Modulation of SCF\(^{\beta-TrCP}\)-dependent I\(\kappa B\alpha\) Ubiquitination by Hydrogen Peroxide*†

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Reactive oxygen species are known to participate in the regulation of intracellular signaling pathways, including activation of NF-\(\kappa B\). Recent studies have indicated that increases in intracellular concentrations of hydrogen peroxide (H\(_2\)O\(_2\)) have anti-inflammatory effects in neutrophils, including inhibition of the degradation of I\(\kappa B\alpha\) after TLR4 engagement. In the present experiments, we found that culture of lipopolysaccharide-stimulated neutrophils and HEK 293 cells with H\(_2\)O\(_2\) resulted in diminished ubiquitination of I\(\kappa B\alpha\) and decreased SCF\(^{\beta-TrCP}\) ubiquitin ligase activity. Exposure of neutrophils or HEK 293 cells to H\(_2\)O\(_2\) was associated with reduced binding between phosphorylated I\(\kappa B\alpha\) and SCF\(^{\beta-TrCP}\) but no change in the composition of the SCF\(^{\beta-TrCP}\) complex. Lipopolysaccharide-induced SCF\(^{\beta-TrCP}\) ubiquitin ligase activity as well as binding of \(\beta-TrCP\) to phosphorylated I\(\kappa B\alpha\) was decreased in the lungs of acatalasemic mice and mice treated with the catalase inhibitor aminotriazole, situations in which intracellular concentrations of H\(_2\)O\(_2\) are increased. Exposure to H\(_2\)O\(_2\) resulted in oxidative modification of cysteine residues in \(\beta-TrCP\). Cysteine 308 in Blade 1 of the \(\beta-TrCP\) \(\beta\)-propeller region was found to be required for maximal binding between \(\beta-TrCP\) and phosphorylated I\(\kappa B\alpha\). These findings suggest that the anti-inflammatory effects of H\(_2\)O\(_2\) may result from its ability to decrease ubiquitination as well as subsequent degradation of I\(\kappa B\alpha\) through inhibiting the association between I\(\kappa B\alpha\) and SCF\(^{\beta-TrCP}\).

Reactive oxygen species (ROS) are generated during normal physiologic processes and participate in the maintenance of cellular homeostasis (1). However, increased production of ROS accompanies pathophysiologic conditions, such as chronic obstructive pulmonary disease, sepsis, and ischemia-reperfusion injury, that are characterized by activation of neutrophils, macrophages, and other cell populations to release cytokines and other proinflammatory mediators, many of which are under the regulatory control of the transcription factor NF-\(\kappa B\) (2–5). Although initial reports indicated that ROS, such as superoxide and hydrogen peroxide (H\(_2\)O\(_2\)), exerted proinflammatory effects through activation of NF-\(\kappa B\), more recent studies have shown that ROS are not only responsible for inducing inflammation but also can have potent anti-inflammatory properties. In particular, increases in intracellular concentrations of H\(_2\)O\(_2\), have been demonstrated to diminish TLR4 (Toll-like receptor 4)-induced activation of NF-\(\kappa B\) and production of proinflammatory cytokines in neutrophils, epithelial cells, and other cell populations (6–8).

NF-\(\kappa B\) p50/p65 heterodimers are retained in the cytoplasm by binding to the inhibitory molecule I\(\kappa B\alpha\) (inhibitor of NF-\(\kappa B\)) (9). However, in response to external stimuli, such as engagement of TLR4, I\(\kappa B\alpha\) is degraded by a three-step process involving phosphorylation by IKK kinases, polyubiquitination by the SCF\(^{\beta-TrCP}\) (Skp1-cullin-F-box/\(\beta\)-transducin repeat-containing protein) complex, and degradation of ubiquitinated I\(\kappa B\alpha\) by the 26 S proteasome, thereby exposing the nuclear localization sequence in NF-\(\kappa B\) and permitting translocation of NF-\(\kappa B\) to the nucleus (10–12). Previous studies from our laboratory showed that exposure of LPS-stimulated neutrophils to H\(_2\)O\(_2\) was associated with diminished degradation of I\(\kappa B\alpha\) and decreased translocation of NF-\(\kappa B\) to the nucleus, providing a potential mechanism for the anti-inflammatory properties of H\(_2\)O\(_2\). Increased intracellular concentrations of H\(_2\)O\(_2\) did not affect LPS-induced activation of IKK or phosphorylation of I\(\kappa B\alpha\) (13). Although H\(_2\)O\(_2\) has been shown to diminish 26 S proteasome activity, at least in part through oxidative modification and S-glutathionylation of the Rpn2 regulatory particle (14), the extent of stabilization of I\(\kappa B\alpha\) in LPS-stimulated neutrophils cultured with H\(_2\)O\(_2\) was greater than that produced by treatment with MG132, a specific inhibitor of the 26 S proteasome (6). Such results suggest that H\(_2\)O\(_2\) may affect additional processes, such as the ubiquitination of I\(\kappa B\alpha\), which are involved in targeting I\(\kappa B\alpha\) for degradation by the 26 S proteasome and which contribute to the activation of NF-\(\kappa B\) (15–17).

