Oxidation of IκBα at Methionine 45 Is One Cause of Taurine Chloramine-induced Inhibition of NF-κB Activation*

Received for publication, November 12, 2002, and in revised form, April 30, 2002
Published, JBC Papers in Press, April 30, 2002, DOI 10.1074/jbc.M10832200

Atsuhito Kanayama‡, Jun-ichiro Inoue‡, Yoshiko Sugita-Konishi‡‡‡, Makoto Shimizu†, and Yusei Miyamoto‡‡‡

From the ‡Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan, the **Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku, Yokohama, Kanagawa 223-8522, Japan, the †††Department of Biomedical Food Research, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan, and the §§Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

A band shift of IκBα was observed in Western blots with Jurkat cells treated with 1 μM taurine chloramine (TauCl) for 1 h. TauCl treatment inhibited tumor necrosis factor α (TNFα)-initiated nuclear factor κB (NF-κB) activation. TauCl did not inhibit either the upstream of IκB kinase (IKK) activation or IKK itself but did inhibit NF-κB activation induced by IKK overexpression. Deletion experiments showed that a TauCl modification site causing the band shift of IκBα is Met45. High performance liquid chromatography and mass spectrometry analyses of a small peptide containing Met45 revealed that TauCl oxidizes Met45. A mutant of IκBα whose Met45 was converted to alanine did not generate a band shift upon TauCl treatment and degraded in response to TNFα stimulation. However, a reporter assay revealed that NF-κB-dependent luciferase expression was not fully recovered in cells transfected with this mutant. These results indicate that Met45 oxidation of IκBα is a molecular mechanism underlying the TauCl-induced inhibition of NF-κB activation. A similar band shift was observed when HL-60 cells expressing myeloperoxidase were treated with 100 μM hydrogen peroxide for 5 min. When rat neutrophils were incubated with bacteria, intracellular taurine decreased interleukin-8 production. Therefore, taurine may help suppress excessive inflammatory reaction in neutrophils.

Taurine, 2-aminoethanesulfonic acid, is a β-amino acid that is synthesized from sulfur-containing amino acids in the body.

* This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan and a research grant from Taisho Pharmaceutical Co., Ltd. (to Y. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Molecular Biology, NA 8.202, University of Texas Southwestern Medical Center, 600 Harry Hines Blvd., Dallas, TX 75390-9148.

‡‡ Present address: Division of Cellular Molecular Biology, Dept. of Cancer Biology, The Inst. of Medical Science, University of Tokyo, 4-6-1 Shirogane, Minato, Tokyo 108-8639, Japan.

‡‡‡ Present address: Division of Microbiology, National Institutes of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan.

§§ To whom correspondence should be addressed: Dept. of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Bioscience Bldg. 402, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan. Tel.: 81-471-36-3628; Fax: 81-471-36-3630; E-mail: yusei74@k.u-tokyo.ac.jp.

1 The abbreviations used are: NF-κB, nuclear factor κB; TNFα, tumor necrosis factor α; IKK, IκB kinase; MPO, myeloperoxidase; TauCl, taurine chloramine; IL-8, interleukin-8; HER2/4, human embryonal kidney 293; FBS, fetal bovine serum; Mg2+, phosphatase-buffered saline without Mg2+ or Ca2+; HFLC, high performance liquid chromatography; PSI, proteasome inhibitor; PDTC, pyrrolidine dithiocarbamate; PMT, phorbol 12-myristate 13-acetate; ABAA, 4-aminobenzoic hydrazide; ARD, ankyrin repeat domain; SRD, signal receiving domain; ROS, reactive oxygen species.
Therefore, the biological function of tau in the neutrophil is not only reduction of the cytotoxicity of HClO but also mitigation of excessive inflammatory reaction. We assume that tau may act as an intermediate regulator between two distinct events in neutrophils during their phagocytosis of bacteria, synthesis of HClO, and NF-κB activation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human acute T cell leukemia Jurkat T and HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA). Promyelocytic leukemia HL-60 cells were obtained from Health Science Research Resources Bank (Osaka, Japan). These three kinds of cells were routinely maintained at 37 °C in a humidified atmosphere of 95% air, 5% CO2, and passed to new culture at a split ratio of 1:10 every week. The composition of culture medium for Jurkat cells was RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The medium pH was adjusted to 7.3 with sodium bicarbonate (Kanto Chemical, Tokyo, Japan). The culture medium for HL-60 cells was made by supplementing one for Jurkat cells with 2 mM glutamine (Kanto Chemical). A culture medium for HEK293 cells was Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, and 6 mM glutamine. The pH of this medium was also adjusted to 7.3 with sodium bicarbonate.

