EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 44, No. 8, 513-520, August 2012

Transient exposure to hydrogen peroxide inhibits the ubiquitination of phosphorylated IκBα in TNFα-stimulated HEK293 cells

Yeji Lee[1], Jin Choi[1], Kyung-Ho Ha[1,2] and Dae-Myung Jue[1,3]

[1]Department of Biochemistry
College of Medicine
The Catholic University of Korea
Seoul 137-701, Korea
[2]Choongang Vaccine Laboratory
Daejeon 305-348, Korea
[3]Corresponding author: Tel, 82-2-2258-7290;
Fax, 82-2-596-4435; E-mail, dmjue@catholic.ac.kr
http://dx.doi.org/10.3858/emm.2012.44.8.058

Accepted 5 June 2012
Available Online 8 June 2012

Abbreviations: DPBS, Dulbecco's phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HEK, human embryonic kidney; ICAM1, intercellular adhesion molecule-1; IKK, IκB kinase; ROS, reactive oxygen species

Abstract

During ischemia-reperfusion injury, brief pre-exposure to oxidative stress renders organs resistant to subsequent severe damage. NF-κB is a transcription factor that is involved in reperfusion-induced inflammatory and immune responses. The activity of NF-κB has been shown to be modulated by oxidative stress in various cell types through different pathways. We studied the effect of pre-exposure to oxidative stress on subsequent NF-κB activation in TNFα-stimulated HEK293 cells. The cells were transiently exposed to 0.5 mM H_2O_2 for 20 min, prior to stimulation with TNFα, and the subsequent expression of NF-κB-dependent genes and the levels of NF-κB signaling molecules were measured. Pre-exposure to H_2O_2 significantly delayed the TNFα-induced expression of an NF-κB reporter gene and inflammatory proteins (intercellular adhesion molecule-1 and IL-1β). The degradation of inhibitor of NF-κB α (IκBα) and the nuclear translocation of NF-κB were also delayed by H_2O_2 treatment, whereas IκBα phosphorylation and IκB kinase activity were not changed. When we examined the ubiquitin/proteosome pathway in H_2O_2-treated cells, we could not detect significant changes in proteosomal peptidase activities, but we were able to detect a delay of IκBα poly-ubiquitination. Our results suggest that transient exposure to oxidative stress temporally inhibits NF-κB-dependent gene expression by suppressing the poly-ubiquitination of phosphorylated IκBα in HEK293 cells.

Keywords: hydrogen peroxide; inflammation; NF-κB; proteasome endopeptidase complex; reactive oxygen species; reperfusion injury; ubiquitin

Introduction

The restoration of the blood supply to an ischemic organ and its subsequent reoxygenation is frequently associated with tissue injury and severe inflammatory response, called reperfusion injury. Reperfusion injury is involved in many diseases such as myocardial infarction, ischemic stroke, acute kidney injury, trauma, circulatory arrest, sickle cell disease and sleep apnea (Eltzschig and Eckle, 2011). In these diseases, the reactive oxygen species (ROS) produced by the reperfused tissues or invading immune cells induce tissue damage, which in turn promotes uncontrolled inflammation and tissue injury by inducing the accumulation and activation of inflammatory and immune cells (Iadecola and Anrather, 2011).

Nuclear factor-κB (NF-κB) transcription factors play a pivotal role in the regulation of cell survival, immune cell maturation and inflammation in various cell types (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). NF-κB activation during the ischemic and inflammatory stages of reperfusion injury has been implicated in both the prevention of tissue damage and the exacerbation of inflammation and immune reactions, depending on the tissue type and the timing of its activation (Van der Heiden *et al.*,

2010; Gordon *et al*., 2011). In unstimulated cells, NF-κB is associated with an inhibitory IκB protein, which inhibits the nuclear localization and thus DNA binding activity of NF-κB. In response to stimuli, including proinflammatory cytokines such as TNFα and IL-1β and endogenous ligands, such as high-mobility group box 1 protein and RNA released upon tissue damage, the IκBs are phosphorylated at Ser-32 and Ser-36 by IκB kinase (IKK); this is one of the major steps of regulation in the NF-κB signaling pathway (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). The phosphorylation of IκB induces its ubiquitination by the E3 ligase βTrCP ($E3RS^{I\kappa B}$) and subsequent degradation by the 26S proteasome, releasing NF-κB, which then enters the nucleus and binds DNA to induce the expression of specific target genes (Ciechanover, 1998; Karin and Ben-Neriah, 2000).

