3-Deazaadenosine, a S-Adenosylhomocysteine Hydrolase Inhibitor, Has Dual Effects on NF-κB Regulation

INHIBITION OF NF-κB TRANSCRIPTIONAL ACTIVITY AND PROMOTION OF IκBα DEGRADATION

(Received for publication, November 19, 1998, and in revised form, March 22, 1999)

Seong-Yun Jeong‡, Sang-Gun Ahn‡, Jeong-Hwa Lee‡, Ho-Shik Kim‡, Jin-Woo Kim§, Hyangshuk Rhim§, Seong-Whan Jeong§, and In-Kyung Kim§‡¶

From the ‡Department of Biochemistry, §Research Institute of Molecular Genetics, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

Previously we reported that 3-deazaadenosine (DZA), a potent inhibitor and substrate for S-adenosylhomocysteine hydrolase inhibits bacterial lipopolysaccharide-induced transcription of tumor necrosis factor-α and interleukin-1β in mouse macrophage RAW 264.7 cells. In this study, we demonstrate the effects of DZA on nuclear factor-κB (NF-κB) regulation. DZA inhibits the transcriptional activity of NF-κB through the hindrance of p65 (Rel-A) phosphorylation without reduction of its nuclear translocation and DNA binding activity. The inhibitory effect of DZA on NF-κB transcriptional activity is potentiated by the addition of homocysteine. Taken together, DZA promotes the proteolytic degradation of IκBα, but not IκBβ, resulting in an increase of DNA binding activity of NF-κB in the nucleus in the absence of its transcriptional activity in RAW 264.7 cells. The reduction of IκBα by DZA is neither involved in IκB kinase complex activation nor modulated by the addition of homocysteine. This study strongly suggests that DZA may be a potent drug for the treatment of diseases in which NF-κB plays a central pathogenic role, as well as a useful tool for studying the regulation and physiological functions of NF-κB.

Nuclear factor-κB (NF-κB),† a ubiquitous transcription factor, is a pivotal regulator of immune responses, inflammation, cell proliferation, oncogenesis, and apoptosis (1–3). A prototype member of the Rel family, hetero- or homodimeric NF-κB, is made from monomers that have a highly conserved approximately 300-amino acid amino-terminal domain, which is called the Rel homology domain, which functions in DNA binding, nuclear translocation, formation of dimers, and IκB binding. Most of the dimeric NF-κB complexes are stored in the cytoplasm of unstimulated cells as inactive complexes through interactions with a group of inhibitory proteins called IκB. Recently, the vertebrate IκB family such as IκBα, IκBβ, Bcl-3, precursor proteins p105 and p100, and IκBε were reported (4, 5). Inactive cytoplasmic NF-κB can be activated by stimulation of cells with a broad range of NF-κB-inducing agents including bacterial lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α). Despite differences among these stimuli, one of their common targets is the cytoplasmic NF-κB-IκB complexes. These signals known to activate NF-κB result in phosphorylation, subsequent ubiquitination, and proteasome-mediated degradation of the IκB proteins, allowing NF-κB to translocate into the nucleus, bind to specific κB sites and thereby activate target genes such as various cytokines, cell adhesion molecules, acute-phase proteins, and immunoreceptors (6). Researchers have long searched to identify IκB kinase which phosphorylates the IκB proteins to initiate the activation cascade of NF-κB. Recently, IκB kinase α (IKKα) and IκB kinase β (IKKβ) were reported as essential kinases for NF-κB activation downstream of TNF-α and interleukin-1 (IL-1) receptors (7–9). Activation of NF-κB could be inhibited through diverse mechanisms by manifold compounds at distinct positions in the activation cascade. One group of NF-κB inhibitors which share the property of being anti-oxidative, includes N-acetyl-L-cysteine (10), acetylsalicylic acid (11), and pyrrolidine dithiocarbamate (12). Some inhibitors interrupt the induced degradation of IκB proteins by the inhibition of 26 S protease (13), or by increase of IκBα synthesis (14, 15). Another group of inhibitors hinders the transcriptional activity of NF-κB already bound to DNA. This group includes SB203580, a specific inhibitor of p38 mitogen-activated protein kinase (16), and elevated intracellular cyclic AMP (cAMP) (17).

3-Deazaadenosine (DZA) was developed to be one of the most potent inhibitors of S-adenosylhomocysteine hydrolase (EC 3.3.1.1) (18). This agent binds to S-adenosylhomocysteine hydrolase resulting in the accumulation of S-adenosylhomocysteine and S-adenosylmethionine, and serves as a substrate for the enzyme resulting in the huge accumulation of 3-deazaadenosylhomocysteine (DZA-Hcy) in cultured cells (18), especially in liver tissue (20). DZA exerts a number of interesting biological properties, such as anti-human immunodeficiency virus (HIV) activity (21, 22), immunosuppressive and anti-inflammatory effects (23, 24), inhibition of lymphocyte-mediated cytolyis (25), inhibition of cytokine expression including TNF-α and IL-1β (26), inhibition of cell adhesion molecule expression (27, 28), and induction of apoptosis in human and mouse leukemia cells (29, 30). Although the wide variety of biological properties underscores that DZA is to be an effective

* This work was supported in part by the funding from the 1995 and 1997 Basic Research Promotion Fund of the Ministry of Education, Korea, and the Korea Science and Engineering Foundation (KOSEF), Cancer Research Center at Seoul National University, Grant 97K4-0401-00-01-0. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea. Tel.: 82-2-590-1175; Fax: 82-2-596-4435; E-mail: ikkim@catholic.ac.kr.

