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Inhibition of LPS-induced NO production by taurine chloramine in macrophages is mediated though Ras-ERK-NF-κB

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Abstract

Taurine is an abundant free amino acid in inflammatory cells that protects cells from inflammatory damages. Although the protection mechanism remains unclear, taurine chloramine (Tau-Cl) produced by the reaction between taurine and hypochlorous acid in neutrophils plays an important role. In this study, we investigated the mechanism(s) by which Tau-Cl inhibits LPS-induced NO production in macrophages. Tau-Cl inhibited LPS-induced iNOS expression and NO production in RAW 264.7 cells. LPS treatment elevated the level of active Ras-GTP, and Tau-Cl inhibited LPS-induced Ras activation. Tau-Cl also inhibited ERK1/2 activation in a dose-dependent manner in both RAW 264.7 cells and murine peritoneal macrophages, whereas it did not exert any effect on p38 MAPK activation. Furthermore, Tau-Cl inhibited NF-κB activation without affecting AP-1 activity. These results suggest that Tau-Cl suppresses LPS-induced NO production by inhibiting specific signaling pathways. Thus, Tau-Cl protects cells from inflammatory injury resulting from overproduction of NO in a signaling pathway-specific manner.

Keywords: Taurine chloramine; Nitric oxide; Ras; ERK1/2; NF-κB; AP-1

1. Introduction

Taurine is one of the most abundant free amino acids present in mammalian tissues and blood cells [1,2]. The intracellular concentration of taurine in mammalian tissue is 10–70 mM and 20–100 μM in human extracellular fluids [3], and this high concentration of taurine regulates many cellular functions, including cell volume, homeostasis, protein stabilization, reproduction, calcium mobilization, and immunity (review in [4,5]). In particular, taurine is claimed to protect cells from inflammatory injury by attenuating the toxicity of hypochlorous acid (HOCl/OCl^-) produced by the myeloperoxidase (MPO) system in neutrophils [6]. Taurine readily reacts with HOCl/OCl^- to form the more stable and less toxic taurine chloramine (Tau-Cl). Tau-Cl is charged, but can be transported into cells in a Na^+- and Cl-dependent manner [7,8]. Within the cell, Tau-Cl modulates production of many pro-inflammatory mediators, such as nitric oxide (NO), superoxide anions, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8, and prostaglandins [9–12]. Nonetheless, the underlying mechanism(s) by which Tau-Cl contributes to anti-inflammatory actions remains unclear.

Lipopolysaccharide (LPS) activates multiple signaling pathways in macrophages, and enhances the production of several inflammatory mediators, such as, NO, TNF-α, and interleukins. One of these LPS-stimulated pathways is the mitogen-activated protein kinase (MAPK) pathway, which includes extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase. The activation of ERK is regulated by a small GTPase $p21^{ras}$ (Ras), which is implicated in the regulation of multiple cellular functions, including proliferation, differentiation, and apoptosis [13]. Ras cycles between inactive GDP-bound and active

Abbreviations: Tau-Cl, taurine chloramine; MPO, myeloperoxidase; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; IL, interleukin; NF-κB, nuclear factor κB; AP-1, activator protein 1; LPS, lipopolysaccharide; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; EMSA, electrophoretic gel mobility shift assay; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; BCA, bicinchoninic acid; MTT, thiazolyl blue tetrazolium bromide; PI, propidium iodide

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GTP-bound states, and transfers signals from receptors and their associated kinases to downstream effectors. LPS initiates the signaling pathway by binding to the surface receptor CD14, and thus is transferred to the LPS receptor complex, Toll-like receptor (TLR)-4 and MD-2. Following LPS binding to TLR-4, the activation is transmitted to Ras, which is localized to the plasma membrane. Ras promotes plasma membrane localization of Raf-1, and Ras-GTP binds to Raf-1, leading to Raf-1 phosphorylation [14,15]. The active Raf-1 phosphorylates and activates MAP/ERK kinase (MEK)1/2, a protein threonine and tyrosine kinase that, subsequently, phosphorylates and activates ERK1/2 [16–18].