SCF E3 ubiquitin ligases consist of three invariant core molecules components, Skp1, cullin-1 (Cul1), and Rbx1 (also known as Roc1 or Hrt1), associated with an F-box protein and ubiquitin-conjugating enzyme (E2) (18, 19). The individual F-box protein and E2 enzyme associated with the SCF ligase complex...
provide substrate specificity. For ubiquitination of IxBα, the specific F-box protein and E2 bound to SCF are β-TrCP and UbcH3/Cdc34, respectively (20–22). Association of phosphorylated IxBα with the β-TrCP component of SCFβ-TrCP permits proper positioning of IxBα for polyubiquitination by UbcH3/Cdc34 (19, 23, 24).

In the present experiments, we examined the ability of H2O2 to modulate IxBα ubiquitination and SCFβ-TrCP activity after TLR4 engagement. We found that exposure to H2O2 or inhibition of catalse, a situation associated with increased intracellular concentrations of H2O2 (6, 13), resulted in diminished association of SCFβ-TrCP with IxBα as well as decreased ubiquitination of IxBα.

**EXPERIMENTAL PROCEDURES**

**Mice**—Male C57BL/6, C3HeB/FeJ, or acatalasemic C3Ga. Cg-Cat B/j mice, 8–12 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were kept on a 12-h/12-h light/dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board-approved protocols (University of Alabama at Birmingham Institutional Animal Care and Use Committee).

**Materials**—Hydrogen peroxide, lipopolysaccharide (LPS; from Escherichia coli O4:B111), 3-amino-1,2,4-triazole (ATZ), and MG132 were purchased from Sigma. Anti-cullin-1, anti-β-TrCP, and anti-c-Myc antibodies were from Zymed Laboratories Inc. (San Francisco, CA). Rabbit anti-IxBα, mouse anti-phospho-IxBα, and mouse anti-ubiquitin antibodies were from Cell Signaling Technology (Danvers, MA). Mouse anti-β-catenin antibodies were from BD Transduction Laboratories (Bar Harbor, ME). The mice were kept on a 12-h/12-h light/dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board-approved protocols (University of Alabama at Birmingham Institutional Animal Care and Use Committee).

**Neutrophil Isolation and Culture**—Bone marrow neutrophils were isolated as described previously (6, 25, 26). Neutrophil purity was consistently >97%, as determined by Wright-Giemsa-stained cytosin preparations. Neutrophils were cultured in RPMI 1640 medium containing 0.5% fetal bovine serum and treated as indicated in the figure legends. Neutrophil viability as determined by trypan blue staining was consistently >95%.

**Cell Culture, Transfection, and Generation of Stable Cell Lines**—Human embryonic kidney cells (HEK 293) cells were maintained in RPMI 1640 (Sigma) containing 10% fetal bovine serum (Atlanta Biologics), and penicillin/streptomycin solution (1:10; Sigma). 293-hTLR4/MD2-CD14 cells, an isolated HEK 293 clone stably transfected with hTLR4, MD2, and CD14 genes (catalog number 293-htlr4-md2-cd14, Invivogen), were maintained according to the manufacturer’s instructions. In experimental procedures, all treatments were performed in serum-free media as described in the figure legends. Cells were transfected using Lipofectamine 2000™ reagent. Stable cell lines overexpressing β-TrCP were generated by transfecting 293-hTLR4/MD2-CD14 cells with β-TrCP-FLAG plasmid DNA using Lipofectamine 2000™ reagent followed by G418 (Sigma) selection.

**Acute Lung Injury Model**—Acute lung injury was induced by intratracheal administration of 1 mg/kg LPS in 50 μl of phosphate-buffered saline as described previously (8, 13, 27, 28). Briefly, mice were anesthetized with isoflurane and then suspended by their upper incisors on a 60° incline board. The tongue was then gently extended, and LPS solution was deposited into the pharynx (8, 25, 29). Mice were pretreated with saline or ATZ (500 mg/kg body weight dissolved in 0.9% saline) intraperitoneally, and 4 h later, LPS (1 mg/kg) was administered intratracheally. Lungs were harvested 24 h after LPS administration.

**Construction of Expression Plasmids and Recombinant Protein Expression**—A full-length human β-TrCP cDNA was purchased from Open Biosystems and cloned into 3FLAG-CMV10 (Sigma) for mammalian expression. Four FLAG-tagged point mutant constructs of β-TrCP-C308A (MB1), C348A (MB2), C471A (MB5), and C511A (MB6) were generated using PCR mutagenesis. An IKKβ cDNA containing N-terminal amino acids 1–420 was obtained from Open Biosystems and cloned into 3FLAG-CMV10. Full-length human ROC1, Skp1, and UBC3/Cdc34 (Open Biosystems) were cloned into pCDNA-Myc vector for mammalian expression as Myc-tagged proteins. A full-length cDNA for β-catenin was purchased from Open Biosystems. The IxBα construct in pET15b was kindly provided by Dr. Gourisankar Ghosh (University of California, San Diego, La Jolla, CA). IxBα and β-catenin were cloned into pGEX vector (GE Healthcare) for bacterial expression as N-terminal GST fusion proteins. GST-tagged recombinant proteins were purified using glutathione-Sepharose (GE Healthcare).

