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ARTICLES

L-Ascorbic Acid Represses Constitutive Activation of NF-κB and COX-2 Expression in Human Acute Myeloid Leukemia, HL-60

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Abstract There is increasing evidence that L-ascorbic acid (LAA) is selectively toxic to some types of cancer cells at pharmacological concentrations, functioning as a pro-oxidant rather than as an anti-oxidant. However, the molecular mechanisms by which LAA initiates cellular signaling leading to cell death are still unclear. In an effort to gain insight into these mechanisms, the effects of LAA on eukaryotic transcription nuclear factor NF-κB and cyclooxygenase-2 (COX-2) expression were investigated. In the present study, LAA suppressed DNA binding activity of NF-κB, composed of a p65/p50 heterodimer, through inhibition of degradation of inhibitory κB-α (IκB-α) and prevention of nuclear translocation of p65. The inhibitory effect of LAA on NF-κB activity was dependent upon glutathione levels in HL-60 cells, as well as generation of H_2O_2 but not superoxide anion. LAA also downregulated the expression of COX-2, which has a NF-κB binding site on its promoter, through repressing NF-κB DNA binding activity. Moreover, cotreatment of 1 μM arsenic trioxide (As_2O_3) with various concentrations of LAA enhanced an LAA-induced repression of NF-κB activity and COX-2 expression. In conclusion, our data suggest that LAA exerts its anti-tumor activity through downregulation of NF-κB activity and COX-2 expression, and these inhibitory effects can be enhanced by co-treatment with As_2O_3. J. Cell. Biochem. 93: 257–270, 2004. © 2004 Wiley-Liss, Inc.

Key words: L-ascorbic acid; NF-κB; COX-2; arsenic; acute myeloid leukemia

L-Ascorbic acid (LAA) is one of the major water-soluble anti-oxidants present in cells and plasma. There are other studies demonstrating that under certain conditions LAA acts as a pro-oxidant and increases DNA damage [Stich et al., 1976; Speit et al., 1980]. Also, there is increasing evidence that LAA is selectively toxic to some types of tumor, functioning as a pro-oxidant rather than as an anti-oxidant [Bram et al., 1980; Bruchelt et al., 1993]. LAA at concentra-

Abbreviations used: AML, acute myeloid leukemia; As_2O_3, arsenic trioxide; BSO, buthionine sulfoximine; COX-2, cyclooxygenase-2; DHA, dehydroascorbic acid; DMSO, dimethyl sulfoxide; DTT, dithiolthreitol; EMSA, electrophoresis mobility shift assay; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; GSH, glutathione; IκB-α, inhibitory κB-α; LAA, L-ascorbic acid; NF-κB, nuclear factor κB; JNK, c-Jun N-terminal kinases; NaN_3, sodium azide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SBA, sodium 5,6-benzylidene-L-ascorbate; SOD, superoxide dismutase; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

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tions of 10 nM to 1 mM induces apoptosis in neuroblastoma, melanoma, and human myelogenous leukemic cell lines within 24 h via induction of oxidative stress [Fujinaga et al., 1994; De Laurenzi et al., 1995]. According to recent in vivo studies, intravenous administration of sodium 5,6-benzylidene-L-ascorbate (SBA) to inoperable cancer patients induces a significant reduction in the tumor volume without any adverse side effects [Sakagami et al., 1991]. Furthermore, our series of studies using an in vitro colony assay has solidly established that LAA is an important modulator of the growth of mouse myeloma and human leukemic cells [Park et al., 1971, 1992; Park and Kimler, 1991]. Another development recently is that arsenic trioxide (As_2O_3) could induce in vitro growth inhibition and/or apoptosis of malignant lymphocytes, myeloma cells, and solid tumor cells such as neuroblastoma [Akao et al., 1998, 1999; Zhu et al., 1999]. Further, the growth inhibition induced by As_2O_3 has been shown to be enhanced by addition of ascorbate to malignant cells, including leukemic and plasmacytoma cells [Dai et al., 1999; Grad et al., 2001]. As_2O_3 induced apoptosis in four human multiple myeolma cell lines. Glutathione (GSH) was shown to be implicated as an inhibitor of As_2O_3-induced cell death either through conjugating or by sequestering reactive oxygen induced by As_2O_3 [Grad et al., 2001]. One possibility was suggested that decreases in GSH were associated with ascorbic acid metabolism [Dai et al., 1999]. Since arsenic toxicity results from forming reversible bonds with active SH groups on regulatory proteins including GSH, intracellular GSH content might be an indicator of sensitivity to As_2O_3 in leukemia cells [Shao et al., 1998]. A subsequent clinical study has shown a promising result in patients with multiple myeloma treated with a combination of As_2O_3 and ascorbate [Bahlis et al., 2001]. Therefore, it would be desirable to elucidate the mechanism(s) by which ascorbate affects the growth of malignant cells, singly and in combination with As_2O_3.

Nuclear transcription factor kappa-B (NF-κB) has been known to regulate expression of many genes in modulating cellular proliferation, inflammatory responses, and apoptosis. NF-κB exists mainly as a hetero- or homodimer consisting of subunits of the Rel family, such as p50, p52, c-Rel, p65 (RelA), or RelB. NF-κB is normally sequestered in the cytoplasm as an inactive complex through binding to an inhibitory protein, IκB. Phosphorylation and subsequent ubiquitination of IκB induced by various extracellular stimuli causes rapid degradation of this inhibitory subunit by proteosomes. Free NF-κB translocates into 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].

N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) is an enzymatic inhibitor that prevents phosphorylation of IκB [Ballif et al., 2001] and also a chymotrypsin-like serine protease inhibitor [Zhu et al., 1997]. TPCK has been used for NF-κB blocking strategies in order to investigate the downstream of NF-κB signaling [Park et al., 2002; Altura and Gebrewold, 2002; Clohisy et al., 2004]. In the present study, we have used this inhibitor in order to investigate whether cyclooxygenase-2 (COX-2) is involved in the downstream of NF-κB blocking induced by LAA.

Recently, many drugs with the ability to inhibit the cyclooxygenase (COX) enzymes have been shown to prevent or delay development of certain tumors. COX, also known as prostaglandin H synthase, is a membrane-bound and bifunctional enzyme that catalyzes the conversion of arachidonic acid to prostaglandin G_2 by its cyclooxygenase activity and prostaglandin G_2 to prostaglandin H_2 by peroxidase activity [Wu, 1996]. COX-1 is constitutively expressed to fulfill its beneficial housekeeping roles in most human tissues; whereas 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 [Ristimaki et al., 1997; Wolff et al., 1998; Chan et al., 1999; Tucker et al., 1999; Prescott and Fitzpatrick, 2000].

