EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 34, No. 5, 332-339, November 2002

Dual effect of oxidative stress on NF-κB activation in HeLa cells

Mi-Sun Byun[1], Kye-Im Jeon[1], Jae-Won Choi[1], Jae-Yong Shim[2] and Dae-Myung Jue[1,3]

[1]Department of Biochemistry
[2]Department of Anesthesiology
College of Medicine, The Catholic University of Korea
505 Banpo-dong, Seocho-gu
Seoul 137-701, Korea
[3]Corresponding author: Tel, 82-2-590-1177;
FAX, 82-2-596-4435; E-mail, dmjue@catholic.ac.kr

Accepted 12 September 2002

Abbreviations: ROS, reactive oxygen species; IKK, IκB kinase; TNF, tumor necrosis factor; IL-1, interleukin-1; LPS, lipopolysaccharide; HA, hemagglutinin; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay.

Abstract

Reactive oxygen species (ROS) has been implicated as an inducer of NF-κB activity in numbers of cell types where exposure of cells to ROS such as H_2O_2 leads to NF-κB activation. In contrast, exposure to oxidative stress in certain cell types induced reduction of tumor necrosis factor (TNF)-induced NF-κB activation. And various thiol-modifying agents including gold compounds and cyclopentenone prostaglandins inhibit NF-κB activation by blocking IκB kinase (IKK). To understand such conflicting effect of oxidative stress on NF-κB activation, HeLa cells were incubated with H_2O_2 or diamide and TNF-induced expression of NF-κB reporter gene was measured. NF-κB activation was significantly blocked by these oxidizing agents, and the inhibition was accompanied with reduced nuclear NF-κB and inappropriate cytosolic IκB degradation. H_2O_2 and diamide also inhibited IKK activation in HeLa and RAW 264.7 cells stimulated with TNF and lipopolysaccharide, respectively, and directly blocked IKK activity *in vitro*. In cells treated with H_2O_2 alone, nuclear NF-κB was induced after 2 h without detectible degradation of cytosolic IκBα or activation of IKK. Our results suggest that ROS has a dual effect on NF-κB activation in the same HeLa cells: it inhibits acute IKK-mediated NF-κB activation induced by inflammatory signals, while longer-term exposure to ROS induces NF-κB activity through an IKK-independent pathway.

Keywords: NF-κB, oxidative stress, hydrogen peroxide, diamide, protein kinases, oxidation-reduction

Introduction

Modulation of proteins and enzyme activities by cellular reduction/oxidation (redox) status is one of the major mechanisms that cells employ to overcome oxidative stress. Although intracellular redox balance is tightly controlled in most cell types, it can be altered by reactive oxygen species (ROS) produced by environmental toxins or in pathologic conditions such as chronic inflammatory diseases and ischemia-reperfusion injury (Halliwell and Gutteridge, 1999). Exposure of cells to oxidative stress can induce alterations in various signal transduction cascades that lead to changes in the activity of related transcription factors (Adler *et al*., 1999; Thannickal and Fanburg, 2000).

The transcription factor NF-κB regulates expression of a wide range of cellular and viral genes and plays important roles in immune and inflammatory responses (Baeuerle and Henkel, 1994). In resting cells, NF-κB proteins are sequestered in the cytosol through interaction with a class of inhibitory proteins called IκBs (IκBα, β, and ε). In response to proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), bacterial lipopolysaccharide (LPS), or viral double-stranded RNA, the IκBs are rapidly phosphorylated at two specific serine residues located at their N-terminal regions. Phosphorylated IκBs then undergo ubiquitination and proteolysis by the 26S proteasome, resulting in release and translocation of NF-κB to the nucleus to induce expression of specific target genes. In the signal pathway for NF-κB activation, phosphorylation of IκBs is likely to be the central point of regulation at which signals from diverse stimuli converge (Barnes and Karin, 1997; May and Ghosh, 1998). Previous studies have identified an IκB kinase (IKK) complex with a molecular mass of 500-900 kDa, which is induced by inflammatory signals and able to phosphorylate the N-terminal serine residues of IκBα and IκBβ involved in NF-κB activation *in vivo* (Chen *et al*., 1996; DiDonato *et al*., 1997). Two subunits of IKK, called IKKα (or IKK1) and IKKβ (IKK2), were shown to play a catalytic role in IκB phosphorylation (DiDonato *et al*., 1997; Mercurio *et al*., 1997; Regnier *et al*., 1997; Woronicz *et al*., 1997; Zandi *et al*., 1997), whereas another subunit IKKγ/ NEMO/IKKAP1 is regulatory and involved in transmission of upstream signals to IKKα and IKKβ (Yamaoka *et al*., 1998; Rothwarf *et al*., 1998; Mercurio

et al., 1999).

