Hydrogen Peroxide Prolongs Nuclear Localization of NF-κB in Activated Cells by Suppressing Negative Regulatory Mechanisms*

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NF-κB transcription factors induce pro-inflammatory molecules (e.g. IL-8) in response to cytokines (e.g. TNFα, IL-1β) or other stimuli. In the basal state, they are sequestered in the cytoplasm by inhibitory IkB proteins. Pro-inflammatory signaling triggers polyubiquitination of intermediaries (e.g. RIP1), which activate IkB kinases that trigger Ser phosphorylation and degradation of IkBα, thereby promoting nuclear translocation of NF-κB. A negative feedback loop exists whereby NF-κB drives resynthesis of IkBα, which promotes export of NF-κB from the nucleus to the cytoplasm. This process relies on Cezanne, a deubiquitinating cytoine protease that stabilizes resynthesized IkBα by removing polyubiquitin from modified intermediaries. H2O2 is generated during inflammation. Here we examined the effects of H2O2 on NF-κB dynamics and pro-inflammatory activation in cultured cells co-stimulated with TNFα or IL-1β. Quantitative reverse transcription-PCR and enzyme-linked immunosorbent assay revealed that H2O2 enhanced the induction of IL-8 by TNFα or IL-1β. We demonstrated by using assays of NF-κB nuclear localization and by imaging of live cells expressing a fluorescent form of NF-κB that H2O2 prolonged NF-κB nuclear localization in cells co-stimulated with TNFα or IL-1β by suppressing its export from the nucleus. We provide evidence that H2O2 suppresses NF-κB export by prolonging polyubiquitination of signaling intermediaries, which promotes Ser phosphorylation and destabilization of newly synthesized IkBα proteins. Finally, we observed that the catalytic activity of Cezanne and its ability to suppress RIP1 polyubiquitination and NF-κB transcriptional activity were inhibited by H2O2. We conclude that H2O2 prolongs NF-κB activation in co-stimulated cells by suppressing the negative regulatory functions of Cezanne and IkBα.

Pro-inflammatory cytokines (e.g. TNFα, IL-1β) drive inflammation by activating the redox-sensitive transcription factor NF-κB, which induces pro-inflammatory molecules including chemokines (e.g. IL-8). In unstimulated cells, NF-κB is sequestered in the cytoplasm through binding to inhibitory IkB molecules, which mask its nuclear localization sequence (1, 2). TNFα or IL-1β activate distinct pathways that converge to activate IkBα kinases (IKK), which phosphorylate IkBα at Ser residues (2–7). This process is regulated by ubiquitin, a protein that can be covalently attached to Lys residues of other cellular proteins in the form of isopeptide-linked polyubiquitin chains (8). Signaling through TNFR or IL-1/Toll-like receptor relies on modification of RIP1 or TRAF6 signaling intermediaries, respectively, with a "non-classical" form of polyubiquitin linked through Lys-63 that triggers the activation of IKKβ (9–13). In addition, polyubiquitin chains linked through Lys-48 play a well recognized role during NF-κB activation by targeting phosphorylated IkBα proteins for proteosomal degradation, thus liberating NF-κB for nuclear entry (2, 14–17).

The resolution of inflammatory responses is regulated by multiple negative feedback mechanisms that act in concert to suppress NF-κB activity. A key step in this process is the induction of IkBα by NF-κB in activated cells. Newly synthesized IkBα proteins translocate to the nucleus, where they target activated NF-κB complexes for export to the cytoplasm (18–21). NF-κB induces other negative regulators of pro-inflammatory signaling including A20 (22) and its sister molecule Cezanne (23), which belong to the OTU family of deubiquitinating cysteine proteases that can cleave ubiquitin monomers from modified proteins (13, 24–26). Both of these proteins can be recruited to activated TNFRs, where they suppress the activity of pro-inflammatory signaling intermediaries by removing polyubiquitin chains from them (13, 26). Thus suppression of TNFR signaling by A20 and Cezanne stabilizes newly synthesized IkBα, which inhibits NF-κB by removing it from the nucleus.

