Hydrogen Peroxide Signaling through Tumor Necrosis Factor Receptor 1 Leads to Selective Activation of c-Jun N-terminal Kinase*

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†‡ The abbreviations used are: JNK, c-Jun N-terminal kinase; IκB, inhibitor of κB; IKK, inhibitor of κB kinase; TNFa, tumor necrosis factor α; TNF-R1, TNF receptor 1; TRADD, TNF-R1-associated death domain; TRAF2, TNF-R-associated factor 2; DD, death domain; RIP, receptor-interacting protein; PBS, phosphate-buffered saline; HAc, hemagglutinin; GST, glutathione S-transferase; TBS, Tris-buffered saline; GA, geldanamycin; CA, constitutively active.

The Lung is an important target for oxidant injury as a consequence of direct inhalation of oxidants or as a result of the production of oxidants during inflammation (1, 2). Although oxidants contribute to tissue damage, these species are also formed in virtually every cell type and are an integral part of normal cell function (3). Oxidants are required for proliferation (4–6), changes in cellular shape (7) and are involved in transcriptional regulation (2). Despite the emerging role of redox signaling in cellular physiology, the exact mechanisms by which oxidants act as signaling molecules are under intense investigation, and many critical targets remain enigmatic.

Binding of tumor necrosis factor-α (TNFα) to its receptor, TNF-R1, results in the activation of inhibitor of κB kinase (IKK) and c-Jun N-terminal kinase (JNK) pathways that are coordinately regulated and important in survival and death. We demonstrated previously that in response to hydrogen peroxide (H2O2), the ability of TNFα to activate IKK in mouse lung epithelial cells (C10) was inhibited and that H2O2 alone was sufficient to activate JNK and induce cell death. In the current study, we investigated the involvement of TNF-R1 in H2O2-induced JNK activation. In lung fibroblasts from TNF-R1-deficient mice the ability of H2O2 to activate JNK was inhibited compared with fibroblasts from control mice. Additionally, in C10 cells expressing a mutant form of TNF-R1, H2O2-induced JNK activation was also inhibited. Immunoprecipitation of TNF-R1 revealed that in response to H2O2, the adapter proteins, TRADD and TRAF2, and JNK were recruited to the receptor. However, expression of the adapter protein RIP, which is essential for IKK activation by TNFα, was decreased in cells exposed to H2O2, and its chaperone Hsp90 was cleaved. Furthermore, data demonstrating that expression of TRAF2 was not affected by H2O2 and that overexpression of TRAF2 was sufficient to activate JNK provide an explanation for the inability of H2O2 to activate IKK and for the selective activation of JNK by H2O2. Our data demonstrate that oxidative stress interferes with IKK activation while promoting JNK signaling, creating a signaling imbalance that may favor apoptosis.

Oxidants have been demonstrated to regulate the activation of c-Jun N-terminal kinase (JNK), as well as the transcription factor NF-κB (2). JNK is a member of the family of mitogen-activated protein kinases, which is well known to be activated by oxidants and a variety of other stresses in many cell types, including lung epithelial cells (1, 8, 9). The contribution of JNK to many phenotypic outcomes, including survival (10–13) and apoptosis (14, 15), appears to depend upon the cell type, stimulus, the duration of JNK activation as well as the engagement of other signaling modules (13). In this regard, oxidant-induced JNK activation has been linked to apoptosis (16, 17). Although oxidants have also been implicated in the activation of the transcription factor NF-κB, currently a number of significant controversies exist around the role of redox events in NF-κB activation (18, 19). Under basal conditions NF-κB is sequestered in the cytoplasm through binding to the inhibitor of κB (IκB) and upon phosphorylation of IκB by IκB kinase (IKK), IκB is rapidly degraded through via the 26 S proteasome, allowing NF-κB to translocate to the nucleus and activate the transcription of over 100 genes, including genes critical to cell survival (20–22).