**In Vitro Phosphorylation of IxBα and β-Catenin**—Phosphorylation of IxBα or β-catenin was performed using 2 μg of GST-tagged substrate protein, 50 ng of IKKβ (Cell Signaling, Danvers, MA), or GSK3β (SignalChem, Richmond, Canada), 50 μl of 1× kinase buffer (Cell Signaling) and 2 mM ATP for 1 h at room temperature. The phosphorylated products were stored at 80 °C until used.

**In Vitro Ubiquitination Assay**—Cultured cells and neutrophils were lysed, or lungs of mice were homogenized in lysis buffer consisting of 50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 10 mM NaF, 2 mM Na3VO4, protease inhibitor mixture (1:100, v/v) (Sigma), and 0.5% Nonidet P-40. Protein concentrations were determined using Bradford’s reagent (Bio-Rad). To immunoprecipitate SCFβ-TrCP, 1 mg of cell lysates or lung homogenates was incubated with anti-ubiquitin-1 mouse monoclonal antibody in 1 ml of lysis buffer containing 5% glycerol for 2 h, followed by the addition of 30 μl of Protein A-Sepharose beads (Sigma) and incubation overnight at 4 °C with continuous stirring. The beads were then washed three times with lysis buffer and twice with ligase buffer (50 mM Tris, pH 7.5, 25 mM MgCl2, 2 mM Na3VO4, 10 mM NaF). Ubiquitination was performed with the washed beads resuspended in 30 μl of solution containing 100 μg of E1-GST (Boston Biochem, Cambridge, MA), 500 ng of His-tagged UbcH3 (Boston Biochem), 1 μg of ubiquitin-FLAG (BioMol, Plymouth Meeting, PA), Energy Regeneration Solution (Boston Biochem) (a mixture that contains MgCl2, ATP, and ATP-regenerating enzymes to recycle hydrolyzed ATP (i.e. AMP and ADP to ATP)), 100 μM ATP, and
Effects of H\(_2\)O\(_2\) and 26 S proteasomal inhibition on IкB\(\alpha\) degradation and cytokine production in LPS-stimulated cells. A–C, neutrophils or 293-hTLR4/MD2-CD14 cells were cultured with H\(_2\)O\(_2\) (0 or 300 \(\mu\)M) for 5 min and then with LPS (0 or 100 ng/ml) for an additional 60 min. In specified experiments, cells were preincubated with the 26 S proteasomal inhibitor MG132 (10 \(\mu\)M) for 1 h before the addition of H\(_2\)O\(_2\) and LPS to the cultures. Representative Western blots show the levels of IкB\(\alpha\) in neutrophils (A) as well as IкB\(\alpha\) (B) and phospho-IкB\(\alpha\) (C) in 293-hTLR4/MD2-CD14 cells. D, neutrophils were cultured with LPS or H\(_2\)O\(_2\), or the combination of H\(_2\)O\(_2\) and LPS for the indicated time period. Western blot analysis of phospho-Ser-32/36IкB\(\alpha\) total IкB\(\alpha\), and actin is shown. E, proteasomal chymotrypsin-like and trypsin-like activity was measured in neutrophils incubated with MG132 (0 or 10 \(\mu\)M) or H\(_2\)O\(_2\) (0, 300, or 500 \(\mu\)M) for 60 min. Values are means \(\pm\) S.D.; *** \(p<0.001\) comparing treatment with H\(_2\)O\(_2\) (300 \(\mu\)M) with MG132 (0 or 10 \(\mu\)M). F, levels of tumor necrosis factor-\(\alpha\) were measured in cell culture supernatants from neutrophils cultured with LPS, H\(_2\)O\(_2\), or H\(_2\)O\(_2\) and LPS for 5 h. In specified experiments, cells were preincubated with MG132 (0 or 10 \(\mu\)M) for 1 h before LPS or H\(_2\)O\(_2\) treatment (means \(\pm\) S.D.; \(n=3\); *** \(p<0.001\) comparing LPS treatment with untreated cells or comparing LPS treatment with cells incubated with either LPS and H\(_2\)O\(_2\) or MG132).

200 ng of phosphorylated substrate for 1 h at 30 °C. The reaction was then stopped by adding SDS-PAGE loading buffer, followed by boiling the samples for 15 min, and resolution on 6% SDS-PAGE. Ubiquitination of substrates was detected by either anti-ubiquitin antibodies or antibodies to IкB\(\alpha\) or \(\beta\)-catenin.

**GST Pull-down of \(\beta\)-TrCP Using Phospho-GST-IкB\(\alpha\) or Phospho-GST-\(\beta\)-Catenin**—Phosphorylated GST-IкB\(\alpha\) or phosphorylated GST-\(\beta\)-catenin (500 ng) was added to 1 mg of protein obtained from cell lysates or lung extracts in 1 ml of lysis buffer containing 5% glycerol. Glutathione-Sepharose beads were added, and the mixture was incubated at 4 °C with continuous stirring for 1 h. The beads were washed three times with lysis buffer, and IкB\(\alpha\)- or \(\beta\)-catenin-bound \(\beta\)-TrCP was analyzed by immunoblotting with specific antibodies to FLAG or \(\beta\)-TrCP.

