Hydrogen peroxide activates IκB kinases through phosphorylation of serine residues in the activation loops

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Abstract The cellular redox state regulates nuclear factor-κB (NF-κB) signaling systems. We investigated the effects of H_2O_2 on inhibitor of NF-κB (IκB) kinases (IKKα and IKKβ), which phosphorylate IκB leading to its degradation and NF-κB activation. Tumor necrosis factor (TNF) stimulation increased IKK activity within 10 min, and then IKK activity decreased gradually within 30 min in HeLa cells. Stimulation of the cells with H_2O_2 induced a slight activation of IKK within 30 min. Furthermore, co-stimulation with TNF suppressed the down-regulation of IKK and sustained the activation for more than 30 min. H_2O_2 also markedly activated IKK in cells that were pretreated with TNF or phorbol myristate acetate. Electrophoretic mobility shift assay revealed that H_2O_2 enhanced TNF-induced NF-κB activation. Studies using IKK mutants and an antibody against phosphorylated IKK proteins revealed that phosphorylation of serine residues, Ser180 of IKKα and Ser181 of IKKβ, in the activation loops was essential for the H_2O_2-mediated activation of IKK. H_2O_2-induced activation of IKKα and IKKβ was reduced by IKKβ and IKKα kinase-negative mutants, respectively, indicating that IKKα and IKKβ were stimulated by H_2O_2 in an interdependent manner. These results suggest that oxidative radical stress has stimulatory effects on NF-κB through the activation of IKK, which is mediated by the phosphorylation of serine residues in the activation loops. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Hydrogen peroxide; IκB kinase; Phosphorylation; NF-κB

1. Introduction

Nuclear factor-κB (NF-κB) is a transcription factor composed of p65 and p50 that has pivotal roles in the immune response, inflammation, development and cell growth. In unstimulated cells, NF-κB is sequestered in the cytoplasm through its tight association with a family of specific inhibitory proteins, called inhibitors of NF-κB (IκB) [1–4]. NF-κB-activating stimuli, such as tumor necrosis factor (TNF) and phorbol myristate acetate (PMA), lead to the phosphorylation of serine residues, Ser32 and Ser36, in the amino-terminal region of IκBα, and negate the inhibitory effects of IκB through its ubiquitination and degradation by proteasomes [1–4]. The released NF-κB translocates to the nucleus and binds to DNA, leading to the expression of a wide range of genes.

Phosphorylation of IκB is mediated by a macromolecular complex called the signalsome with a molecular mass of 500–900 kDa, which is composed of three major proteins, IKKα, IKKβ and IKKγ/NEMO (NF-κB essential modulator). Both IKKα and IKKβ are serine/threonine kinases [5–9], whereas IKKγ lacks kinase activity but is required for the activation of IKKα and IKKβ in vivo [10,11]. IKKα and IKKβ are highly homologous with the characteristic structure of an N-terminal protein kinase domain that contains a canonical mitogen-activated protein kinase kinase activation loop motif of the sequence Ser-X-X-X-Ser, as well as leucine-zipper and helix-loop-helix motifs [4]. Phosphorylation of the serine residues in the activation loop, i.e. Ser176 and Ser180 in IKKα and Ser177 and Ser181 in IKKβ, is thought to be important for the regulation of their kinase activity. Indeed, replacement of Ser177 and Ser181 in IKKβ with alanine prevents TNF-induced activation of this kinase [7,12]. Similarly, a mutation of Ser176 to alanine also inactivates IKKα [13]. Although it is not yet known, MEKK3 is the most plausible candidate for activating IKK, because TNF-induced activation of IKK and NF-κB is reduced in MEKK3 knock-out mice [14]. The serine residues in the activation loops as well as Ser23 in IKKα are important for the regulation of IKK kinase activity, because protein kinase B (PKB)/Akt phosphorylates Ser23 to activate IKKα [15,16].

After stimulation with TNF, IKKα and IKKβ activity rapidly increase within 5–15 min, and then gradually decrease within 30–120 min in most cell types [5,6,12]. Biochemical studies revealed that TNF activates the heterodimeric IKK complex in a directional manner: TNF triggers the activation of IKKα, which then stimulates IKKβ [17,18]. The mechanism of IKK downregulation is not fully understood, but recent studies suggest that at least two mechanisms are involved: autophosphorylation at the C-terminal regions of IKK molecules [12] and dephosphorylation through protein phosphatase 2A (PP2A) [5].

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radical (OH), are generated through multiple sources, including the electron transport reaction in mitochondria, or γ-ray irradiation of the cells. ROS are also produced in phagocytotic cells, such as neutrophils and macrophages, through NADPH oxidase at the site

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Abbreviations: EMSA, electrophoretic mobility shift assay; IKK, IκB kinase; IκB, inhibitor of NF-κB; IL-1, interleukin-1; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; PKB/Akt, protein kinase B/Akt; PMA, phorbol myristate acetate; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; TNF, tumor necrosis factor

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of inflammation. Cells possess several anti-oxidants to protect against the cytotoxic effects of ROS, and maintain the cellular redox in the reduced state. Recent studies revealed that the cellular redox state is essential not only for cell survival, but also for cellular signaling linked to cell growth and cell differentiation [19–21].