Although there is much convincing evidence that LAA selectively exerts its cytotoxic effects on many tumors, the mechanism by which LAA initiates cellular signaling that leads to cell death is still unclear. Since NF-κB is a ubiquitous transcription factor likely to be involved in tumor cell survival and death, and since COX-2 with its expression dependent on NF-κB is also

involved in tumor pathogenesis, we sought mechanism(s) mediated through these two factors. In addition, we investigated whether cotreatment of As_2O_3 enhances LAA action.

METHODS AND EXPERIMENTAL PROCEDURES

Reagents

LAA, As_2O_3, buthionine sulfoximine (BSO), dithiothreitol (DTT), catalase, sodium azide (NaN_3), and superoxide dismutase (SOD) and TPCK were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640, fetal bovine serum (FBS), and antibiotics were obtained from Gibco-BRL (Rockville, MD). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LAA, 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), HL-60 cells (from American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS 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 LAA and As_2O_3 or DMSO without TPCK. The amount of DMSO in the cell culture medium never exceeded a final concentration of 0.5%.

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 400 μl of buffer A, 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 for 6 min and the supernatant stored at −70°C as a cytosolic extract. After measurement of protein content, the pellet was resuspended in 100 μl of ice-cold buffer C, 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 [Dent and Latchman, 1993]. The protein content of the final extracts was estimated using the BCA kit according to the manufacturer's protocol from Bio-Rad (Richmond, CA).

Electrophoretic 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 γ-[^{32}P] ATP 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. Antibody (5 or 10 μg) 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, 2 μl of 0.1% bromophenol blue was added and samples were electrophoresed through a 6% nondenaturing polyacrylamide gel at 150 V at room temperature. Finally, the gel was dried and exposed onto X-ray film.

Measurement of Proliferation

HL-60 cells (3×10^4) were cultured in 96-well flat-bottom microtiter plates (Costar, Cambridge, 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 test. The difference were judged to be statistically significant if $P < 0.05$.

Measurement of Apoptosis

Apoptosis was monitored by DNA fragmentation assay. For DNA fragmentation assay, DNA

was extracted using a Puregene DNA isolation kit (Gentra system, Minneapolis, MN) according to the manufacturer's protocol. Briefly, 5×10^6 HL-60 cells were washed and harvested. Cell pellets were resuspended in 600 μl of cell lysis solution containing 3 μl of RNase solution (4 mg per ml) and incubated at 37°C for 1 h. Samples were treated with 200 μl of protein precipitation solution and centrifuged at 15,000 g for 3 min. Isopropanol (600 μl) was added to the supernatant and the sample centrifuged at 15,000*g* for 10 min. The pellet was washed with 70% ethanol and analyzed on a 1.8% agarose gel containing ethidium bromide. Electrophoresis was carried out in TAE buffer (40 mM Tris-acetate, 1 mM EDTA).

Total Cell Lysate Extraction and Western Blot Analysis

HL-60 cells (5×10^6) were harvested and washed, and pellet was 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. After centrifuging for 30 min, the supernatant was collected, aliquoted, and stored at −70°C. 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 1 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).

RESULTS

LAA With or Without 1 μM As_2O_3 Suppresses NF-κB DNA-Binding Activity

We investigated whether LAA, with or without 1 μM 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 almost completely downregulated by treatment of LAA, with or without 1 μM As_2O_3, in a concentration- and time-dependent manner (Fig. 1A,B).

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

The most prominent form of NF-κB/Rel proteins has been described as a heterodimer of p50 and p65. 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 LAA with or without 1 μM As_2O_3 was indeed NF-κB existing as a heterodimer of p50 and p65 subunits.

LAA Inhibits NF-κB Activation by Preventing the Degradation of IκB-α and the Translocation of p65 Subunit but Does Not Directly Disrupt Binding of NF-κB to DNA

In an attempt to understand the mechanism underlying the inhibitory effects of LAA on NF-κB activation, HL-60 cells were treated with LAA at concentrations which 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. LAA inhibited the degradation of IκB-α and the translocation of p65 to the nucleus in a concentration-dependent manner (Fig. 3A). According to our previous results, high concentration of As_2O_3 also showed direct inhibition of DNA binding activity of NF-κB. Thus, we investigated whether the inhibition of NF-κB activation by LAA was due to direct disruption of DNA binding of NF-κB. Nuclear extract from untreated HL-60 cells was incubated with