Previous studies have shown that ROS-induced oxidative stress plays various inhibitory or stimulatory roles in NF-κB signaling (Morgan and Liu, 2011). It has been suggested that oxidative stress induces NF-κB activation in that hydrogen peroxide (H_2O_2) can induce NF-κB activation in lymphocytes and monocytes, and various antioxidants inhibit NF-κB activation in cells stimulated with TNFα, IL-1, LPS and phorbol esters (Schreck *et al*., 1991, 1992). Early studies implicated the H_2O_2-induced phosphorylation of Tyr-42 on IκBα instead of IKK-mediated Ser-32/36 phosphorylation in NF-κB activation, whereas recent reports suggested that H_2O_2 also activates NF-κB by modulating IKK activity (reviewed by Gloire *et al*., 2006; Morgan and Liu, 2011). Although these results suggest that a shift in the cellular redox equilibrium to an oxidized state promotes NF-κB activation, it was also shown that exposure to H_2O_2 and other oxidizing agents suppressed TNFα-induced NF-κB activation in various cell types by inactivating upstream kinase IKK (Korn *et al*., 2001; Byun *et al*., 2002; Levrand *et al*., 2005; Loukili *et al*., 2010). This inhibitory effect was suggested to occur through the oxidation of Cys-179 within the β subunit of IKK (Reynaert *et al*., 2004, 2006). ROS are also known to directly modify NF-κB subunits p50 and p65, inhibiting their DNA-binding abilities (Toledano *et al*., 1993). Moreover, it has been reported that the sustained exposure of lens epithelial cells to H_2O_2 inhibited NF-κB activation by inhibiting the proteasomal peptidase and thus stabilizing IκBα (Wu *et al*., 2009).

In a previous study transient oxidative stress was used to test the protective effect of ischemic preconditioning (Zahler *et al*., 2000). Thus, we investigated the effect of transient oxidative stress on NF-κB activation in human embryonic kidney (HEK) 293 cells stimulated with TNFα. Pre-exposure to H_2O_2 inhibited TNFα-induced NF-κB activation by delaying the degradation of IκBα, but it did not inhibit TNFα-induced IκBα phosphorylation and IKK activation. Examination of the ubiquitin-proteasome pathway revealed that H_2O_2 inhibits the poly-ubiquitination of IκBα, whereas proteasomal peptidase activities were not changed, indicating that transient oxidative stress temporally inhibits NF-κB activation by blocking IκBα ubiquitination in TNFα-stimulated HEK293 cells.

Results

Transient H_2O_2 delays TNFα-induced NF-κB activation

To determine the effect of transient oxidative stress on TNFα-induced NF-κB activity, HEK293 cells were incubated with H_2O_2 for 20 min. Then, the medium was exchanged with medium containing TNFα, and NF-κB-dependent gene expression was measured and calibrated with β-galactosidase expressed under the β-actin promoter (Figure 1). TNFα induced a 6.5-fold increase of NF-κB reporter expression after 4 h, whereas the pre-incubation of cells with 0.1 mM H_2O_2 resulted in an approximately 50% decrease in reporter expression. No further decreases were observed at higher doses of H_2O_2 (Figure 1A). Cell viability was decreased by approximately 25% in cells pre-exposed to H_2O_2 after 4 h of TNFα stimulation, and it was further decreased to approximately 50% after 24 h (Figure S1). To understand the time-dependent effects of H_2O_2 on NF-κB activation, we measured reporter expression at various time points after the addition of TNFα to cells pretreated with 0.5 mM H_2O_2 (Figure 1B). The suppressive effect of H_2O_2 on TNFα-induced NF-κB activation appeared at up to 4 h of TNFα stimulation, and inhibition was no longer significant after 8 h of TNFα stimulation. When we measured the mRNAs of inflammatory proteins, IL-1β and intercellular adhesion molecule-1 (ICAM1), which are known NF-κB transcriptional targets (Karin and Ben-Neriah, 2000; Van der Heiden *et al*., 2010), we observed similar delays in expression induced by pre-exposure to H_2O_2 (Figure 1C). Interestingly, while the suppression of IL-1β expression by H_2O_2 was fully recovered after 4 h, ICAM1 expression remained reduced compared to that in control cells even after 12 h of TNFα treatment. Overall, these results suggested that the transient exposure of cells to oxidative stress temporally suppresses NF-κB activation and NF-κB-dependent gene expression.

