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Taurine Chloramine Inhibits Inducible Nitric Oxide Synthase and TNF-α Gene Expression in Activated Alveolar Macrophages: Decreased NF-κB Activation and IκB Kinase Activity[1]

Madhabi Barua,[2] Yong Liu,[2] and Michael R. Quinn[3]

Taurine prevents tissue damage in a variety of models that involve inflammation, including oxidant-induced lung damage. The mechanism of protection is uncertain, but is postulated to involve the actions of taurine chloramine (Tau-Cl) derived via halide-dependent myeloperoxidase associated with neutrophils. Understanding the influence of Tau-Cl on the production of inflammatory mediators by alveolar macrophages provides an opportunity for determining the mechanism of Tau-Cl action. The effects of Tau-Cl were evaluated on the production of NO and TNF-α in NR8383, a cloned cell line derived from rat alveolar macrophages (RAM), and in primary cultures of RAM. Production of NO and TNF-α, and expression of inducible NO synthase was inhibited by Tau-Cl in activated NR8383 cells as well as in RAM. Temporal (2, 4, 8, 24 h) expression of inducible NO synthase and TNF-α mRNAs was reduced by Tau-Cl in NR8383 cells. Tau-Cl depressed NF-κB migration into the nucleus of activated NR8383 cells and caused a more sustained presence of IκB in the cytoplasm. Stabilization of cytoplasmic IκB-α in Tau-Cl-treated cells resulted from decreased phosphorylation of IκB-α serine-32 and a lower activity of IκB kinase (IKK). Additional experiments demonstrated that Tau-Cl does not directly inhibit IKK activity. These results suggest that Tau-Cl exerts its effects at some level upstream of IKK in the signaling pathway and inhibits production of inflammatory mediators through a mechanism that, at least in part, involves inhibition of NF-κB activation. ***The Journal of Immunology*****, 2001, 167: 2275–2281.**

Taurine, a free amino acid not incorporated into protein, protects against tissue damage in a variety of models that share inflammation as a common pathogenic feature (1). This has been particularly well documented in animal models of lung injury. Prophylactic administration of taurine protects lung from damage induced by inhalation exposure to NO_2 (2) or O_3 (3, 4) and by intratracheal instillation of bleomycin (5), amidarone (6), or paraquat (7). All of these models of lung damage involve an influx of neutrophils followed by macrophages and production of inflammatory mediators.

Although the mechanism of taurine protection is uncertain, the ability of taurine to attenuate the highly toxic effects of HOCl/OCl^- produced by activated polymorphonuclear leukocytes, eosinophils, and basophils is thought to be important. Taurine reacts with HOCl/OCl^- to form taurine chloramine (Tau-Cl),[4] an oxidant that is far less reactive and more stable than HOCl/OCl^- (8–11). Although the detoxification of HOCl/OCl^- by Tau-Cl formation has been postulated to account for the protective effects of taurine, more recent studies suggest that Tau-Cl may be a significant biological effector molecule. This is supported by reports that Tau-Cl inhibits production of NO, TNF-α, and other proinflammatory mediators by activated cells from a variety of different tissues (1, 12–17). However, the mechanism of Tau-Cl action has not been determined and the effects of Tau-Cl on activated alveolar macrophages have not been reported, except in preliminary form (16).

Production of proinflammatory mediators is primarily regulated at the level of gene transcription through the activity of several transcription factors. Of prominent importance in this regard is the nuclear transcription factor NF-κB because NF-κB is ubiquitously involved in regulating the expression of inducible NO synthase (iNOS), TNF-α, and several other proinflammatory genes (18–20). NF-κB is a hetero- or homodimeric protein consisting of various combinations of subunits belonging to the Rel family: p65 (Rel A), p50, p52, Rel B, and cRel. The p65/p50 dimer is the prototypical form of NF-κB in the nucleus of most activated cells and is the most potent *trans* activator of proinflammatory gene expression (18, 21). In unstimulated cells, NF-κB is sequestered in the cytoplasm as a complex with a member of the IκB family; IκB-α is the most well characterized. Upon stimulation, IκB-α is phosphorylated at serine residues 32 and 36, ubiquitinated, and degraded by the 26S proteasome (22–24). Degradation of IκB-α unmasks the nuclear localization sequence of NF-κB, allowing translocation into the nucleus and binding to the promoter region of target genes (25).

Phosphorylation of IκB is catalyzed by IκB kinase (IKK)-α and IKK-β, which are components of a large (800 kDa) multiprotein complex referred to as IKK. Other components of IKK include NF-κB essential modifier and IKK complex-associated protein (22, 26, 27). The IKK complex is a common mediator of several upstream signaling cascades that regulates IKK-α and IKK-β

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[4] Abbreviations used in this paper: Tau-Cl, taurine chloramine; iNOS, inducible NO synthase; IKK, IκB kinase; MG-132, N-CBZ-Leu-Leu-Leu-A1; RAM, rat alveolar macrophage.

