

Transient exposure to hydrogen peroxide inhibits the ubiquitination of phosphorylated I κ B α in TNF α -stimulated HEK293 cells

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<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, I κ B kinase; ROS, reactive oxygen species

Abstract

During ischemia-reperfusion injury, brief pre-exposure to oxidative stress renders organs resistant to subsequent severe damage. NF- κ B is a transcription factor that is involved in reperfusion-induced inflammatory and immune responses. The activity of NF- κ B 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- κ B activation in TNF α -stimulated HEK293 cells. The cells were transiently exposed to 0.5 mM H₂O₂ for 20 min, prior to stimulation with TNF α , and the subsequent expression of NF- κ B dependent genes and the levels of NF- κ B signaling molecules were measured. Pre-exposure to H₂O₂ significantly delayed the TNF α -induced expression of an NF- κ B reporter gene and inflammatory proteins (intercellular adhesion molecule-1 and IL-1 β). The degradation of inhibitor of NF- κ B α (I κ B α) and the nuclear

translocation of NF- κ B were also delayed by H₂O₂ treatment, whereas I κ B α phosphorylation and I κ B kinase activity were not changed. When we examined the ubiquitin/proteasome pathway in H₂O₂-treated cells, we could not detect significant changes in proteosomal peptidase activities, but we were able to detect a delay of I κ B α poly-ubiquitination. Our results suggest that transient exposure to oxidative stress temporally inhibits NF- κ B-dependent gene expression by suppressing the poly-ubiquitination of phosphorylated I κ B α in HEK293 cells.

Keywords: hydrogen peroxide; inflammation; NF- κ B; 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- κ B (NF- κ B) 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- κ B 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- κ B is associated with an inhibitory I κ B protein, which inhibits the nuclear localization and thus DNA binding activity of NF- κ B. In response to stimuli, including proinflammatory cytokines such as TNF α and IL-1 β and endogenous ligands, such as high-mobility group box 1 protein and RNA released upon tissue damage, the I κ Bs are phosphorylated at Ser-32 and Ser-36 by I κ B kinase (IKK); this is one of the major steps of regulation in the NF- κ B signaling pathway (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). The phosphorylation of I κ B induces its ubiquitination by the E3 ligase β TrCP (E3RS^{I κ B}) and subsequent degradation by the 26S proteasome, releasing NF- κ B, 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- κ B signaling (Morgan and Liu, 2011). It has been suggested that oxidative stress induces NF- κ B activation in that hydrogen peroxide (H₂O₂) can induce NF- κ B activation in lymphocytes and monocytes, and various antioxidants inhibit NF- κ B activation in cells stimulated with TNF α , IL-1, LPS and phorbol esters (Schreck *et al.*, 1991, 1992). Early studies implicated the H₂O₂-induced phosphorylation of Tyr-42 on I κ B α instead of IKK-mediated Ser-32/36 phosphorylation in NF- κ B activation, whereas recent reports suggested that H₂O₂ also activates NF- κ B 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- κ B activation, it was also shown that exposure to H₂O₂ and other oxidizing agents suppressed TNF α -induced NF- κ B activation in various cell types by inactivating upstream kinase IKK (Korn *et al.*, 2001; Byun *et al.*, 2002; Levrant *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- κ B 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 H₂O₂ inhibited NF- κ B activation by inhibiting the proteasomal peptidase and thus stabilizing I κ B α (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- κ B activation in human embryonic kidney (HEK) 293 cells stimulated with TNF α . Pre-exposure

to H₂O₂ inhibited TNF α -induced NF- κ B activation by delaying the degradation of I κ B α , but it did not inhibit TNF α -induced I κ B α phosphorylation and IKK activation. Examination of the ubiquitin-proteasome pathway revealed that H₂O₂ inhibits the poly-ubiquitination of I κ B α , whereas proteasomal peptidase activities were not changed, indicating that transient oxidative stress temporally inhibits NF- κ B activation by blocking I κ B α ubiquitination in TNF α -stimulated HEK293 cells.