The NF-κB signaling system is subject to complicated redox regulation in a cell type-specific manner [22]. In lymphoid cells, a potent oxidant, pervanadate, induces phosphorylation at a tyrosine residue, Tyr42, in IκB, which in turn leads to the dissociation of IκB from NF-κB [23–25]. Conversely, treatment of cells with reductants such as *N*-acetylcysteine (NAC) and pyrrolidinedithiocarbamate, or overexpression of a reducing molecule, thioredoxin, suppresses extracellular stimuli-induced NF-κB activation [26–28]. In addition, NAC suppresses TNF-induced activation of IKK in HeLa cells [28]. Several discrepant results have been reported on redox regulation of the NF-κB signaling system: H_2O_2 suppressed cytokine-induced activation of IKK, leading to attenuation of NF-κB activation [29], and oxidative modification of a cysteine residue in the activation loop of IKK leads to its inactivation [30]. Thus, it remains unclear how ROS regulate NF-κB activity in the cells. The present study examined the effects of H_2O_2 on IKK, and demonstrated that oxidative radical stress activates IKK through the phosphorylation of serine residues in the activation loop.

2. Materials and methods

2.1. Materials

TNFα was obtained from Upstate Biotechnology (Lake Placid, NY, USA). PMA and anti-Flag M2 antibodies were obtained form Sigma (St. Louis, MO, USA). Anti-IKKα, anti-IKKβ, anti-IKKγ, anti-HA and anti-IκBα antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphoserine (Ser180) of IKKα/anti-phosphoserine (Ser181) of IKKβ antibody was obtained from Cell Signaling (Beverly, MA, USA). The plasmids, pRKF-IKKα and pRKHA-IKKβ, have been described previously [28]. The gene for IKKγ was amplified from a human cDNA library by polymerase chain reaction (PCR) using a set of two oligonucleotides, GGATCCATGAATAGGCACCTCTGGAAGAG and CTACTCAATGCACTCCATGACATGTATCT, and was cloned in an expression vector to generate a plasmid pRKF-IKKγ. Substitution mutations of IKKα and IKKβ were introduced by PCR using oligonucleotides, which harbor mutations in the corresponding sites. pRKF-IKKαEE and pRKHA-IKKβEE encode constitutively active IKK mutants in which the serine residues in the activation loops are replaced by glutamic acid residues. The kinase inactive mutants, IKKαK44A and IKKβ K44A, in which the lysine residues at position 44 are substituted with alanine, were kindly provided by Dr. David V. Goeddel (Tularik, CA, USA).

2.2. Cell culture and transfection

HeLa cells were cultured in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin at 37°C in 5% CO_2 in air. The cells (5×10^5 cells) in a 35-mm dish were transfected with plasmids using Lipofectin (Gibco BRL) following the manufacturer's instructions.

2.3. Electrophoretic mobility shift assay (EMSA) and Western blot analysis

The cells were solubilized with a buffer (10 mM HEPES/NaOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.4% NP-40, 0.5 mM APMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM dithiothreitol (DTT)), and then centrifuged at $10\,000\times g$ for 15 min. The pellets were resuspended in a buffer (20 mM HEPES/NaOH, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 μg/ml antipain, 1 mM DTT), and then centrifuged at $15\,000\times g$ for 15 min. The supernatants were used as the nuclear extracts. The nuclear extracts (10 μg of protein) were incubated with the ^{32}P-labeled probes (100 000 cpm) of the NF-κB binding site (AGCTTCAGAGGGGACTTTCCGAGAGGTCGA) in 20 μl of buffer (20 mM HEPES/NaOH, pH 7.9, 5% glycerol, 1 mM EDTA, 100 μg/ml poly(dI-dC)) for 20 min at room temperature. Then the samples were electrophoresed on an 8% polyacrylamide gel and analyzed using an Imaging plate (Fuji Film). For preparation of cell extracts, cells were solubilized with buffer A (20 mM Tris–HCl, pH 7.4, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 mM NaF, 1 mM APMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM DTT, and 1% Triton X-100), and then centrifuged at $15\,000\times g$ for 20 min. The supernatants were used as the cell extracts. Western blot analysis was performed using an ECL membrane and a Western Blotting Detection System (Amersham Pharmacia) according to the manufacturer's instructions.

2.4. IKK kinase assay

The kinase activity of IKK was analyzed by immune complex kinase assay using a substrate, GST-IκBα(1–55), as described previously [24]. Briefly, the cells were solubilized in ice-cold buffer A, and then centrifuged at $15\,000\times g$ for 20 min. IKKα and IKKβ were recovered from the lysates by immunoprecipitation, and then the immune complexes were incubated with 10 μl reaction buffer containing 20 mM HEPES/NaOH, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 100 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM DTT, 100 μM ATP, 0.05 μCi [γ-^{32}P]ATP, 5 μg GST-IκBα(1–55) at 30°C for 20 min. After SDS–polyacrylamide gel electrophoresis (PAGE), the phosphorylation of GST-IκBα was estimated by Imaging plate (Fuji Film).

2.5. [^{32}P]Orthophosphate labeling of Flag-IKKα and HA-IKKβ in cells

HeLa cells (5×10^5 cells) in a 35-mm dish were transfected with a plasmid encoding Flag-IKKα or HA-IKKβ together with a control vector or a plasmid encoding HA-IKKβK44A or Flag-IKKαK44A. After incubation for 24 h, the medium was changed to phosphate-free Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 0.1% dialyzed fetal bovine serum containing 100 μCi/ml [^{32}P]-orthophosphate, and then further incubated for 4 h. The cells were washed with an ice-cold buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl), and then solubilized with 0.5 ml ice-cold buffer A. Following centrifugation at $15\,000\times g$ for 20 min, transfected Flag-IKKα and HA-IKKβ proteins were recovered from the cell lysates by immunoprecipitation.