† The abbreviations used are: NF-κB, nuclear factor-κB; DZA, 3-deazaadenosine; Hcy, homocysteine; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IKK, IκB kinase; PBMC, human peripheral blood mononuclear cells; DZA-Hcy, 3-deazaadenosylhomocysteine; IL-1, interleukin-1; DZAr, 3-deaza(-2′)-aristeromycin; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; PKCα, catalytic subunit of protein kinase A; GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HIV, human immunodeficiency virus.

This paper is available on line at http://www.jbc.org

18981
Materials and Methods

Chemicals—DZA, 3-deaza(-±)-aristeromycin (DZAr), and DZA-hey were donated by Dr. Chiang of the Walter Reed Army Institute of Research, Washington, D.C. Hey-thiolactone and LPS (Escherichia coli, No. 0127 B-8) were purchased from Sigma. Recombinant glutathione S-transferase (GST) fusion protein of human TNF-α and GST-human LeBα were kindly provided by Dr. Dae-Myung Jue in our department. Unless specified otherwise, all reagents were purchased from Sigma.

Cell Culture—Mouse macrophage RAW 264.7, human monocytic THP-1, and SV40-transformed African green monkey kidney COS-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). PBMC were isolated from defibrinated blood using Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) by density-gradient separation, followed by adherence to tissue culture dish for 2 h at 37 °C. Nonadherent cells were removed by washing the monolayer four times with phosphate-buffered saline. All cells were cultured in RPMI 1640 medium supplemented with 20 mM HEPES, 25 mM sodium bicarbonate, 50 μg/ml gentamicin, and 10% heat-inactivated fetal bovine serum (HyClone Laboratories Inc., Logan, UT) at 37 °C in an atmosphere of 5% CO₂.

Northern Blot Analysis—Total cellular RNA was isolated from cells at 2 h after LPS (1 μg/ml) stimulation according to a previously described method (31). Five μg of total RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde. Northern blot analysis was performed as described (26).

Preparation of Cytoplasmic Fraction and Nuclear Extract—After RAW 264.7 cells were stimulated with LPS (1 μg/ml) for 1 h and COS-7 cells were stimulated with recombinant GST-human TNF-α protein (50 ng/ml) for 1 h, cells were washed twice with phosphate-buffered saline. Cytosolic fraction and nuclear extract for Western blot analysis and electrophoretic mobility shift assay (EMSA) were prepared as described by Dignam et al. (32). Concentration of protein was determined using a protein assay reagent (Pierce).

EMSA—Five μg of nuclear protein and 1 μg of poly(dI-dC) (Roche Molecular Biochemicals, Mannheim, Germany)-labeled probes were prepared from each specific primer and cDNA fragments of amplimer sets (CLONTECH, Palo Alto, CA) by polymerase chain reaction.

Preparation of Cytosolic Fraction and Nuclear Extract—After RAW 264.7 cells were stimulated with LPS (1 μg/ml) for 1 h and COS-7 cells were stimulated with recombinant GST-human TNF-α protein (50 ng/ml) for 1 h, cells were washed twice with phosphate-buffered saline. Cytosolic fraction and nuclear extract for Western blot analysis and electrophoretic mobility shift assay (EMSA) were prepared as described by Dignam et al. (32). Concentration of protein was determined using a protein assay reagent (Pierce).

EMSA—Five μg of nuclear protein and 1 μg of poly(dI-dC) (Roche Molecular Biochemicals) per reaction were incubated for 15 min at room temperature with NF-κB consensus sequence (Santa Cruz Biotechnology, Santa Cruz, CA), which was 3’-end labeled with 32P in binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 0.2% (w/v) Tween 20, 30 mM KCl). After the binding reaction, samples were analyzed by electrophoresis on a 6% native polyacrylamide gel that was run in 0.5X Tris borate-EDTA (TBE) buffer, pH 8.0, and then the gel was subjected to autoradiography. For competition, 50-fold unlabeled NF-κB or AP-1 consensus sequence (Santa Cruz Biotechnology) were used.

Western Blot Analysis—Ten μg of proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for NF-κB proteins or 12.5% SDS-PAGE for IκB proteins, and transferred to a nitrocellulose membrane in transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol) at 500 mA for 4 h. Western blot analysis was performed as described (26). All primary antibodies used in this study (anti-p65 (C-20), anti-p50 (NLS), anti-IκBα/B/MAD-3 (C-21), and anti-IκBβ (C-20)) were purchased from Santa Cruz Biotechnology.

Results

LPS-induced Expression of TNF-α mRNA Is Inhibited by DZA—Transcription of TNF-α (C-20) and IL-1β was inhibited by DZA in a dose-dependent manner in RAW 264.7 cells stimulated by LPS (26). To investigate the effects of DZA on the expression of TNF-α in other types of cells, THP-1 cells and PBMC were pretreated with increasing concentrations of DZA for 1 h, and stimulated by the addition of LPS at a final concentration of 1 μg/ml. As shown in Fig. 1, LPS stimulation increased the steady-state levels of TNF-α mRNA as early as 2 h. Treatment of DZA inhibited the LPS-induced expression of TNF-α mRNA dose-dependently in both THP-1 cells and PBMC in the same manner as in RAW 264.7 cells. Expression of β-actin mRNA as a control was not affected by DZA in any of the cells. These results show that DZA efficiently inhibits the LPS-induced expression of TNF-α mRNA, and this potency is not only restricted to murine macrophage cells.