Preparation of TauCl—TauCl was freshly prepared by adding equimolar NaOCl (Kanto) dropwise to 5 ml of 80 mM tauine (Kanto) in 0.05 m sodium phosphate buffer (pH 8.3) before use, as described previously (13). Each preparation of TauCl was monitored by ultraviolet absorption spectra (200–300 nm) to confirm the production of monoclonal antibody as well as the absence of dichloramine or unreacted NaOCl. The concentration of TauCl was determined using the molar extinction coefficient (ε280 nm = 415 M–1 cm–1).

Western Blot Analysis—Cell extracts were prepared as described previously (14). The extracts were boiled and fractionated by SDS-PAGE (12.5%). To detect a small change in the molecular weight of IκB like its molecular weight increase due to phosphorylation, we used a large gel consisting of ~10 cm separating gel and overran until IκB came close to the bottom of the gel (14). In most experiments, the primary and secondary antibodies used were rabbit anti-IκBα antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and donkey anti-rabbit IgG antibody linked to horseradish peroxidase (Amersham Biosciences), respectively. When mouse anti-FLAG antibody was used as a primary antibody, the secondary antibody was rabbit anti-mouse IgG horseradish peroxidase-conjugated antibody. Proteins on the membrane were visualized by an enhanced chemiluminescent method with a kit (Amersham Biosciences).

Electrophoretic Mobility Shift Assay—Cells were rinsed with ice-cold phosphate-buffered saline without Mg2+ or Ca2+ (PBS(−)), and nuclear extracts were prepared as described previously (15, 16). An oligonucleotide probe containing the sequence of NF-κB binding site (sense, 5′-ggttCAGAGGGGACTTTCCGAGAGG-3′; antisense, 5′-tgacaCTCTCCTGAAAAGCTCTCCGTGaaT-3′) was labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega, Madison, WI). The labeled oligonucleotide probe was digested by thrombin. The peptide fragment consisting of IκBα was added to the reaction solution, and the reaction was carried out at 25°C for 15 min. The reaction solution was separated by HPLC with an octadecyl column (Senshu Scientific Co., Tokyo, Japan). The separation run was recorded with a UV detector at 215 nm.

Reporter Assay—A reporter gene and 0.5 μg of a reporter gene and 0.5 μg of pGL3-B-tk-luc or five binding sites of cAMP-responsive element-binding protein (5×-CMV-Luc) were co-transfected with 0.1 μg of a reporter gene and 0.5 μg of pGL3-B-tk-luc and 5 μg of the reporter gene and 0.5 μg of pGL3-B-tk-luc. 280 (M45A)) were prepared as described previously (18). A wild type deletion mutant consisting of the amino acids 1–280 (IκBα 1–280 (wild type)) and a corresponding point-mutated IκBα (IκBα 1–280 (M45A)) were prepared by PCR as described above. A vector pME was used to express wild type and mutants of IκBα in Jurkat cells (14).

Measurement of MPO Activity and Taurine Concentration in Jurkat and HL-60 Cells—Cells (3×105) in 50 mM potassium phosphate buffer (pH 6.0) were homogenized for 10 s three times with 5-s intervals using a Polytron-type homogenizer. The MPO activity was determined using a method previously described (19). The concentration of protein in the cell homogenate was determined by the Lowry method (20).

Cells (1×107) were rinsed twice with PBS(−) to remove extracellular taurine and digested with 5% trichloroacetic acid. Cell debris was removed by centrifugation at 20,000×g for 5 min. The amount of taurine in the supernatant was determined by an amino acid analyzer (Hitachi, Tokyo, Japan). The cell volume (1×107 cells) was estimated by subtracting freeze-dried weight from wet weight. The intracellular concentration of taurine was obtained by dividing the amount of intracellular taurine by the cell volume.

Isolation of Rat Neutrophils and Quantification of IL-8—Sprague-Dawley male rats (10 weeks old) were intraperitoneally administrated with 40 ml of 1% casein (Wako) in PBS(−). Eighteen hours later, neutrophils were collected from the abdominal cavity by washing with PBS(−). Collected neutrophils were rinsed twice with PBS(−), resuspended in 1 ml of neutrophil isolation buffer (5 mM CaCl2, 5 mM MgCl2, and 10 mM HEPES Tris, pH 7.3), and placed on ice. The cell suspension was mixed with 0.5 ml of a hypotonic buffer to reduce intracellular taurine. The composition of hypotonic buffer was 95 mM KC1, 1.3 mM CaCl2, 0.5 mM MgCl2, and 10 mM HEPES Tris, pH 7.3, whose osmolality was 198 ± 1 mosM kg−1 (n = 3). To load taurine, cells whose taurine content was reduced were incubated for 30 min with a hypertonic buffer (150 mM NaCl, 1.3 mM CaCl2, and 0.5 mM MgCl2 containing 100 mM tauine, buffered with 10 mM HEPES Tris, pH 7.3, 280 (M45A) was made by site-directed mutagenesis using a method previously described by Kunkel et al. (18). A wild type deletion mutant consisting of the amino acids 1–280 (IκBα 1–280 (wild type)) and a corresponding point-mutated IκBα (IκBα 1–280 (M45A)) were prepared by PCR as described above. A vector pME was used to express wild type and mutants of IκBα in Jurkat cells (14).

