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

Tetsuya Ogino [a,*], Mutsumi Hosako [a], Kazuhisa Hiramatsu [a], Masako Omori [a], Michitaka Ozaki [b], Shigeru Okada [a]

[a] Department of Pathological Research, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata, Okayama 700-8558, Japan
[b] Department of Organ Transplantation and Regenerative Medicine, Hokkaido University Graduate School of Medicine, N-15, W-7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan

## 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.

## 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].

* Corresponding author. Tel.: +81 86 235 7145; fax: +81 86 235 7148.
E-mail address: togino@md.okayama-u.ac.jp (T. Ogino).

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-Iso-leucyl-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 $1 \times 10^6$ 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 $500 \times g$ 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-

stimulated Jurkat cells in the kinase buffer as described above. After the phosphorylation reaction, GST-IκB was adsorbed to the glutathione agarose beads, washed twice with phosphate-buffered saline (PBS) and used as a substrate. The phosphorylation of the substrate at Ser32/36 was confirmed by Western blot. The phosphatase activity was estimated by the dephosphorylation of this phosphorylated GST-IκB. The cytosolic fraction of the control cells and NH$_2$Cl-treated cells were extracted by three cycles of freeze–thawing in PBS containing protease inhibitor cocktail (Complete mini, Roche Diagnostics, Indianapolis, IN). Then, the extracts were added with the phosphatase substrate and incubated for 90 min at 37 °C. The remaining phosphorylation of GST-IκB was detected by Western blotting.

### 2.7. Oxidation and in vitro phosphorylation of IκB

The phosphorylation at Ser32/36 was compared between NH$_2$Cl-oxidized and non-oxidized IκB in two ways. Firstly, recombinant GST-IκB (0.1 mg/ml in PBS) was oxidized in vitro by various doses of NH$_2$Cl, and then phosphorylated by the TNFα-stimulated Jurkat cell lysate as described above. Secondly, NF-κB–IκB complex was immunoprecipitated from NH$_2$Cl (70 μM)-oxidized and control Jurkat cells using anti-NF-κB p65 antibody-conjugated agarose, and then phosphorylated by recombinant active IκB kinase α. In both cases, the phosphorylation was detected by Western blot using anti-phospho-IκBα (Ser32/36) antibody. The amino acid composition of oxidized and control IκB was also compared using rh-IκBα. Especially, methionine oxidation to methionine sulfoxide was studied after alkaline hydrolysis using an amino acid analyzer (Hitachi, L-8500, Tokyo, Japan) at the Okayama University Central Research Laboratory as described previously [36].

### 2.8. Image analysis and statistical analysis

For Western blot and autoradiogram images, each band densities were measured using ImageJ software (http://rsb.info.nih.gov/ij/), and expressed as % of the sum of total band densities. Results were tabulated for the indicated number of experimental samples. Group means were compared using Student's $t$ test. Analysis of variance (ANOVA) with Tukey's test was also used for multiple comparison using Statcel QC software (OMS publishing Inc., Saitama, Japan). The $P$ values less than 0.05 were considered to be significantly different.

## 3. Results

### 3.1. Inhibition of TNFα-induced NF-κB Activation by NH$_2$Cl

Stimulation with TNFα (20 ng/ml) for 2 h induced marked activation of NF-κB in Jurkat cells, and this activation was significantly inhibited by the NH$_2$Cl pretreatment (Fig. 1). The NF-κB band was identified by the supershift using antibodies against either the p50 or p65 subunits of NF-κB (Fig. 1A). This band disappeared by excess of unlabeled consensus oligonucleotide but not by mutated oligonucleotide, which further confirmed the specificity.

To elucidate how NH$_2$Cl inhibited TNFα-induced NF-κB activation, the phosphorylation and degradation of IκB was studied. TNFα significantly enhanced the phosphorylation of IκB protein, which was detected in the presence of proteasome inhibitor (Fig. 2). This TNFα-induced IκB phosphorylation was significantly reduced in NH$_2$Cl (70 μM)-pretreated cells (Fig. 2).

