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Raf-Independent and MEKK1-Dependent Activation of NF-κB by Hydrogen Peroxide in 70Z/3 pre-B Lymphocyte Tumor Cells

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Abstract We have previously demonstrated that hydrogen peroxide (H_2O_2) treatment of murine 70Z/3 pre-B lymphocytes inhibits the immune response to lipopolysaccharide by attenuating signaling through c-Jun N-terminal kinase (JNK) activation. In the present study, we further examined the signaling intermediates responsible for immunosuppression by H_2O_2, focusing on NF-κB, a dimeric transcription factor whose activation is implicated in a number of immune response. Treatment of 70Z/3 pre-B cells with H_2O_2 caused activation of NF-κB in the nuclei by detection of NF-κB specific DNA binding, concomitant with phosphorylation of IκBα. H_2O_2 stimulation of NF-κB occurred within 20 min of treatment, reached maximum level at 60 min, and sustained for 2 h or more. Especially, MEK1 may contribute to H_2O_2-induced NF-κB activation as shown in the inhibition of NF-κB binding activity by the MEK1 inhibitor, PD 98059, and H_2O_2-induced MEK1 activation. However, H_2O_2 exhibited no effect on the activity of Raf-1 kinase, which was an upstream activator of MEK1. Furthermore, B-58l and α-hydroxyfarnesylphosphonic acid, two inhibitors of Ras, did not block NF-κB activation. In addition, the transient transfection of a dominant negative Ras (RasN17) construct showed a negligible inhibitory effect on the activation of NF-κB by H_2O_2. Instead, treatment of 70Z/3 cells with H_2O_2 resulted in the activation of MAPKkinase kinase 1 (MEKK1) as well as JNK. Therefore, our data suggest that H_2O_2 regulates the activity of NF-κB by MEK1 activation through MEKK1-dependent but Ras/Raf-independent mechanism. J. Cell. Biochem. 88: 545–556, 2003.

Key words: hydrogen peroxide; NF-κB; MEKK1; Raf; IκB kinase

Activation of the transcription factor, NF-κB has been shown to be a key component of immune response [Ghosh et al., 1998]. Our previous results demonstrated that paclitaxel-induced immune suppression was associated with NF-κB activation via conventional PKC isotypes in lipopolysaccharide-stimulated 70Z/3 pre-B lymphocyte tumor cells. [Lee and Jeon, 2001]. NF-κB is composed of two subunits of p50 and 65 kDa, which is present constitutively in the cytoplasm in an inactive state by inhibitory proteins, IκB [Baldwin, 1996]. Upon activation, IκB undergoes phosphorylation and degradation, and the NF-κB heterodimer translocates into the nucleus, where it binds to DNA and activates transcription [Rice and Ernst, 1993]. Recently, it has been widely known that a high molecular weight IκB kinase (IKK) complex, consisting of IKK-α, IKK-β, NF-κB-inducing kinase (NIK) and two adaptor or scaffold proteins is involved in IκB phosphorylation [Woronicz et al., 1997].

Independent studies have also demonstrated that MAPK kinase kinase 1 (MEKK1), which acts as an upstreamactivator of c-Jun N-terminal kinase (JNK), activates IKK activity [Lee et al., 1997]. MEKK1 has been mainly implicated in the activation of the JNK and the extracellular signal-regulated protein kinase (ERK) pathways based on its ability to activate these kinases when overexpressed [Lange-Carter et al., 1993; Minden et al., 1994]. Most attention has focused on JNK, in part because MEKK1 can induce apoptosis under certain circumstances and JNK has been implicated in

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this process, and because Raf isoforms appear to be the major kinase for the ERK pathway [Dentet al., 1992; Kyriakis et al., 1992]. Each MAPK kinase (MAPKK), however, can be activated by more than one MAPK kinase kinase (MEKK), increasing the complexity and diversity of MAPK signalling. In particular, activation of ERK by growth factors depends on the Raf-1, but other MEKKs may activate ERK in response to pro-inflammatory stimuli [Chang and Karin, 2001]. Also, the stimuli by serum and lysophosphatidic acid activate JNK and ERK at least in part through MEKK1 [Yujiri et al., 1998].

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) exert multiple regulatory effects on cell behavior and function as a second messenger [Finkel, 1998]. Especially, many cell types produce H_2O_2 in response to a variety of growth factor such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [Sundaresan et al., 1995; Bae et al., 1997]. In addition, it has been demonstrated that H_2O_2 has been known to stimulate phosphorylation of the three identified MAP kinases, ERK, JNK, and p38 [Guyton et al., 1996; Irani et al., 1997]. However, apart from functioning as second oxygen messenger of signal transduction, reactive oxygen species exert direct toxic effects on cell behavior. Especially, human lymphocytes are quite susceptible to oxidative stress [El-Hag et al., 1986], although reactive oxygen species can act as a trigger for lymphocyte activation [Rosa, 1997]. Dumont et al. reported that H_2O_2 elicited apoptosis through activation of caspase-3 and NF-κB followed by elevated expression of p53 in human T cell line CEM C7 [Dumont et al., 1999]. Our previous reports also demonstrated that H_2O_2 inhibited the immune response to lipopolysaccharide by attenuating signaling through JNK activation in 70Z/3 pre-B cells [Lee and Yea, 2000].

Here we show that H_2O_2 may induce immunomodulatory effects through the activation of NF-κB in a Ras/Raf-independent, MEKK1-dependent manner that involves degradation of IκBα in 70Z/3 pre-B lymphocytes.

MATERIALS AND METHODS

Materials

Anti-Raf, anti-IKKα, anti-IKKβ, and anti-MEKK1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific antibodies were obtained from Cell Signaling Technology (Beverly, MA). Protein A-agarose was from Roche Molecular Biochemicals (Indianapolis, IN). B-581 and α-hydroxyfarnesylphosphonic acid were from Biomol (Plymouth meeting, PA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and DMRIE-C reagent were purchased from Life Technologies, Inc. (Rockville, MD). Reagents for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were from Bio-Rad (Hercules, CA). [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). GF 109203X, Gö 6976, PD 98059, and SB 203580 were purchased from Calbiochem (San Diego, CA). H_2O_2 was obtained from Sigma (St. Louis, MO).

Mammalian Cell Culture and Transient Transfection

The murine pre-B cell line, 70Z/3 (ATCC TIB 152), was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium supplemented with 100 units of penicillin/ml, 100 units of streptomycin/ml, 2 mM L-glutamine, and 10% FCS. 70Z/3 pre-B cells were transiently transfected with a dominant negative Ras construct using DMRIE-C reagent (GIBCO-BRL). All assays were performed within 48 h after transfection, and the experiments were repeated at least twice.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as previously described, and gel mobility shift assays were performed [Lee and Yang, 2000]. Briefly, 5 μg of nuclear extracts were incubated with 2 μg of poly (dI-dC) (Sigma) and ^{32}P-end-labeled DNA probe (double-stranded synthetic 26-bp oligonucleotides GATCTCAGAGGGGACTTTCCGAAGAGA containing the κB enhancer of immunoglobulin κ light chain gene). The identity of the shifted bands was confirmed by competition with unlabeled oligomer containing NF-κB site.