3. Results

3.1. H_2O_2 induced slight IKK activation, but this activation was not sufficient for NF-κB activation in HeLa cells

Treatment of HeLa cells with TNF induced a marked activation of IKKα and IKKβ within 10 min, and then the activity of these kinases gradually decreased within 30 min (Fig. 1A), consistent with previous studies [5,6,12]. H_2O_2 induced a slight gradual activation of these kinases within 30 min (Fig. 1A). Western blotting analysis indicated that TNF induced rapid phosphorylation of serine residues, Ser180 of IKKα and Ser181 of IKKβ, in the activation loops (Fig. 1B). H_2O_2 phosphorylated these serine residues as well, but this phosphorylation was induced more slowly than TNF-mediated phosphorylation (Fig. 1B). Western blotting analysis also indicated that TNF induced phosphorylation of IκB, as revealed by the mobility shift on SDS–PAGE in the presence of a proteasome inhibitor, zLLLH, and degradation within 10 min (Fig. 1C). In contrast, H_2O_2 did not induce phosphorylation or degradation of IκB in the cells (Fig. 1C). Consistent with the lack of IκB degradation, H_2O_2 did not activate NF-κB (see Fig. 3, later). These results suggest that H_2O_2 alone induces a slight activation of IKK, but this activation is not sufficient for the NF-κB activation.

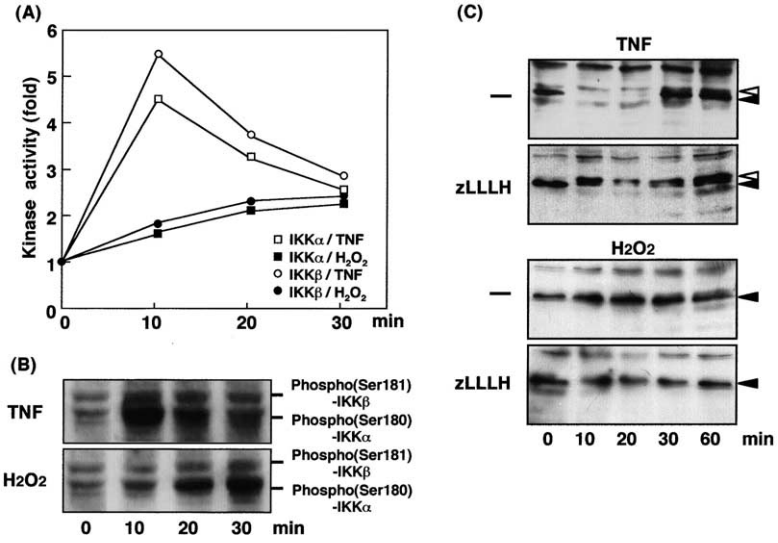

Fig. 1. H_2O_2 induced slight IKK activation. A: HeLa cells were stimulated with 3 mM H_2O_2 or 20 ng/ml TNF. At the indicated time, cell lysates were prepared and IKKα or IKKβ activities were estimated. B: The cells were treated with TNF or H_2O_2, and then the phosphorylation levels of serine residues, Ser180 of IKKα and Ser181 of IKKβ, in the activation loops were investigated by Western blotting using a specific antibody against phosphorylated IKK. C: The cells were treated with TNF or H_2O_2 in the presence or absence of 10 μM zLLLH for the indicated times, and then cellular proteins were analyzed by Western blotting using anti-IκB antibody. The black arrowheads indicate IκB and the white arrowheads indicate the phosphorylated form of IκB.

3.2. H_2O_2 enhanced TNF-induced IKK activation

When cells were stimulated by TNF together with H_2O_2, downregulation of IKKα and IKKβ was suppressed, and increased levels of these kinase activities were sustained for more than 30 min (Fig. 2A). Furthermore, H_2O_2 markedly activated IKKα and IKKβ in cells pretreated with TNF or PMA for 30 or 60 min (Fig. 2B). To elucidate the effects of H_2O_2 on the signalsome, we immunoprecipitated IKKγ and estimated the activity of IKKγ-associated IKKα and IKKβ. This assay revealed that TNF rapidly activated IKKγ-associated IKKα and IKKβ, and then the activity of these kinases decreased gradually following the activation (Fig. 2C). In contrast, H_2O_2 slowly induced a slight activation of these kinases. When the cells were treated with H_2O_2 after 10 min of TNF stimulation, downregulation of the kinase was suppressed, and the activated levels of the kinases were sustained for more than 30 min (Fig. 2C). Furthermore, H_2O_2 led to marked activation when the cells were pretreated with TNF or PMA for 30 or 60 min (Fig. 2C). Thus, H_2O_2 exhibited a marked stimulatory effect on IKKα and IKKβ when cells were co-stimulated with TNF or PMA. Consistent with the results of this kinase assay, EMSA revealed that co-stimulation of the cells with 1.0 mM H_2O_2 slightly but significantly enhanced TNF-induced NF-κB activation (Fig. 3).