Results

Transient H₂O₂ delays TNF α -induced NF- κ B activation

To determine the effect of transient oxidative stress on TNF α -induced NF- κ B activity, HEK293 cells were incubated with H₂O₂ for 20 min. Then, the medium was exchanged with medium containing TNF α , and NF- κ B-dependent gene expression was measured and calibrated with β -galactosidase expressed under the β -actin promoter (Figure 1). TNF α induced a 6.5-fold increase of NF- κ B reporter expression after 4 h, whereas the pre-incubation of cells with 0.1 mM H₂O₂ resulted in an approximately 50% decrease in reporter expression. No further decreases were observed at higher doses of H₂O₂ (Figure 1A). Cell viability was decreased by approximately 25% in cells pre-exposed to H₂O₂ after 4 h of TNF α stimulation, and it was further decreased to approximately 50% after 24 h (Figure S1). To understand the time-dependent effects of H₂O₂ on NF- κ B activation, we measured reporter expression at various time points after the addition of TNF α to cells pretreated with 0.5 mM H₂O₂ (Figure 1B). The suppressive effect of H₂O₂ on TNF α -induced NF- κ B activation appeared at up to 4 h of TNF α stimulation, and inhibition was no longer significant after 8 h of TNF α stimulation. When we measured the mRNAs of inflammatory proteins, IL-1 β and intercellular adhesion molecule-1 (ICAM1), which are known NF- κ B transcriptional targets (Karin and Ben-Neriah, 2000; Van der Heiden *et al.*, 2010), we observed similar delays in expression induced by pre-exposure to H₂O₂ (Figure 1C). Interestingly, while the suppression of IL-1 β expression by H₂O₂ was fully recovered after 4 h, ICAM1 expression remained reduced compared to that in control cells even after 12 h of TNF α treatment. Overall, these results suggested that the transient exposure of cells to oxidative stress temporally suppresses NF- κ B activation and NF- κ B-dependent gene expression.

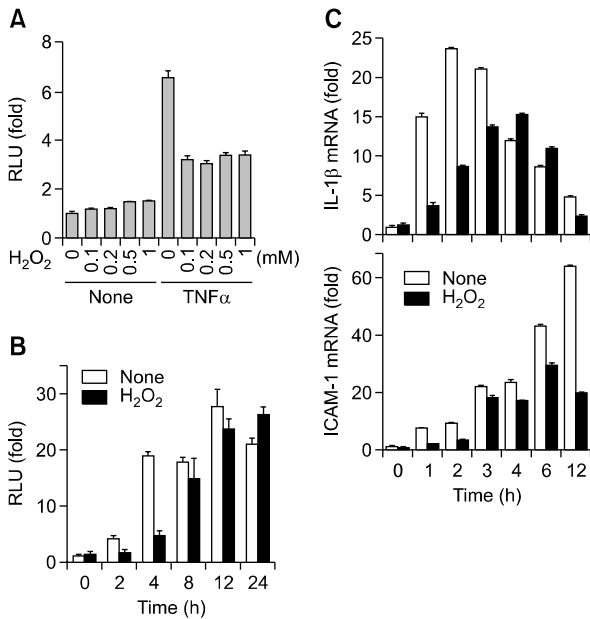


Figure 1. The transient inhibition of TNF α -induced NF- κ B activation by H₂O₂. (A) HEK293 cells were transfected with an NF- κ B reporter construct (I κ B-Luc) together with a control expression vector (β -actin/ β -galactosidase) and incubated for 2 days. The cells were incubated with various doses of H₂O₂ in for 20 min in DPBS/D-glucose, washed and stimulated with TNF α (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 H₂O₂ (0.5 mM) for 20 min and then stimulated with TNF α 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-1 β (upper panel) and ICAM-1 (lower panel) to calculate the mRNA level, which was then normalized to GAPDH ($n = 3$).

H₂O₂ 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 I κ B α 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; Levrant *et al.*, 2005; Loukili *et al.*, 2010). To determine whether transient exposure to H₂O₂ inhibited NF- κ B activation by blocking IKK activity, the IKK complex was isolated from HEK293 cells that had been sequentially treated with H₂O₂ and TNF α , and its kinase activity was measured in a reaction mixture containing [γ -³²P]ATP and GST-I κ B α (Figure 2). Our results showed that TNF α -induced IKK activity was not inhibited by the transient exposure of cells to up to 1 mM H₂O₂ (Figure 2A). The time course for the activation of IKK by TNF α stimulation in cells pre-exposed to H₂O₂ was also not different from that of non-treated control cells, and the IKK kinase