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phosphorylation of IκB. In the present study, Tau-Cl is demonstrated to inhibit production of NO and TNF-α by activated rat alveolar macrophages (RAM) in culture. Our results show that Tau-Cl inhibits the expression of iNOS and TNF-α genes by attenuating the NF-κB signal transduction pathway at a point that is above the level of IKK activation.

Materials and Methods

Materials

Monoclonal Ab against iNOS was purchased from Transduction Laboratories (Lexington, KY). Polyclonal Ab to p65, p50, cRel, IκB-α, IκB-β, IKK-β, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), as was mAb against IKK-α. Ab against phosphorylated IκB-α and GST-IκB-α were obtained from New England Biolabs (Beverly, MA). Culture medium and rat recombinant IFN-γ were purchased from Life Technologies (Grand Island, NY). LPS W (*Escherichia coli* 0111:B4) was purchased from Difco (Detroit, MI) and heat-inactivated FBS was purchased from Gemini Bio-Products (Calabasas, CA). Plasmids containing cDNA probe for iNOS (murine), TNF-α (murine), and GAPDH (rat) were graciously provided by Dr. C. Nathan (Cornell University, New York, NY), Chiron (Emeryville, CA), and Dr. R. Dong (London University, London, U.K.), respectively. NaOCl was purchased from Fisher Scientific (Fairlawn, NJ). Taurine, MG-132 (N-CBZ-Leu-Leu-Leu-A1) and other chemicals were obtained from Sigma (St. Louis, MO). Tau-Cl was freshly synthesized on the day of use by adding equimolar amounts of NaOC1 dropwise to taurine at pH 8.3 and was authenticated by measuring its UV absorption spectra (190–350 nm) which assured monochloramine formation and the absence of dichloramine, NH_2Cl, and unreacted $HOCl/OCl^-$ (15, 28).

Alveolar macrophage cultures

Bronchoalveolar cells were obtained as described previously (29), with some modifications. Briefly, adult female Sprague Dawley rats (Taconic Farms, Germantown, NY) were anesthetized by an injection (i.p.) of sodium pentobarbital. The thoracic cavity was opened, the trachea was cannulated, and the lungs were lavaged with calcium and magnesium-free HBSS. The lungs were massaged with each lavage and were sequentially washed until 60 ml of lavage fluid was accumulated. Cells were collected by centrifugation (250 × *g*), washed in HBSS, and a differential cell count was performed. Cells were resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, 10% FCS, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were placed into tissue culture plates and incubated at 37°C in 5% CO_2 for 1 h before nonadherent cells were removed by washing with medium. Adherent macrophages (RAM) continued in culture until used.

NR8383 cells, a clonal cell line derived from RAM, were obtained from the American Type Culture Collection (Manassas, VA) and were grown in flasks containing Ham's F-12 nutrient mixture supplemented with 15% FBS, 2 mM glutamine, and antibiotics. Experiments were conducted in DMEM supplemented with heat-inactivated 2% FBS, 1% penicillin, and 1% streptomycin at a density of 1×10^7 cells/100-mm diameter culture dish or 1×10^6/well in six-well plates for both cell types. Cells were activated with LPS (1 μg/ml) and IFN-γ (10 U/ml), and Tau-Cl was added immediately thereafter.

Nitrite and TNF-α measurements

Samples (100 μl) of conditioned medium were mixed with equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 10 min before measuring OD at 550 nm using sodium nitrite as standard (16). Concentrations of TNF-α were determined by ELISA using an immunoassay kit specific for rat TNF-α according to the manufacturer's instructions (BioSource International, Camarillo, CA).

Northern blot analyses

Northern blot analyses were conducted as previously described (16). Briefly, total RNA was extracted from NR8383 cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH), size fractioned by electrophoresis in 1% agarose-formaldehyde gel, transferred to Nytran membrane, and cross-linked to the membrane by UV irradiation. Blots were prehybridized in ExpressHyb Hybridization Solution (Clontech Laboratories, Palo Alto, CA) for 1 h (68°C) before hybridization with [^{32}P]dCTP random prime-labeled cDNA at 68°C for 16–18 h. Blots were washed three times at room temperature in 2× SSC containing 0.5% SDS followed by two washes at 50°C in 0.1× SSC containing 0.1% SDS. Membranes were stripped of cDNA probe between sequential hybridizations. RNA hybridized with cDNA probe was visualized by phosphor imager analyses and by autoradiography using Kodak XAR-5 film. Autoradiograms were analyzed by computer-assisted densitometry.

Western blot analyses

Cells were collected, centrifuged, washed in PBS, and lysed in radioimmunoprecipitation assay buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing aprotinin (5 U/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), PMSF (1 mM), sodium orthovanadate (1 mM), and sodium fluoride (1 mM). After centrifugation, cell lysates were diluted in SDS containing sample preparation buffer, subjected to SDS-PAGE, and transferred to nitrocellulose filters. Filters were blocked in a solution of PBS containing 5–10% nonfat dry milk or BSA and 0.05% Tween 20. After incubations with the indicated primary and secondary Abs, reactive bands were visualized by ECL (Amersham, Arlington Heights, IL). Protein concentrations were determined by bicinchoninic acid with BSA as standard (Pierce, Rockford, IL).