The degradation of IκB was studied in the absence of proteasome inhibitor. Interestingly, NH$_2$Cl treatment resulted in a duplication of IκB band, in which slow-moving band appeared on SDS-PAGE. This slow-moving band was likely to represent oxidized IκB, because NH$_2$Cl-oxidized recombinant human IκB also showed similar band-shift on SDS-PAGE, as described later in Fig. 10A. Without NH$_2$Cl
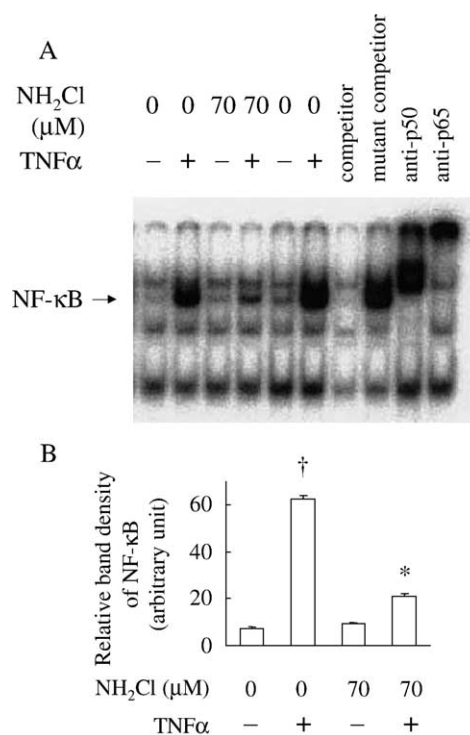


Fig. 1. NF-κB activation by TNFα and its inhibition by NH$_2$Cl. Jurkat cells ($1 \times 10^6$ cells/ml) were treated with NH$_2$Cl (70 μM) for 10 min at 37 °C, then the medium was changed and stimulated with TNFα (20 ng/ml) for 2 h. Nuclear protein was extracted and analyzed by electrophoretic mobility shift assay. (A) Representative autoradiogram. Competitor lane contains 25 times excess of non-radiolabeled oligonucleotide. (B) Relative band densities (mean±S.D.) from 3 independent experiments. †: Significantly increased from NH$_2$Cl (0 μM), TNFα (−) samples ($P<0.05$). *: Significantly decreased from NH$_2$Cl (0 μM), TNFα (+) samples ($P<0.05$).

pretreatment, TNFα significantly reduced IκB band density, which indicated protein degradation (Fig. 3A). This TNFα-induced IκB degradation was significantly suppressed by NH$_2$Cl pretreatment (Fig. 3A). In addition, when the two IκB band densities were compared separately, the lower band decreased significantly by TNFα, whereas the upper band did not (Fig. 3B).

### 3.2. Effects of NH$_2$Cl on the IκB kinase and phosphoprotein phosphatase activity

As NH$_2$Cl inhibited TNFα-induced IκB phosphorylation, IκB kinase activity was measured. Without NH$_2$Cl treatment, the cell lysate from TNFα-stimulated cells catalyzed $^{32}$P incorporation into GST-IκB, which indicated the IκB kinase activation (Fig. 4). Interestingly, a similar degree of IκB kinase activity was observed in NH$_2$Cl pretreated, TNFα-stimulated cell lysate (Fig. 4), which indicated that the IκB kinase activity did not change significantly by NH$_2$Cl.

