Interleukin-1β Induction of NFκB Is Partially Regulated by H₂O₂-mediated Activation of NFκB-inducing Kinase*

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Reactive oxygen species (ROS) have been demonstrated to act as second messengers in a number of signal transduction pathways, including NFκB. However, the mechanism(s) by which ROS regulate NFκB remain unclear and controversial. In the present report, we describe a mechanism whereby interleukin-1β (IL-1β) stimulation of NFκB is partially regulated by H₂O₂-mediated activation of NIK and subsequent NIK-mediated phosphorylation of IKKα. IL-1β induced H₂O₂ production in MCF-7 cells and clearance of this ROS through the expression of GPx-1 reduced NFκB transcriptional activation by inhibiting NIK-mediated phosphorylation of IKKα. Although IκKα and IKKβ were both involved in IL-1β-mediated activation of NFκB, only the IκKα-dependent component was modulated by changes in H₂O₂ levels. Interestingly, in vitro reconstitution experiments demonstrated that NIK was activated by a very narrow range of H₂O₂ (1–10 μM), whereas higher concentrations (100 μM to 1 mM) inhibited NIK activity. Treatment of cells with the general Ser/Thr phosphatase inhibitor (okadaic acid) lead to activation of NFκB and enhanced NIK activity as a IκKα kinase, suggesting that ROS may directly regulate NIK through the inhibition of phosphatases. Recruitment of NIK to TRAF6 following IL-1β stimulation was inhibited by H₂O₂ clearance and Rac1 siRNA, suggesting that Rac-dependent NADPH oxidase may be a source of ROS required for NIK activation. In summary, our studies have demonstrated that redox regulation of NIK by H₂O₂ is mechanistically important in IL-1β induction of NFκB activation.
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NIK has been shown to interact with IKKα in yeast two-hybrid systems (35) and has the potential to specifically phosphorylate IKKα (36). It has been suggested that ROS play an important role in the activation of NFκB (37), however, this concept remains controversial (7). Various forms of ROS, including superoxide anion (O2-) and hydrogen peroxide (H2O2), have been implicated in cell signaling. ROS are tightly controlled in cells by a group of antioxidant factors, including glutathione peroxide (GPx), superoxide dismutase (SOD), catalase, peroxiredoxins, and small factors such as glutathione and thioredoxin. Among these, GPx is a group of selenoenzymes responsible for reducing various hydroperoxides in the presence of the reduced form of glutathione (38). At least four GPx isoforms have been identified (39). GPx-1 is the isoform that exists in the cytoplasm, and it uses glutathione to degrade H2O2 into water.

A number of studies have revealed that ROS are involved in IL-1β signal transduction. For example, IL-1β stimulates ROS generation in a number of cell systems (40–42). In addition, studies that have used chemical antioxidants, such as diamide, N-acetyl-L-cysteine, and phenylarsine oxide, have shown significant effects on IL-1β-induced signaling (43, 44). Although there remain disagreements over the applications of these antioxidants that might weaken this argument (7), a number of studies have also used specific ROS modulation enzymes to confirm the importance of ROS. For example, specific antioxidant enzymes, such as GPx-1 and Mn-SOD, have demonstrated strong inhibitory effects on TNFα- or IL-1β-induced signal transduction (6, 45). In addition, factors that control ROS generation by cells have been shown to influence IL-1β induction of NFκB and the transcriptional activation of downstream genes (46, 47). However, the mechanisms of ROS action in the IL-1β pathway remain poorly defined.

In this report, we have investigated the role of cellular H2O2 in IL-1β induction of NFκB. Using GPx-1 expression to modulate cellular H2O2, we have demonstrated that H2O2 plays an important role in NIK activation of IKKα following IL-1β stimulation. We found that H2O2 imparts its activation on this cascade by promoting TRAF6 association with NIK and potentially inhibiting phosphatases that inactivate NIK. Reconstitution experiments demonstrated that a very narrow range of H2O2 concentration (1–10 μM) facilitate NIK activation, whereas higher levels of H2O2 inhibit NIK activity. These findings shed light on the molecular mechanism by which ROS act to regulate NFκB activation by IL-1β.

**EXPERIMENTAL PROCEDURES**

Recombinant Adenoviral Vector Infection and siRNA Transfection—Adenoviral infections were performed in serum-free medium for 2 h at an m.o.i. of 500 particles/cell, followed by the addition of an equal volume of fresh media containing 20% fetal bovine serum. Cells were fed with fresh media at 24 h and cells were analyzed at 48 h post-infection. These conditions produced >90% transduction with recombinant adenovirus, as assessed with Ad.CMV-GFP reporter gene expression. Eight different types of recombinant adenoviruses were used, including: Ad.BglII (empty control vector that does not express a transgene), Ad.NFκBLC (a NFκB-responsive luciferase reporter vector) (48), Ad.GPX-1 (GPx-1 tagged with a c-Myc epitope at the N terminus) (6), Ad.IKKα(KM) (a dominant negative mutant form of IKKα) (6), Ad.IKKβ(ΔN) (a dominant negative mutant form of IKKβ) (6), Ad.NIK(DN) (a truncated dominant negative NIK adenoviral vector kindly provided by Dr. Robert Schwabe at the University of North Carolina) (49, 50), Ad.IκBαAS (an adenoviral vector that expresses the antisense IκBα cDNA and activates NFκB by reducing levels of the IκBα repressor) (51), and Ad.IκBαS/A (an adenoviral vector that expresses a dominant negative IκBα mutant (S32/36A) that inhibits NFκB activation by preventing IKK-mediated phosphorylation of IκBα) (52). For NFκB transcriptional luciferase assays, MCF-7 cells were infected with Ad.NFκBLuc at an m.o.i. of 500 particles/cell 24 h prior to experimental treatments. Human NIK siRNA and Rac1 siRNA were purchased from a library of pre-screened siRNAs made by Santa Cruz Biotechnology, and transfections were performed following the manufacturer’s protocol.