Reactive oxygen species (ROS) such as hydrogen peroxide are generated in inflamed tissues and may contribute to the pathogenesis of chronic inflammatory diseases including atherosclerosis, rheumatoid arthritis, and chronic obstructive pulmonary disease (27–29). Although numerous studies have identified a role for ROS in regulating signaling to NF-κB (30),

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‡The abbreviations used are: TNF, tumor necrosis factor; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; IKK, IkBα kinases; ROS, reactive oxygen species; CM-H2DCFDA, 5-(and 6-) chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate; GFP, green fluorescent protein; dsRed, Discosoma species red fluorescent protein; HA, hemagglutinin; Ub, ubiquitin; VME, vinylmethyl; CT, cycle threshold.
their potential effects on negative regulators of NF-κB have received little attention. Nevertheless, emerging reports suggest that ROS may enhance cellular activation by suppressing the activity of anti-inflammatory enzymes such as histone deacetylases (31) and mitogen-activated protein (MAP) kinase phosphatases (MKPs) (32). Here we demonstrate that H₂O₂ can suppress the negative regulatory functions of 1κBα and Cezanne, thus prolonging NF-κB nuclear localization and pro-inflammatory transcriptional responses in activated cells.

**EXPERIMENTAL PROCEDURES**

*Reagents and Antibodies—* Human recombinant TNFα and IL-1β were obtained commercially (R&D, Minneapolis, MN). Anti-RelA (sc372; Santa Cruz Biotechnology, Santa Cruz, CA), anti-1κBα (sc-847; Santa Cruz Biotechnology), anti-phospho1κBα Ser-32/Ser-36 (Cell Signaling Technology), anti-RIP1 (BD Biosciences Pharmingen, Oxford, UK), anti-HA (Roche Applied Science, Burgess Hill, UK), anti-tubulin (Sigma-Aldrich), and anti-lamin B antibodies (Santa Cruz Biotechnology) were obtained commercially. The generation of anti-Cezanne antibodies has been described (26). 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was obtained from Invitrogen. Other reagents were obtained from Sigma-Aldrich unless otherwise stated.

*Plasmids—* Expression vectors encoding HA epitope-tagged Cezanne (pHM6-Cezanne) or GFP-tagged Cezanne (pEGFP-Cezanne) have been described (23). An expression vector containing a dsRed-tagged version of RelA (pRelA-dsRed) was described previously (33).

*Cell Lines and Transfection—* HeLa and A549 cells were cultured using Dulbecco’s modified Eagle’s medium, 10% fetal-calf serum, supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin. Transient transfection of cells was achieved using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

*Measurement of Intracellular ROS—* Intracellular ROS were measured in live cells using CM-H₂DCFDA, a probe that forms a fluorescent adduct (dichlorofluorescein) when oxidized. Cells were incubated with 5 μM CM-H₂DCFDA for 15 min and then washed with phosphate-buffered saline prior to experimentation. Fluorescence was quantified by flow cytometry.

*ELISA to Detect IL-8—* The levels of IL-8 proteins in cell culture supernatants were quantified using a commercial enzyme-linked immunosorbenent assay (ELISA, human IL-8 Duol Set; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Absorbance (OD) was measured at 450 nm on a microtiter plate reader. Concentrations were obtained by interpolation from standard curves as described previously (34).

*Comparative Real-time PCR—* Transcript levels were quantified by comparative real-time PCR using gene-specific primers for IL-8 (sense, 5′-TGCGAAGGAGTGCAAAAG-3′; antisense, 5′-CTCCACACACCTCTCGC-3′), 1κBα (sense, 5′-CTGATGTCAGAGTGATCTACAG-3′; antisense, 5′-CCTGACTACTGTGCCTGATAG-3′), and β-actin (sense, 5′-CTGGAACGGTGAGGTGACA-3′; antisense, 5′-AAGGGACTTCTCTGTAACATGCA-3′) purchased from Sigma-Aldrich. Quantitation of GNB2L1 was carried out using primers obtained from Applied Biosystems (Foster City, CA).