In Vitro Raf-1 Kinase Assay

The cell lysates were prepared as previously described [Lee and Jeon, 2001]. Immunoprecipitation was performed on the whole cell lysates using anti-Raf and protein A-agarose beads. After incubation for 2 h at 4°C,

immunoprecipitates were washed twice with ice-cold lysis buffer. After washing with kinase buffer (20 mM Tris, pH 7.4, 20 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$), Raf-1 kinase activity was assayed by phosphorylation of the recombinant MEK1 in the presence of PD 98059, which was used to inhibit the autokinase activity of MEK1. The samples were resolved by SDS–PAGE, and phosphoproteins visualized by autoradiography.

IκB Kinase (IKK) Assay

For IKK assay, both anti-IKKα and IKKβ antibodies were used to immunoprecipitate both kinases. After immunoprecipitation, kinase assay was performed as described elsewhere [Lee and Jeon, 2001]. Briefly, the kinase reaction was performed in 30 μl of kinase buffer (20 mM HEPES, pH 7.8, 10 mM $MgCl_2$, 100 μM Na_3VO_4, 20 mM β-glycerophosphate, 2 mM DTT, 50 mM NaCl) for 30 min at 30°C in the presence of 10 μM ATP-10 μCi of [γ-^{32}P]ATP (10 Ci/mmol) (NEN Life Science Products) and 500 ng of the substrate GST-IκBα (Santa Cruz Biotechnology). The reactions were terminated with 4 × Laemmli sample buffer. Proteins were analyzed on 12.5% SDS–polyacrylamide gels, dried, and visualized by autoradiography.

Immunoblot Analysis

Once 70Z/3 pre-B cells reached subconfluence, the cells were incubated for additional times in the presence of H_2O_2. The cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested by resuspension of the cell pellet in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM β-glycerophosphate, and 2 mM sodium fluoride). Cell lysates were clarified by centrifugation at 15,000×g for 10 min at 4°C, and their protein concentrations were determined with the aid of a BCA protein assay reagent kit (Pierce; Rockford, IL). For immunoblotting, cell lysates were denatured in Laemmli sample buffer, and resolved by SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose, and immunoblot analysis was performed using specific antibodies as indicated. Immune complexes on nitrocellulose were detected by enzyme-linked chemiluminescence (Amersham Pharmacia Inc., Piscataway, NJ).

RESULTS

H_2O_2-Induced NF-κB Activation

In agreement with other reports [True et al., 2000] suggesting that reactive oxygen intermediates play a critical role in activation of NF-κB induced by TNF and other agents, H_2O_2 increased NF-κB binding activity in nuclear extracts of 70Z/3 pre-B cells. Identity of the shifted bands was confirmed by competition with unlabeled oligomer containing NF-κB site. The kinetics of NF-κB activation in 70Z/3 cells following H_2O_2 exposure were shown in Figure 1A. H_2O_2-induced NF-κB activation occurred within 20 min of treatment, reached maximum level at 60 min, and sustained for 2 h or more. DNA binding activity of NF-κB was gradually increased at 100–300 μM H_2O_2 and sharply decreased at 1 mM (Fig. 1B).

Effect of H_2O_2 on IκB Kinase and IκBα Phosphorylation

The event that occurs during activation of NF-κB is the phosphorylation of IκB that serve to target it for ubiquitination and degradation. Therefore, we determined IκB phosphorylation using a phospho-specific anti-IκBα antibody that detects IκBα only when activated by phosphorylation at Ser-32. Treatment of cells with H_2O_2 led to an increase of phosphorylated IκBα reaching a plateau between 5 and 30 min (Fig. 2A). Next, to determine whether IκBα phosphorylation by H_2O_2 results from upstream IKK complex, which phosphorylate the residues in IκB molecule [Karin, 1999; Israel, 2000], we directly measured IKK complex kinase activity by immunoprecipitating both IKKα and IKKβ from 70Z/3 cells that are treated with H_2O_2. As shown in Figure 2B, H_2O_2 elicited a slight and transient IKK activation.

Involvement of MEK1/ERK Pathway in H_2O_2-Induced NF-κB Activation

To further explore the mechanism responsible for H_2O_2-induced NF-κB activation, several inhibitors of tyrosine kinase and PKC were utilized (Fig. 3). A conventional PKC inhibitor, Gö 6976 partially inhibited H_2O_2-induced NF-κB activation. Although GF 109203X inhibits conventional PKCs as well as novel PKCs, it is unlikely to be effective as much as Gö 6976 in the inhibition of conventional PKCs. No apparent inhibition in H_2O_2-induced NF-κB activation was observed with either genistein