Cell Culture and Treatment—MCF-7 cells (a human breast cancer cell line obtained from ATCC) were chosen for studies due to their low level of endogenous GPx-1. MCF-7 cells were grown in minimal essential medium with Eagle’s salts and L-glutamine, 1% minimal essential medium non-essential amino acids, 10% fetal bovine serum, 1% penicillin/streptomycin. For H2O2 treatment, concentrated H2O2 (30%) (Fisher Scientific, Fair Lawn, NJ) was diluted to 1 mM with deionized H2O and added to fresh medium at a final concentration of 1 mM. The spent medium was removed from MCF-7 cells and quickly replaced with medium containing 1 mM H2O2. After incubation at 37°C for 1 h (or otherwise indicated), the medium was changed to fresh medium without H2O2, and incubation of cells at 37°C was continued. IL-1β inductions were performed using 1 ng/ml human IL-1β (R&D Systems, Minneapolis, MN) for the indicated times. Control MCF-7 cells were also fed fresh medium but did not receive any treatment. Cells were harvested for luciferase assays at 6 h following H2O2 or IL-1β treatments. MCF-7 cells were washed twice with ice-cold PBS and were prepared for each assay accordingly.

Western Blots—Cell lysates were prepared and normalized for protein concentrations using a Bio-Rad Kit (Bio-Rad, Philadelphia, PA). Western blotting was performed using standard protocols. In brief, 50 μg of crude proteins for each condition were separated on a denaturing 10% SDS-PAGE and transferred to nitrocellulose (Hybond C, Amer- sham Biosciences). The membranes were then blocked and probed with primary antibody for 1 h at room temperature using dilutions suggested by the manufacturer. After being washed with blocking buffer several times, the membranes were probed with the appropriate dilution of secondary antibody. Immunoreactive proteins were detected using either an enhanced chemiluminescence ECL (Amer sham Biosciences) and exposure to x-ray film, or an Odyssey Infrared Imaging System (LI-COR Biotech, Lincoln, NE). The antibodies against IKKα, IKKβ, NIK, GST, FLAG, IL-1R1, MEKK1, and TRAF6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rac1 antibody was purchased from BD Transduction Laboratories (Lexington, KY).

Luciferase Assays—Luciferase activity was measured using a kit from Promega (Madison, WI) according to the manufacturer’s instructions. MCF-7 cells were infected with Ad.NFκBLCuc 24 h prior to treatment. Ad.NFκBLCuc contains the luciferase gene driven by four tandem copies of the NFκB consensus sequence fused to a TATA-like promoter from the herpes simplex virus thymidine kinase gene. 5 μg of total protein from each sample was used to perform the luciferase assays.

Immunoprecipitation—Cell samples were washed with ice-cold PBS twice and were lysed with radioimmuno precipitation assay buffer (150 mM NaCl, 50 mM Tris, pH 7.2, 1% deoxycholate, 1% Triton X-100, 0.1% SDS) at 4°C for 30 min. Protein concentrations were determined using a Bio-Rad kit and 500 μg of cellular protein, and 5 μl of primary antibody was mixed with 1 ml of radioimmuno precipitation assay buffer at 4°C for 1 h. Then 50 μl of Protein-A-agarose beads (Santa Cruz Biotechnology) was added to the mixture, and the mixture was rotated for 4 h. The beads were spun down at 5000 rpm for 5 min at 4°C and washed with ice-cold PBS three times prior to analysis of immunoprecipitates.
In Vitro Kinase Assay—Kinases (NIK, MEKK1, IKKα, or IKKβ) were immunoprecipitated with their respective antibodies and then mixed with 1 μg of the appropriate protein substrate (IKKα, IKKβ, or IkBα) in 0.3 ml cold ATP, 10 μCi of [γ-32P]ATP, and 10 μl of kinase buffer (40 mM Hepes, 1 mM β-glycerophosphate, 1 mM nitrophenol phosphate, 1 mM Na3VO4, 10 mM MgCl2, and 2 mM dithiothreitol). Reaction mixtures were incubated at 30 °C for 30 min (or shorter times as indicated), and reactions were then terminated by the addition of SDS-PAGE loading buffer at 98 °C for 5 min. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane and exposed to x-ray film.

Detection of Cellular ROS Production Using H2DCFDA—Stock solutions of H2DCFDA (Molecular Probes, Eugene, OR) were generated in Me2SO at a concentration of 50 μg/ml immediately prior to use. Cells were washed three times with PBS prior to simultaneous treatment with H2DCFDA (10 μM) and IL-1β (1 ng/ml) for 20 min in PBS at 37 °C in the dark. For samples infected with adenoviral vectors or transfected with siRNAs, this was done 48 h prior to stimulation with IL-1β. When DPI (10 μM) was used to inhibit NADPH oxidases, it was added at the time of IL-1β stimulation. Cells were washed in PBS at 20 min post-stimulation and then were fixed for 10 min in 4% paraformaldehyde. Cells were subsequently mounted in 4',6-diamidino-2-phenylindole containing antifadent and were examined by fluorescent microscopy for DCF signal. Exposure times were constant for all experimental samples.

