

Suppression of the TNF α -Induced Increase in IL-1 α Expression by Hypochlorite in Human Corneal Epithelial Cells

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PURPOSE. In response to injury, activated neutrophils release tumor necrosis factor (TNF)- α and myeloperoxidase (MPO). TNF α in turn causes human corneal epithelial cells to secrete interleukin (IL)-1 α , whereas MPO results in formation of HClO/OCI⁻. The effect of HClO/OCI⁻ on the expression of the IL-1 α gene and protein is unknown. The current study was undertaken to examine in immortalized human corneal epithelial cells whether NaOCl alters TNF α -induced increases in expression of IL-1 α gene and protein.

METHODS. Semiquantitative RT-PCR and ELISA characterized IL-1 α gene and protein expression, respectively. TNF α -induced nuclear transfer of nuclear factor (NF)- κ B was measured by electrophoretic mobility shift assay (EMSA). The α isoform of inhibitory protein κ B (I κ B α) was identified by Western blot analysis.

RESULTS. Exposure to NaOCl (0.75 mM) for 10 minutes caused suppression of TNF α -induced increases in IL-1 α mRNA and protein, declines in NF κ B nuclear transfer, and a modification of I κ B α , based on a bandshift detected by Western blot analysis. Modified I κ B α became resistant to TNF α -induced proteolysis. Methionine sulfoxide reductase A (MsrA, 10 μ M) eliminated the NaOCl-induced I κ B α bandshift.

CONCLUSIONS. NaOCl oxidizes I κ B α at methionine residues and thereby suppresses dissociation of I κ B α from NF κ B. Decreased dissociation could in turn suppress TNF α -induced activation of NF κ B, resulting in declines in expression of IL-1 α gene and protein. These effects suggest that release of HClO/OCI⁻ in vivo by activated neutrophils may counterbalance TNF α -induced NF κ B-dependent secretion of IL-1 α and suppress an excessive inflammatory reaction. (*Invest Ophthalmol Vis Sci.* 2002;43:3190-3195)

The corneal epithelium provides a barrier function against infection through the maintenance of tight junctional integrity. If the tight junctions are disrupted as a result of wound-

ing, stromal infection may occur during the healing process if tight junctional re-formation is delayed or inadequate. For the barrier function to be reestablished, the tight junctions must have adequate resistance through the formation of close apposition between neighboring cells in the most superficial cell layer. Formation of tight junctional complexes depends on a myriad of interactions among different cytokine receptor-linked second-messenger cascades that mediate control of corneal epithelial growth, differentiation, and cell survival. In addition, timely closure of epithelial wounds is dependent on the interplay between the release of cytokines, not only from epithelial cells, but also from the stromal keratocytes and immune system cells and on cytokines secreted from corneal nerves and the lacrimal and accessory lacrimal glands. Should the tight junctional integrity remain compromised, tear fluid containing cytokines may travel through the junctions in excessive amounts into the stroma. Some of them may set into play events leading to the breakdown of the stromal matrix and inflammation followed by immunologic responses. One outcome could be infiltration of neutrophils resulting in ulceration of the diseased or damaged tissues.¹⁻³ These considerations have prompted an intense effort to gain further understanding of how to control cytokine-mediated responses resulting from infection.

Activated neutrophils infiltrate and aggregate at a corneal inflammatory site. They secrete large amounts of myeloperoxidase (MPO) and produce hydrogen peroxide in phagolysosomes and the extracellular compartment.⁵ MPO catalyzes the production of hypochlorous acid (HClO/OCI⁻) from hydrogen peroxide and the chloride ion.⁶⁻⁸ HClO/OCI⁻ is a potent antimicrobial agent that affects both the emitting and surrounding cells at an inflammatory site as a result of oxidizing a wide range of biomolecules, particularly thiols, methionine, and amines.⁹ In some other tissues, formation of taurine chloramine (TauCl) has been detected. TauCl is a major chlorination product and is a more stable but less toxic oxidant than HClO/OCI⁻.^{7,9,10}

Recent studies have suggested that TauCl has a significant role as an immunomodulator. It can inhibit (1) the production in macrophages of nitric oxide and tumor necrosis factor (TNF)- α ¹¹; (2) the production in bone marrow-derived dendritic cells of TNF α , interleukin (IL)-6, -10, and -12, prostaglandin (PG)E₂, and nitrite¹²; (3) the production in glioma cells of monocyte chemoattractant protein-1 and macrophage inflammatory protein-2¹³; and (4) the production in rheumatoid arthritis fibroblast-like synoviocytes of IL-6 and 8.¹⁴ Because the transcription of the genes of these inflammatory proteins is controlled by the transcription factor nuclear factor (NF)- κ B,¹⁴⁻¹⁷ TauCl and its source HClO/OCI⁻ elicit their effects through NF κ B-associated cell-signaling pathways.