DNA Binding Activity of NF-κB Is Increased by DZA—Since the activation of transcription factor NF-κB has been shown to be indispensable for TNF-α expression induced by LPS (35), we examined the effect of DZA on DNA binding activity of NF-κB in the nucleus of RAW 264.7 cells by EMSA, using 32P-labeled NF-κB specific oligonucleotides. An inducible protein-DNA complex was observed in the nuclear extracts from LPS-stimulated RAW 264.7 (Fig. 2A). Unexpectedly, cells pretreated with DZA revealed no significant decrease of the LPS-induced

The Effects of 3-Deazaadenosine on NF-κB Regulation

Drug for treatment of many human diseases, the action mechanism of DZA is not yet fully understood.

Previously, we reported that DZA inhibits LPS-induced TNF-α and IL-1β transcription in mouse macrophage RAW264.7 cells (26). In this study, elementary experiments confirming DZA inhibition of TNF-α transcription in human monocyteic macrophage THP-1 cells and human peripheral blood monocytes (PBMC) encouraged us to investigate the effect of DZA on NF-κB activation with the intention of understanding the cellular mechanism of DZA. DZA potently inhibited the transcriptional activity of NF-κB through the hindrance of κB phosphorylation without reduction of nuclear translocation and DNA binding activity of NF-κB in the nucleus. These results strongly suggest that DZA may serve as a potent drug for the treatment of diseases in which NF-κB plays an important pathogenic role, as well as a useful tool for studying the regulation and physiological functions of NF-κB.

The Effects of 3-Deazaadenosine on NF-κB Regulation

Drug for treatment of many human diseases, the action mechanism of DZA is not yet fully understood.

Previously, we reported that DZA inhibits LPS-induced TNF-α and IL-1β transcription in mouse macrophage RAW264.7 cells (26). In this study, elementary experiments confirming DZA inhibition of TNF-α transcription in human monocyteic macrophage THP-1 cells and human peripheral blood monocytes (PBMC) encouraged us to investigate the effect of DZA on NF-κB activation with the intention of understanding the cellular mechanism of DZA. DZA potently inhibited the transcriptional activity of NF-κB through the hindrance of κB phosphorylation without reduction of nuclear translocation and DNA binding activity of NF-κB in the nucleus. These results strongly suggest that DZA may serve as a potent drug for the treatment of diseases in which NF-κB plays an important pathogenic role, as well as a useful tool for studying the regulation and physiological functions of NF-κB.
DNA binding activity of NF-κB, even though expression of the TNF-α gene was potently inhibited by exposure to DZA. Moreover, DNA binding activity was potentiated in nuclear extracts dose dependently by DZA, irrespective of LPS stimulation. In competition experiments, a 50-fold amount of unlabeled NF-κB-specific oligonucleotide absolutely inhibited typical binding activities, but the same amount of unlabeled AP-1 oligonucleotide failed to inhibit binding activities, confirming their specificities (Fig. 2B). These results suggest that the inhibition of TNF-α gene expression by DZA occurred without down-regulation of NF-κB DNA binding activity, and that DZA increases DNA binding activity of NF-κB in RAW 264.7 cells. Since the presence of a reducing agent such as DTT in preparation of the nuclear extract and the execution of EMSA could mask lost DNA binding activity of NF-κB, we repeated the EMSA assay without DTT (Fig. 2C). Removal of DTT from the assay could not modulate each of the DNA binding activities which were presented by DTT in an assay with DTT, indicating that the DZA inhibition of TNF-α gene expression is not caused by a loss of NF-κB DNA binding activity.

**Nuclear Translocation of NF-κB Is Increased by DZA**—We examined the effects of DZA on the translocation of p65 (Rel-A), p50 (NF-κB1), and c-Rel into the nucleus of RAW 264.7 cells stimulated with or without LPS using Western blot analysis. LPS stimulation increased protein levels of each NF-κB subunit in the nucleus. Treatment of DZA at a concentration of 100 μM induced an increase of Rel family proteins in the nucleus of RAW 264.7 cells regardless of LPS stimulation (Fig. 3A). These results are in exact agreement with the results in Fig. 2A, which shows that DZA induces nuclear translocation of NF-κB and potentiates its LPS-induced translocation in RAW 264.7 cells. We next established the modulation of p65 in the nucleus of RAW 264.7 cells by DZA at various concentrations or various time intervals. Similar to the results in Fig. 2A, DZA at 100 μM concentration increased the levels of p65 in the nucleus regardless of LPS stimulation (Fig. 3B). Fig. 3C shows the modulation of p65 level in the nucleus by treatment of DZA at various time intervals. p65 in the nucleus increased remarkably at 60 and 120 min after treatment with DZA, even though there was a slight decrease at 15 min. The time-response effect of DZA on the translocation of p65 did not correlate with the time-response effect of DZA on NF-κB DNA binding activity. However, the amount of cytoplasmic p65 decreased proportionally to the increase of nuclear p65 by DZA as measured in a Western blot analysis of total cellular extracts and cytoplasmic fractions (data not shown). These results demonstrate that DZA increases the amount of p65 nuclear translocation in RAW 264.7 cells, and that the increase of p65 in the nucleus by DZA is caused only by...
nuclear translocation, and not by the enhancement of p65 expression.