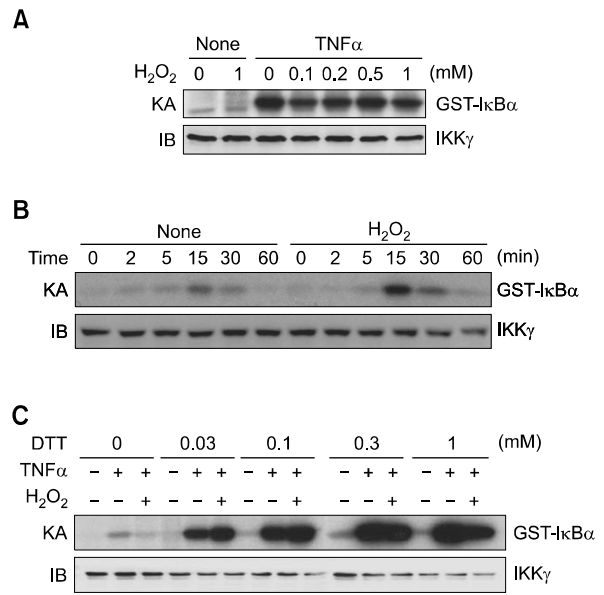


Figure 2. IKK activity was not obviously inhibited by H₂O₂. (A) HEK293 cells were exposed to various doses of H₂O₂ for 20 min, washed and stimulated with TNF α (20 ng/ml) for 10 min. IKK complexes in the cell lysate were immunoprecipitated with anti-IKK γ antibody. An *in vitro* kinase assay (KA) was carried out using [γ -³²P]ATP and GST-I κ B α (1-54) as substrates. The IKK γ in the kinase assay mixture was measured by immunoblot (IB) analysis. (B) The cells were exposed to H₂O₂ (0.5 mM) for 20 min and stimulated with TNF α for various times. IKK activity was determined as in (A). (C) The cells were exposed to H₂O₂ (0.5 mM) for 20 min and stimulated with TNF α 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.

activity at 15 min was somewhat elevated by H₂O₂ 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 H₂O₂-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 H₂O₂ to the level of activity in untreated control cells.

To elucidate the cause of the reduced NF- κ B activity in cells pre-exposed to H₂O₂, we then determined the levels of proteins involved in the NF- κ B signaling pathway and their modifications (Figure 3). Immunoblotting analysis of cytosolic I κ B α revealed that its degradation upon TNF α stimulation was blocked by pre-exposure to H₂O₂ (Figure 3A). The detection of I κ B α phosphorylated at Ser-32 and Ser-36 using a phospho-specific antibody revealed

that TNF α -induced I κ B α phosphorylation was not inhibited by pre-exposure to H₂O₂. Consistent with the H₂O₂-induced inhibition of I κ B α degradation, an increase in the nuclear level of NF- κ B subunit p65 was inhibited by pre-exposure to H₂O₂. When we measured time-dependent changes in NF- κ B signaling proteins after TNF α stimulation, pre-exposure of cells to H₂O₂ significantly delayed I κ B α degradation, whereas I κ B α phosphorylation was not changed in the same cells (Figure 3B). The increase of nuclear p65 was delayed, again reflecting the delayed degradation of I κ B α . Similarly, electrophoretic mobility assays (EMSA) of nuclear extract prepared from cells pre-exposed to H₂O₂ showed a delay in the appearance of κ B-sequence binding activity compared with non-exposed TNF α -stimulated control cells (Figure 3C).

H₂O₂ inhibits the poly-ubiquitination of phosphorylated I κ B α

Our results suggested that transient exposure to H₂O₂ inhibits the TNF α -induced activation of NF- κ B in HEK293 cells by blocking the degradation of phosphorylated I κ B α . Because the signal-induced degradation of I κ B α is known to occur through the ubiquitin-proteasome pathway, we measured proteasomal enzyme activity and I κ B α ubiquitination in cells pre-exposed to H₂O₂ (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 H₂O₂ 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 H₂O₂ treatment, but these differences were not statistically significant ($P > 0.05$ by Student's *t*-test). We then monitored the ubiquitination of I κ B α by immunoblotting analysis (Figures 4B and 4C). The stimulation of cells with TNF α in the absence of the proteasomal inhibitor MG-132 induced the time-dependent degradation of I κ B α . However, when the cells were stimulated with TNF α in the presence of MG-132, high-molecular-weight poly-ubiquitinated I κ B α appeared after 5 min and remained undegraded. When the cells were pre-exposed to H₂O₂ and stimulated with TNF α in the presence of MG-132, the poly-ubiquitination of I κ B α 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 TNF α stimulation. In contrast, pre-exposure to H₂O₂ resulted in an approximately