High Performance Liquid Chromatography (HPLC) and Mass Spectrometry Analysis—A peptide fragment consisting of IκBα 43–50 was synthesized as one with acetylated N-terminal end (Ac-εEQMLKELQ). This peptide fragment at 1 μM was incubated with 400 μM TauCl for 30 min at 37 °C. The reaction solution was separated by HPLC with an octadecyl sulfonate C18 column (Senshu Scientific Co., Tokyo, Japan). The separation run was recorded with a UV detector set at 215 nm. The absorption spectra (200–280 nm) of each cell lysate was diluted with 450 μl of each cell lysate was diluted with 450 μl of 100 mM sodium phosphate buffer (pH 7.0). When the mixture turned yellow after a certain incubation at 37 °C, the reaction was stopped with 500 μl of 1× Na2CO3. The β-galactosidase activity was spectrophotometrically determined at 430 nm. Because the transfection efficiency estimated by β-galactosidase activity did not widely deviate, luciferase activity was not normalized with the transfection efficiency.

IKK Assay—After Jurkat cells (8×105) were treated with TauCl and TNFα, cell lysate was made as described previously (17). The lysate was centrifuged at 13,000×g for 15 min. The supernatant was cleaned by a 1-h incubation with 2 μM of normal rabbit serum and 30 μg of a 50:50 (v/v) slurry of Protein G-Sepharose beads and centrifuged again at 20,000×g for 1 min. The anti-IKK1 (1.5 μg) antibody (Santa Cruz Biotechnology) was added to the resultant supernatant. Then 20 μl of the 50:50 (v/v) slurry of Protein G-Sepharose beads was added again. The mixture was rotated for 1 h and centrifuged at 2,000×g for 1 min. The final pellet was incubated at 37 °C for 30 min with 3 μg of IκBα in the presence of 20 μM ATP and 5 μg of [γ-32P]ATP. IκBα was fractionated on 10% polyacrylamide-SDS gel and phosphorylation was detected by imaging analysis with BAS 3000 (Fuji, Tokyo, Japan).

Preparation of Human IκBα Mutants—Deletion mutants of IκBα were prepared by a PCR method. All PCR products of IκBα cDNA fragments were verified by nucleotide sequencing. To subclone IκBα deletion mutants, pGEX (Amersham Biosciences) was used. Their overproduction as glutathione S-transferase fusion proteins was carried out with Escherichia coli BL21 strain (Promega, Madison, WI). IκBα-glutathione S-transferase fusion protein was purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech). IκBα mutants were released from glutathione S-transferase by digestion with thrombin protease (Amersham Pharmacia Biotech) and concentrated using Centricron (Millipore Corp., Bedford, MA).

A point mutation of IκBα whose Met83 was converted to alanine (IκBα M83A) was made by site-directed mutagenesis using a method previously described by Kunkel et al. (18). A wild type deletion mutant consisting of the amino acids 1–280 (IκBα 1–280 (wild type)) and a corresponding point-mutated IκBα (IκBα 1–280 (M83A)) were prepared by PCR as described above. A vector pME was used to express wild type and mutants of IκBα in Jurkat cells (14).
Taurine was replaced with raffinose in the hypertonic buffer for cells whose taurine content was kept low. Cells were resuspended in RPMI 1640 containing 0.25% bovine serum albumin (Sigma), whose pH was adjusted to 7.3 with 5 mM sodium bicarbonate. An aliquot of neutrophil suspension (200 μl of 1 × 10^6 cells/ml) was mixed with the same volume of E. coli K88 suspension (200 μl of 1 × 10^6 cells/ml) to initiate phagocytosis. Therefore, the multiplicity of infection was 10. After incubation for the desired time, neutrophils and bacteria were precipitated by centrifugation at 20,000 x g, and the supernatant was used to determine IL-8 by an enzyme-linked immunosorbent assay with the rat GRO/CINC-1 detection kit (Amer- sham Biosciences).