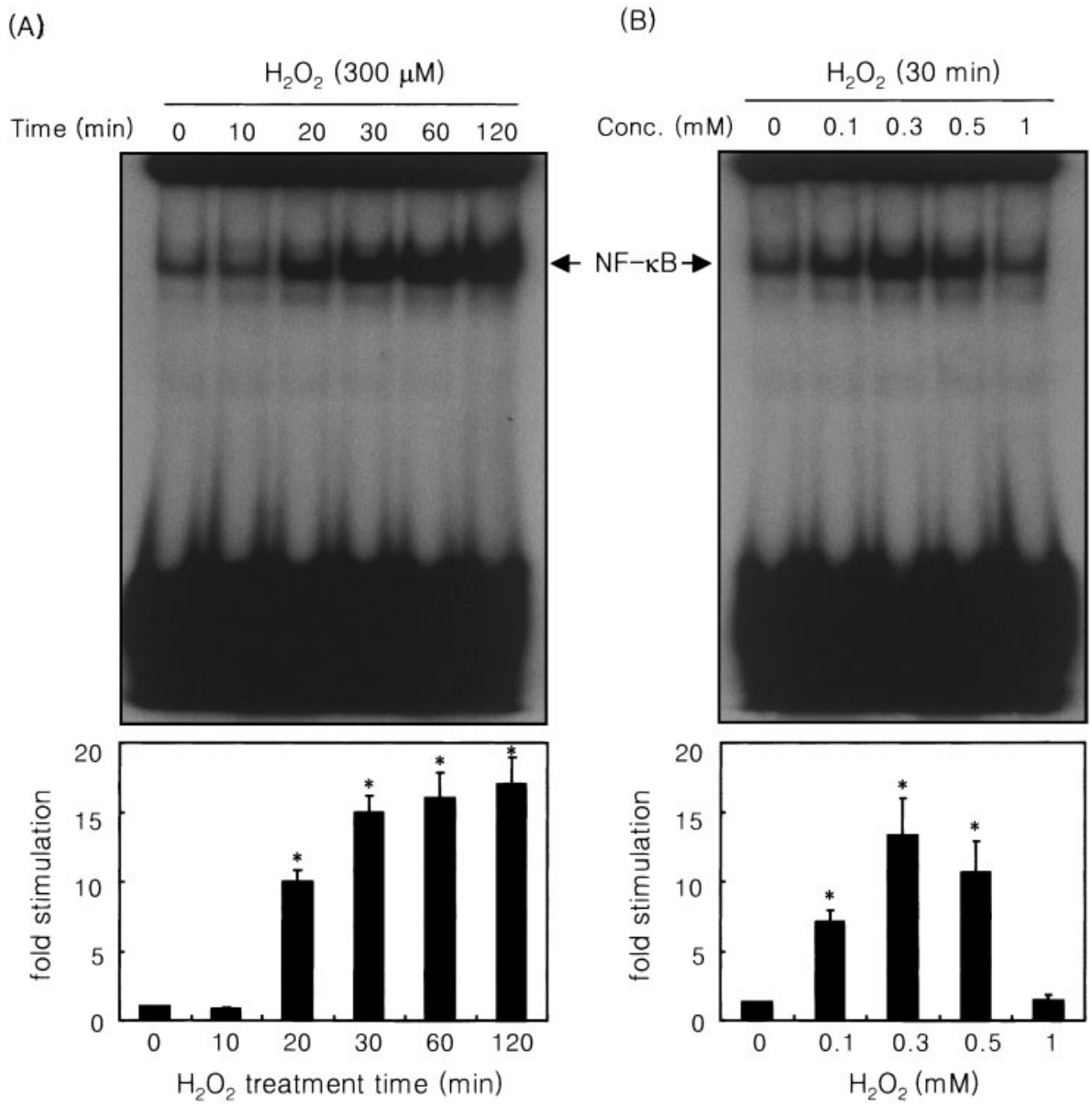


Fig. 1. NF-κB activation by hydrogen peroxide (H_2O_2). Murine 70Z/3 pre-B cells were treated with 300 μM H_2O_2 for the indicated time (**A**), or at indicated concentrations for 30 min (**B**). Nuclear extracts were prepared, incubated with a ^{32}P-labeled κB probe, and analyzed by EMSA. The positions of NF-κB complexes are shown. Identity of the shifted bands was confirmed by competition with unlabeled oligomer containing NF-κB site. The **upper panel** presented is representative of three independent experiments. The **lower panel** represents data quantitated by densitometry, expressed as fold stimulation. The values are the mean of three separate experiments with error bar representing the standard deviations (*$P < 0.05$).

(tyrosine kinase inhibitor) or GF 109203X (pan PKC inhibitor), which were pretreated the cells for 2 h before H_2O_2 treatment. On the other hand, since previous reports showed that MAP kinase pathway is important in the activation of NF-κB [Nakano et al., 1998], we also investigated the effect of PD 98059 (MEK inhibitor), or SB 203580 (p38 kinase inhibitor) on NF-κB activation by H_2O_2. PD 98059 markedly inhibited H_2O_2-induced NF-κB activation suggesting a critical role for MEK1. U0126, another selective MEK inhibitor, also effectively reduced the activation of NF-κB (data not shown). In contrast, SB 203580 enhanced rather than inhibited H_2O_2-induced NF-κB activation. This finding is consistent with the reported results that SB203580 enhanced NF-κB transcriptional activity [Birkenkamp et al., 2000].

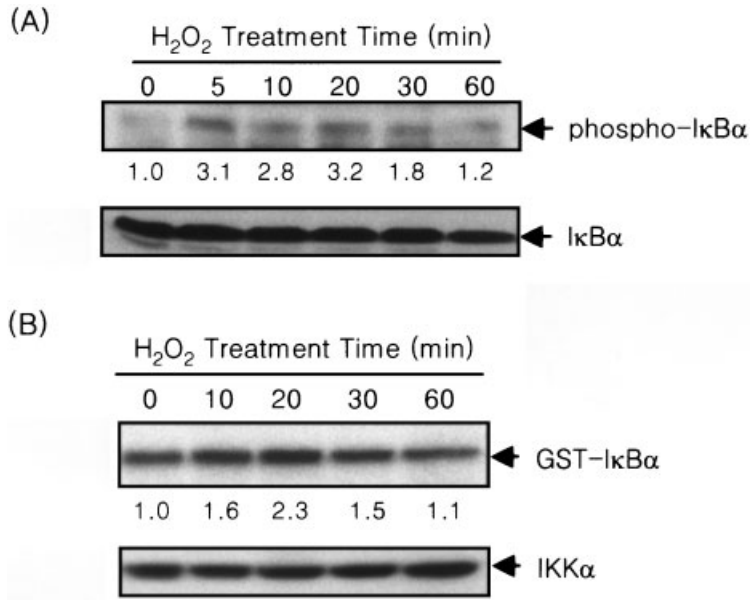


Fig. 2. Effect of H_2O_2 on IκB-α phosphorylation. **A**: 70Z/3 pre-B cells were treated with 300 μM H_2O_2 for the indicated time, and the whole cell lysates were analyzed by Western blot. To detect H_2O_2-induced IκB phosphorylation, it was used a polyclonal phospho-specific anti-IκB-α antibody which detects IκB-α only when activated by phosphorylation at Ser-32 (**upper panel**). In parallel experiments, immunoblot analysis was also performed using anti-IκB-α to show similar amounts of proteins in all lanes (**lower panel**). **B**: 70Z/3 pre-B cells were treated as indicated above. Both anti-IKKα and IKKβ antibodies were used to immunoprecipitate both kinases from the cell lysates. The immunoprecipitates of IKK complex were analyzed in the in vitro kinase assay using GST-IκBα as a substrate (**upper panel**). Equal amounts of the immunoprecipitated kinase complex present in each lane were confirmed by immunoblotting for IKKα (**lower panel**). Numbers listed below each band indicate value quantified by densitometry of immunoblots, expressed as fold change in phosphorylation, in which the phosphorylation observed in unstimulated cells was defined as 1. The results presented are representative of three independent experiments.