As the phosphorylation status depends on a balance between kinase and phosphatase, the decrease in the IκB phosphorylation might be a result of increased protein phosphatase activity. To address this possibility, phosphorylated GST-IκB was incubated with cytosolic fraction and its dephosphorylation was compared. The phosphorylated GST-IκB showed a definite
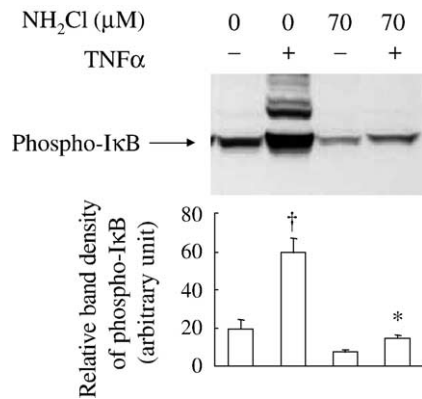
Fig. 2. NH$_2$Cl inhibited TNFα-induced IκB phosphorylation. Jurkat cells (1 × 10$^6$ cells/ml) were pretreated with proteasome inhibitor for 30 min at 37 °C, and then treated with NH$_2$Cl (70 μM) for 10 min at 37 °C. The medium was changed and stimulated with TNFα (20 ng/ml) for 10 min. Thirty micrograms of lysate proteins were separated by 12.5% SDS-PAGE and blotted on a nitrocellulose membrane. The upper picture is a representative Western blot image. The lower bar diagram shows relative band densities (mean ± S.D.) from 5 independent experiments. †: Significantly increased from NH$_2$Cl (0 μM), TNFα (−) samples (P < 0.05). *: Significantly decreased from NH$_2$Cl (0 μM), TNFα (+) samples (P < 0.05).
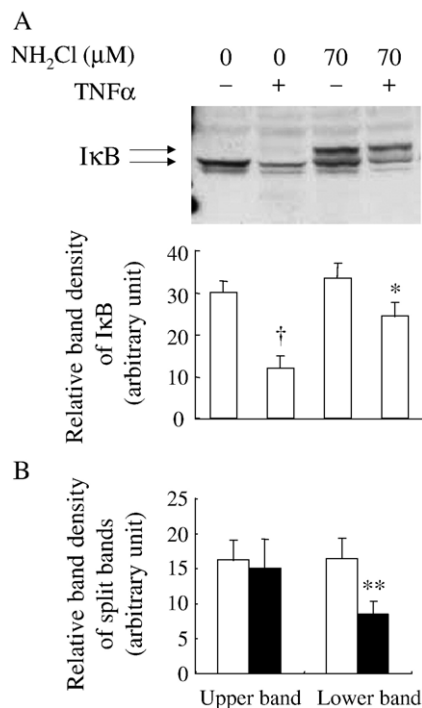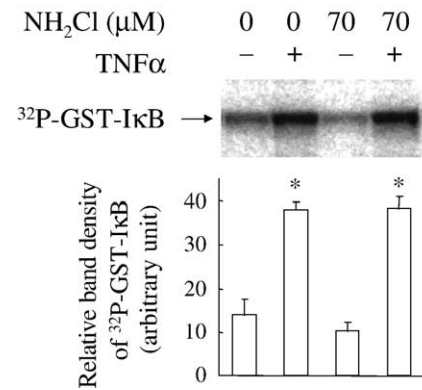


Fig. 4. NH$_2$Cl did not inhibit TNFα-induced IκB kinase activation. Jurkat cells were treated as described in Fig. 3 legend. Cellular protein was extracted with the lysis buffer supplemented with 0.42 M NaCl and 10% (v/v) glycerol. IκB kinase complex was immunoprecipitated from 250 μg of lysate protein using 2 μg of anti-IκB kinase antibody and protein A-agarose beads, then incubated with GST-IκB and [γ-$^{32}$P]-ATP in a kinase buffer for 30 min at 37 °C. The protein was separated by 10% SDS-PAGE and $^{32}$P incorporation was detected by a phosphoimager. The bar diagram shows relative band densities (mean ± S.D.) from 3 independent experiments. *: Significantly increased by TNFα (P < 0.05). NH$_2$Cl pretreatment had no significant effects.

band by anti-phospho-IκB antibody (Fig. 5). After the incubation with cytosolic fraction, the band densities did not show significant decrease (Fig. 5), which indicated that the phosphatase activity was below the detectable limit in all samples, and not likely to be the cause of decrease in IκB phosphorylation.

### 3.3. Effects of IκB oxidation on its phosphorylation in vitro

To study if the direct oxidation of IκB affected its phosphorylation, recombinant GST-IκB was treated with NH$_2$Cl and then phosphorylated by the TNFα-stimulated Jurkat cell lysate. Without NH$_2$Cl treatment, GST-IκB was phosphorylated efficiently at Ser32/36 (Fig. 6). This phosphorylation occurred less efficiently when the GST-IκB was