RESULTS

Cellular H2O2 Influences IL-1β-mediated NFκB Activation—Previous studies have demonstrated that IL-1β stimulation of various cell types leads to cellular ROS production (53–55). Others have also demonstrated that H2O2 influences NFκB transcriptional activation following a number of stimuli, including TNF, UV, and IL-1β (3, 6). Furthermore, direct treatment of cells with H2O2 has the ability to activate NFκB (6, 56, 57). However, one study has also suggested that NFκB activation by IL-1β occurs in the absence of induced ROS in epithelial cells (42). In the present study, we sought to better understand potential mechanisms by which H2O2 influences IL-1β-mediated activation of NFκB. The MCF-7 breast cancer cell line was chosen for these studies, because it expresses a very low level of endogenous GPx-1 (an antioxidant enzyme responsible for degrading cellular H2O2 to water) and enables efficient modulation of cellular H2O2 levels through the overexpression of recombinant human GPx-1 (6).

We first sought to confirm that IL-1β stimulation of MCF-7 cells led to an increase in cellular H2O2. Indeed, our studies demonstrated that IL-1β stimulation of MCF-7 cells enhanced H2DCFDA fluorescence, suggesting that H2O2 is elevated following IL-1β treatment (Fig. 1A). Furthermore, infection with recombinant adenovirus expressing GPx-1 (Ad.GPx-1), but not the control Ad.BglII empty vector, significantly attenuated H2DCFDA fluorescence following IL-1β stimulation (Fig. 1A). These findings confirmed that IL-1β stimulates cellular H2O2 production in MCF-7 cells and that ectopic expression of GPx-1 could successfully modulate the cellular redox environment.

We next evaluated the extent to which GPx-1 expression could modulate IL-1β induction of NFκB using a NFκB-dependent luciferase reporter. Indeed, we observed that IL-1β induction of NFκB at 6 h was significantly attenuated following infection with Ad.GPx-1 virus, as compared with the infection with an empty adenoviral control vector (Ad.BglII) (Fig. 1B). In contrast, ectopic expression of GPx-1 had no effect on baseline transcriptional activity of NFκB. As a control for the ability of GPx-1 expression to modulate H2O2 induction of NFκB, we
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FIGURE 2. GPx-1-mediated clearance of H$_2$O$_2$ inhibits NFkB activation upstream to IkBa stabilization. MCF-7 cells were infected with the indicated recombinant adenovirus vectors at an m.o.i. of 500 particles/cell for each virus. After 24 h, cells were re-infected with Ad.NFkBLuc for 24 h at 500 particles/cell prior to direct analysis (A) or stimulation with IL-1$\beta$ (1 ng/ml) (B and C). A, the ability of antisense IxBa mRNA expression (Ad.IxBa(AS)) to constitutively induce NFkB transcriptional activation was assessed in the presence of Ad.BglII (control vector) or Ad.GPx-1 co-infection. B and C, NFkB transcriptional activation was evaluated at 6 h following treatment with IL-1$\beta$ by assessing the relative luciferase activity in 5 $\mu$g of protein lysate. In panel B, the mean relative light units (RLU/min) (±S.E., n = 3) are given for each sample as an index of NFkB transcriptional activation. In C, the mean change (±S.E., n = 3) in NFkB transcriptional activation following IL-1$\beta$ stimulation is given (the baseline level of luciferase activity, as determined from Ad.BglII-infected cells in the absence of IL-1$\beta$, was subtracted from all experimentally induced values). Statistical comparisons of marked groups using the Student’s $t$ test are given with $p$ values.

H$_2$O$_2$ Influences NFkB Activation Upstream of IkBa Phosphorylation—

We next sought to investigate the molecular mechanism through which H$_2$O$_2$ influences IL-1$\beta$-mediated NFkB transcriptional activation. IkBa plays a pivotal role in NFkB activation; phosphorylation of IkBa on two serines (Ser-32/Ser-36) leads to its disassociation from NFkB in the cytoplasm, proteasome-dependent degradation of IkBa, and mobilization of NFkB to the nucleus. To evaluate whether H$_2$O$_2$ influences NFkB activation downstream of IkBa (i.e. after NFkB dissociates from IkBa and moves to the nucleus), we used an antisense IkBa cDNA expressed from recombinant adenovirus (Ad.IxBa(AS)) that represses IkBa protein levels and induces nuclear translocation of NFkB (51). As previously shown in HeLa cells (51), infection with Ad.IxBa(AS) significantly reduced the level of IkBa protein in MCF-7 cells (data not shown) and led to the induction of NFkB transcriptional activity in the absence of cytokine stimulation (Fig. 2A). In the presence of antisense IkBa mRNA, overexpression of GPx-1 did not alter NFkB transcriptional activity, suggesting that H$_2$O$_2$ levels do not directly influence the transcriptional activity of the NFkB complex once it has been mobilized from IkBa.

We next sought to evaluate whether H$_2$O$_2$ might influence IL-1$\beta$-dependent NFkB activation by regulating factors that phosphorylate IkBa. Indeed, the majority of NFkB activation following IL-1$\beta$ stimulation is mediated by IkBa serine phosphorylation, as indicated by a nearly complete block in NFkB activation by adenoviral expression of the dominant negative IkBa mutant (IkBaS32/36A) (Fig. 2B). Furthermore, overexpression of GPx-1 and IkBaS32/36A gave a similar level of inhibition as seen with IkBaS32/36A alone, suggesting that GPx-1 regulates components of the NFkB pathway upstream of IkBa. Based on previous reports for other stimuli demonstrating redox-dependent activation of the IKK complex (6, 48, 58–61), we reasoned that H$_2$O$_2$ might also influence activation of the IKK complex following IL-1$\beta$ stimulation.