It has been suggested that intracellular ROS levels regulate NF-κB activation, in that H_2O_2 can induce NF-κB activation in lymphocytes and monocytes (Schreck *et al*., 1991; Meyer *et al*., 1993; Manna *et al*., 1998). In these cells, NF-κB activation by diverse signals such as TNF, IL-1, LPS, and phorbol esters was inhibited by various structurally unrelated antioxidants (Schreck *et al*., 1992), and by overexpression of antioxidant enzymes (Schreck *et al*., 1991; Kretz-Remy *et al*., 1996; Jin *et al*, 1997; Manna *et al*., 1998). Many NF-κB-activating signals also induce increase of intracellular ROS levels, further supporting the involvement of ROS in the pathway for NF-κB activation (Schreck *et al*., 1992). Although molecular mechanisms involved in ROS-induced NF-κB activation is still largely unknown, recent studies with lymphocytes exposed to oxidative stress suggested that NF-κB is activated through a pathway that involves phosphorylation of Tyr-42 on IκBα instead of IKK-mediated serine phosphorylation (Koong *et al*., 1994; Imbert *et al*., 1996; Mukhopadhyay *et al*., 2000; Schoonbroodt *et al*., 2000). These results suggest that a shift in the cellular redox equilibrium to an oxidized state accompanies and promotes NF-κB activation through an IKK-independent mode in certain types of cells. However, it was also shown that pre-exposure of various types of cells to H_2O_2 or thiol-oxidizing agents, including phenylarsine oxide, pervanadate, and diamide, suppressed TNF-induced NF-κB activation and blocked phosphorylation and degradation of IκBα (Singh *et al*., 1996; Lahdenpohja *et al*., 1998; Zahler *et al*., 2000). Our previous study showed that thiol-binding metal compounds such as gold, zinc, and copper inhibit NF-κB activation in LPS-stimulated macrophages by blocking IκBα degradation and IKK activation (Jeon *et al*., 2000). *In vitro* IKK activity was also suppressed by these metal compounds as well as other thiol-reactive agents, and required the presence of reducing agent dithiothreitol in the reaction mixture. Recently other thiol-reactive compounds such as cyclopentenone prostaglandins (prostaglandin A_1 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2), arsenite, and lipid peroxidation product, 4-hydroxy-2-nonenal, were also shown to inhibit NF-κB and IKK activation in cells stimulated with TNF, IL-1, or phorbol esters (Kapahi *et al*., 2000; Rossi *et al*., 2000; Straus *et al*., 2000; Ji *et al*., 2001). Experiments with mutant IKK protein showed that cyclopentenone prostaglandins and arsenite block IKK activity by covalently modifying Cys-179 on the activation loop of IKKβ (Kapahi *et al*., 2000; Rossi *et al*., 2000). Taken together, these observations suggest that oxidative stress has opposite effects on NF-κB activity depending on the signaling pathway employed for NF-κB activation.

Here we examined the effect of an oxidative stress elicited by addition of exogenous H_2O_2 and thiol-oxidizing agent diamide on NF-κB activation in TNF-stimulated HeLa cells. Our results show that both agents suppressed TNF-induced NF-κB transcriptional activity, increase of nuclear NF-κB, and degradation of cytosolic IκBα. H_2O_2 and diamide also inhibited IKK activation in TNF-stimulated HeLa cells, and preincubation with these oxidizing agents suppressed IKK activity *in vitro*. In cells treated with H_2O_2 alone, nuclear NF-κB was induced after 2 h without detectible degradation of IκBα or IKK activation. Our data imply that oxidative stress suppresses NF-κB activation in TNF-stimulated HeLa cells by blocking IKK activity, while H_2O_2 on its own induces NF-κB activity through an IKK-independent mechanism.

Materials and Methods

Materials

Antibodies to IκBα (C-21), p65/RelA (SC-109), IKK (M-280), IKK (H-4), and hemagglutinin (HA) tag (Y-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag (M2) and anti-β-actin antibodies were from Stratagene (La Jolla, CA) and Sigma (St. Louis, MO), respectively. H_2O_2 was purchased from Junsei Chemical (Tokyo, Japan) and diluted to a 400 mM stock before use. Diamide was from Sigma and dissolved in phosphate-buffered saline (PBS) at a 1 M stock. Recombinant GST-IκBα containing N-terminal 54 residues of IκBα and recombinant human TNF were prepared by expression in *Escherichia coli* as described previously (Jeon *et al*., 2000). HeLa human epithelial cells and RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (ATCC, Manassa, MD). HeLa cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). RAW 264.7 cells were grown in the RPMI 1640 medium supplemented with 5% fetal calf serum, 20 mM Hepes and gentamicin (50 μg/ml).

NF-κB reporter gene assay

A reporter plasmid IgκB-Luc containing a κB sequence upstream of a minimal interleukin-8 promoter and a luciferase gene was kindly provided by Dr. T. H. Lee (Yonsei University, Seoul, Korea). HeLa cells grown in a 12-well culture plate (2×10^5 cells/well) were transfected with 120 ng of IgκB-Luc, together with 200 ng of β-actin promoter-driven β-galactosidase expression plasmid. Transfected cells were incubated in the presence or absence of oxidizing agents and stimulated with TNF (10 ng/ml). After 4 h, the

cells were washed with PBS and lysed in reporter lysis buffer (Promega, Madison, WI) by freezing and thawing. Luciferase assays were performed with a luminometer (Turner Designs, Sunnyvale, CA), and activity was normalized to β-galactosidase activity.