**Other Chemical Reagents**—A proteasome inhibitor (PSI), Z-Ile-Glu(OEt)-Ala-Leu-CHO, was obtained from Bachem (Budendorf, Switzerland). Pyrrolidine dithiocarbamate (PDTC), phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from Sigma. 4-Aminobenzoic hydrazide (ABAH) was from Aldrich. Alkaline phosphatase (calf intestine) was purchased from Takara. All other reagents used were analytical grade.

**RESULTS**

**TauCl-induced Interruption of NF-κB Activation**—To investigate the effect of TauCl on NF-κB activation, we paid attention to IκBα, which is a key protein in the cascade of NF-κB activation. We used the Jurkat T cell line, because this cell line was one of the most characterized cell lines with regard to molecular mechanisms of NF-κB activation. Western blot analysis of IκBα was performed for 15 min after stimulation with 20 ng/ml TNFα. When the cells were stimulated with TNFα, a band shift of IκBα due to its phosphorylation by IKK was observed at 5 min, and both original and shifted bands disappeared at 10 and 15 min (Fig. 1A). Treatment with 1 mM TauCl for 1 h prior to TNFα stimulation brought about a shift in the band of IκBα. Although IκBα also survived for 15 min when cells were pretreated for 1 h with 100 μM PSI and 300 μM PDTC, transient patterns of IκBα after TNFα stimulation were different from that observed in TauCl pretreatment. These results indicate that TauCl pretreatment increased resistance of IκBα to degradation induced by TNFα. However, TauCl seems to inhibit IκBα degradation differently from PSI or PDTC.

We studied the effect of TauCl on IκBβ. Jurkat cells were stimulated with a combination of PMA and ionomycin to initiate the degradation of IκBα and IκBβ at one time (Fig. 1B, Control). IκBα and IκBβ disappeared for 30 min and 2 h after stimulation, respectively. When cells were pretreated with 1 mM TauCl for 1 h, IκBβ degraded, whereas IκBα did not. These results indicate that modification of IκBα by TauCl is specific to α isofrom.

To examine if TauCl-induced inhibition of IκBα degradation leads to interruption of nuclear transfer of NF-κB, an electrophoretic mobility shift assay was performed. After TNFα stimulation, the amount of NF-κB translocated into the nucleus increased for 30 min (Fig. 1C). However, a 1-h pretreatment with 1 mM TauCl drastically decreased NF-κB translocation into the nucleus. We performed a reporter assay to reveal that TauCl inhibits NF-κB-dependent gene expression. Luciferase activity in cells transfected with 3×κB-tk-luc was decreased by TauCl in a dose-dependent manner, while no effects of TauCl on luciferase transcription were observed in cells transfected with 5×CRE-tk-luc (Fig. 1D). These results indicate that inhibition of IκBα degradation by TauCl leads to inhibition of nuclear transfer of NF-κB and subsequent NF-κB-dependent transcription.

We examined effects of TauCl treatment on IKK activity and NF-κB activation triggered by overexpression of IKKβ. IκBα was phosphorylated by in vitro incubation with IKK immunoprecipitated from Jurkat cells stimulated with TNFα (Fig. 2A). A similar phosphorylation was observed, although IKK was extracted from cells treated with 1 mM TauCl for 1 h prior to TNFα stimulation. This means that TauCl does not inhibit either the signal pathway upstream of IKK activation triggered by TNFα stimulation or IKK itself. When IKKβ was overexpressed in HEK293 cells, NF-κB was activated, and luciferase
expression was increased by ~30-fold (Fig. 2B). TauCl treatment inhibited two-thirds of this increase of NF-κB-dependent luciferase expression. The results indicate that TauCl interacts with the signal pathway downstream rather than upstream of IKK activation.

Characterization of TauCl-induced Modification of IκBα—Because TauCl treatment caused a band shift of IκBα similar in molecular size to that of phosphorylated IκBα at Ser32/Ser36, we assumed that TauCl might modify IκBα at these serine residues. A FLAG-tagged IκBα mutant whose Ser32/Ser36 are converted to alanine (FLAG-IκBα (S32A/S36A)) was overexpressed in Jurkat cells, treated with 1 mM TauCl for 1 h, and immunoprecipitated with anti-FLAG antibody. Western blot analysis revealed that TauCl treatment induced band shift of FLAG-IκBα (S32A/S36A), indicating that modification of IκBα by TauCl does not occur at Ser32/Ser36 (Fig. 3A).

To examine whether TauCl-induced band shift is due to phosphorylation, cell extracts were treated for 30 min with 10 units of alkaline phosphatase before electrophoresis. Alkaline phosphatase treatment dephosphorylated IκBα that had been phosphorylated at Ser32/Ser36 by IKK activated by TNFα (Fig. 3B). However, a similar alkaline phosphatase treatment of the extract from cells pretreated for 1 h with 1 mM TauCl did not affect shifted bands of IκBα, showing that phosphorylation is not a cause of TauCl-induced IκBα band shift.