LPS-induced NF-κB Transcriptional Activity Is Inhibited by DZA, and This Inhibitory Effect Is Augmented by the Addition of Hcy—Since DZA is known to inhibit the expression of TNF-α mRNA without a diminution of NF-κB nuclear translocation and DNA binding activity, we proved the effects of DZA on the NF-κB transcriptional activity by transient transfection experiments of RAW 264.7 cells using reporter gene constructs carrying two copies of the wild-type (J16) NF-κB binding sequence or mutant (J32) NF-κB binding sequence in front of the CAT gene, which have been shown to specifically respond to NF-κB activation (33). LPS stimulation of cells transfected with J16 resulted in a 4.7-fold induction of CAT expression (Fig. 4A), whereas no induction of CAT expression by LPS was observed in cells transfected with J32. Treatment of the cells with 100 μM DZA for 1 h before LPS stimulation resulted in a drastic inhibition of LPS-induced CAT expression. LPS-induced transcriptional activity of NF-κB was dose dependently inhibited by DZA (Fig. 4B). These results strongly suggest that DZA potently inhibits NF-κB transcriptional activity in RAW 264.7 cells stimulated by LPS, even though it more intensely provokes the nuclear translocation and DNA binding activity of NF-κB. The effect of Hcy on DZA inhibition of NF-κB transcriptional activity was examined. There was a tendency to inhibit NF-κB transcriptional activity more than the inhibition observed by DZA alone (Fig. 4B). These results imply that DZA inhibition of NF-κB transcriptional activity is involved in the accumulation of DZA-Hcy in RAW 264.7 cells. To determine the effect of DZA on CAT expression induced by expression of p65, RAW 264.7 cells were co-transfected with CAT-reporter plasmids and pSVL65 that express large amounts of p65. As a control, cells were transfected with pSVL substituting for pSVL65. After 6 h of transfection, one group of cells was exposed for 18 h to 100 μM DZA and the other was not (Fig. 4C). Expression of p65 induced a 5.9-fold increase of CAT protein, driven from J16 under the absence of DZA treatment, but did not induce CAT protein expression from J32. The expression of CAT protein was completely inhibited from the cells transfected with pSVL65 when DZA was treated into cells at the concentration of 100 μM, although the induction of CAT expression in the cells transfected with pSVL 65 was higher than that in the cells only stimulated with LPS. The production of p65 was not affected by DZA as monitored by Western blot analysis using anti-p65 (data not shown). These results, together with the results in Fig. 4, A and B, showing DZA inhibition of LPS-induced NF-κB transcriptional activity, suggest that DZA directly inhibits the transcriptional activity of p65 but not at the signal cascade of LPS-induced NF-κB activation. To elucidate whether any of the observed effects could be due to DZA-Hcy, cells to be exposed to 100 μM DZA for 1 h before LPS stimulation were pretreated with a more specific and potent inhibitor of S-adenosylhomocysteine hydrolase, DZAn, to block the intracellular accumulation of DZA-Hcy (Fig. 4D). Treatment of DZAn almost completely abrogated the inhibition of NF-κB transcriptional activity in cells treated with 100 μM DZA, indicating a central role for DZA-Hcy in the DZA inhibition of NF-κB transcriptional activity. No inhibition of NF-κB transcriptional activity was observed in cells treated with DZAn alone. To investigate whether exogenous DZA-Hcy is capable of inhibition, cells were exposed to 100 μM DZA-Hcy for 1 h before LPS stimulation. As shown in Fig. 4D, exogenous DZA-Hcy failed to inhibit NF-κB transcriptional activity in the cells stimulated with LPS, demonstrating that DZA-Hcy is incapable of inhibition when given exogenously.

LPS-induced Phosphorylation of p65 Is Inhibited by DZA—To determine if DZA might inhibit the functional activation of NF-κB by modifying phosphorylation of p65, we examined the phosphorylation of p65 in LPS-stimulated RAW 264.7 cells in the presence or absence of DZA. As shown in Fig. 5, LPS induced a strong phosphorylation of p65. In cells pretreated with 100 μM DZA, however, LPS-induced phosphorylation of p65 was markedly reduced. A low constitutive phosphorylation of p65 was observed in unstimulated cells, which was reduced by treatment of the cells with DZA alone. These results strongly suggest that DZA inhibits the transcriptional activity of NF-κB by the hindrance of p65 phosphorylation required for the functional activation of NF-κB.