Cytoplasmic and nuclear protein preparations

Cytoplasmic (postnuclear) and nuclear protein fractions were prepared according to Schreiber et al. (30), with modifications. Cells were washed twice in PBS and suspended in buffer A. After incubating for 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6% (v/v) and the samples were vigorously vortexed before centrifuging at 16,000 × *g* for 30 s at 4°C. The supernatant cytoplasmic fraction was removed and stored at −80°C until used. The nuclear pellet was suspended in buffer C (30) and vigorously vortexed intermittently for 15 min before centrifuging at 16,000 × *g* for 20 min at 4°C. The supernatant nuclear protein extract was stored at −80°C until used.

NF-κB EMSAs

Protein-DNA binding interactions were performed by incubating (22°C, 15 min) 3–5 μg of nuclear protein with 1 ng of ^{32}P-labeled (50,000 cpm) double-stranded oligonucleotide probe (custom synthesized by Bioserve Biotechnologics, Laurel, MD) in 20 mM Tris-HC1 (pH 7.9) containing 50 mM NaC1, 2 mM $MgC1_2$, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 0.5% BSA, and 2 μg of poly(dI-dC) in a final volume of 20 μl. For gel supershift analyses, Abs were included in the above reaction mixture and incubated at 4°C for 90 min before addition of the ^{32}P-labeled oligonucleotide probe, followed by incubation at 22°C for 15 min. The protein-DNA complexes were fractionated on 6% native polyacrylamide gels run in 0.5× TBE buffer (Tris-borate-EDTA). Probes were labeled using the Klenow fragment of DNA polymerase and [^{32}P]dCTP. The consensus NF-κB sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′ was used as probe for EMSA. The mutant NF-κB probe used for competition in EMSA had the same sequence with the exception of a single base (underlined) that was changed to C. Gels were dried and bands were visualized by autoradiography.

Immunoprecipitation and kinase assays

Immunoprecipitation of IKK from cell lysates (800 μg of protein) was accomplished using anti-mouse IKK-α mAb and ultralink immobilized protein A/G (Pierce) incubated overnight at 4°C. Immunoprecipitates were washed five times with radioimmunoprecipitation assay buffer and twice with kinase reaction buffer (50 mM Tris-HCl (pH 8.00) containing 50 mM magnesium chloride, 3 μM okadaic acid, and 300 nM ATP). Enzyme assays were performed by adding 1 μg of GST-IKB-α and 6.8 μCi of [γ-^{32}P]ATP (7000Ci/mmol) to the kinase reaction buffer containing the immunoprecipitates and incubating at 30°C for 30 min. The reaction was stopped by the addition of 2× SDS sample preparation buffer followed by boiling for 5 min. The samples were subjected to SDS-PAGE, transferred to nitrocellulose filters, analyzed by autoradiography, and finally subjected to immunoblotting.

Results

Addition of Tau-Cl to cultured cells at the time of activation with LPS (1 μg/ml) and IFN-γ (10 U/ml) resulted in a dose-dependent inhibition of NO production, as measured by NO_2^- medium accumulation over 24 h (Fig. 1). Tau-Cl inhibited NO production by NR8383 and by primary cultures of RAM with similar potency ($IC_{50} \approx 0.5$ mM) and efficacy. Western blot analyses of cell lysates revealed that iNOS protein was reduced in both cell types by