Cell extracts were first boiled for 5 min to inactivate enzymes and then treated for 30 min with TauCl in the range of 0–0.4 mM. Boiling reduced the sensitivity of IκBα to TauCl, but TauCl still generated the band shift (Fig. 3C). This experiment suggests that any enzymatic reaction may not be involved in band shift formation of IκBα by TauCl.

Identification of TauCl-modified Site in IκBα—The modification site of IκBα by TauCl was investigated to gain insight into molecular mechanisms of TauCl-mediated inhibition of IκBα degradation. Two deletion mutants of IκBα 43–180 and 47–180 synthesized in bacteria were treated with 400 μM TauCl in vitro and fractionated on 12.5% polyacrylamide/SDS gel. The deletion mutant of IκBα 47–180 did not show its band shift upon TauCl treatment, although band shift was observed with wild type IκBα 1–317 and the other mutant of IκBα 43–180 (Fig. 4A). We speculated that one of the TauCl modification sites exists in the region of amino acid residues 43–46 of IκBα. Therefore, we made three more deletion mutants of IκBα 43–48, 45–180, and 46–180 and repeated a similar TauCl treatment experiment. Mutants of IκBα 44–180 and 45–180 exhibited band shift, whereas IκBα 46–180 did not (Fig. 4B). We assumed that a TauCl modification site might be Met45 of IκBα.

Analysis of TauCl-induced Modification of IκBα Met45—A small peptide of IκBα 43–50 with acetylated N-terminal end, Ac-EQMLKELQ, was used to understand how IκBα Met45 is modified by TauCl. The chromatogram of this peptide showed a major peak at the retention time of 22 min (Fig. 5A, Control).
ment peptide of Ac-EQMVKELQ (1 mM) was treated with 400–180, were synthesized, and 5 µg of each protein was incubated with 400 µM TauCl for 30 min at 37 °C followed by SDS-PAGE. The degree of band shift was observed by gel staining with Coomassie Brilliant Blue. B, additional deletion mutants of IκBα (IκBα 44–180, 45–180, and 46–180) were fractionated by SDS-PAGE after a similar TauCl treatment and stained with Coomassie Brilliant Blue.

This was designated as the intact peptide peak. A small peak at 2 min was due to phosphate in a buffer. Treatment of the peptide for 30 min with 400 µM TauCl resulted in the appearance of another peak at 15 min with decreasing the intact peptide peak (Fig. 5A, TauCl). This means that TauCl treatment increases the hydrophobicity of the peptide.

For further analysis, the intact peptide peak at 22 min and another peak at 15 min generated by TauCl treatment were collected and subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. TauCl treatment increased the molecular weight of the peptide by a factor of ~16 (Fig. 5B). Because the increased molecular weight of 16 is equal to that of one oxygen atom, the modification of the peptide by TauCl is estimated to be oxidation of IκBα Met45 to methionine sulfoxide. Other small peaks usually observed in this type of mass spectrometry are ghosts.

**Resistance of Oxidized Met45 to TNFα-induced IκBα Degradation**—To confirm that Met45 of IκBα is an amino acid residue that is modified by TauCl, Western blot analysis was performed with Jurkat cells transfected with pME-IκBα 1–280 (wild type and M45A). For control, pME-IκBα 1–280 (wild type) was used. These deletion mutants were used to distinguish mutant IκBα from endogenous full-length IκBα. When cells expressing IκBα 1–280 (wild type) and (M45A) were stimulated with 20 ng/ml TNFα, these IκBαs degraded for 20 min (Fig. 6A). Upon 1-h pretreatment with 2 mM TauCl, two shifted bands of IκBα 1–280 (wild type) and (M45A) were generated. Either shifted or original band did not degrade like endogenous full-length wild type IκBα. However, IκBα 1–280 (M45A) was degraded by TNFα stimulation regardless of a similar treatment with TauCl.

Using 5×κB-tk-luc, reporter assay was performed in Jurkat cells transfected with pME-IκBα 1–280 (wild type and M45A). Treatment with 2 mM TauCl for 1 h inhibited TNFα-induced luciferase activity by a factor of 90% in cells expressing IκBα 1–280 (wild type) (Fig. 6B). In cells expressing IκBα 1–280 (M45A), inhibition by TauCl was less (70%), although not completely reversed. To evaluate expression levels of IκBα 1–280 (wild type) and (M45A), a Western blot was performed. Band densities were less than that of endogenous IκBα (Fig. 6C). The low level of expression of IκBα (M45A) relative to endogenous IκBα may account for failure in full recovery of luciferase expression in cells transfected with pME-IκBα 1–280 (M45A).
Methionine 45 Oxidation of IκBα by Taurine Chloramine

