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Oxidative modification of IκB by monochloramine inhibits tumor necrosis factor α-induced NF-κB activation

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Abstract

We have previously reported that monochloramine (NH_2Cl), a neutrophil-derived oxidant, inhibited tumor necrosis factor α (TNFα)-induced expression of cell adhesion molecules and nuclear factor-κB (NF-κB) activation (Free Radical Research 36 (2002) 845–852). Here, we studied the mechanism how NH_2Cl inhibited TNFα-induced NF-κB activation, and compared the effects with taurine chloramine (Tau–NHCl). Pretreatment of Jurkat cells with NH_2Cl at 70 μM resulted in suppression of TNFα-induced IκB phosphorylation and degradation, and inhibited NF-κB activation. In addition, a slow-moving IκB band appeared on SDS-PAGE. By contrast, Tau–NHCl for up to 200 μM had no effects. Interestingly, NH_2Cl did not inhibit IκB kinase activation by TNFα. Protein phosphatase activity did not show apparent change. When recombinant IκB was oxidized by NH_2Cl in vitro and phosphorylated by TNFα-stimulated Jurkat cell lysate, its phosphorylation occurred less effectively than non-oxidized IκB. In addition, when NF-κB–IκB complex was immunoprecipitated from NH_2Cl-treated cells and phosphorylated in vitro by recombinant active IκB kinase, native IκB but not oxidized IκB was phosphorylated. Amino acid analysis of the in vitro oxidized IκB showed methionine oxidation to methionine sulfoxide. Although Tau–NHCl alone had little effects on TNFα-induced NF-κB activation, simultaneous presence of Tau–NHCl and ammonium ion significantly inhibited the NF-κB activation, probably through the conversion of Tau–NHCl to NH_2Cl. These results indicated that NH_2Cl inhibited TNFα-induced NF-κB activation through the oxidation of IκB, and that NH_2Cl is physiologically more relevant than Tau–NHCl in modifying NF-κB-mediated cellular responses.

Keywords: Monochloramine; NF-κB; IκB; Methionine sulfoxide; TNFα; Inflammation

1. Introduction

Reactive oxygen species (ROS) are involved in signal transduction in various situations [1,2]. In some cases, ROS are produced inside cells by enzymes such as NADPH oxidase homologue [3], and in others, ROS come from extracellular space. Acute inflammation is one example where ROS production is enhanced and also various cytokines are working for the proper tissue repair. Recent evidences suggested that ROS can work as inflammatory mediators that modify cellular responses to various cytokines [4]. Activated neutrophils are among the most potent sources of ROS in the body. When neutrophils are activated, NADPH oxidase complex produce superoxide anion [5,6]. Several oxidants, such as hydrogen peroxide, hypochlorous acid and hydroxyl radical are derived from superoxide in the reaction catalyzed by superoxide dismutase, myeloperoxidase and transition metals. Chloramine derivatives are among the neutrophil-derived oxidants, and they are produced in the reaction of amino compounds with hypochlorous acid [7–9]. Chloramines are interesting oxidants that may affect cellular signal transduction [10,11]. The chemical and biological properties of chloramine vary greatly depending on the original molecular structure. For example, ammonia-monochloramine (NH_2Cl) is membrane-permeable and readily reacts with intracellular molecules, whereas taurine–chloramine (Tau–NHCl) is practically membrane-impermeable [12].

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When considering the signal transduction at inflammatory site, TNFα-induced nuclear factor κB (NF-κB) activation is of particular importance. TNFα is one of the major inflammatory cytokines, and various cells, such as macrophages, neutrophils, T cells and fibroblasts, produce TNFα [13]. On the other hand, almost all somatic cells express TNFα receptor and TNFα has diverse biological effects [14,15]. In endothelial cells, TNFα stimulates the synthesis of cell adhesion molecules, such as E-selectin and ICAM-1, and it is mediated by NF-κB activation [16,17].

NF-κB is a pleiotropic transcription factor, which is activated by a variety of stimulation such as cytokines, growth factors, immune complexes and inflammatory stimulations. In the dormant state, NF-κB exists in the cytoplasm as a complex with its inhibitory protein, IκB [18]. When the cells are stimulated, IκB is phosphorylated at Ser 32/36 by IκB kinase [19], then ubiquitinylated and digested by proteasome [20,21]. IκB degradation leads to a release of active NF-κB complex, which translocates to the nucleus and stimulates transcriptional activation of various genes.

Oxidative stress may have various effects on NF-κB activation. For example, some reports showed that H_2O_2 stimulated NF-κB activation [22–24], whereas others indicated that ROS did not mediate its activation [25,26]. Recently, Kanayama et al. reported that Tau–NHCl (1 mM) oxidized Met45 of IκB, which resulted in the inhibition of TNFα-induced NF-κB activation and a band shift of IκB on SDS-PAGE [27]. Sodium hypochlorite (0.75 mM) also showed similar effects on cultured corneal epithelial cells [28]. We have previously reported that NH_2Cl at substantially lower dose (50–70 μM) inhibited TNFα-induced NF-κB activation and expression of E-selectin and ICAM-1 in human umbilical vein endothelial cells [29].

In this paper, we studied the mechanism how NH_2Cl inhibited TNFα-induced NF-κB activation, and found that direct oxidation of IκB resulted in the decrease in phosphorylation of IκB by IκB kinase complex, which resulted in the inhibition of IκB degradation and NF-κB activation. Our results also indicated that NH_2Cl was biologically more relevant than Tau–NHCl, because the effects were observed at much lower dose, and that ammonium ion enhanced the inhibition of NF-κB activation by Tau–NHCl, probably through its conversion to NH_2Cl.