During inflammation, activated neutrophils infiltrate the cornea and can induce the epithelium to express IL-1 α ,^{18,19} -1 β ,^{18,20} -6, and -8²⁰ and TNF α .^{20,21} Expression of their genes is ultimately dependent on dissociation of the inhibitory protein κ B (I κ B) from NF κ B, which results in activation of

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Supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (YM).

Submitted for publication March 21, 2002; revised May 22, 2002; accepted June 6, 2002.

Commercial relationships policy: N.

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NF κ B.^{14,22,23} In the present study, we investigated the effect of NaOCl on expression in the epithelium of (1) gene and protein of the proinflammatory cytokine IL-1 α , (2) nuclear transfer of NF κ B, and (3) oxidation status of the α isoform of IkB ($\text{IkB}\alpha$).

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 mixture and fetal bovine serum (FBS) were from Iwaki (Tokyo, Japan). Cholera toxin, insulin, gentamicin, RNA extraction buffer (TRIzol), oligo dT primer, and RNase H⁻ reverse transcriptase (SuperScript II) were from Invitrogen (Groningen, The Netherlands). Human epidermal growth factor (EGF) was obtained from BD Biosciences (Bedford, MA), recombinant human TNF α and an immunoassay system (An'Alyza) from Genzyme Techne (Minneapolis, MN), recombinant RNasin RNase inhibitor from Promega (Madison, WI), and NaOCl from Wako (Tokyo, Japan). Alkaline phosphatase (AP) and calpain inhibitor I were purchased from Roche Diagnostics Corp. (Mannheim, Germany), a protein assay kit from Bio-Rad Laboratories (Hercules, CA), and polyvinylidene difluoride membranes from Millipore (Bedford, MA). The affinity-purified rabbit polyclonal antibody for $\text{IkB}\alpha$ was from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-rabbit IgG linked to horseradish peroxidase, an enhanced chemiluminescence (ECL) Western blot detection system, and [γ -³²P] adenosine triphosphate (ATP) were from Amersham Pharmacia Biotech (Buckinghamshire, UK). T4 oligonucleotide kinase was from Takara Biomedical (Shiga, Japan), and DNA (HotStartTaq) polymerase was from Qiagen (Chatsworth, CA).

Culture of Human Corneal Epithelial Cells

Santen Pharmaceutical Co., Ltd. (Osaka, Japan) generously provided simian virus (SV)40-immortalized human corneal epithelial (HCE) cells. They were cultured in DMEM/Ham's F-12 mixture medium, supplemented with 10% FBS, 5 $\mu\text{g}/\text{mL}$ cholera toxin, 10 ng/mL human EGF, and 40 ng/mL gentamicin. Cultures were incubated at 37°C under fully humidified 95% air and 5% CO₂. All experiments were performed with HCE cells seeded at a density of 8×10^5 cells per 35-mm dish and cultured overnight.

Cell Treatment with OCl⁻ and Stimulation with TNF α

NaOCl was diluted to 40 mM with 60 mM sodium phosphate buffer (pH 8.3) consisting of 60 mM Na₂HPO₄ and 60 mM NaH₂PO₄. Cells were exposed to various concentrations of NaOCl for 10 minutes in a 37°C incubator. After the treatment, cells were washed with phosphate-buffered saline (PBS). To activate NF κ B, HCE cells were exposed to 30 ng/mL TNF α for various periods after treatment with NaOCl.