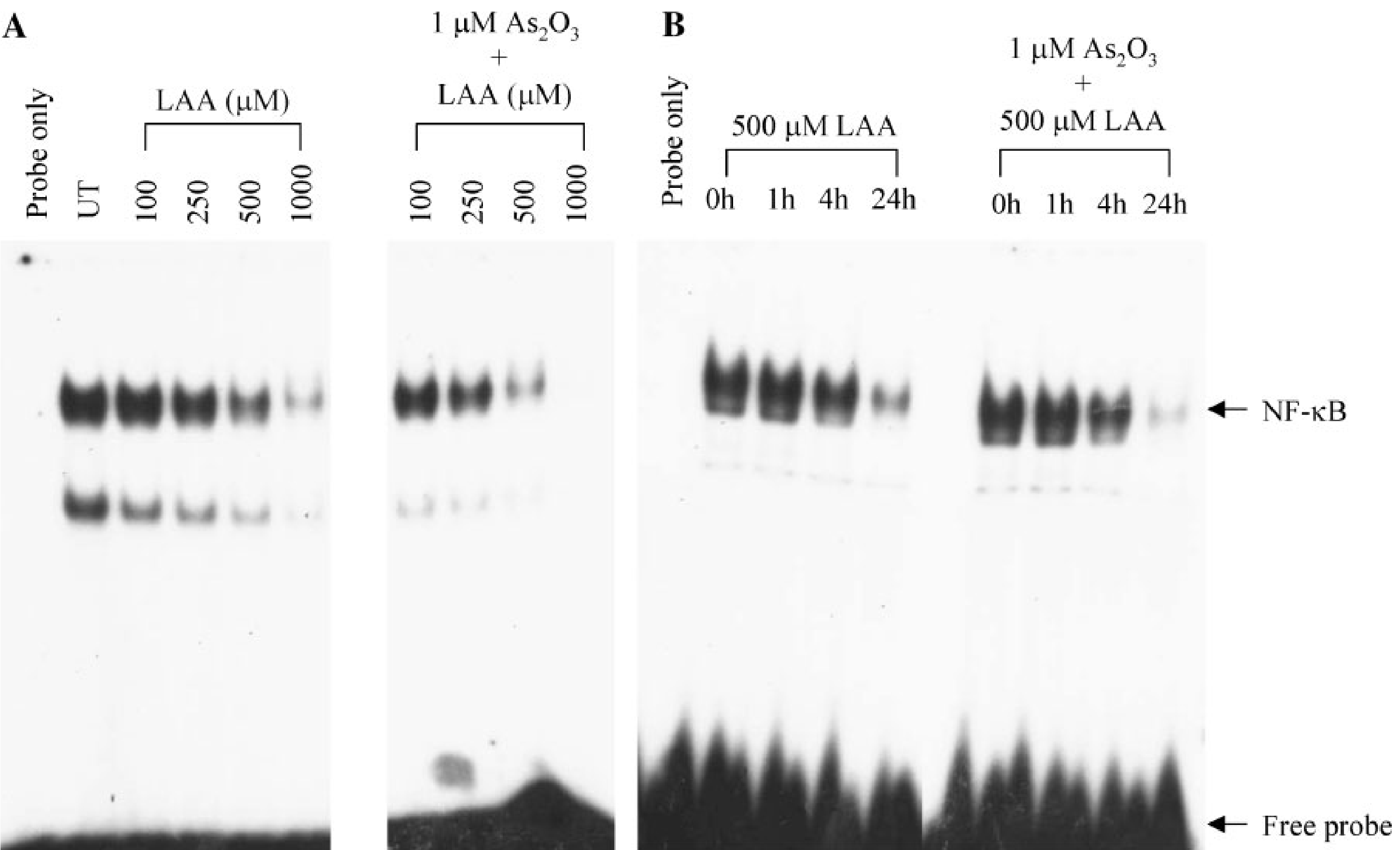

Fig. 1. LAA with or without 1 μM As_2O_3 suppresses NF-κB DNA-binding activity. Cells were harvested and nuclear extract was extracted. EMSA was performed as described in Materials and Methods. UT, untreated cell. Results from one of three similar experiments are shown. **A**: Concentration-dependence of NF-κB activity suppression by LAA, with or without 1 μM As_2O_3. HL-60 cells (7×10^6) were cultured in various concentrations of LAA, with or without 1 μM As_2O_3 as indicated, for 24 h. **B**: Time-dependence of NF-κB activity suppression by LAA, with or without 1 μM As_2O_3. HL-60 cells (7×10^6) were cultured in 500 μM LAA, with or without 1 μM As_2O_3, for various time periods as indicated.

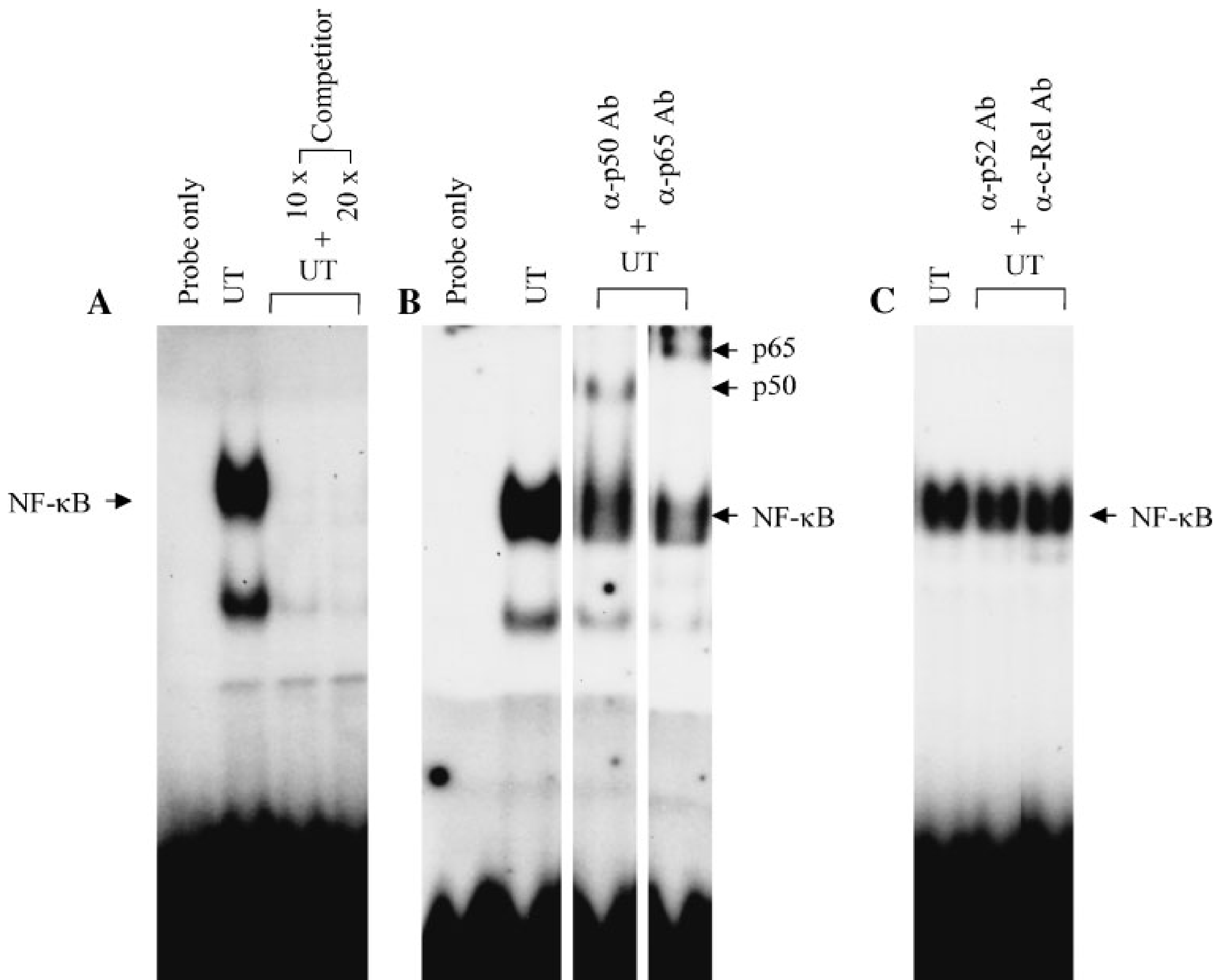

Fig. 2. Constitutively activated NF-κB consists of p50/p65 subunits. Nuclear extract (10 μg) from untreated HL-60 cells was incubated with 10- or 20-fold unlabeled NF-κB oligonucleotide, α-p50, α-p65, α-p52, or α-c-Rel antibody, as indicated. EMSA was performed as described in Materials and Methods.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

500 μM LAA and NF-κB probe at room temperature in vitro before EMSA was performed. Interestingly, LAA did not inhibit the ability of NF-κB to bind DNA directly in vitro (Fig. 3B, lane 3). These findings indicated that LAA inhibited the constitutive NF-κB activation by preventing IκB-α degradation and translocation of p65 subunits, but not by direct disruption of NF-κB DNA-binding activity.