Total RNA was extracted and reverse-transcribed as described previously (35). Real-time PCR was carried out using the iCycler system (Bio-Rad) and SYBR green master mix (Bio-Rad) according to the manufacturer’s instructions. Reactions were incubated at 95 °C for 3 min before thermal cycling at 95 °C for 10 s and 56 °C for 45 s. Reactions were performed in triplicate. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product (CT) as described previously (35). CT was calculated for the genes of interest and for the housekeeping gene β-actin. For each cDNA sample, the CT for β-actin was subtracted from the CT for each gene of interest to give the parameter ΔCT, thus normalizing the initial amount of RNA used. The amount of each target was calculated as 2^-ΔΔCT, where ΔΔCT is the difference between the ΔCT of the two cDNA samples to be compared.

*Assays of NF-κB Intracellular Localization—* Intracellular localization of endogenous RelA was assessed by immunostaining of paraformaldehyde-fixed cells using anti-RelA antibodies and Alexa Fluor 568-conjugated secondary antibodies followed by laser-scanning confocal microscopy (LSM 510 META; Zeiss, Oberkochen, Germany) following Ref. 35. Image analysis was performed using Velocity software (Improvision, Coventry, UK) to calculate the ratio of RelA present in the nucleus when compared with the cytoplasm. Alternatively, levels of endogenous RelA were measured in cytosolic or nuclear lysates prepared using the NucBuster kit (Novagen, San Diego, CA) by Western blotting using anti-RelA antibodies, horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection.

The intracellular localization of RelA was also assessed in real time using cells cultured on glass coverslips in 60-mm dishes. They were transfected with 0.5 μg of mammalian expression vector containing RelA-dsRed and allowed to recover for 48 h. The localization of RelA-dsRed in live cells was assessed using a laser-scanning confocal microscope with a specialized stage that maintained cultures at 37 °C in a 5% CO₂ environment (LSM 510 META; Zeiss, Oberkochen, Germany). Images were made every 3–5 min following stimulation. Image analysis was performed using Velocity software (Improvision, Coventry, UK) to calculate the proportion of RelA-dsRed in the nucleus at multiple time points following stimulation. The duration of nuclear localization was defined as the time interval at which the proportion of nuclear RelA exceeded 20%.

*Assay of NF-κB Transcriptional Activity—* NF-κB transcriptional activity was measured using an NF-κB reporter (pGL2) as described previously (26). Cells were co-transfected with pNF-luc and pRL-TK (encoding Renilla luciferase to normalize transfection efficiency) using Lipofectamine and incubated for 16 h. Cells were then treated with TNFα (10 ng/ml) for 16 h before measurement of NF-κB activity. Firefly and Renilla luciferase activity was assessed using the Dual-Luciferase reporter assay kit (Promega, Madison, WI) and luminescence counter (Topcount microplate scintillation; Packard).

*Precipitation of TNF Receptor Complexes—* HEK293 cells were treated with biotinylated TNFα (200 ng/ml) for varying durations and then lysed using 50 mM Tris (pH 7.6), 137 mM

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**NF-κB Export Regulation by H₂O₂**

**FIGURE 1.** H₂O₂ enhanced induction of IL-8 in response to TNFα or IL-1β. A, HeLa cells were stimulated with IL-1β (20 ng/ml) for 4 h either in the absence or in the presence of varying concentrations of H₂O₂. B and C, HeLa cells were stimulated with IL-1β (20 ng/ml) or TNFα (10 ng/ml) for varying times either in the presence or in the absence of 100 μM H₂O₂. A–C, IL-8 transcript levels were quantified by comparative real-time PCR and were normalized by measuring β-actin (A and B) or GNB2L1 (C) transcript levels. Mean values calculated from triplicate wells were pooled from three independent experiments and are shown with standard deviations. D, HeLa or A549 cells were stimulated with IL-1β (20 ng/ml) for 4 h either in the presence or in the absence of H₂O₂ (100 μM) or remained untreated as a control. E, A549 cells were pretreated with N-acetyl-cysteine (NAC, 10 mM) for 1 h or remained untreated as indicated. They were then stimulated with TNFα (10 ng/ml) for 4 h either in the presence or in the absence of H₂O₂, or remained untreated as a control. D and E, IL-8 protein levels in cell culture supernatants were quantified by ELISA. Mean values calculated from triplicate wells were pooled from three independent experiments and are shown with standard deviations. F and G, intracellular ROS were measured in HeLa cells, which were preincubated with a redox-sensitive probe (CM-H₂DCFDA) and then treated with IL-1β (20 ng/ml) and/or H₂O₂ (100 μM) for varying times. Mean fluorescence intensities (MFI) were calculated from triplicate measurements of 10⁶ cells and are shown with standard deviations. G, HeLa cells were stimulated with TNFα (10 ng/ml) either in the presence or in the absence of 100 μM H₂O₂. After 2 h, cultures were co-treated with actinomycin D (Act D) to inhibit transcription, and IL-8 transcripts were quantified at varying times by comparative real-time PCR and were normalized by measuring glyceraldehyde-3-phosphate dehydrogenase transcript levels. Data shown represent the proportion of IL-8 mRNA at each time point when compared with IL-8 mRNA levels at the time of actinomycin D treatment. Mean values calculated from triplicate wells were pooled from three independent experiments and are shown with standard deviations.