Fig. 3. NH$_2$Cl inhibited TNFα-induced IκB degradation. Jurkat cells (1 × 10$^6$ cells/ml) were treated with NH$_2$Cl (70 μM) for 10 min at 37 °C, and then the medium was changed and treated with TNFα (20 ng/ml) for 10 min. Protein samples were separated by 12.5% SDS-PAGE and blotted on a nitrocellulose membrane. A: The bar diagram shows relative band densities (mean ± S.D.) from 5 independent experiments. The two IκB bands were measured together. †: Significantly decreased from NH$_2$Cl (0 μM), TNFα (−) samples (P < 0.05). *: Significantly higher than NH$_2$Cl (0 μM), TNFα (+) samples (P < 0.05). B: The two IκB bands were measured separately. White column: NH$_2$Cl (70 μM), TNFα (−) samples. Black column: NH$_2$Cl (70 μM), TNFα (+) samples. **: Significantly decreased by TNFα (P < 0.05).
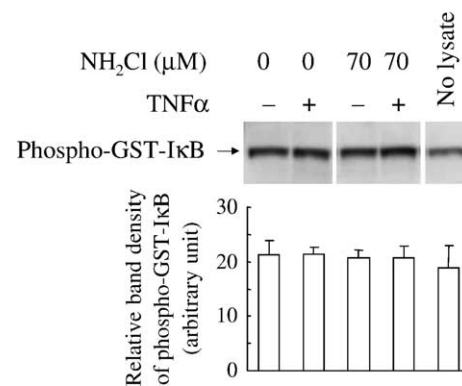


Fig. 5. Phosphatase activity that dephosphorylate IκB. GST-IκB was phosphorylated as described in Materials and methods and used as a phosphatase substrate. The phosphorylated GST-IκB was incubated for 90 min at 37 °C with cytosolic fraction of the cells, which have been treated as indicated. After the reaction, the remaining phosphorylation was detected by phospho-IκB (Ser32/36) antibody. The bar diagram shows relative band densities (mean ± S.D.) from 3 independent experiments. No significant difference was found.
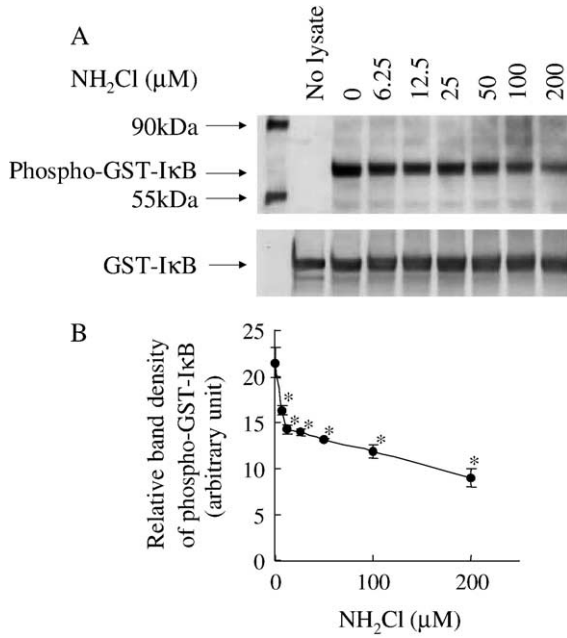
Fig. 6. Effects of in vitro oxidation of GST-IκB on its phosphorylation. GST-IκB (0.1 mg/ml, 70 kDa) was oxidized in vitro by indicated concentrations of NH$_2$Cl, and then they were incubated with TNFα-stimulated Jurkat cell lysate in kinase buffer for 30 min at 37 °C. The phosphorylation was detected by anti-phospho-IκBα (Ser32/36) antibody. A: Representative Western blot image. Lower panel showed the same load of the protein. B: Relative band densities (mean±S.D.) from 3 independent experiments. *: Significantly decreased from "0 μM" samples (P<0.05).

oxidized by NH$_2$Cl, and this effect depended on the dose of NH$_2$Cl (Fig. 6).

In addition, when the NF-κB–IκB complex was recovered from NH$_2$Cl-treated and control Jurkat cells, and phosphorylated in vitro using recombinant active IκB kinase α, only single phospho-IκB band was observed regardless of NH$_2$Cl treatment, despite that NH$_2$Cl-treated cells showed two IκB bands (i.e. oxidized and native IκB) (Fig. 7). The results indicated that IκB which was oxidized in the cell was hardly phosphorylated by IκB kinase.
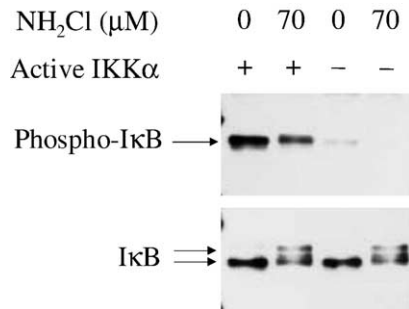


