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Arsenic Trioxide Represses Constitutive Activation of NF-κB and COX-2 Expression in Human Acute Myeloid Leukemia, HL-60

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Abstract It has been proposed that eukaryotic nuclear transcripton factor nuclear factor kappa-B (NF-κB) and cyclooxygenase-2 (COX-2) are implicated in the pathogenesis of many human diseases including cancer. Arsenic has been widely used in medicine in Oriental countries. Recent studies have shown that arsenic trioxide (As_2O_3) could induce in vitro growth inhibition and apoptosis of malignant lymphocytes, and myeloma cells. However, the molecular mechanisms by which As_2O_3 initiates cellular signaling toward cell death are still unclear. In the present study, the effects of As_2O_3 on NF-κB and COX-2 expression in HL-60 cells were investigated. As_2O_3 suppressed DNA-binding activity of NF-κB composed of p65/p50 heterodimer through preventing the degradation of IκB-α and the nuclear translocation of p65 subsequently as well as interrupting the binding of NF-κB with their consensus sequences. Inhibitory effect of As_2O_3 on NF-κB DNA activity was dependent upon intracellular glutathione (GSH) and H_2O_2 level, but not superoxide anion. Futhermore, we found that As_2O_3 also downregulated the expression of COX-2, which has NF-κB binding site on its promoter through repressing the NF-κB DNA-binding activity. J. Cell. Biochem. 94: 695–707, 2005.

Key words: arsenic trioxide; HL-60; NF-κB; COX-2

Nuclear factor kappa-B (NF-κB) has been known to regulate expression of many genes in modulating cellular proliferation, inflammatory responses, and apoptosis [Sen and Packer, 1996; Barnes and Karin, 1997]. NF-κB exists mainly as a hetero- or homo-dimer consisting of subunits of Rel family such as p50, p52, c-Rel, p65 (RelA), or RelB. NF-κB is normally sequestered in the cytoplasm as an inactive complex by binding to an inhibitory protein, IκB. IκB tightly modulates NF-κB activity by preventing nuclear translocation of NF-κB and inhibiting DNA binding. When cells are exposed to various extracellular stimuli, IκB is rapidly degraded by proteosomes following its phosphorylation and subsequent ubiquitination. Hence, free NF-κB translocates to the nucleus, where it binds to its binding sites in the promoter of target genes, thereby controlling their expression [Beg and Baltimore, 1996; Grimm et al., 1996].

Recently, many drugs with the ability to inhibit the cyclooxygenase (COX) enzymes have been shown to prevent or delay development of certain tumors. COX is membrane-bound and bifunctional enzyme showing its cyclooxygenase activity and peroxidase activity [Wu, 1996].

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COX-1 is constitutively expressed to fulfill its beneficial housekeeping roles in most human tissues; whereas cyclooxygenase-2 (COX-2), with multiple binding sites on its promoter for various transcription factors including NF-κB, is not constitutively expressed in most normal tissues but it is rapidly induced by cytokines, tumor promoters, growth factor, and oncogenes [Prescott and Fitzpatrick, 2000]. The expression of COX-2 has been shown to be abnormally or overexpressed in various human tumors, including breast, head and neck, lung, pancreatic, and gastric cancers [Subbaramaiah et al., 1996; Ristimaki et al., 1997; Shen et al., 1997; Chan et al., 1999; Tucker et al., 1999].

Arsenic has been widely used in medicine to cure ulcer, rheumatism, arthritis, cancer, etc. in Oriental countries. In the early 1900s, Fowler's solution containing inorganic arsenic compound was used as a remedy for leukemia patients [Kandal and Leroy, 1937]. Recent studies have shown that arsenic trioxide (As_2O_3) could induce in vitro growth inhibition and apoptosis of malignant lymphocytes, myeloma cells, and some solid tumor cell lines such as oesophageal carcinoma and neuroblastoma [Akao et al., 1998, 1999; Shen et al., 1999; Zhu et al., 1999]. It was reported that As_2O_3 induced complete remissions inpatients with acute promyelocytic leukemia (APL) [Shen et al., 1997]. Clinical response is associated with incomplete cytodifferentiation and the induction of apoptosis with caspase activation in leukemic cells [Soignet et al., 1998]. Though much convincing evidence has suggested that As_2O_3 exerts cytotoxicity selectively to many tumors, the mechanism by which As_2O_3 initiates cellular signaling toward cell death is still unclear. Here, we investigated the effects of As_2O_3 on NF-κB DNA binding activity and COX-2 expression in HL-60 cells.

MATERIALS AND METHODS

Reagents

As_2O_3, buthionine sulfoximine (BSO), dithiothreitol (DTT), catalase, sodium azide (NaN_3), and superoxide dismutase (SOD), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640, fetal bovine serum, and antibiotics were obtained from Gibco-BRL (Rockville, MD). All antibodies for electrophoretic mobility shift assay (EMSA) and Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). As_2O_3, BSO, DTT, catalase, NaN_3, and SOD were dissolved in phosphate-buffered saline (PBS). TPCK was dissolved in dimethyl sulfoxide (DMSO).

Cell Culture

Human acute myeloid leukemia (AML) cell line, HL-60 cells (from American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified 5% CO_2 incubator. Control cultures received the same amount of PBS without As_2O_3 or DMSO without TPCK. The amount of DMSO in the cell culture medium never exceeded a final concentration of 0.5%. Bone marrow (BM) from normal adults that was collected by aspiration was used following dilution 5:1 with medium 199 containing 50 U/ml heparine. This mixture was layered onto an equal volume of Ficoll-Plague, centrifuged at 450 G for 30 min at 18°C and the cell layer collected. Control cultures for BM cells received the same amount of PBS with 10 ng/ml GM-CSF.

Preparation of Nuclear and Cytosolic Extracts From HL-60 Cells

HL-60 cells (1×10^7) were lysed by incubation at 4°C for 10 min in buffer consisting of 10 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysate was centrifuged and the supernatant stored at −70°C as a cytosolic extract. After measurement of protein content, the pellet was resuspended in ice-cold buffer consisting of 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF. After incubation at 4°C for 20 min, the extract was centrifuged for 6 min, and the supernatant was collected, aliquoted, and stored at −70°C as a nuclear extract [Krappmann et al., 1999]. The protein content of the final extracts was estimated using the BCA kit according to the manufacturer's protocol from Bio-Rad (Richmond, CA).

Electrophoresis Mobility Shift Assay (EMSA)

EMSA was performed using a DNA-protein binding detection kit for NF-κB binding according to the manufacturer's protocol from Gibco-BRL (Rockville, MD) with minor modifications.

Briefly, the NF-κB oligonucleotide was labeled with [^{32}P]-γATP by T4 polynucleotide kinase and purified on a Nick column (Pharmacia Biotech., Uppsala, Sweden). The binding reaction was carried out in 25 μl of mixture containing 5 μl of incubation buffer (10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, and 0.1 mg sonicated salmon sperm DNA per ml), 10 μg of nuclear extract, and 100,000 cpm of the labeled probe. A 10- or 20-fold excess of unlabeled NF-κB oligonucleotide as a competitor was added after the binding reaction where necessary for competition assay. Five or ten μg of antibody for p50, p52, c-Rel, or p65 was added after binding reaction, where necessary for super-shift assay. After 20 min of incubation at room temperature, samples were electrophoresed through a 6% nondenaturing polyacrylamide gel. For quantitative analysis, protein–DNA complex was excised and quantified by liquid scintillation counting.

