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Original Contribution

Sustained oxidative stress inhibits NF-κB activation partially via inactivating the proteasome

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ABSTRACT

NF-κB is a family of important transcription factors involved in many cellular functions, such as cell survival, proliferation, and stress responses. Many studies indicate that NF-κB is a stress-sensitive transcription factor and its activation is regulated by reactive oxygen species. In previous studies, we and others demonstrated that this transcription factor can be activated by transient oxidative stress. However, the effects of sustained oxidative stress on NF-κB activation are not clear. The objective of this study was to determine the effects of sustained oxidative stress on NF-κB activation and to elucidate the signaling events affected by sustained oxidative stress. Human lens epithelial cells (HLEC) that were subjected to 4 h of continuous influx of hydrogen peroxide were used to investigate the effects of sustained oxidative stress on NF-κB activation. The data showed that, unlike transient oxidative stress, sustained exposure of HLEC to physiologically relevant levels of H_2O_2 (50–100 μM for 4 h) did not induce the degradation of I-κB and activation of NF-κB, but attenuated TNFα-induced degradation of I-κB and activation of NF-κB. Sustained exposure of HLEC to these levels of H_2O_2 also inactivated proteasome activity by 50–80%. Consistent with the role of the proteasome in degradation of I-κB and activation of NF-κB, treatment of HLEC with proteasome inhibitors also attenuated TNFα-induced I-κB degradation and NF-κB activation. The data also indicate that activation of NF-κB is essential for the cells to recover from oxidative stress. Inhibiting NF-κB activation during recovery from transient oxidative stress significantly reduced the cell viability. Together, these data indicate that sustained oxidative stress may inactivate the proteasome and subsequently inhibit NF-κB activation by impeding the degradation of I-κB. The oxidative inactivation of the proteasome and subsequent impairment of NF-κB activation may contribute to the death of lens epithelial cells, a common feature associated with cataract.

Nuclear factor (NF)-κB is a class of transcription factors that can both enhance and repress gene expression by binding to discrete DNA sequences, known as κB elements, in promoters and enhancers. In mammalian cells, there are five NF-κB family members, RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), and they form various complexes of NF-κB homo- or heterodimers [1,2]. The genes regulated by NF-κB include those controlling programmed cell death (apoptosis), cell adhesion, proliferation, inflammation, cellular-stress response, and tissue remodeling [3–6]. However, the expression of these genes is tightly coordinated with the activity of many other signaling pathways. Therefore, the outcome of NF-κB activation depends on the nature and the cellular context of its induction. In unstimulated cells, NF-κB complexes are retained in the cytoplasm by a family of inhibitory proteins known as inhibitors of NF-κB (I-κB's). Activation of NF-κB typically involves the phosphorylation of I-κB by the I-κB kinase (IKK) complex, which results in I-κB degradation by the ubiquitin–proteasome pathway (UPP). This releases NF-κB and allows it to translocate into the nucleus and bind to promoters or enhancers of the target genes. Many antiapoptotic genes are regulated in an NF-κB-dependent manner, so that NF-κB activation would favor cell survival.

NF-κB is generally considered a central regulator of stress responses, because it can be activated by hundreds of different stimuli, such as lipopolysaccharide (LPS), tumor necrosis factor α (TNFα), and other proinflammatory cytokines, as well as environmental stress. Reactive oxygen species have long been recognized to play an important role in NF-κB activation [7]. It was proposed that reactive oxygen species are common secondary messengers of many different stimuli that activate NF-κB [8]. For example, LPS binds to TLR4 and activates NADPH oxidase, which releases H_2O_2 and activates NF-κB [9]. Consistent with the role of reactive species in NF-κB, treatments of cells with antioxidants, such as *N*-acetyl-L-cysteine and pyrrolidine dithiocarbamate, block NF-κB activation induced by phorbol ester, IL-1, TNFα, and LPS [10]. Treatment of cells with exogenous H_2O_2 can

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also result in NF-κB activation in many, but not all, cell types [10–13]. In addition, reactive oxygen species also modulate NF-κB activation that is induced by other stimuli. In both MCF-7 and HeLa cells, moderate levels of extracellular H_2O_2 (up to 25 μM) alone do not activate NF-κB. However, these concentrations of H_2O_2 stimulate TNFα-induced NF-κB activation [13]. In contrast, treatment of the cells with higher concentrations of H_2O_2 attenuates TNFα-induced activation of NF-κB [13].