Fig. 7. Phosphorylation of IκB from NH$_2$Cl-treated Jurkat cells by IκB kinase. Cytosolic fraction was prepared from NH$_2$Cl (70 μM)-treated and control Jurkat cells by 3 cycles of freeze thawing in PBS containing protease inhibitors (Complete mini), and then NF-κB–IκB complex was immunoprecipitated from 1.5 mg protein using anti-NF-κB p65 antibody-conjugated agarose beads. The beads were washed 3 times with PBS and phosphorylated by recombinant active IκB kinase α (IKKα, 100 ng/40 μl) in kinase buffer (40 μl, containing 100 μM ATP) for 20 min at 30 °C. After the reaction, the beads were washed once with PBS, and analyzed by SDS-PAGE.
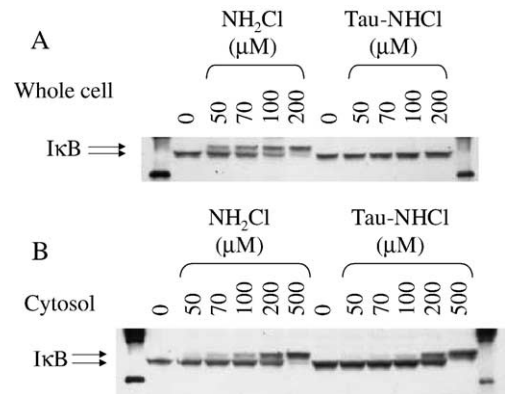


Fig. 8. IκB band-shift by NH$_2$Cl and Tau−NHCl. (A) Jurkat cells were treated with the indicated concentration of NH$_2$Cl or Tau−NHCl for 10 min at 37 °C, then the cells were collected, and the cellular protein was analyzed by Western blotting. (B) Cytosolic fraction was extracted from $1 \times 10^7$ cells by three cycles of freeze thawing, and the samples (2.5 mg protein/ml) were treated with the indicated concentrations of NH$_2$Cl. Then the protein was analyzed by Western blotting.

### 3.4. NH$_2$Cl, but not Tau−NHCl, inhibited TNFα-induced NF-κB activation

As Tau−NHCl has been reported to inhibit TNFα-induced NF-κB activation at much higher dose [27], the inhibitory effects of NH$_2$Cl and Tau−NHCl were compared. When the whole cell
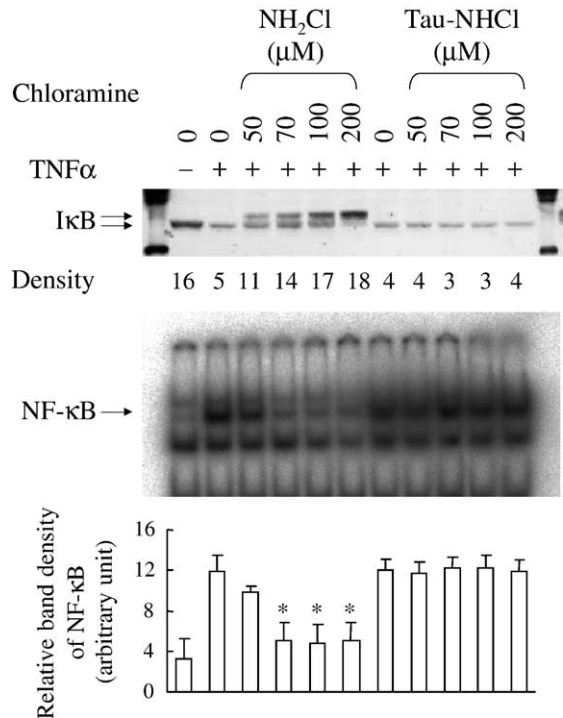