To approach this question, we used two dominant negative adenoviral vectors that express mutant forms of either the IKKa (Ad.IKKaKM) or IKKB (Ad.IKKBKA) subunits of the IKK complex. Results from these analyses demonstrated that Ad.IKKaKM or Ad.IKKBKA infection of MCF-7 cells inhibited ~50% of IL-1$\beta$-dependent NFkB activation (Fig. 2C). The level of inhibition observed by overexpression of GPx-1 was similar to that seen with IKKaKM and slightly less than that seen with IKKBKA. Co-infection with Ad.IKKaKM and Ad.IKKBKA gave nearly complete inhibition of NFkB activation, suggesting that these two subunits of the IKK complex primarily control NFkB activation by IL-1$\beta$. To address whether GPx-1-sensitive signaling was directed through IKKa or IKKB, we performed co-expressing studies with each of these IKK mutants and GPx-1. We reasoned that if H$_2$O$_2$ influenced IKKa activation, inhibition of NFkB in the presence of IKKaKM would be unaffected by GPx-1 co-expression. Furthermore, if this hypothesis was correct, we would expect to observe enhanced inhibition of NFkB following infection with Ad.IKKBKA plus Ad.GPx-1, as compared with Ad.IKKBKA or Ad.GPx-1 infection alone. The reverse scenario would be true if H$_2$O$_2$ selectively influenced IKKB activation (i.e. Ad.IKKaKM plus Ad.GPx-1 infection would provide an enhanced level of inhibition,
as compared with each vector alone). Our results from these experiments (Fig. 2C) demonstrated significant synergism in the inhibition of IL-1β-dependent NFκB activation in the presence of both IKKα/β and GPx-1 expression, as compared with each individually. In contrast, inhibition of NFκB was similar following IKKαKM plus GPx-1 overexpression, as compared with expression of IKKαKM or GPx-1 alone (Fig. 2C). These findings suggest that GPx-1 acts to inhibit IKKα, but not IKKβ, activation following IL-1β stimulation. Because both IKKα and IKKβ contribute to NFκB activation by IL-1β, we conclude that ROS control only half of the NFκB activation pathways in response to IL-1β.

H₂O₂ Regulates IKK Kinase Activity—Given that GPx-1 expression appeared to modulate IKKα activation following IL-1β stimulation, we next sought to directly confirm that H₂O₂ could activate the IKK complex in vivo. To this end, we used in vitro kinase assays to monitor IKK activity following IL-1β stimulation and compared these results to that observed following direct H₂O₂ stimulation of MCF-7 cells. This assay utilized immunoprecipitated IKKα or IKKβ, followed by in vitro phosphoroylation of GST-ÎºκBα in the presence of [γ-³²P]ATP. Following IL-1β stimulation, both IKKα and IKKβ kinase activities were substantially increased, peaking at ~15–30 min following stimulation (Fig. 3A). The total cellular protein levels of both kinases were also examined, and no changes occurred following IL-1β stimulation (Fig. 3C). However, because IKKα and IKKβ form a complex in vivo (as evident by immunoprecipitation of IKKα with an IKKβ-specific antibody, Fig. 3C), it was impossible to separate the extent to which IKKα and/or IKKβ was activated in the IKK complex. Similar changes in IKKα- and IKKβ-mediated GST-ÎºκBα phosphorylation were seen following treatment of cells with 1 mM H₂O₂ (Fig. 3B). Collectively, these studies demonstrated that H₂O₂ can directly activate the IKK complex in vivo and suggested a plausible mechanism for IL-1β-induced regulation of NFκB.

H₂O₂-dependent Activation of NIK Contributes to NFκB Activation by IL-1β—We next sought to investigate the molecular mechanism by which H₂O₂ preferentially modulated IKKα following IL-1β stimulation. Previous reports have implicated the redox-dependent activation of IKK following H₂O₂ treatment of cells (62). Furthermore, NIK has previously been demonstrated to preferentially phosphorylate IKKα over IKKβ (36). Hence, NIK was an obvious potential candidate for the redox regulation of NFκB by IL-1β. To investigate the involvement of NIK in NFκB activation following IL-1β stimulation, we utilized a dominant negative NIK mutant (49, 50). Adenovirus-mediated expression of this NIK mutant in MCF-7 cells (Fig. 4A) significantly inhibited both H₂O₂- and IL-1β-mediated activation of NFκB (Fig. 4B). To evaluate whether the GPx-1-sensitive component of NFκB activation by IL-1β was mediated through NIK, we performed co-infection experiments with Ad.NIK(DN) and Ad.GPx-1 virus. Results from these experiments demonstrated that expression of dominant negative NIK or GPx-1 significantly inhibited NFκB activation to similar extents following H₂O₂ (Fig. 4C) or IL-1β stimulation (Fig. 4D). Importantly, the level of NFκB inhibition seen following combined infection of cells with Ad.NIK(DN) and Ad.GPx-1 virus was similar to that seen following infection with each vector alone for both H₂O₂ (Fig. 4C) and IL-1β (Fig. 4D) stimulation. These results support the hypothesis that NIK plays an important role in NFκB activation by IL-1β.