H_2O_2 Induces NF-κB Activation Through MEK1/ERK Signaling Independent of Ras-Raf

Since Ras/Raf signaling has been shown to be upstream activators of MEK1/ERK pathway, we further investigated the involvement of Ras/Raf in the activation of NF-κB in 70Z/3 cells exposed to H_2O_2. In this study, 70Z/3 pre-B cells were transiently transfected with a dominant negative Ras construct (RasN17), which blocks endogenous Ras function interfering with upstream activation of Ras proteins [Chen et al., 1994]. As previously reported [Pahan et al., 2000], the expression of a dominant negative mutant of Ras demonstrated inhibitory effects on NF-κB activation induced by lipopolysaccharide used as a positive control, indicating that the transfected cells expressed RasN17 proteins sufficient for inhibiting Ras function. However, transient transfection of RasN17 had little inhibitory effect on H_2O_2-induced NF-κB activation (Fig. 4A). In addition, B-581 and α-hydroxyfarnesylphosphonic acid, which block the Ras localization and function by inhibiting farnesyltransferase activity, showed no inhibitory effect on H_2O_2-induced NF-κB activation (Fig. 4B). Moreover, contrary to the idea that Raf-1 is involved in NF-κB activation [Flory et al., 1998], H_2O_2 did not activate Raf-1 detectably (Fig. 4C). LPS, used as a positive control, demonstrated activating effects on Raf-1. Since the electrophoretic mobility of Raf-1 has been known to be an adequate monitor for the activation state of Raf-1, we also investigated the mobility shift of the C-terminal fragment of Raf-1 (RfIII) expressed in 70Z/3 cells. It has been previously reported that this 33 kDa RfIII exhibited serum- and phorbol ester-induced shift in gel mobility that mimicked the shift observed with full-length Raf-1 [Olah et al., 1995]. A significant shift in electrophoretic mobility was observed with RfIII after LPS treatment of the overexpressor cells. However, H_2O_2 showed no effect on RfIII mobility shift (Fig. 4C). These results imply that MEK1/ERK signaling, which is associated with H_2O_2-induced NF-κB activation, is distinct from Ras/Raf.

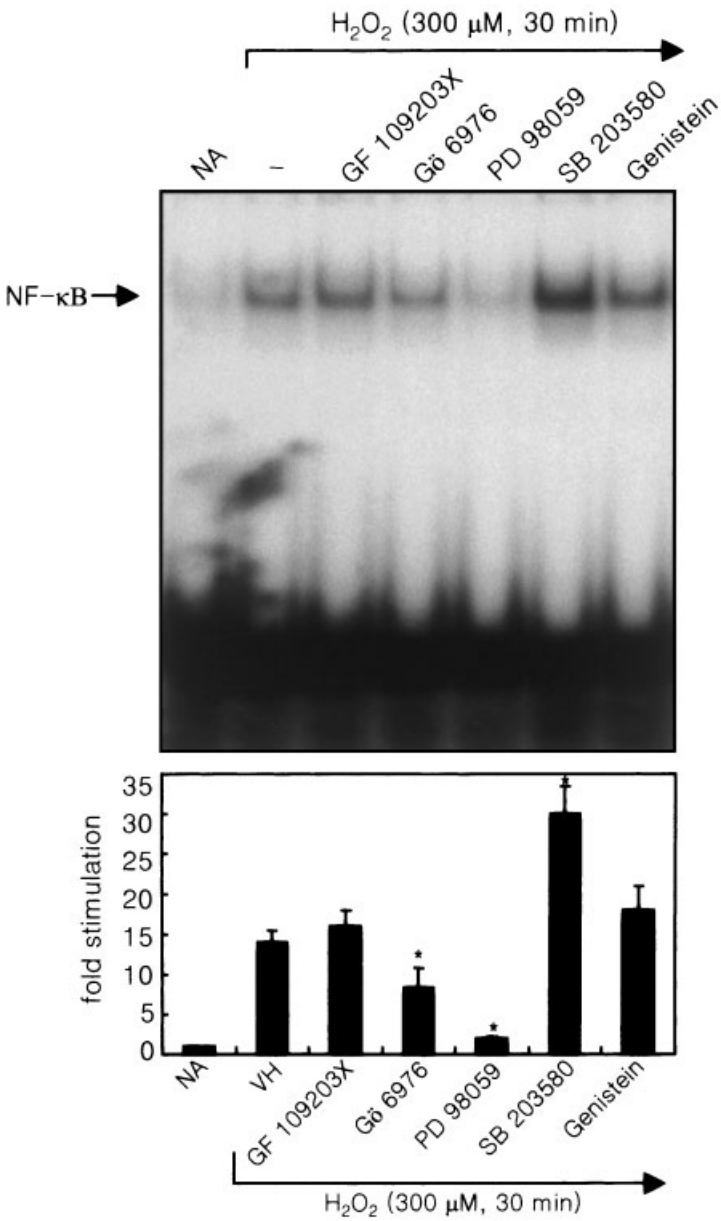
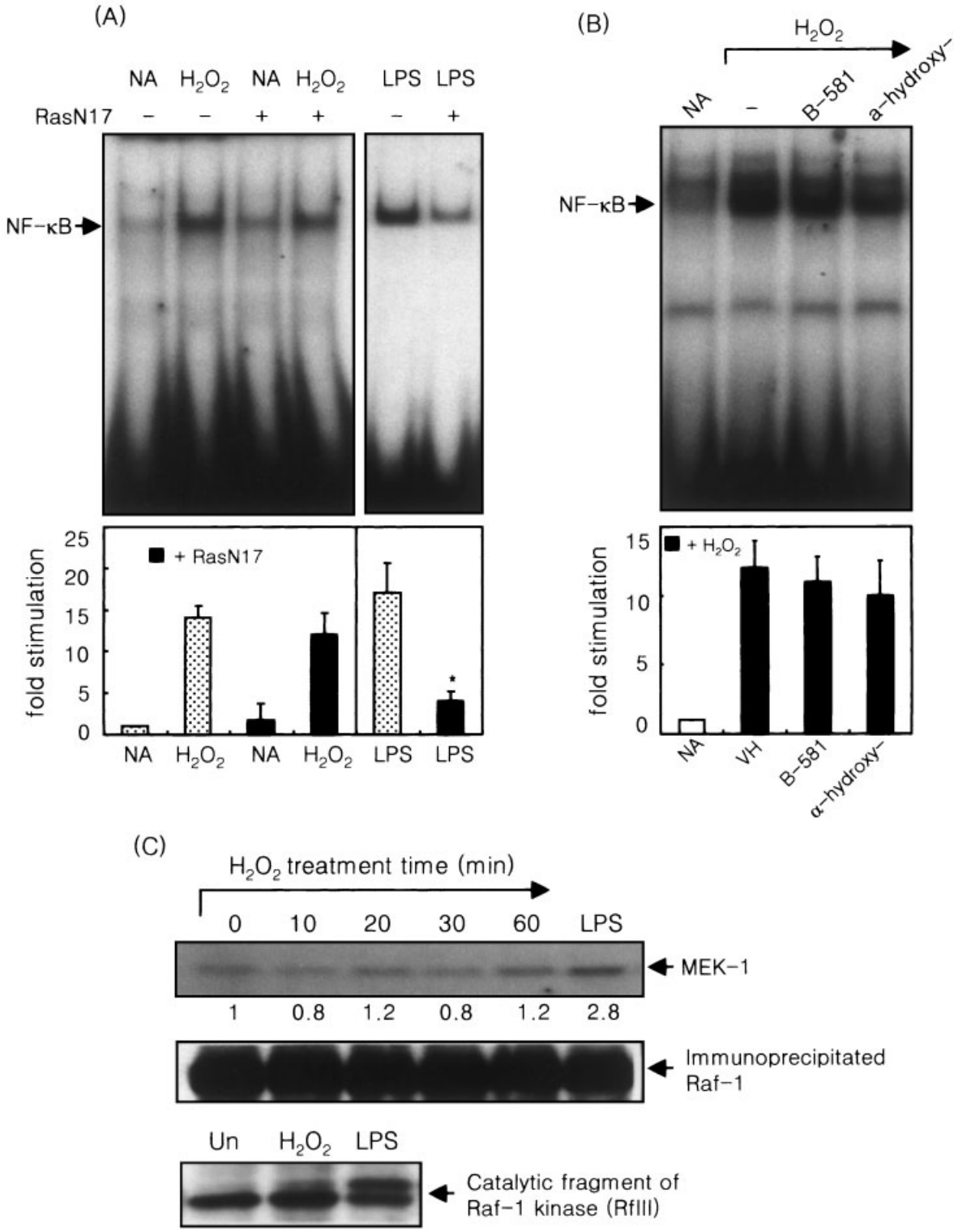