3.3. H_2O_2 activates IKKα and IKKβ through the phosphorylation of serine residues in the activation loop

We then analyzed the effects of H_2O_2 on ectopically overexpressed IKKα and IKKβ. H_2O_2 led to gradual activation of transfected IKKα and IKKβ (Fig. 4A) in a dose dependent manner in which the activities reached maximum levels at 1–5 mM H_2O_2 (Fig. 4B). To elucidate the mechanism of the H_2O_2-mediated activation, we analyzed the effects of H_2O_2 on

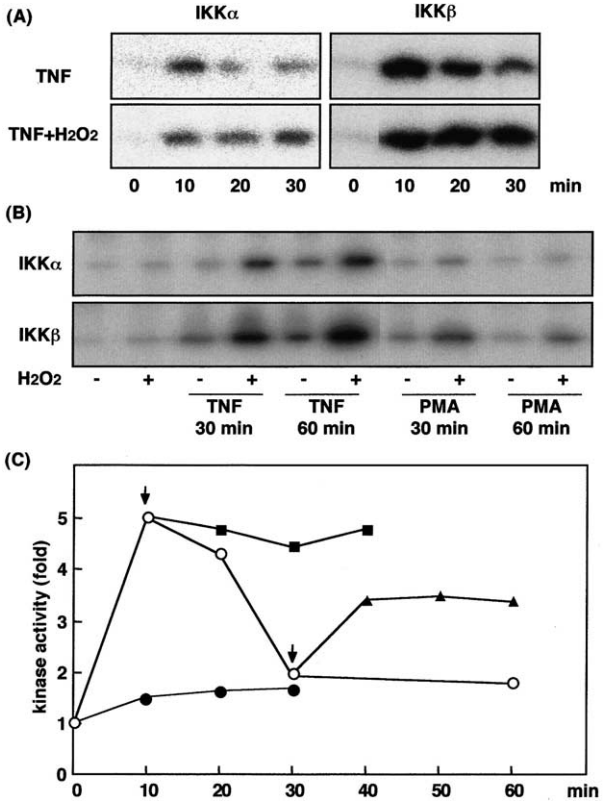

Fig. 2. Stimulatory effects of H_2O_2 on TNF- and PMA-induced IKK activation. A: The cells were stimulated with 20 ng/ml TNF with or without 3 mM H_2O_2. At the indicated time, cell lysates were prepared and IKKα or IKKβ activities were estimated. B: The cells were pretreated with 20 ng/ml TNF or 1 μg/ml PMA for 30 or 60 min. Then the cells were further incubated with or without 3 mM H_2O_2 for 30 min, and the IKKα and IKKβ kinase activity was estimated. C: The cells were stimulated with TNF alone (open circles), H_2O_2 alone (closed circles), or H_2O_2 following pretreatment with TNF for 10 min (squares) or 30 min (triangles). At the indicated time, IKKγ was recovered from cell lysates by immunoprecipitation using anti-IKKγ antibody. The kinase activity associated with IKKγ was estimated.

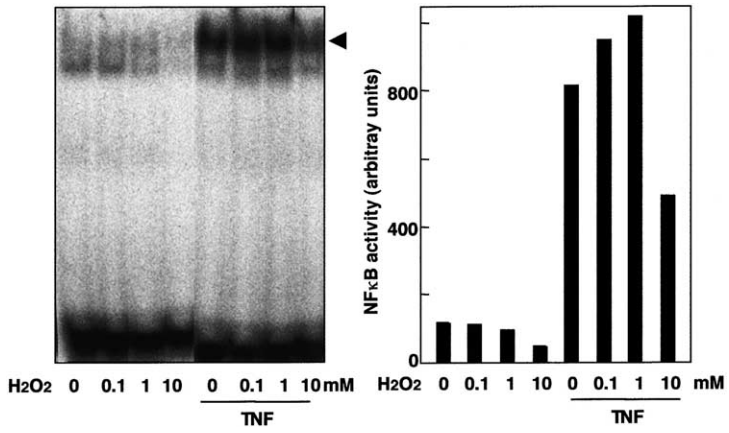

Fig. 3. Effects of H_2O_2 on NF-κB in the cells. The cells were treated with various concentrations of H_2O_2 together with 20 ng/ml TNF for 60 min. Then nuclear extracts were prepared, and the DNA binding activity of NF-κB was analyzed by EMSA.

several IKK mutants. PκB/Akt is activated in response to oxidative radical stress [31], and this kinase activates IKKα through the phosphorylation of Ser23 [15,16]. This finding suggests that H_2O_2 activates IKK by the phosphorylation of Ser23 through PKB/Akt. This was not the case, however, because treatment of the cells with H_2O_2 activated IKKα23A in which Ser23 was replaced with alanine (Fig. 4C), whereas H_2O_2 did not activate IKKαK44A and IKKβK44A in which Lys44, conserved and essential lysine residues in the kinase domain were replaced with alanine (Fig. 4C). As shown in Fig. 1B, H_2O_2 induced phosphorylation of serine residues in the activation loops of IKK as well as TNF. To clarify the role of these serines, we analyzed the effects of H_2O_2 on IKK mutants in which the serine residues, Ser176 and Ser180 in IKKα and Ser177 and Ser181 in IKKβ, were replaced with alanine. IKKα176A and IKKβ177A were activated in response to H_2O_2 (Fig. 4C). Thus, although there was no doubt about the role of Ser176 in IKKα and Ser177 in IKKβ in the regulation of these kinases, these residues were dispensable for H_2O_2-mediated activation of IKKα and IKKβ. In contrast,