The UPP is a highly regulated protein degradation system, which selectively degrades abnormal proteins and many cellular regulatory proteins [14]. An active UPP is essential for the cells to withstand and recover from various environmental stresses [15,16]. The UPP plays an important role in the signaling of NF-κB activation [17,18]. For example, during TNFα-induced NF-κB activation, TNFα binding to TNF receptor 1 results in ubiquitination of receptor-interacting protein 1 (RIP1) [19,20]. TRAF2 is a ubiquitin ligase (E3) for RIP1 ubiquitination via the K63-linked polyubiquitin chain [21,22], and A20 is the deubiquitinating enzyme that disassembles the K63-linked polyubiquitin chain. Ubiquitinated RIP1 recruits IKK complexes through its interaction with IKKγ/Nemo in the IKK complex [22–24] and leads to eventual activation of IKKβ by phosphorylation of serines 177 and 181 in the activation loop [25]. Activated IKK complex phosphorylates I-κBα at two serine sites (S32 and S36) [26], which triggers its ubiquitination and degradation by the 26S proteasome [27–29]. The degradation of I-κB releases NF-κB and allows it to enter the nucleus, bind to specific promoters or enhancers, and turn on the transcription of target genes [3].

Environmental factors associated with enhanced risk for age-related cataract include sunlight [30,31], smoking [32], and exposure to other toxic chemicals [31]. On a biochemical level, all of these factors increase the oxidative burden on the lens. In experimental animals as well as in humans, cataract can be induced by exposure to a high-oxygen atmosphere [33,34]. Oxidative stress is a known causal factor for cataractogenesis [35]. Reactive oxygen species, such as O_2^-, H_2O_2, and $OH^·$, can be generated through exposure to light if a photosensitizer, such as riboflavin, is present in any ocular fluid or tissues such as in the aqueous humor [36]. If not efficiently quenched, the photochemically generated reactive oxygen species will damage cellular constituents or disrupt the signal transduction pathways. Epithelial cells are the primary targets of these oxidative assaults by reactive species because they are closest to the aqueous humor. Although the physiological levels of H_2O_2 in the aqueous and lenses remain controversial, as high as 80 μM H_2O_2 in the aqueous and 100 μM H_2O_2 in the lenses have been reported [37,38]. In most cases cataract is triggered by damage to the lens epithelial cells by the reactive oxygen species that are generated in the aqueous humor [39–42]. Therefore, lens epithelial cells are a physiologically relevant model to study the effects of sustained oxidative stress on cellular functions.

As in other cell types, low levels of reactive oxygen species serve as signaling messengers for lens epithelial cell proliferation [43,44]. We also demonstrated that transient exposure of lens epithelial cells to H_2O_2 results in activation of NF-κB [11]. It has been demonstrated that sustained oxidative stress inactivates many enzymes in cells. However, it remains largely unknown how sustained oxidative stress affects cell signaling pathways. In this study, we investigated the effects of physiologically relevant sustained oxidative stress on NF-κB activation in human lens epithelial cells (HLEC). We found that sustained exposure of HLEC to H_2O_2 did not activate NF-κB, but attenuated TNFα-induced NF-κB activation. We found that oxidative inactivation of the proteasome is one of the underlying mechanisms for the attenuated I-κB degradation and subsequent NF-κB activation in response to sustained oxidative stress. Furthermore, we demonstrated that activation of NF-κB is essential for HLEC to recover from oxidative stress. Inhibiting NF-κB activation during recovery from transient oxidative stress significantly reduced cell viability. Together, these data indicate that oxidative inactivation of the proteasome in HLEC not only impairs the protein quality control system [45–51], it also accounts for the impairment of cell signaling for NF-κB activation. Impairment of either the protein quality control mechanism or the cell signaling pathway by sustained oxidative stress may contribute to the death of lens epithelial cells, a common pathological feature of cataract.

Materials and methods

Materials

All materials used for SDS–PAGE were purchased from Bio-Rad Laboratories (Hercules, CA, USA). TNFα was purchased from R&D Systems (Minneapolis, MN, USA). MG132, Bay 11-7082, and BMS-345541 were obtained from Calbiochem–Novabiochem (La Jolla, CA, USA). Dulbecco's modified Eagle medium (DMEM), fetal calf serum, nonessential amino acid solution, and antibiotics for cell cultures were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies to p65, phosphorylated I-κB, total I-κB, phosphorylated Akt (Thr 473), and total Akt were purchased from Cell Signaling Technology (Danvers, MA, USA); monoclonal antibody to β-actin was purchased from Sigma–Aldrich (St. Louis, MO, USA). MTS assay reagents were purchased from Promega (Madison, WI, USA). HRP-conjugated anti-rabbit secondary antibody was from Jackson ImmunoResearch (West Grove, PA, USA). All other chemicals were obtained from Sigma–Aldrich, and were of the highest purity available.