Redox Activation of NIK Preferentially Regulates IKKα following IL-1β Stimulation—Our data thus far have implicated NIK in the redox-dependent activation of NFκB by IL-1β. Furthermore, our results suggest that activation of IKKα was involved in this pathway of redox activation. To this end, we hypothesized that H₂O₂ activation of NIK enhanced its ability to phosphorylate IKKα. Given the apparent specificity of the redox component of IL-1β signaling for the IKKα subunit of the IKK complex (Fig. 2C), we first sought to evaluate whether NIK specifically controlled IKKα activation in response to IL-1β or H₂O₂ stimulation. To this end, we generated bacterial GST fusion proteins for both IKKα and IKKβ and evaluated the ability of NIK to phosphorylate these two proteins following IL-1β or H₂O₂ stimulation. Generation of full-length IKKα and IKKβ GST fusion protein was not successful due to bacterial toxicity. As an alternative strategy, we generated truncated IKKα and IKKβ GST fusion proteins that comprised their respective activation domains phosphorylated by IKK kinases. For IKKα, this region included the sequence between Gly-131 to Trp-205, a 75-amino acid peptide containing Ser-176 and Ser-180 sites known to be phosphorylated and critical for IKKα activity. Similarly, an IKKβ fusion was generated from Ala-132 to Trp-206. This 75-amino-acid peptide contained Ser-177 and Ser-181 sites known to be phosphorylated and critical for IKKβ activity (Fig. 5A). These two fusion proteins were purified (Fig. 5B) and used for in vitro kinase assays following H₂O₂ or IL-1β treatment. Results from these studies demonstrated that H₂O₂ and IL-1β treatments stimulated immunoprecipitated NIK to phosphorylate GST-IKKα, but not GST-IKKβ (Fig. 5C). Furthermore, the extent of NIK activation by these two stimuli (Fig. 5C) was reflected in their respective abilities to activate NFκB in transcriptional assays (Fig. 4B) (i.e. IL-1β > H₂O₂). To confirm that the inability of NIK to phosphorylate GST-IKKβ was not the result of poor fusion protein quality, we evaluated the ability of MEKK1 to phosphorylate GST-IKKβ following TNFα treatment. MEKK1 has been shown to preferentially phosphorylate IKKβ (36). Indeed, immunoprecipitated MEKK1, from TNFα-treated cells, had a greater ability to phosphorylate GST-IKKβ as compared with GST-IKKα (Fig. 5C). These results confirmed that both
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**FIGURE 4.** Adenovirus-mediated expression of a dominant negative NIK mutant reduces NFκB transcriptional activation following H2O2 or IL-1β treatment of MCF-7 cells. A, MCF-7 cells were infected at an m.o.i. of 500 particles/cell with Ad.NIK(DN) or Ad.LacZ (negative control) adenoviral vectors. At 48 h post-infection, total cell lysates were prepared and analyzed by Western blotting to detect the HA-tagged NIK mutant. B, the effect of NIK(DN) overexpression on NFκB transcriptional activation following H2O2 or IL-1β treatment was evaluated in MCF-7 cells using a NFκB driving luciferase reporter assay. MCF-7 cells were infected with Ad.NIK(DN) or Ad.BglII (control virus) at 500 particles/cell. At 24 h post-infection, cells were re-infected with Ad.NFκB-Luc (500 particles/cell) for an additional 24 h prior to stimulation with H2O2 (1 mM) or IL-1β (1 ng/ml). Cells were harvested 6 h after treatment, and luciferase activity was determined. Results depict the mean relative luciferase units (RLU) per minute (± S.E., n = 3). C and D, MCF-7 cells were infected with Ad.BglII, Ad.GPx-1, and/or Ad.NIK(DN) at an m.o.i. of 500 particles/cell of each virus. At 24 h post-infection, cells were re-infected with Ad.NFκB-Luc (500 particles/cell) for an additional 24 h prior to stimulation with H2O2 (1 mM) or IL-1β (1 ng/ml). Cells were harvested 6 h after treatment, and luciferase activity was determined. Results depict the mean change (± S.E., n = 3) in NFκB transcriptional activation following IL-1β and H2O2 stimulation. Paired comparisons (*, †) demonstrated significant differences (p < 0.05), as assessed by the Student’s t test.

GST-IKKβ and GST-IKKα were receptive kinase substrates and demonstrated that IL-1β and H2O2 treatments stimulate NIK to preferentially phosphorylate IKKα.

To provide further evidence that NIK predominantly signals through IKKα in the context of IL-1β-stimulated NFκB activation in vivo, we investigated whether expression of dominant negative NIK enhanced inhibition of NFκB in the presence of IKKα or IKKβ/A dominant mutants. Results from these experiments (Fig. 5D) demonstrated that Ad.NIK(DN) infection was only able to augment inhibition of IL-1β-mediated NFκB activation in the presence of Ad.IKKα or Ad.IKKβ/A but not Ad.IKKακM. These results mirrored those seen following co-infection with the IKK mutants and GPx-1 (Fig. 2C), suggesting that NIK acts predominantly through IKKα, but not IKKβ/A, in vivo following IL-1β stimulation to induce NFκB.

To directly evaluate the redox dependence of NIK activation following IL-1β stimulation, we next asked whether GPx-1 overexpression inhibited the ability of NIK to phosphorylate GST-IKKα in an *in vitro* kinase assay. As a control, we first tested whether activation of NIK, following treatment of MCF-7 cells with H2O2, would be inhibited by GPx-1 overexpression. Indeed, as shown in Fig. 5E, immunoprecipitated NIK from H2O2-treated cells had significantly attenuated GST-IKKα kinase activity following Ad.GPx-1 infection. Similarly, IL-1β-stimulated NIK kinase activity was significantly reduced in the presence of GPx-1 expression (Fig. 5E). Cumulatively, these data provide strong support that NIK activation following IL-1β stimulation is redox regulated by H2O2.