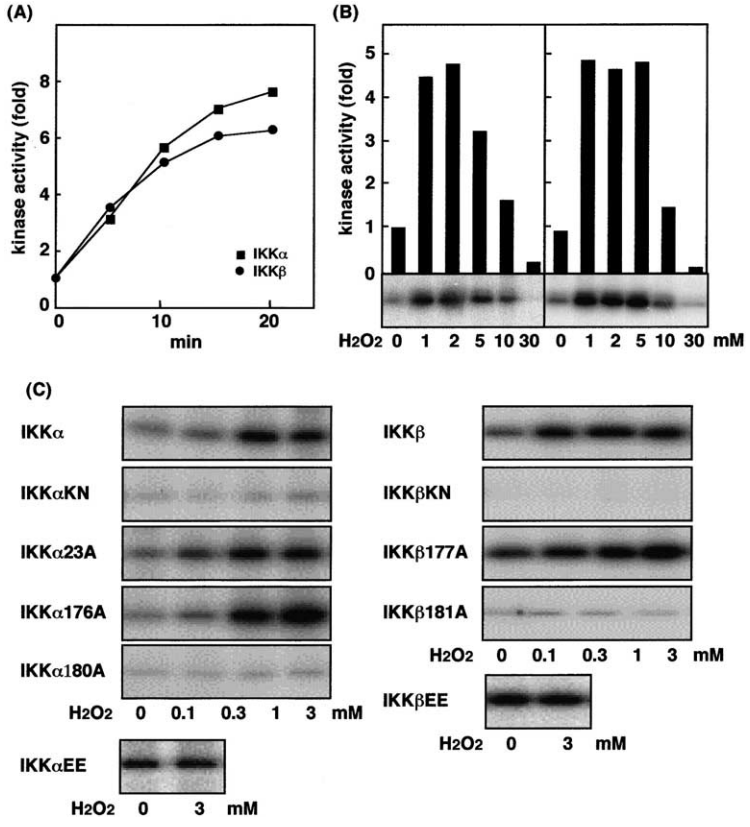

Fig. 4. Functional analysis of H_2O_2-induced activation of transfected IKKα and IKKβ mutants. A and B: The cells were transfected with an expression plasmid for Flag-IKKα or HA-IKKβ, and incubated for an additional 24 h, and then kinase activity was estimated. The cells were treated with 3 mM H_2O_2 for the indicated time (A), or stimulated with various concentrations of H_2O_2 for 30 min (B). C: The cells were transfected with an expression plasmid for wild-type or mutant Flag-IKKα and HA-IKKβ, and further incubated for 24 h. Then the cells were treated with various concentrations of H_2O_2 for 30 min and the kinase activity was estimated.