2. Materials and methods

2.1. Materials

Recombinant human TNFα was obtained from Peprotech, Inc. (London, England). Full length recombinant human IκBα (rh-IκBα) and glutathione-S-transferase-tagged IκB (GST-IκB) were from BIOMOL (Plymouth Meeting, PA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. [γ-^{32}P]ATP was from Perkin Elmer (Yokohama, Japan). Antibodies against IκB-α (sc-847 and sc-1643), NF-κB p50 (sc-7178) and p65 (sc-109G), IκB kinase α and α/β (sc-7218, sc-7607) were from Santa Cruz Biotechnology, and anti-phospho-IκBα (Ser32/36) monoclonal antibody (#9246) was from Cell Signaling Technology (Beverly, MA). Anti-NF-κB p65-conjugated agarose was from Santa Cruz Biotechnology. Recombinant active IκB kinase α was from Upstate (Lake Placid, NY). NF-κB consensus oligonucleotide (E3292) and mutant oligonucleotide (sc-2511) were from Promega (Madison, WI) and Santa Cruz Biotechnology, respectively. Proteasome inhibitor (Benzyloxycarbonyl-L-Isoleucyl-γ-t-Butyl-L-Glutamyl-L-Alanyl-L-Leucinal) was from Peptide Institute, Inc. (Osaka, Japan). Other chemicals were of analytical grade or better. Approximately 5 mM of monochloramine (NH_2Cl) and taurine–chloramine (Tau–NHCl) were prepared fresh just before experiments and the concentration was determined by the UV absorption spectra as described previously [30].

2.2. Cell culture and chloramine pretreatment

Jurkat T cell, a human acute T cell leukemia cell line, was obtained from Hayashibara Biochemical Laboratories Inc (Fujisaki Cell Center; Okayama, Japan). The cell culture medium was RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 110 mg/l sodium pyruvate (from Life Technologies, Inc.; Gaithersburg, MD, USA). Cells were grown in a CO_2 incubator containing 5% CO_2 at 37 °C.

For chloramine pretreatment, cells were suspended in the fresh medium at 1×10^6 cells/ml. Then, 50–500 μM of either NH_2Cl or Tau–NHCl (i.e., 50–500 nmol/10^6 cells) were added and incubated for 10 min at 37 °C. Where indicated, the medium was supplemented with 5 mM of ammonium chloride. The treated cells were separated from the medium by centrifugation at 500×*g* for 5 min, then resuspended in a fresh medium and stimulated with TNFα (20 ng/ml) for indicated times before the experiments.

2.3. Electrophoretic mobility shift assay (EMSA) for NF-κB

Electrophoretic mobility shift assay was performed as described previously [31,32]. Protein samples were prepared either from whole cell lysate [33] or from nuclear fraction [31] at the indicated times. Protein concentration was determined by Bradford method [34]. Equal amount of protein samples were incubated with ^{32}P-end-labeled NF-κB consensus oligonucleotide for 30 min at 25 °C. The DNA–protein complex formed was separated from free oligonucleotide by 6% native polyacrylamide gels. Binding specificity was confirmed by the competition with excess amount of unlabeled consensus oligonucleotide or with mutant oligonucleotide. For supershift assays, nuclear extracts prepared from TNFα treated cells were incubated with antibodies to either p50 or p65 NF-κB subunits for 45 min at room temperature before the complex was analyzed by EMSA. The incorporation of ^{32}P was measured by a phosphoimager (BAS-2000, Fuji Photo Film Co. Ltd., Tokyo, Japan).

2.4. Western blot analysis

The cells were collected 10 min after the addition of TNFα, washed with ice-cold PBS, and cellular proteins were extracted in a lysis buffer (20 mM HEPES–NaOH (pH 7.4), 40 mM β-glycerophosphate, 20 mM NaF, 1 mM Na_3VO_4, 20 mM p-nitrophenyl phosphate, 1 mM dithiothreitol, 0.1% (v/v) Nonidet-P40, 10 μg/ml each of leupeptin, aprotinin, pepstatin, and 1 mM phenylmethylsulfonyl-fluoride). For the detection of phosphorylated form of IκB, the cells were preincubated with 50 μM of proteasome inhibitor (Z-Ile-Glu(OBu^t)-Ala-LeuH) for 30 min before chloramine treatment. The samples (typically 30 μg protein/well) were separated by 12.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Immunoreactive proteins were detected using the enhanced chemiluminescence system (NEN, Boston, MA).

2.5. In vitro IκB kinase assay

The IκB kinase activity was measured as reported previously [33] with minor modification. Briefly, IκB kinase complex was immunoprecipitated from the cell lysate, and reacted with synthetic substrate (GST-IκB) and [γ-^{32}P] ATP in a kinase buffer (HEPES–NaOH pH 7.4, 20 mM; $MgCl_2$, 10 mM; EDTA, 1 mM; EGTA, 1 mM; p-nitrophenylphosphate, 20 mM; β-glycerophosphate, 20 mM; sodium orthovanadate, 0.1 mM; DTT, 1 mM) [35]. After the reaction, the samples were separated by SDS-PAGE and the incorporation of ^{32}P was measured by a phosphoimager.

2.6. Phosphoprotein phosphatase activity

The substrate for the phosphatase assay was prepared by phosphorylating the GST-IκB in the presence of ATP and the whole cell lysate from TNFα-