Electrophoretic mobility shift assay (EMSA) and immunoblot analysis

Nuclear and cytoplasmic extracts were prepared from HeLa cells as described previously (Jeong and Jue, 1997). The oligonucleotide containing consensus recognition sequence for NF-κB or C/EBP were obtained from Santa Cruz Biotechnology, and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Binding reaction was performed with 5 μg of nuclear extract as described (Jeong and Jue, 1997) and the reaction products were analyzed by electrophoresis on a 4% polyacrylamide gel in 0.25×TBE buffer (22.5 mM Tris-HCl, pH 8.5, 22.5 mM borate, 0.5 mM EDTA). Immunoblot analyses of IκBα and other proteins were performed with corresponding antibodies and visualized by ECL detection kit (Amersham, Buckinghamshire, UK) (Jeong and Jue, 2000).

In vitro IKK assay

HeLa and RAW 264.7 cells grown in 100-mm plates were treated with various agents and washed 3 times with ice-cold PBS containing 1 mM Na_3VO_4, and 5 mM EDTA. The cells were scraped and suspended in lysis buffer (0.75 ml) containing 20 mM Tris-HCl, 0.5 M NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM 4-nitrophenylphosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany), and 1 mM dithiothreitol (Jeon *et al.*, 2000). After incubation for 20 min on ice, the lysate was cleared by centrifugation at 20,000 *g* for 15 min. The supernatant fraction was analyzed for protein with bicinchoninic acid reagent (Pierce, Rockford, IL). An aliquot (120 μg protein) of the lysate was immunoprecipitated with anti-IKKα antibody. *In vitro* kinase assay was performed with immune complexes and bacterially synthesized GST-IκBα proteins (1 μg) in 15 μl of kinase buffer containing 20 mM Hepes (pH 7.7), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP, 1-5 μCi of [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, complete EDTA-free protease inhibitor cocktail, and 1 mM dithiothreitol at 30°C for 30-60 min. Samples were analyzed by 12.5% SDS-PAGE and autoradiography. Phosphorylation of GST-IκBα was quantitated in a phosphorimage analyzer (BAS-2500, Fujifilm, Tokyo, Japan).

Results

Inhibition of TNF-induced NF-κB activation by H_2O_2 and diamide

To determine the effect of oxidative stress on TNF-induced NF-κB activation, we measured expression of NF-κB reporter gene in HeLa cells treated with H_2O_2 and diamide (Figure 1). TNF induced about 6-fold increase of reporter gene expression, whereas incubation of cells with oxidizing agents resulted in a dose-dependent suppression of NF-κB activation. The inhibition was observed at 0.1 and 0.3 mM concentrations of H_2O_2 and diamide, respectively. In cells treated with 3 mM H_2O_2 or 1 mM diamide, NF-κB activity was completely suppressed in both TNF-stimulated and unstimulated cells. The inhibitory effect of oxidizing agents was also observed when cells were washed after preincubation with oxidizing agents and then stimulated with TNF (data not shown). The expression level of control β-galactosidase was not significantly different between cells incubated in the presence or absence of oxidizing agents, indicating that the decrease of reporter gene expression in cells exposed to oxidative stress was not caused by general cytotoxicity (Figure 1B).

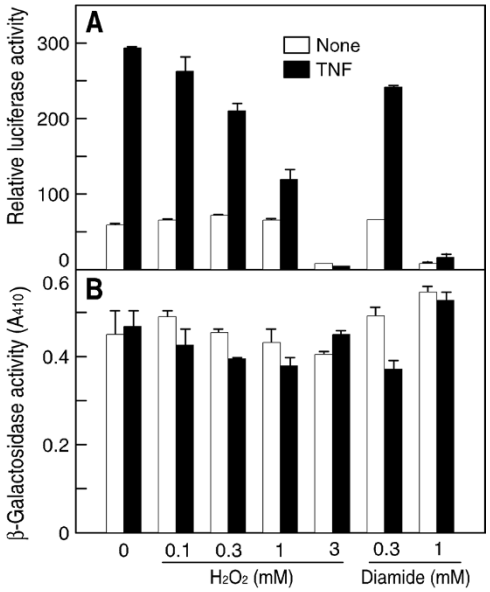

Figure 1. H_2O_2 and diamide inhibit TNF-induced NF-κB reporter gene expression. HeLa cells were cotransfected with NF-κB reporter construct (IgκB-Luc) and control β-galactosidase expression plasmid and incubated for 44 h. The cells were treated with various doses of H_2O_2 or diamide for 10 min, and incubated in the presence (solid bar) or absence (blank bar) of TNF (20 ng/ml) for 4 h before cell lysis. Relative luciferase activity, determined using β-galactosidase activity as a normalizing factor, is shown in (A), and β-galactosidase activity in (B). The data are presented as mean ± SD of triplicate cultures.