In this study, we found that when Jurkat cells were treated with TauCl, IκBα was band-shifted and became resistant to TNFα stimulation (Fig. 1A). A similar treatment of Jurkat cells with TauCl caused the suppression of NF-κB nuclear translocation and consequent NF-κB-dependent gene expression (Fig. 1, C and D). Recently, Barua et al. (22) have shown that TauCl inhibits NF-κB-dependent gene expression of inducible nitric-oxide synthase and TNFα in activated alveolar macrophages, because IκBα degradation is inhibited by TauCl treatment. In this respect, our observation is consistent with their results (22). They speculated that TauCl may interact with a certain upstream step of IKK because TauCl did not directly inhibit IKK activity in an in vitro assay. In Fig. 1B, IκBβ was degraded by TNFα stimulation in TauCl-treated Jurkat cells, while IκBα did not degrade. An expressed deletion mutant of IκBα 1–280 (M45A) also degraded after TNFα stimulation regardless of a similar treatment with TauCl (Fig. 6A). Since the activation of IKK is required for degradation of IκBβ and the IκBα mutant,

**FIG. 8. Effects of intracellular taurine on IL-8 release from rat neutrophils.** Rat neutrophils whose taurine content was changed were incubated with bacteria for 1, 3, 5, and 7 h. When ABAH was used, cells were treated with 500 μM of this MPO inhibitor for 30 min prior to bacterial stimulation. ABAH was also present during incubation with bacteria. The supernatant was used to determine IL-8. The data are given as means, with bars representing S.D. (n = 3). Open circles, taurine-reduced cells; filled circles, taurine-reloaded cells; half-filled circles, taurine-reloaded cells treated with ABAH. cells were treated with 20 ng/ml TNFα for 15 min. However, the nuclear transfer of NF-κB was decreased by treatment for 5 min with 100 μM H2O2 but recovered by the 30-min pretreatment and presence of 500 μM ABAH during TNFα stimulation. This suggests that IκBα modified by intracellularly synthesized TauCl inhibits NF-κB nuclear translocation.

**Effect of Intracellular Taurine on IL-8 Release from Rat Neutrophils Stimulated with Bacteria—**To demonstrate that intracellular taurine really affects the NF-κB-dependent production of inflammatory cytokine, the release of IL-8 was measured with rat neutrophils. As shown in Fig. 8, the release of IL-8 initiated by bacteria was significantly higher in taurine-reduced neutrophils (1 ± 1 unit, n = 11) than reloaded ones (26 ± 9 unit, n = 11) (p < 0.05). Furthermore, when taurine-reloaded neutrophils were treated with 500 μM ABAH before and during incubation with bacteria, the release of IL-8 was significantly increased for 3–7 h (p < 0.05). Because neutrophils express MPO, intracellular taurine reacts with HClO produced during phagocytosis of bacteria and forms TauCl. Intracellularly synthesized TauCl seems to decrease NF-κB-dependent IL-8 production.

**DISCUSSION**

In this study, we found that when Jurkat cells were treated with TauCl, IκBα was band-shifted and became resistant to TNFα stimulation (Fig. 1A). A similar treatment of Jurkat cells with TauCl caused the suppression of NF-κB nuclear translocation and consequent NF-κB-dependent gene expression (Fig. 1, C and D). Recently, Barua et al. (22) have shown that TauCl inhibits NF-κB-dependent gene expression of inducible nitric-oxide synthase and TNFα in activated alveolar macrophages, because IκBα degradation is inhibited by TauCl treatment. In this respect, our observation is consistent with their results (22). They speculated that TauCl may interact with a certain upstream step of IKK because TauCl did not directly inhibit IKK activity in an in vitro assay. In Fig. 1B, IκBβ was degraded by TNFα stimulation in TauCl-treated Jurkat cells, while IκBα did not degrade. An expressed deletion mutant of IκBα 1–280 (M45A) also degraded after TNFα stimulation regardless of a similar treatment with TauCl (Fig. 6A). Since the activation of IKK is required for degradation of IκBβ and the IκBα mutant,
we do not agree with their speculation that TauCl negatively regulates IKK in its upstream. Furthermore, Fig. 2, A and B, clearly shows that TauCl does not inhibit either the signal pathway upstream of IKK activation or IKK itself but interrupts the signal pathway after IKK activation. In our in vitro experiments with cell extracts and synthesized IxBa fragment peptides, TauCl treatment also caused IxBa band shift, showing that TauCl can directly modify the IxBa molecule (Fig. 3, B and C, and Fig. 4). We used a large gel whose length was ~10 cm and overran to separate shifted bands from the original band of IxBa as previously described (14). When we used a mini gel, we could not observe the band shift of IxBa (data not shown). Our results show that a direct modification of IxBa by TauCl that is a cause of its band shift is a crucial step for increase in IxBa resistance to degradation initiated by TNFα stimulation.