Cell culture and treatments

HLEC (SRA 01/04) were routinely maintained at 37°C under 5% CO_2 atmosphere and were cultured with DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

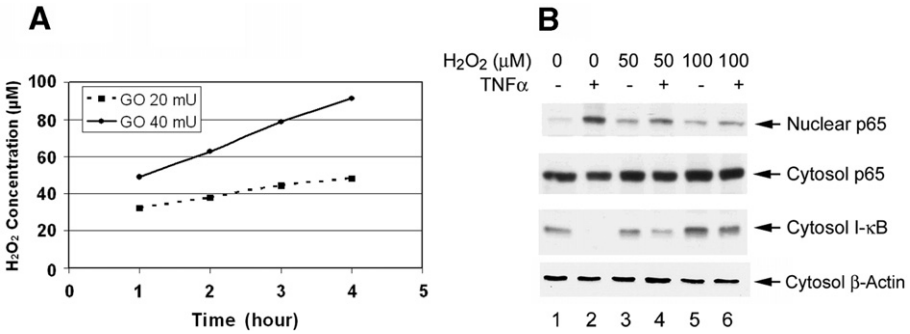

Fig. 1. Continuous influx of H_2O_2 downregulates TNFα-induced NF-κB activation. Confluent HLEC were cultured in the presence of 0, 20, and 40 mU/ml glucose oxidase for 4 h. (A) H_2O_2 levels in the medium were determined every hour during the incubation. This resulted in maximal concentrations of H_2O_2 in the medium of 0, 50, and 100 μM, respectively. The cells were then treated with or without 10 ng/ml TNFα in an oxidant-free medium for 30 min. (B) The levels of NF-κB (p65) in the nucleus and cytosol were determined by Western blotting. The levels of I-κBα and β-actin in the cytosol were also determined by Western blotting. The data are representative of three independent experiments.

Sustained exposure to oxidative stress was achieved by incubating cells with glucose oxidase (20 or 40 mU/ml) in a serum-, pyruvate-, and phenol red-free medium, supplemented with D-glucose (4500 mg/L). This produced sustained levels of H_2O_2 in the medium. Levels of H_2O_2 in the medium were determined by a colorimetric method as described previously [46]. In the presence of HLEC, 20 and 40 mU/ml glucose oxidase in the medium maintained the H_2O_2 concentrations at 30–50 and 50–100 μM, respectively (Fig. 1A). To study the effects of sustained oxidative stress on NF-κB activation, HLEC that were exposed to H_2O_2 for 4 h were incubated in fresh oxidant-free medium and treated with 10 ng/ml TNFα for 0, 15, and 30 min. The cells were then collected by scraping in PBS and were fractioned into cytosolic and nuclear fractions. To study the effect of proteasome inhibition on NF-κB activation, the cells were treated with 10 μM MG132 for 30 min before the addition of TNFα (10 ng/ml). The cells were then collected at 0, 15, and 30 min after the addition of TNFα and fractioned into cytosolic and nuclear fractions.

Preparation of nuclear extracts and DNA-binding assays

After treatment with TNFα, cells were rinsed with ice-cold PBS containing 1 mM EDTA and collected by scraping. After a brief centrifugation, the cell pellet was suspended in 200 μl ice-cold hypotonic lysis buffer (10 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.3% NP-40, 1 mM DTT, 1 mM AEBSF). After 15 min incubation on ice, the cell suspensions were vortexed for 10 s and centrifuged at 15,000 *g* for 15 s to recover intact nuclei. The nuclear pellets were resuspended in 200 μl of the above buffer without NP-40 and then centrifuged for 20 s. The resultant nuclear pellets were then resuspended in 20 μl ice-cold nuclear protein extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, and a cocktail of protease inhibitors) and incubated on ice for an additional 30 min, being vortexed every 10 min for 5 s. The samples were then centrifuged at 15,000 *g* for 5 min. The resultant supernatants, the source of DNA-binding proteins, were mixed 1:1 with ice-cold 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1% NP-40 and stored at −70°C until use. Protein concentrations in the extracts were determined by the Coomassie Plus protein assay kit (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as the standard. Electrophoretic mobility shift assays were used to determine the DNA-binding activity of NF-κB in the nuclear extracts. Briefly, equal amounts of nuclear extract (2 μg protein) from each sample were incubated with 10 fmol of ^{32}P-labeled oligonucleotide (1×10^5 cpm) specific for NF-κB in 20 μl binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 4% glycerol, 1 μg/ml poly(dI–dC), 1 μg/ml BSA) for 20 min at room temperature. The DNA–protein complexes formed were resolved on 5% nondenaturing polyacrylamide gels using 0.5×TBE (45 mM Tris–borate and 1 mM EDTA). Autoradiography was performed to visualize the shifted DNA–protein complexes.

Proteasome activity and lactate dehydrogenase activity assays

HLEC pellets were suspended in 25 mM Tris–HCl buffer (pH 7.6) containing 1 mM DTT. The cell suspensions were incubated on ice for 30 min, being vortexed every 10 min for 10 s. After centrifugation at 15,000 *g* for 10 min at 4°C, the supernatants were collected and used to measure activities of the proteasome and lactate dehydrogenase. The three peptidase activities of the proteasome were determined by using fluorogenic peptides as substrates. Succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC) was used for the chymotrypsin-like activity, *N*-*t*-butyloxycarbonyl-Leu-Ser-Thr-Arg-amidomethylcoumarin (LSTR-AMC) was used for the trypsin-like activity, and benzyloxycarbonyl-Leu-Leu-Glu-amidomethylcoumarin (LLE-AMC) was used for the peptidylglutamyl peptide hydrolase activity. The mixture, containing 20 μg of cell supernatant in 25 mM Tris–HCl, pH 7.6, was incubated at 37°C with the appropriate concentrations of peptide substrate (LLVY-AMC at 25 μM, LLE-AMC and LSTR-AMC at 40 μM) in a buffer containing 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN_3, and 0.04% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate. The final volume of the assay was 200 μl. Enzymatic kinetics were measured with a temperature-controlled microplate fluorometric reader (25 °C). Excitation/emission wavelengths were 380/440 nm. The lactate dehydrogenase activity was determined using CytoTox96 nonradioactive cytotoxicity assay kits (obtained from Promega) according to the manufacturer's instruction.