LAA Exerts its Inhibitory Effect Through Modulation of GSH

Recently, we found that GSH had a protective role against LAA effects of inducing proliferation inhibition in HL-60 cells (Fig. 4). Therefore, we examined whether the inhibitory effect of LAA on NF-κB activity was correlated with modulation of GSH level. HL-60 cells were cultured with 250 μM LAA, with or without 1 mM BSO or 0.2 mM DTT, for 24 h and then EMSA was performed. As shown in Figure 5, addition of 1 mM BSO to 250 μM LAA synergistically suppressed NF-κB activity, comparing to the extent how 1 mM BSO or 250 μM LAA alone decreased NF-κB activity. In contrast, treatment of 0.2 mM DTT completely abolished the 250 μM LAA-induced downregulation of NF-κB activity. DTT (0.2 mM) alone did not affect or very slightly increased NF-κB activity (Fig. 5B). These results indicated that inhibitory effect of LAA on NF-κB activity was dependent on GSH level confirming that intracellular GSH played

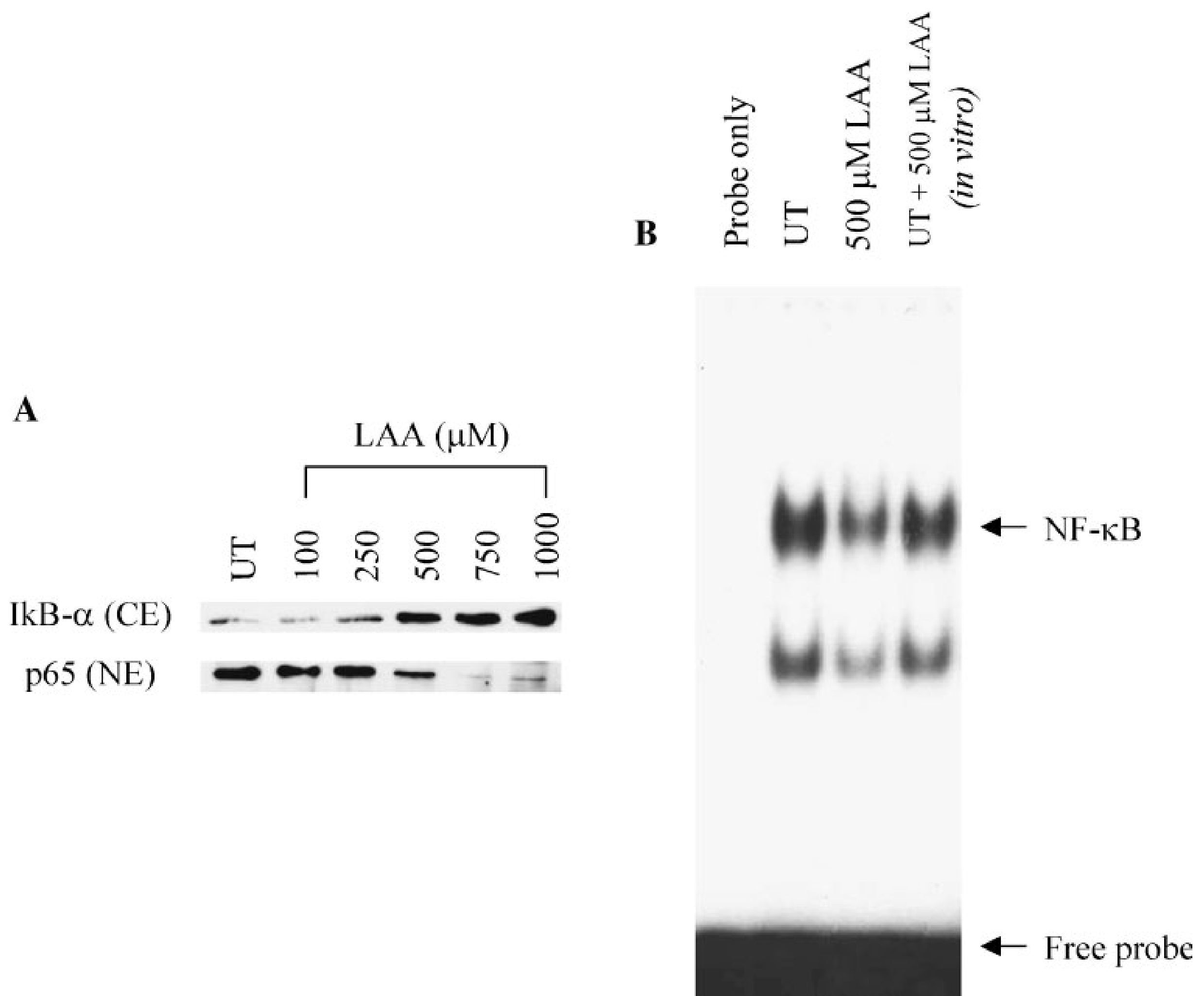

Fig. 3. LAA inhibits NF-κB activation by preventing the degradation of IκB-α and the translocation of p65 subunit but not direct-interruption of DNA binding of NF-κB to their consensus sequences. UT, untreated cell. Results from one of three similar experiments are shown. **A**: LAA inhibits translocation of p65 into nucleus through preventing degradation of IκB-α. HL-60 cells (7×10^6) were cultured in the various concentrations of LAA as indicated for 24 h. Cells were harvested and nuclear and cytoplasmic extracts were extracted. Nuclear and cytoplasmic extracts were assayed for IκB-α and for p65 by Western blot analysis using α-IκB-α and α-p65 antibodies, respectively. NE, nuclear extract; CE, cytoplasmic extract. **B**: LAA does not directly prevent NF-κB DNA-binding activity to their consensus sequence. HL-60 cells were treated with or without 500 μM LAA for 24 h. Nuclear extracts (10 μg) were subjected to EMSA as described in Materials and Methods. For **lane 3**, the nuclear extract from untreated cells was incubated with 500 μM LAA in vitro.

a critical role in the inhibitory effect of LAA suppressing NF-κB activity.