Fig. 9. NH$_2$Cl, but not Tau−NHCl, inhibited TNFα-induced NF-κB activation. Jurkat cells were treated with the indicated concentration of NH$_2$Cl or Tau−NHCl for 10 min at 37 °C, and then the medium was changed and stimulated with TNFα (20 ng/ml) for 10 min. Cellular protein was extracted with the lysis buffer supplemented with 0.42 M NaCl and 10% (v/v) glycerol. Relative band densities (arbitrary unit) were indicated for IκB Western blot. The two IκB bands were measured together. The bottom bar diagram shows relative band densities of NK-κB (mean±S.D.) from 3 independent experiments. *: Significantly decreased from chloramine (0 μM), TNFα (+) samples (P<0.05).

was treated with $NH_2Cl$ at 50–200 µM, IκB showed a gradual band-shift, whereas Tau–NHCl at the same concentration showed no band shift (Fig. 8A). However, when the cell membrane was disrupted by three cycles of freeze thawing, and the cytosolic fraction was treated with chloramine, both $NH_2Cl$ and Tau–NHCl induced a similar degree of IκB band-shift (Fig. 8B). TNFα-induced NF-κB activation was also compared between $NH_2Cl$ and Tau–NHCl treated cells. As expected, $NH_2Cl$ at 70 µM or more inhibited NF-κB activation significantly (Fig. 9). By contrast, Tau–NHCl for up to 200 µM inhibited neither NF-κB activation nor IκB degradation (Fig. 9).

### 3.5. Oxidation of rh-IκBα by $NH_2Cl$ resulted in methionine oxidation and a band shift on SDS-PAGE

Oxidative modification of IκB by $NH_2Cl$ was studied using rh-IκBα. When rh-IκBα (0.12 mg/ml) was treated with indicated concentrations of $NH_2Cl$, the rh-IκBα gradually shifted to a slow-moving band as observed in the whole cell sample (Fig. 10A). Treatment of 0.19 mg/ml of rh-IκBα with 70 µM $NH_2Cl$, in which the molar ratio of $NH_2Cl$ to rh-IκBα was approximately 14:1, resulted in almost complete band shift (data not shown). The amino acid composition of this oxidized and control rh-IκBα was compared after alkaline hydrolysis. The oxidized IκB showed a disappearance of methionine with an appearance of methionine sulfoxide peaks (Fig. 10B). Other amino acid peaks were almost constant. These results were
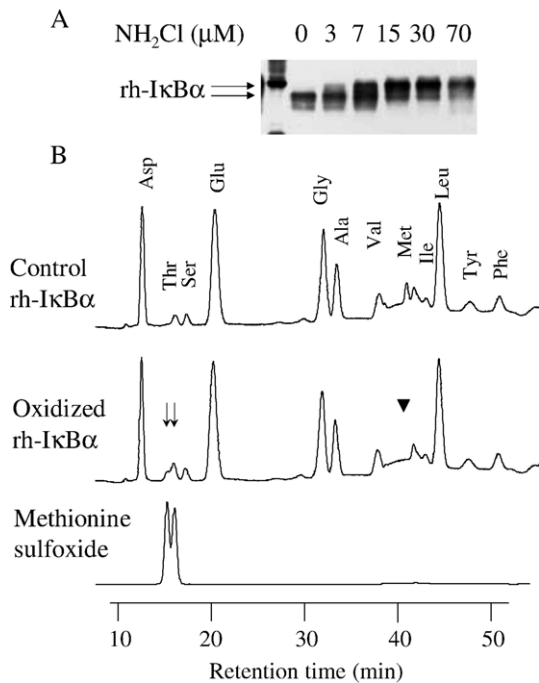