The activation of MAPK leads to activation of transcription factors, such as, nuclear factor (NF)-κB, activator protein 1 (AP-1), activating transcription factor 2, and cAMP-responsive element binding protein. Among these transcription factors, NF-κB and AP-1 are largely involved in expression of pro-inflammatory genes [19,20]. Recently, it has been shown that Tau-Cl inhibits activation of NF-κB by decreasing phosphorylation of IκBα serine 32 [21], or by promoting oxidation of IκBα at methionine 45 [22]. Because LPS is known to promote the DNA binding activity of NF-κB and AP-1 that leads to production of inducible nitric oxide synthase (iNOS) in macrophages, and because the activation of these transcription factors is under the control of p38 and ERK signaling pathways, we investigated the effect of Tau-Cl on LPS-induced activation of Ras, MAPK (ERK and p38), and regulation of NF-κB and AP-1 that lead to iNOS expression and NO production.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit polyclonal antibodies against ERK1/2, p38, phospho-ERK1/2, phospho-p38, c-jun and c-fos were purchased from New England Biolabs (Beverly, MA). Mouse monoclonal antibody against iNOS from Transduction Laboratories (Lexington, KY), and antibodies to p65 and p50 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Fetal bovine serum (FBS) was from HyClone (Logan, UT), and penicillin and streptomycin were from GibcoBRL (Grand Island, NY). Oligonucleotide primers were purchased from TaKaRa (Shuzo, Shiga, Japan) and Tib Molbiol (Eresburgstrabe, Berlin, Germany), and [γ-^{32}P]ATP (3000 Ci/mmol) was from NEN (Boston, MA). Tau-Cl was freshly synthesized on the day of use by adding equimolar amounts of NaOCl (Aldrich Chemical, Milwaukee, MI) to taurine. The authenticity of Tau-Cl formation was monitored by UV absorption (200–400 nm) [23]. Endotoxin-free or low endotoxin grade water and buffers were used.

2.2. RAW 264.7 cell culture and preparation of peritoneal macrophages

The murine macrophage cell line, RAW 264.7 cells (ATCC, Manassas, VA) were grown in DMEM supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin and 10% FBS at 37 °C in 5% CO_2.

C57BL/6J mice (8–16 weeks old) were obtained from Jackson Laboratories (Bar Harbor, MA) and injected with 1 ml of 3% thioglycolate. Murine peritoneal macrophages were obtained 96 h after thioglycolate injection by peritoneal lavage with cold HBSS (without Ca^{2+}, Mg^{2+} and phenol red), suspended in DMEM containing 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin. After allowing adherence overnight at 37 °C in 5% CO_2, nonadherent cells were removed by washing.

2.3. RT-PCR

Total RNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Reverse transcription was performed with 500 ng total RNA, according to the instructions provided by TaKaRa. PCR amplification of iNOS mRNA was carried out with the following primers, 5′-AGA CTG GAT TTG GCT GGT CCC TCC-3′ (forward) and 5′-AGA ACT GAG GGTACA TGC TGGAGC C-3′ (reverse). The resulting 527 bp product was resolved by electrophoresis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the loading control.

2.4. Western blot analysis

RAW 264.7 cells were treated with the appropriate stimulus for various durations, washed with ice-cold HBSS, and then harvested by gentle scraping. Cells were lysed in a lysis buffer containing 20 mM Tris–Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 μg/ml chymostatin, 2 mM PMSF, 10 μM leupeptin and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). Protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Lysates were mixed with 5× Laemmli sample buffer and subjected to 10% SDS-PAGE. Separated proteins were transferred onto nitrocellulose membrane (MSI, Westborough, MA), and non-specific binding was blocked with 6% non-fat milk dissolved in TBST buffer (10 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Blots were probed with iNOS antibody and developed using the ECL method (Amersham, Arlington Heights, IL).