**Imaging of DCF Fluorescence**—Intracellular levels of ROS, including hydrogen peroxide, were measured using the redox-sensitive probe DCFH-DA (30) in conjunction with fluorescent microscopy (1, 6, 8, 31–33). Briefly, neutrophils, HEK 293 cells, or 293-hTLR4/MD2-CD14 cells (~80% confluent) were incubated in a 4-well chambered coverglass (Nalge; Naperville, IL) with dichlorofluorescein diacetate (10 \(\mu\)M) for 60 min, followed by treatment with various concentrations of H\(_2\)O\(_2\) or LPS at 37 °C. Images were acquired by single bidirectional scans of live cells using a Leica DMI8RE inverted epifluorescence/Nomarski microscope outfitted with Leica TCS NT laser confocal optics. The pinhole setting was 0.2 Airy units, and laser excitation was set for 5% to avoid dye photobleaching. The levels of fluorescence were averaged using SimplePCI software (Compix; Cranberry Township, PA). Images were processed using IPLab Spectrum and Adobe Photoshop (Adobe Systems) software.

**Cytokine Enzyme-linked Immunosorbent Assay**—Levels of tumor necrosis factor-\(\alpha\) from neutrophils into culture media were determined using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapois, MN), according to the manufacturer's instructions and as previously described (7, 8, 13, 34).

**Measurement of Proteasome Activity**—26 S proteasomal chymotrypsin-like and trypsin-like activity was measured in HEK 293 cells using the fluorogenic peptide substrate succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin or \(\alpha\)-butoxy-carbonyl-Leu-Arg-Arg-7-amido-4-methylcoumarin, respectively, as previously described (13, 35–38).

**Labeling of \(\beta\)-TrCP Free Cysteine Thiols**—The extent of free (unoxidized) cysteine residues within \(\beta\)-TrCP was determined using the biotinylated iodoacetyl ethylenediamine (BIAM)-labeling assay (14, 39–42). Briefly, cell lysates (0.5 mg/ml) obtained from HEK 293 cells that transiently expressed \(\beta\)-TrCP-FLAG were incubated with BIAM (100 \(\mu\)M) for 30 min at room temperature, and then excess BIAM was removed by passing the extracts through Bio-Gel P10. Next, BIAM-protein conjugates were precipitated with streptavidin-agarose for 1 h at 4 °C. Samples were washed four times with lysis buffer containing 0.05% SDS and then subjected to reducing SDS-PAGE.
**H₂O₂ Inhibits SCFβ-TrCP Activity**

and Western blot analysis with antibodies to β-TrCP or FLAG peptide. Cells were also directly incubated with BIAM (200 μM) for 20 min, and then cell lysates were passed through Bio-Gel P10 to remove excess BIAM. The amount of BIAM-β-TrCP adduct formation was determined using streptavidin-agarose pull-down followed by Western blot analysis with antibodies specific to β-TrCP or FLAG peptide.

**Statistical Analyses**—For each experiment, neutrophils were isolated and pooled from groups of mice (n = 3–4), and all conditions were studied at the same time. Data are presented as means ± S.D. for each experimental group. One-way analysis of variance, the Tukey-Kramer multiple comparison test (for multiple groups), or Student’s t test (for comparisons between two groups) were used. p < 0.05 was considered significant.

**RESULTS**

**Inhibitory Effects of Hydrogen Peroxide on TLR4-induced IkBα Degradation**—In recent studies, we demonstrated that increased intracellular levels of H₂O₂ were associated with diminished degradation of IkBα and reduced 26 S proteasomal activity in LPS-stimulated neutrophils (6, 8). As shown in Fig. 1, A–C, whereas exposure of LPS-stimulated neutrophils or 293-hTLR4/MD2-CD14 cells to H₂O₂ resulted in diminished degradation of IkBα, the extent of IkBα stabilization in H₂O₂-treated cells was greater than that found after blockade of 26 S proteasomal function with MG132 in the absence of H₂O₂. These results suggested that the mechanisms by which H₂O₂ prevents IkBα degradation may extend beyond proteasomal inhibition.

Because phosphorylation of IkBα is required for its polyubiquitination and subsequent degradation by the 26 S proteasome, we hypothesized that a mechanism by which H₂O₂ might diminish TLR4-induced degradation of IkBα was through inhibiting IkBα phosphorylation. To examine this possibility, 293-hTLR4/MD2-CD14 cells were stimulated with LPS in the presence or absence of H₂O₂. As shown in Fig. 1C, H₂O₂ exposure appeared to have no effect on LPS-induced phosphorylation of IkBα. Although exposure to LPS or LPS and H₂O₂ both resulted in enhanced phosphorylation of IkBα, the amount of total IkBα decreased in LPS-treated cells but not in those treated with both LPS and H₂O₂ (Fig. 1D). Next, we examined the effects of H₂O₂ and MG132 on 26 S proteasomal activity. As shown in Fig. 1E, incubation of HEK 293 cells with MG132 resulted in inhibition of trypsin-like and chymotrypsin-like proteasomal activity; such inhibition of 26 S proteasomal function was more pronounced in cells treated with MG132 as compared with that found in cells treated with H₂O₂ alone (Fig. 1E). As shown in Fig. 1F, preincubation with H₂O₂ or MG-132 had inhibitory effects on the release of tumor necrosis factor-α from LPS-stimulated neutrophils.