Fig. 10. In vitro oxidation of rh-IκBα and amino acid analysis. Full-length recombinant human IκBα in PBS was oxidized in vitro by $NH_2Cl$ for 10 min. (A) Western blot analysis showed that oxidized IκBα (0.12 mg protein/ml) moved more slowly than control IκBα on SDS-PAGE. (B) IκBα (0.19 mg protein/ml) in PBS was oxidized by $NH_2Cl$ (70 µM) for 10 min. The oxidized and control samples were alkaline hydrolyzed for 18 h, acidified by HCl and subjected to amino acid analysis. Arrowhead: disappearance of methionine peak, Double arrow: methionine sulfoxide peaks.
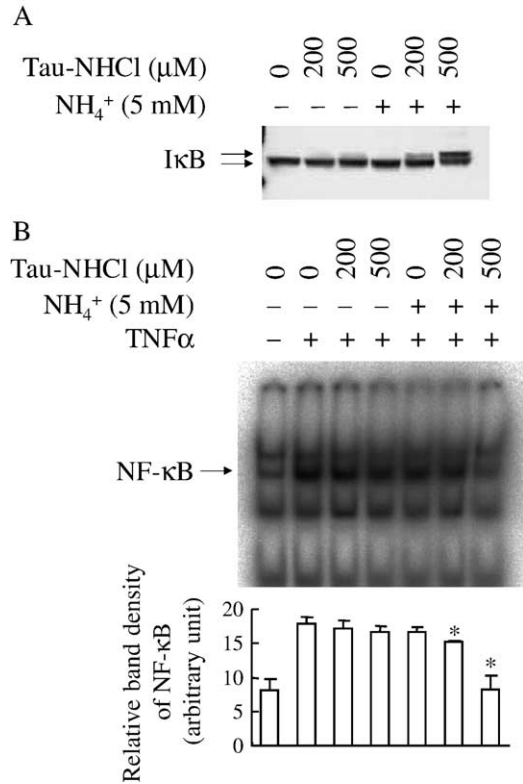


Fig. 11. Ammonium ion enhanced Tau–NHCl-induced IκB band-shift and inhibition of NF-κB activation. Jurkat cells were treated with the indicated concentrations of Tau–NHCl for 10 min at 37 °C in the presence or absence of 5 mM ammonium ion. A: Protein samples were analyzed by Western blot using anti-IκB antibody. B: After Tau–NHCl treatment, the medium was changed and the cells were stimulated with TNFα (20 ng/ml) for 10 min. NF-κB activation was studied by electrophoretic mobility-shift assay. The bar diagram shows relative band densities of NK-κB (mean±S.D.) from 3 independent experiments. *: Significantly decreased from Tau–NHCl (0 µM), $NH_4^+$ (+), TNFα (+) samples ($P < 0.05$).

consistent with previous reports, which showed that IκB oxidation at Met45 by Tau–NHCl caused a band shift of IκB on SDS-PAGE [27,28].

### 3.6. Ammonium ion enhanced the inhibition of NF-κB activation by Tau–NHCl

As Tau–NHCl may transfer chlorine to ammonium ion to form $NH_2Cl$ [8], and millimolar concentration of ammonium ion exist in gastrointestinal tract [37,38], the effects of ammonium ion on Tau–NHCl-induced inhibition of NF-κB activation was studied. Although Tau–NHCl alone had little effect on the IκB band-shift even at 500 µM, the presence of 5 mM ammonium ion in the medium markedly enhanced this band-shift (Fig. 11A). Consistently, TNFα-induced NF-κB activation was also inhibited significantly by Tau–NHCl only in the presence of ammonium ion (5 mM) (Fig. 11B).

## 4. Discussion

$NH_2Cl$ significantly inhibited TNFα-induced NF-κB activation in Jurkat cells, and this inhibition was accompanied by

the decrease in IκB phosphorylation and degradation. This finding was consistent with our previous report using human umbilical vein endothelial cells [29]. Our results indicated that direct oxidation of IκB, especially at Met residues, was the cause of $NH_2Cl$-induced inhibition of NF-κB activation. These findings were consistent with a previous report, which showed that Tau–NHCl (1 mM) oxidized Met45 of IκB, and this oxidation resulted in the inhibition of TNFα-induced NF-κB activation and a band shift of IκB on SDS-PAGE [27]. As IκB kinase activity was not inhibited, the target of $NH_2Cl$ probably located downstream of IκB kinase activation. We further demonstrated that oxidized IκB was a poorer substrate for IκB kinase. Actually, when GST-IκB was oxidized by $NH_2Cl$ in vitro, it became more resistant to be phosphorylated at Ser32/36. Moreover, when NF-κB–IκB complex was recovered from $NH_2Cl$-treated Jurkat cells and phosphorylated by recombinant active IκB kinase, the phosphorylation of oxidized IκB was not detectable, while native IκB was phosphorylated efficiently. Although it must be studied further why oxidized IκB is a poorer substrate for IκB kinase, it was reported that amino terminal of IκB (1−54) was sufficient to interact with IκB kinase complex [39]. Therefore, Met45 oxidation is likely to alter the surface charge of IκB, thereby affecting the interaction with IκB kinase. In addition, the slow-moving IκB band, which probably represents oxidized IκB, was more resistant to degradation upon TNFα stimulation. These results suggested that oxidized IκB became resistant to stimulation-induced phosphorylation and degradation, and the subsequent NF-κB activation was inhibited.