Total RNA Extraction and Semiquantitative Reverse Transcription-Polymerase Chain Reaction

In preparation for RT-PCR, the cellular RNA was isolated with RNA extraction reagent (TRIzol). Single-stranded cDNA was prepared by RT with isolated total RNA used as a template with oligo dT primer, dNTP mix, recombinant RNasin RNase inhibitor and RNase H⁻ reverse transcriptase. PCR was performed with DNA polymerase (HotStartTaq) with primers based on published sequences as follows: for IL-1 α : sense, 5'-caaggagagcatggtgtagtagcaaccaacg-3', and antisense, 5'-tagtgccgtgagttccagaagaagaggagg-3'¹⁸; for β -actin: sense, 5'-accaactgggacgacatggag-3' and antisense, 5'-cgtgaggatcctcatgaggtagc-3'.²⁴ PCR was initiated in a thermal cycler programmed at 95°C for 15 minutes, and then at 94°C for 1 minute, 65°C for 1 minute, and 72°C for 2 minutes (30 cycles). The sizes of the PCR products for IL-1 α and β -actin were 407 and 354 bp, respectively. After PCR, the content of each tube was analyzed by 1.5% agarose gel electrophoresis. The bands were visualized with ethidium bromide. The density of bands was determined

using an image-analysis program provided by Scion Corp. (Frederick, MD).

Enzyme-Linked Immunosorbent Assay

After treatment, cells were collected in PBS and centrifuged at 1000g for 5 minutes at 4°C. Cell lysis solution, containing 50 mM Tris(hydroxymethyl)aminomethane (Tris)/Cl (pH 7.6), 300 mM NaCl, and 0.5% Triton X-100, was added to pelleted cells and kept for 2.5 hours on ice with frequent vortexing. The lysates were centrifuged at 16,000g for 5 minutes, and the resultant supernatant was diluted with PBS. The concentration of IL-1 α was colorimetrically measured at 450 nm by an ELISA system (An'Alyza). The cellular protein concentration in cell lysates was determined with a protein assay kit with bovine serum albumin used as a standard.

Electrophoretic Mobility Shift Assay

For the purification of nuclei, pelleted cells were resuspended in buffer A, consisting of 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM dithiothreitol (DTT), buffered with 10 mM HEPES-KOH (pH 7.9) for 10 minutes on ice. Lysis was completed by vortexing for 30 seconds. The lysates were centrifuged at 16,000g for 30 seconds at 4°C. The purified nuclear pellet was resuspended in buffer B (25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 M EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM DTT, and 20 mM HEPES-KOH (pH 7.9), with frequent vortexing for 20 minutes on ice. After centrifugation at 16,000g for 2 minutes, the supernatant was saved to obtain a nuclear extract. The protein amount was determined with the protein assay kit. An oligonucleotide probe containing the sequence of the NF κ B binding site (sense, 5'-agcttCAGAGGGGACTTCCGAGAGG-3' antisense, 5'-tcgaCCTCTCGGAAAGTCCCCTCTGa-3') was labeled with [γ -³²P]-ATP, using T4 oligonucleotide kinase, which was incubated with 5 μg nuclear extract for 20 minutes at room temperature. The NF κ B that bound to the labeled oligonucleotide probe was fractionated on 4% polyacrylamide gel and visualized by autoradiography.

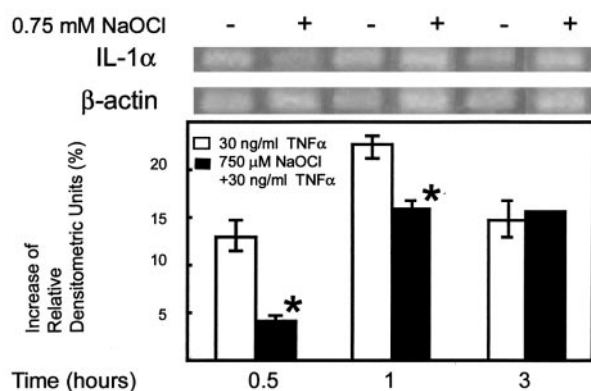
Western Blot Analysis

Pelleted cells were resuspended in TNE buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40) containing 20 μM PMSF and 10 mg/mL calpain inhibitor I. The suspension was vortexed for 30 seconds and centrifuged at 16,000g for 5 minutes at 4°C. The supernatant was then added to an equal volume of 2 $\times$ gel loading buffer (100 mM Tris-Cl [pH 6.8], 4% SDS, 20% glycerol, 0.5 mg/mL bromophenol blue, and 10% β -mercaptoethanol) and boiled for 5 minutes. The protein assay kit was used to determine cellular protein content. A constant amount of extract (60 μg) was separated on a 12% polyacrylamide gel and electrotransferred to PVDF membranes. To be able to detect a small change in apparent molecular weight of $\text{IkB}\alpha$, for example, an increase due to phosphorylation, the assay was continued until $\text{IkB}\alpha$ reached close to the bottom of an elongated separating gel (approximately 10 cm long).²⁵ Membranes were blocked in 5% nonfat milk in wash buffer (10 mM Tris-Cl [pH 7.5], containing 100 mM NaCl and 0.1% Tween 20) and immunoblotted with the affinity-purified rabbit polyclonal antibody for $\text{IkB}\alpha$ at a dilution of 1:400. Immuno-complexes were detected by binding to donkey anti-rabbit IgG linked to horseradish peroxidase at a dilution of 1:500, followed by use of the ECL Western blot detection assay kit.