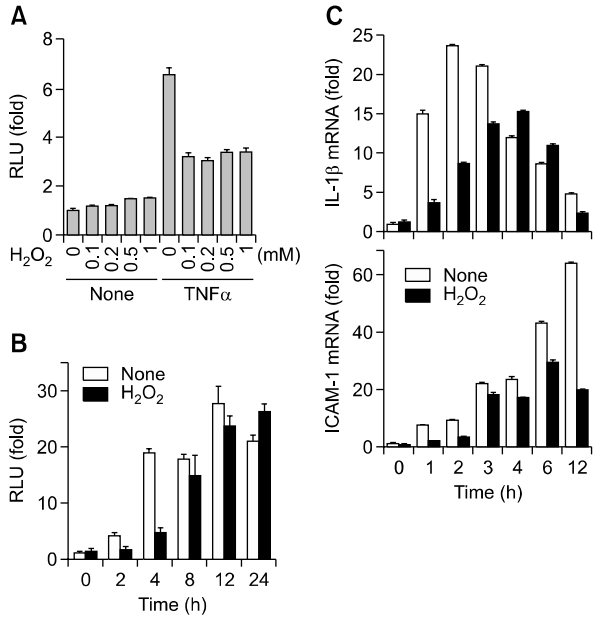

Figure 1. The transient inhibition of TNFα-induced NF-κB activation by H_2O_2. (A) HEK293 cells were transfected with an NF-κB reporter construct (IgκB-Luc) together with a control expression vector (β-actin/β-galactosidase) and incubated for 2 days. The cells were incubated with various doses of H_2O_2 in for 20 min in DPBS/D-glucose, washed and stimulated with TNFα (20 ng/ml) in DMEM/10% FBS for 4 h. The luciferase activity in the cell lysate was determined by luminometry and normalized to the β-gal activity (n = 3). (B) The cells were incubated with or without H_2O_2 (0.5 mM) for 20 min and then stimulated with TNFα for various times, and the luciferase activity was measured (n = 3). (C) The cells were treated as in (B), and cellular total RNA was isolated. cDNA was synthesized using MMLV reverse transcriptase, and quantitative real-time PCR was performed with primer and probe sets for IL-1β (upper panel) and ICAM-1 (lower panel) to calculate the mRNA level, which was then normalized to GAPDH (n = 3).

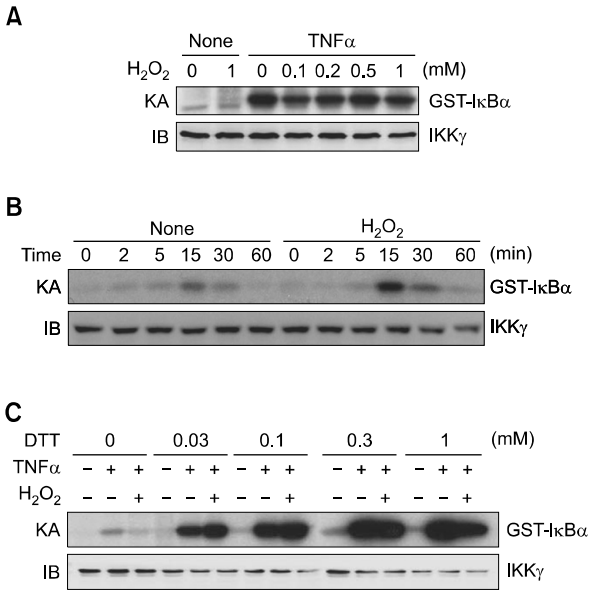

Figure 2. IKK activity was not obviously inhibited by H_2O_2. (A) HEK293 cells were exposed to various doses of H_2O_2 for 20 min, washed and stimulated with TNFα (20 ng/ml) for 10 min. IKK complexes in the cell lysate were immunoprecipitated with anti-IKKγ antibody. An *in vitro* kinase assay (KA) was carried out using [γ-^{32}P]ATP and GST-IκBα(1-54) as substrates. The IKKγ in the kinase assay mixture was measured by immunoblot (IB) analysis. (B) The cells were exposed to H_2O_2 (0.5 mM) for 20 min and stimulated with TNFα for various times. IKK activity was determined as in (A). (C) The cells were exposed to H_2O_2 (0.5 mM) for 20 min and stimulated with TNFα for 10 min. The cells were lysed in cell lysis buffer containing various concentrations of DTT. Immunoprecipitation and *in vitro* kinase assays were performed as in (A) using buffers containing the indicated concentrations of DTT.

H_2O_2 does not inhibit IκBα phosphorylation but delays NF-κB nuclear localization

Previous studies have shown that the IKK complex, which catalyzes the signal-induced phosphorylation of IκBα on specific serine residues, is susceptible to inactivation by ROS and reactive nitrogen species (Korn *et al*., 2001; Byun *et al*., 2002; Reynaert *et al*., 2004; Levrand *et al*., 2005; Loukili *et al*., 2010). To determine whether transient exposure to H_2O_2 inhibited NF-κB activation by blocking IKK activity, the IKK complex was isolated from HEK293 cells that had been sequentially treated with H_2O_2 and TNFα, and its kinase activity was measured in a reaction mixture containing [γ-^{32}P]ATP and GST- IκBα (Figure 2). Our results showed that TNFα-induced IKK activity was not inhibited by the transient exposure of cells to up to 1 mM H_2O_2 (Figure 2A). The time course for the activation of IKK by TNFα stimulation in cells pre-exposed to H_2O_2 was also not different from that of non-treated control cells, and the IKK kinase activity at 15 min was somewhat elevated by H_2O_2 pre-treatment (Figure 2B). Because a previous study showed that the inhibitory effect of ROS on IKK activity depended on the concentration of the reducing agent in the enzyme reaction mixture (Korn *et al*., 2001), we lysed the cells, immunoprecipitated IKK complex, and determined its kinase activity in buffers containing different concentrations of dithiothreitol (DTT) (Figure 2C). Although an H_2O_2-induced decrease in kinase activity was observed in IKK prepared in buffers without added DTT, even 0.03 mM DTT was sufficient to recover IKK activity in cells pre-treated with H_2O_2 to the level of activity in untreated control cells.