2.5. Production of nitric oxide

The amount of nitrite, a stable end product of NO, present in the conditioned media was determined by Griess reaction. Briefly, the conditioned media (100 μl) from

RAW 264.7 cells stimulated with LPS (1 μg/ml) for 20 h were reacted with an equal volume of Griess for 10 min at room temperature. The absorbance was measured at 550 nm using a Power Wavex 340 ELISA reader (Bio-Tek instruments, Winoosk, VT). In some experiments, MEK inhibitor PD98059 and p38 inhibitor SB203580 were used.

2.6. MAPK activation

To examine the extent of LPS-derived MAPK activation, the phosphorylation of ERK and p38 was measured as previously described [24]. Briefly, RAW 264.7 cells and murine peritoneal macrophages (1×10^7) treated with taurine and Tau-Cl for 20 min prior to LPS stimulation were lysed in 200 μl of lysis buffer supplemented with 50 mM NaF and 2 mM Na_3VO_4 for 30 min at 4 °C. Cell lysates were clarified by centrifuging at 18,000 × *g* for 2 min at 4 °C. Samples (10 μg) were heated with 5× Laemmli sample buffer for 5 min at 95 °C, resolved on 10% SDS-PAGE, and then immunoblotted as described above. Phosphorylation was detected using phospho-specific ERK1/2 and p38 antibodies, and the total contents of ERK1/2 and p38 were detected using antibodies against ERK1/2 and p38.

2.7. Ras activation

To detect the effect of Tau-Cl on Ras activation, the affinity precipitation (or pull-down) assay was performed. Cells were serum-starved for 24 h, then stimulated with LPS for 2–15 min. In some experiments, cells were treated with 1.0 mM Tau-Cl for 20 min prior to LPS treatment. After washing with PBS, cells (1×10^7) were directly lysed in Mg^{2+} lysis/wash buffer (MLB) containing Complete™ protease inhibitor (Roche, Mannheim, Germany). Ras activation was subsequently determined using the Ras activation assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's protocol. Briefly, cell lysates were incubated with 10 μg of glutathione-agarose beads conjugated to a glutathione-*S*-transferase (GST) fusion protein of the Ras-binding domain of Raf-1 for 1 h at 4 °C. Beads were washed with MLB and active Ras proteins were resolved on a 12% SDS-PAGE, and probed with anti-Ras antibody.

2.8. Preparation of nuclear extracts

Nuclear extracts were prepared by cell lysis and then by nuclear lysis. Briefly, cells were suspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 μM leupeptin and 0.2 mM PMSF) and vortexed for 15 s. After incubating for 20 min at 4 °C, cell lysates were centrifuged at 10,000 × *g* for 6 min. The nuclear pellet was suspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 μM leupeptin and 0.2 mM PMSF) for 40 min on ice, and centrifuged at 10,000 × *g* for 20 min. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA).

2.9. Electrophoretic mobility shift assay (EMSA)

Protein–DNA binding activity for NF-κB and AP-1 was determined. Briefly, 10–20 μg of nuclear protein was incubated with 0.25 μg of poly(dI-dC) (Amersham) and ^{32}P-labeled (5000 cpm) DNA probe in binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 5% glycerol and 0.5 mM DTT) for 30 min at 30 °C. The protein–DNA complexes were analyzed on 5% native polyacrylamide gels. For the supershift experiment, antibodies were included in the above reaction mixture and incubated at 4 °C for 3 h before addition of the ^{32}P-labelled DNA probe. The oligonucleotide sequences used to detect the activities of NF-κB and AP-1 are as follows: NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (sense) and 5′-GCC TGG GAA AGT CCC CTC AAC T-3′ (antisense); and AP-1, 5′-CTA GTG ATG AGT CAG CCG GAT C-3′ (sense), 5′-GAT CCG GCT GAC TCA TCA CTA G-3′ (antisense).