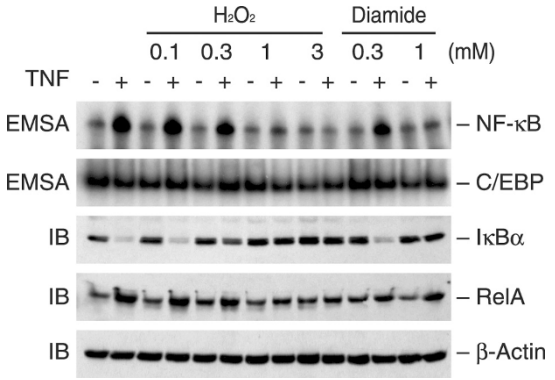

Figure 2. Oxidizing agents block TNF-induced increase in nuclear κB-binding activity, while suppressing degradation of cytosolic IκBα. HeLa cells were treated with H_2O_2 or diamide as described in Figure 1, stimulated with TNF for 15 min, and nuclear and cytosolic extracts were prepared. Nuclear extracts were incubated with ^{32}P-labeled NF-κB (first panel) or C/EBP probes (second panel), and analyzed by EMSA. Immunoblotting analysis of p65/RelA in the nuclear extract and IκBα and β-actin in the cytosolic extract was performed with specific antibodies by chemiluminescence reaction. The data represent three experiments.

Since TNF-induced NF-κB activation is known to depend on phosphorylation and degradation of inhibitory IκB proteins and translocation of NF-κB to the nucleus, we tested the effect of oxidizing agents on the level of these proteins in TNF-stimulated HeLa cells. Determination of nuclear κB-binding activity by EMSA showed low level of NF-κB in unstimulated cells, while it was induced remarkably by TNF (Figure 2, first panel). H_2O_2 or diamide suppressed TNF-induced κB-binding activity in a dose-dependent manner. Supershift analysis with specific antibodies demonstrated that the κB-binding protein induced by TNF was heterodimer of p50/NFKB1 and p65/RelA subunits (data not shown). TNF and oxidizing agents did not alter the level of nuclear C/EBP, demonstrating a specific effect of these agents on NF-κB activity (Figure 2, second panel). Immunoblot analysis of nuclear p65/RelA protein also showed suppression of TNF-induced increase of this protein by H_2O_2 or diamide, indicating that the decrease of nuclear κB-binding activity was caused by reduced translocation of NF-κB protein to the nucleus (Figure 2, third panel). The decrease of nuclear NF-κB in cells treated with oxidizing agents was accompanied by reduced degradation of IκBα in the cytosol (Figure 2, fourth panel), while β-actin level remained constant (Figure 2, fifth panel).

Oxidizing agents inhibit signal-induced IKK activation and *in vitro* IKK activity

Degradation of IκBα protein occurs after signal-induced phosphorylation at specific serine residues by

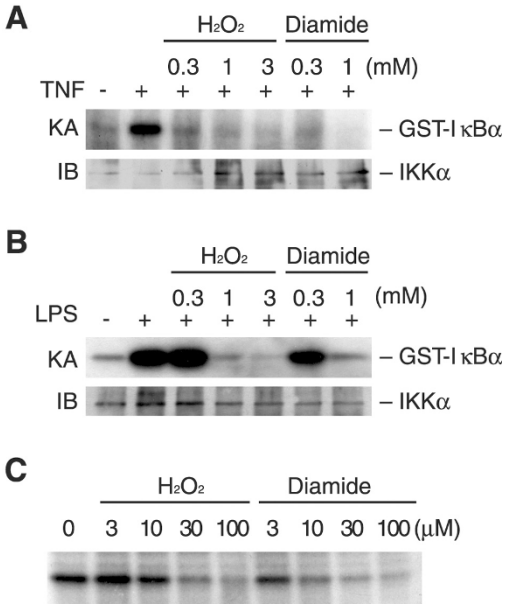

Figure 3. Oxidizing agents inhibit signal-induced IKK activation and *in vitro* IKK activity. (A) HeLa cells pretreated with various doses of H_2O_2 or diamide for 10 min were incubated in the presence or absence of TNF (20 ng/ml) for 5 min before cell lysis. IKK was immunoprecipitated with anti-IKKα antibody and used in *in vitro* kinase reactions with 1 μg of GST-IκBα (aa 1-54) and [γ-^{32}P]ATP in a buffer containing 1 mM dithiothreitol. The reaction products were separated by SDS-PAGE, transferred to polyvinylidone fluoride membranes, and subjected to autoradiography. The levels of IKKα in each reaction mixture were determined by immunoblotting analysis with anti-IKKα antibody. (B) RAW 264.7 macrophages were treated with various doses of H_2O_2 or diamide for 10 min and stimulated with LPS (1 μg/ml) for 15 min. IKK assay and immunoblotting analysis were performed as described in (A). (C) IKK complex was obtained by immunoprecipitation from HeLa cells treated with TNF (20 ng/ml) for 5 min. The immune complex was incubated with various doses of H_2O_2 or diamide for 10 min in kinase buffer without reducing agent. The IKK immune complex was washed once in kinase buffer containing 1 mM dithiothreitol and kinase reactions were conducted. The data represent two experiments.