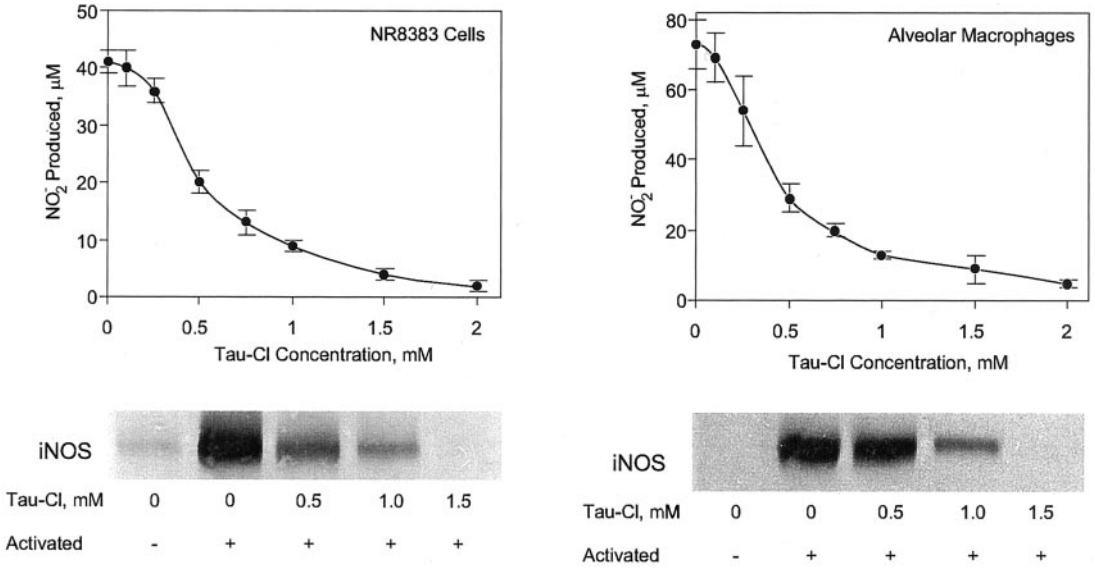

FIGURE 1. Tau-Cl inhibits production of NO_2^- and expression of iNOS protein in NR8383 cells and in primary cultures of RAM. Tau-Cl was added to the culture medium at the time of activation (LPS, 1 μg/ml + IFN-γ, 10 U/ml). After 24 h, samples of conditioned medium were analyzed for NO_2^- content using Griess reagent and cell lysates were prepared for Western blot analysis of iNOS. Values represent mean ± SD of triplicate samples. Similar results were obtained in 4–10 additional independent experiments.

Tau-Cl in a concentration-dependent manner (Fig. 1). Whereas unactivated NR8383 cells expressed low basal levels of iNOS protein, unactivated primary cultures of RAM did not. In addition, unlike unactivated primary RAM cultures, unactivated NR8383 cells produced low concentrations (4–6 μM) of NO_2^-. Production of TNF-α by both cell types was also dose-dependently inhibited by Tau-Cl (Fig. 2). Unactivated NR8383 and RAM did not secrete detectable amounts of TNF-α into the medium. Cell viability was unaffected by the range of Tau-Cl concentrations used in these studies (data not shown) as measured by trypan blue exclusion and by conversion of the tetrazolium salt MTS into a formazan product using a kit from Promega (Madison, WI). Further studies of the molecular mechanism of Tau-Cl inhibition of NO and TNF-α production were conducted using the clonal cell line NR8383.

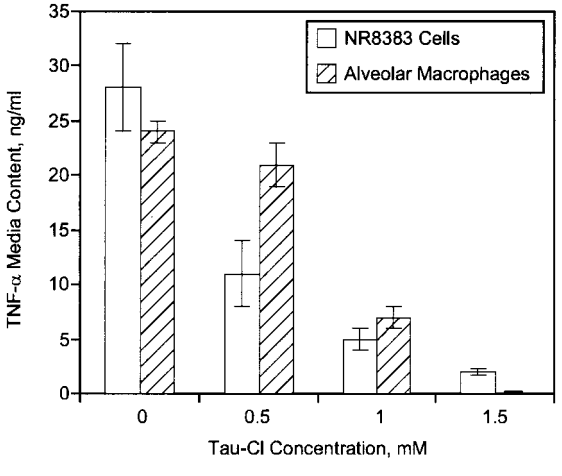

FIGURE 2. Tau-Cl inhibits production of TNF-α in activated rat NR8383 and alveolar macrophages. Cells were exposed to Tau-Cl in the culture medium at the time of activation and medium content of TNF-α was measured by ELISA 24 h later. Values represent the mean ± SD of triplicate samples. Similar results were obtained in three additional independent experiments.

The temporal expression of iNOS and TNF-α mRNAs was evaluated by Northern blots (Fig. 3). Neither iNOS nor TNF-α mRNAs were detected in preparations from unactivated cells. Transcripts for TNF-α were expressed earlier than iNOS transcripts in activated NR8383 cells and Tau-Cl concentration-dependently inhibited expression of both mRNAs. Tau-Cl appeared to exert its most profound effects during early phases of message expression, regardless of the relatively different temporal patterns, i.e., at 2–4 h for TNF-α and 4–8 h for iNOS mRNA. These results suggested that early events in gene transcription may be affected by Tau-Cl.

Although transcriptional regulation of proinflammatory gene expression is mediated by several transcription factors, the involvement of NF-κB appears to be obligatory in most cell types. Since iNOS and TNF-α gene expression is regulated by NF-κB, the effects of Tau-Cl on NF-κB-binding activity were examined in nuclear protein extracts of NR8383 cells by EMSA (Fig. 4). The binding activity of NF-κB recovered from the nucleus increased within 30 min of activation, relative to that of unactivated cells, and cumulatively increased over the remaining 24 h of activation. Nuclear protein extracts from cells activated in the presence of Tau-Cl (1.0 mM) exhibited greatly reduced NF-κB-binding activity relative to that of activated cells at all times examined. In addition, a second band of NF-κB-binding activity was evident after 3, 6, and 24 h of activation, but was not recovered from the nuclear protein fraction of Tau-Cl-treated cells. Supershift EMSA analyses of nuclear protein extracts prepared from NR8383 cells activated for 3 h demonstrated that the upper NF-κB band consisted of p50/p65 (Fig. 5). This was evident because Ab against p50 and Ab against p65, but not cRel, supershifted the upper band. The lower NF-κB band is a homodimer consisting of p50 subunits because p50 Ab selectively supershifted the entire complex while p65 and cRel Abs were ineffective. Specificity of NF-κB binding was determined by preincubating nuclear protein extracts with excess (50-fold) unlabeled NF-κB probe or with a one-base mutant NF-κB probe as competitor before incubating with radiolabeled probe and conducting the EMSA. Similar results were obtained with Tau-Cl-treated cells, i.e., the identity and specificity of p50/p65 was verified (data not shown) but the lower band (p50/p50)