Treatment of Cell Lysates with AP and MsrA

After exposure to 0.75 mM NaOCl for 10 minutes, cells were collected in PBS, centrifuged at 1000g for 5 minutes, and resuspended in 30 μL cold TNE buffer. The cell suspension was vortexed for 30 seconds and centrifuged at 16,000g for 5 minutes. The pH value of 30 μL of the supernatant was adjusted to 9.7 with 4.5 μL 0.1 N NaOH, then mixed with 10 U AP and incubated at 37°C for 30 minutes. The reaction was terminated by adding 4.5 μL 0.1 N HCl. To treat with methionine sulfoxide reductase (MsrA), cell lysates were prepared in 30 μL cold

A



B

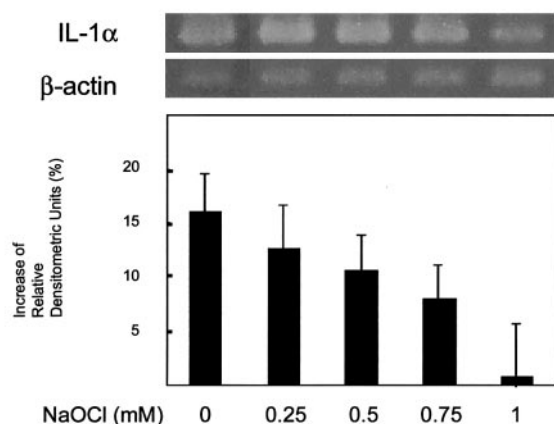


FIGURE 1. Pretreatment with NaOCl inhibited transcription of IL-1 α . (A) Cells that were seeded at a density of 8×10^5 cells per 35-mm dish and cultured overnight were treated with NaOCl (0.75 mM) or vehicle (60 mM sodium phosphate buffer) for 10 minutes at 37°C. Cells were then washed with PBS, fresh medium was added containing TNF α (30 ng/mL), and cells were incubated at 37°C. At the indicated times, cells were harvested for total RNA extraction. (B) The concentration of NaOCl was varied up to 1 mM. After incubation with TNF α (30 ng/mL) for 1 hour, cells were subjected to total RNA extraction. Detection of IL-1 α and β -actin mRNA was performed by semiquantitative RT-PCR. All the results were normalized in comparison with β -actin, and basal message levels without stimulation with TNF α were subtracted. Data are the mean $\pm$ SEM of results in three independent experiments. *Significantly different from cells treated with TNF α by Student's *t*-test unpaired analysis ($P < 0.05$).

TNE buffer, supplemented with 15 mM DTT. Recombinant yeast MsrA was prepared as reported previously.²⁶ Cell lysates were treated with 10 μ M MsrA for 15 minutes at 37°C. To evaluate the effects of DTT on the shifted band, cell lysates were incubated for 30 minutes at 37°C without MsrA. After these enzyme treatments, each sample was mixed with 2 $\times$ gel loading buffer and boiled for 5 minutes for Western blot analysis.

RESULTS

Effect on Transcription of IL-1 α

To examine whether NaOCl affects transcription of IL-1 α , we determined the amount of IL-1 α message by semiquantitative RT-PCR. A 10-minute pretreatment with 0.75 mM NaOCl had no effect on the basal levels of expression of IL-1 α mRNA at 0 hours (no stimulation with TNF α ; data not shown). These basal