The experiments shown in Fig. 1, A–D, indicate that although H₂O₂ appeared to be a less potent inhibitor of 26 S proteasomal activity than was MG132, exposure of LPS-treated cells to H₂O₂ appeared to stabilize IkBα levels to a greater extent than did MG132. These results suggest that in addition to its inhibitory actions on the 26 S proteasome, H₂O₂ also may affect upstream events that result in IkBα stabilization, such as through modulating IkBα ubiquitination.

**FIGURE 2. Exposure to H₂O₂ diminishes SCFβ-TrCP-dependent ubiquitination of IkBα.** A and B, levels of ubiquitinated IkBα (Ub-IkBα) were determined in neutrophils (A) or in 293-hTLR4/MD2-CD14 cells that transiently expressed Myc-tagged IkBα, IKKβ-FLAG, or Ub-HA. In B, cells were treated with combinations of H₂O₂, LPS, and MG132 as indicated, and cell extracts were subjected to immunoprecipitation (IP) with anti-ubiquitin antibodies. Representative Western blots show the amounts of Ub-IkBα conjugates detected with antibodies specific for phospho-IkBα. Two additional experiments provided similar results. C and D, neutrophils (C) or 293-hTLR4/MD2-CD14 cells stably expressing β-TrCP-FLAG (D) were cultured with H₂O₂ (0 or 300 μM) for 60 min, and then the SCFβ-TrCP complex was immunoprecipitated from cell extracts using anti-cullin-1 antibodies, followed by detection of SCFβ-TrCP-dependent ubiquitination of phosphorylated recombinant IkBα. Representative Western blots show levels of Ub-IkBα as detected with anti-phospho-IkBα antibodies. A second experiment provided similar results.

**Effects of H₂O₂ on IkBα Ubiquitination by SCFβ-TrCP—**Ubiquitination of IkBα occurs after TLR4 engagement (43) and is required for the processing and degradation of IkBα by the 26 S proteasome. As shown in Fig. 2, A and B, exposure of neutrophils or 293-hTLR4/MD2-CD14 cells to LPS resulted in increased ubiquitination of IkBα, an effect that was enhanced by blockade of 26 S proteasomal activity following the addition of the proteasomal inhibitor MG132 to the cultures. In contrast, LPS-induced ubiquitination of IkBα was diminished in a concentration-dependent manner after exposure of cells to H₂O₂.

The E3 ubiquitin ligase SCFβ-TrCP is responsible for the ubiquitination of IkBα (15, 44, 45). Therefore, given the diminished ubiquitination of IkBα in H₂O₂-exposed cells, it seemed possible that H₂O₂ might affect SCFβ-TrCP activity. As shown in Fig. 2, C and D, SCFβ-TrCP isolated from neutrophils or 293-hTLR4/
MD2-CD14 cells cultured with H$_2$O$_2$ was less able to ubiquitinate IκBα than was SCF$^{β$-TrCP} purified from cells that had not been exposed to H$_2$O$_2$.

In initial experiments to determine the mechanism for the inhibitory actions of H$_2$O$_2$ on SCF$^{β$-TrCP} ubiquitin ligase activity, we examined the effect of H$_2$O$_2$ on cullin-1 neddylation, a process that has been shown to result in enhanced SCF$^{β$-TrCP} activity (45). Despite the reduction of SCF$^{β$-TrCP} activity under cellular incubation of HEK 293 cells with H$_2$O$_2$ resulted in increased cullin-1 neddylation (Fig. 3A). Western blot analysis with anti-Myc antibodies. A second experiment provided similar results.

Because the H$_2$O$_2$-induced decrease in SCF$^{β$-TrCP} ubiquitin ligase activity might be due to alterations in the composition of the SCF$^{β$-TrCP} complex, we determined binding of β-TrCP to Skp1, cullin-1 to Skp1, and cullin-1 to Roc1 in HEK 293 cells that were transfected with β-TrCP-FLAG and Skp1-Myc, cullin-1-FLAG and Skp1-Myc, or cullin-1-FLAG and Roc1-Myc were treated with H$_2$O$_2$ (0, 100, 300, and 500 μM) for 1 h. Western blots show the amounts of SCF$^{β$-TrCP} components before (cell extract) or after immunoprecipitation with anti-FLAG-agarose beads (IP: Flag). After immunoprecipitation, the amounts of β-TrCP-FLAG associated with Skp1-Myc (B) or of cullin-1-FLAG associated with Skp1-Myc (C) or of cullin-1-FLAG associated with Roc1-Myc (D) were determined by Western blot analysis with anti-Myc antibodies. A second experiment provided similar results.

As shown in Fig. 4A, incubation of the 293-hTLR4/MD2-CD14 cells with H$_2$O$_2$ diminished binding of IκBα to β-TrCP in LPS-stimulated cells. Similar results were found after cell extracts from 293-hTLR4/MD2-CD14 cells stably expressing β-TrCP-FLAG were incubated with phosphorylated IκBα tagged with GST (Fig. 4B).