The signaling events that are required for the activation of JNK and NF-κB have been investigated in great detail using the ligand tumor necrosis factor-α (TNFα). TNFα binds as a trimer to three TNF-R1 monomers causing aggregation of the intracellular death domains. The death domain (DD) containing protein, TRADD, binds directly to the DD of TNF-R1 and then recruits the adaptor molecule (23). TRAF2. TRAF2 is essential for JNK activation as well as the recruitment of the IKK complex to the receptor (24). Receptor-interacting protein (RIP) is recruited to TNF-R1 through interaction with TRADD and is essential for the activation of IKK (24). Additionally, TRADD can directly interact with FADD, which is involved in executing caspase-dependent cell death. Recently it has been demonstrated that activation of IKK and JNK following stimulation of TNF-R1 is coordinately regulated and that the activation of NF-κB regulates the extent and duration of JNK activation. Transcription of NF-κB-driven anti-apoptotic genes such as X-IAP is critical in preventing sustained activation of JNK and also in promoting survival (25, 26). Additionally, transient activation of JNK can also mediate survival signaling via JunD, which collaborates with NF-κB to increase the expression of the survival gene cIAP-2 (13). Conversely, when NF-κB-dependent gene transcription is prevented, JNK activa-
In this study, we have investigated the role of TNF-R1, an inflammatory cytokine, in vitro activity assessed by an in vitro kinase assay. Cells were treated with either pCDNA3 or p60/H9004/H9260, either 10 ng/ml TNF for 15 min or 300 μM H2O2 for 1 h. Lysates were harvested, HA-JNK immunoprecipitated, and JNK activity assessed by an in vitro kinase assay.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—A line of spontaneously transformed mouse alveolar type II epithelial cells (C10) was propagated in CRML-100 medium containing 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. Murine recombinant TNFα was purchased from Calbiochem. The JNK, TRAF2, TRADD, and Hsp90 antibodies were purchased from Santa Cruz. NF-κB activation is prolonged allowing the execution of TNF-induced apoptosis (25, 26).

Previously we demonstrated that the oxidant hydrogen peroxide (H2O2) inhibits IKK, activates JNK, and causes apoptosis (1, 8). In the present study, we have investigated the role of TNF-R1 in the activation of JNK by H2O2. We demonstrate here that H2O2 signals to JNK via TNF-R1 and TRAF2 and that IKK and NF-κB activation are prevented as a result of degradation of the adaptor protein, RIP, and cleavage of its chaperone, Hsp90.

RESULTS

H2O2 Signaling via TNF-R1

A. WT

B. WT p60ΔCD

nt

5% milk in tris-buffered saline (TBS). Levels of HA were detected with a monoclonal antibody (12CA5, Roche Applied Science). All other proteins were detected with antibodies from Santa Cruz according to the following protocol. Membranes blocked overnight in TBS/milk were washed two times for 15 min in TBS containing 0.05% Tween 20 and incubated with the primary antibody for 1 h at 4°C. Membranes were washed three times for 20 min in TBS/Tween 20 and incubated with a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After a 30-min wash with TBS/Tween 20, conjugated peroxidase was detected by ECL according to the manufacturer’s instructions (Amersham Biosciences). For immunoprecipitations, cells were grown to confluence in 100-mm dishes, washed three times with PBS, and treated with test agents in PBS. Cells were lysed in immunoprecipitation buffer (50 mM Hepes, pH 7.4, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1% aprotinin), and lysates were incubated with TNF-R1 antibody for 2 h with rocking at 4°C. After incubation with antibody, protein G-agarose beads (Invitrogen) were added for 1 h. Precipitates were washed three times with lysis buffer, and 2× Laemmli was added following boiling, loading onto a 10% polyacrylamide gel, and Western analysis.

Assessment of NF-κB Transcriptional Activity—C10 cells were transiently transfected with a 6×B-tk-luc plasmid containing 6 NFκB DNA elements and either TRAF2 or pCDNA3. Cells were treated with 1 ng/ml TNF for 4 h. Cells were lysed in Luciferase Assay Lysis Buffer (Promega, Madison, WI), and a luciferase assay was performed as previously described (8).

Activation of JNK and IKK following TNF-R1 activation is prolonged allowing the execution of TNF-induced apoptosis (25, 26). These results demonstrate that the ability of H2O2 to activate JNK was substantially decreased in TNF-R1−/− cells compared with wild type controls at time points ranging from 15 min to 2 h. As expected, TNFα-induced activation of JNK was also abrogated in TNF-R1−/− fibroblasts. Additionally, overexpression of a truncated form of TNF-R1 (p60ΔCD), which lacks the intracellular death domain in C10 cells also resulted in inhibition of the TNFα- or H2O2- induced activation of JNK (Fig. 1B). These results demonstrate that H2O2-induced activation of JNK requires TNF-R1.
H$_2$O$_2$ Signaling via TNF-R1

H$_2$O$_2$ Causes Degradation of RIP and Cleavage of Hsp90—Despite the dependence of TNF-R1 in the H$_2$O$_2$-induced activation of JNK, H$_2$O$_2$ does not activate IKK or NF-$k$B (8). The activation of IKK by TNF is required for the recruitment of RIP and Hsp90 (30) to the TNF-R1 DD. Hsp90 and RIP form a complex; destabilization of Hsp90 leads to RIP degradation and consequently prevents the activation of IKK and NF-$k$B (31). We therefore investigated whether the lack of IKK activation in cells treated with H$_2$O$_2$ was due to destabilization of RIP and/or Hsp90 and assessed the levels of TRAF2, RIP, and Hsp90 by Western blot analysis. As seen in Fig. 4A, TRAF2 levels were not affected by H$_2$O$_2$ or TNF in a time frame of up to 2 h. Evaluation of Hsp90 revealed that a lower molecular weight species of Hsp90 was present after treatment with H$_2$O$_2$ for 2 h (Fig. 4B) and first appeared after 30 min of exposure (data not shown). This Hsp90 cleavage product did not appear in cells treated with TNFα (Fig. 4B). In agreement with these findings, RIP levels were markedly decreased in cells treated with H$_2$O$_2$ for 2 h, whereas TNFα exposure did not affect RIP expression compared with sham controls (Fig. 4C, and data not shown). Because RIP and Hsp90 are required for IKK activation, their destabilization by H$_2$O$_2$ provides a plausible explanation for the inability of H$_2$O$_2$ to activate IKK.