Measurement of Proliferation

HL-60 cells (3×10^4) were cultured in 96-well flat-bottom microtiter plates (Costar, Cambrige, MA) in 0.2 ml of RPMI 1640 containing antibiotics (5 μg gentamycin per ml) and 10% FBS. Cultures were incubated at 37°C with 5% CO_2 for 24 h and were pulsed with 1 μCi of [^{3}H]-thymidine (specific activity of 2 Ci per mmol; New England Nuclear, Boston, MA) during the last 4 h of the culture period. Cultures were harvested and [^{3}H]-thymidine incorporation was determined by liquid scintillation counter. The statistical significance of the differences between treated and untreated samples was evaluated using Student's *t*-test. The difference were judged to be statistically significant if $P < 0.05$.

Measurement of Apoptosis

Apoptosis was monitored by DNA fragmentation assay and flow cytometry with propium iodide (PI)-staining. For DNA fragmentation assay, DNA was extracted using a Puregene DNA isolation kit (Gentra system, Minneapolis, MN) according to the manufacturer's protocol. Electrophoresis was carried out in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). For flow cytometry, 1×10^6 HL-60 cells were washed

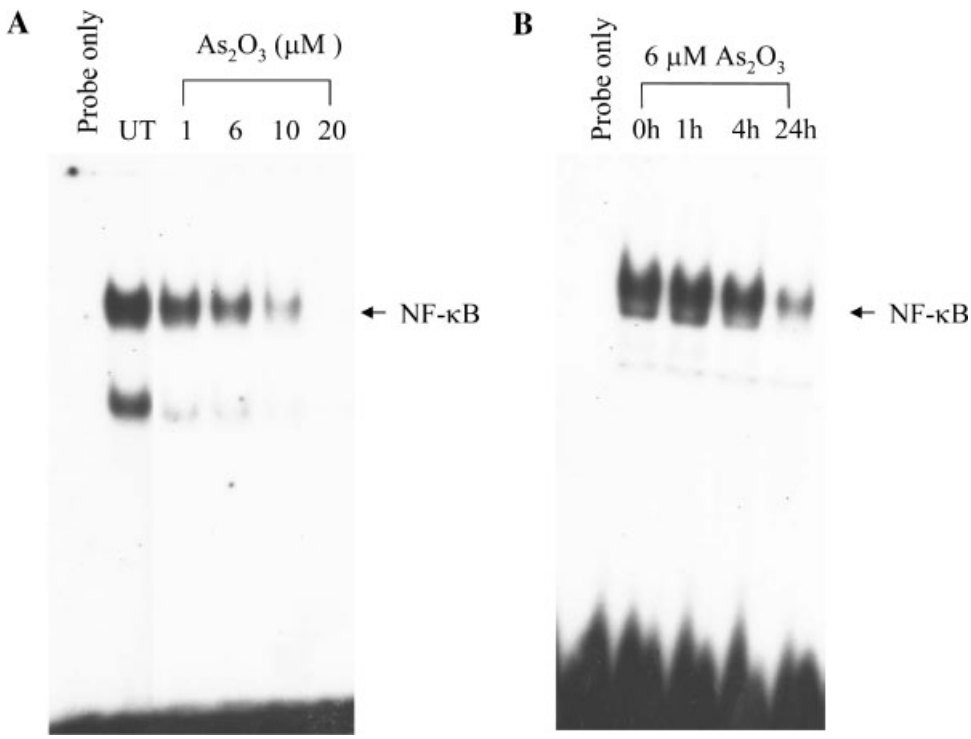

Fig. 1. As_2O_3 suppresses NF-κB DNA-binding activity. EMSA was performed as described in Materials and Methods. UT, untreated. Results from one of three similar experiments are shown. **A**: Concentration-dependence of NF-κB activity suppression by As_2O_3. HL-60 cells were cultured in various concentrations of As_2O_3, as indicated, for 24 h. **B**: Time-dependence of NF-κB activity suppression by As_2O_3. HL-60 cells were cultured in 6 μM As_2O_3, for various time periods as indicated.

with PBS and fixed with 2 ml of ice-cold 70% ethanol for overnight. The cells were wahed with PBS and treated with 200 μl of reaction solution (100 μg/ml RNase and 50 μg/ml PI) for 20 min at 37°C with dark condition. DNA content and distribution was analyzed by flow cytometry.

Total Cell Lysate Extraction and Western Blot Analysis

HL-60 cells (5×10^6) were harvested and suspended with 1.0 ml of ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng PMSF per ml, 0.03% aprotinin, and 1 μM sodium orthovanadate) and incubated on ice for 30 min. The protein content of the final extracts was estimated using the BCA kit according to the manufacturer's protocol (Bio-Rad, Richmond, CA). Total cell lysates for measuring COX-2 levels or nuclear and cytoplasmic extracts for measuring p65 and IκB-α levels were subjected to 12% SDS-polyacrylamide gel electrophoresis. After a 3-h transfer of the gel to PVDF membrane (Amersham Life Sciences, Arlington Heights, IL), the blots were blocked with 5% fat-free dry milk in PBS containing 0.1% Tween-20 for 2 h at room temperature and then washed in the same buffer. Each protein level was detected with each antibody (Santa Cruz Biotech., Santa Cruz, CA). The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL).

Measurement of Intracellular Total Glutathione (GSH) and Oxidized Form (GSSG)

Intracellular GSH contents were measured using a GSH:GSSG ratio assay kit (Calbiochem, San Diego, CA). In brief, for the accurate determination of the low amount of GSSG in cells, 5×10^6 cells were harvested and treated with a thiol-scavenging reagent to prevent oxidation of GSH to GSSG during sample preparation. Cells were freeze-thawed and separated by centrifugation. Supernatant was used for GSSG measurement according to the manufacturer's instruction, while the pellet was dissolved in 1 M NaOH and analyzed for protein by Bio-Rad protein assay (Bio-Rad Laboratories, CA). To