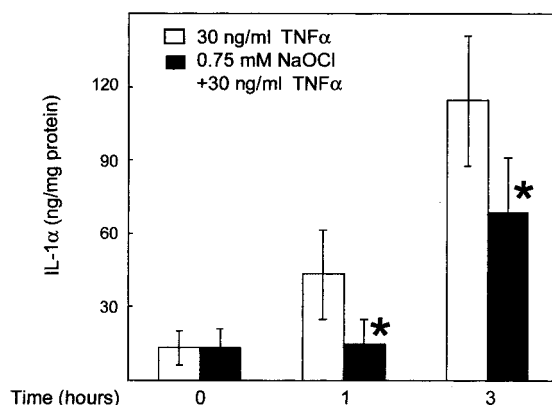
levels were subtracted, and increased message levels were plotted. Exposure for 1 hour to TNF α (30 ng/mL) increased expression of IL-1 α mRNA, but it decreased at 3 hours (Fig. 1A). Even though NaOCl pretreatment did not affect this transient pattern of change, it significantly decreased TNF α -induced increase in expression of IL-1 α mRNA at 30 minutes and 1 hour ($P < 0.05$), whereas no difference was observed at 3 hours. TNF α stimulation-dependent expression of the IL-1 α gene was decreased by approximately 70% at 30 minutes.

The concentration-dependent effects of pretreatment with NaOCl on the expression of the IL-1 α gene were studied. NaOCl at up to 1 mM decreased TNF α -induced increases in transcription of IL-1 α in a dose-dependent manner (Fig. 1B). These declines suggest that NaOCl may suppress activation of NF κ B.

Effect on Expression of IL-1 α Protein

An ELISA was used to determine the effects of a 10-minute pretreatment with 0.75 mM NaOCl on the TNF α (30 ng/mL)-

A



B

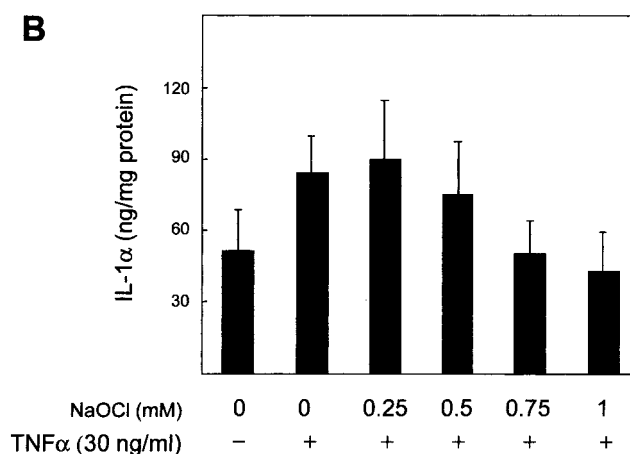


FIGURE 2. Pretreatment with NaOCl inhibited IL-1 α protein expression. (A) Cells were treated with NaOCl (0.75 mM) or vehicle (60 mM sodium phosphate buffer) for 10 minutes at 37°C. Cells were then washed with PBS, and fresh medium was added containing TNF α (30 ng/mL) and incubated at 37°C. At the indicated times, cells were harvested and lysed. (B) The concentration of NaOCl was varied up to 1 mM. After incubation with TNF α (30 ng/mL) for 1 hour, cell lysates were prepared. The amount of IL-1 α in cell lysates was measured by ELISA. Protein amounts are expressed in nanograms per milligram of total cellular protein assayed in corresponding cellular lysates. Data are the mean $\pm$ SEM of results from three independent experiments. *Significantly different from cells treated with TNF α by Student's *t*-test unpaired analysis ($P < 0.05$).

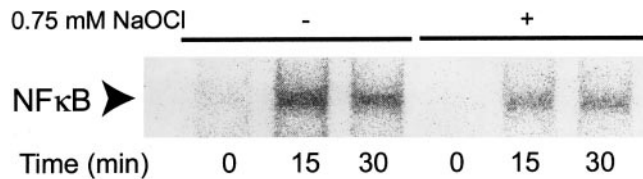


FIGURE 3. Pretreatment with NaOCl repressed TNF α -induced nuclear transfer of NF κ B. Cells were treated with vehicle (60 mM sodium phosphate buffer) or with NaOCl (0.75 mM) for 10 minutes at 37°C and washed with PBS. Fresh medium containing TNF α (30 ng/mL) was added and cells incubated for up to 30 minutes at 37°C. Nuclear extracts were prepared for EMSA. Similar results were obtained in three independent experiments.

induced increases in expression of IL-1 α protein. At 1 hour, the TNF α -induced increase without NaOCl pretreatment was 2.3-fold larger than with NaOCl pretreatment (Fig. 2A). After 3 hours, it declined to less than 1.8-fold. Dose-dependent inhibition of expression of IL-1 α protein by pretreatment with NaOCl was observed at doses up to 1 mM (Fig. 2B).