TNF-induced NF-κB Transcriptional Activity Is Inhibited by DZA—We tried to determine if DZA could inhibit NF-κB transcriptional activity without affecting nuclear translocation in non-hematopoietic cells stimulated by other inducers of NF-κB. TNF-α stimulation of COS-7 cells prominently induced the nuclear translocation of NF-κB and the DNA binding activity of NF-κB, which was not observed in unstimulated cells (Fig. 6A). The addition of 100 μM DZA to COS-7 cells before TNF-α stimulation had no effect on nuclear translocation or DNA binding activity of NF-κB, suggesting that DZA did not affect TNF-α-induced nuclear translocation of NF-κB in COS-7 cells. DZA alone did not induce the nuclear translocation of NF-κB in the cells. In transient transfection experiments of COS-7 cells using reporter gene constructs (J16 or J32), TNF-α stimulation of cells transfected with J16 resulted in CAT expression that was 2.6-fold greater (Fig. 6B). No induction of CAT expression by TNF-α was observed in cells transfected with J32. Treatment of these cells with 100 μM DZA for 1 h before TNF-α stimulation resulted in complete inhibition of CAT protein expression. These results reveal that DZA is a common and potent inhibitor of NF-κB transcriptional activity induced by distinct stimuli. The effect of Hcy on DZA inhibition of NF-κB transcriptional activity was examined. As shown in Fig. 6C, DZA dose dependently inhibited the transcriptional activity of NF-κB in cells stimulated by TNF-α. Combination of DZA and Hcy more potently inhibited NF-κB transcriptional activity than inhibition observed by DZA alone. These results show that DZA inhibition of NF-κB transcriptional activity is involved in the accumulation of DZA-Hcy in COS-7 cells stimulated with TNFα. Cells co-transfected with pSVL65 and CAT-reporter plasmid (J16 or J32) showed a 3.0-fold expression of CAT protein driven from J16, but not from J32 (Fig. 6D). 100 μM DAZa strongly inhibited the induction of CAT protein in cells expressing p65, suggesting that DZA directly inhibits the transcriptional activity of p65.

DZA Induces Proteolytic Degradation of IκBo, but Not IκBo− Translocation of NF-κB into the nucleus is linked to proteolytic degradation of IκB proteins (1), and as we have described above, DZA increases NF-κB nuclear translocation in RAW 264.7 cells. To test whether DZA promotes proteolytic degradation of IκB proteins, RAW 264.7 cells were pretreated with DZA following stimulation with or without LPS. As shown in Fig. 7A, the level of IκBo protein was notably reduced by treatment of DZA alone for 2 h at a concentration of 100 μM. The IκBo protein was fully recovered at 1 h after LPS stimulation without DZA, since IκBo is autoregulated through the activation of NF-κB (3, 36). Notably, DZA pretreatment before LPS prevented synthesis of IκBo which was recovered when treated with LPS alone, indicating that DZA interfered with the synthesis of IκBo because the transcriptional activity of NF-κB was inhibited by DZA. Resynthesis of IκBo after stimulation of LPS was more potently prevented by the addition of Hcy plus DZA (data not shown). We also examined the effect of DZA on IκBo levels in RAW 264.7 cells at various time inter-
DZA - - + +
LPS - + - +
p65 - - - -

**FIG. 5.** LPS-induced phosphorylation of p65 is inhibited by DZA. Phosphate-labeled RAW 264.7 cells were pretreated with or without DZA (100 μM) for 1 h, and stimulated with the addition of LPS (1 μg/ml). After incubation for 1 h, p65 was recovered by immunoprecipitation using specific antibody and fractionated by SDS-PAGE, followed by autoradiography. Similar results were obtained in an independent experiment.

vals. IxBα levels were reduced from their level at 60 min to their level at 120 min after treatment with DZA, and this reduction was continued to 240 min (Fig. 7B). Thus, these results demonstrate that DZA promotes the degradation of IxBα and prevents its resynthesis. Furthermore, these results reasonably support that DZA increases nuclear translocation and DNA binding activity of NF-κB in RAW 264.7 cells. A different IxB isoform, IxBβ is one of the major regulators of NF-κB activity, whose kinetics is slower than that of IxBα in response to NF-κB inducers such as LPS (6). We investigated the effect of DZA on IxBβ in RAW 264.7 cells (Fig. 7, C and D). Stimulation of LPS for 1 h without pretreatment of DZA led to the disappearance of IxBβ, which would normally exist in Western blot analysis using unstimulated RAW 264.7 cells (Fig. 7C). The disappearance of IxBβ was also observed in cells pretreated for 1 h with DZA at increasing concentrations following stimulation with LPS. Interestingly, treatment of 100 μM DZA alone for 2 h at several concentrations could not reduce the level of IxBβ (Fig. 7C), and IxBβ remained continuously until 4 h after exposure of 100 μM DZA (Fig. 7D), implying that DZA cannot lead to IxBβ down-regulation, and the down-regulation of IxBβ is not involved in enhanced nuclear translocation of NF-κB, mediated by DZA in RAW 264.7 cells.

**DZA-induced Proteolytic Degradation of IxBα Is Neither Modulated by Hcy nor Involved in IKK Complex Activation—**

The addition of 100 μM Hcy potentiated the inhibitory effect of DZA on NF-κB transcriptional activity. To investigate whether Hcy can potentiate the effect of DZA on proteolytic degradation of IxBα in RAW 264.7 cells, cells were treated with increasing concentrations of DZA in the presence or absence of 100 μM Hcy. As shown in Fig. 8A, additional treatment of Hcy could not modulate the proteolytic effect of DZA on IxBα. The treatment of Hcy alone could not reduce IxBα at all. In addition, the synergic effect of Hcy was not observed in cells treated with 100 μM DZA for increasing times (Fig. 8B). These results indicate that DZA-induced proteolytic degradation of IxBα in RAW 264.7 cells is dissociated from the accumulation of DZA-Hcy. Recently, it was reported that IKK complexes consisting of IKKα and IKKβ control phosphorylation of IxB proteins in cytokine-induced NF-κB activation pathway (7–9). We tried to determine if IKK complexes can participate in the down-regu-

