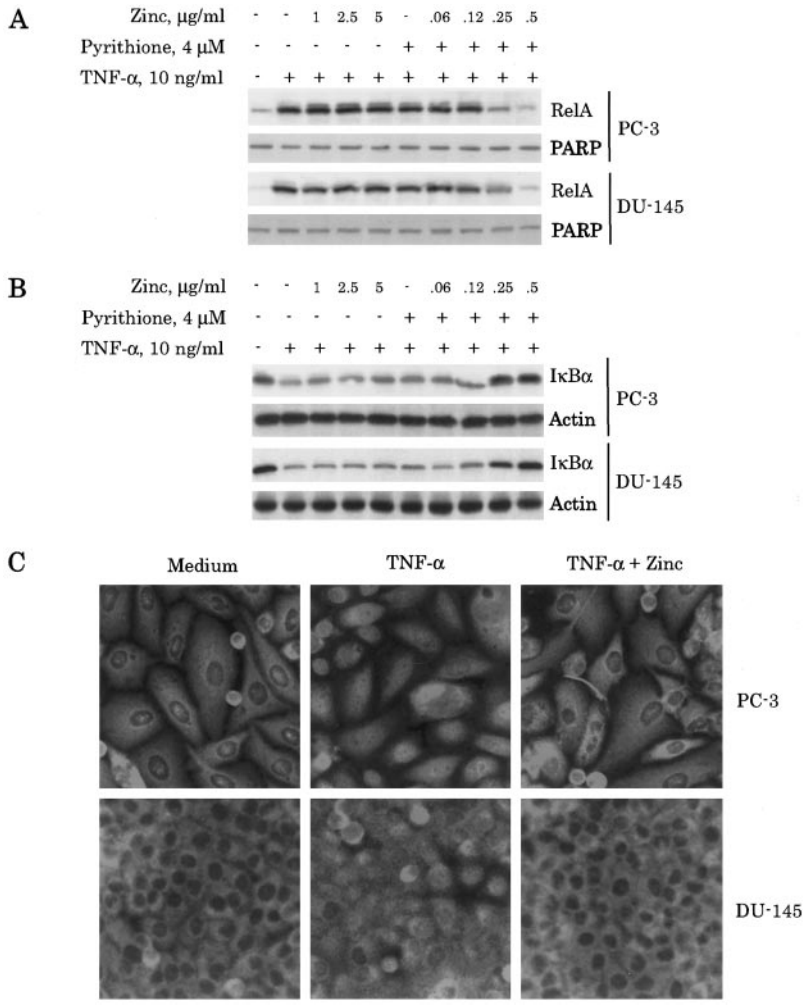

Fig. 1 *A,* zinc suppresses nuclear translocation of RelA PC-3 and DU-145 cells. Cells were preincubated with the indicated concentrations of zinc in the form of $ZnSO_4$ with or without 4 μM pyrithione for 30 min, followed by incubation with 10 ng/ml of TNF-α for 30 min. RelA protein levels were determined by Western blotting analysis with specific antibodies in nuclear extracts. Expression of poly(ADP-ribose) polymerase (*PARP*) was used to control equal protein loading. *B,* zinc blocks degradation of IκBα in prostate cancer cells. Cytoplasmic extracts from the same samples were subjected to SDS-PAGE, followed by Western blotting with anti-IκBα antibody. Expression of actin was used to control equal protein loading. *C,* immunohistochemical staining of PC-3 and DU-145 cells. Cells were preincubated with 0.5 μg/ml of zinc and 4 μM pyrithione for 30 min, followed by incubation with 10 ng/ml of TNF-α for 30 min. Cells were fixed and stained with anti-RelA antibody.

amounts of proteins (20 μg) were mixed with an equal volume of 2× Laemmli sample buffer, boiled, and resolved by electrophoresis in 10% SDS-PAGE gels. The proteins were transferred from the gel to a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad; 15 V, 3 mA/cm^2 for 24 min). Membranes were incubated in blocking solution containing 5% nonfat dry milk overnight to inhibit nonspecific binding. The membranes were then incubated with specific antibody (1–3 μg/ml) for 2 h. After washing in Tris/0.1% Tween 20 for 30 min, membranes were incubated for another 30 min with horseradish peroxidase-conjugated secondary antibody. The membranes were then washed and developed with enhanced chemiluminescence (ECL Western Blotting Kit; Amersham, Arlington Heights, IL).