To elucidate the cause of the reduced NF-κB activity in cells pre-exposed to H_2O_2, we then determined the levels of proteins involved in the NF-κB signaling pathway and their modifications (Figure 3). Immunoblotting analysis of cytosolic IκBα revealed that its degradation upon TNFα stimulation was blocked by pre-exposure to H_2O_2 (Figure 3A). The detection of IκBα phosphorylated at Ser-32 and Ser-36 using a phospho-specific antibody revealed

that TNFα-induced IκBα phosphorylation was not inhibited by pre-exposure to H_2O_2. Consistent with the H_2O_2-induced inhibition of IκBα degradation, an increase in the nuclear level of NF-κB subunit p65 was inhibited by pre-exposure to H_2O_2. When we measured time-dependent changes in NF-κB signaling proteins after TNFα stimulation, pre-exposure of cells to H_2O_2 significantly delayed IκBα degradation, whereas IκBα phosphorylation was not changed in the same cells (Figure 3B). The increase of nuclear p65 was delayed, again reflecting the delayed degradation of IκBα. Similarly, electrophoretic mobility assays (EMSA) of nuclear extract prepared from cells pre-exposed to H_2O_2 showed a delay in the appearance of κB-sequence binding activity compared with non-exposed TNFα-stimulated control cells (Figure 3C).

H_2O_2 inhibits the poly-ubiquitination of phosphorylated IκBα

Our results suggested that transient exposure to H_2O_2 inhibits the TNFα-induced activation of NF-κB in HEK293 cells by blocking the degradation of phosphorylated IκBα. Because the signal-induced degradation of IκBα is known to occur through the ubiquitin-proteasome pathway, we measured proteasomal enzyme activity and IκBα ubiquitination in cells pre-exposed to H_2O_2 (Figure 4). The proteasomal chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase activities in the cell extract were determined using specific fluorogenic substrates. As shown in Figure 4A, the incubation of cells with 0.5 mM H_2O_2 did not significantly change the activities of the three proteasomal peptidases evaluated. Both chymotrypsin-like and peptidylglutamyl peptide hydrolase activities were decreased by approximately 30% after 15 min of H_2O_2 treatment, but these differences were not statistically significant ($P > 0.05$ by Student's *t*-test). We then monitored the ubiquitination of IκBα by immunoblotting analysis (Figures 4B and 4C). The stimulation of cells with TNFα in the absence of the proteasomal inhibitor MG-132 induced the time-dependent degradation of IκBα. However, when the cells were stimulated with TNFα in the presence of MG-132, high-molecular-weight poly-ubiquitinated IκBα appeared after 5 min and remained undegraded. When the cells were pre-exposed to H_2O_2 and stimulated with TNFα in the presence of MG-132, the poly-ubiquitination of IκBα was obvious only after 30 min. Immunoblotting analysis with anti-ubiquitin antibody revealed that the cellular level of total ubiquitinated proteins was largely unchanged after TNFα stimulation. In contrast, pre-exposure to H_2O_2 resulted in an approximately

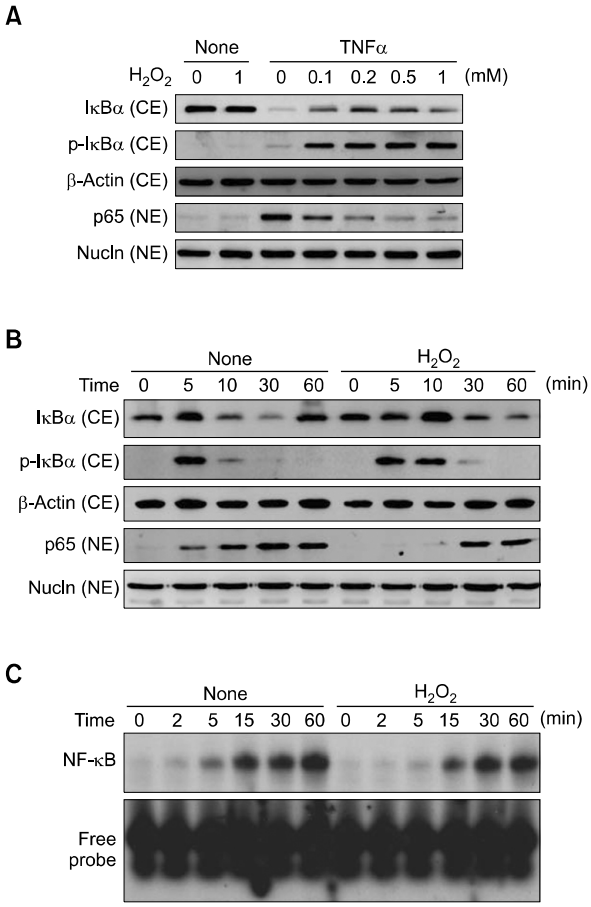

Figure 3. The effect of H_2O_2 on the TNFα-induced phosphorylation and degradation of IκBα, and the nuclear translocation of p65. (A) HEK293 cells were treated with various doses of H_2O_2 for 20 min and stimulated with TNFα (20 ng/ml) for 10 min. The levels of IκBα and phosphorylated IκBα (p-IκBα) in the cytoplasmic extract (CE) and p65 in the nuclear extract (NE) were determined by immunoblotting analysis using specific antibodies. β-Actin and nucleoporin (Nucln) were used as control proteins for the cytoplasmic and nuclear extracts, respectively. (B) The cells were exposed to H_2O_2 (0.5 mM) for 20 min and stimulated with TNFα for various times. The changes in the levels of proteins in the cytoplasmic and nuclear extracts were determined by immunoblotting analysis as described in (A). (C) Nuclear extracts prepared from cells treated as in (B) were used to determine NF-κB-DNA binding activity by EMSA. The upper panel shows the retarded NF-κB band, and the lower panel shows unbound free probe.