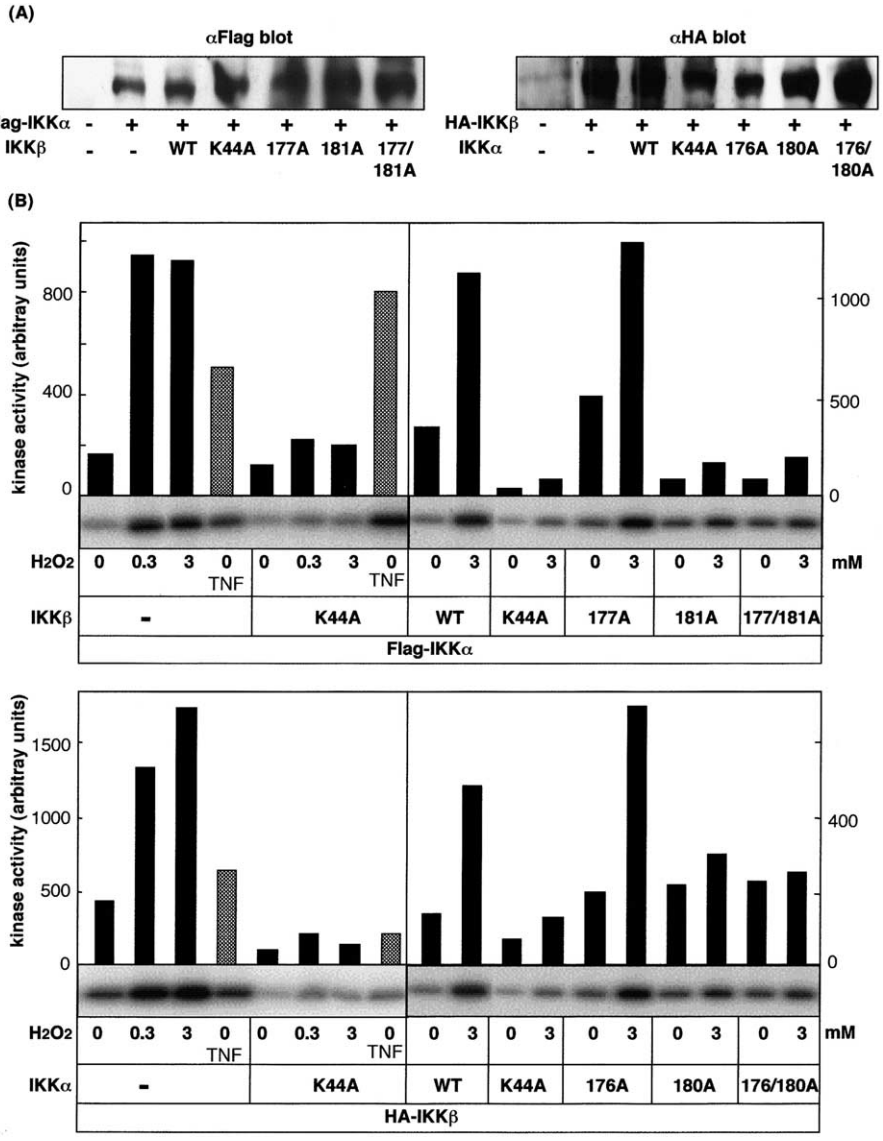

Fig. 5. H_2O_2 stimulated IKKα and IKKβ in an interdependent manner. The cells were transfected with an expression plasmid for Flag-IKKα or HA-IKKβ (0.5 μg) together with a control vector or a plasmid encoding wild-type or mutant IKKβ or IKKα (0.5 μg), and further incubated for 24 h. A: The expression levels of transfected IKK were estimated by Western blotting. B: The cells were treated with various concentrations of H_2O_2 for 30 min, or with 20 ng/ml TNF for 10 min. Then the kinase activities of the transfected Flag-IKKα or HA-IKKβ were estimated.

H_2O_2-induced activation of IKK was abolished in IKKα180A and IKKβ181A, indicating that Ser180 of IKKα and Ser181 of IKKβ were essential for the H_2O_2-induced activation (Fig. 4C). H_2O_2 did not activate either IKKαEE or IKKβEE in which Ser176 and Ser180 in IKKα or Ser177 and Ser181 in IKKβ were substituted with glutamic acid, respectively. Together, these findings suggest that H_2O_2 activates IKKα and IKKβ through the phosphorylation of these serine residues in the activation loop.

3.4. *IKKα and IKKβ were stimulated by H_2O_2 in an interdependent manner*

Activation of the heterodimeric IKKα/IKKβ complex by TNF or other stimuli characteristically proceeds in a sequential manner, and activation of IKKβ is dependent on the IKKα kinase activity [17,18]. To elucidate whether H_2O_2-mediated IKK activation is directional, IKKα and IKKβ activities were estimated in cells co-transfected with mutant and wild-type IKKα and IKKβ. Co-transfection of mutant IKK did not change the expression levels of the transfected IKKα and IKKβ (Fig. 5A). IKKαK44A suppressed TNF-induced activation of IKKβ, whereas IKKβK44A did not inhibit IKKα, indicating that TNF activates IKKβ through IKKα (Fig. 5B). In contrast, both IKKαK44A and IKKβK44A suppressed H_2O_2-induced activation of IKKβ and IKKα, respectively (Fig. 5B). IKKα180A and IKKβ181A, which were H_2O_2-unresponsive mutants (see Fig. 4C), also suppressed H_2O_2-induced activation of IKKβ and IKKα, respectively (Fig. 5B). Furthermore, double mutants IKKβ177/181A and IKKα176/180A, in which two serine residues in the activation loop were exchanged for alanine, also had inhibitory effects on IKKα and IKKβ, respectively (Fig. 5B). IKKβ177A and IKKα176A, which can be activated by H_2O_2 (see Fig. 4C), did not inhibit the H_2O_2-induced activation of IKKα and

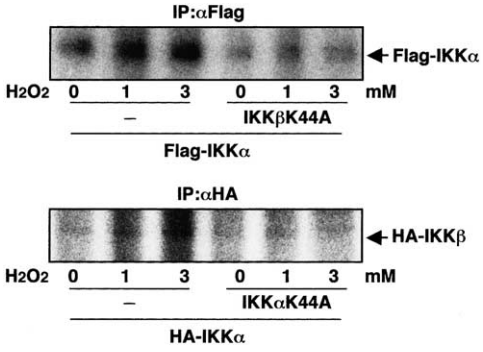

Fig. 6. H_2O_2 induced IKKα and IKKβ phosphorylation in an interdependent manner. The cells were transfected with an expression plasmid for Flag-IKKα or HA-IKKβ (0.5 μg) together with a control vector or a plasmid encoding IKKβK44A or IKKα K44A (0.5 μg), respectively. After 24 h, cells were labeled with [^{32}P]orthophosphate for 4 h, and then stimulated with various concentrations of H_2O_2 for 30 min. Immunoprecipitation was performed with anti-Flag or anti-HA antibody, and the phosphorylation levels of IKK were analyzed using SDS–PAGE and autoradiography.

IKKβ (Fig. 5B). We then analyzed the effects of H_2O_2 on the phosphorylation of IKKα and IKKβ in vivo by [^{32}P]orthophosphate labeling of the cells. H_2O_2 induced phosphorylation of the transfected IKKα and IKKβ (Fig. 6). IKKβK44A inhibited the phosphorylation of IKKα, and IKKαK44A inhibited the phosphorylation of IKKβ (Fig. 6). Thus, the H_2O_2-induced phosphorylation of IKKα is dependent on IKKβ, and vice versa. These results indicate that IKKα and IKKβ are stimulated by H_2O_2 in an interdependent manner.