IKK (Barnes and Karin, 1997; May and Ghosh, 1998). To determine whether H_2O_2 and diamide inhibit signal pathway leading to IKK activation, HeLa cells, with or without pretreatment with oxidizing agents, were stimulated with TNF. The cell lysate was immunoprecipitated with anti-IKKα antibody and incubated with GST-IκBα and [γ-^{32}P]ATP to evaluate incorporation of ^{32}P into the fusion protein. IKK activity was not detected in unstimulated cells, and H_2O_2 and diamide alone did not induce IKK activation (Figure 3A and data not shown; see also Figure 5). IKK activity was induced in TNF-stimulated cells, and pretreatment of cells with H_2O_2 and diamide inhibited TNF-induced IKK activation. To determine whether these oxidizing agents also block IKK activation induced by other signal in other type of cell, IKK activity was measured in RAW 264.7 macrophages stimulated with LPS.

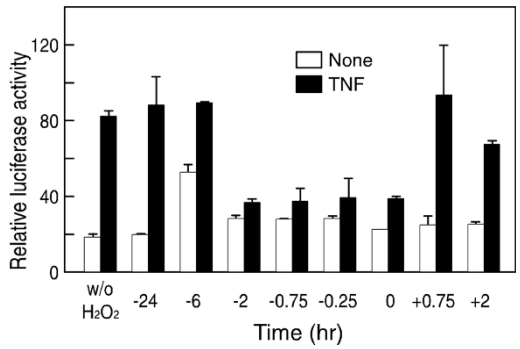

Figure 4. Time-dependent inhibition of TNF-induced NF-κB activation and induction of NF-κB by H_2O_2. HeLa cells were cotransfected with IgκB-Luc and control β-galactosidase expression plasmid and incubated for 48 h. The cells were added with H_2O_2 (1 mM) at the times indicated before or after addition of TNF (20 ng/ml). After incubation in the presence (solid bar) or absence (blank bar) of TNF for 4 h, the cells were lysed and relative luciferase activity was determined using β-galactosidase activity as a normalizing factor. The data shown in the left are obtained from cells incubated in the absence of H_2O_2 (w/o H_2O_2). The data are presented as mean ± SD of triplicate cultures.

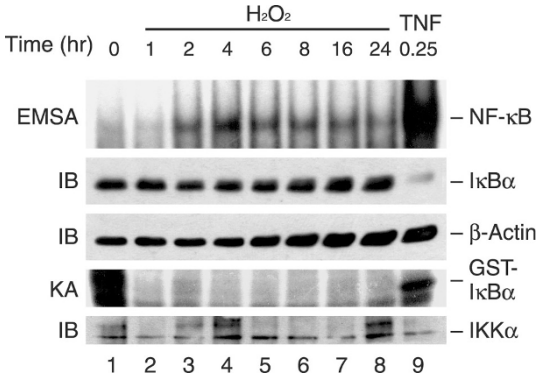

Figure 5. H_2O_2 induces NF-κB activation in the absence of IκBα degradation. HeLa cells were incubated with H_2O_2 (1 mM) (lanes 2-8) or TNF (20 ng/ml) (lane 9) for times indicated and the nuclear and cytosolic extracts were prepared. The nuclear extracts were incubated with ^{32}P-labeled NF-κB probe, and analyzed by EMSA (first panel). The cytosolic extracts were used to measure the levels of IκBα and β-actin by immunoblotting with specific antibodies (second and third panels). IKK was immunoprecipitated with anti-IKKα antibody and used in kinase reactions with GST-IκBα and [γ-^{32}P] ATP, and phosphorylated GST-IκBα was detected by autoradiography (fourth panel). The levels of IKK in each reaction mixture were determined by immunoblotting analysis with anti-IKKα antibody (fifth panel). The data represent three experiments.

RAW 264.7 cells showed low basal IKK activity even when they were not stimulated, and stimulation of cells with LPS induced significant IKK activity (Figure 3B). Preincubation of cells with H_2O_2 or diamide suppressed LPS-induced IKK activation. Our results demonstrate that oxidizing agents inhibit NF-κB activation by blocking IKK activation, and their inhibitory effect appears in different signal pathways of IKK activation.

To test whether inhibition of IKK activation by H_2O_2 and diamide occurs through direct inhibition of IKK activity, IKK complex was isolated from TNF-stimulated HeLa cells and incubated with various doses of oxidizing agents for 10 min. The IKK complex was then washed in the kinase buffer containing 1 mM dithiothreitol and kinase activity was measured. Our result shown in Figure 3C revealed that IKK complex isolated from TNF-stimulated HeLa cells was inhibited by both H_2O_2 and diamide in a dose-dependent manner. Inhibitory effect of both oxidizing agents appeared over 3-100 μM concentration range and higher doses of H_2O_2 and diamide completely blocked IKK activity (data not shown).