Overexpression of TRAF2 Activates JNK and Inhibits the Ability of TNFα to Activate IKK and NF-$k$B—The results presented above demonstrate that in response to H$_2$O$_2$, TRADD and TRAF2 are recruited to the TNF-R1, whereas RIP and Hsp90, which are essential for IKK activation, are destabilized. The absence of RIP at TNF-R1 has been demonstrated to enhance recruitment of TRADD and TRAF2, suggesting a competition for binding at TNF-R1 between TRADD/TRAF2 and RIP (24). Therefore, an enhanced presence of TRAF2 at TNF-R1 in response to H$_2$O$_2$ may sustain JNK activation while inhibiting IKK. To confirm that the effects of H$_2$O$_2$ in C10 cells can be mimicked by TRAF2 accumulation at the receptor, we overexpressed wild-type TRAF2 and analyzed the activity of IKK, NF-$k$B, and JNK. TRAF2 overexpression was sufficient to inhibit the ability of TNFα to activate IKK and NF-$k$B (Fig. 5, A and B). Moreover, TRAF2 overexpression also led to activation of JNK under base-line conditions (Fig. 5C). Lastly, we determined whether H$_2$O$_2$-induced degradation of RIP and cleavage of Hsp90 would alter JNK and IKK activation in response to TNFα. As expected, pretreatment of C10 cells with H$_2$O$_2$ for 2 h, the time point associated with RIP degradation and Hsp90 cleavage (Fig. 4), led to a complete inhibition of IKK activation by TNFα. Although under these conditions JNK activation by H$_2$O$_2$ was not longer observed, pretreatment with H$_2$O$_2$ enhanced JNK activation in response to TNFα (Fig. 6). These data confirm that H$_2$O$_2$ causes a signaling imbalance at TNF-R1, leading to preferential activation of JNK, while inhibiting IKK.
Although it has been established that H$_2$O$_2$ is a signaling molecule that can activate JNK (8, 16, 32), the exact mechanism by which this occurs has not been elucidated. We demonstrate here that H$_2$O$_2$, in concentrations observed under inflammatory conditions (33–35), activates JNK in a TNF-R1-dependent manner.

**A**

**IP:** TNF-R1  
**IB:** TRADD  
1min  
5min  

-  
+  
-  

-  
+  
-  

**TNF**  
H$_2$O$_2$  

**B**

**Sham**  
**TNF 5min**  
**H$_2$O$_2$ 5min**  

-  
+  
-  

-  
+  
-  

**TNF-R1 IP:**  

-  
+  
-  

**IB:** TRADD  
1min  
5min  

-  
+  
-  

-  
+  
-  

**C**

**IP:** TNF-R1  
**IB:** TRAF2  
1min  
5min  

-  
+  
-  

-  
+  
-  

**TNF**  
H$_2$O$_2$  

**D**

**Sham**  
**TNF 5min**  
**H$_2$O$_2$ 5min**  

-  
+  
-  

-  
+  
-  

**TNF-R1 IP:**  

-  
+  
-  

**IB:** TRAF2  
1min  
5min  

-  
+  
-  

-  
+  
-  

**E**

**IP:** TNF-R1  
1V: HA-JNK  

**IVK:**  

30min  
60min  

-  
+  
-  

-  
+  
-  

-  
+  
-  

**TNF**  
H$_2$O$_2$  

**DISCUSSION**

Although it has been established that H$_2$O$_2$ is a signaling molecule that can activate JNK (8, 16, 32), the exact mechanism by which this occurs has not been elucidated. We demonstrate here that H$_2$O$_2$, in concentrations observed under inflammatory conditions (33–35), activates JNK in a TNF-R1-dependent manner.
dependent manner and, consequently, that H$_2$O$_2$-induced JNK is substantially decreased in cells lacking TNF-R1 or in cells expressing a truncated version of TNF-R1 lacking the intracellular death domain. We also provided evidence for the presence of TRAF2 and TRADD at TNF-R1 under base-line conditions, in agreement with recent reports demonstrating that adaptor proteins can be present in nonstimulated cells (36). We were only able to detect small changes in the recruitment of endogenously expressed adaptor proteins to TNF-R1 after stimulation of the lung epithelial cells examined here, in agreement with findings by others (19) but in contrast to reports that use expression cassettes or immune cells (24). Interestingly, analogous to observations demonstrating the presence of IKK at TNF-R1, we provide evidence here that JNK can also be recruited to and is active at TNF-R1.