Immunohistochemistry. PC-3 and DU-145 cells were grown on sterile glass slides, followed by treatment with 10 ng/ml TNF-α with or without preincubation with 0.5 μg/ml of $ZnSO_4$ and 4 μM pyrithione for 30 min. Slides were washed with PBS, fixed in −10°C methanol for 5 min, and air-dried. Specimens were then incubated in blocking solution containing 10% serum, washed with PBS and incubated with 1 μg/ml of RelA antibody. After washing with three changes of PBS, the specimens were incubated for 45 min with 3 μg/ml of FITC-conjugated secondary antibody and examined using fluorescence microscope.

Measurement of Apoptosis. DNA fragmentation was detected using the APO-BRDU kit (The Phoenix Flow Systems, Inc.) according to the protocols provided with the kit.

RESULTS

Physiological Concentrations of Zinc Inhibit NF-κB Activation in PC-3 and DU-145 Human Prostate Cancer Cells. Multiple studies have established the role of NF-κB-regulated genes in malignant transformation (2), progression of cancer to hormone-independent growth (3, 15–18), and in resistance to therapeutic regimens in a wide variety of tumors (6, 19, 20). Reports from several laboratories revealed that thiol-reactive metals suppress NF-κB activity in a dose-dependent manner (21–24). However, it is important to note that in most of these studies, nonphysiological levels of zinc were used, which were much higher than circulating levels of zinc [the plasma zinc level is ~1 μg/ml, of which about 66% is mobile, transportable zinc available for uptake by cells (11)], and represent

zinc concentrations that cells would never be exposed to *in situ*. Our preliminary data show clearly that physiological levels of zinc (0.25–0.5 μg/ml) inhibit activation of NF-κB in prostate cancer cells. Two androgen-independent prostate cancer cell lines, PC-3 and DU-145, were pretreated with various concentrations of zinc with or without 4 μM zinc ionophore, pyrithione, followed by stimulation with 10 ng/ml of TNF-α. Prostate cancer cells have lost the ability to accumulate zinc; therefore, pyrithione was used to facilitate zinc transport across the cell membrane. The findings presented in Fig. 1*A* demonstrate that in the presence of pyrithione, 0.5 μg/ml of zinc almost completely abolished TNF-α-induced nuclear accumulation of RelA in both PC-3 and DU-145 cell lines. We also determined whether physiological zinc levels prevent the degradation of the inhibitory subunit IκBα. Cytoplasmic extracts from the same samples were subjected to SDS-PAGE, followed by Western blotting with anti-IκBα antibody. Stimulation of PC-3 and DU-145 cells with TNF-α resulted in the rapid degradation of IκBα. In prostate cancer cells pretreated with zinc, the lack of nuclear accumulation of RelA corresponded with failure to degrade IκBα (Fig. 1*B*). As an additional approach to evaluate the effect of physiological zinc levels on NF-κB activation in prostate cancer cells, we performed immunohistochemical staining of PC-3 and DU-145 cells using anti-RelA antibody. The results presented in Fig. 1*C* confirm that physiological levels of zinc suppress TNF-α-induced nuclear translocation of RelA in both cell lines.