TNF α -Induced Nuclear Transfer of NF κ B

Expression of the IL-1 α gene depends on activation of NF κ B.²² EMSA was performed to determine whether NaOCl suppresses nuclear transfer of NF κ B. Figure 3 shows that the amount of NF κ B transferred to the nucleus over 30 minutes gradually increased in response to stimulation with TNF α . In contrast, pretreatment with NaOCl for 10 minutes markedly suppressed transfer of NF κ B. This result indicates that NaOCl-induced suppression of expression of the IL-1 α gene and protein initiated by stimulation with TNF α is appreciably due to its inhibition of the nuclear transfer of NF κ B.

NaOCl-Induced Modification of I κ B α

In unstimulated cells, NF κ B is in its inactive state; it is associated with I κ B and sequestered in the cytoplasm.²⁷ Stimulation of TNF α can initiate the degradation of I κ B, resulting in its dissociation from NF κ B, which leads to nuclear translocation

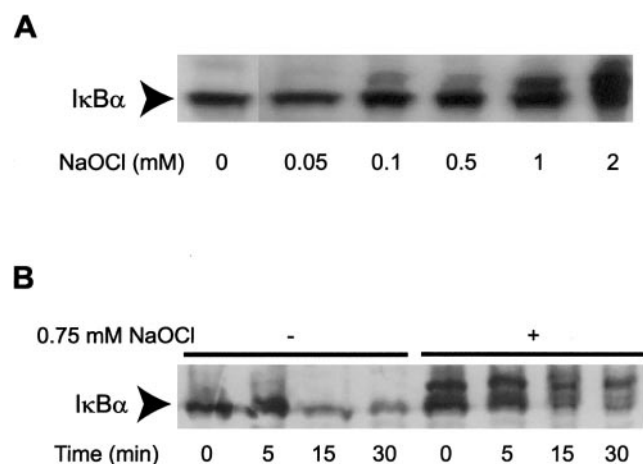


FIGURE 4. NaOCl induced I κ B α band-shifting. Cells were treated with each concentration of NaOCl for 10 minutes at 37°C. (A) The concentration of NaOCl was varied up to 2 mM. Cell lysates were made without stimulation with TNF α . (B) Incubation with TNF α (30 ng/mL) was performed for up to 30 minutes at 37°C. At the indicated times, cell lysates were prepared. Each lane was loaded with 60 μ g protein. Bands reacting with primary antibody specific for I κ B α were visualized with ECL. Similar results were obtained in three independent experiments.

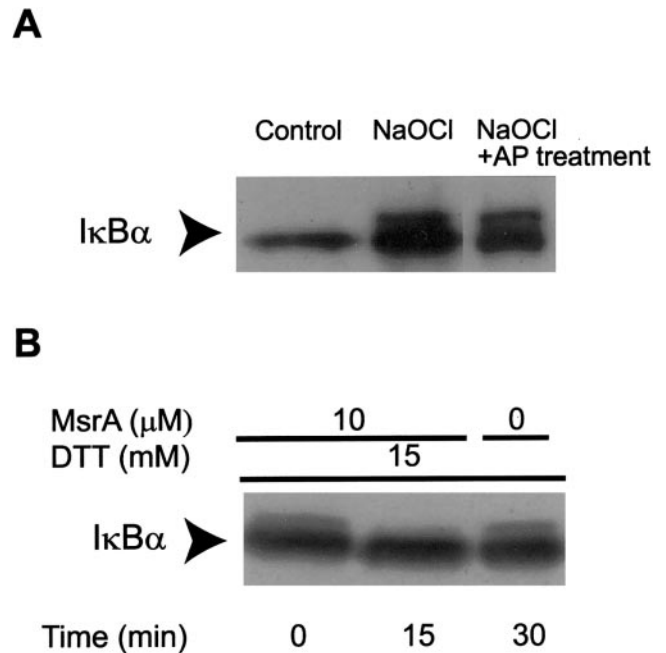


FIGURE 5. Effects on NaOCl-induced I κ B α band-shifting of treatment of cell lysate with AP and MsrA. Cells were treated with NaOCl (0.75 mM) for 10 minutes at 37°C. (A) Pelleted cells were lysed in TNE buffer. Cell lysates (30 μ L each) were adjusted to pH 9.7, 10 U AP was added, and cell lysates incubated for 30 minutes at 37°C. Treatment was terminated by neutralization of lysates. (B) Pelleted cells were lysed in TNE buffer containing 15 mM DTT. Cell lysates were treated with 10 μ M MsrA for 15 minutes at 37°C. To evaluate effects of DTT on the shifted band, cell lysates were incubated for 30 minutes at 37°C without MsrA. Each lane was loaded with 60 μ g protein. Bands that reacted with primary antibody specific for I κ B α were visualized with ECL. Similar results were obtained in three independent experiments.