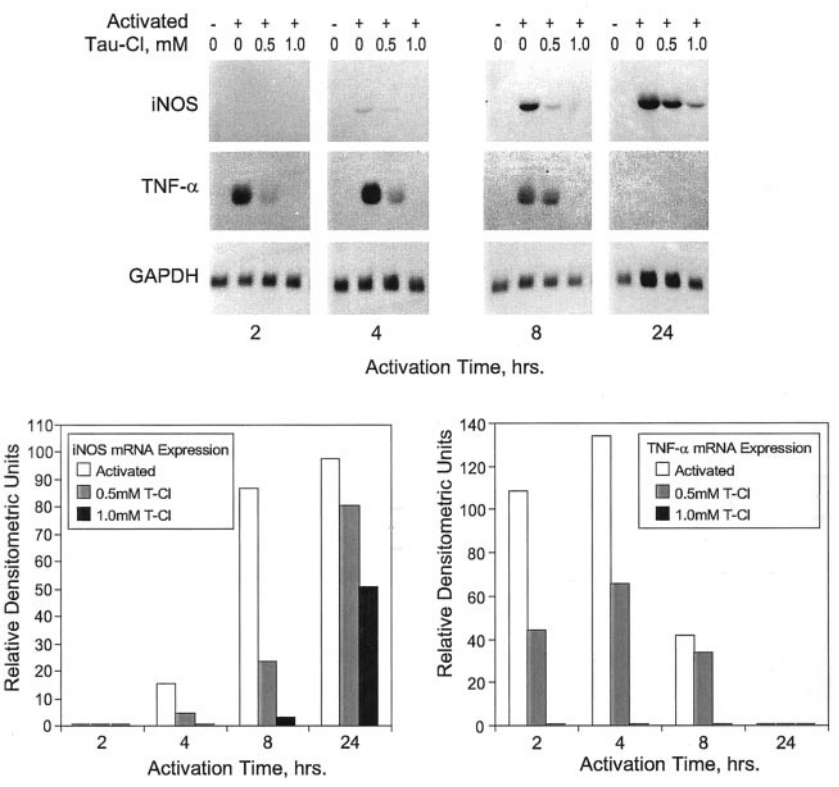

FIGURE 3. Kinetics of iNOS, TNF-α, and GAPDH mRNA expression in NR8383 cells treated as described in Fig. 1 legend. Total RNA fractions were size fractionated on 1% agarose-formaldehyde gels before transfer to Nytran membranes. Blots were sequentially probed with each [^{32}P]dCTP random prime-labeled cDNA probe with stripping between assays. RNA hybridized with cDNA probe was visualized after autoradiography and analyzed by computer-assisted densitometry. Bands were normalized to the amount of GAPDH applied to each lane and quantified as relative densitometric units. Similar results were obtained in three additional independent experiments.

was not observed (Fig. 4). These results indicated that Tau-Cl was either inhibiting NF-κB binding to its nuclear recognition sites or was inhibiting some earlier event in the process of NF-κB activation.

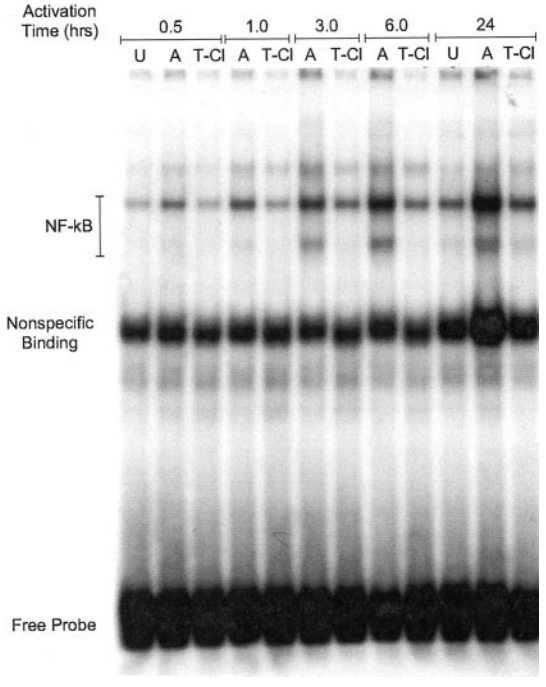

FIGURE 4. Kinetics of NF-κB nuclear protein binding in NR8383 cells. Cells were treated for the indicated times before preparing nuclear protein extracts for analyses by EMSA. U, Unactivated cells; C, control activated cells; T-Cl, cells exposed to 1.0 mM Tau-Cl with activation for various lengths of time. Similar results were obtained in three additional independent experiments.