Western blot analysis

To determine the levels of phosphorylated proteins, cells were rinsed once with ice-cold PBS and immediately collected in SDS-gel loading buffer. Cell lysates were then denatured at 100°C for 3 min. Because SDS-gel loading buffer is not compatible with most of the commonly used protein quantification methods, we assessed the protein concentrations in the lysate by determining the densities of the protein bands on the gel after separation on SDS–PAGE and staining with Coomassie Brilliant Blue R-250. To determine levels of proteins in the cytosolic and nuclear fractions, cytosol and nuclear preparations were mixed with an equal volume of 2×SDS-gel loading buffer. Equal amounts of proteins (20 μg) were resolved on 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with rabbit polyclonal antibodies against I-κBα (1:1000 dilution), phosphorylated Akt (Thr 473) (1:1000 dilution), and total Akt (1:1000 dilution) and mouse monoclonal antibodies against phosphorylated I-κBα (Ser32/36) (1:1000 dilution) and β-actin (1:10,000 dilution). After incubation with the corresponding HRP-conjugated secondary antibodies, the specific bound antibody was visualized using a Super Signal chemiluminescence detection kit (Pierce).

Cell viability assays

HLEC at 90–95% confluence in 96-well plates were treated with 0–400 μM H_2O_2 in a serum-, pyruvate-, and phenol red-free medium for 1 h. The cells were then allowed to recover for 16 h in an oxidant-free medium in the presence or absence of 5 μM Bay 11-7082 or 5 μM BMS-345541, two specific inhibitors of NF-κB activation. Cell viability was determined before and after 16 h recovery in oxidant-free medium with MTS assay as described [52].

Statistical analyses

Statistical analysis was performed using Student's *t* test assuming equal variances for all data points (comparison of two groups) or by two-way ANOVA with Tukey's post hoc test (comparison of multiple groups). All the statistical analyses were performed using Systat software (SYSTAT 11). A $p<0.05$ was considered statistically significant.

Results

Sustained oxidative stress attenuates TNFα-induced p65 nuclear translocation

In unstimulated cells, NF-κB is associated with I-κB and retained in the cytoplasm. Upon various stimulations, I-κB is phosphorylated and degraded by the UPP, and NF-κB enters into the nucleus and triggers the expression of a large number of genes. To study the effects of sustained oxidative stress on NF-κB activation, we first determined the effects of sustained exposure to H_2O_2 on NF-κB (p65) nuclear translocation. Confluent HLEC were cultured in the presence of 0, 20, and 40 mU/ml glucose oxidase for 4 h. This maintained H_2O_2 levels in the medium of 0, 30–50, and 50–100 μM, respectively (Fig. 1A). The

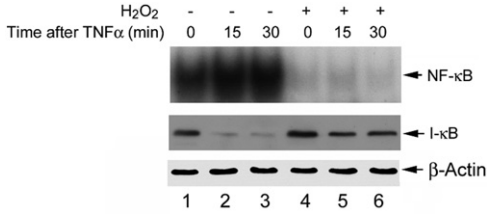

Fig. 2. Sustained exposure to H_2O_2 attenuates TNFα-induced NF-κB activity. Confluent lens epithelial cells were cultured in the presence or absence of 40 mU/ml glucose oxidase for 4 h. The cells were then treated with 10 ng/ml TNFα for 0, 15, and 30 min. The DNA-binding activity of NF-κB in the nuclear extracts was determined by electrophoretic mobility shift assays (top). The levels of I-κBα and β-actin in the cytosol were determined by Western blotting (middle and bottom, respectively). The data are representative of three independent experiments.

cells were then treated with or without 10 ng/ml TNFα in an oxidant-free medium for 30 min. The levels of NF-κB (p65) in the nuclear and cytosolic fractions were determined by Western blotting. As in other cell types, the majority of p65 was localized in the cytosol of unstimulated cells (Fig. 1B). Sustained exposure of HLEC to H_2O_2 increased the levels of p65, but did not alter the cellular localization of p65 (Fig. 1B, compare lanes 3 and 5 with 1). Upon stimulation with TNFα, nuclear p65 levels increased and cytosolic p65 levels decreased (Fig. 1B, compare lane 2 with 1), indicating that p65 was translocated into the nucleus in response to TNFα stimulation. In contrast to p65, I-κBα was detected only in the cytosolic fraction (Fig. 1B), not in the nuclear fraction (data not shown). Associated with p65 nuclear translocation was the dramatic decrease in I-κBα levels in the cytosol (Fig. 1B, compare lane 2 with 1). Sustained exposure to H_2O_2 attenuated the TNFα-induced decrease in the levels of I-κBα and nuclear translocation of p65 in a dose-dependent manner (Fig. 1B, compare lanes 4 and 6 with 2). These data indicate that sustained exposure of HLEC to H_2O_2 may attenuate NF-κB activation.