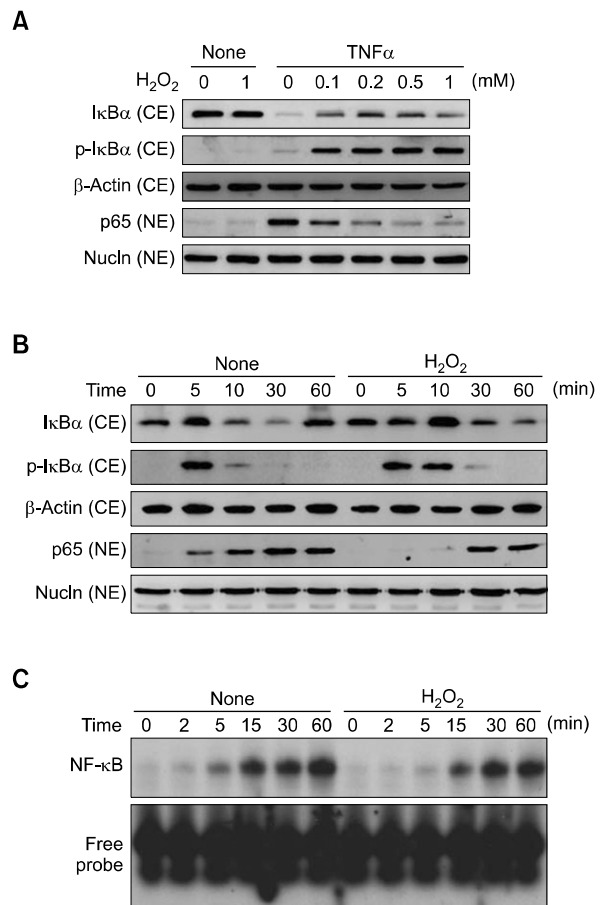


Figure 3. The effect of H₂O₂ on the TNF α -induced phosphorylation and degradation of I κ B α , and the nuclear translocation of p65. (A) HEK293 cells were treated with various doses of H₂O₂ for 20 min and stimulated with TNF α (20 ng/ml) for 10 min. The levels of I κ B α and phosphorylated I κ B α (p-I κ B α) 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 H₂O₂ (0.5 mM) for 20 min and stimulated with TNF α 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- κ B-DNA binding activity by EMSA. The upper panel shows the retarded NF- κ B 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 H₂O₂.

Discussion

In this study, the transient oxidative stress elicited by exposing cells to H₂O₂ was found to temporally inhibit NF- κ B reporter expression in TNF α -stimulated HEK293 cells. The inhibitory effect of H₂O₂ appeared