LAA Inhibits the Activation of NF-κB Through H_2O_2 Generation but not Superoxide Anion

As shown in Figure 4, our [^{3}H]-thymidine incorporation results also showed that treatment with catalase completely abrogated, but treatment with NaN_3 enhanced, the inhibitory effect of LAA on the growth of HL-60 cells. Moreover, treatment with SOD did not (or only slightly) affect the inhibitory effect of LAA (data not shown). Therefore, we examined whether the generation of H_2O_2 stimulated by LAA affected NF-κB activity. HL-60 cells were cultured with LAA, 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/ml catalase, 100 μM NaN_3, or 1,000 U/ml SOD alone did not affect NF-κB activity (Fig. 6B–D). Surprisingly, the strong inhibitory effect of 500 μM LAA on NF-κB activity (Fig. 6A) was completely abrogated by addition of 500 U/ml catalase (Fig. 6B); whereas

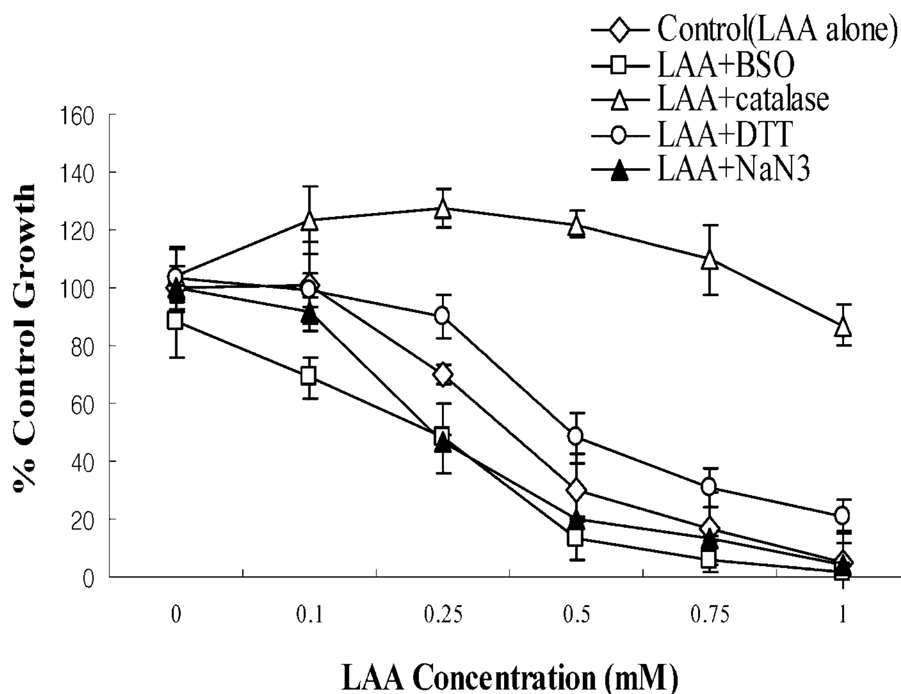

Fig. 4. Effect of BSO, catalase, DTT, and NaN_3 on LAA-induced inhibition of cell proliferation. Data represent the mean ± SE of triplicate determinations and are representative of three experiments. Results are presented relative to cell growth under control conditions (absence of LAA or other chemicals). HL-60 cells (3×10^4/well) were cultured in various concentrations of LAA in the presence or absence of 1 mM BSO or 0.2 mM DTT or 500 U/ml catalase or 100 μM NaN_3 for 24 h. Cell proliferation was determined by measuring [^{3}H]-thymidine uptake.

the addition of 100 μM NaN_3 (Fig. 6C) or 1,000 U/ml SOD (Fig. 6D) enhanced the inhibitory effect of LAA on NF-κB activity. These data were consistent with our recent [^{3}H]-thymidine incorporation results (Fig. 4). Based on these findings, we can postulate that LAA inhibits the growth of HL-60 cells through repressing NF-κB activity via modulation of GSH levels and generation of H_2O_2.

Blockade of NF-κB Activity by TPCK Causes Growth Arrest and Apoptosis of HL-60 Cells

As shown in Figure 1, treatment with LAA demonstrates a concentration- and time-dependent inhibition of NF-κB activity. Then, we investigated the role of NF-κB on the growth of HL-60 cells. HL-60 cells were cultured with 10 μM TPCK (a specific NF-κB inhibitor) for 24 h. EMSA, ^{3}[H]-thymidine incorporation assays, and DNA fragmentation assays were carried out. Treatment with 10 μM TPCK induced strong repression of NF-κB activity and growth arrest as well as apoptosis of HL-60 cells (Fig. 7A–C). These finding indicated that NF-κB plays a pivotal role in the growth of HL-60 cells and can be considered as a critical target for the growth inhibition and apoptosis of HL-60 cells.

LAA, With or Without 1 μM As_2O_3, Inhibits Expression of COX-2 Through Suppression of NF-κB DNA Binding Activity

Recently, it was reported that the signals leading to *src*-induced COX-2 transcription involve both Ras/MEKK-1/JNK and Ras/Raf-1/ERK pathways [Xie and Herschman, 1996]. Moreover, the COX-2 gene contains many consensus elements for various kinds of transcription factors, including a NF-κB binding site located −223/−214 bp upstream from the transcription start site [Yamamoto et al., 1995]. Therefore, we examined whether LAA, with or without 1 μM As_2O_3, could repress expression of COX-2. As shown in Figure 8A,B, LAA with or without 1 μM As_2O_3 inhibited the expression of COX-2 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 10 μM TPCK also induced repression of COX-2 expression (Fig. 8C). Combined with the finding that 10 μM TPCK suppressed NF-κB activity