H_2O_2 on its own induces NF-κB activation in delayed time

To understand time-dependent effect of oxidative stress on TNF-induced NF-κB activation, H_2O_2 was added to HeLa cells at different time intervals before or after addition of TNF, and expression of NF-κB reporter gene was measured 4 h after TNF treatment. Another group of cells was incubated only with H_2O_2 in the absence of TNF stimulation. Suppressive effect of H_2O_2 on TNF-induced NF-κB activation appeared when it was added to the cells simultaneously with TNF or within 2 h before TNF addition (Figure 4). Inhibition of reporter gene expression by H_2O_2 detected was no more detected when the cells were exposed to H_2O_2 for more than 6 h or H_2O_2 was added 45 min after addition of TNF. On the contrary, H_2O_2 on its own induced significant reporter gene expression after 10 h (-6 h in Figure 4), and the level returned to basal level after 28 h (-24 h in Figure 4).

EMSA was performed to determine whether the induction of NF-κB reporter gene in H_2O_2-treated HeLa cells is associated with increase in nuclear κB-binding activity. H_2O_2-induced nuclear κB-binding activity detected after 2 h peaked at 4 h, and then gradually decreased up to 24 h (Figure 5). However, even in cells treated with H_2O_2 for 4 h, the level of NF-κB in the nucleus was far lower than cells induced with TNF for 15 min (compare lane 4 with lane 9 in Figure 5). Immunoblot analysis showed that H_2O_2 did not induce significant degradation of cytosolic IκBα, whereas it was degraded almost completely in TNF-treated cells (Figure 5). Control β-actin level was not changed by either H_2O_2 or TNF. IKK activation was also not detected in H_2O_2-treated cells, indicating that H_2O_2-induced NF-κB activation is not mediated by IKK activation.

Discussion

In this study, an oxidative stress elicited by addition of H_2O_2 or diamide was found to inhibit NF-κB reporter gene expression in TNF-stimulated HeLa cells. The inhibitory effect of oxidizing agents was associated with suppression of TNF-induced increase in nuclear κB-binding activity and p65/RelA protein, and degradation of cytosolic IκBα. At similar concentration ranges, H_2O_2 and diamide also blocked IKK activation in TNF-induced HeLa cells and also in LPS-stimulated macrophages. In previous studies, pre-exposure of Jurkat T cells or endothelial cells to H_2O_2 suppressed TNF-induced activation of NF-κB, and expression of NF-κB-dependent genes by these cells (Lahdenpohja *et al*., 1998; Zahler *et al*., 2000). Recently, H_2O_2 was also shown to inhibit IKK activation in transformed alveolar epithelial cells stimulated with TNF or IL-1β (Korn *et al*., 2001). These results indicate that oxidizing agents inhibit NF-κB activation by blocking activation of IKK induced by TNF or other inflammatory stimuli. However, when higher doses of H_2O_2 (3 mM) or diamide (1 mM) were added to the cells, basal NF-κB activity in unstimulated cells as well as that induced by TNF was suppressed. The inhibition did not seem to be caused by cytotoxic effect of high-dose oxidizing agents, because expression of control β-galactosidase was similar to other cells. Although the underlying mode of suppression of basal NF-κB activity is not clear from our result, it is possible that treatment of cells with excess oxidizing agents leads to inactivation of other mediator of NF-κB-dependent gene expression in addition to IKK. In this regard, it was shown that reduced cysteine residue of p50/NFKB1 subunit of NF-κB is required for its binding to DNA (Toledano and Leonard, 1991; Matthews *et al*., 1992; Toledano *et al*., 1993), and expression of an NF-κB target gene (Hayashi *et al*., 1993). Oxidation of this cysteine might be responsible for suppression of basal NF-κB activity in cells treated with high doses of H_2O_2 or diamide.

Our result showed that H_2O_2 and diamide inhibit activation of IKK in both TNF-stimulated HeLa cells and LPS-induced RAW 264.7 macrophages, indicating that oxidizing agents target a common step in both signaling pathways for NF-κB activation. When IKK complex purified from TNF-stimulated HeLa cells was incubated with H_2O_2 and diamide, both agents directly blocked enzyme activity. A similar effect of H_2O_2 was shown in a previous study, employing IKK complex isolated from alveolar epithelial cells (Korn *et al*., 2001). These results suggested that the inhibitory effect of oxidizing agents on IKK induction appears through oxidative inactivation of IKK. In our previous study, *in vitro* IKK activity was shown to require the presence of reducing agent dithiothreitol in the reaction buffer (Jeon *et al*., 2000). Moreover, various thiol-binding metal compounds, such as gold, zinc, copper and arsenite, and thiol-modifying agents, including *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, cyclopentenone prostaglandins, and 4-hydroxy-2-nonenal, commonly blocked *in vitro* IKK activity (Jeon *et al*., 2000; Kapahi *et al*., 2000; Rossi *et al*., 2000; Straus *et al*., 2000; Ji *et al*., 2001). It is not clear whether all of these thiol-oxidizing and thiol-reactive agents inhibit IKK by modifying the same residue in IKK. However, these results indicate that IKK has a cysteine sulfhydryl group, which is critical for enzyme activity and susceptible to oxidative stress or thiol-reactive agents.