2.10. MTT reduction assay and flow cytometric analysis

To determine whether the taurine and Tau-Cl concentrations (0–1.0 mM) used in the current experiments caused cell death or programmed cell death, we determined cell viability using the conventional MTT (thiazolyl blue tetrazolium bromide) reduction assay. Briefly, cells (1.5×10^5) were incubated in the presence or the absence of taurine or Tau-Cl for 20 h at 37 °C in 5% CO_2. Thereafter, 10 μl of MTT solution (5 mg/ml) was added to each well. After 2 h incubation at 37 °C, media were removed, and cells were dissolved in DMSO. The absorbances at 550 nm were measured using VersaMax microplate reader and associated Softmax software (Molecular Devices, Sunnyvale, CA). The apoptotic cells were measured using propidium iodide (PI) nuclear staining. Briefly, ethanol fixed cells were incubated with 50 μg/ml PI containing 50 μg/ml RNase A and 1% Triton X-100 for 10 min at room temperature in the dark, centrifuged, and washed three times with PBS. Cells were resuspended in PBS and run in a FACScan cytometer (BD Bioscience, San Jose, CA). The percentage of cells with low PI staining (sub-G1 phase) was determined by examining FL-2A histograms using CellQuest Pro software (BD Bioscience).

2.11. Statistical analysis

The two-tailed Student's *t*-test (paired) was performed using Microsoft Excel software (Redmond, WA). Data are

expressed as mean ± S.D., and a p-value < 0.05 was considered significant.

3. Results

3.1. Tau-Cl inhibited LPS-induced iNOS expression and NO production in RAW 264.7 cells

It was previously shown that Tau-Cl inhibits the LPS and interferon (IFN)-γ-dependent production of pro-inflammatory mediators in murine macrophages [9–11,21,25]. This inhibition of LPS/IFN-γ-induced NO production by Tau-Cl resulted from inhibition of iNOS transcription, translation, and enzyme activity [9,25]. In this study, we observed that LPS (1 μg/ml) stimulation enhanced expression of iNOS mRNA and protein, and NO production, respectively within 2, 5, and 10 h in RAW 264.7 cells, (Fig. 1A–C), yet a higher concentration of LPS (10 μg/ml) did not further increase NO production (data not shown). In support of previous results, Tau-Cl inhibited LPS-induced iNOS expression and NO production in a dose-dependent manner. However, taurine had no significant inhibitory effect on LPS-induced iNOS expression and NO production (Fig. 1D, E). These results are consistent with our previous report [11] that showed that Tau-Cl inhibited LPS and IFN-γ-induced NO production in murine peritoneal macrophages.

3.2. Tau-Cl inhibited phosphorylation of ERK but not p38 MAPK

As a possible mechanism by which Tau-Cl inhibits LPS-induced NO production, we hypothesized that Tau-Cl may interfere with the MAPK signaling pathway. To verify the role of MAPK in LPS-induced NO production, we examined the effect of the MEK inhibitor, PD98059 and the p38 inhibitor, SB203580. The LPS-induced NO production was inhibited by both PD98059 and SB203580 in a dose-dependent manner (Fig. 2A). This observation contrasts to the result seen by Chen and Wang [26] that showed that activation of p38 rather than ERK was implicated in LPS-stimulated NO production. Next, we examined the effect of Tau-Cl on LPS-induced phosphorylation of ERK and p38. Twenty minutes pretreatment of Tau-Cl prior to 15 min LPS stimulation caused marked inhibition of phosphorylation of ERK1/2 in both RAW 264.7 cells and murine peritoneal macrophages, without affecting phosphorylation of p38 (Fig. 2B, C). The inhibitory effect provided by Tau-Cl was compared to that of PD98059 and SB203580, two specific inhibitors of MEK1/2 and p38, respectively. 1.0 mM Tau-Cl selectively inhibited ERK1/2 phosphorylation to an extent similar to 10 μM PD98059 (Fig. 2D). SB203580 had no inhibitory effect on ERK1/2 phosphorylation. We also examined whether Tau-Cl alone would induce ERK activation in RAW 264.7 cells, because Midwinter et al. [27] suggested that Tau-Cl alone at 20–