4. Discussion

H_2O_2 stimulates IKKα and IKKβ in HeLa cells as shown in the present study, and a reductant, NAC, suppresses TNF-induced NF-κB activation through inhibition of IKKα and IKKβ [28]. Together, these results suggest that IKKα and IKKβ have a pivotal role in the redox regulation of NF-κB. Several signaling proteins are also involved in the redox regulation of NF-κB. Oxidative radical stress activates NF-κB through phosphorylation of Tyr42 in IκB in lymphoid cells, indicating that protein tyrosine kinase is also involved in the NF-κB activation [23–25]. Thus, oxidative radical stress stimulates the NF-κB signaling system through multiple pathways, including IKK, in a cell type-specific manner.

The above lines of evidence indicate that oxidative radical stress acts positively on NF-κB in the cells. There are discrepant results, however, on the effects of oxidative radical stress on the NF-κB signaling system. Thiol-reactive metal compounds, such as auranofin, inhibit NF-κB activation by inhibiting IKK activity [32]. Thiol-reactive arsenite suppresses IKK activation through oxidative modification of a critical cysteine residue, Cys179, in the activation loop of IKKβ [30]. Thus, the reduced state of the cysteine residue on IKK molecules is a requisite for the kinase activity. Indeed, we also observed that H_2O_2 inactivated purified IKK in vitro (data not shown). These properties, however, are compatible with the stimulatory effects of H_2O_2 on IKK in the cells, because the IKK activity is suppressed by H_2O_2 at high concentrations (see Fig. 4B). This suppression might reflect the inactivation of IKK by oxidative modification. Korn et al. reported that H_2O_2 suppressed TNF-induced activation of IKK in the cells [29]. They examined the effects of H_2O_2 after a relatively short period (5 min) after TNF stimulation, however, whereas we investigated the IKK activity after a longer period. Indeed, we also observed that pretreatment of the cells with H_2O_2 partially suppressed the initial activation of IKK triggered by TNF, and the phosphorylation and degradation of IκB were slightly attenuated within 20 min after TNF stimulation, consistent with Korn et al. (data not shown). IKK activity increased gradually, and then reached higher levels than in the absence of H_2O_2 within 30 min after TNF stimulation. In addition, they showed that H_2O_2 suppressed TNF-induced NF-κB activation using a luciferase assay. This suppression, however, might not be due to the inactivation of IKK, because H_2O_2 suppressed the expression of the luciferase gene of the NF-κB reporter plasmid at the transcriptional or translational step (data not shown). Furthermore, EMSA revealed that H_2O_2 stimulated the nuclear translocation of NF-κB, as shown in Fig. 3.

An intriguing finding is that H_2O_2 stimulates ectopically overexpressed IKK in a manner different from TNF. TNF activates IKKα and IKKβ in a sequential manner, whereas H_2O_2 stimulates these kinases in an interdependent manner (Figs. 5 and 6). Although it remains unknown how H_2O_2 activates IKK, the interdependent activation of IKKα and IKKβ possibly reflects the differential mechanism of IKK activation between cytokines and oxidative radical stress. A plausible model is that H_2O_2 activates the upstream kinases, such as MEKK3, and then stimulates IKK. Alternatively, oxidative radical stress might stimulate IKK through the suppression of protein phosphatase, such as PP2A, because these enzymes are easily inactivated by oxidative modification [33]. Supporting this model, H_2O_2 had stimulatory effects on IKK when phosphorylated IKK and PP2A were incubated together in vitro (data not shown).

In some cell types, ROS act as second messengers for NF-κB activation [34–39]. Recently, Bonizzi et al. reported that interleukin-1 (IL-1) activated NF-κB through the production of ROS in a cell type-specific manner [37,38]. They showed that IL-1 stimulated the production of ROS by 5-lipoxygenase in lymphoid cells and by NADPH oxidase in monocytic cells [37,38]. It remains unclear, however, how ROS activate the NF-κB signaling system. One possible mechanism is that ROS stimulate tyrosine protein kinases that lead to the phosphorylation of Tyr32 on IκB, thus activating NF-κB. In addition, the stimulatory effects of H_2O_2 on IKKα and IKKβ, as shown in the present study, might be an alternative mechanism by which ROS act as second messengers to activate NF-κB.

Phagocytic cells including neutrophils produce ROS at sites of inflammation. It is possible that ROS then synergistically enhance IKK activation triggered by cytokines and other stimuli, and stimulate gene expression through NF-κB. Consistent with this model, H_2O_2 enhanced lipopolysaccharide-induced gene expression of IL-8 through stimulation of NF-κB [40]. Furthermore, it has been suggested that ROS produced by NADPH oxidase in neutrophils stimulate TNF-induced NF-κB activation and intracellular adhesion molecule-1 expression in endothelial cells [41]. Thus, ROS might have an important role in several biologic responses, such as inflam-

mation and immune responses through redox regulation of IKK.