**Narrow Ranges of H2O2 Facilitate NIK Activation in the Presence of Other Cellular Factors**—We hypothesized that H2O2 might enhance NIK activation during IL-1β signaling through one of three mechanisms: 1) by direct action of H2O2 on NIK to enhance its kinase activity, 2) by activating unknown redox effectors within the cell that activate NIK, and/and or 3) through H2O2-mediated inhibition of phosphatases that inactivate NIK. To approach the first hypothesis, we attempted to directly activate immunoprecipitated NIK from unstimulated MCF-7 cells with increasing concentrations of H2O2. Results from these experiments demonstrated that a very narrow range (1–10 μM) of H2O2 was capable of activating NIK to phosphorylate GST-IKKα in *vitro* (Fig. 6A, compare lanes 2 and 3 to lane 1). Higher concentrations of H2O2 (100 μM and 1 mM) did not give rise to NIK activation in this assay (lanes 4 and 5). However, the extent of NIK activation following *in vitro* treatment with 1–10 μM H2O2 was still significantly lower than the level of NIK activation seen following direct treatment of MCF-7 cells with 1 mM H2O2 for 30 min (Fig. 6A, lane 1). These findings support the notion that other cellular factors may be required to facilitate the redox-dependent activation of NIK and that these factors are predominantly lost during the immunoprecipitation of inactive NIK.

To test whether other cellular factors were required for the H2O2-dependent activation of NIK, we performed *in vitro* NIK activation assays in crude cell lysates harvested from both untreated and H2O2-treated MCF-7 cells. Crude lysates were first treated with 1 μM to 1 mM H2O2 for 30 min, and then NIK was immunoprecipitated and assayed for its ability to phosphorylate GST-IKKα. Crude lysates generated from cells treated with 1 mM H2O2 served as an internal control for the maximal achievable NIK activation at each *in vitro* concentration of H2O2 used to stimulate NIK in crude lysates. Several interesting findings emerged from these studies (Fig. 6B). First, as seen following *in vitro* exposure of immunoprecipitated NIK (Fig. 6A, lanes 2 and 3), only 1 and 10 μM concentrations of H2O2 were able to activate NIK in crude lysates from...
FIGURE 5. Redox activation of NIK preferentially regulates IKKα-mediated induction of NfκB following H2O2 or IL-1β treatment of MCF-7 cells. A, diagram of bacterial-derived GST-IKK fusion proteins used for assessing IKK kinase activities in relationship to the full-length IKK proteins. The activation domains that contain Ser phosphorylation sites (*) are gray, and GST is black. For IKKa, a 75-aa fragment (from Gly-131 to Trp-205) within its activation domain was used (lanes 1-4). For IKKβ, a 75-aa fragment (from Ala-132 to Trp-206) in the activation domain was used (lanes 5-8). B, SDS-PAGE Coomassie Blue gel of purified GST fusion proteins. C, immunoprecipitated (IP) NIK or MEKK1 from MCF-7 cells treated with H2O2 (1 mM), IL-1β (1 ng/ml), or TNFα (0.5 ng/ml) for the indicated times was used in an in vitro kinase assay with [γ-32P]ATP and GST-IKKα or GST-IKKβ. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were then analyzed by autoradiography followed by Western blotting with anti-GST. D, MCF-7 cells were co-infected with Ad.NIK(DN) and Ad.IKKα(KM) or Ad.IKKβ(KA) at an m.o.i. of 500 particles/cell of each virus. Ad.BglII infection was used as a negative control. At 24 h post-infection, cells were re-infected with Ad.NFκB-luc (500 particles/cell) for an additional 24 h prior to stimulation with IL-1β (1 ng/ml) for 6 h. Cells were then harvested, and luciferase activity was determined as an index of NFκB transcriptional activity. Results depict the mean change (±S.E., n = 3) in NFκB transcriptional activation following IL-1β stimulation. Statistical comparisons using the Student’s t test are marked for p value grouped comparisons. E, MCF-7 cells were infected with Ad.GPx-1 or Ad.BglII at 500 particles/cell. At 48 h post-infection, cells were treated with H2O2 (1 mM) or IL-1β (1 ng/ml) for 30 min and lysates were prepared for in vitro kinase assays. NIK was immunoprecipitated and then incubated with [γ-32P]ATP and GST-IKKα for 30 min in 30°C in kinase buffer. The reactions were analyzed by SDS-PAGE and transferred to nitrocellulose membrane prior to autoradiography.

unstimulated cells (Fig. 6B, compare lanes 8 and 10 to lane 2). Exposure of unstimulated crude lysates to 10 μM H2O2 (lane 8) achieved a similar level of activation as seen following in vivo exposure of cells to 1 mM H2O2 (lane 1). Second, exposure of crude lysates to higher concentrations of H2O2 (100 μM and 1 mM), inhibited NIK activation, as evident by a decline in phosphorylated GST-IKKα in samples derived from cells pretreated with 1 mM H2O2 (compare lanes 1, 7, and 9 to lanes 3 and 5). This inhibition was also evident in lysates derived from untreated cells treated with increasing concentrations of H2O2 (compare lanes 4, 6, 8, and 10). In summary, these results substantiate the findings that only very narrow ranges of H2O2 can activate NIK and that unknown cytoplasmic factors enhance the redox-dependent activation of NIK.