Enhanced Intracellular H$_2$O$_2$ Concentrations in Vivo Are Associated with Diminished SCF$^{β$-TrCP} Activity and Association with IκBα—Intracellular concentrations of H$_2$O$_2$ are increased in acatalasemic mice and in mice treated with ATZ, a specific inhibitor of catalase (13). These conditions therefore permit exploration of the effects of increased intracellular H$_2$O$_2$ on SCF$^{β$-TrCP} activity under in vivo conditions. As shown in Figs. 5 and 6, SCF$^{β$-TrCP} isolated from the lungs of acatalasemic mice or mice treated with ATZ is less able to ubiquitinate IκBα and also demonstrates diminished binding to phosphorylated IκBα as compared with SCF$^{β$-TrCP} from control mice (Figs. 5B and 6D). In addition, intratracheal administration of LPS resulted in enhanced ubiquitination of IκBα in the lungs of control mice but not in the lungs of mice treated with ATZ before LPS exposure (Fig. 6A). Although SCF$^{β$-TrCP} isolated from the lungs of LPS-exposed mice showed increased ability to ubiquitinate either phosphorylated IκBα or phosphorylated β-catenin, there was no increase in ubiquitin ligase activity of SCF$^{β$-TrCP} that had been purified from mice treated with ATZ before LPS exposure (Fig. 6B and C). These results are consistent with the ability of increased intracellular H$_2$O$_2$ levels to inhibit SCF$^{β$-TrCP} activity and decrease IκBα turnover even under basal conditions (Figs. 5A and 6D).

Cysteine Thiols of β-TrCP Undergo Oxidative Modification after Exposure to H$_2$O$_2$—Reactive oxygen species (and particularly H$_2$O$_2$) can affect intracellular signaling through posttranslational protein modification and particularly through oxidation of cysteine thiols (1, 42, 46, 47). To determine if cellular exposure to H$_2$O$_2$ produces modification of cysteines in β-TrCP, we labeled free, unmodified cysteine residues in β-TrCP fragments and then treated the cells with increasing doses of H$_2$O$_2$ followed by immunoprecipitation of IκBα.
cysteines in Blades 1, 2, 5, and 6, and then the FLAG-tagged mutants or wild type β-TrCP were expressed in HEK 293 cells.

As shown in Fig. 8B, mutation of cysteine 308 in Blade 1 of β-TrCP to alanine reduces binding of phosphorylated IκBα with β-TrCP, whereas mutation of cysteine 348 in Blade 2, cysteine 471 in Blade 5, or cysteine 511 in Blade 6 in the WD40 domain of β-TrCP did not affect binding between β-TrCP and phosphorylated IκBα. These results demonstrate that cysteine 308 in Blade 1 of β-TrCP participates in binding between β-TrCP and phosphorylated IκBα. Of note, whereas exposure of cells to H$_2$O$_2$ increased intracellular oxidation of DCF-DA, an indicator of intracellular ROS formation, transient expression or β-TrCP or exposure to LPS had no effects on DCF-DA oxidation (supplemental Fig. 1).

**DISCUSSION**

Previous studies have shown that increased intracellular concentrations of H$_2$O$_2$ exert anti-inflammatory effects on TLR4 induced neutrophil activation (51, 52). Exposure of neutrophils to H$_2$O$_2$ stabilizes cytoplasmic concentrations of IκBα, both in resting cells and after TLR4 engagement, and also diminishes LPS-induced nuclear translocation of NF-κB (6–8, 13). Although initial experiments with HeLa cells suggested that exposure to H$_2$O$_2$ enhanced nuclear translocation of NF-κB, subsequent studies (51, 53–56) in other cell populations found the opposite effect, with H$_2$O$_2$ inhibiting NF-κB activation. Such disparate findings suggested that the role of H$_2$O$_2$ in affecting pathways relating to the activation of NF-κB is likely to be cell type-specific. Several potential mechanisms for the ability of H$_2$O$_2$ to inhibit degradation of IκBα and to diminish activation of NF-κB have been proposed (6, 13, 57–59). Phosphorylation of serine 32 and 36 in IκBα is an indicator of intracellular ROS formation, transient expression or β-TrCP or exposure to LPS had no effects on DCF-DA oxidation. **(Fig. 1)**

**FIGURE 4.** H$_2$O$_2$ diminishes SCF$^\beta$-TrCP-dependent binding to IκBα. A, 293-hTLR4/MD2-CD14 cells transiently expressing β-TrCP-FLAG, IκBα-Myc, and IKKβ-FLAG were treated with combinations of H$_2$O$_2$, LPS, and MG132 as noted in the figure. The amounts of IκBα associated with β-TrCP were determined after pull-down with anti-IκBα antibodies (IP: IκBα) with Western blot analysis using anti-FLAG antibodies. Representative Western blots show the level of IκBα, β-TrCP-FLAG, and actin before (cell extract) or after IκBα immunoprecipitation (IP: IκBα). B, 293-hTLR4/MD2-CD14 cells stably expressing β-TrCP-FLAG were treated with H$_2$O$_2$ (0, 100, 300, and 500 μM) for 1 h, and then the cell extracts were incubated with phosphorylated GST-IκBα, followed by pull-down with glutathione-Sepharose. Western blots show the amount of β-TrCP-FLAG bound to GST-IκBα (pull down) and amounts of GST-IκBα and β-TrCP-FLAG in the cell extracts before pull-down. The optical density (means ± S.D.) obtained from three independent experiments (A and B) is provided (*, p < 0.05, comparing the amount of IκBα associated with β-TrCP in cells incubated with 300 or 500 μM H$_2$O$_2$ with that found in untreated cells).