**RESULTS**

H₂O₂ Enhanced Transcriptional Activation of Pro-inflammatory Genes in Response to TNFα or IL-1β—We examined whether H₂O₂ could regulate pro-inflammatory activation of HeLa or A549 cells, either alone or in combination with IL-1β or TNFα. We observed by comparative real-time PCR that the application of inhibitors (Roche Applied Science, Basel, Switzerland). Lysates were clarified by low speed centrifugation and precleared using protein-G-Sepharose before precipitation of TNFR complexes using streptavidin-coated beads (Roche Applied Science). Beads were then washed extensively using lysis buffer. Precipitated material or lysates were analyzed by Western blotting using specific primary antibodies, horse-radish peroxidase-conjugated secondary antibodies, and chemiluminescent detection.

**Assay of Cezanne Catalytic Activity**—The catalytic activity of Cezanne was assessed by measuring its capacity to bind to a ubiquitin-derived probe (HAUbVME), which contained a thiol-reactive vinylmethyl ester group at the C terminus (36). It was chosen because of its ability to bind covalently to the catalytic cysteine of Cezanne (25). The probe is tagged with HA epitope to facilitate detection and is ~10 kDa in size. Cytosolic lysates were made from cells expressing GFP-tagged Cezanne using 50 mM Tris (pH 7.6), 0.2% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. Probe was applied to cytosolic lysates, and reactions were incubated at 37 °C for 1 h. Probe-Cezanne conjugates were detected by Western blotting using anti-HA epitope antibodies (1:1000; Roche Applied Science), horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection.

**Statistics**—Differences between samples were analyzed using a paired Student’s t test and analysis of variance (*, p < 0.05, **, p < 0.01, ***, p < 0.001, as shown in the figure legends).
H$_2$O$_2$ alone (6–100 μM) had little or no effect on IL-8 transcript levels in HeLa cells (Fig. 1A, compare lane 1 with lanes 6–8). However, H$_2$O$_2$ significantly enhanced the production of IL-8 transcripts in a concentration-dependent manner in cells that were co-treated with IL-1β (Fig. 1A, compare lane 2 with lanes 3–5) without affecting cell proliferation or survival (data not shown). Thus we used H$_2$O$_2$ at 100 μM in subsequent experiments. In time course experiments, we observed that co-stimulation with H$_2$O$_2$ significantly enhanced the induction of IL-8 transcripts by IL-1β or TNFα at both early and late time points (Fig. 1B, compare lanes 4, 6, 8, and 10 with lanes 5, 7, 9, and 11; Fig. 1C, compare lanes 3 and 5 with lanes 4 and 6). Similarly, we observed by ELISA of cell culture supernatants that the application of H$_2$O$_2$ significantly enhanced the production of IL-8 protein in cells that were co-treated with IL-1β (Fig. 1D, compare lanes 3 and 4 with lanes 7 and 8) or TNFα (Fig. 1E, compare lanes 5 and 7).