**FIG. 4.** LPS-induced NF-κB transcriptional activity is inhibited by DZA in RAW 264.7 cells. The cells were transiently transfected with reporter gene constructs carrying two copies of the wild type (J16, ■) NF-κB binding sequence or mutant type (J32, □) NF-κB binding sequence in front of the CAT gene. A, the effect of DZA on NF-κB transcriptional activity. At 18 h after transfection with J16 (■) or J32 (□), cells were treated with or without 100 μM DZA for 1 h before stimulation. After 18 h of stimulation by the addition of LPS (1 μg/ml), cells were lysed to determine the expression of CAT. B, the effect of Hcy on the inhibitory effect of DZA. At 18 h after transfection with J16, cells were treated for 1 h with or without DZA at the indicated dosages (μM) in the absence (■) or presence (□) of Hcy (100 μM). After 18 h of stimulation by the addition of LPS (1 μg/ml), cells were lysed to determine the expression of CAT. C, the effect of DZA in the cells transfected with pSVL65 expressing p65. The cells were co-transfected with pSVL65 and J16 (■), or with pSVL65 and J32 (□). As a control, cells were transfected with pSVL substituting pSVL65. At 6 h after transfection, cells were treated with or without 100 μM DZA, and incubated for a further 18 h. And then cells were lysed to determine the expression of CAT. D, the effect of DZA derivatives. At 18 h after transfection with J16, cells were treated for 1 h with 100 μM DZA or 100 μM DZA-Hcy before stimulation. For the DZAri treatment, 100 μM DZAri was given 1 h before DZA treatment. After 18 h of stimulation by the addition of LPS (1 μg/ml), cells were lysed to determine the expression of CAT. Determination of CAT activity was performed using CAT enzyme-linked immunosorbent assay kit. Values are mean ± S.D. (n = 3).
The Effects of 3-Deazaadenosine on NF-κB Regulation

FIG. 6. TNF-induced NF-κB transcriptional activity is inhibited by DZA in COS-7 cells. A, the effect of DZA on NF-κB binding activity. The cells were pretreated with or without 100 μM DZA for 1 h before stimulation, and then stimulated by the addition of recombinant GST-human TNF-α (50 ng/ml). Nuclear extracts were prepared at 1 h after stimulation. 5 μg of each nuclear protein were subjected to a DNA binding reaction with 32P-P-end-labeled NF-κB consensus sequence, and then DNA-protein complexes were separated by native polyacrylamide gel electrophoresis. B, the effect of DZA on NF-κB transcriptional activity. The cells were transiently transfected with reporter gene constructs carrying two copies of the wild type (J16, □) or mutant type (J32, □) NF-κB binding sequence in front of the CAT gene. At 18 h after transfection, cells were treated with or without 100 μM DZA for 1 h before stimulation. After 18 h of stimulation by the addition of TNF-α (50 ng/ml), cells were lysed to determine the expression of CAT. C, the effect of Hcy on the inhibitory effect of DZA. At 18 h after transfection with J16, cells were treated for 1 h with or without DZA at the indicated dosages (μM), in the absence (■) or presence (□) of Hcy (100 μM). After 18 h of stimulation by the addition of TNF-α (50 ng/ml), cells were lysed to determine the expression of CAT. D, the effect of DZA in the cells transfected with pSVL65 expressing p65. The cells were co-transfected with pSVL65 and J16 (□), or with pSVL65 and J32 (□). As a control, cells were transfected with pSVL substituting pSVL65. At 6 h after transfection, cells were treated with or without 100 μM DZA, and incubated for a further 18 h. And then cells were lysed to determine the expression of CAT. Determination of CAT expression was performed using CAT enzyme-linked immunosorbent assay kit. Values are mean ± S.D. (n = 3).

FIG. 7. DZA induces proteolytic degradation of IκBo. A, dose-response effect of DZA on IκBo in RAW 264.7 cells. The cells were pretreated with DZA at the indicated concentrations (μM) for 1 h, and then stimulated by the addition of LPS (1 μg/ml) or not stimulated. After 1 h, cytosolic fractions were prepared. 10 μg of each protein was separated by SDS-PAGE, and then Western blot analysis with specific antibody against IκBo was performed. B, time-response effect of DZA on IκBo. RAW 264.7 cells were treated with 100 μM DZA for the indicated times. At the end of the times, cytosolic fractions were prepared. 10 μg of each protein was separated by SDS-PAGE, and then Western blot analysis with specific antibody against IκBo was performed. C, dose-response effect of DZA on IκBβ in RAW 264.7 cells. The cells were pretreated with DZA at the indicated concentrations (μM) for 1 h, and then stimulated by the addition of LPS (1 μg/ml) or not stimulated. After 1 h, cytosolic fractions were prepared. 10 μg of each protein was separated by SDS-PAGE, and then Western blot analysis with specific antibody against IκBβ was performed. D, time-response effect of DZA on IκBβ. RAW 264.7 cells were treated with 100 μM DZA for the indicated times. At the end of the times, cytosolic fractions were prepared. 10 μg of each protein was separated by SDS-PAGE, and then Western blot analysis with specific antibody against IκBβ was performed. Data illustrated are from a single experiment and are representative of a total of three separate experiments.