Fig. 3. MEK/ERK pathway is required for NF-κB activation. 70Z/3 pre-B cells were pretreated with either GF 109203X (1 μM), Gö 6976 (1 μM), PD 98059 (50 μM), SB 203580 (30 μM), or genistein (60 μM) for 2 h before stimulation with H_2O_2 for 30 min. EMSA was performed on nuclear extracts. Identity of the shifted bands was confirmed by competition with unlabeled oligomer containing NF-κB site. Results of one representative experiment of three are shown in the **upper panel**. The **lower panel** represents data quantitated by densitometry, expressed as fold stimulation. The values are the mean of three separate experiments with error bar representing the standard deviations (*$P < 0.05$).

Fig. 4. Ras/Raf pathway is not involved in H_2O_2-induced NF-κB activation. **A**: 70Z/3 pre-B cells were transfected with control vector or dominant negative Ras (RasN17). After 48 h, transfected cells were treated with either LPS or H_2O_2 for 30 min. Nuclear extracts were prepared, incubated with a ^{32}P-labeled κB probe, and analyzed by EMSA. **B**: 70Z/3 pre-B cells were pretreated (+) or not (–) with either B-581 (50 μM) or α-hydroxyfarnesylphosphonic acid (10 μM) for 24 h before treatment with H_2O_2 for 30 min. EMSA was performed on nuclear extracts. The **lower panels** of (A) and (B) represent data quantitated by densitometry, expressed as fold stimulation. The values are the mean of three separate experiments with error bar representing the standard deviations (*$P < 0.05$). **C**: 70Z/3 pre-B cells were incubated in the presence of 0.5% serum for 24 h to minimize serum effect on Raf-1 kinase and then treated with either 300 μM H_2O_2 for the indicated time, or 1 μg/ml LPS for 20 min. For in vitro Raf-1 kinase assays, Raf-1 immunoprecipitates were incubated in kinase buffer containing 10 μCi of [γ-^{32}P]ATP in the presence of MEK1 as a substrate. Samples were resolved by electrophoresis on 10% SDS–PAGE, and the radioactivity incorporated into the ^{32}P-labeled MEK-1 protein was determined by autoradiography. In the **middle panel**, immunoprecipitates were prepared in parallel with those assayed for kinase activity, separated by SDS–PAGE, transferred to filters, and probed with anti-Raf antibody. In the **bottom panel**, 70Z/3 cells expressing C-terminal Raf fragment (RfIII) were incubated in the presence of 0.5% serum for 24 h prior to treatment with either vehicle, H_2O_2 (300 μM, 30 min), or LPS (1 μg/ml, 20 min). Proteins were separated by electrophoresis on SDS–polyacrylamide gels (12.5%). The electrophoretic mobility of RfIII was examined by immunoblot analysis using the anti-Raf antibody. The results presented are representative of three independent experiments.


(A)
NA
H2O2
NA
H2O2
LPS
LPS
RasN17
–
–
+
+
–
+
NF-κB
fold stimulation
25
20
15
10
5
0
+ RasN17
NA
H2O2
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H2O2
LPS
LPS
(B)
H2O2
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B-581
a-hydroxy-
NF-κB
fold stimulation
15
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VH
B-581
α-hydroxy-
(C)
H2O2 treatment time (min)
0
10
20
30
60
LPS
MEK-1
1
0.8
1.2
0.8
1.2
2.8
Immunoprecipitated
Raf-1
Un
H2O2
LPS
Catalytic fragment of
Raf-1 kinase (RfIII)

Fig. 4.

MEKK1 Pathway is Required for Hydrogen Peroxide-Induced NF-κB Activation

Since MEKK1 is implicated in the activation of IKK [Lee et al., 1997], the effect of H_2O_2 on MEKK1 activation was measured in 70Z/3 pre-B lymphocytes. We directly measured MEKK1 activity by immunoprecipitation with an antibody against MEKK1, followed by in vitro autophosphorylation assay. Despite few published direct demonstration, it is assumed that autoactivation of MEKK1 via autophosphorylation of Thr-575 might be an immediate response to initial kinase activation [Deak and Templeton, 1997]. The autophosphorylation of MEKK1 was first observed 10 min after treatment of 70Z/3 pre-B cells with H_2O_2, reaching a maximum at 30 min (Fig. 5A). The activation of JNK, which is a downstream effector of MEKK1, was also detectable in cells treated with H_2O_2 (Fig. 5B). Additionally, SEK1 and MEK1 activation was estimated by measuring the level of phosphorylation in whole cell lysates using an antibody raised against phosphorylated peptides that correspond to the active forms of SEK1 and MEK1. Surprisingly, H_2O_2 appeared to have no effect on SEK1 phosphorylation (Fig. 5C) despite that SEK1 is a downstream kinase of MEKK1. Rather, treatment of pre-B cells with H_2O_2 substantially increased MEK1 phosphorylation. Moreover, the kinetics of H_2O_2-induced MEK1 phosphorylation was well correlated with those of NF-κB binding activity. Phosphorylation of MEK1 was first observed 20 min after treatment of 70Z/3 pre-B cells with H_2O_2 and sustained for 2 h or more (Fig. 5D). When western blotting was performed, no change in the level of MEK1 protein was observed throughout the treatment period. Thus, these data suggest that MEK1 rather than SEK1 is involved in H_2O_2-induced activation of NF-κB that is dependent of MEKK1.