The above studies suggested that H2O2 was able to activate NIK to phosphorylate IKKa. However, an alternative possibility was that some other unknown redox-regulated IKKa kinases (i.e. TAK1, etc.) might associate with NIK and thereby co-immunoprecipitate in our kinase assays and complicate the assignment of GST-IKKα phosphorylation to NIK. To formally address this possibility, we expressed FLAG-tagged wild type NIK (WT-NIK) or the dominant negative NIK mutant (DN-NIK) and assessed IKKa kinase activity following immunoprecipitation of the FLAG tag from H2O2-treated MCF-7 cell lysates. We reasoned that if H2O2 activated an alternative IKKa kinase that associated with NIK, this would be revealed as residual H2O2-induced IKKa kinase activity in precipitates of the kinase-dead DN-NIK mutant. Results from these experiments are shown in Fig. 6C. Immunoprecipitated FLAG-tagged WT-NIK demonstrated a significant increase in its ability to phosphorylate GST-IKKα following H2O2 treatment (Fig. 6C, compare lanes 7 to 8). This level of IKKa kinase activity was similar to what had been seen following immunoprecipitation of endogenous NIK (compare lanes 1 to 2). In contrast, FLAG immunoprecipitates from LacZ (negative control without a FLAG tag) or FLAG-tagged DN-NIK transfected cells failed to demonstrate H2O2-induced IKKa kinase activity (lanes 3–6). These studies suggest that other H2O2-activated IKKa kinases likely do not associate with NIK. However, given that the DN-NIK construct is a truncation mutant (49, 50), we cannot currently rule out that an alternative IKKa kinase might associate with the deleted region of NIK. However, we failed to see TAK1 (a known alternative IKKa kinase) association with NIK in MCF-7 cells prior to or following IL-1 or H2O2 treatment (data not shown), suggesting that if this occurs it does not involve TAK1.

The third potential mechanism by which H2O2 might enhance NIK activation during IL-1β signaling includes H2O2-mediated inhibition of phosphatases that inactivate NIK. NIK is known for its ability to auto-phosphorylate, so redox regulation by phosphatases seems reasonable
Redox Activation of NIK

(62). Although it is well recognized that H2O2-dependent inactivation of phosphatases plays important roles in signaling (45, 63), information on phosphatase regulation of NIK is lacking. To approach this question we assessed the effects of okadaic acid (a general Ser/Thr phosphatase inhibitor) on both NFκB activation and the activation of NIK to phosphorylate GST-IKKβ. Indeed, treatment of cells with increasing concentrations of okadaic acid significantly increased the transcriptional activation of NFκB in MCF-7 cells (Fig. 7A). Similarly, okadaic acid treatment of MCF-7 cells significantly enhanced the IKKα kinase activity of immunoprecipitated NIK (Fig. 7B) and also enhanced the association of NIK with TRAF6 (Fig. 7C, compare lanes 1 and 7). These findings suggest that certain Ser/Thr phosphatases may indeed play a role in NIK activation and its association with TRAF6; as such, Ser/Thr phosphatases are potential targets of H2O2-mediated inhibition following IL-1β stimulation.

H2O2 Modulates NIK Association with TRAF6—Results thus far have demonstrated that IL-1β-mediated activation of NIK leads to enhanced IKKα phosphorylation and is partially responsible for activation of NFκB. In vitro, the process of NIK activation by H2O2 appears to require unknown cellular factors and may also involve inhibition of phosphatases in vivo. TRAF6, which recruits NIK to the IL-1 receptor complex, is an integral part of generating an active IKKα kinase complex following IL-1β stimulation (64). To this end, we sought to investigate whether H2O2 modulated the association of NIK with TRAF6. Such a mechanism could explain why cell lysate was required for maximal H2O2-mediated activation of immunoprecipitated NIK (Fig. 6, A and B). We evaluated the extent to which IL-1β or H2O2 treatment enhanced the association of NIK with immunoprecipitated TRAF6. Results from these experiments demonstrated that IL-1β or H2O2 stimulation of MCF-7 cells increased the association of NIK with TRAF6 (Fig. 7C). Furthermore, degradation of H2O2 by Gpx-1 expression inhibited this association (Fig. 7C, lanes 3 versus 4 and lanes 5 versus 6). As expected, the increased association between TRAF6 and NIK promoted by phosphatase inhibition (i.e. OA treatment) was unaffected by Gpx-1-mediated clearance of H2O2 (Fig. 7C, lanes 7 versus 8). Together with earlier studies, these experiments provide strong support that H2O2 regulates NIK activity by modulating the association between NIK and TRAF6.

Rac1 and NADPH Oxidase Control the Redox-dependent Association of NIK with TRAF6—The source of ROS generation following IL-1β stimulation remains complex and controversial. Several studies have indirectly implicated NADPH oxidases as a ROS source, based on the ability of diphenyleneiodonium (a NADPH oxidase inhibitor, DPI) to prevent ROS-dependent activation of IL-1β induced genes such as E-selectin, inducible nitric-oxide synthase, c-fos, and collagenase (46, 65, 66). However, others have suggested that 5-lipoxygenase may be involved in IL-1β induction of ROS in lymphoid cells, while NADPH oxidase plays a selective role in monocytic cell-induced ROS following IL-1β stimulation (42). Rac1, a small GTPase, plays a central role in cellular ROS generation through certain NADPH oxidases (67). Rac1 has also been linked to IL-1β induction of p65NFκB in a murine thymoma cell line (47). However, it has been suggested that in epithelial cells, Rac1 and NADPH oxidase do not play a role in NFκB activation by IL-1β (42). Given the controversy surrounding potential sources of ROS following IL-1β stimulation, we sought to investigate the potential role of Rac1/
NADPH oxidase in the IL-1β-induced ROS found in MCF-7 mammary epithelial cells.

Using Rac1 siRNA to inhibit Rac1 and DPI to inhibit NADPH oxidase, we investigated the role of Rac1/NADPH oxidase in ROS production following IL-1β stimulation. Results from H2DCFDA staining demonstrated that both DPI and Rac1 siRNA effectively reduced ROS production in MCF-7 cells following IL-1β stimulation (Fig. 8A). No inhibition in ROS was seen following transfection with a scrambled siRNA control. Rac1 siRNA also effectively inhibited total Rac1 protein levels in cell lysates (Fig. 8B). These findings provide strong evidence that a Rac1-regulated NADPH oxidase controls ROS production following IL-1β stimulation.