Cysteine 308 in the Blade 1 β-TrCP β-Propeller Region Is Required for Binding between β-TrCP and Phosphorylated IκBα—β-TrCP contains a total of 25 cysteine residues. The binding site of β-TrCP to phosphorylated substrates, including phosphorylated IκBα, has been localized to the top face of the WD40 domain of β-TrCP (19, 48–50). There are a total of 12 cysteines in the seven blades of the β-TrCP WD40 β-propeller region that are likely to interact with phosphorylated substrates, including phosphorylated IκBα; of these 12 cysteines, only four are likely to exist as thiolate anions as a result of being close to positively charged amino acids (i.e. Cys-Leu in Blade 1, Leu-Cys-Leu in Blade 2, Arg-Cys-Leu in Blade 5, and Arg-Cys-X-Arg in Blade 6) (Fig. 8A). These four cysteines in Blades 1, 2, 5, and 6 are therefore potentially more vulnerable to oxidation by H$_2$O$_2$ and were targets for site-directed mutagenesis in β-TrCP. In these experiments, Cys → Ala point mutations in β-TrCP were generated in the targeted location of NF-κB, subsequent studies (51, 53–56) in other cell populations found the opposite effect, with H$_2$O$_2$ inhibiting NF-κB activation. Such disparate findings suggested that the role of H$_2$O$_2$ in affecting pathways relating to the activation of NF-κB is likely to be cell type-specific. Several potential mechanisms for the ability of H$_2$O$_2$ to inhibit degradation of IκBα and to diminish activation of NF-κB have been proposed (6, 13, 57–59). Phosphorylation of serine 32 and 36 in IκBα by the IKK complex is required to initiate the subsequent ubiquitination and degradation of IκBα in the 26 S proteasome (60–62). Although previous studies in C10 and aortic smooth muscle cells demonstrated that H$_2$O$_2$ treatment resulted in inhibition of IKKβ (52), we did not find any effects of H$_2$O$_2$ exposure on IKK activity in neutrophils (7, 51). Similarly, the present studies did not demonstrate any alterations in the phosphorylation of IκBα after incubation of 293-hTLR4/MD2-CD14 cells with H$_2$O$_2$. JOURNAL OF BIOLOGICAL CHEMISTRY

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From the article:

Our laboratory and others have demonstrated that increased intracellular levels of H$_2$O$_2$ result in inhibition of 26 S proteasomal function in neutrophils or other cell populations (6, 63). If inhibition of the 26 S proteasome was the primary mechanism leading to stabilization of intracellular levels of I$k$B$_\alpha$ in H$_2$O$_2$-treated cells, then one would also expect to find increased concentrations of polyubiquitinated I$k$B$_\alpha$ after H$_2$O$_2$ exposure. However, as shown in the present experiments, incubation of LPS-stimulated neutrophils or 293-hTLR4/MD2-CD14 cells with H$_2$O$_2$ resulted in diminished ubiquitination of I$k$B$_\alpha$, consistent with inhibition of ubiquitin ligase activity. Our studies also found that exposure of neutrophils or HEK 293 cells to H$_2$O$_2$ resulted in inhibition of the activity of SCF$\beta$-TrCP, the specific E3 ubiquitin ligase responsible for ubiquitination of I$k$B$_\alpha$, thereby providing a mechanism that may explain the ability of increased intracellular concentrations of H$_2$O$_2$ to prevent degradative activity of I$k$B$_\alpha$, activation of NF-$\kappa$B, and expression of NF-$\kappa$B proinflammatory genes, such as tumor necrosis factor-$\alpha$, after TLR4 engagement (Fig. 9).

Umbiquitination of target proteins is a multistep process. After activation of an E1 enzyme, ubiquitin is transferred to an active cysteine of a ubiquitin-conjugating enzyme (E2). A ubiquitin ligase (E3) then transfers ubiquitin from the E2 ubiquitin-conjugating enzyme to the substrate for RING finger E3 ligases or by facilitating the transfer of ubiquitin-conjugating enzyme to the target protein either by forming an E3-ubiquitin thioester intermediate target protein either by forming an E3-ubiquitin thioester intermediate. The E1 enzyme then transfers ubiquitin from the E2 ubiquitin-conjugating enzyme to the target protein either by forming an E3-ubiquitin thioester intermediate. In the case of HECT E3 ubiquitin ligases or by facilitating the transfer of ubiquitin directly from the E2 to the substrate for RING finger E3 ubiquitin ligases (60, 61). SCF$\beta$-TrCP, a RING finger E3, appears to be specific for ubiquitination of phosphorylated I$k$B$_\alpha$ as well as for phosphorylated $\beta$-catenin (11, 24, 64, 65). SCF$\beta$-TrCP includes several structural and functional components: $\beta$-TrCP, a F-box protein that binds to phosphorylated I$k$B$_\alpha$ and $\beta$-catenin; the adapter protein SKP1,
H$_2$O$_2$ Inhibits SCF$^\beta$-TrCP Activity