We next examined whether the effects of H$_2$O$_2$ were mediated via elevated levels of intracellular ROS. Using a fluorescent probe, it was demonstrated that levels of intracellular ROS were elevated rapidly by the application of exogenous H$_2$O$_2$ (either in the presence or in the absence of IL-1β) and were sustained for at least 360 min (Fig. 1F). In contrast, treatment of cells with IL-1β alone did not elevate intracellular ROS (Fig. 1F).

Moreover, we observed that the capacity of H$_2$O$_2$ to enhance IL-8 induction in response to TNFα was suppressed in cultures that were pretreated with the anti-oxidant N-acetyl-cysteine (NAC) (10 mM, Fig. 1E, compare lanes 7 and 8), confirming that H$_2$O$_2$ exerts its modulatory effects on pro-inflammatory signaling by altering intracellular redox status.

The potential effects of H$_2$O$_2$ on IL-8 mRNA stability were assessed using actinomycin D to block de novo transcription in activated cells. We observed that H$_2$O$_2$ did not alter the rate of decay of IL-8 transcript levels in cells co-treated with TNFα (Fig. 1G). Taken together, these observations suggest that H$_2$O$_2$ can enhance transcriptional activation of IL-8 in response to IL-1β or TNFα by altering intracellular redox.

**H$_2$O$_2$ Prolongs Nuclear Localization and Enhances DNA Binding of NF-κB in Activated Cells**—We examined whether H$_2$O$_2$ modulated NF-κB activation by IL-1β or TNFα, which is a central event in IL-8 induction. Western blotting or immunostaining revealed that RelA accumulated in the nucleus in response to 15–30 min (Fig. 2A, upper panel, and Fig. 2B, compare lanes 1 and 2) or TNFα (Fig. 2A, compare lanes 6 and 7) but subsequently localized to the cytoplasm following 120 min of treatment (Fig. 2A, compare lanes 2 and 3 with lanes 7 and 8; Fig. 2B, compare lanes 2 and 3). Co-stimulation with H$_2$O$_2$ and IL-1β or with H$_2$O$_2$ and TNFα led to increased nuclear localization of NF-κB at late (120–240 min) time points (Fig. 2A, compare lanes 3 and 5 and lanes 8 and 10; Fig. 2B, compare lanes 3 and 5) without significantly affecting nuclear RelA expression at early time points (30 min) (Fig. 2A, compare lanes 2 and 4 and lanes 7 and 9; Fig. 2B, compare lanes 2 and 4). Thus we conclude that co-stimulation with H$_2$O$_2$ significantly enhanced nuclear localization of NF-κB and binding to DNA sequences in cells that were co-stimulated with IL-1β or TNFα for 120–240 min. These findings were supported by in vitro assays, which revealed that H$_2$O$_2$ altered the kinetics of NF-κB activation in response to IL-1β by
enhancing DNA binding following 120 or 240 min of co-treatment (data not shown).