DISCUSSION

In this study, we demonstrated the dual effects of DZA on the regulation of NF-κB, inhibition of NF-κB transcriptional activity, and promotion of IκBo degradation. A hypothetical diagram of the effects of DZA is illustrated in Fig. 9. There have been many reports that described some interesting properties of DZA, including the inhibitory effect on the expression of cytokines and cell adhesion molecules, the induction of apoptosis and an anti-HIV effect. The involvement of NF-κB in the expression of numerous cytokines and adhesion molecules which promote several diseases is well known (1). Our previous study (26) and this paper shows that DZA potently inhibited the transcription of cytokines such as TNF-α and IL-1β. Additionally, other researchers reported that the adherence of cells was inhibited through the suppression of adhesion molecule expression of CAT. Determination of CAT expression was performed using CAT enzyme-linked immunosorbent assay kit. Values are mean ± S.D. (n = 3).
NF-κB have described a role for NF-κB in the growth of cells. Additionally, recent reports (38–41) have performed.

Fractions were prepared. Western blot analysis using specific antibody against IκBα molecules. How DZA inhibits the expression of cytokines and adhesion molecules was accomplished using recombinant protein of GST-IκBα prepared, and IKK complexes were recovered by immunoprecipitation. At the end of the times, cytosolic fractions were prepared. 10 μg of each protein was subjected to Western blot analysis with specific antibody against IκBα. The IKK assay was accomplished using recombinant protein of GST-IκBα as a substrate. Similar results were obtained in an independent experiment.

**FIG. 8.** DZA-induced proteolytic degradation of IκBα is neither modulated by Hcy nor involved in IKK complex activation. A. RAW 264.7 cells were treated with increasing concentrations of DZA for 2 h in the presence or absence of 100 μM Hcy, and then cytosolic fractions were prepared. 10 μg of each protein was subjected to Western blot analysis using specific antibody against IκBα. B, the cells were treated for the indicated times with 100 μM DZA, in the absence or presence of Hcy (100 μM). At the end of the times, cytosolic fractions were prepared. 10 μg of each protein was separated by SDS-PAGE, and then Western blot analysis with specific antibody against IκBα was performed. C, the cells were treated with 100 μM DZA or LPS (1 μg/ml) for the indicated times. At the end of the times, total cell lysates were prepared, and IKK complexes were recovered by immunoprecipitation. IKK assay was accomplished using recombinant protein of GST-IκBα as a substrate. Similar results were obtained in an independent experiment.

**FIG. 9.** Hypothetical diagram illustrating the effects of DZA on NF-κB regulation.

Expression by DZA (27, 28). Even though they provided for the possibility of DZA as a potent therapeutic agent for diseases in which these cytokines and adhesion molecules play a central pathogenic role, the mechanism has not been elucidated. Since these cytokines and adhesion molecules are included in specific target genes of NF-κB, this study successfully explains how DZA inhibits the expression of cytokines and adhesion molecules.

Beg et al. (37) reported that a phenotype of the p65−/− mice died during embryonic development through massive apoptosis of hepatocytes, suggesting that NF-κB might play an important role in protecting cells from apoptosis, instead of promoting growth of cells. Additionally, recent reports (38–41) have described a role for NF-κB in blocking apoptosis induced by TNF.

Interestingly, this protective function of NF-κB was apparent not only against TNF but also against cells treated with ionizing radiation or chemotherapeutic agents (39). On the other hand, the role of the Rel proteins in oncogenic transformation is quite well established, and it is now clear that Rel proteins malignantly transform and immortalize cells by inducing expression of specific genes. These findings suggest the possibility that some of the target genes for Rel-mediated oncogenesis and for NF-κB-mediated blockage of apoptosis may be identical. Thus, it is strongly suggested that agents which inhibit NF-κB transcriptional activity may have both direct anti-cancer effects and effects that facilitate apoptosis in tumor cells. Recently, we reported that DZA induced apoptosis in lymphocytic mouse leukemia L1210 cells (30). The report showed that even though DZA raised the NF-κB binding activity during apoptosis induction, c-myc transcription that is a downstream target gene of NF-κB (1, 42) was markedly reduced at the early stage of apoptosis induction. Similarly, the reduction of c-myc transcription in DZA-induced apoptosis was also observed in human promyelocytic leukemia HL-60 cells by other researchers (43, 44). Collectively, it is reasonable that DZA inhibition of NF-κB transcriptional activity may entirely serve as a potent inducing factor or may participate at least in part of the induction of apoptosis by DZA in tumor cells. Finally, these findings point to DZA as a useful chemotherapeutic agent which may prevent malignant transformation of normal cells into tumor cells and induce apoptosis of tumor cells through the inhibition of NF-κB transcriptional activity.

NF-κB, whose enhancer elements are located in HIV-long terminal repeats, is a key molecule of HIV replication for the pathogenesis of AIDS (45), suggesting that NF-κB may be a target for the therapy of AIDS. Previously, other researchers described that DZA may have an anti-HIV effect (21, 22), but the mechanism of this effect has not been understood. The finding of DZA inhibition of NF-κB transcriptional activity described in this study explains how DZA can exert an anti-HIV effect as described by other researchers. The inhibition of NF-κB transcriptional activity by DZA suggests that DZA may inhibit the replication of HIV, implying that DZA may be a useful drug for the therapeutic treatment of AIDS.