In HeLa cells treated with H_2O_2 alone, nuclear κB-binding activity was induced after 2 h and increased expression of NF-κB reporter gene was detected after 10 h. Several groups have reported that H_2O_2 can induce NF-κB activation in HeLa cells (Meyer *et al*., 1993; Wang *et al*., 1998), while it was not confirmed in other study (Li and Karin, 1999), suggesting that other factors such as intracellular redox status is important for H_2O_2-induced NF-κB response. Our result shows that induction of NF-κB occurs at delayed time and the level was much lower compared with that induced by TNF (Figure 5). Moreover, degradation of IκBα and activation of IKK were not detected in H_2O_2-treated cells, while TNF induced both IκBα degradation and IKK activation. In previous studies, H_2O_2-induced NF-κB activation in various cell types including endothelial cells and epithelial cells was also shown to occur in the absence of IκBα degradation (Milligan *et al*., 1998; Canty *et al*., 1999). These results suggest that oxidative stress in HeLa cells activate NF-κB through an alternative pathway independent of IκBα degradation and IKK activation. In previous studies with Jurkat T cells and U937 cells, NF-κB activation after hypoxia-reoxygenation or pervanadate treatment was shown to occur through phosphorylation of IκBα at Tyr-42, instead of phosphorylation at serine residues mediated by IKK (Imbert *et al*., 1996; Mukhopadhyay *et al*., 2000). Recently, H_2O_2-induced NF-κB activation in murine T lymphocytes was also shown to depend on phosphorylation of Tyr-42, but not on serine residues of IκBα (Schoonbroodt *et al*., 2000). These results suggest that NF-κB activation in H_2O_2-treated HeLa cells also rely on Tyr-42 phosphorylation of IκBα. However, we could not detect increase in IκBα tyrosine phosphorylation in H_2O_2-treated HeLa cells, and the signaling pathway for NF-κB activation by oxidizing agents in these cells is not understood (data not shown).

In summary, our results showed a dual effect of oxidative stress on NF-κB activation in HeLa cells. Acute exposure of cells to oxidizing agents inactivates IKK, thus suppressing acute NF-κB activation induced by inflammatory cytokines or bacterial LPS. However,

oxidative stress on its own induces NF-κB activation in delayed time through an IKK-independent mode. These results indicate that ROS produced in various pathologic conditions, such as ischemia-reperfusion and chronic inflammatory diseases, might play a complex role in regulation of NF-κB-dependent gene expression.

Acknowledgements

This work was supported by grants from the Ministry of Health and Welfare, Republic of Korea (02-PJ1-PG3-20908-0023). We thank Dr. T. H. Lee for IgκB-Luc.

References

Adler V, Yin Z, Tew KD, Ronai Z. Role of redox potential and reactive oxygen species in stress signaling. Oncogene 1999;18:6104-11

Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141-79

Barnes PJ, Karin M. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997;336:1066-71

Canty TG Jr, Boyle EM Jr, Farr A, Morgan EN, Verrier ED, Pohlman TH. Oxidative stress induces NF-κB nuclear translocation without degradation of IκBα. Circulation 1999;100: II361-4

Chen ZJ, Parent L, Maniatis T. Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. Cell 1996;84:853-62

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature 1997;388:548-54

Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine, 3rd Ed., Oxford University Press, Oxford

Hayashi T, Ueno Y, Okamoto T. Oxidoreductive regulation of nuclear factor κB. Involvement of a cellular reducing catalyst thioredoxin. J Biol Chem 1993;268:11380-8

Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF. Tyrosine phosphorylation of IκBα-activates NF-κB without proteolytic degradation of IκBα. Cell 1996;86:787-98

Jeon KI., Jeong JY, Jue DM. Thiol-reactive metal compounds inhibit NF-κB activation by blocking IκB kinase. J Immunol 2000;164:5981-9

Jeong JY, Jue DM. Chloroquine inhibits processing of tumor necrosis factor in lipopolysaccharide-stimulated RAW 264.7 macrophages. J Immunol 1997;158:4901-7

Jeong JY, Jue DM. Murine pro-tumor necrosis factor expressed in Saccharomyces cerevisiae HF7c localized to membrane/particulate. Exp Mol Med 2000;32:142-50

Ji C, Kozak KR, Marnett LJ. IκB kinase, a molecular target for inhibition by 4-hydroxy-2-nonenal. J Biol Chem 2001;276: 18223-8