20% decrease in total protein ubiquitination, which was not recovered in cells incubated for 1 h in the absence of H_2O_2.

Discussion

In this study, the transient oxidative stress elicited by exposing cells to H_2O_2 was found to temporally inhibit NF-κB reporter expression in TNFα-stimulated HEK293 cells. The inhibitory effect of H_2O_2 appeared

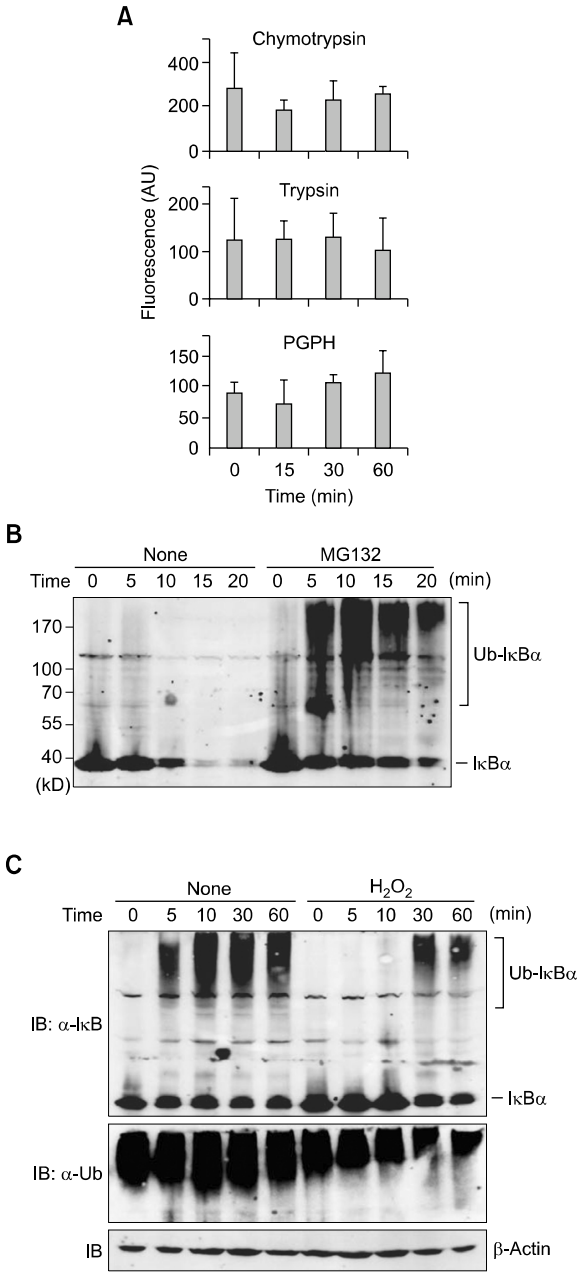

Figure 4. The transient inhibition of IκBα poly-ubiquitination by H_2O_2. (A) HEK293 cells were incubated with H_2O_2 (0.5 mM) for various times, and the cells were lysed in hypotonic buffer to prepare cell extract. Chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase (PGPH) peptidase activities were measured using fluorogenic peptide substrates (n = 3). (B) HEK293 cells were incubated with or without MG-132 (20 μM) for 1 h, and stimulated with TNFα (20 ng/ml) for up to 20 min. The cells were lysed, and immunoblotting analysis was performed using the anti-IκBα antibody. (C) Cells were incubated with MG-132 for 1 h, and a group of cells was exposed to 0.5 mM H_2O_2 for 20 min. After stimulation with TNFα, cells were lysed at various times. The cell lysate was analyzed by immunoblotting using anti-IκBα (upper panel), anti-ubiquitin (middle panel) and anti-β-actin (lower panel) antibodies.

even at low concentrations (0.1 mM) of H_2O_2 (Figure 1A), suggesting that the NF-κB signaling pathway responds sensitively to changes in redox state induced by various pathologic conditions. Pre-exposure to H_2O_2 inhibited the expression of an NF-κB-reporter genes and IL-1β and ICAM-1 in response to TNFα, but their expression was recovered to the level of non-treated control cells after a lag period (Figures 1B and 1C). The differences in the time required to reach maximal expression between H_2O_2-treated and non-treated cells were approximately 4 h for the luciferase reporter and approximately 2 h for IL-1β and ICAM-1 mRNA. These results suggest that the inhibitory effect of transient oxidative stress on NF-κB activation is temporary and that the NF-κB signaling molecule that was modified by ROS recovers its original activity *via* cellular anti-oxidants and anti-oxidant enzymes.