A

pull down: streptavidin

B

input: cell extract

C

pull down: streptavidin

FIGURE 7. Exposure to H$_2$O$_2$ induces oxidation of $\beta$-TrCP cysteine thiols. A, cell extracts obtained from HEK 293 cells transiently expressing $\beta$-TrCP were incubated with H$_2$O$_2$ and BIAM. BIAM adduct formation in $\beta$-TrCP was determined by pull-down with streptavidin-agarose followed by Western blotting with $\beta$-TrCP antibody. $\beta$-HEK 293 cells transiently expressing $\beta$-TrCP were treated with H$_2$O$_2$ (300 $\mu$M) for 20 min followed by culture with BIAM (200 $\mu$M) for an additional 20 min. A representative Western blot shows the amount of total $\beta$-TrCP in the cell extract (left; input) and the BIAM-$\beta$-TrCP adduct formation obtained after precipitation with streptavidin-agarose (right; pull down). A second experiment provided similar results.

FIGURE 8. Cys-308 in $\beta$-TrCP is involved in the binding of phosphorylated IxB$\alpha$. A, schematic diagram of the human $\beta$-TrCP gene showing the three primary domains: dimerization domain, F-box domain, and substrate binding WD40 domain ($\beta$-propeller region). Partial amino acid sequence of Blade 1, Blade 2, Blade 5, and Blade 6 is shown, and the cysteines targeted for mutagenesis are underlined. B, a representative Western blot (bottom) shows the concentrations of FLAG-tagged wild type $\beta$-TrCP or mutant $\beta$-TrCP with cysteine to alanine changes, specifically C308A (MB1), C348A (MB2), C478A (MB5), or C511A (MB6) transiently expressed in HEK 293 cells (input). Cell extracts were incubated with recombinant phospho-IxB$\alpha$-GST and then subjected to glutathione-Sepharose precipitation (pull down). The amounts of $\beta$-TrCP associated with GST-phospho-IxB$\alpha$ were determined by Western blot analysis with anti-FLAG antibodies (top). Two additional experiments provided similar results. Mean optical density for IxB$\alpha$ associated with $\beta$-TrCP (wild type (WT)) or $\beta$-TrCP MB1 is shown in C (means ± S.D.; n = 3; * p < 0.05). aa, amino acids; WT, wild type.

which binds to $\beta$-TrCP as well as the NH$_2$-terminal region of cullin-1; and the RING finger protein Rbx1/Roc1/Hrt1, which binds to the COOH-terminal region of cullin-1 and also recruits the E2 enzyme UbcH3/Cdc34 that ubiquitinates phosphorylated IxB$\alpha$ and phosphorylated $\beta$-catenin (19, 48–50).

In the present experiments, we found that exposure of 293-hTLR4/MD2-CD14 cells to H$_2$O$_2$ was associated with diminished activity of SCF$^\beta$-TrCP. Because phosphorylated IxB$\alpha$ was used in ubiquitination assays containing purified E1 and E2, the decreased ubiquitination of IxB$\alpha$ found with SCF$^\beta$-TrCP incubates protein function (46, 68), it is possible that cysteines in $\beta$-TrCP that are critical for binding IxB$\alpha$ are oxidized in H$_2$O$_2$-exposed cells. In these studies, exposure of $\beta$-TrCP to H$_2$O$_2$ resulted in diminished BIAM adduct formation, consistent with oxidative modification of cysteine residues in $\beta$-TrCP.

Using site-directed mutagenesis of specific cysteines in the $\beta$-TrCP WD40 region that are likely to interact with phosphorylated IxB$\alpha$, we found that Cys-308 in Blade 1 of the $\beta$-TrCP $\beta$-propeller region is required for optimal binding between phosphorylated IxB$\alpha$ and $\beta$-TrCP and is likely to be involved in
the H$_2$O$_2$-induced reduction in binding between SCF$^{\beta}$-TrCP and phosphorylated I$_B$$\alpha$. These results are consistent with the previously reported findings that alkylation of cysteine thiols of $\beta$-TrCP by N-ethylmaleimide significantly diminished ubiquitination of I$_B$$\alpha$, whereas alkylation of cullin-1 and Roc1 with N-ethylmaleimide had no effect on I$_B$$\alpha$ ubiquitination (20).

Exposure of the lungs to LPS during Gram-negative pneumonia, during systemic Gram-negative infections, or through environmental factors produces acute inflammatory changes associated with neutrophil migration into the pulmonary parenchyma and airways, release of reactive oxygen species and proinflammatory cytokines, and the development of lung injury (69–71). Catalase facilitates the conversion of H$_2$O$_2$ to H$_2$O and O$_2$, and intracellular H$_2$O$_2$ concentrations are increased in neutrophils from acatalasemic mice or mice treated with amitriazole (13). The present experiments, showing diminished ubiquitination activity through diminishing association with I$_B$$\alpha$ and prevention of nuclear translocation of NF-$\kappa$B.

Despite these findings, there was no evidence of nuclear translocation of NF-$\kappa$B in H$_2$O$_2$-treated HEK 293 cells, indicating that the inhibition of I$_B$$\alpha$ ubiquitination and proteasomal degradation results in maintenance of cytoplasmic levels of I$_B$$\alpha$ and prevention of nuclear translocation of NF-$\kappa$B.

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