To determine the effects of Tau-Cl on the migration of NF-κB into the nucleus, Western blot analyses of nuclear protein fractions were conducted (Fig. 6). Accumulation of p50 and p65 NF-κB subunits in the nuclear protein fraction was greatly reduced in Tau-Cl-treated cells relative to control NR8383 cells over the entire 24 h of activation. No changes in p50 or p65 were detected in

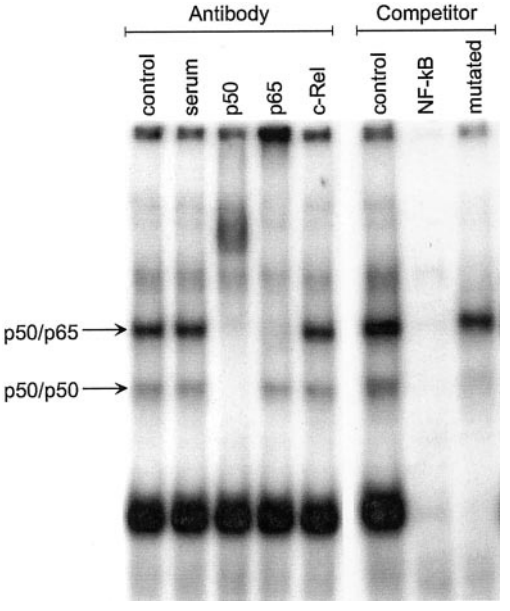

FIGURE 5. Characterization of NF-κB complexes was assessed by supershift assays using preimmune serum and Abs (Santa Cruz Biotechnology) against NF-κB proteins. Specificity was determined using 50-fold excess of unlabeled NF-κB or a single-base mutated NF-κB probe as competitor. Nuclear protein extracts were prepared 3 h after activation. Similar results were obtained in three additional independent experiments.

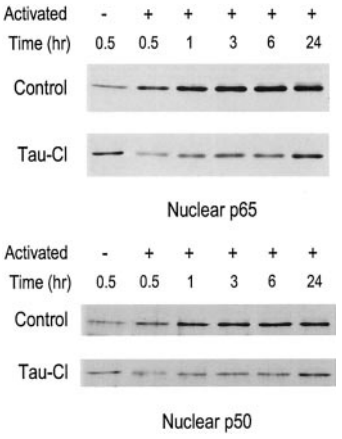

FIGURE 6. Tau-Cl inhibits the migration of p50 and p65 into the nucleus of activated NR8383 cells. Cells were treated for the times indicated before preparing nuclear protein extracts for analyses by Western blot using Abs (Santa Cruz Biotechnology) specific for p50 and p65. Similar results were obtained in two additional independent experiments.

cytosolic fractions when measured over time or as a result of Tau-Cl treatment (data not shown). These results suggested that Tau-Cl was not inhibiting the binding of NF-κB to its nuclear recognition site per se, but was inhibiting some aspect of NF-κB activation in the cytoplasm. This was evaluated by determining the presence of cytosolic IκB-α in the same cell preparations as used for Fig. 6. Control cells exhibited a rapid disappearance of cytosolic IκB-α during the first 1 h of activation with gradual reappearance occurring between 3 and 24 h of activation (Fig. 7). Cells activated in the presence of Tau-Cl (1.0 mM) did not exhibit this transient change in cytoplasmic IκB-α, but demonstrated increased IκB-α content 24 h after activation similar to that of control activated cells. These results suggest that in Tau-Cl-treated cells IκB was not being degraded and resynthesized, but was being retained as a complex with NF-κB in the cytoplasm.

As a prerequisite for NF-κB translocation to the nucleus, cytosolic IκB must be phosphorylated, ubiquitinated, and degraded by the 26S proteasome (22–24). To address the effects of Tau-Cl on this event, we used MG-132, a peptide that blocks proteasome activity, thus allowing phosphorylated IκB to accumulate in the cytoplasm (31, 32). Preliminary studies demonstrated that a 60-min preincubation (37°C, 5% CO_2) of NR8383 cells with 10 μM MG-132 before activation was adequate for inhibiting degradation of phosphorylated IκB. Treatment with MG-132 also inhibited NO production, measured 24 h later by activated cells (data not shown). The effects of Tau-Cl on phosphorylation of IκB-α were determined by using cells that were preincubated (1 h, 37°C, 5% CO_2) with 10 μM MG-132 or vehicle (DMSO, 0.25% (v/v) final concentration) before activation with LPS + IFN-γ. Tau-Cl was added at the time of activation. Cell lysates were prepared 1 h later and analyzed by Western blot using Abs specific for IκB-α phosphorylated at serine residue 32 (Fig. 8). Preparations from activated cells contained reduced amounts of unphosphorylated IκB-α and increased p-IκB-α relative to unactivated cells. Preincubation with MG-132 (10 μM) for 1 h before activation greatly enhanced the p-IκB-α signal, whereas cells activated in the presence of Tau-Cl (1 mM) accumulated only low amounts of p-IκB. Cells activated in the presence of Tau-Cl maintained unphosphorylated IκB-α levels that were similar to those of unactivated controls.