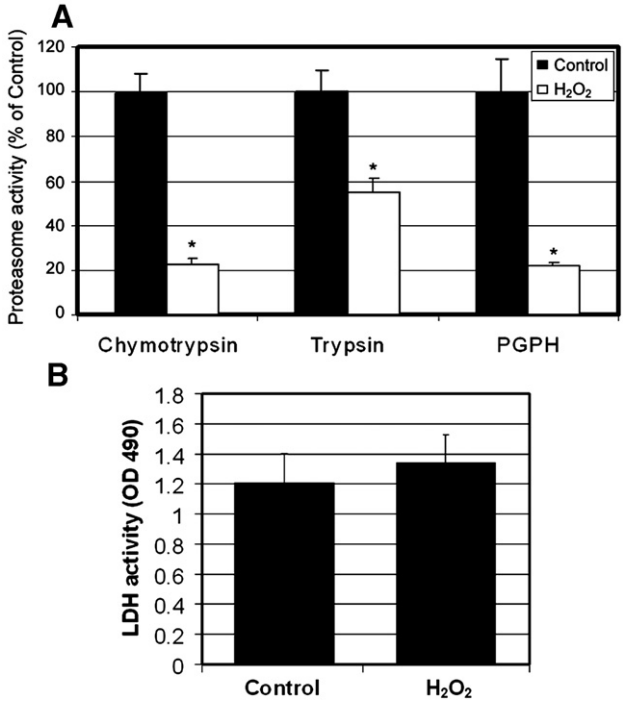

Fig. 3. Sustained exposure to H_2O_2 inactivates the proteasome. Confluent lens epithelial cells were cultured in the presence and absence of 40 mU/ml glucose oxidase for 4 h. The cells were collected and lysed in a hypotonic buffer with vortexing. After centrifugation the resultant supernatants were used to determine activities of (A) proteasome and (B) lactate dehydrogenase. Chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase (PGPH) peptidase activities of the proteasome were determined using fluorogenic peptides as substrates. Enzymatic kinetics were measured with a temperature-controlled microplate fluorometric reader. Excitation/emission wavelengths were 380/440 nm. *$p<0.01$ in comparison between control and chronically oxidized cells. The data are summarized from two independent experiments, each was done in triplicate.

Sustained oxidative stress decreases TNFα-induced NF-κB activation

To evaluate further the effects of sustained oxidative stress on NF-κB activation, HLEC were exposed to 0 or 100 μM H_2O_2 for 4 h and then treated with 10 ng/ml TNFα in an oxidant-free medium for 0, 15, and 30 min. The pilot experiments showed that TNFα-induced NF-κB activation was detectable as early as 5 min upon stimulation and reached peak levels by 30–60 min. The signal declined thereafter and was barely detectable by 4 h. Therefore, we chose 15- and 30-min time points in these experiments. As shown in Fig. 2, treatment of nonstressed HLEC with TNFα for 15 and 30 min resulted in a strong DNA-binding activity of NF-κB (compare lanes 2 and 3 with 1). Exposure of HLEC to 100 μM H_2O_2 for 4 h blocked the TNFα-induced increase in DNA-binding activity of NF-κB (Fig. 2, compare lanes 5 and 6 with 2 and 3). Basal DNA-binding activity of NF-κB was also substantially reduced in cells that were exposed to sustained oxidative stress (Fig. 2, compare lane 4 with 1). The time-dependent decrease in levels of I-κB was also significantly attenuated in oxidized cells (Fig. 2, middle). These data confirm that sustained exposure of HLEC to H_2O_2 impairs TNFα-induced NF-κB activation.

Sustained oxidative stress inactivates the proteasome in HLEC

Ubiquitin-dependent degradation of I-κB is required for NF-κB nuclear translocation and DNA binding. The UPP also plays an important role in the activation of IKK, the upstream kinase involved in NF-κB activation. To determine whether the oxidation-induced attenuation of I-κB degradation in response to TNFα stimulation was