H₂O₂ Suppresses Export of RelA from the Nucleus in IL-1β-treated Cells—To assess directly the potential effects of H₂O₂ on NF-κB dynamics, we used laser-scanning confocal microscopy to monitor the intracellular localization of a fluorescent form of RelA (RelA-dsRed) in real time in living cells. Analysis of multiple cells revealed that RelA-dsRed translocated from the cytoplasm to the nucleus in response to IL-1β and that peak nuclear fluorescence occurred following an average of 48 min of treatment (Fig. 3A, compare panels 1 and 3; Fig. 3B, treatment 1; Fig. 3C, left panel, lane 1). This was followed by export of RelA-dsRed from the nucleus to the cytoplasm (Fig. 3A, compare panels 3 and 6; Fig. 3B). Thus RelA-dsRed was localized to the nucleus in IL-1β-treated cells for an average of 146 min (Fig. 3C, right panel, lane 1). Treatment with H₂O₂ alone did not stimulate nuclear translocation of RelA-dsRed (data not shown). However, H₂O₂ altered NF-κB dynamics in response to IL-1β by modestly delaying import of RelA-dsRed in a concentration-dependent manner. Thus peak nuclear fluorescence occurred at later time points in cells co-treated with IL-1β and H₂O₂ (Fig. 3A, compare panels 3 and 9; Fig. 3B, Fig. 3C, left panel, compare lane 3 with lane 1). We observed that H₂O₂ also suppressed the export of RelA-dsRed from the nucleus (Fig. 3A, compare panels 6 and 12; Fig. 3B). Thus the average duration of RelA-dsRed nuclear localization in response to IL-1β was significantly elevated by co-treatment with H₂O₂ in a concentration-dependent manner (Fig. 3C, right panel). We conclude that H₂O₂ enhances and prolongs pro-inflammatory activation in response to IL-1β or TNFα by delaying the export of NF-κB from the nucleus.
H$_2$O$_2$ Promotes Ser-32/Ser-36 Phosphorylation and Degradation of Newly Synthesized IkB$_\alpha$ Proteins—We reasoned that H$_2$O$_2$ may prolong NF-kB nuclear localization by suppressing the expression or stability of newly synthesized IkB$_\alpha$ molecules, which normally inhibit pro-inflammatory activation by exporting NF-kB complexes from the nucleus to the cytoplasm. Western blotting of cytosolic lysates revealed that IkB$_\alpha$ was degraded rapidly (15 min) in response to IL-1$\beta$ (Fig. 4A, compare lanes 1 and 2) or TNF$\alpha$ (compare lanes 6 and 7) and that this process was not suppressed by co-treatment with H$_2$O$_2$ (Fig. 4A, compare lanes 2 and 4 and lanes 7 and 9). After 60–120 min, IkB$_\alpha$ protein levels were restored fully in cells treated with IL-1$\beta$ or TNF$\alpha$ (Fig. 4A, compare lanes 2 and 3 with lanes 7 and 8) but only partially restored in cells that were co-treated with H$_2$O$_2$ (Fig. 4A, compare lanes 3 and 5 with lanes 8 and 10).

Comparative real-time PCR revealed that co-treatment with H$_2$O$_2$ enhanced the induction of IkB$_\alpha$ transcripts by IL-1$\beta$ or TNF$\alpha$ (Fig. 4B). We conclude therefore that H$_2$O$_2$ suppresses newly synthesized IkB$_\alpha$ at a post-transcriptional level. We next examined whether H$_2$O$_2$ regulates phosphorylation of resynthesized IkB$_\alpha$ proteins at Ser-32/Ser-36, a modification that is known to target IkB$_\alpha$ for proteasomal degradation. We observed that serine phosphorylation of newly synthesized IkB$_\alpha$ was significantly greater in cells co-treated for 1 h with H$_2$O$_2$ and IL-1$\beta$ when compared with cells treated with IL-1$\beta$ alone (Fig. 4C, compare lanes 5 and 7). Thus we conclude that H$_2$O$_2$ suppresses export of RelA from the nucleus in activated cells by promoting Ser phosphorylation and destabilization of newly synthesized IkB$_\alpha$ proteins.

H$_2$O$_2$ Prolongs TNF$\alpha$ Signaling by Inhibiting an Anti-inflammatory Deubiquitinating Enzyme—We examined whether phosphorylation and destabilization of newly synthe-
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sized IκBα by H$_2$O$_2$ in activated cells was associated with prolonged activity of upstream signaling intermediaries. The effects of H$_2$O$_2$ on the recruitment of RIP1 to the TNFR and subsequent polyubiquitination were assessed following purification of TNFR complexes using biotinylated TNFα. Western blotting revealed that TNFα induced transient polyubiquitination of RIP1 in TNFR complexes, which was observed following 2 or 5 min of stimulation but had declined by 20 min (Fig. 5A, compare lanes 1, 2, 6, and 10). Co-treatment of cells with H$_2$O$_2$ altered the kinetics of RIP1 polyubiquitination in response to TNFα by significantly reducing RIP1 modification following 2 or 5 min of co-treatment (compare lanes 2 and 3 with lanes 6 and 7) and significantly enhancing it at 20 min (compare lanes 10 and 11). Thus although TNFR signaling to NF-κB was initially retarded, it was significantly prolonged in cells that were co-treated with H$_2$O$_2$.