Previous studies, including one from our research group, have shown that IKK in various cell types is susceptible to inactivation by ROS such as H_2O_2, nitric oxide (NO) and peroxynitrite (Korn *et al*., 2001; Byun *et al*., 2002; Reynaert *et al*., 2004; Levrand *et al*., 2005; Loukili *et al*., 2010). It was suggested that the ROS-induced inhibition of IKK is caused by the oxidative modification of Cys-179 of the IKKβ subunit of the IKK complex (Reynaert *et al*., 2004, 2006), which is located in the activation loop of IKKβ and plays a crucial role in the regulation of enzyme activity and signal-induced enzyme activation (Byun *et al*., 2006). Interestingly, when we measured the *in vitro* activity of IKK complexes prepared from HEK293 cells transiently exposed to H_2O_2, we could not detect a significant change in its activity compared with that of non-treated control cells (Figure 2A). Our results showed that the IKK activity of H_2O_2-pre-exposed cells at 15 min after TNFα stimulation was elevated compared to that of non-treated control cells (Figure 2B). Recently, Loukili *et al*. (2010) showed that oxidants induce the hyperactivation of IKK by blocking protein phosphatase 2A, thus stabilizing active phosphorylated IKK, possibly explaining our result. Because the *in vitro* IKK assay is performed in the presence of exogenously added reducing agents such as DTT, it is possible that the oxidized IKK in the cell is re-reduced during enzyme isolation and assays, and thus not representative of actual intracellular IKK activity. Indeed, we observed H_2O_2-induced decrease of enzyme activity in IKK prepared in the absence of DTT (Figure 2C), although this also does not reflect its actual intracellular activity. In our immunoblot analysis of IκBα phosphorylated on Ser-32 and Ser-36, pre-exposing cells to H_2O_2 did not significantly change the level and timing of IκBα

phosphorylation (Figures 3A and 3B), suggesting that IKK activity is not modulated by H_2O_2 pre-exposure in these cells.

Whereas the phosphorylation of IκBα was not changed by pre-exposure of cells to H_2O_2, the TNFα-induced degradation of IκBα was delayed (Figures 3A and 3B). In the same cells, increases in nuclear NF-κB p65 and κB-binding activity were delayed by H_2O_2 treatment *via* similar modes (Figures 3B and 3C). These results indicated that the effect of H_2O_2 was mediated through blocking the degradation of phosphorylated IκBα, whereas the DNA-binding ability of NF-κB was not changed by H_2O_2. Because the degradation of phosphorylated IκBα occurs *via* the ubiquitin-proteasome pathway (Ciechanover, 1998; Karin and Ben-Neriah, 2000), we examined proteasomal peptidase activities and the ubiquitination of IκBα in H_2O_2-treated cells. Our results showed that although proteasomal peptidase activities were not changed, the TNFα-induced poly-ubiquitination of IκBα was delayed by H_2O_2 pre-exposure for a time period (10-30 min) similar to the delay in IκBα degradation and p65 nuclear translocation (Figures 3 and 4). These results clearly indicated that the delay of NF-κB activation that occurs in cells exposed to H_2O_2 is caused by the inhibition of IκBα poly-ubiquitination and suggested two different possible modes for the inhibition of IκBα poly-ubiquitination by H_2O_2: the transient modification of IκBα or the transient inhibition of IκBα ubiquitinating enzyme(s). In a previous study, exposure of cells to various nitrosative stresses induced S-nitrosylation of the Bcl-2 protein, leading to its decreased ubiquitination and degradation *via* ubiquitin-proteasome pathway, which in turn inhibited apoptosis (Azad *et al*., 2010). In contrast, NO-induced S-nitrosylation modulates the ubiquitin E3 ligase activity of parkin, contributing to the accumulation of neurotoxic protein aggregates within neurons and glial cells in Parkinson's disease and other neurodegenerative disorders (Chung *et al*., 2004; Yao *et al*., 2004). Although our results do not conclusively determine which mode is responsible for the H_2O_2-induced inhibition of IκBα ubiquitination, these results suggested that it was caused by a similar modification of IκBα or IκBα ubiquitinating enzyme(s).

The results shown in Figure 4C suggest that the inhibitory effect of H_2O_2 on IκBα ubiquitination is specific and not due to a general inhibition of protein ubiquitination. IκBα poly-ubiquitination was blocked by H_2O_2 and then slowly recovered to normal levels over a 30 min period; in the same cells, the level of total ubiquitinated proteins was not changed by H_2O_2 pre-exposure and slowly decreased thereafter. These results suggest that H_2O_2 acts *via* two different modes: immediate and sensitive inhibition for IκBα ubiquitination, and slow and partial inhibition for general protein ubiquitination.