Jin DY, Chae HZ, Rhee SG, Jeang KT. Regulatory role for a novel human thioredoxin peroxidase in NF-κB activation. J Biol Chem 1997;272:30952-61

Kapahi P, Takahashi T, Natoli G, Adams SR, Chen Y, Tsien RY, Karin M. Inhibition of NF-κB activation by arsenite through reaction with a critical cysteine in the activation loop of IκB kinase. J Biol Chem 2000;275:36062-6

Koong AC, Chen EY, Giaccia AJ. Hypoxia causes the activation of nuclear factor κB through the phosphorylation of IκBα on tyrosine residues. Cancer Res 1994;54:1425-30

Korn SH, Wouters EF, Vos N, Janssen-Heininger YM. Cytokine-induced activation of nuclear factor-κB is inhibited by hydrogen peroxide through oxidative inactivation of IκB kinase. J Biol Chem 2001;276:35693-700

Kretz-Remy C, Mehlen P, Mirault ME, Arrigo AP. Inhibition of IκBα-phosphorylation and degradation and subsequent NF-κB activation by glutathione peroxidase overexpression. J Cell Biol 1996; 133:1083-93

Lahdenpohja N, Savinainen K, Hurme M. Pre-exposure to oxidative stress decreases the nuclear factor-κB-dependent transcription in T lymphocytes. J Immunol 1998;160:1354-8

Li N, Karin M. Is NF-κB the sensor of oxidative stress? FASEB J 1999;13:1137-43

Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-B and activated protein-1. J Biol Chem 1998; 273:13245-54

Matthews JR, Botting CH, Panico M, Morris HR, Hay RT. Inhibition of NF-κB DNA binding by nitric oxide. Nucleic Acids Res 1996;24:2236-42

May MJ, Ghosh S. Signal transduction through NF-κB. Immunol Today 1998;19:80-8

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science 1997;278:860-6

Mercurio F, Murray BW, Shevchenko A, Bennett BL, Young DB, Li JW, Pascual G, Motiwala A, Zhu H, Mann M, Manning AM. IκB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Mol Cell Biol 1999;19:1526-38

Meyer M, Schreck R, Baeuerle PA. H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J 1993;12:2005-15

Milligan SA, Owens MW, Grisham MB. Differential regulation of extracellular signal-regulated kinase and nuclear factor-κB signal transduction pathways by hydrogen peroxide and tumor necrosis factor. Arch Biochem Biophys 1998;352:255-62

Mukhopadhyay A, Manna SK, Aggarwal BB. Pervanadate-induced nuclear factor-κB activation requires tyrosine phos-

phorylation and degradation of IκBα. Comparison with tumor necrosis factor-α. J Biol Chem 2000;275:8549-55

Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M. Identification and characterization of an IκB kinase. Cell 1997;90:373-83

Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, Santoro MG. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature 2000; 403:103-8

Rothwarf DM, Zandi E, Natoli G, Karin M. IKK-γ is an essential regulatory subunit of the IκBα kinase complex. Nature 1998;395:297-300

Schoonbroodt S, Ferreira V, Best-Belpomme M, Boelaert JR, Legrand-Poels S, Korner M, Piette J. Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of IκBα in NF-κB activation by an oxidative stress. J Immunol 2000;164:4292-300

Schreck R, Albermann K, Baeuerle PA. Nuclear factor κB: an oxidative stress-responsive transcription factor of eukaryotic cells. Free Radic Res Commun 1992;17:221-37

Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 1991;10:2247-58

Singh S, Darnay BG, Aggarwal BB. Site-specific tyrosine phosphorylation of IκBα negatively regulates its inducible phosphorylation and degradation. J Biol Chem 1996;271: 31049-54

Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, Glass CK. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits multiple steps in the NF-κB signaling pathway. Proc Natl Acad Sci USA 2000;97:4844-9

Toledano MB, Ghosh D, Trinh F, Leonard WJ. N-terminal DNA-binding domains contribute to differential DNA-binding specificities of NF-κB p50 and p65. Mol Cell Biol 1993; 13:852-60

Toledano MB, Leonard WJ. Modulation of transcription factor NF-κB binding activity by oxidation-reduction In vitro. Proc Natl Acad Sci USA 1991;88:4328-32

Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signaling pathways on cell survival. Biochem J 1998;333:291-300

Woronicz JD, Gao X, Cao Z, Rothe M, Goeddel DV. IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. Science 1997;278:866-9

Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, Kirk HE, Kay RJ, Israel A. Complementation cloning of NEMO, a component of the IκB kinase complex essential for NF-κB activation. Cell 1998;93:1231-40

Zahler S, Kupatt C, Becker BF. Endothelial preconditioning by transient oxidative stress reduces inflammatory responses of cultured endothelial cells to TNF-α. FASEB J 2000;14: 555-64

Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IκB kinase complex (IKK) contains two kinase subunits, IKK and IKK, necessary for IκB phosphorylation and NF-κB activation. Cell 1997;91:243-52