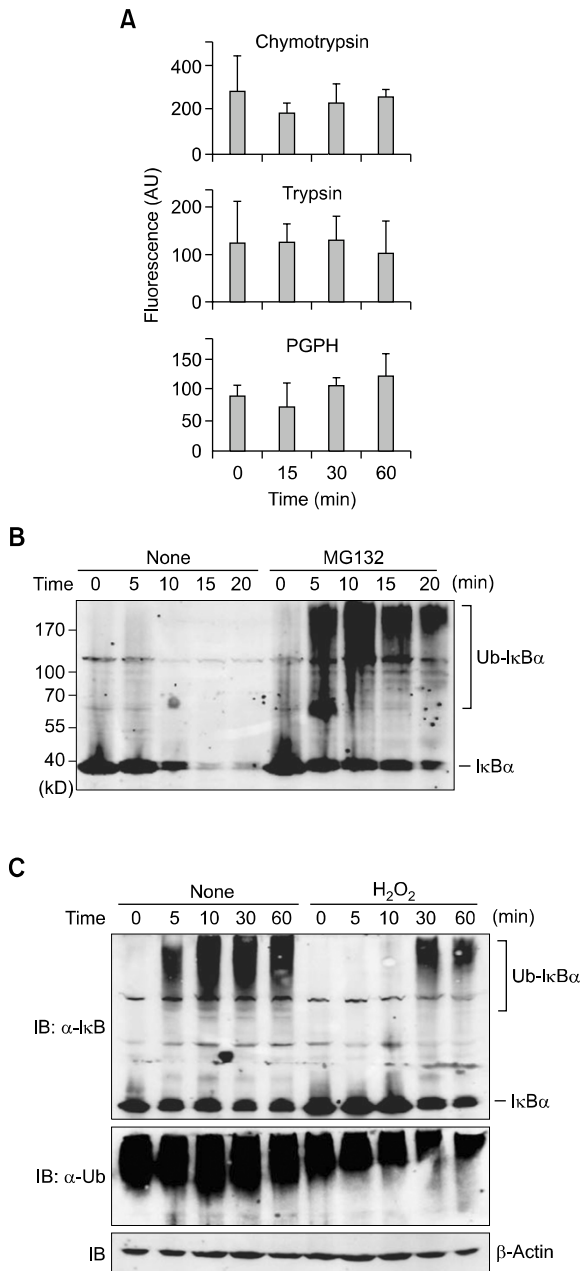


Figure 4. The transient inhibition of IκBα poly-ubiquitination by H₂O₂. (A) HEK293 cells were incubated with H₂O₂ (0.5 mM) for various times, and the cells were lysed in hypotonic buffer to prepare cell extract. Chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase (PGPH) peptidase 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 H₂O₂ 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 H₂O₂ (Figure 1A), suggesting that the NF-κB signaling pathway responds sensitively to changes in redox state induced by various pathologic conditions. Pre-exposure to H₂O₂ 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 H₂O₂-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 H₂O₂, nitric oxide (NO) and peroxynitrite (Korn *et al.*, 2001; Byun *et al.*, 2002; Reynaert *et al.*, 2004; Levrant *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 H₂O₂, 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 H₂O₂-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 H₂O₂-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 H₂O₂ did not significantly change the level and timing of IκBα

phosphorylation (Figures 3A and 3B), suggesting that IKK activity is not modulated by H₂O₂ pre-exposure in these cells.

Whereas the phosphorylation of I κ B α was not changed by pre-exposure of cells to H₂O₂, the TNF α -induced degradation of I κ B α was delayed (Figures 3A and 3B). In the same cells, increases in nuclear NF- κ B p65 and κ B-binding activity were delayed by H₂O₂ treatment *via* similar modes (Figures 3B and 3C). These results indicated that the effect of H₂O₂ was mediated through blocking the degradation of phosphorylated I κ B α , whereas the DNA-binding ability of NF- κ B was not changed by H₂O₂. Because the degradation of phosphorylated I κ B α occurs *via* the ubiquitin-proteasome pathway (Ciechanover, 1998; Karin and Ben-Neriah, 2000), we examined proteasomal peptidase activities and the ubiquitination of I κ B α in H₂O₂-treated cells. Our results showed that although proteasomal peptidase activities were not changed, the TNF α -induced poly-ubiquitination of I κ B α was delayed by H₂O₂ pre-exposure for a time period (10-30 min) similar to the delay in I κ B α degradation and p65 nuclear translocation (Figures 3 and 4). These results clearly indicated that the delay of NF- κ B activation that occurs in cells exposed to H₂O₂ is caused by the inhibition of I κ B α poly-ubiquitination and suggested two different possible modes for the inhibition of I κ B α poly-ubiquitination by H₂O₂: the transient modification of I κ B α or the transient inhibition of I κ B α 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 H₂O₂-induced inhibition of I κ B α ubiquitination, these results suggested that it was caused by a similar modification of I κ B α or I κ B α ubiquitinating enzyme(s).

The results shown in Figure 4C suggest that the inhibitory effect of H₂O₂ on I κ B α ubiquitination is specific and not due to a general inhibition of protein ubiquitination. I κ B α poly-ubiquitination was blocked by H₂O₂ 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 H₂O₂ pre-exposure and slowly decreased thereafter. These results suggest that H₂O₂ acts *via* two

different modes: immediate and sensitive inhibition for I κ B α 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 H₂O₂ for 5 min. They observed that the TNF α -induced expression of cell-adhesion molecules and inflammatory cytokines was inhibited by this pre-exposure to H₂O₂ and suggested that this phenomenon occurred due to the inhibition of NF- κ B activity. Our results showed that transient pre-exposure to H₂O₂ inhibits TNF α -induced NF- κ B activation by temporarily blocking the ubiquitination of phosphorylated I κ B α , whereas IKK activity and I κ B α phosphorylation remain largely intact in the same cells. These results suggest that modulation of I κ B α ubiquitination by ROS plays a critical role in regulating the NF- κ B-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 IKK γ , p65/RelA, I κ B α , and ubiquitin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-Ser-32 and phospho-Ser-36 I κ B α , 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)-I κ B α containing the N-terminal 54 residues of I κ B α and recombinant human TNF α 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- κ B reporter assay

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

2006). Kinase activity was measured in reaction mixtures containing 10 μM ATP, [γ -³²P]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 IκB-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 -³²P]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).

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