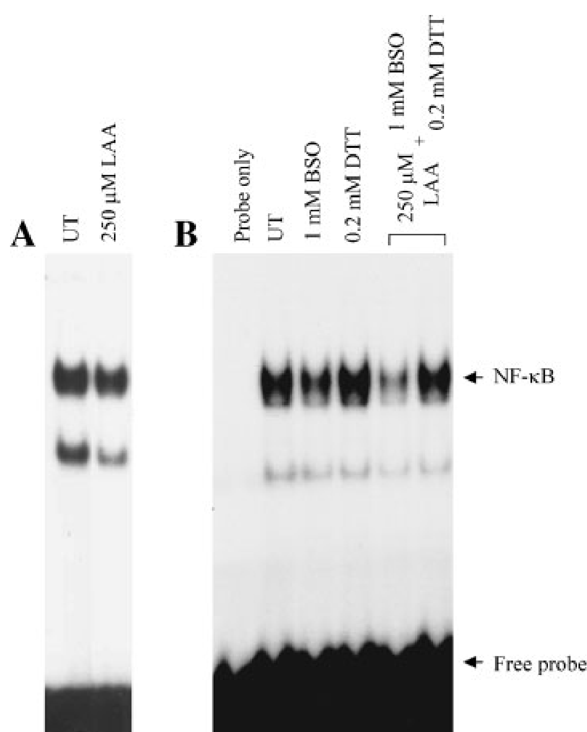

Fig. 5. Inhibitory effect of LAA on NF-κB activity is correlated with modulating GSH level in HL-60 cells. **A**: 250 μM LAA suppresses NF-κB activity. This result is from Figure 5 as a control to compare the extent of downregulation of NF-κB activity with results in (**B**). B: DTT blocks but BSO enhances suppression of NF-κB activity induced by 250 μM LAA. HL-60 cells (7×10^6) were cultured in the presence and absence of 1 mM BSO or 0.2 mM DTT, without or with 250 μM LAA, as indicated for 24 h. Cells were harvested and nuclear extracts prepared. Nuclear extracts (10 μg) were incubated with radiolabeled NF-κB oligonucleotide at room temperature for 20 min and EMSA was performed as described in Materials and Methods. UT, untreated cell. Results from one of three similar experiments are shown.

(Fig. 7A), these results indicate that NF-κB regulated the expression of COX-2 via its binding sites on the promoter of COX-2. Hence, downregulation of NF-κB by LAA, with or without 1 μM As_2O_3, resulted in the inhibition of COX-2 expression.

DISCUSSION

Recent advances in our understanding of the biochemical and molecular bases for inflammatory and carcinogenic processes revealed the involvement of activation of NF-κB by cytokines, reactive oxygen species (ROS), and a variety of chemical agents. Constitutive NF-κB activation is associated with proliferation and survival of certain tumor cells [Li et al., 1997; Sovak et al., 1997] 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 LAA with or without 1 μM 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).

These results are consistent with previous data that up to 25% of hematopoietic malignancies in adults contain rearrangements or amplifications of members of the NF-κB/IκB gene families [Luque and Gelinas, 1997]. Inhibition of NF-κB strongly increases the apoptosis rate and slows down cell proliferation in Hodgkin/Reed-Sternberg cells [Bargou et al., 1997].

We examined the mechanism by which LAA repressed NF-κB DNA binding activity. LAA inhibited the degradation of IκB-α and the translocation of p65 to the nucleus in a concentration-dependent manner (Fig. 3A). This result is consistent with previous reports that the repression of NF-κB is mediated through blockade of IκB-α degradation, which in turn prevents translocation of p65 to the nucleus [Thanos and Maniatis, 1995]. In another study, selective inhibition of Rel/NF-κB signaling in murine skin through targeted overexpression of a super-repressor form of IκB-α resulted in spontaneous development of squamous carcinomas [Hogerlinden et al., 1999]. Moreover, functional blockade of NF-κB by expressing dominant negative mutant IκB-α in transgenic mouse epidermis caused pronounced hyperplasia [Seitz et al., 1998].

High concentrations of As_2O_3 also showed direct inhibition of DNA binding activity of NF-κB (unpublished data). However, LAA could not affect a direct disruption of NF-κB binding to its consensus sequence (Fig. 3B). Based on these findings, we conclude that constitutive NF-κB activation of HL-60 cells is suppressed by LAA via inhibition of translocation of p65 resulting from the blocking of IκB-α degradation, but without direct disruption of NF-κB DNA binding activity.