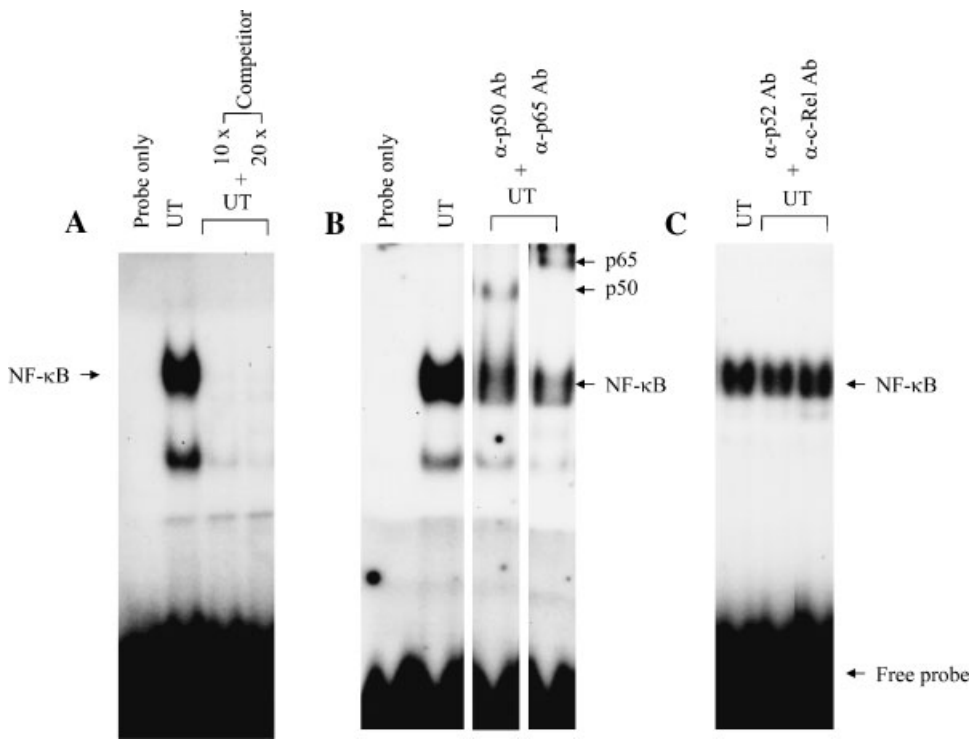

Fig. 2. Constitutively activated NF-κB consists of p50/p65 subunits. Nuclear extract (10 μg) from untreated HL-60 cells was incubated with 10- or 20-fold unlabeled NF-κB oligonucleotide, p50, p65, p52, or c-Rel antibody, as indicated. EMSA was performed as described in Materials and Methods. UT, untreated. Results from one of three similar experiments are shown. **A**: Competition and (**B**, **C**) super-shift analysis of NF-κB activity.

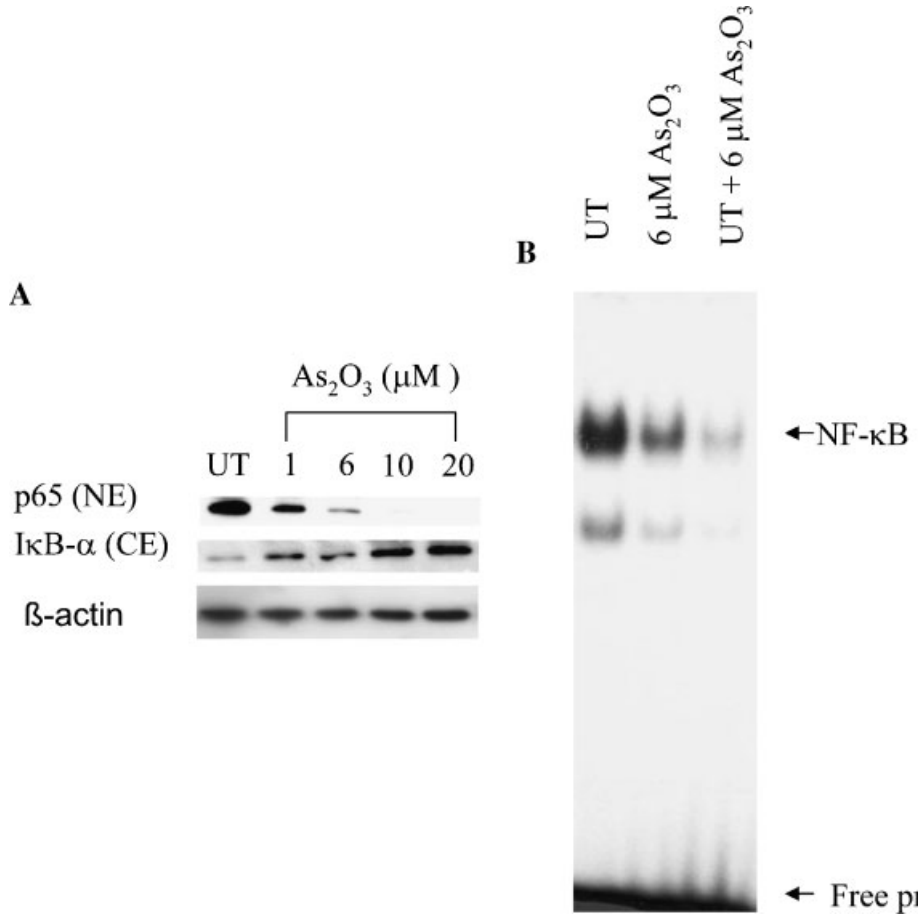

Fig. 3. As_2O_3 inhibits NF-κB activation by preventing the degradation of IκB-α and the translocation of p65 subunit as well as direct-interruption of DNA binding of NF-κB to their consensus sequences. UT, untreated. Results from one of three similar experiments are shown. **A**: As_2O_3 inhibits translocation of p65 into nucleus through preventing degradation of IκB-α. HL-60 cells were cultured for 24 h and harvested. Nuclear and cytoplasmic extracts were assayed by Western blot analysis using IκB-α and p65 antibodies, respectively. NE, nuclear extract; CE, cytoplasmic extract. **B**: As_2O_3 (6 μM) directly prevents NF-κB DNA-binding activity to its consensus sequence. HL-60 cells were treated with or without As_2O_3 for 24 h. Nuclear extracts (10 μg) were subjected to EMSA. For lane 3, the nuclear extract from untreated cells was incubated with 6 μM As_2O_3 in vitro.

measure the total GSH level, cells were assayed using the same methods without treatment with thiol-scavenging reagent.

Determination of Intracellular H_2O_2 Accumulation

Intracellular H_2O_2 production was measured using H_2DCFDA (Sigma, St. Louis, MO), an H_2O_2 sensitive fluorescent dye. Briefly, cells (5×10^5) were incubated in 0.5 μM H_2DCFDA for 30 min and incubated in the presence or absence of LAA for 1 h. Cells were washed and resuspended in PBS, and then analyzed by flow cytometry. H_2DCFDA diffuses into cells and, after conversion by non-specific esterases, reacts with H_2O_2 so as to form a fluorescent molecule [Grad et al., 2001]. The channel number of the peak of the fluorescence intensity distribution is used as a measure of the intracellular H_2O_2 content. To compare levels of H_2O_2, the peak for treated cells was expressed as relative to the peak for control cells.

RESULTS

As_2O_3 Suppresses NF-κB DNA-Binding Activity

We investigated whether As_2O_3 inhibits activation of NF-κB in HL-60 cells. This was analyzed by EMSA using ^{32}P-labeled oligonucleotide that contains NF-κB binding sites. Unstimulated HL-60 cells had a high basal level of NF-κB activity, which was downregulated by treatment of As_2O_3, in a concentration- and time-dependent manner (Fig. 1A,B).