Ischemic preconditioning is a strategy in which brief, transient episodes of ischemia attenuate tissue injury during subsequent ischemia and reperfusion (Eltzschig and Eckle, 2011). Previous studies have revealed that the oxygen-dependent induction of hypoxia-inducible factor-1 and the resulting adenosine receptor signaling play a critical role in ischemic preconditioning (Eckle *et al*., 2007, 2008). As an *in vitro* model to test the role of oxidative stress that occurs during preconditioning, Zahler *et al*. (2000) transiently exposed endothelial cells to 1 mM H_2O_2 for 5 min. They observed that the TNFα-induced expression of cell-adhesion molecules and inflammatory cytokines was inhibited by this pre-exposure to H_2O_2 and suggested that this phenomenon occurred due to the inhibition of NF-κB activity. Our results showed that transient pre-exposure to H_2O_2 inhibits TNFα-induced NF-κB activation by temporarily blocking the ubiquitination of phosphorylated IκBα, whereas IKK activity and IκBα phosphorylation remain largely intact in the same cells. These results suggest that modulation of IκBα ubiquitination by ROS plays a critical role in regulating the NF-κB-dependent expression of inflammatory and immune genes in reperfusion injury and other acute and chronic inflammatory diseases.

Methods

Cells and reagents

HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA), and maintained in DMEM supplemented with 10% heat-inactivated FBS, and antibiotics. Antibodies against IKKγ, p65/RelA, IκBα, and ubiquitin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-Ser-32 and phospho-Ser-36 IκBα, β-actin, and nucleoporin were obtained from Cell Signaling Technology (Beverly, MA), Sigma (St. Louis, MO), and BD Biosciences (San Jose, CA), respectively. Recombinant glutathione S-transferase (GST)-IκBα containing the N-terminal 54 residues of IκBα and recombinant human TNFα were expressed in *Escherichia coli* as described previously (Ha *et al*., 2009). Hydrogen peroxide was purchased from Junsei Chemical (Tokyo, Japan) and diluted fresh in Dulbecco's phosphate-buffered saline (DPBS) containing 5 mM D-glucose prior to use. The fluorogenic substrates for proteasomal peptidase and MG-132 were obtained from Sigma.

IKK assay and NF-κB reporter assay

Cell extract preparation and anti-IKKγ immunoprecipitation were performed as previously described (Byun *et al*.,

2006). Kinase activity was measured in reaction mixtures containing 10 μM ATP, [γ-^{32}P]ATP (3 μCi) and GST-IκBα (2 μg) (Ha *et al*., 2009). The reaction products were analyzed by SDS-PAGE on a 10% gel and electrophoretically transferred to a PVDF membrane. Phosphorylated GST-IκBα was visualized by autoradiography and quantitated in a phosphor image analyzer (Fujifilm, Tokyo, Japan). The NF-κB reporter gene assay was performed in HEK293 cells transfected with IgκB-Luc, as described previously (Byun *et al*., 2002). A β-Actin promoter-driven β-galactosidase expression plasmid was used for the normalization of luciferase activity.

Immunoblotting analysis

The proteins in the cell extracts were analyzed by immunoblotting and detected using the WEST-one detection system (Intron Biotechnology, Seongnam, Korea) according to the recommended procedure. For the analysis of ubiquitinated proteins, cells grown on a 6-well plate were rinsed with DPBS and lysed in 60 μl of 1× SDS sample buffer. The lysed cells were scraped off the plate, transferred to a microtube, and boiled for 5 min. The cell lysate was cleared by centrifugation at 10,000 *g* for 2 min, and 30 μl of the lysate was analyzed *via* SDS-PAGE on an 8% gel and subsequent immunoblotting.

Electrophoretic mobility shift assay

Nuclear and cytoplasmic extracts were prepared from HEK293 cells as described previously (Byun *et al*., 2006). An oligonucleotide containing the consensus recognition sequence was obtained from Santa Cruz Biotechnology and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The binding reaction was performed with 10 μg of nuclear extract and the reaction products were analyzed by electrophoresis on a 6% polyacrylamide gel in 0.5× TBE buffer (45 mM Tris-HCl, pH 8.5, 45 mM borate and 1 mM EDTA). The gel was dried under a vacuum, and radioactive bands were detected by autoradiography.

Analysis of mRNA

Total cellular RNA was isolated using an RNA isolation kit (RNA Stat-60, amsbio, Abingdon, UK) and cDNA was synthesized using M-MLV reverse transcriptase (Promega, Seoul, Korea). IL-1β, ICAM-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were measured by quantitative RT-PCR using a kit (qPCR Mix, QARTA Bio, Fremont, CA) and a real-time PCR machine (Applied Biosystems 7300). The PCR primers and fluorescent probes used are presented in Table S1.

Assay of proteasome activity and cell viability

The peptidase activities of three proteasome components were measured using a fluorogenic substrate, as described previously (Wu *et al*., 2009). Cells were lysed in 25 mM Tris-HCl buffer (pH 7.6) containing 1 mM DTT, and the lysate was cleared by centrifugation at 15,000 *g* for 10 min. The cell extract was incubated with succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC), N-t-butyloxycarbonyl-Leu-Ser-Thr-Arg-amidomethylcoumarin (LSTR-AMC), or benzyloxycarbonyl-Leu-Leu-Glu-amidomethylcoumarin (LLE-AMC) to determine chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase activity, respectively. Each assay mixture contained 10 μg protein of cell extract, and enzyme activity was measured at 25°C in a temperature-controlled microplate fluorometric reader (Victor 3, PerkinElmer, Waltham, MA) using excitation/emission wavelengths of 380/440 nm. Cell viability was measured using a kit (Cell Counting Kit-8, Dojindo Lab, Rockville, MD) according to the manufacturer's recommendations.