Zinc Decreases c-IAP2 Expression and Activates JNK in PC-3 and DU-145 Cells. NF-κB regulates expression of numerous genes with known antiapoptotic activity (25–27). The findings presented in Fig. 2*A* demonstrate that in PC-3 and DU-145 cells, physiological concentrations of zinc (0.5 μg/ml), noted to reduce nuclear accumulation of RelA, decrease constitutive expression of the antiapoptotic protein c-IAP2 in a time-dependent manner. We also examined the status of JNK activation in prostate cancer cells in response to zinc treatment because repression of JNK activation by NF-κB participates in the antiapoptotic effect of this transcription factor (28). Therefore, inhibition of NF-κB by zinc might endorse JNK activation with potential promoting effects on apoptotic pathways. Cells were treated for various periods of time with TNF-α, zinc, or a combination of TNF-α and zinc, and JNK activation was determined by Western blot analysis using antibodies that specifically recognize the active phosphorylated forms of JNK. Pretreatment with zinc induced prolonged JNK activation in both PC-3 and DU-145 cells in the presence and absence of TNF-α, whereas treatment with TNF-α alone induced minimal and transient JNK activation detectable only in DU-145 cells (Fig. 2*A*). Importantly, pyrithione alone had no effect on the status of JNK activation and c-IAP2 expression (Fig. 2*B*).

Zinc Sensitizes PC-3 Cells to TNF-α and Paclitaxel-mediated Apoptosis. Recent studies demonstrated that inhibition of NF-κB sensitizes prostate cancer cells to TNF-α-mediated apoptosis (7). Given our findings of a zinc inhibitory effect on NF-κB activation and c-IAP2 expression and its stimulatory effect on JNK activation, we examined whether zinc sensitizes prostate cancer cells to apoptosis induced by TNF-α and the anticancer agent paclitaxel. TNF-α and paclitaxel alone did not induce apoptosis in PC-3 cells as defined by TUNEL

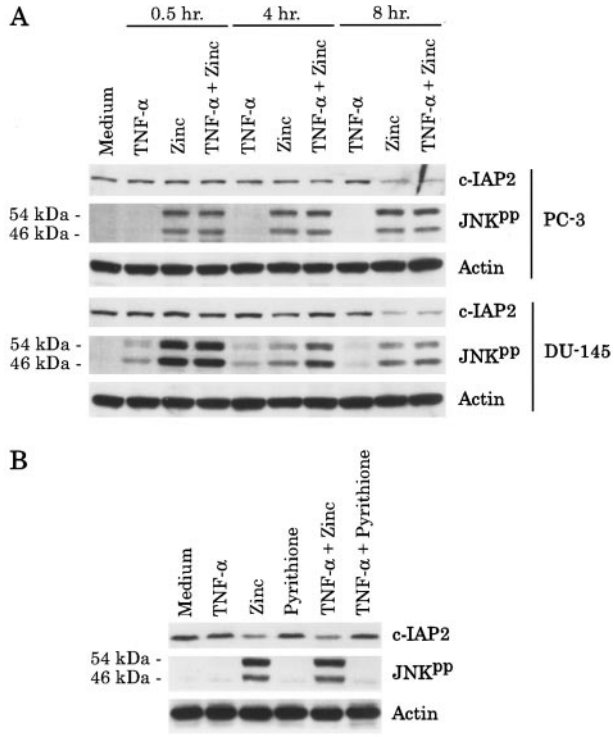

Fig. 2 Physiological concentrations of zinc decrease c-IAP2 expression and activate JNK in PC-3 and DU-145 cells. Cells were treated with zinc (0.5 μg/ml) and pyrithione (4 μM) with or without TNF-α (10 ng/ml) or with TNF-α alone for the indicated periods of time. Expression of c-IAP2 protein and phosphorylated forms of JNK was examined by Western blotting analysis using anti-c-IAP2 antibody and antibodies that specifically recognize the phosphorylated forms of JNK. Expression of actin was used to control equal protein loading.