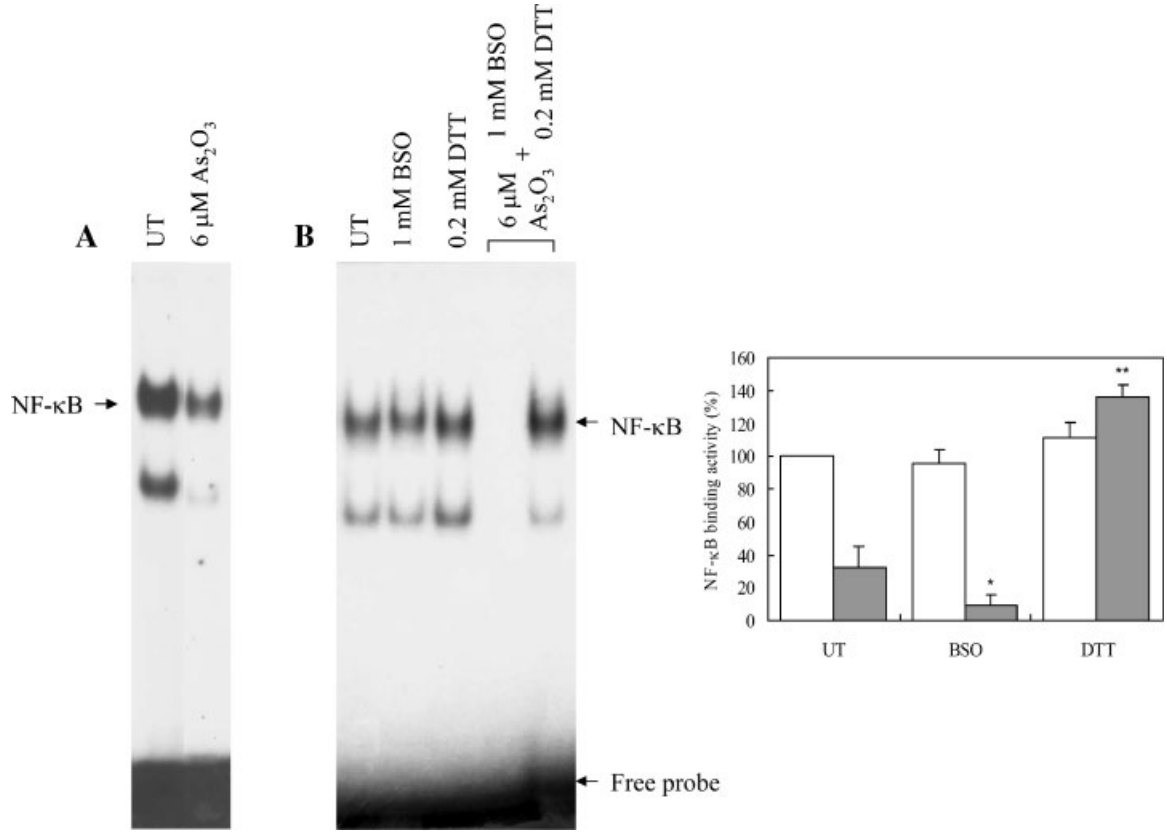

Fig. 4. Inhibitory effect of 6 μM As_2O_3 on NF-κB activity is correlated with modulating intracellular GSH level in HL-60 cells. **A**: DTT blocks but BSO enhances suppression of NF-κB activity induced by 6 μM As_2O_3. HL-60 cells (7×10^6) were cultured with or without 6 μM As_2O_3 in the presence or absence of 1 mM BSO or 0.2 mM DTT as indicated for 24 h. EMSA was performed as described. UT, untreated. Results from one of three similar experiments are shown. **B**: Quantitative analysis was performed and results were expressed as relative activity to untreated control. Asterisks denote statistically significant difference between As_2O_3 treatment and each control condition ($P < 0.01$).

Constitutively Activated NF-κB in HL-60 Cells Consists of p50/p65 Subunits

To ascertain the specificity as well as the identity of NF-κB in HL-60 cells, EMSA was conducted with excess amount of unlabeled NF-κB oligonucleotide or antibodies against the typical NF-κB subunits p50, p52, p65, or c-Rel. Incubation of unstimulated nuclear extracts with 10- and 20-fold excess unlabeled NF-κB oligonucleotide before EMSA abolished the activation of NF-κB DNA binding (Fig. 2A), indicating that the retarded band observed by EMSA was indeed NF-κB. Moreover, incubation of unstimulated nuclear extracts with an antibody against either p50 or p65 shifted the band with the higher molecular weight (Fig. 2B), but an antibody against either p52 or c-Rel did not (Fig. 2C). These results indicate that the NF-κB complex inactivated by As_2O_3 was indeed NF-κB existing as a heterodimer of p50 and p65 subunits.

As_2O_3 Inhibits NF-κB Activation by Preventing the Degradation of IκB-α and the Translocation of p65 Subunit as Well as Inhibiting the Binding Between NF-κB and DNA

In an attempt to understand the mechanism underlying the inhibitory effects of As_2O_3 on NF-κB activation, HL-60 cells were treated with As_2O_3 at concentrations that resulted in the inhibition of NF-κB activation. Western blot analysis was performed to examine the degradation of the inhibitory factor IκB-α and the nuclear translocation of the functionally active subunit p65. As_2O_3 inhibited the degradation of IκB-α and the translocation of p65 to the nucleus in a concentration-dependent manner (Fig. 3A). Moreover, high concentration of As_2O_3 also showed direct inhibition of DNA binding activity of NF-κB. Nuclear extract from untreated HL-60 cells was incubated with 6 μM As_2O_3 and NF-κB probe at room temperature in vitro before EMSA was performed. Interestingly,