When we first observed TauCl-induced band shifts of IxBa, we thought that TauCl inhibited IKK or proteasome, modified IKK phosphorylation sites Ser32/Ser36, or caused phosphorylation of other sites, because the band shift by TauCl was similar to that by phosphorylation in molecular size. When Jurkat cells were treated with the combination of PMA and ionomycin to degrade IxBa and IxBβ together, IxBβ degraded even in the presence of TauCl, whereas IxBα did not (Fig. 1B). This result precluded the possibility of inhibition of IKK or proteasome by TauCl, because IKK and proteasome both are required to degrade IxBβ. When Jurkat cells expressing FLAG-IxBα (S32A/S36A) were treated with TauCl, IxBa mutant exhibited the band shift (Fig. 3A). Therefore, it is very hard to think that phosphorylation sites of IxBα, Ser32/Ser36, were modified by TauCl. An idea of phosphorylation at other sites by TauCl was also excluded by the results shown in Fig. 3B. Fig. 3C indicates that the modification of IxBα by TauCl is a non-enzymatic reaction. We carried out in vitro experiments using synthesized IxBa deletion mutants and fragment peptide and came to the conclusion that the oxidation of IxBa at Met45 by TauCl is one cause of its band shift (Figs. 4 and 5). Because methionine sulfoxide is a product when one oxygen molecule reacts with methionine, we deduced that Met45 is oxidized to methionine sulfoxide.

It is still unclear whether Met45 is the only oxidation site by TauCl. Because two shifted bands of IxBa were observed when cells were treated with TauCl or H2O2 (Figs. 1A, 6A, and 7B), TauCl may oxidize another methionine residue of IxBa, besides Met45. If one of two shifted bands resulted from phosphorylation, the number of the shifted bands would decrease when cell extracts were treated with alkaline phosphatase. Therefore, we expect that another methionine residue is oxidized. As shown in Fig. 1B, TauCl treatment did not generate a band shift of IxBβ, whereas a similar treatment generated two shifted bands of IxBa. We think that this IxBa-specific oxidation results from different location of methionine residues between IxBa and -β. IxBa consists of a centrally located ankyrin repeat domain (ARD); a signal-receiving domain (SRD) from the amino terminus to ARD; and a proline-, glutamic acid-, serine-, and threonine-rich region from ARD to the carboxyl terminus (23). IxBa contains six methionine residues, Met1, Met13, Met37, and Met45 in SRD and Met91 and Met79 in ARD. On the other hand, IxBβ has a Met1 in SRD and all of the others, Met45, Met222, Met271, and Met282, in ARD. Probably methionine residues in SRD are more susceptible to oxidation than those in ARD because SRD is jutting out on the surface of IxBα-NF-κB complex. Thus, besides Met45, Met13, or Met37 of IxBa may be a feasible oxidation site. In Fig. 4A, only one shifted band was observed when full-length IxBa (residues 1–317) was treated with 400 μM TauCl in vitro. We assume that one shifted band is the upper one of two shifted bands probably due to a high concentration of TauCl at 400 μM in vitro treatment, because two shifted bands of IxBa were observed by in vitro treatment with 100 μM TauCl (Fig. 3C). Furthermore, the distance between the original and shifted band of full-length IxBa is larger than that of a deletion mutant of IxBa 43–180 (Fig. 4A). These data support an idea that another oxidation site is Met13 or Met37.

Even if Met13 or Met37 is oxidized by TauCl, one may think that this oxidation site may not be crucial in degradation of IxBa because a mutant of IxBa 1–280 (M45A) expressed in Jurkat cells did not generate a band shift upon TauCl treatment and degraded in response to TNFα stimulation (Fig. 6A). However, in a reporter assay, luciferase expression was not fully recovered in cells expressing this mutant (Fig. 6B). The density ratio of endogenous over exogenous IxBa bands was ~3, indicating that expressed IxBa 1–280 (wild type) and (M45A) were only 25% of total IxBa. Therefore, even if expressed IxBa 1–280 (M45A) fully degrades upon TNFα stimulation after TauCl treatment, the maximal restoration of luciferase expression might not be more than 25%. However, unless the restoration of luciferase expression by overexpression of the mutant becomes 100%, there is still a possibility of other mechanisms, including oxidation of IxBα at Met13 or Met37. Therefore, we have to clearly state here that oxidation of IxBa at Met45 may be only one cause of TauCl-induced interruption of NF-κB activation.