assay (Fig. 3*A*). However, we found that preincubation of PC-3 cells with 0.5 μg/ml of zinc in the presence of pyrithione does increase their sensitivity to cell death when subsequently stimulated with either TNF-α or paclitaxel (Fig. 3*A*). As expected, pyrithione alone did not sensitize PC-3 cells to the cytotoxic agents. To assess the potential involvement of caspase family members in the cell death process, we tested whether the pan-caspase inhibitor Z-VAD-fmk would prevent DNA fragmentation in prostate cancer cells simultaneously treated with zinc and TNF-α or paclitaxel. Fig. 3*B* demonstrates that Z-VAD-fmk efficiently blocked apoptosis in PC-3 cells treated with either zinc/TNF-α or zinc/paclitaxel.

DISCUSSION

Aberrant transcriptional regulation of NF-κB-dependent genes is believed to be a major event contributing to malignant transformation and progression of the prostate cancer phenotype. Recent studies suggest that thiol-reactive metals including zinc may act to suppress NF-κB activity by blocking IκB kinase (29). However, in most of these studies, nonphysiological levels of zinc were used, which were much higher than the plasma level of zinc of ~1 μg/ml (30). Therefore, we investigated the effect of physiological concentrations of zinc on the status of NF-κB activation in prostate cancer cells. Our experiments