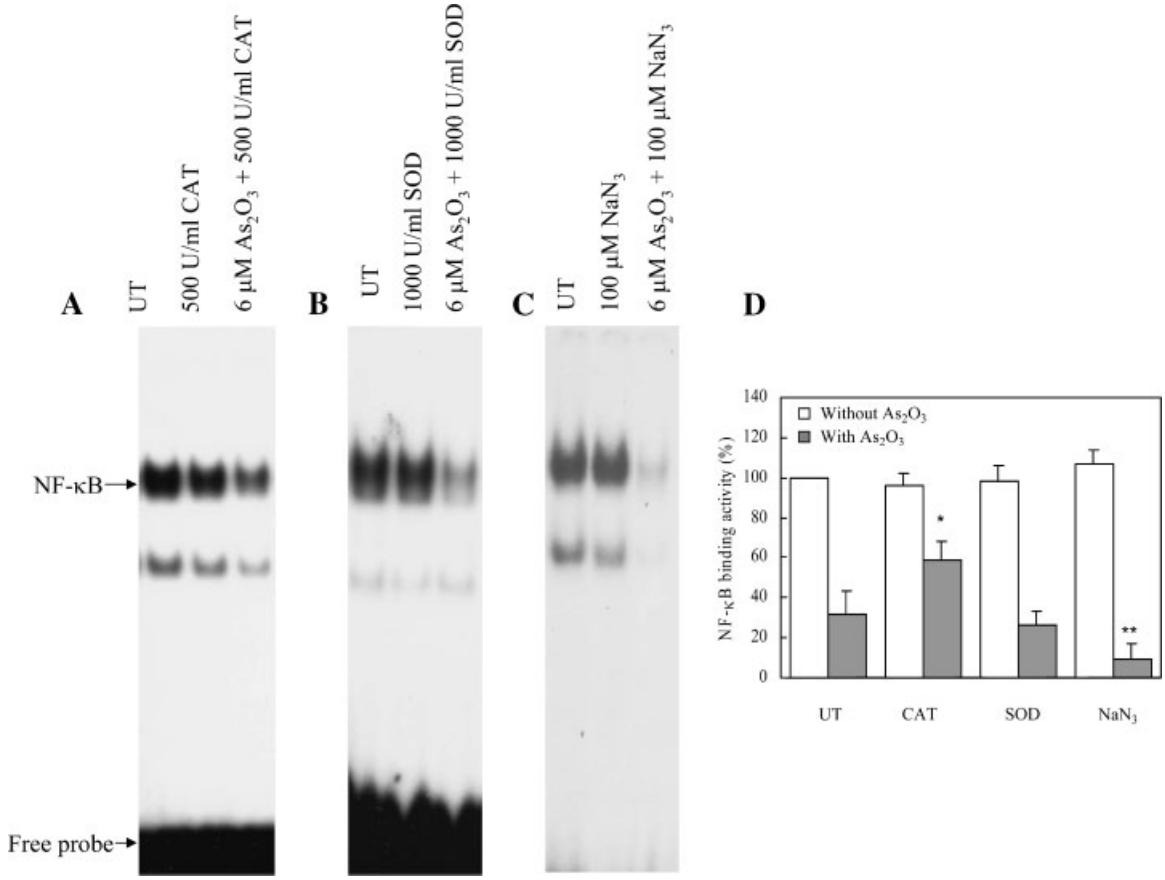

Fig. 5. The elevated H_2O_2 level promotes NF-κB inhibitory activity induced by As_2O_3. HL-60 cells were cultured with or without 6 μM As_2O_3, in the presence or absence of 500 U catalase per ml, 100 μM NaN_3, or 1,000 U SOD per ml, as indicated for 24 h. Nuclear extracts (10 μg) were incubated with radiolabeled NF-κB oligonucleotide at room temperature for 20 min and EMSA was performed. Control: untreated. Results from one of three similar experiments are shown. **A**: Inhibitory effects induced by 6 μM As_2O_3 are made less sensitive by catalase; **B** SOD do not affect suppression of NF-κB activity induced by 6 μM As_2O_3, but **C** NaN_3 accelerates 6 μM As_2O_3-induced suppression of NF-κB activity. **D**: Results were expressed as relative activity to untreated control. Asterisks denote statistically significant difference between As_2O_3 treatment and each control condition ($P < 0.05$).

As_2O_3 markedly inhibited the ability of NF-κB to bind DNA directly in vitro (Fig. 3B, lane 3). These findings indicated that As_2O_3 inhibited the constitutive NF-κB activation by preventing the IκB-α degradation and the translocation of p65 subunits as well as direct-interruption of NF-κB DNA binding activity to their consensus sequences.

Inhibitory Effect of As_2O_3 on NF-κB Activity Is Correlated With Modulation of GSH Level in HL-60 Cells

We examined whether the inhibitory effect of As_2O_3 on NF-κB activity was correlated with modulation of GSH level by treatment with BSO, which is a GSH synthetic inhibitor, or DTT, which is a reducing agent. HL-60 cells were cultured with 6 μM As_2O_3, at which concentration NF-κB activity and cell proliferation were inhibited by 50%, with or without 1 mM BSO or 0.2 mM DTT, for 24 h and then EMSA was performed. As shown in Figure 4, addition of 1 mM BSO to 6 μM As_2O_3 synergistically suppressed NF-κB binding activity, even though 1 mM BSO alone only slightly decreased NF-κB activity. In contrast, treatment of 0.2 mM DTT completely abolished the 6 μM As_2O_3-induced downregulation of NF-κB activity. DTT (0.2 mM) alone did not affect or slightly increased NF-κB activity (Fig. 4A). Quantitative data are shown in Figure 4B. These results indicated that inhibitory effect of As_2O_3 on NF-κB activity was dependant on GSH level confirming that intracellular GSH played a suppressive role in the inhibitory effect of As_2O_3 on NF-κB activity.

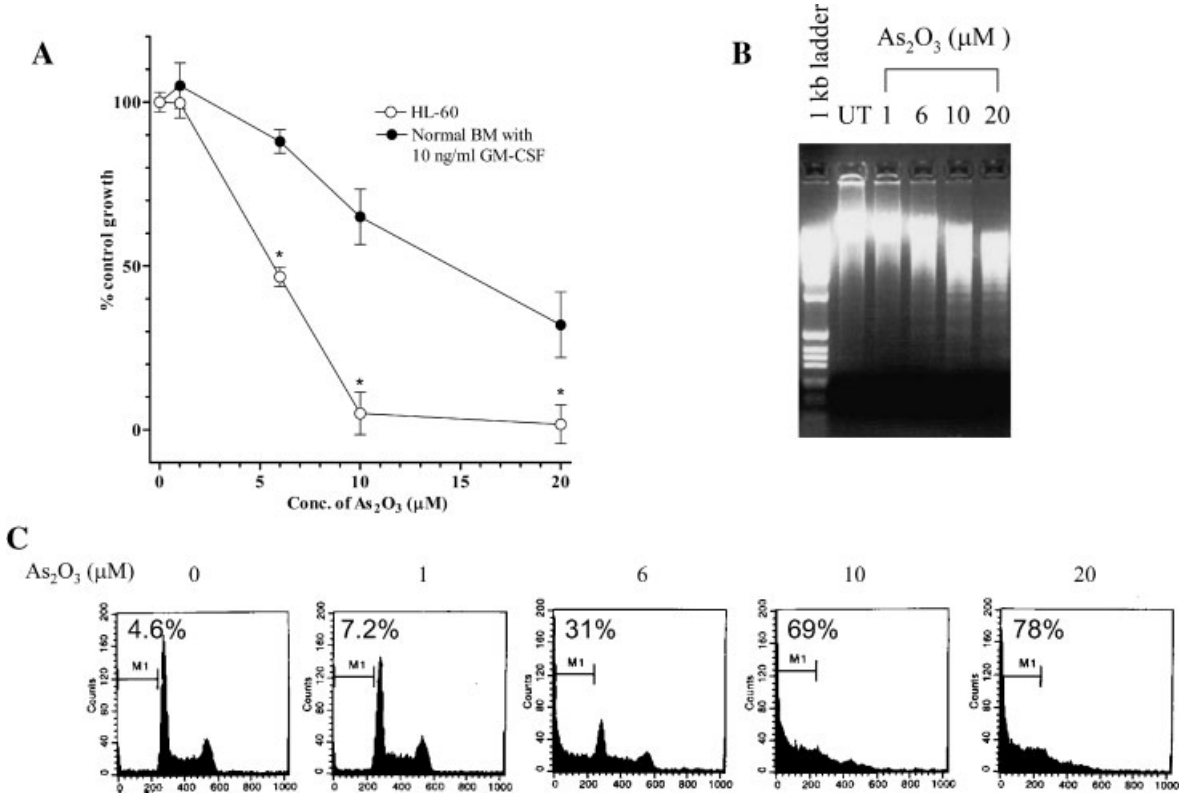

Fig. 6. As_2O_3 induces proliferation inhibition as well as apoptosis in HL-60 cells. **A**: Effect of As_2O_3 on the growth of HL-60 cells and normal BM cells. HL-60 cells and BM cells were cultured with various concentrations of As_2O_3 as indicated for 24 h. Cell proliferation was assessed by [^{3}H]-thymidine uptake assay and expressed as relative to the untreated controls. Data represent the mean ± SD of triplicate determinations and are representative of three experiments. Results are presented as percent of control taken as 100%. Asterisks denote statistically significant difference between normal BM and HL-60 cells ($P < 0.01$). **B**: As_2O_3 causes the apoptosis of HL-60 cells in a dose-dependent manner. HL-60 cells were cultured in the absence or presence of various concentrations of As_2O_3 as indicated for 24 h. Cells were harvested and DNA was extracted and analyzed by agarose gel electrophoresis. UT, untreated. **C**: FACS analysis of apoptotic cells with PI staining. Cells were treated for 24 h with the indicated various concentrations of As_2O_3 and then evaluated for DNA content after PI staining by FACS. M1, apototic DNA.