The export of NF-κB from the nucleus to the cytoplasm in chronically stimulated cells relies on the induction of Cezanne, a deubiquitinating cysteine protease that suppresses TNFR signaling to IκBα by cleaving ubiquitin from modified RIP1 (26). Given the exquisite sensitivity of cysteine proteases to oxidative stress, we reasoned that H$_2$O$_2$ may prolong RIP1 polyubiquitination by inhibiting the catalytic activity of Cezanne. Western blotting revealed that overexpression of Cezanne significantly reduced the buildup of polyubiquitinated RIP1 in cells treated with TNFα at 5 min but not 20 min (Fig. 5A, compare lanes 6 and 8 with lanes 10 and 12) but had little or no effect in cells that were co-treated with TNFα and H$_2$O$_2$ at both 5 and 20 min (compare lanes 7 and 9 and lanes 11 and 13). In addition, we observed by reporter gene assay that H$_2$O$_2$ significantly reduced the capacity of overexpressed Cezanne to suppress NF-κB transcriptional activity in response to TNFα (Fig. 5B, compare lanes 3 and 6). We conclude therefore that H$_2$O$_2$ suppresses the capacity of Cezanne to inhibit TNFR signaling to NF-κB in cultured cells.

We assessed the effects of H$_2$O$_2$ on the catalytic activity of Cezanne using an assay employing a ubiquitin-derived thiol-reactive probe (HAUbVME), which is known to bind specifically to the catalytic cysteine of deubiquitinating enzymes (36). Incubation of probe with lysates from cells transfected with GFP-Cezanne revealed a prominent band corresponding to the catalytic cysteine of deubiquitinating enzymes (36). Reactive probe (HAUbVME), which is known to bind specifically to the catalytic cysteine of deubiquitinating enzymes (36). Western blotting revealed that overexpression of Cezanne in response to TNFα (Fig. 5B, compare lanes 3 and 6). We conclude therefore that H$_2$O$_2$ suppresses the capacity of Cezanne to inhibit TNFR signaling to NF-κB in cultured cells.

We assessed the effects of H$_2$O$_2$ on the catalytic activity of Cezanne using an assay employing a ubiquitin-derived thiol-reactive probe (HAUbVME), which is known to bind specifically to the catalytic cysteine of deubiquitinating enzymes (36). Western blotting revealed that overexpression of Cezanne in response to TNFα (Fig. 5B, compare lanes 3 and 6). We conclude therefore that H$_2$O$_2$ suppresses the capacity of Cezanne to inhibit TNFR signaling to NF-κB in cultured cells.

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DISCUSSION

Chronically inflamed tissues are characterized by a complex milieu of pro-inflammatory cytokines (e.g. TNFα and IL-1β) and ROS (e.g. H$_2$O$_2$). A potential role of ROS in the pathogenesis of chronic inflammatory diseases has been suggested from numerous in vivo studies in which inflammatory processes have been suppressed by administration or overexpression of antioxidant enzymes (37–41) or have been enhanced by deletion of genes encoding antioxidants (42, 43).

Studies of cultured cells have revealed that ROS have profound effects on numerous physiological activities including pro-inflammatory activation, which can be altered by ROS through poorly defined mechanisms. Numerous reports have indicated that NF-κB-dependent transcription is a redox-sensitive process (30). However, the effects of oxidative stress on cellular activation are complex and vary between particular types of cells, ROS, and co-activating stimuli. For example, H$_2$O$_2$ has been shown to be a direct inducer of NF-κB transcriptional activity and pro-inflammatory activation in some cell types (e.g. T-cells, skeletal muscle myotubes, and human breast MCF-7 cells) but not others (e.g. human microvessel endothelial cells and A549 epithelial cells) (44–46). In addition, H$_2$O$_2$ can enhance or prolong cellular activation in response to pro-inflammatory cytokines in several cell types (47, 48).

The molecular mechanisms by which ROS alter signaling to NF-κB remain uncertain and are likely to operate at multiple levels. Previous studies have suggested that H$_2$O$_2$ stimulates IκB phosphorylation at Tyr-42 via Syk protein-tyrosine kinase, which leads to disassociation of NF-κB, which can subsequently enter the nucleus (45). Others have shown that H$_2$O$_2$ can activate IKK by triggering phosphorylation of serine residues in the activation loop (49), a process that leads to serine phosphorylation and destabilization of IκBα. In addition, NF-κB proteins are themselves sensitive to oxidation, a modification that decreases DNA binding (50).