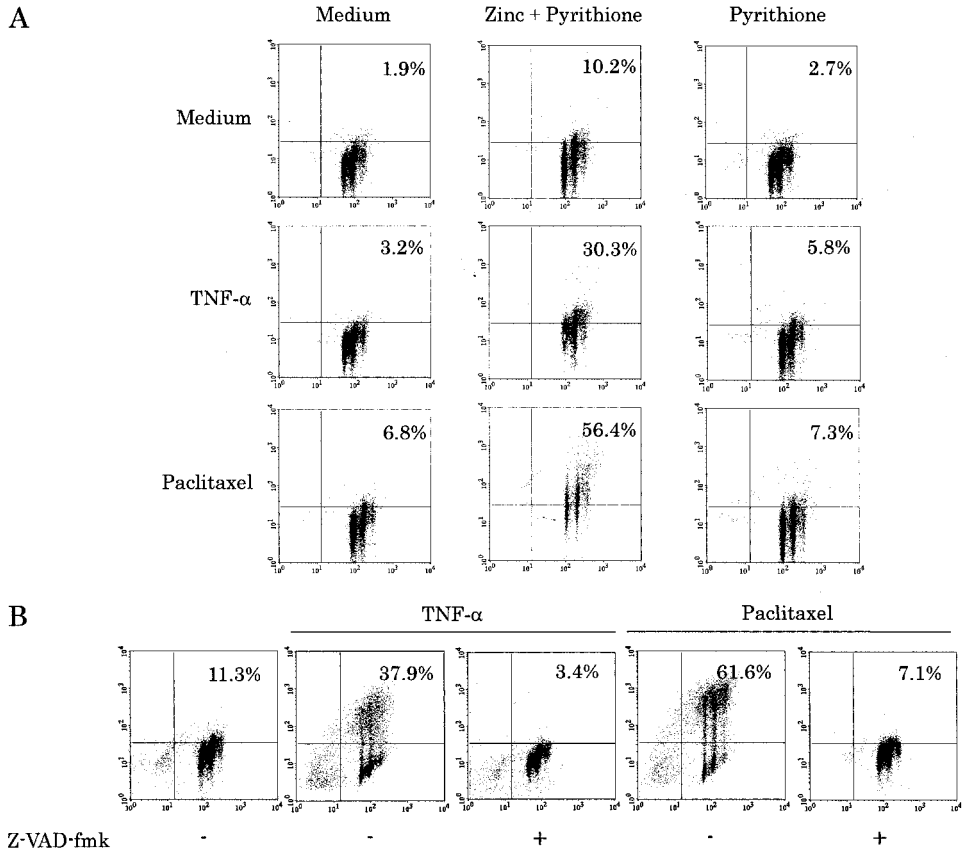

Fig. 3 *A*, zinc sensitizes PC-3 cells to TNF-α and paclitaxel-mediated apoptosis. PC-3 cells were preincubated with zinc (0.5 μg/ml) and pyrithione (4 μM) for 30 min, followed by stimulation with TNF-α (10 ng/ml) or paclitaxel (50 nM) for 24 h. The percentage of apoptotic cells was determined by TUNEL assay, followed by flow cytometry analysis as indicated in "Materials and Methods." *X axis*, DNA content; *Y axis*, TUNEL staining intensity. *B*, concomitant treatment with zinc and TNF-α or paclitaxel induces caspase-dependent apoptosis in PC-3 cells. PC-3 cells were cultured in the presence or absence of pan-caspase inhibitor Z-VAD-fmk (60 μM) for 30 min and then incubated with zinc (0.5 μg/ml) and pyrithione (4 μM) for 30 min, followed by stimulation with TNF-α (10 ng/ml) or paclitaxel (50 nM) for 24 h. The percentage of apoptotic cells was determined by TUNEL assay, followed by flow cytometry analysis. *X axis*, DNA content; *Y axis*, TUNEL staining intensity.

clearly demonstrate that physiological levels of zinc (0.25–0.5 μg/ml) inhibit activation of NF-κB in prostate cancer cells. Importantly, without pyrithione, zinc had no effect on RelA translocation and IκBα degradation, even when it was used at 5 μg/ml. Pyrithione alone also had no effect on the status of NF-κB activation (Fig. 1, *A* and *B*).

Several reports suggest that the decreased zinc level in malignant prostate tissue is not entirely attributable to a decreased availability of zinc in circulation (11, 30). Specialized mechanisms are required for both zinc uptake and release (31). Genes that are involved in mammalian zinc transport have been cloned recently (30, 32, 33). The *Arabidopsis* ZIP1 has been characterized as a plasma membrane-associated zinc uptake transporter that leads to the cellular accumulation of zinc (34). The human homologue of this gene, hZIP1, is widely expressed in mammalian tissues and is believed to be the major zinc transporter for many human cell types (33). Expression of hZIP1 in prostate cancer cells is regulated by testosterone and prolactin. Both hormones at physiological concentrations increased zinc accumulation in LNCaP and PC-3 cells (30). Overexpression of hZIP1 and the other member of the ZIP superfamily, hZIP2, in human K562 erythroleukemia cells resulted in an elevated zinc uptake activity not found in untransfected cells (32, 33). Therefore, strategies aimed at the overexpression of zinc uptake transporters in prostate cancer cells may represent a novel therapeutic approach for the treatment of hormone-refractory cancers.

It is well established that certain concentrations of zinc may trigger loss of mitochondrial potential and induce apoptosis in prostate cancer cells (12, 13). It is important to note that in our experiments, NF-κB inhibition was observed at zinc concentrations that had no significant effect on cell death rate within 24 h of treatment (Figs. 1 and 3). These results indicate that suppression of NF-κB by zinc is not simply a byproduct of the molecular events triggered by the cell death process, but rather it is a direct and independent effect of zinc. Thus, lower concentrations of zinc might have a more specific effect on prostate cancer cell evolution and sensitivity to cytotoxic agents via inhibition of NF-κB-dependent events, whereas higher concentrations of zinc may directly activate apoptotic pathways via alternative mechanisms.

Multiple studies established a central role of the NF-κB-regulated genes in neoplastic transformation, metastatic tumor progression, and evolution of cancer cells to hormone-independent growth (2, 3, 8). Overexpression of antiapoptotic proteins controlled by NF-κB family members has also been implicated as a key element of drug resistance in a wide variety of tumors (6, 20). Therefore, reestablishment of high intracellular zinc levels may have important implications for inhibiting angiogenic and metastatic potential of prostate cancer cells, suppressing tumor progression to an androgen-independent state, and sensitizing cancer cells to cytotoxic agents.

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