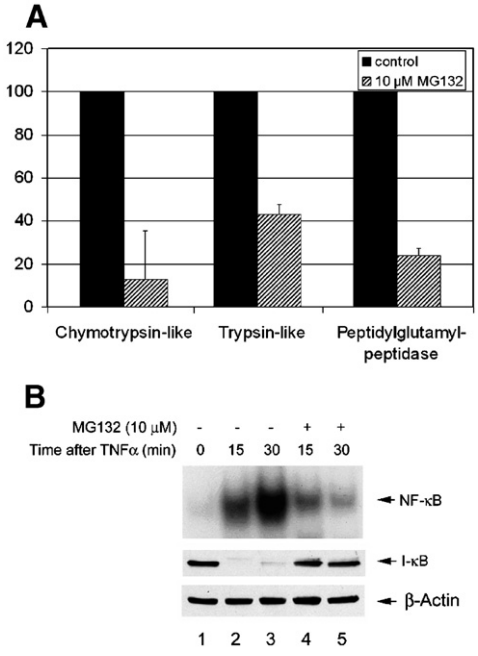

Fig. 4. Proteasome inhibition downregulates TNFα-induced NF-κB activity. (A) Confluent lens epithelial cells were first cultured in the presence or absence of 10 μM MG132 for 30 min. This concentration of MG132 inhibited the three peptidase activities of the proteasome by 60–85%. (B) The cells were then treated with10 ng/ml TNFα for 0, 15, and 30 min. The DNA-binding activity of NF-κB in the nuclear extracts was determined by electrophoretic mobility shift assays (top). Levels of I-κBα and β-actin in the cytosol were determined by Western blotting (middle and bottom). The data are representative of three independent experiments.

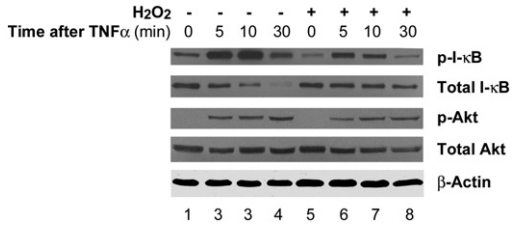

Fig. 5. Sustained exposure to H_2O_2 also impairs the TNFα-induced phosphorylation of I-κBα. Confluent lens epithelial cells were cultured in the presence or absence of 40 mU/ml glucose oxidase for 4 h. The cells were then incubated with 10 ng/ml TNFα for 0, 5, 10, and 30 min. Levels of phosphorylated I-κBα and phosphorylated Akt in the cells were determined by Western blotting analysis using a monoclonal antibody against phosphorylated I-κBα (Ser32/36) or rabbit polyclonal antibodies against phosphorylated Akt (Thr473). The levels of total I-κBα, Akt, and β-actin were determined and used as the loading controls. The data are representative of three independent experiments.

related to impairment of UPP activity, we examined peptidase activities of the proteasome. As shown in Fig. 3A, all three peptidase activities of the proteasome decreased dramatically in H_2O_2-exposed cells. Whereas the trypsin-like activity decreased ~50% in response to exposure to 100 μM H_2O_2 for 4 h, the chymotrypsin-like activity and peptidylglutamyl peptide hydrolase activity decreased by 80%. The decline in proteasome activity was not due to oxidation-induced cell death, because >95% of the stressed cells were viable after 4 h exposure to H_2O_2 (data not shown). Furthermore, the activity of an unrelated enzyme, lactate dehydrogenase, did not decrease under the same conditions (Fig. 3B). These data suggest that the proteasome is one of the cellular components that are susceptible to sustained oxidative stress.

Chemical inhibition of the proteasome partially prevents TNFα-induced I-κBα degradation and NF-κB activation

The coincidence of oxidative inactivation of the proteasome and attenuation of degradation of I-κBα prompted us to hypothesize that oxidative inactivation of the proteasome may be responsible for the oxidation-induced attenuation of I-κBα degradation and NF-κB activation. To test this hypothesis, we examined the effects of chemical inhibition of the proteasome on TNFα-stimulated I-κBα degradation and NF-κB activation. As shown in Fig. 4A, treatment of the cells with 10 μM MG132 inhibited the peptidase activities of the proteasome by 60–85%, which was similar to that of cells exposed to sustained oxidative stress. NF-κB DNA-binding activity increased significantly upon TNFα stimulation. When pretreated with MG132, the TNFα-induced NF-κB binding was substantially reduced (Fig. 4B, top). The rate of TNFα-induced degradation of I-κBα was also decreased when the cells were treated with the proteasome inhibitor. These data indicate that oxidative inactivation of the proteasome may be one of the mechanisms that are responsible for the attenuation of NF-κB activation upon sustained oxidative stress.

Sustained oxidative stress reduces TNFα-induced phosphorylation of I-κBα

The data presented above indicate that sustained oxidative stress almost completely blocked the TNFα-induced NF-κB activation, but proteasome inhibition only partially prevented this activation. This suggests that oxidative inactivation of the proteasome is only partially responsible for the impairment of NF-κB activation by sustained oxidative stress. To start investigating the effect of sustained oxidation on cell signaling involved in NF-κB activation, we determined the effect of sustained oxidative stress on TNFα-induced phosphorylation of I-κBα. As shown in Fig. 5, sustained oxidative stress significantly reduced the levels of phosphorylated I-κBα, indicating that the signaling pathways that lead to I-κBα phosphorylation were also impaired by sustained oxidative stress. In contrast, the phosphorylation of Akt was not affected by this level of oxidative stress (Fig. 5). These data indicate that the NF-κB signaling pathway is more susceptible to oxidative stress than the Akt signaling pathway. From these data, we conclude that the attenuation of NF-κB activation upon sustained oxidative stress was due not only to the inactivation of the proteasome, but also to damage to other steps of the signaling pathway.