Supplemental data

Supplemental data include a figure and a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-8-06.pdf.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology of the Republic of Korea (NRF2011-0024179).

References

Azad N, Iyer A, Vallyathan V, Wang L, Castranova V, Stehlik C, Rojanasakul Y. Role of oxidative/nitrosative stress-mediated Bcl-2 regulation in apoptosis and malignant transformation. Ann N Y Acad Sci 2010;1203:1-6

Byun MS, Jeon KI, Choi JW, Shim JY, Jue DM. Dual effect of oxidative stress on NF-κB activation in HeLa cells. Exp Mol Med 2002;34:332-9

Byun MS, Choi J, Jue DM. Cysteine-179 of IκB kinase β plays a critical role in enzyme activation by promoting phosphorylation of activation loop serines. Exp Mol Med 2006;38:546-52

Chung KK, Thomas B, Li X, Pletnikova O, Troncoso JC, Marsh L, Dawson VL, Dawson TM. S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. Science 2004;304:1328-31

Ciechanover A. The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J 1998;17:7151-60

Eckle T, Krahn T, Grenz A, Kohler D, Mittelbronn M, Ledent C, Jacobson MA, Osswald H, Thompson LF, Unertl K, Eltzsching HK. Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptor. Circulation 2007;115:1581-90

Eckle T, Kohler D, Lehmann R, El Kasmi KC, Eltzsching HK. Hypoxia-inducible factor-1 is central to cardioprotection: a new paradigm for ischemic preconditioning. Circulation 2008;118:166-75

Eltzschig HK, Eckle T. Ischemia and reperfusion--from mechanism to translation. Nat Med 2011;17:1391-401

Gloire G, Legrand-Poels S, Piette J. NF-κB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 2006;72:1493-505

Gordon JW, Shaw JA, Kirshenbaum LA. Multiple facets of NF-κB in the heart: to be or not to NF-κB. Circ Res 2011;108: 1122-32

Ha KH, Byun MS, Choi J, Jeong J, Lee KJ, Jue DM. N-Tosyl-L-phenylalanine chloromethyl ketone inhibits NF-κB activation by blocking specific cysteine residues of IκB kinase β and p65/RelA. Biochemistry 2009;48:7271-8

Hayden MS, Ghosh S. Shared principles in NF-κB signaling. Cell 2008;132:344-62

Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med 2011;17:796-808

Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu Rev Immunol 2000;18: 621-63

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

Loukili N, Rosenblatt-Velin N, Rolli J, Levrand S, Feihl F, Waeber B, Pacher P, Liaudet L. Oxidants positively or negatively regulate nuclear factor κB in a context-dependent manner. J Biol Chem 2010;285:15746-52

Levrand S, Pesse B, Feihl F, Waeber B, Pacher P, Rolli J, Schaller MD, Liaudet L. Peroxynitrite is a potent inhibitor of NF-κB activation triggered by inflammatory stimuli in cardiac and endothelial cell lines. J Biol Chem 2005;280:34878-87

Morgan MJ, Liu ZG. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res 2011;21:103-15

Reynaert NL, Ckless K, Korn SH, Vos N, Guala AS, Wouters EF, van der Vliet A, Janssen-Heininger YM. Nitric oxide represses inhibitory κB kinase through S-nitrosylation. Proc Natl Acad Sci USA 2004;101:8945-50

Reynaert NL, van der Vliet A, Guala AS, McGovern T, Hristova M, Pantano C, Heintz NH, Heim J, Ho YS, Matthews DE, Wouters EF, Janssen-Heininger YM. Dynamic redox control of NF-κB through glutaredoxin-regulated S-glutathionylation of inhibitory κB kinase β. Proc Natl Acad Sci USA 2006;103:13086-91

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

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

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

Vallabhapurapu S, Karin M. Regulation and function of NF-κB transcription factors in the immune system. Annu Rev Immunol 2009;27:693-733

Van der Heiden K, Cuhlmann S, Luong le A, Zakkar M, Evans PC. Role of nuclear factor κB in cardiovascular health and disease. Clin Sci (Lond) 2010;118:593-605

Wu M, Bian Q, Liu Y, Fernandes AF, Taylor A, Pereira P, Shang F. Sustained oxidative stress inhibits NF-κB activation partially *via* inactivating the proteasome. Free Radic Biol Med 2009;46:62-9

Yao D, Gu Z, Nakamura T, Shi ZQ, Ma Y, Gaston B, Palmer LA, Rockenstein EM, Zhang Z, Masliah E, Uehara T, Lipton SA. Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. Proc Natl Acad Sci USA 2004;101:10810-4

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