DISCUSSION

In the present study, we showed that H_2O_2 was able to stimulate the translocation of NF-κB to the nucleus in 70Z/3 pre-B cells. However, contrary to other reports [Bowie and O'Neill, 2000] that NF-κB activation by H_2O_2 was slow (120 min) and transient, H_2O_2-induced NF-κB activation occurred within 20 min of treatment, reached maximum level at 60 min, and sustained for 2 h or more. In addition, treatment of cells with H_2O_2 led to an increase of phosphorylated IκBα. Moreover, the present study showed that H_2O_2 elicited a slight and transient IKK activation. The results suggested by others [Jaspers et al., 2001], in which treatment with H_2O_2 increased IKK activity, concomitant with phosphorylation and ubiquitination of IκBα, showed consistency with our results.

MEKK1, which acts as an upstream activator of JNK, is implicated in the activation of IKK [Lee et al., 1997; Nakano et al., 1998]. In this study, we found that H_2O_2 increased the autophosphorylation of MEKK1 as well as phosphorylation of JNK. Despite few published direct demonstration, it is assumed that autoactivation of MEKK1 via autophosphorylation of Thr-575 might be an immediate response to initial kinase activation [Deak and Templeton, 1997]. The kinetics of H_2O_2-induced MEKK1 phosphorylation was well correlated with those of NF-κB binding activity, suggesting that MEKK1 positively regulate NF-κB activation in pre-B cells exposed to H_2O_2. However, H_2O_2 appeared to have no effect on SEK1 phosphorylation despite that the activation signal of MEKK1 passes through SEK1. Instead, MEK1, which is an upstream activator of ERK, was phosphorylated by H_2O_2 treatment. This result suggests that MEK1 may contribute to H_2O_2-mediated NF-κB activation as shown in the inhibition of H_2O_2-induced NF-κB binding activity by MEK inhibitor, PD 98059 (Fig. 3), although Janssen-Heininger et al. [1999] showed the dose-dependent activation of NF-κB after exposure to PD 98059. In contrast, Raf-1, which has been implicated in the activation of MEK1, is unlikely to be involved in H_2O_2-induced NF-κB activation because H_2O_2 exhibited no effect on Raf-1 kinase activity. This finding would be consistent with the reported results that Raf-1 did not phosphorylate a purified GST-IκB—a fusion protein [Hambleton et al., 1995]. In addition, a dominant negative Ras (RasN17) showed negligible inhibitory effects on H_2O_2-induced NF-κB activation implying a role for Ras/Raf signaling far more distal to NF-κB activation by H_2O_2, although several observations have demonstrated that Ras is an important sensor of redox stress [Lander et al., 1995]. Thus, H_2O_2-mediated MEKK1 activation seems to be attributable to the activation of MEK1/ERK pathway. In fact, the catalytic domain of MEKK1 activates MEK1 by phosphorylating it on the same sites that Raf-1 phosphorylates,