of NF κ B. To determine whether NaOCl affects I κ B, we characterized the dose-dependent effects of NaOCl on levels of I κ B α in the cytoplasm, by using Western blot analysis (Fig. 4A). The results show that throughout the range from 0.05 to 2 mM NaOCl the I κ B α band broadened and gradually shifted its position.

Effect on TNF α -Induced Proteolysis of I κ B α

TNF α elicits responses in many systems through activation of NF κ B. This occurs as the result of its dissociation from I κ B α . To determine whether NaOCl alters I κ B α and thereby protects I κ B α from undergoing proteolysis, we performed Western blot analysis on I κ B α after its exposure to TNF α , with or without pretreatment with 0.75 mM NaOCl. The results shown in Figure 4B indicate time-dependent effects of TNF α on the level of I κ B α . It is evident that without pretreatment with NaOCl the intensity of the I κ B α band gradually lessened over a 30-minute period. However, with pretreatment a prominent, shifted, higher-molecular-weight I κ B α band remained for 30 minutes. The lower-molecular-weight, unmodified I κ B α band waned, without NaOCl pretreatment. These results suggest NaOCl-induced modification of I κ B α protects it from the degradative processes activated by TNF α .

Effect of AP on Modification of I κ B α

Because the band-shifting and -broadening caused by NaOCl was similar to that observed after TNF α -induced phosphorylation of serines 32 and 36 on I κ B α , we examined whether exposure of cell lysates to AP before electrophoresis would

cause the effect induced by NaOCl to disappear.^{28,29} The results shown in Figure 5A indicate that the band-shifting and -broadening of I κ B α caused by NaOCl was nearly the same as that obtained with NaOCl and AP. This similarity suggests that I κ B α band-shifting and -broadening is not the result of NaOCl induced phosphorylation.

Effect of MsrA on NaOCl-Induced Alteration of I κ B α

Exposure to HClO/CIO⁻ can oxidize proteins at their methionine residues.³⁰ To validate that the changes shown in Figures 4A and 4B reflect NaOCl-induced methionine oxidation, cell lysates were exposed to MsrA (Fig. 5B). MsrA was used, because in vitro it selectively reduces either free methionine sulfoxide (Met[O]) or protein-bound Met(O) to methionine.²⁴ After exposure to 0.75 mM NaOCl, the cells were lysed in TNE buffer containing 15 mM DTT and then exposed for 15 minutes to the same buffer, which contained 10 μ M MsrA.³¹ In the presence of 10 μ M MsrA and 15 mM DTT band-shifting was less at 15 minutes. However, in the absence of MsrA, DTT by itself failed to lessen NaOCl-induced band-shifting, even for a longer exposure time of 30 minutes. These results suggest that NaOCl-induced band-shifting of I κ B α reflects selective oxidation of methionine residues that is often seen in other proteins exposed to an oxidative stress.^{30,32}

DISCUSSION

During corneal inflammation, numerous pro- and anti-inflammatory cytokines are secreted. Two proinflammatory cytokines released by corneal epithelial cells are TNF α and IL-1 α . Another effect in vivo that one or both of them may have is degradation of the corneal matrix in that they stimulate activity and expression of matrix metalloproteinase (MMP)-9 in cultured HCE cells.^{33,34} Besides release of cytokines in the epithelium, infiltrating neutrophils are activated and release MPO during inflammation. It has been suggested that release of MPO mediates the production of HClO/CIO⁻ to hinder bacterial growth at the site of infection.⁵ Because it has been shown in other tissues that MPO, through the formation of HClO/CIO⁻, inhibits release of cytokines during an inflammatory response, we determined in the present study in HCE cells whether NaOCl (i.e., HClO/CIO⁻) affects activation of NF κ B and expression of the IL-1 α gene and protein in TNF α -stimulated HCE cells. NaOCl transiently suppressed the responses elicited by TNF α . Such declines may counterbalance excessive stromal degradation as well as the heightened inflammatory reaction resulting from release of TNF α .