As_2O_3-Induced Inactivation of NF-κB Activity Is Correlated With H_2O_2 Level

We examined whether the H_2O_2 level affected NF-κB activity inhibition induced by As_2O_3. HL-60 cells were cultured with 6 μM As_2O_3, with or without 500 U/ml catalase, 100 μM NaN_3, or 1,000 U/ml SOD, for 24 h; and then EMSA was performed. Treatment of 500 U catalase per ml, 100 μM NaN_3, or 1,000 U SOD per ml alone did not affect NF-κB activity (Fig. 5). While the addition of 500 U catalase made NF-κB inactivation less sensitive to As_2O_3 (Fig. 5A), the inhibitory effect of 6 μM As_2O_3 on NF-κB activity was enhanced by addition of 100 μM NaN_3, which inhibited catalase activity and promoted H_2O_2 level (Fig. 5C). The inhibitory effect of As_2O_3 on NF-κB activity was not affected by the 1,000 U SOD/ml (Fig. 5B). Based on these findings, we can postulate that As_2O_3 inhibits the growth of HL-60 cells through repressing NF-κB activity via inhibitory modulation of GSH levels and accumulation of H_2O_2.

As_2O_3 Induces Growth Arrest and Apoptosis of HL-60 Cells

In subsequent studies, we sought to elucidate the functional relevance of this inhibition of the NF-κB activity caused by As_2O_3. In our experiment, HL-60 cells and BM cells were cultured with various concentrations of As_2O_3 for 24 h. As_2O_3 caused the growth inhibition of HL-60 cells stronger than normal BM cells (Fig. 6A). Such a growth inhibitory effect was accompanied by cell apoptosis, as shown in Figure 6B,C. These results indicated that As_2O_3 could induce growth inhibition and apoptosis of HL-60.

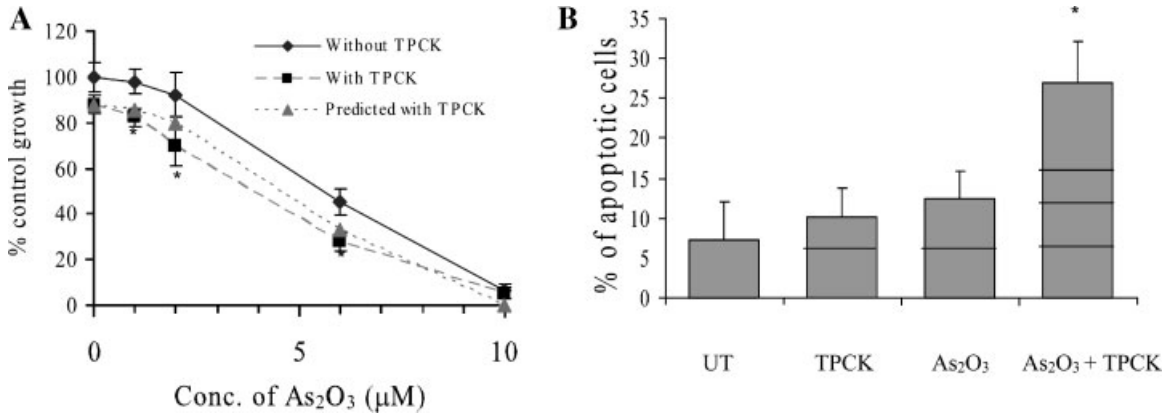

Fig. 7. Blockade of NF-κB activity by TPCK enhances the growth inhibitory effects of As_2O_3 on cell proliferation and As_2O_3-induced apoptosis. **A**: HL-60 cells (3×10^4) were incubated for 24 h in the presence or absence of the indicated dose of As_2O_3, with or without 2 μM TPCK. Cell proliferation was assessed by [^{3}H]-thymidine uptake assay and expressed as relative to the untreated controls. Data represent the mean ± SD of triplicate determinations. Predicted values were plotted from the % control growth value by subtracting 12% (assuming independent action), which could be ascribed to 2 μM TPCK. Asterisks denote statistically significant difference between predicted value and experimental value ($P < 0.05$). **B**: HL-60 cells (2×10^6) were cultured with or without 2 μM As_2O_3 and/or 2 μM TPCK for 24 h. Cells were analyzed for apoptosis by flowcytometry after staining with PI. The data are expressed as % PI-unstained peak and represent means ± SD of triplicate determinations. Asterisks denote statistically significant difference between predicted value (sum of apoptotic cell percentage, ascribed to TPCK or As_2O_3 alone) and experimental value ($P < 0.01$).

Next, we sought to determine whether inhibition of As_2O_3-mediated NF-κB activation affected cell growth inhibitory effect and apoptosis. First, cells were treated with As_2O_3 in the presence or absence of 2 μM TPCK, which selectively blocked NF-κB activation and cell proliferation was assayed using [^{3}H]-thymidine uptake method. Concomitant treatment of the cells with the NF-κB inhibitor TPCK enhanced the cell proliferation inhibitory effects of As_2O_3 (especially above a concentration of 5 μM) (Fig. 7A). Treatment with 2 μM TPCK alone or 2 μM As_2O_3 alone showed 12% and 8% inhibitory effect, respectively, on proliferation compared with untreated cells, whereas concomitant treatment of cells with TPCK and As_2O_3 at doses of 2 μM further augmented the proliferation inhibition (32%) (Fig. 7A). These results suggest that the NF-κB plays a positive regulatory role on the induction of As_2O_3-mediated growth inhibition. Next, we perform further studies to define whether such effects are due to enhancement of As_2O_3-induced apoptosis. Treatment of cells with 2 μM TPCK alone slightly altered the frequency of apoptotic cells compared with untreated cells. Treatment of cells with 2 μM As_2O_3 resulted in 11.8% inducton of apoptosis, while concomitant treatment of cells with the NF-κB inhibitor further enhanced As_2O_3-induced cell apoptosis up to 26.5% (Fig. 7B).