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References

[1] Miyamoto, S. and Verma, I.M. (1995) Cancer Res. 66, 255–292.
[2] Baldwin Jr., A.S. (1996) Annu. Rev. Immunol. 14, 649–683.
[3] Ghosh, S., May, M.J. and Kopp, E.B. (1998) Annu. Rev. Immunol. 16, 225–260.
[4] Karin, M. and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663.
[5] DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E. and Karin, M. (1997) Nature 388, 548–554.
[6] Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M. and Karin, M. (1997) Cell 91, 243–252.
[7] Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J., Young, D.B., Barbosa, M., Mann, M., Manning, A. and Rao, A. (1997) Science 278, 860–866.
[8] Regnier, C.H., Song, H.Y., Gao, X., Goeddel, D.V., Cao, Z. and Rothe, M. (1997) Cell 90, 373–383.
[9] Woronicz, J.D., Gao, X., Cao, Z., Rothe, M. and Goeddel, D.V. (1997) Science 278, 866–869.
[10] Rothwarf, D.M., Zandi, E., Natoli, G. and Karin, M. (1998) Nature 395, 297–300.
[11] Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S.T., Weil, R., Agou, F., Kirk, H.E., Kay, R.J. and Israel, A. (1998) Cell 93, 1231–1240.
[12] Delhase, M., Hayakawa, M., Chen, Y. and Karin, M. (1999) Science 284, 309–313.
[13] Ling, L., Cao, Z. and Goeddel, D.V. (1998) Proc. Natl. Acad. Sci. USA 95, 3792–3797.
[14] Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z. and Su, B. (2001) Nat. Immunol. 2, 620–624.
[15] Romashkova, J.A. and Makarov, S.S. (1999) Nature 401, 86–90.
[16] Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M. and Donner, D.B. (1999) Nature 401, 82–85.
[17] O'Mahony, A., Lin, X., Geleziunas, R. and Greene, W.C. (2000) Mol. Cell. Biol. 20, 1170–1178.
[18] Yamamoto, Y., Yin, M.J. and Gaynor, R.B. (2000) Mol. Cell. Biol. 20, 3655–3666.
[19] Finkel, T. (1998) Curr. Opin. Cell Biol. 10, 248–253.
[20] Kamata, H. and Hirata, H. (1999) Cell Signal. 11, 1–14.
[21] Rhee, S.G. (1999) Exp. Mol. Med. 31, 53–59.
[22] Li, N. and Karin, M. Is NF-κB The sensor of oxidative stress?, (1999) FASEB J. 13, 1137–1143.
[23] Imbert, V., Rupec, R.A., Livolsi, A., Pahl, H.L., Traenckner, E.B., Mueller-Dieckmann, C., Farahifar, D., Rossi, B., Auberger, P., Baeuerle, P.A. and Peyron, J.F. (1996) Cell 86, 787–798.
[24] Beraud, C., Henzel, W.J. and Baeuerle, P.A. (1999) Proc. Natl. Acad. Sci. USA 96, 429–434.
[25] Schoonbroodt, S., Ferreira, V., Best-Belpomme, M., Boelaert, J.R., Legrand-Poels, S., Korner, M. and Piette, J. (2000) J. Immunol. 164, 4292–4300.
[26] Hirota, K., Murata, M., Sachi, Y., Nakamura, H., Takeuchi, J., Mori, K. and Yodoi, J. (1999) J. Biol. Chem. 274, 27891–27897.
[27] Takeuchi, J., Hirota, K., Itoh, T., Shinkura, R., Kitada, K., Yodoi, J., Namba, T. and Fukuda, K. (2000) Antioxid. Redox Signal. 2, 83–92.
[28] Oka, S., Kamata, H., Kamata, K., Yagisawa, H. and Hirata, H. (2000) FEBS Lett. 472, 196–202.
[29] Korn, S.H., Wouters, E.F., Vos, N. and Janssen-Heininger, Y.M. (2001) J. Biol. Chem. 276, 35693–35700.
[30] Kapahi, P., Takahashi, T., Natoli, G., Adams, S.R., Chen, Y., Tsien, R.Y. and Karin, M. (2000) J. Biol. Chem. 275, 36062–36066.
[31] Shaw, M., Cohen, P. and Alessi, D.R. (1998) Biochem. J. 336, 241–246.
[32] Jeon, K.I., Jeong, J.Y. and Jue, D.M. (2000) J. Immunol. 164, 5981–5989.
[33] Rusnak, F. and Reiter, T. (2000) Trends Biochem. Sci. 25, 527–529.
[34] Muroi, M., Muroi, Y. and Suzuki, T. (1994) J. Biol. Chem. 269, 30561–30568.
[35] Schenk, H., Hexel, K., Baeuerle, P.A., Droge, W. and Schulze-Osthoff, K. (1995) EMBO J. 14, 3731–3740.
[36] Kurata, S., Matsumoto, M., Tsuji, Y. and Nakajima, H. (1996) Eur. J. Biochem. 239, 566–571.
[37] Bonizzi, G., Piette, J., Schoonbroodt, S., Greimers, R., Havard, L., Merville, M.P. and Bours, V. (1999) Mol. Cell. Biol. 19, 1950–1960.
[38] Bonizzi, G., Piette, J., Merville, M.P. and Bours, V. (2000) Biochem. Pharmacol. 59, 7–11.
[39] Sanlioglu, S., Williams, C.M., Samavati, L., Butler, N.S., Wang, G., McCray Jr., P.B., Ritchie, T.C., Hunninghake, G.W., Zandi, E. and Engelhardt, J.F. (2001) J. Biol. Chem. 276, 30188–30198.
[40] Tanaka, C., Kamata, H., Takeshita, H., Yagisawa, H. and Hirata, H. (1997) Biochem. Biophys. Res. Commun. 232, 568–573.
[41] Fan, J. and Mali, A.B. (2002) J. Biol. Chem. 277, 3404–3411.