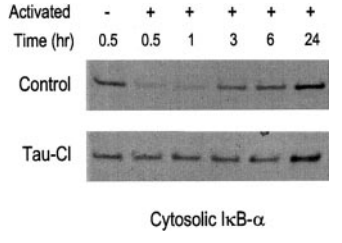

FIGURE 7. Tau-Cl inhibits degradation of IκB in the cytosolic fraction of NR8383 cells after various times of activation. Each lane was loaded with 10 μg of protein. Bands reacting with primary Ab specific for IκB-α (Santa Cruz Biotechnology) were visualized using ECL. Similar results were obtained in three additional independent experiments.

To further evaluate the effects of Tau-Cl on phosphorylation of IκB-α, cells were activated in the presence of various concentrations of Tau-Cl for 30 and for 60 min (Fig. 9). Preincubation of cells in the presence of 10 μM MG-132 greatly enhanced the p-IκB-α signal in activated cells relative to controls, with more p-IκB-α accumulating after 60 min of activation than after 30 min. Tau-Cl dose-dependently inhibited accumulation of p-IκB-α after 30 min of activation and inhibition persisted for up to 60 min. These results (Figs. 8 and 9) suggested that Tau-Cl attenuated NF-κB activation primarily by inhibiting the phosphorylation of IκB-α rather than by affecting ubiquitination or 26S proteasome activity.

Since IκB-α is phosphorylated by the IKK multiprotein complex, the effect of Tau-Cl on the status of intrinsic cellular IKK activation was determined (Fig. 10*A*). NR8383 cells were activated in the presence of various concentrations of Tau-Cl for 1 h. The IKK complex was immunoprecipitated from cell lysates and analyzed for IKK activity using GST-IκB-α as substrate. Blots used for autoradiograms were subsequently probed for IKK-β and IKK-α by Western blot (Fig. 10*A*). Cells activated in the presence of Tau-Cl had greatly reduced IKK activity as compared with that of activated control cells (*A, second lane*) even though the relative amounts of immunoprecipitated IKK-β and IKK-α were similar among the conditions used in this experiment. Immunoprecipitated IKK also contained IKK-γ in equivalent amounts across treatment conditions (M. R. Quinn, M Barua, and V. Serban, unpublished observations). Additional experiments were conducted to more stringently test the effect of Tau-Cl on IKK activity. IKK was immunoprecipitated from cell lysates of activated cells and Tau-Cl was added at the initiation of the assay for IKK activity (Fig. 10*B*). The activity of IKK was unimpaired by the presence of Tau-Cl. These results suggest that Tau-Cl exerts its effect by inhibiting upstream signaling pathways that activate IKK, rather than by directly inhibiting IKK activity.

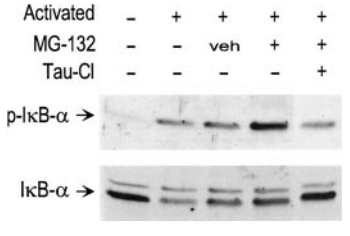

FIGURE 8. Effects of Tau-Cl on phosphorylation of IκB-α in NR8383 cells. Cells were exposed to medium alone, MG-132 (10 μg), or to vehicle (DMSO, 0.25% (v/v) final concentration) for 1 h (37°C, 5% CO_2) before addition of LPS + IFN-γ. Tau-Cl (1.0 mM) was added at the time of activation and cell lysates were prepared 1 h thereafter. Each lane was loaded with 20 μg of protein and analyzed by Western blotting. Bands reacting with primary Abs against phosphorylated IκB-α (*upper panel*) and IκB-α (*lower panel*) was visualized by ECL. Similar results were obtained in three additional independent experiments.

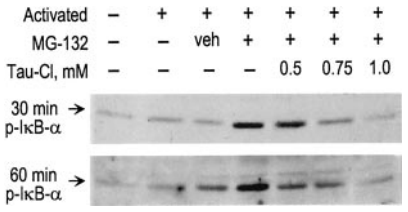

FIGURE 9. Tau-Cl inhibits phosphorylation of IκB-α in MG-132-treated NR8383 cells. Cells were exposed to MG-132 (10 μg) or vehicle (DMSO, 0.25% (v/v) final concentration) for 1 h (37°C, 5% CO_2) before addition of LPS + IFN-γ and various concentrations of Tau-Cl. Cell lysates were prepared 30 min and 60 min after activation and phosphorylated IκB-α was analyzed by Western blot. Bands were visualized by ECL. Similar results were obtained in three additional independent experiments.

Discussion

Tau-Cl, formed in the presence of taurine and neutrophil-associated halide-dependent myeloperoxidase, inhibits the production of proinflammatory mediators by activated cells derived from various sources (1, 12–17). It has been proposed that this action of Tau-Cl represents a major mechanism by which taurine protects against tissue damage in several animal model systems (1, 12–17, 33). Taurine protection against inflammatory lung damage has been well documented (2–7, 34, 35). In all of these models, production of inflammatory mediators by alveolar macrophages is thought to play an important role in the ensuing pathology. Since determining the effects of Tau-Cl on production of inflammatory mediators by alveolar macrophages is crucial to understanding the mechanism of action, we conducted studies using primary cultures of RAM and NR8383 cells. The cell line NR8383 retains many of the characteristics of RAM (36, 37) and has been useful for other molecular studies of alveolar macrophage function (38–40).