Here we examined the effects of H$_2$O$_2$ on cellular activation in response to TNFα or IL-1β. We chose to treat cells with up to 100 μM H$_2$O$_2$ because we have previously detected this concentration in supernatants from cultures of activated primary human macrophages. We observed that H$_2$O$_2$ and pro-inflammatory cytokines (IL-1β, TNFα) induced IL-8 in synergy in cultured epithelial cells. We also describe a novel mechanism for the pro-inflammatory effects of H$_2$O$_2$, which significantly altered NF-κB dynamics in co-stimulated cells by causing it to be retained in the nucleus for prolonged periods. Prior reports suggest that timely NF-κB export from the nucleus is essential for suppression of pro-inflammatory processes (18–21). The corollary is that retention of nuclear NF-κB in cells exposed to ROS may lead to persistent cellular activation and chronic inflammation.

Activation of NF-κB leads to the de novo production of IκBα, which participates in a negative feedback mechanism by binding nuclear NF-κB and exporting it from the nucleus to the cytoplasm (18, 21). Our study suggests that H$_2$O$_2$ alters NF-κB dynamics in activated cells by triggering Ser-32/Ser-36 phos-
phorylation and subsequent destabilization of newly synthesized IkBα proteins. This concept is consistent with previous observations that co-stimulation with H2O2 can prolong IKK activation in response to TNFα (49). Thus sustained pro-inflammatory signaling in activated cells exposed to H2O2 may suppress newly synthesized IkBα proteins and prevent NF-κB export.

To define the level at which H2O2 prolongs pro-inflammatory signaling, we assessed its effects on activation of RIP1, which is a key component of the TNFR complex. Recent studies have revealed that TNF signaling relies on modification of RIP1 by polyubiquitin chains that trigger the activation of IKKβ and TAK1 (11, 13). We demonstrate for the first time that the kinetics of RIP1 polyubiquitination at the TNFR in response to TNFα treatment was altered by co-treatment with H2O2. Analysis of precipitated TNFR complexes revealed that modification of RIP1 was initially delayed by co-treatment with H2O2. The underlying molecular mechanism is uncertain, but we speculate that it could be due to the inhibition by H2O2 of ubiquitin-activating enzymes that are known to rely on redox-sensitive Cys residues for catalysis (8). However, the physiologic relevance of delayed pro-inflammatory signaling in cells co-treated with H2O2 remains uncertain as we did not detect a delay in subsequent transcriptional activity (Fig. 1B). Importantly, we observed that RIP1 polyubiquitination in response to TNFα was significantly prolonged in cells that were co-treated with H2O2. Thus it is likely that H2O2 prolongs NF-κB nuclear localization by prolonging TNFR signaling in co-treated cells, which promotes Ser phosphorylation and degradation of newly synthesized IkBα.

Our group has recently identified a cysteine protease called Cezanne that functions as a negative regulator of TNFR signaling by cleaving ubiquitin monomers from polyubiquitinated RIP1, thus stabilizing IkBα for NF-κB suppression (23, 24, 26). Thus Cezanne inhibits the induction of IL-8 by TNFα by shortening the duration of TNFR signaling (26). Given the sensitivity of cysteine proteases to oxidative stress, we reasoned that H2O2 may prolong signaling to NF-κB by inhibiting the activity of Cezanne. Consistent with this hypothesis, we observed that the catalytic activity of Cezanne and its ability to suppress RIP1 polyubiquitination and NF-κB transcriptional activity were inhibited by H2O2. We conclude that the ability of H2O2 to prolong TNFR signaling can potentially be explained by its inhibitory effects on Cezanne activity.

In summary, we describe a novel mechanism for the pro-inflammatory effects of H2O2, which prolonged nuclear localization of NF-κB in activated cells by suppressing the negative regulatory functions of IkBα and Cezanne. Our novel findings that ROS can inhibit an anti-inflammatory deubiquitinating enzyme are consistent with an emerging theme that oxidative stress can promote chronic inflammation by inhibiting the catalytic activity of anti-inflammatory enzymes (31, 32).

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