Naumann and Scheidereit (46) described that p65 is strongly phosphorylated during the activation of NF-κB in vivo. A recent study demonstrated that the transcriptional activity of NF-κB is regulated through phosphorylation of p65 by cAMP-independent activation of a catalytic subunit of protein kinase A (PKAc) associated with IκB proteins by a mechanism which signals caused degradation of IκB proteins results in the activation of PKAc and subsequent phosphorylation of p65 (47). In this study, we showed that DZA disturbed the phosphorylation of p65 induced by LPS. So, we suggest a possibility that accumulated cellular DZA-Hcy, caused by treatment of cells with DZA, might inhibit the activation of PKAc associated with IκB proteins, or interrupt the PKAc-mediated phosphorylation of p65 directly or indirectly. SB203580, a specific inhibitor of p38 mitogen-activated protein kinase, and elevated intracellular cAMP has been represented as an inhibitor of NF-κB, which inhibit NF-κB transcriptional activity already bound to DNA in the nucleus (16, 17). Even though, DZA and these inhibitors share a similarity that all of them inhibit NF-κB transcriptional activity without affecting its nuclear translocation and DNA binding activity, there is a definite difference in the mechanism of NF-κB inhibition. In contrast to SB203580 and elevated cellular cAMP, DZA inhibits the phosphorylation of p65.

This study indicates that DZA inhibits NF-κB transcriptional activity through a hindrance of p65 phosphorylation by
the accumulation of DZA-Hcy in cells. Nevertheless, we cannot totally rule out that DZA independent of cellular DZA-Hcy accumulation, may have interfered with NF-κB transcriptional activity. In the report by Backlund et al. (48), treatment with 100 μM DZA resulted in significant accumulation of DZA-Hcy in RAW 264.7 cells. The addition of Hcy to DZA increased the accumulation of DZA-Hcy to about 5 times the level caused by DZA alone. In contrast to a greatly increased accumulation of DZA-Hcy by the addition of Hcy to DZA treatment in the cells, we observed a slightly potentiated inhibition of NF-κB transcriptional activity by the addition of Hcy to DZA.

We described that DZA promotes the proteolytic degradation of IkBα, but not IkBβ in RAW 264.7 cells, leading to an increase in nuclear translocation and DNA binding activity. Increased DNA binding activity of NF-κB in the nucleus by DZA was also observed in DZA-induced apoptosis in L1210 cells (30), and DZA might reduce IkBα in this case. However, a DZA-mediated increase of NF-κB DNA binding activity in the nucleus was not discernable in COS-7 cells, suggesting that DZA-mediated IkBα degradation is cell-type specific. A number of kinases have been suggested to phosphorylate IkBα, and recently a high molecular mass, approximately 700 kDa, kinase complex termed IKK (7–9) was identified. However, DZA mediates IkBα degradation through an IKK-independent pathway and DZA can stimulate other kinase pathways. We conclude this because IKK was not activated at all by DZA while it was intensely activated by LPS in this study. One notable fact is that DZA stimulates proteolytic degradation of IkBα but not IkBβ. Among kinases which have been reported to induce degradation of IkB proteins, including IKK, none can selectively differentiate between IkBα and IkBβ. Thus, we expect that DZA might be a valuable probe to identify kinases that function with only IkBα.

In conclusion, we demonstrate that DZA has dual effects on NF-κB regulation. One is an inhibitory effect on NF-κB transcriptional activity already bound to target DNA, and another is the promotion of proteolytic degradation of IkBα. Although the wide variety of biological properties observed with DZA has emphasized that DZA is an effective drug for treatment of human diseases including inflammation, infections, and tumors, the mechanism and target molecules of DZA’s action have never been elucidated. This study is the first to demonstrate that DZA promotes the proteolytic degradation of IkBα in this study. We described that DZA promotes the proteolytic degradation of IkBα, but not IkBβ in RAW 264.7 cells, suggesting that DZA-mediated IkBα degradation is cell-type specific. A number of kinases have been suggested to phosphorylate IkBα, and recently a high molecular mass, approximately 700 kDa, kinase complex termed IKK (7–9) was identified. However, DZA mediates IkBα degradation through an IKK-independent pathway and DZA can stimulate other kinase pathways. We conclude this because IKK was not activated at all by DZA while it was intensely activated by LPS in this study. One notable fact is that DZA stimulates proteolytic degradation of IkBα but not IkBβ. Among kinases which have been reported to induce degradation of IkB proteins, including IKK, none can selectively differentiate between IkBα and IkBβ. Thus, we expect that DZA might be a valuable probe to identify kinases that function with only IkBα.

In conclusion, we demonstrate that DZA has dual effects on NF-κB regulation. One is an inhibitory effect on NF-κB transcriptional activity already bound to target DNA, and another is the promotion of proteolytic degradation of IkBα. Although the wide variety of biological properties observed with DZA has emphasized that DZA is an effective drug for treatment of human diseases including inflammation, infections, and tumors, the mechanism and target molecules of DZA’s action have never been elucidated. This study is the first to demonstrate that NF-κB is a specific target molecule of DZA and suggests that DZA may be a potent drug for the treatment of diseases in which NF-κB plays an important pathogenic role, as well as a useful tool for studying the regulation and physiological functions of NF-κB.

Acknowledgment—We appreciate Dr. David Baltimore, California Institute of Technology, Pasadena, CA, for the kind donations of J16 and J32.