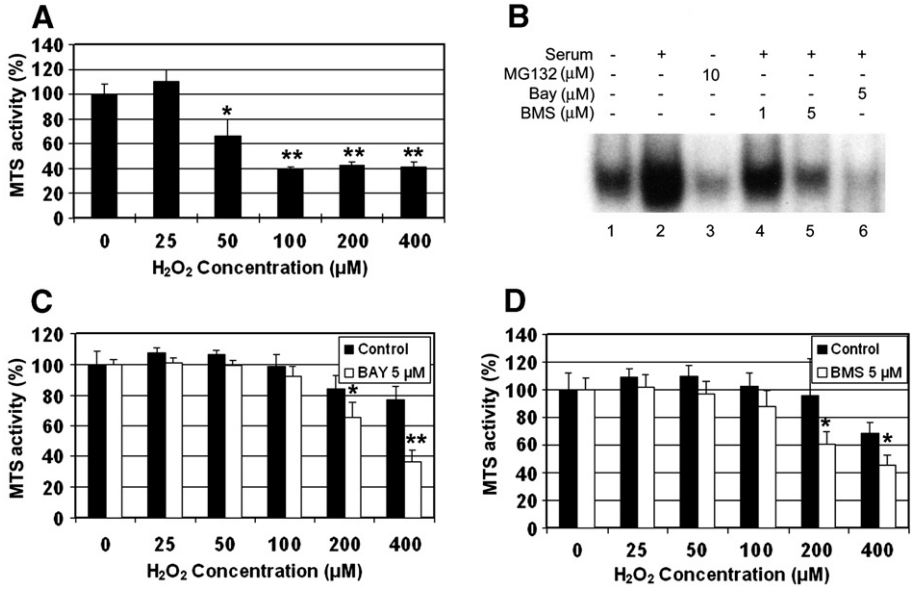

Fig. 6. Inhibition of NF-κB activation during recovery from transient oxidative stress potentiates cytotoxicity of oxidative stress. Subconfluent HLEC were treated with the indicated concentrations H_2O_2 for 1 h and then allowed to recover in oxidant-free medium for 16 h in the absence or presence of 5 μM Bay 11-7082 or 5 μM BMS-345541, two classic NF-κB inhibitors. The cell viability was determined by MTS assay. (A) MTS activity immediately after exposure to H_2O_2. (B) The effects of MG132, Bay 11-7082, and BMS-345541 on NF-κB activation. (C) MTS activity after 16 h recovery in the presence or absence of 5 μM Bay 11-7082. (D) MTS activity after 16 h recovery in the presence or absence of 5 μM BMS 345541. Data in (A, C, and D) are summaries of two independent experiments, each done in triplicate. *$p<0.05$ and **$p<0.01$ compared with cells treated with the same levels of H_2O_2. The data in (B) are representative of two independent experiments.

NF-κB activation is essential for HLEC to recover from oxidative stress

It has been demonstrated that NF-κB activation in general has prosurvival functions in response to various stressors. To determine whether NF-κB activation plays a role in HLEC's recovery from transient oxidative stress, we determined the effects of NF-κB inhibitors (Bay 11-7082 and BMS-345541) [53,54] on recovery from transient oxidative stress. To do so, subconfluent (90–95% confluence) HLEC were treated with 0–400 μM H_2O_2 for 1 h and then allowed to recover in oxidant-free medium in the absence or presence of 5 μM Bay 11-7082 or 5 μM BMS-345541 for 16 h. Transient exposure to as low as 50 μM H_2O_2 resulted in a decrease in MTS activity (Fig. 6A). Exposure to a single bolus of 100 μM or higher doses of H_2O_2 reduced the MTS activity by 60% (Fig. 6A). The decrease in MTS activity immediately after transient oxidative stress was mainly due to oxidative inhibition of the mitochondrial dehydrogenases, which were measured by the MTS assay, because 95% of the cells were viable as judged by trypan blue staining (data not shown). When the cells were allowed to recover in an oxidant-free medium for 16 h, the MTS activity recovered to >70% of the control levels (Figs. 6C and 6D). As we and others demonstrated previously [15,52], transient exposure of the cells to lower levels of H_2O_2 stimulated cell growth (Figs. 6C and 6D). In the absence of NF-κB inhibitors during the recovery period, the cells could withstand transient exposure of 100 μM H_2O_2 without significant loss of cell viability. When exposed to 200 and 400 μM H_2O_2, the cell viability decreased to ~80 and 75% of the controls, respectively (Figs. 6C and 6D). As shown in Fig. 6B, treatment of the cells with 5 μM Bay 11-7082 or 5 μM BMS-345541 inhibited NF-κB activation by ~90 and ~70%, respectively. However, treatment of cells with these levels of NF-κB inhibitors in the absence of oxidative stress had no significant effects on cell viability. In contrast, treatment of oxidatively stressed cells with these levels of NF-κB inhibitors significantly increased the cytotoxicity of H_2O_2 (Figs. 6C and 6D), indicating that activation of NF-κB is essential for the cells to recover from transient oxidative stress.