Among various amino acids, Met and Cys have been reported to be particularly vulnerable to oxidative modification by chloramines [40]. Under the experimental condition of Fig. 10A, rh-IκBα concentration was 0.12 mg/ml, which equals to 3.3 μM, assuming that the molecular weight of IκB was 36,000. One IκB has 6 Met and 8 Cys, and one $NH_2Cl$ can oxidize one Met or two Cys. Thus, 10 times excess of $NH_2Cl$ is sufficient to oxidize all Met and Cys of IκB. Fig. 10A showed that IκB almost completely shifted with 30 μM of $NH_2Cl$, which was consistent with the above calculation. Interestingly, 7 μM of $NH_2Cl$ already induced a substantial band shift, which indicated that there might be critical Met and/or Cys residues that contributed significantly to band shift and were oxidized more readily than others. This assumption is also consistent with the previous report that showed Met45 oxidation induced IκB band shift [27]. In the case of Fig. 10B, the molar ratio of $NH_2Cl$ to rh-IκBα was approximately 14:1, and this $NH_2Cl$ dose was more than enough to oxidize all Met and Cys, if the oxidant reacted solely to these two amino acids. Consistently, the amino acid analysis of $NH_2Cl$-oxidized rh-IκBα showed almost complete disappearance of Met and appearance of two small peaks near Thr. The latter peaks are likely to represent methionine sulfoxide, which shows two peaks due to diastereomers. Although Cys was destroyed by alkaline hydrolysis and could not be detected [41], in vitro oxidation of rh-IκBα may also oxidize Cys residues. However, we previously reported that cellular thiol contents did not change significantly by $NH_2Cl$ (70 μM) treatment in Jurkat cells and endothelial cells [29,36]. The protein-bound thiol also showed no significant change. This apparent discrepancy may be explained by the rapid turnover of glutathione. Many cells maintain millimolar levels of glutathione, and oxidized thiol may quickly reduced by glutathione, which is then replenished by glutathione synthase system [42].

We also reported that membrane permeable $NH_2Cl$ was effective at substantially lower dose than Tau–NHCl, and Tau–NHCl may need to be converted to membrane permeable species to have significant effects. $NH_2Cl$ significantly inhibited the TNFα-induced NF-κB activation as low as 70 μM, while Tau–NHCl showed no significant inhibition even at 500 μM. Tau–NHCl did not show definite band-shift of IκB at this concentration, which indicated that IκB was not oxidatively modified. The difference is probably because of the difference in membrane permeability. Actually, when the cell membrane was disrupted by freeze thawing and cytoplasmic proteins were directly exposed to chloramines, both $NH_2Cl$ and Tau–NHCl induced a similar band-shift of IκB. These results indicated that extracellular taurine can prevent oxidative modification of intracellular proteins by sequestering oxidants in the extracellular space as Tau–NHCl. By contrast, when Tau–NHCl is produced inside the cell, it can oxidize intracellular proteins and may affect cellular responses.

It is noteworthy that Tau–NHCl may be transformed to $NH_2Cl$ when excess ammonium ion is present [8], and other chlorine transfer reactions between amines or amino acids have also been reported [43]. Our result also indicated that Tau–NHCl at 500 μM effectively inhibited TNFα-induced NF-κB activation when 5 mM of ammonium ion was added in the medium. Millimolar range of ammonium ion generally exists inside the gastrointestinal tract [37,38], and substantial numbers of neutrophils also migrate to the gut in various inflammatory conditions [44]. In addition, Tau–NHCl has been reported to be one of the major chloramines produced by activated neutrophils [45]. Under these conditions, relatively inert Tau–NHCl can work as a pro-oxidant through the conversion to $NH_2Cl$. Thus, $NH_2Cl$ may be a physiologically relevant oxidant that inhibits NF-κB-mediated cellular responses at micromolar concentration, and when excess of ammonium ion exists, other chloramine derivatives, such as Tau–NHCl, may also inhibit NF-κB activation through the conversion to $NH_2Cl$.

## Acknowledgments

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