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]. It is well known

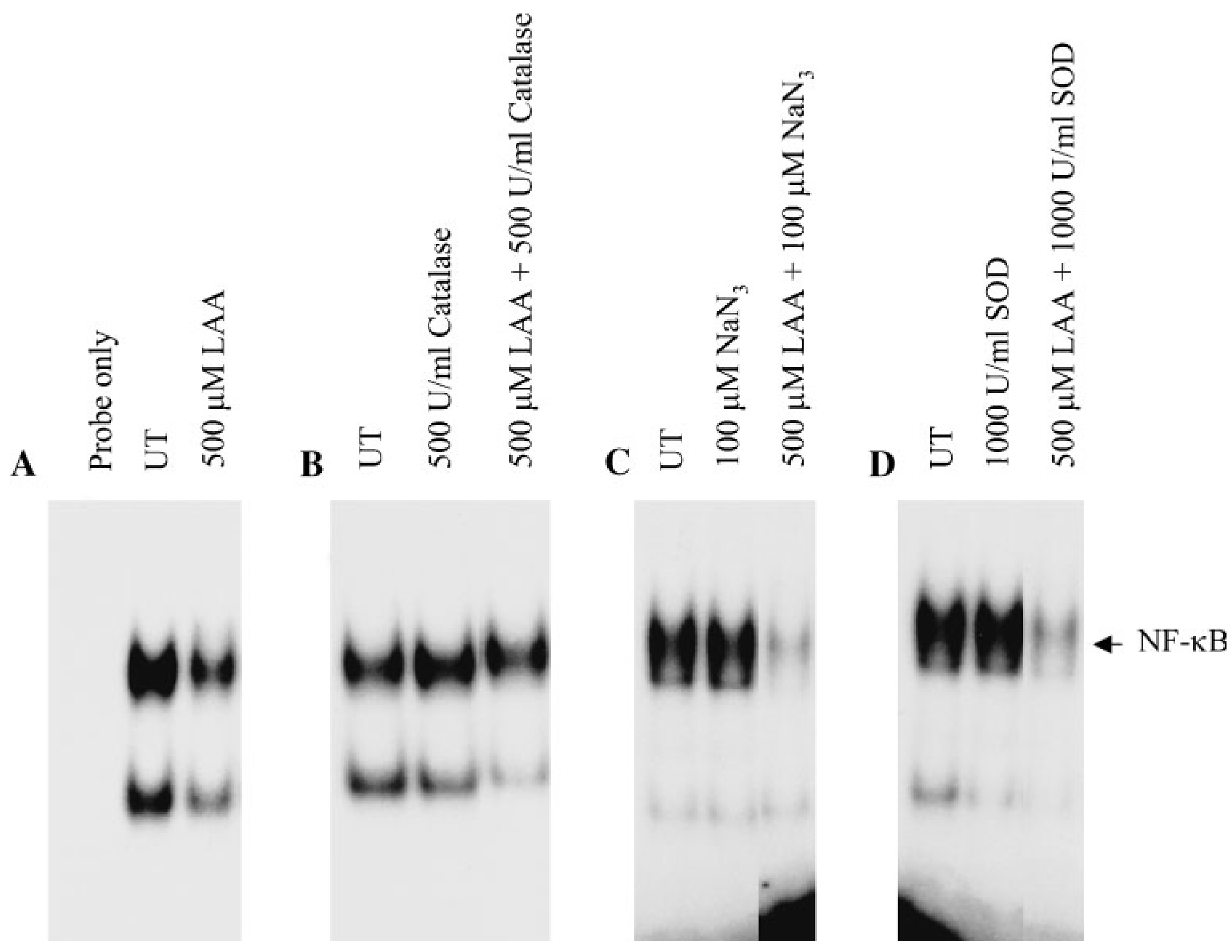

Fig. 6. LAA inhibits the activation of NF-κB through H_2O_2 generation but not superoxide anion. HL-60 cells (7×10^6) were cultured with or without 500 μM LAA, in the presence or absence of 500 U catalase per ml, 100 μM NaN_3, or 1,000 U SOD per ml, as indicated for 24 h. Cells were harvested and nuclear extracts were extracted. Nuclear extracts (10 μg) were incubated with radiolabeled NF-κB oligonucleotide at room temperature for 20 min and EMSA was performed as described in Materials and Methods. UT, untreated cell. Results from one of three similar experiments are shown. **A**: LAA (500 μM) suppresses NF-κB activity. This result is from Figure 5 as a control to compare the extent of downregulation of NF-κB DNA binding activity with results in (**B**), (**C**), and (**D**). Catalase (B) completely abrogates but NaN_3 (C) and SOD (D) augment suppression of NF-κB activity induced by 500 μM LAA.

that the majority of LAA is oxidized extracellularly to dehydroascorbic acid (DHA) [Agus et al., 1999], transported into the cell by glucose transporters and then rapidly reduced to LAA by a GSH-dependent mechanism [Vera et al., 1995]. LAA and GSH have many actions in common and can act complementarily. An important function of GSH is the elimination of ROS and the reduction of DHA [Meister, 1994]. We investigated that LAA-induced repression of NF-κB is correlated with the modulation of GSH and ROS levels. We found that treatment with DTT (Fig. 5B) or catalase (Fig. 6B) abolished the LAA-induced repression of NF-κB activity; but BSO (Fig. 5B), NaN_3 (Fig. 6C), or SOD (Fig. 6D) enhanced it.

These results are completely consistent with our recent [^{3}H]-thymidine incorporation results that LAA induced growth arrest and apoptosis of HL-60 cells via repression of GSH function and generation of H_2O_2.

LAA and SBA induced apoptosis of HL-60 cells in a concentration-dependent manner through the production of H_2O_2, which was increased with incubation time, and reached a plateau level after 30–60 min. A recent finding that higher amount of catalase almost completely eliminated the cytotoxic activity of LAA and SBA further supports our conclusion [Tajima et al., 1998]. Based on these findings, modulation of intracellular GSH and H_2O_2 levels appears to be very important for LAA-induced