Effect of As_2O_3 on Proliferation Inhibition Is Correlated With Modulation of GSH Level and H_2O_2 Level in HL-60 Cells

As shown in Figure 8A, addition of 1 mM BSO enhanced the growth inhibition of HL-60 cells induced by As_2O_3. However, treatment of 0.2 mM DTT markdely blocked the As_2O_3-induced growth inhibition of HL-60 cells. This finding indicated that As_2O_3-induced growth inhibition of HL-60 cells through repression of GSH function. While inhibitory effect of As_2O_3 was not affected by addition of 500 U/ml catalase, elevation of H_2O_2 through blocking the intracellular catalase by addtion of 100 μM NaN_3 synergistically enhanced that of As_2O_3 (Fig. 8B). These results showed that although As_2O_3 does induce proliferation inhibition without H_2O_2, the inhibitory effect of As_2O_3 is amplified under oxidative condition with H_2O_2. As shown in Figure 8B, inhibitory effect of As_2O_3 was not affected by addition of 1,000 U/ml SOD. This result indicated that superoxide anion also was not correlated with the inhibitory effect of As_2O_3. It implicated that As_2O_3 might be mainly exert its inhibitory effect through repression of GSH function. These were completely matched with our data that As_2O_3-induced repression of the NF-κB activity was modulated by intracellular GSH level, but not by superoxide anion. As shown in Figure 8C, incubation with As_2O_3 for

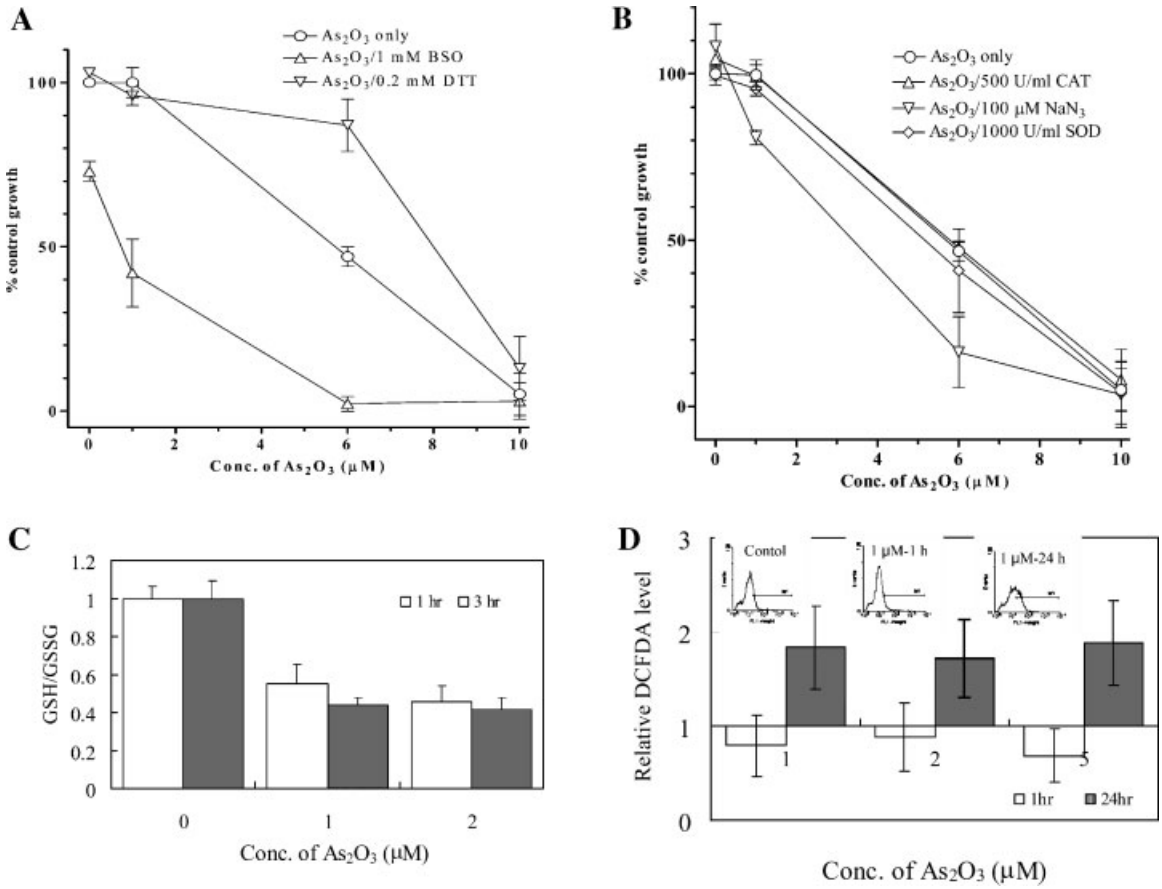

Fig. 8. **A**: HL-60 cells were cultured in various concentrations of As_2O_3 in the presence or absence of 1 mM BSO or 0.2 mM DTT as indicated for 24 h. Cell proliferation was assessed by [^{3}H]-thymidine uptake assay and expressed as relative to the untreated controls. Data represent the mean ± SD of triplicate determinations. **B**: HL-60 cells were cultured in various concentrations of As_2O_3 in the presence or absence of 500 U/ml catalase, 1,000 U/ml SOD, or 100 μM NaN_3 as indicated for 24 h. Cell proliferation was assessed by [^{3}H]-thymidine uptake assay and expressed as relative to the untreated controls. Data represent the mean ± SD of triplicate determinations and are representative of three experiments. **C**: HL-60 cells were incubated with the indicated concentrations of As_2O_3 for 1 or 3 h, and the intracellular GSH and GSSG levels were estimated as described in Materials and Methods. Ratios of GSH to GSSG are expressed as relative to untreated control. Values are means ± SD of three experiments. **D**: HL-60 cells were pretreated with H_2DCFDA probe for 30 min and then treated with the indicated concentrations of As_2O_3 for 1 or 24 h. The oxidized DCF was analyzed by flow cytometry. M1 gate represents cells undergoing H_2O_2 accumulation and is presented as a percentage of the total events collected. Values are expressed as relative to untreated control. Results are from one representative experiment of three.

1 h or 3h resulted in the decrease of GSH/GSSG ratio in HL-60 cell lines. Intracellular H_2O_2 accumulation was also observed in these cells after the treatment with As_2O_3 for 24 h (Fig. 8D).

As_2O_3 Inhibits Expression of COX-2 Through Suppression of NF-κB DNA Binding Activity

As shown in Figure 9A, As_2O_3 inhibited the expression of COX-2, which contains consensus element for NF-κB binding site in a concentration-dependent manner. Next, we investigated whether downregulation of COX-2 expression is correlated with inhibition of NF-κB activity by TPCK. Treatment with 2 μM TPCK slightly induced repression of COX-2 expression (Fig. 9B). However, 1 μM As_2O_3 combined with 2μM TPCK more highly suppressed NF-κB activity than either used alone (Fig. 9B). These results indicate that NF-κB regulated the expression of COX-2 via its binding sites on the promoter of COX-2 (Fig. 9). Hence, downregulation of NF-κB by As_2O_3 resulted in the inhibition of COX-2 expression.