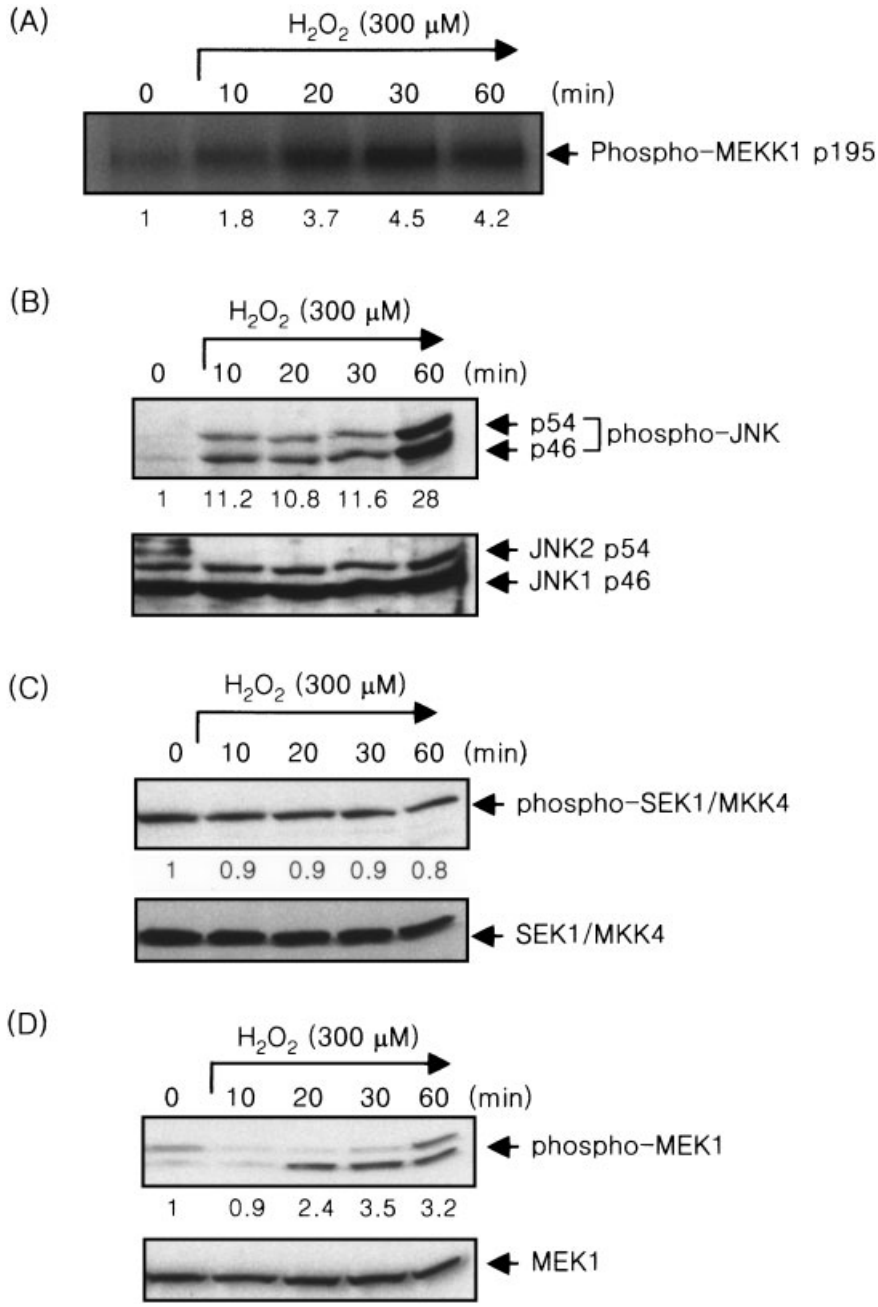

Fig. 5. The activation of multiple pathways including MEKK1/JNK and MEK1 by H_2O_2. **A**: Murine 70Z/3 pre-B cells were treated with 300 μM H_2O_2 for the indicated time, and in vitro autokinase assays were performed on the immunoprecipitated MEKK1 proteins to determine MEKK1 autophosphorylation. The autophosphorylation of MEKK1 is indicated by arrow. **B–D**: H_2O_2-induced phosphorylations of JNK, SEK1, and MEK1 were investigated using antibodies that detect kinases only when activated by phosphorylation. Murine 70Z/3 pre-B cells were stimulated with 300 μM H_2O_2 for the indicated time, and the whole cell lysates were analyzed by Western blot. The phosphorylated forms of JNK (B), SEK1 (C), and MEK1 (D) were detected with immunoblotting using anti-phospho-JNK (Thr183/Tyr185), anti-phospho-SEK1 (Thr261) and anti-phospho-MEK1 (Ser217/221) antibodies. The same blot was stripped and then reprobed with anti-JNK, anti-SEK1, and anti-MEK1 antibodies to show similar expression level of each protein in all lanes. In all experiments, 70Z/3 pre-B cells were incubated in the presence of 0.5% serum for 24 h prior to H_2O_2 treatment to minimize serum effect. Numbers listed below each band indicate value quantified by densitometry of immunoblots, expressed as fold change in phosphorylation, in which the phosphorylation observed in unstimulated cells was defined as 1. Results of one representative experiment of three are shown.

and MEKK1 activates ERK directly through MEK1 [Gardner et al., 1994; Karandikar et al., 2000]. INF-α was also known to regulate the activity of the MEK/ERK pathway through a Ras/Raf-independent mechanism [Romerio et al., 2000]. On the other hand, it has been recently reported that JNK could be activated by a constitutively active MEK1 besides SEK1 [Bagowski et al., 2001]. However, in our study, MEK1 did not phosphorylate JNK in vitro (data not shown), suggesting that an indirect pathway may connect the MEK1/ERK cascade to the JNK cascade. Therefore, our examination of MAPK pathways revealed that MEKK1-dependent MEK1/ERK and JNK signaling are required for H_2O_2-induced NF-κB activation, such as okadaic acid-induced IL-6 gene expression, which is mediated through the ERK1/2 and JNK pathway-dependent activation of NF-κB transcriptional capacity [Tuyt et al., 1999].

Since redox signaling has been shown to activate NF-κB by pathway that does not involve phosphorylation or degradation of IκBα [Imbert et al., 1996; Flohe et al., 1997], we also examined an alternative pathway to NF-κB activation. An alternative route to NF-κB activation, independent of IκB phosphorylation, involves the kinase Tpl-2/Cot (tumour progression locus 2 kinase) which can phosphorylate p105, leading to its processing into p50. In addition, Tpl-2 phosphorylates and activates both MEK-1 and SEK-1 in vitro [Hagemann et al., 1999]. However, the Cot response by H_2O_2 was not detectable in 70Z/3 pre-B cells (data not shown).

Many results have demonstrated a critical role for the PKC isoforms in the NF-κB pathway at the level of IKK activation and IκB degradation [Lallena et al., 1999; Trushin et al., 1999]. In this study, a highly selective cell-permeable conventional PKC inhibitor, Gö 6976 partially inhibited H_2O_2-induced NF-κB activation. Conversely, treatment of cells with GF 109203X, which inhibits the conventional and novel isoforms, had little or no effect on H_2O_2-induced NF-κB activation. Although GF 109203X inhibits conventional PKCs in addition to novel PKCs, it is unlikely to be effective as much as Gö 6976 in the inhibition of conventional PKCs. Therefore, we can not exclude the possibility that conventional PKCs play, in part, an important role in the H_2O_2-induced NF-κB activation. However, Kaul et al. reported that conventional PKC activity may modulate basal NF-κB activity but does not participate in H_2O_2-stimulated NF-κB activation [Kaul et al., 1998], implying that inhibitory effect of Gö 6976 is due to the inhibition of basal NF-κB activity.

Originally, it has been known that NF-κB activation suppresses the signals for cell death. However, Lin et al. [1999] reported the paradoxical role of NF-κB in apoptosis, functioning as both a pro-apoptotic and anti-apoptotic regulatory factor within a single cell type. Our results lead to conclusion that Ras/Raf-independent, MEKK1-dependent MEK1 and JNK activation caused NF-κB activation, resulting in immune suppression in 70Z/3 pre-B cells. Further experiments are required to determine if the dominant negative mutant of MEKK1 prevents H_2O_2 from activating MEK1 and NF-κB.

REFERENCES

Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB, Rhee SG. 1997. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. J Biol Chem 272: 217–221.

Bagowski CP, Xiong W, Ferrell JE. 2001. c-Jun N-terminal kinase activation in *Xenopus laevis* eggs and embryos. A possible non-genomic role for the JNK signaling pathway. J Biol Chem 276:1459–1465.

Baldwin JAS. 1996. The NF-κB and IκB proteins: New discoveries and insights. Annu Rev Immunol 14:649–683.

Birkenkamp KU, Tuyt LML, Lummen C, Wierenga ATJ, Kruijer W, Vellenga E. 2000. The p38 MAP kinase inhibitor SB203580 enhances nuclear factor-kappa B transcriptional activity by a non-specific effect upon the ERK pathway. Br J Pharmacol 131:99–107.

Bowie A, O'Neill LAJ. 2000. Oxidative stress and nuclear factor-κB activation. Biochem Pharmacol 59:13–23.

Chang L, Karin M. 2001. Mammalian MAP kinase signalling cascades. Nature 410:37–40.

Chen S-Y, Huff SY, Lai C-C, Der CJ, Powers S. 1994. Ras-G15A protein shares highly similar dominant-negative biological properties with Ras-17N and forms a stable, guanine-nucleotide resistant complex with CDC25 exchange factor. Oncogene 9:2691–2698.

Deak JC, Templeton DJ. 1997. Regulation of the activity of MEK kinase 1 (MEKK1) by autophosphorylation within the kinase activation domain. Biochem J 322:185–192.

Dent P, Haser W, Haystead TAJ, Vincent LA, Roberts TM, Sturgill TW. 1992. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science 257:1404–1407.

Dumont A, Hehner SP, Hofmann TG, Ueffing M, Droge W, Schmitz ML. 1999. Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-κB. Oncogene 18:747–757.

El-Hag A, Lipsky PE, Bennett M, Clark RA. 1986. Immunomodulation by neutrophil myeloperoxidase and hydrogen peroxide: Differential susceptibility of human lymphocyte functions. J Immunol 136:3420–3426.

Finkel T. 1998. Oxygen radicals and signaling. Curr Opin Cell Biol 10:248–253.

Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L. 1997. Redox regulation of NF-κB activation. Free Radic Biol Med 22:1115–1126.

Flory E, Weber CK, Chen P, Hoffmeyer A, Jassoy C, Rapp UR. 1998. Plasma membrane-targeted Raf kinase activates NF-κB and human immunodeficiency virus type 1 replication in T lymphocytes. J Virol 72:2788–2794.