The H$_2$O$_2$-induced destabilization of RIP and cleavage of its chaperone Hsp90, both of which are essential in the activation of IKK, provide a plausible explanation for the selective activation of JNK, but not IKK, by H$_2$O$_2$. A similar disruption of adaptor protein recruitment to the death domain of TNF-R1 has been demonstrated in response to geldanamycin (GA), a disruptor of Hsp90, which leads to the destabilization of RIP and consequently inhibits the activation of IKK and NF-$\kappa$B by TNF-$\alpha$ (30, 31). In agreement with our observations on H$_2$O$_2$, GA did not affect the stability of TRAF2 nor did it affect JNK activation. GA-induced RIP degradation involves the proteasome and appears to occur in a caspase- and lysosome-independent manner (31). It remains to be determined whether these events are also involved in RIP degradation by H$_2$O$_2$. It is of interest to note that GA brings about a disruption of endothelial nitric-oxide synthase, causing it to produce superoxide, the precursor of H$_2$O$_2$ (37). Thus, it is conceivable that H$_2$O$_2$ production may also contribute to RIP degradation and NF-$\kappa$B repression after GA treatment (31), although this possibility remains to be tested formally.

In cells overexpressing TRAF2, the ability of TNF-$\alpha$ to activate IKK and NF-$\kappa$B is inhibited, whereas JNK was constitutively active. In contrast to our observations, others have shown that TRAF2 overexpression leads to NF-$\kappa$B activation (22, 38–40). The discrepancy of their findings compared with our study may stem from the cell type and experimental conditions used. For example, it is possible that IKK activation by TRAF2 was transient, as opposed to JNK, and that we missed its elevated activity, or that the levels of TRAF2 expression conditions used. For example, it is possible that IKK activation by TRAF2 was transient, as opposed to JNK, and that we missed its elevated activity, or that the levels of TRAF2 expression.

Fig. 5. Overexpression of TRAF2 activates JNK and inhibits the ability of TNF-$\alpha$ to activate IKK and NF-$\kappa$B. A, C10 cells were transfected with 1 $\mu$g of TRAF2 or 1 $\mu$g of pcDNA3 in the presence of HA-IKK.B. Cells were treated with 10 ng/ml TNF-$\alpha$ for 5 min. HA-IKK.B was immunoprecipitated using and HA-specific antibody, an IKK activity was assessed in an in vitro kinase assay. B, C10 cells were transfected with 1 $\mu$g of NF-$\kappa$B-luc or 1 $\mu$g of pcDNA3 in the presence of 1 $\mu$g of TRAF2. Cells were treated with 1 ng/ml TNF-$\alpha$ for 4 h, and NF-$\kappa$B activity was detected in a luciferase assay. C, C10 cells were transfected with 1 $\mu$g of pcDNA or 1 $\mu$g of TRAF2 in the presence of 1 $\mu$g of HA-JNK1. HA-JNK1 was immunoprecipitated and JNK activity assessed in an in vitro kinase assay. The lower panel demonstrates the expression of HA-JNK.

Fig. 6. H$_2$O$_2$ disrupts TNF-$\alpha$-dependent signaling. Cells were exposed to 500 $\mu$M H$_2$O$_2$ for 2 h and then to 10 ng/ml TNF-$\alpha$ for an additional 5 min (top panel) or 15 min (middle panel) for the assessment of IKK or JNK, respectively, via in vitro kinase assays. The bottom panel demonstrates JNK levels, as a loading control.
TNFα-induced NF-κB activation upstream by interfering with the formation of the TNF-R1 signaling complex. It is plausible that H₂O₂ can affect binding of TNFα to its receptor or can promote its shedding. Recent observations demonstrate that redox events affect the binding of TNFα to TNF-R1 (19), and H₂O₂-induced shedding of soluble TNF-R1 has been demonstrated in lung epithelial cells (42). However, it is unlikely that these events have contributed to our present observations, as we have demonstrated that JNK activation by TNFα is enhanced in cells pretreated with H₂O₂ (Fig. 6), illustrating that oxidant-induced JNK activation can mediate apoptosis itself can alter the redox potential of the cell and uses oxidants to signal (for review, see Ref. 27). It is conceivable that the extent of oxidant production, including H₂O₂, dictates whether TNF-R1 activation by TNFα will lead to cell death or survival. Our present findings point to a putative proapoptotic role for oxidants under inflammatory conditions by causing direct signaling through TNF-R1, in addition to promoting TNFα-induced apoptosis via c-Jun N-terminal kinase.

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