Given that Rac1 was in part required for the stimulation of ROS following IL-1β treatment of MCF-7 cells, we next sought to better understand if Rac1 was also required for NIK recruitment to TRAF6 following IL-1β stimulation. Rac1 siRNA indeed reduced the ability of NIK to associate with TRAF6 following IL-1β stimulation (Fig. 8C). However, this inhibition was not seen following transfection with a scrambled siRNA control. Combined with earlier results, these data suggest that Rac1-mediated H2O2-dependent activation of NIK, through the inhibition of phosphatases, promotes association of TRAF6 with NIK.

Data demonstrating ligand-independent association of activated NIK with TRAF6 following okadaic acid or H2O2 treatment (Fig. 7) suggested that NIK can associate with TRAF6 prior to its recruitment to the receptor. However, it remained unclear if the redox-dependent activation of NIK was required for TRAF6 recruitment to IL-1R1. To address this question, we used NIK and Rac1 siRNAs to modulate the formation of NIK-TRAF6 complex formation following IL-1β stimulation. Results from these experiments demonstrated that NIK siRNA effectively inhibited total NIK in cell lysates (Fig. 8B), and as expected, also prevented NIK recruitment to TRAF6 following IL-1β stimulation (Fig. 8C). Importantly, inhibition of NIK protein levels had no effect on steady-state levels of TRAF6. To address whether NIK was required for TRAF6 recruitment to IL-1R1, we performed IL-1R1 pull-down assays in the presence of NIK, Rac1, or scrambled siRNAs (Fig. 8D). If the redox-sensitive complex formation between TRAF6 and NIK was absolutely required for binding of TRAF6 to IL-1R1, we would anticipate that both NIK and Rac1 siRNAs would prevent TRAF6 recruitment to IL-1R1. As shown in Fig. 8D, this was not the case. Rac1 siRNA effectively inhibited both the recruitment of NIK and TRAF6 to IL-1R1. However, NIK inhibition did not alter TRAF6 recruitment to IL-1R1 following IL-1β stimulation. This finding provides strong evidence that NIK binding to TRAF6 is not required for the recruitment of TRAF6 to ligand activated IL-1R1. Given that the association of TRAF6 with NIK can be directly activated by H2O2 or okadaic acid in the absence of a ligand signal, our data suggest that redox activation of NIK likely promotes TRAF6-NIK complex formation both prior to and following TRAF6 recruitment to the IL-1 receptor.

**DISCUSSION**

The mechanism by which ROS stimulates the NFκB pathway remains quite complex and multifaceted. IL-1β induction of the NFκB pathway is one example for which molecular mechanisms of ROS action...
FIGURE 8. Rac1 regulates NADPH oxidase-mediated ROS production and the recruitment of TRAF6 and NIK to IL-1R1 following IL-1β stimulation. A, MCF-7 cells were assessed for H2O2 production following IL-1β (1 ng/ml) stimulation for 20 min in the presence of H2DCFDA (10 µM). Treatment groups are marked on the fluorescent images and included: 1) transfection with Rac1-siRNA or scrambled siRNA 48 h prior to IL-1β stimulation, and 2) DPI (10 µM) or vehicle (Ctrl) treatment at the time of IL-1β stimulation. 4',6-Diamidino-2-phenylindole was included in the mounting media for identification of nuclei. B, MCF-7 cells were transfected with Rac1-siRNA, NIK-siRNA, or scrambled siRNA (scr-siRNA). Western blots for total cellular Rac1, NIK, and actin in the absence of IL-1 stimulation are shown at 48 h post-transfection. C, MCF-7 cells were transfected with Rac1-siRNA, NIK-siRNA, or scrambled siRNA for 48 h prior to IL-1β (1 ng/ml) stimulation for 20 min. TRAF6 was immunoprecipitated from cell lysates, and Western blots (WB) for TRAF6 and NIK are shown. D, MCF-7 cells were transfected with Rac1-siRNA, NIK-siRNA, or scrambled siRNA for 48 h prior to IL-1β (1 ng/ml) stimulation for 20 min and immunoprecipitation of IL-1R1. Western blots for NIK, TRAF6, and IL-1R1 are given for the various treatment groups as indicated following immunoprecipitation of IL-1R1.

In vitro reconstitution experiments attempting to directly activate immunoprecipitated NIK with H2O2 demonstrated that one or more cellular factors are required for the redox activation of NIK to phosphorylate GST-IKKα (Fig. 6). In vivo, H2O2 promoted NIK association with TRAF6 in the absence of a ligand signal, and GPx-1 expression inhibited IL-1β-induced TRAF6-NIK association and NIK activation (Figs. 7C and 5E). Given the close correlation between redox activation of NIK and its association with TRAF6, we anticipate that TRAF6 is a required cellular component necessary for NIK activation as an IKKζ kinase. The ability of okadaic acid to directly promote NIK kinase activity and association with TRAF6, in the absence of a ligand signal, suggests that H2O2-mediated inhibition of protein phosphatases may be responsible for the redox activation of NIK. Hence, we favor a model whereby TRAF6 must recruit to NIK prior to phosphorylating IKKζ. Although NIK binding to TRAF6 may be necessary for an active IKKζ kinase complex, this association was not necessary for TRAF6 to recruit to IL-1R1; TRAF6 effectively recruited to IL-1 stimulated IL-1R1 in the absence of NIK.

Cumulatively, these studies place NIK as a central redox-regulated signaling molecule in IL-1-mediated activation of NFκB. We propose a model whereby IL-1 induces Rac1-dependent ROS production through NADPH oxidase, which in turn leads to NIK activation through the inhibition of protein phosphatases and the recruitment of NIK/TRAF6 complexes to the IL-1 receptor.