Gardner AM, Vaillancourt RR, Lange-Carter CA, Johnson GL. 1994. MEK-1 phosphorylation by MEK kinase, Raf, and mitogen-activated protein kinase: Analysis of phosphopeptides and regulation of activity. Mol Cell Biol 5:193–201.

Ghosh S, May MJ, Kopp EB. 1998. NF-κB and Rel proteins: Evolutionarily conserved mediators of immune responses. Annu Rev Immunol 16:225–260.

Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ. 1996. Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury. J Biol Chem 271:4138–4142.

Hagemann D, Tropmair J, Rapp UR. 1999. Cot protooncoprotein activates the dual specificity kinases MEK-1 and SEK-1 and induces differentiation of PC12 cells. Oncogene 18:1391–1400.

Hambleton J, McMahon M, DeFranco AL. 1995. Activation of Raf-1 and mitogen-activated protein kinase in murine macrophages partially mimics lipopolysaccharide-induced signaling events. J Exp Med 182:147–154.

Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF. 1996. Tyrosine phosphorylation of IκBα activates NFκB without proteolytic degradation of IκBα. Cell 86:787–798.

Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, Sundaresan M, Finkel T, Goldschmidt-Clermont PJ. 1997. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. Science 275:1649–1652.

Israel A. 2000. The IKK complex: An integrator of all signals that activate NF-κB? Trends Cell Biol 10:129–133.

Janssen-Heininger YNW, Macara I, Mossman BT. 1999. Cooperativity between oxidants and tumor necrosis factor in the activation of nuclear factor (NF)-κB: Requirement of Ras/mitogen-activated protein kinases in the activation of NF-κB by oxidants. Am J Respir Cell Mol Biol 20:942–952.

Jaspers I, Zhang W, Fraser A, Samet JM, Reed W. 2001. Hydrogen peroxide has opposing effects on IKK activity and IκBα breakdown in airway epithelial cells. Am J Respir Cell Mol Biol 24:769–777.

Karandikar M, Xu S, Cobb MH. 2000. MEKK1 binds Raf-1 and the ERK2 cascade components. J Biol Chem 275: 40120–40127.

Karin M. 1999. The beginning of the end: IκB kinase (IKK) and NF-κB activation. J Biol Chem 274:27339–27342.

Kaul N, Gopalakrishna R, Gundimeda U, Choi J, Forman HJ. 1998. Role of protein kinase C in basal and hydrogen peroxide-stimulated NF-κB activation in the murine macrophage J774A.1 cell line. Arch Biochem Biophys 350:79–86.

Kyriakis JM, App H, Zhang X-F, Banerjee P, Brautigan DL, Rapp UR, Avruch J. 1992. Raf-1 activates MAP kinase-kinase. Nature 358:417–421.

Lallena M-J, Diaz-Meco MT, Bren G, Paya CV, Moscat J. 1999. Activation of IκB kinase β by protein kinase C isoforms. Mol Cell Biol 19:2180–2188.

Lander HM, Ogiste JS, Teng KK, Novogrodsky A. 1995. p21ras as a common signaling target of reactive free radicals and cellular redox stress. J Biol Chem 270: 21195–21198.

Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260:315–319.

Lee M, Jeon YJ. 2001. Paclitaxel-induced immune suppression is associated with NF-κB activation via conventional PKC isotypes in lipopolysaccharide-stimulated 70Z/3 pre-B lymphocyte tumor cells. Mol Pharmacol 59:248–253.

Lee M, Yang KH. 2000. 2-acetylaminofluorene suppresses immune response through the inhibition of nuclear factor-κB activation during the early stage of B cell development. Toxicol Lett 114:173–180.

Lee M, Yea SS. 2000. Hydrogen peroxide inhibits the immune response to lipopolysaccharide by attenuating signaling through c-Jun N-terminal kinase and p38 associated with protein kinase C. Immunopharmacology 48: 165–172.

Lee FS, Hagler J, Zhijian J, Chen ZJ, Maniatis T. 1997. Activation of the IκB kinase complex by MEKK1, a kinase of the JNK pathway. Cell 88:213–222.

Lin B, Williams-Skipp C, Tao Y, Schleicher MS, Cano LL, Duke RC, Scheinman RI. 1999. NF-κB functions a both a pro-apoptotic and anti-apoptotic regulatory factor within a single cell type. Cell Death Differ 6:570–582.

Minden A, Lin A, McMahon M, Lange-Carter C, Derijard B, Davis RJ, Johnson GL, Karin M. 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science 266:1719–1723.

Nakano H, Shindo M, Sakon S, Nishinaka S, Mihara M, Yagita H, Okumura K. 1998. Differential regulation of IκB kinase α and β by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci USA 95:3537–3542.

Olah Z, Ferrier A, Lehel C, Anderson WB. 1995. The 33-kDa C-terminal domain of Raf-1 protein kinase exhibits a Ras-independent serum- and phorbol ester-induced shift in gel mobility. Biochem Biophys Res Commun 214:340–347.

Pahan K, Liu X, McKinney MJ, Wood C, Sheikh FG, Raymond JR. 2000. Expression of a dominant-negative mutant of p21(ras) inhibits induction of nitric oxide synthase and activation of nuclear factor-κB in primary astrocytes. J Neurochem 74:2288–2295.

Rice NR, Ernst MK. 1993. In vivo control of NF-κB activation by IκB-α. EMBO J 12:4685–4695.

Romerio F, Riva A, Zella D. 2000. Interferone-α2b reduces phosphorylation and activity of MEK and ERK through a Ras/Raf-independent mechanism. Br J Cancer 83:532–538.

Rosa LF. 1997. Effect of adrenaline on lymphocyte metabolism and function. A mechanism involving cAMP and hydrogen peroxide. Cell Biochem Funct 15:103–112.

Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. 1995. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science 270:296–299.

True AL, Rahman A, Malik AB. 2000. Activation of NF-κB induced by H_2O_2 and TNF-α and its effects on ICAM-1 expression in endothelial cells. Am J Physiol Lung Cell Mol Physiol 279:L302–L311.

Trushin SA, Pennington KN, Algeciras-Schimnich A, Paya CV. 1999. Protein kinase C and calcineurin synergize to activate IκB kinase and NF-κB in T lymphocytes. J Biol Chem 274:22923–22931.

Tuyt LML, Dokter WHA, Birkenkamp K, Koopmans SB, Lummen C, Kruijer W, Vellenga E. 1999. Extracellular-regulated kinase 1/2, Jun N-terminal kinase, and c-Jun are involved in NF-κB-dependent IL-6 expression in human monocytes. J Immunol 162:4893–4902.

Woronicz JD, Gao X, Cao Z, Rothe M, Goeddel DV. 1997. IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. Science 278:866–870.

Yujiri T, Sather S, Fanger GR, Johnson GL. 1998. Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science 282:1911–1914.