DISCUSSION

Constitutive NF-κB activation is associated with proliferation and survival of certain tumor cells [Li et al., 1997; Sovak et al., 1997]

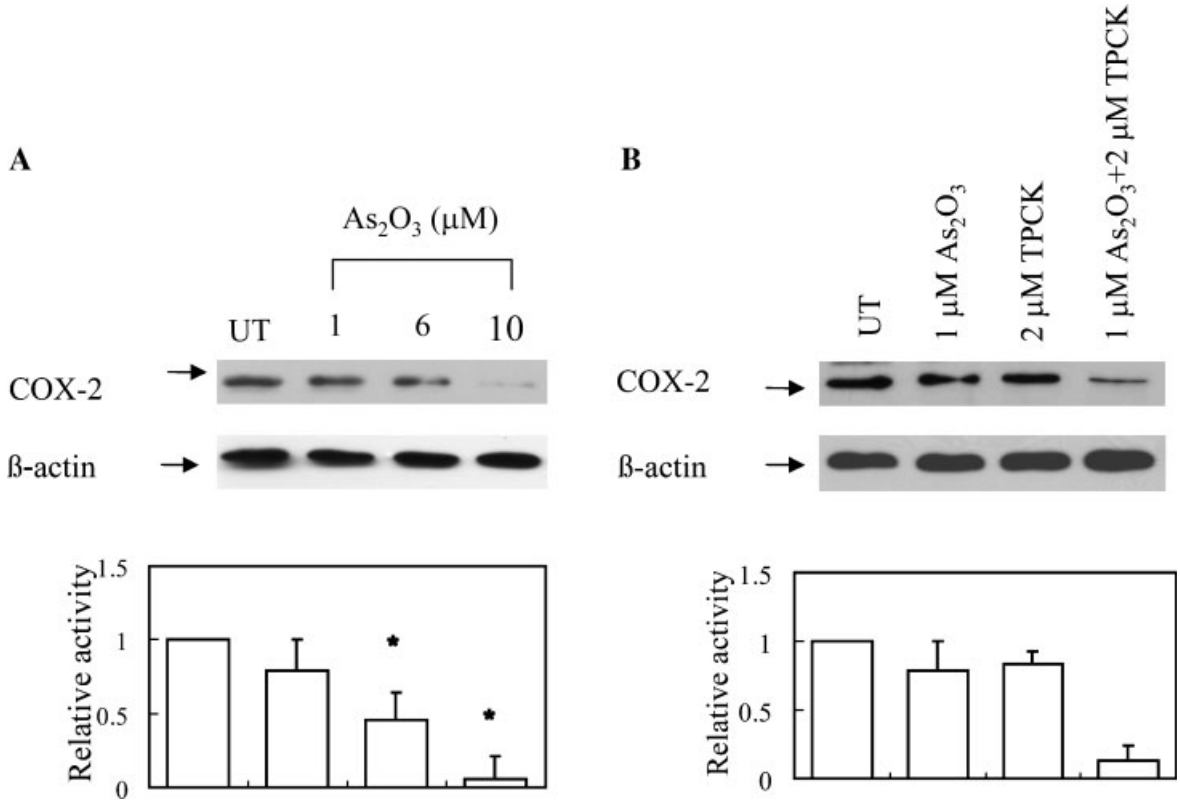

Fig. 9. **A**: As_2O_3 represses constitutive expression of COX-2 in a dose-dependent manner. HL-60 cells (5×10^6) were cultured with various concentrations of As_2O_3 as indicated for 24 h, respectively. Cells were harvested and total extracts were analyzed by Western blot analysis using COX-2 antibody. UT, untreated. Results from one of three experiments are provided. Densitometric analysis was performed on the autoradiograph for quantitative analysis using the TotalLab Image Analysis software (Nonlinear Dynamics Ltd., UK). Signal intensities are expressed in arbitrary units. β-actin was used to normalize the protein level. Values are expressed as relative to untreated control. Each data point represents a mean of three individual values and standard deviations. Asterisks denote statistically significant differences between As_2O_3 treatment and control condition ($P < 0.01$, ANOVA followed by Dunnett's test using MINITAB). **B**: TPCK inhibits COX-2 expression. HL-60 cells were cultured with and without 2 μM TPCK for 24 h.

and also causes resistance to apoptosis [Giri and Aggarwal, 1998]. In the present study, we demonstrated that NF-κB was constitutively activated in untreated HL-60 cells and almost completely downregulated by treatment of As_2O_3 in a concentration- and time-dependent manner (Fig. 1A,B). Moreover, we verified that this NF-κB was composed of p50 and p65 subunits (Fig. 2). This strongly implies a critical role of NF-κB in leukemia cell survival.

It is well known that redox status is important in regulating cellular signaling pathways during oxidative stress and can influence gene transcription by modulating various transcription factors [Flohe et al., 1997]. An important function of GSH is the elimination of ROS [Meister, 1994]. Intracellular GSH content might be indicator of sensitivity of As_2O_3 in leukemia cells [Shao et al., 1998] because arsenic can form reversible bonds with SH groups of GSH [Lee et al., 1989]. We also found that treatment with DTT abolished the As_2O_3-induced repression of NF-κB activity; but BSO, NaN_3 enhanced it (Figs. 4 and 5). Addition of SOD (Fig. 5C) did not affect NF-κB activity downregulation induced by As_2O_3.

These results are completely consistent with our [^{3}H]-thymidine incorporation results that As_2O_3 induced proliferation inhibition of HL-60 cells via repression of GSH function (Fig. 8). It was suggested that catalase activity is also very important for the sensitivity of cells to As_2O_3 in addition to intracellular GSH content and confirmed our result that addition of NaN_3 enhanced inhibitory effect of As_2O_3-repressing NF-κB activity (Fig. 5C). Based on these findings, modulation of intracellular GSH and H_2O_2 levels appears to be very important for As_2O_3-induced repression of NF-κB activity, which might then induce apoptosis in HL-60 cells.

Some studies suggested that arsenite induced NF-κB activation in cultured endothelial cells [Barchowsky et al., 1996; Li et al., 2002]. In these studies, activation of NF-κB by arsenite occured as maximal levels within 30–60 min and returned to almost normal level after 4 hr of treatment. As suggested, it seems to be an immediate early response in attempting to protect the cells from arsenic-induced stress by upregulating the expression of a resistance factor. In contrast to this response, chronic exposure to low levels of arsenic can cause inhibition of cell growth. In the present study, suppression of NF-κB activity by treatment with arsenic for 24 h was investigated.

Our results show that inhibition of NF-κB activity, proliferation inhibition, and apoptosis of HL-60 cells by As_2O_3 was enhanced by the specific NF-κB inhibitor TPCK. These results, taken together, imply that NF-κB plays an important role in growth of HL-60 cells, and suppression of this transcription factor by As_2O_3 could account for the induction of growth inhibition and apoptosis.

As shown in Figure 9, As_2O_3 downregulated the expression of COX-2, which was a target gene of NF-κB. These findings are consistent with previous reports that COX-2 may be implicated in several human tumors, including breast, head and neck, lung, pancreatic, and gastric cancers [Subbaramaiah et al., 1996; Ristimaki et al., 1997; Wolff et al., 1998; Chan et al., 1999; Tucker et al., 1999]. In conclusion, As_2O_3 in a concentration-dependent manner repressed the expresson of COX-2 by inhibition of NF-κB activity through repression of GSH function and accumulation of H_2O_2.

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