The present studies demonstrate that Tau-Cl dose-dependently inhibits production of NO and TNF-α and expression of iNOS protein in activated RAM. Tau-Cl inhibits production of these proinflammatory mediators primarily through depressing iNOS and TNF-α gene transcription as evidenced by decreased expression of iNOS and TNF-α mRNAs in activated NR8383 cells. Studies using other cell types also suggest that Tau-Cl inhibits expression of inflammatory genes (1, 12–17) and this appears to occur without affecting protein synthesis in general (1, 17). Although the effects of Tau-Cl on protein synthesis were not directly evaluated in the present study, Tau-Cl elicited increased production of inducible heat shock protein 70 in NR8383 cells 3–6 h after activation (M. R. Quinn, Y. Liu, and M. Barua, unpublished observations). This suggests that the present results cannot be accounted for by a general suppression of gene transcription or protein synthesis by Tau-Cl.

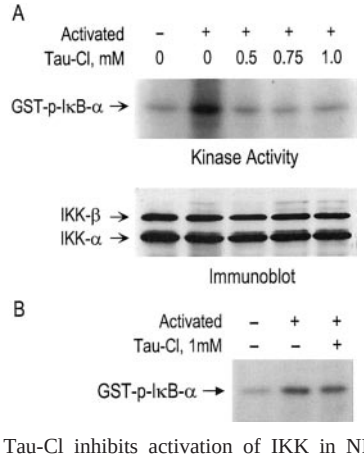

FIGURE 10. Tau-Cl inhibits activation of IKK in NR8383 cells. *A*, NR8383 cells were activated in the presence of various concentrations of Tau-Cl for 1 h (37°C, 5% CO_2) and IKK was immunoprecipitated from cell lysates using IKK-α Ab. Unactivated NR8383 cells served as an additional control. Activity of immunoprecipitated IKK was measured using GST-IκB-α as substrate and GST-p-IκB-α was visualized by autoradiography. Relative amounts of IKK-α and IKK-β in the precipitated complexes were determined by Western blots and were visualized by ECL. *B*, IKK was immunoprecipitated from activated NR8383 cells and IKK activity was measured in the absence or presence of 1 mM Tau-Cl added to the assay mixture. IKK activity was assessed using GST-IκB-α as described for *A*. Similar results were obtained in three additional independent experiments.

Further studies demonstrated that the transcriptional effects of Tau-Cl on genes regulating expression of iNOS and TNF-α result, in part, from decreased translocation of NF-κB into the nucleus of activated cells. Transcription of iNOS and TNF-α genes is critically dependent on the NF-κB transcription factor signaling pathway (41–43). Decreased p50/p65 nuclear binding in Tau-Cl-treated cells is of particular interest because NF-κB with this subunit composition potently *trans*-activates target genes, whereas the p50 homodimer is thought to suppress or to exert relatively low *trans* activation (18, 21). Inhibition of NF-κB activation was accounted for by stabilization of cytosolic IκB in cells activated in the presence of Tau-Cl. The release of cytosolic NF-κB from the NF-κB-IκB complex requires phosphorylation of IκB by the IKK multiprotein complex, followed by ubiquitination and degradation by the 26S proteasome (22–25). Our results suggest that the kinase activity of IKK was attenuated in cells that were activated in the presence of Tau-Cl through a mechanism that most likely involves upstream signaling pathways. This is substantiated by the lack of effects of Tau-Cl on IKK activity when Tau-Cl is directly added to the IKK assay mixture containing IKK immunoprecipitated from activated NR8383 cells. Although it is possible that Tau-Cl interferes with the regulatory interaction between NF-κB essential modifier (IKK-γ) and IKK-β (26, 44), it seems more likely that Tau-Cl interferes with one of the key upstream kinases in the signaling pathway, e.g., NF-κB-inducing kinase or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (23, 27), or some as yet to be identified kinase (45).

The present results are consistent with reports (1, 17) that Tau-Cl exerts its most potent inhibitory effects on production of proinflammatory mediators when present around (±2 h) the time of activation, but is relatively ineffective if added 16–14 h later. Recently, it was reported that taurine administration, in combination with niacin, protected mice from bleomycin-induced lung damage (5, 34, 35). The protective effects were associated with diminished NO production, accompanied by decreased iNOS mRNA and iNOS protein expression in extracts of whole lung. In addition, nuclear protein extracts prepared from whole lung exhibited decreased NF-κB binding (EMSA) accompanied by increased IκB-α in mice treated with taurine and niacin (35). Although these changes were not localized to a specific cell type, alveolar macrophages and neutrophils would be expected to be present and to contribute to the pathology observed in this model. It is intriguing to speculate that the mechanism of Tau-Cl action described in the present communication may explain, in part, the reported protective effects of taurine (34, 35). Although we have not evaluated the combined effects of taurine and niacin on any of the parameters measured in the present study, taurine alone has been demonstrated to be without effect (Refs. 1, 12, and 17; M. R. Quinn, unpublished observations).

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