There are two possible reasons why oxidized Met45 disturbs IxBa degradation. One possibility is that oxidized Met45 constructs a structural obstacle, which prevents IKK from approaching IxBa phosphorylation sites, Ser32 and Ser36. The other possibility is that even if IKK can phosphorylate oxidized IxBa, the F-box protein necessary for ubiquitination that occurs at Lys37 and Lys37 of IxBa cannot recognize the phosphorylated IxBa (24). Because Met13 and Met37 are also located close to IxBa phosphorylation and ubiquitination sites, their oxidation may have a similar disturbing effect on IxBa degradation. Further experiments are required to understand detail molecular mechanisms of IxBa resistance induced by TauCl.

Reactive oxygen species (ROS) such as H2O2, hydroxyl radical, and superoxide anion are generated during the course of normal metabolism. These oxidants modify a variety of cellular constituents, protein, lipid, DNA, and so on, which causes cellular damage. All amino acids can be oxidized with ROS. However, methionine as well as cysteine residues possess sulfor-containing side chains that are the most sensitive to the ROS-mediated oxidation. Involvement of cysteine residues in intracellular redox regulation is well known. Oxidation of cysteine thiol most likely forms disulfide, which glutathione easily reduces. However, function of methionine residues in redox regulation has been left unclear. A recently published review article suggests that methionine residues play an important role in redox regulation via a redox of methionine and methionine sulfoxide (25). There are two representative proteins whose functional activity is regulated by this redox system, Shaker potassium channel (26) and calmodulin (27). Both are involved in cellular excitability. This study provides the first evidence for the regulation of NF-κB activation by oxidation of a methionine residue of IxBa. Although TauCl is known to be able to oxidize proteins by in vitro treatment (28), it has not been demonstrated that methionine oxidation by TauCl takes a part in intracellular events. Because it has been reported that methionine sulfoxide reductase is present in white blood leukocytes (29), NF-κB activation might be under the methionine redox control. It was shown that TauCl selectively reacts with IxBa. IxBa degradation is important in rapid response to phag-
ocytosis, but IκBβ is related to continuous NF-κB activation (30, 31). Therefore, we assume that intracellular taurine may function to suppress undesired rapid inflammatory reaction in neutrophils. The concentration of taurine is ~50 μM in the extracellular space (32). MPO is released to the extracellular space of inflammatory sites when neutrophils are broken by dramatic activation. This situation leads to the extracellular formation of TauCl. Although the membrane permeability of TauCl is low, extracellular TauCl can diffuse into surrounding cells and inhibit NF-κB activation (33). Taking these data together, we assume that in inflammatory sites as well as neutrophils, taurine can suppress the inflammation in which ROS is generated to an excessive extent.

Acknowledgments—We thank H. Katayama and Dr. H. Nagasawa for expert technical assistance in mass spectrometry analyses and Dr. Michael Karin for providing pBclact-3HAIIKβ.

REFERENCES
1. Zelikovic, I., and Chesney, R. W. (1989) in New Protective Roles for Selected Nutrients (Spiller, G. A., and Scala, J., eds.) pp. 253–294, Alan R. Liss, Inc., New York
2. Fukuda, K., Hirai, Y., Yoshida, H., Nakajima, T., and Usui, T. (1982) Clin. Chem. 28, 1758–1761
3. McDonald, P. P., and Cassatella, M. A. (1997) FEBS Lett. 412, 538–586
4. Thomas, E. L., Grisham, M. B., and Jefferson, M. M. (1986) Methods Enzymol. 12, 569–586
5. Baeuerle, P. A. (1993) Nature 365, 182–185
6. Lloyd, A. R., and Oppenheim, J. J. (1992) Immunol. Today 13, 169–172
7. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) Blood 92, 3007–3017
8. Stapleton, P. O., O’Flaherty, L., Redmond, H. P., and Bouchier-Hayes, D. J. (1998) J. Parenter. Enteral. Nutr. 22, 42–48
9. Huxtable, R. J. (1992) Physiol. Rev. 72, 101–163
10. Ogino, T., Kobuchi, H., Sen, C. K., Roy, S., Packer, L., and Maguire, J. J. (1997) J. Biol. Chem. 272, 26247–26252
Oxidation of IκBα at Methionine 45 Is One Cause of Taurine Chloramine-induced Inhibition of NF-κB Activation
Atsuhiro Kanayama, Jun-ichiro Inoue, Yoshioko Sugita-Konishi, Makoto Shimizu and Yusei Miyamoto

J. Biol. Chem. 2002, 277:24049-24056.
doi: 10.1074/jbc.M110832200 originally published online April 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110832200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 8 of which can be accessed free at http://www.jbc.org/content/277/27/24049.full.html#ref-list-1