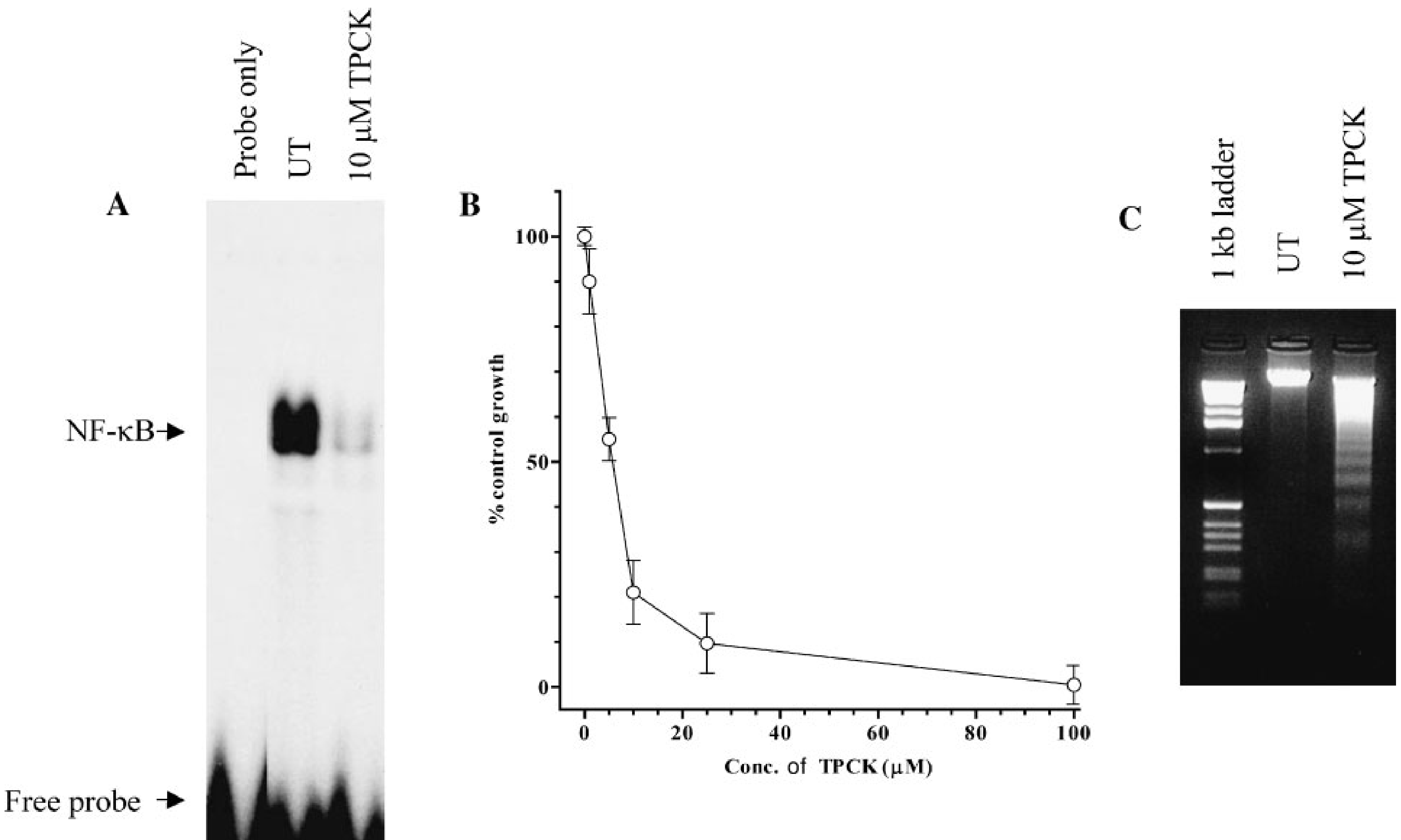

Fig. 7. Blockade of NF-κB activity by TPCK, a specific NF-κB inhibitor, causes growth arrest and apoptosis in HL-60 cells. **A**: TPCK downregulates NF-κB activity. HL-60 cells (7×10^6) were cultured in the absence or presence of 10 μM TPCK for 24 h, nuclear extracts were prepared, and EMSA was performed. Results from one of three similar experiments are shown. **B**: TPCK causes the growth inhibition of HL-60 cells. HL-60 cells (3×10^4/well) were cultured with various concentrations of TPCK as indicated for 24 h. Cells were pulsed with [^{3}H]-thymidine during the last 4 h of culture. Data represent the mean ± SE of triplicate determinations and are representative of three experiments. Results are presented as percent of control, which was the proliferation of HL-60 cells treated with 0.5% DMSO only and which resulted in an activity of 81298 ± 2374 cpm. **C**: TPCK causes the apoptosis of HL-60 cells. HL-60 cells (2×10^6) were cultured in the presence or absence of 10 μM TPCK for 24 h. Cells were harvested and DNA was extracted and analyzed by agarose gel electrophoresis. **Lane 1**: 1-kb marker. Control: untreated (0.5% DMSO). Results from one of three similar experiments are shown.

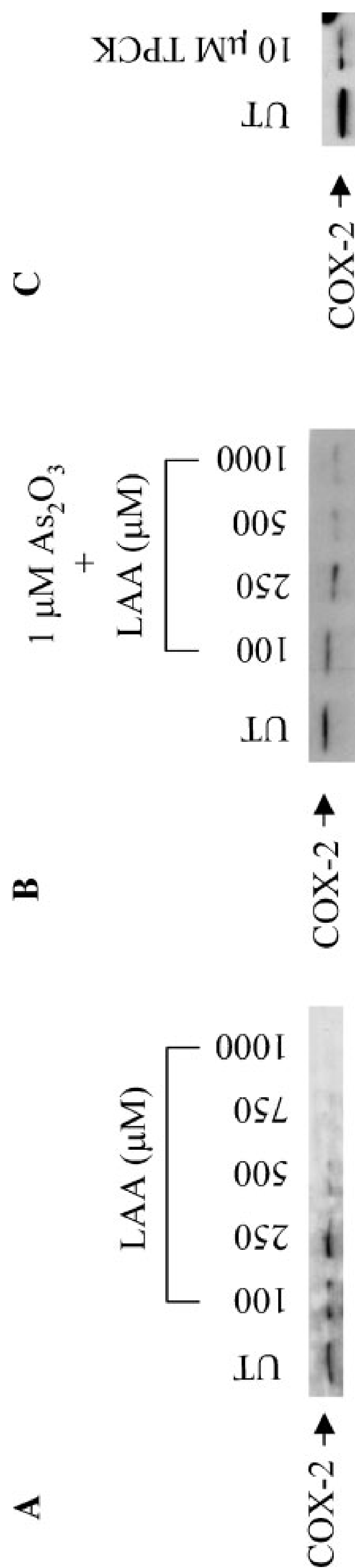

Fig. 8. LAA with or without 1 μM As_2O_3 inhibits the expression of COX-2 through suppression of NF-κB DNA binding activity. Control: untreated. Results from one of three similar experiments are shown. LAA, with (**A**) or without (**B**) 1 μM As_2O_3, represses constitutive expression of COX-2 in a concentration-dependent manner. HL-60 cells (5×10^6) were cultured with various concentrations of LAA, with or without 1 μM As_2O_3 as indicated, for 24 h, respectively. Cells were harvested and total extracts were extracted and analyzed by Western blot analysis using COX-2 antibody. **C**: TPCK inhibits COX-2 expression. HL-60 cells (5×10^6) were cultured with or without 10 μM TPCK for 24 h. Cells were harvested and total extract was extracted and analyzed by Western blot analysis using COX-2 antibody.

repression of NF-κB activity, which might then induce apoptosis in HL-60 cells.

Our results show that inhibition of NF-κB activity (Fig. 7A) by the specific NF-κB inhibitor TPCK caused growth arrest (Fig. 7B) and apoptosis of HL-60 cells (Fig. 7C) in a concentration-dependent manner. 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 LAA could account for the induction of growth inhibition and apoptosis.

As shown in Figure 8, LAA with or without 1 μM As_2O_3 downregulated the expression of COX-2 in a concentration-dependent manner through blockade of NF-κB DNA binding activity (Fig. 7A). This result demonstrated that suppression of NF-κB activity (Fig. 6) induced by LAA prevented the expression of COX-2. It implies that constitutive activation of NF-κB induces COX-2 expression, which might induce the proliferation of HL-60 cells. Increased levels of COX-2 serve to decrease the intracellular levels of free arachidonic acid and thereby prevent apoptosis. Moreover, prostaglandins modulate most cellular processes that control cell growth and differentiation, and especially prostaglandin E2 inhibits apoptosis by inducing expression of the Bcl-2 proto-oncogene [Sheng et al., 1998].

The property of arsenic could also be represented in our recent results that As_2O_3 downregulated NF-κB activity by preventing the degradation of IκB-α and the translocation of the p65 subunit, as well as direct disruption of NF-κB DNA-binding through binding to SH-groups in the DNA-binding region of NF-κB. These results support the concept that intracellular GSH level could be associated with the sensitivity of cells to As_2O_3-induced apoptosis. Moreover, As_2O_3 inhibits glutatathione peroxidase activity, important in elimination of H_2O_2, and thus increases cellular H_2O_2 content in NB4 cells [Dai et al., 1999]. LAA synergism of As_2O_3-induced apoptosis in NB4 cells is mediated by H_2O_2 production because it is inhibited by catalase. The catalase activity was also very important for the sensitivity of cells to As_2O_3 in addition to intracellular GSH content. As conformed to these postulations, we showed here that cotreatment with 1 μM As_2O_3 enhanced the inhibitory effect of LAA (Figs. 1 and 8) via increasing inhibition of GSH function and generation of H_2O_2.

In conclusion, LAA in a concentration-dependent manner repressed the expresson of COX-2 by inhibition of NF-κB activity through repression of GSH function and generation of H_2O_2. Moreover, since As_2O_3 inhibits activity of GSH, which is a proton donor for the Glutathione peroxidase-catalyzed breakdown of H_2O_2, by binding to the SH group of GSH, this contributes to the accumulation of intracellular H_2O_2. For this reason, we postulate that cotreatment with 1 μM As_2O_3 can synergistically enhance the ability of LAA to suppress NF-κB activity and COX-2 expression.

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