Discussion

Cataract is one of the most common age-related eye diseases and oxidative stress is an important factor in cataractogenesis [35,55]. Chronic oxidative stress not only causes protein damage and precipitation in lens fibers, it also contributes to the dysfunction and apoptotic death of lens epithelial cells [56–58]. The death of epithelial cells is also a common feature of cataractous lenses in both humans and experimental animals [59–64]. NF-κB is a class of transcription factors that regulate the expression of many prosurvival genes, such as survivin, Bcl-2, and inhibitors of apoptosis [65–67]. In this study, we investigated the effects of sustained oxidative stress on NF-κB activation. We found that sustained oxidative stress impairs cell signaling involved in NF-κB activation. Oxidative inactivation of the proteasome is one of the mechanisms that underlie the oxidative impairment of NF-κB activation. We also demonstrated that inhibition of NF-κB activation during recovery from transient oxidative stress reduces the viability of stressed cells. These data indicate that attenuation of NF-κB activation by sustained oxidative stress may contribute to the death of lens epithelial cells associated with cataractogenesis.

The relationship between oxidative stress and NF-κB activation has been studied extensively. However, the question of whether H_2O_2 stimulates or inhibits NF-κB remains controversial. Many studies showed that transient exposure to H_2O_2 stimulates NF-κB activation [10–12]. However, opposite results have also been reported [13,68,69]. NF-κB activation has a dual and opposite dependence on oxidative events, because its translocation is favored by oxidative events in the cytosol, whereas binding to DNA requires a reductive environment in the nucleus [70,71]. Therefore, low levels of oxidative stress stimulate NF-κB activation, whereas higher levels of oxidative exposure can turn a potential positive stimulus into an inhibitory effect. The present work indicates that the duration of oxidative stress also has a great impact on NF-κB activation. Whereas transient oxidative stress may activate this transcription factor, sustained exposure to a relatively lower level of oxidants may impair NF-κB activation.

The UPP plays important roles in diverse cellular functions, including protein quality control, cell cycle regulation, and signal transduction [14,72]. A functional UPP is required for the cells to cope with various stresses, including heavy metals [73,74], amino acid analogs, and oxidative stress [15,16]. However, the UPP itself is also a target of such stresses. All three classes of ubiquitination enzymes (E1, E2, and E3) have a cysteine in their active site, and therefore the activities of these enzymes are subject to redox regulation [75,76]. In addition, other types of modifications, such as S-nitrosylation, can inactivate these enzymes [77]. Reactive oxygen species and reactive lipid peroxidation products, such as 4-hydroxynonenal, also impair the proteasome [78–83]. Therefore, a sustained oxidative insult may also damage or impair the function of critical components of the UPP [75–81,84]. We demonstrated in retinal pigment epithelial cells that the proteasome is the most susceptible component of the UPP to physiological relevant oxidative stress [51].

The UPP is an important protein quality control system [47,83,85], which selectively degrades mutant, misfolded, or damaged proteins [46,48,86]. It has been thought that the failure of timely removal of abnormal or damaged proteins by the UPP may account for the proteasome inhibitor-induced intolerance to various environmental stresses [15,16]. The present work indicates that the attenuation of NF-κB activation via proteasome inhibition is another reason for oxidative stress-induced cytotoxicity.

Oxidative inactivation of the proteasome and the resultant impairment of I-κB degradation only partially account for the attenuation of TNFα-induced NF-κB activation. It seems that other steps in the cell signaling for NF-κB activation are also affected by sustained oxidative stress. For example, TNFα-induced I-κBα phosphorylation was also reduced by chronic oxidative stress. The IKK-mediated phosphorylation of I-κBα is a prerequisite for the ubiquitination and degradation of I-κBα [2]. Comparative analysis of the structures of the kinase domains of IKKs and related kinases indicates that oxidation of Cys-179 in either IKKα or IKKβ would lead to its inhibition [69,87]. Sustained oxidative stress may increase the levels of oxidized GSH in the cells. It has been demonstrated that high levels of oxidized GSH hinder the activity of enzymes involved in ubiquitination and, accordingly, block proteolysis of I-κBα [69,76,88]. Ubiquitination is required not only for proteasomal degradation of phosphorylated I-κBα, but also for phosphorylation of I-κBα, because ubiquitination is involved in controlling IKK activity [17,18]. Sustained oxidative stress may also regulate NF-κB function via other mechanisms, such as directly modulating the transcription activity of NF-κB [88]. Further studies to elucidate the signaling events that are affected by sustained oxidative stress are warranted to understand comprehensively the molecular mechanisms of oxidative regulation of NF-κB. A comprehensive understanding of NF-κB activation may not only shed light on the pathogenesis of many stress-related diseases, but will also provide clues to the pharmaceutical treatment of such diseases.

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