Release of TNF α by activated neutrophils at the site of infection initiates an inflammatory response by stimulating surrounding cells to produce and release cytokines.³ In the corneal epithelium, it has been shown that exposure to TNF α causes these cells to increase expression of IL-1 α gene and protein.⁴ Release of IL-1 α from these cells is a key factor in the regulation of inflammation and wound healing on the ocular surface.¹⁸ For IL-1 α gene and protein expression to be stimulated, the transcription factor NF κ B must first be activated.²² Its activation is dependent on the dissociation of I κ B α from NF κ B. The expression of IL-1 α gene and protein occurs after phosphorylation and proteolysis of I κ B α .

In the present study, we assessed the effects of NaOCl on the integrity of I κ B α , the activation of NF κ B, and expression of the IL-1 α gene and protein. The results shown in Figure 4A indicate that NaOCl shifted the position of the I κ B α band in a dose-dependent manner. This band-shifting appeared to result from the oxidation of a methionine residue on I κ B α , because band-shifting was lessened by treatment with MsrA (Fig. 5B).

Phosphorylation of I κ B α does not appear to account for the bandshift, because it occurred despite treatment with AP (Fig. 5A). The results shown in Figure 3 suggest that TNF α -induced proteolysis of I κ B α is less pronounced after transient exposure to NaOCl. This result could mean that methionine oxidation protects I κ B α from undergoing phosphorylation, ubiquitination, and subsequent proteolysis and stabilizes its interaction with NF κ B. The results shown in Figure 3 are in agreement with this suggestion, because preexposure for 10 minutes to NaOCl suppressed activation of NF κ B. The results in Figures 1 and 2 show that inhibition of NF κ B resulting from a 10-minute exposure to NaOCl inhibited the expression of the IL-1 α gene and protein. Furthermore, the results shown in Figures 1 and 2 are consistent with the time course of NaOCl-induced inhibition of NF κ B nuclear transfer shown in Figure 3.

In other studies, there is evidence to suggest that the biological mediator of increases in HClO/CIO⁻ is TauCl, which is formed from the reaction of taurine with HClO/CIO⁻.⁷ TauCl inhibits the production and release of cytokines from lymphocytes, glioma cells, and rheumatoid arthritis fibroblast-like synoviocytes.¹¹⁻¹⁴ Kontny et al.¹⁴ showed that TauCl reduces the activation of transcription factors such as NF κ B and activating protein (AP)-1 in rheumatoid arthritis fibroblast-like synoviocytes and agree with our finding that NaOCl suppresses NF κ B activation (Fig. 3). Barua et al.²³ recently showed in macrophages that TauCl inhibits inducible (i)NOS and expression of the TNF α gene by inhibiting upstream signaling pathways that activate I κ B kinase. Because taurine is present in the corneal epithelium and TauCl spontaneously forms from taurine and HClO/CIO⁻, the final mediator may be TauCl. However, it is not yet known whether TauCl is intracellularly synthesized.

Methionine oxidation of various proteins is reported to alter their characteristics.³⁵ For instance, exposure to oxidative stress leads to methionine oxidation of the HIV-2 protease and its inactivation, which can be partially reversed with MsrA.³⁵ Previously, modification of I κ B α by TauCl was not described, whereas we detected NaOCl-induced alteration of I κ B α . It is possible that our ability to detect such a modification is due to a difference in electrophoresis procedures. Presumably, the band-shifting that we detected was the result of methionine oxidation, and it was lessened by treatment with MsrA. Because we detected expression of the MsrA gene in HCE cells with RT-PCR, it is possible that intracellular methionine oxidation may occur in vivo and be reversible (data not shown).

In summary, in HCE cells TNF α induced activation of NF κ B, which resulted in increases in expression of the IL-1 α gene and protein. This response can be transiently suppressed in HCE cells by NaOCl (i.e., HClO/CIO⁻), which suggests that the time course and magnitude of an immune response in vivo may depend on a host of feedback interactions between various mediators. This suppression occurs as the result of the oxidation of methionine residues on I κ B α , which in turn inhibits activation of NF κ B by TNF α . These results shed new sight on the regulation of the innate immune response in corneal epithelium.

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