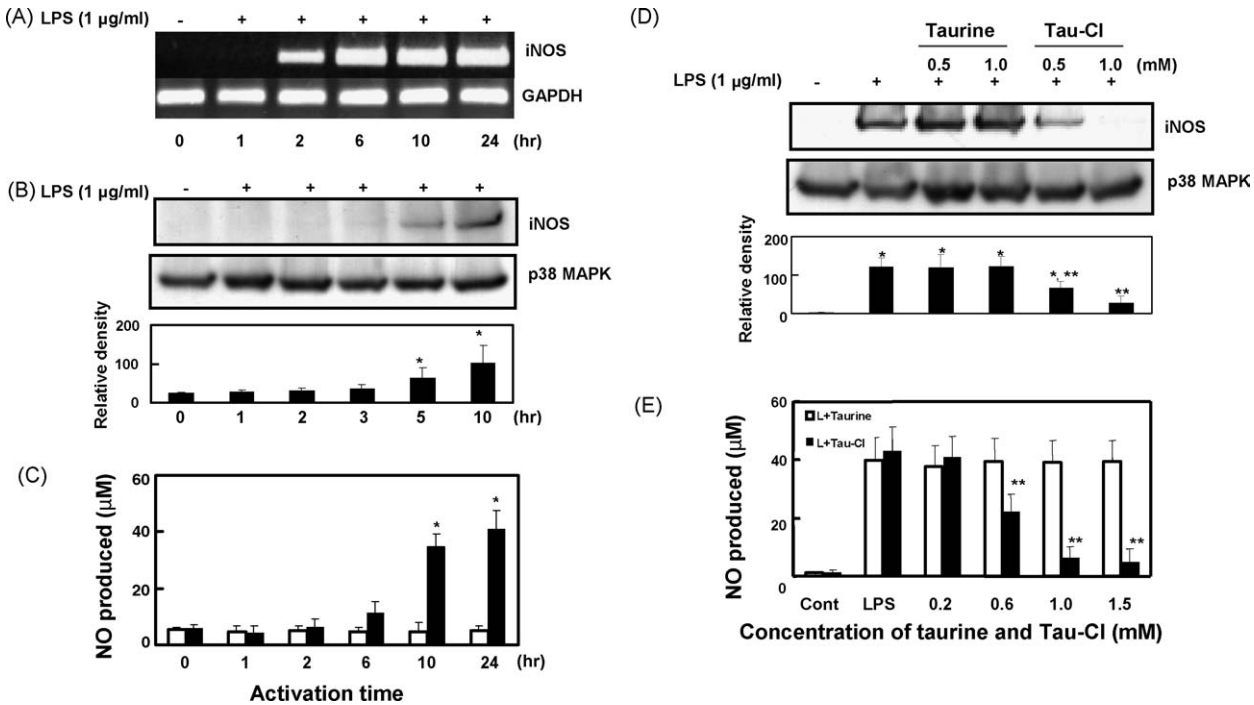

Fig. 1. Tau-Cl inhibits LPS-induced iNOS expression and NO production in RAW 264.7 cells. Bar graphs represent the densitometry of gel or blot from all experiments in each group, and error bars show mean ± S.D. (A) LPS-induced increase of iNOS mRNA was determined by RT-PCR ($n = 3$). (B) LPS-induced iNOS expression was measured using specific antibody for iNOS, and blot was reprobed with p38 MAPK antibody to show equal loading ($n = 3$, $^{*}p < 0.05$). (C) LPS-derived NO production was measured by Griess reaction ($n = 2$, $^{*}p < 0.05$). (D and E) Cells were treated with taurine or Tau-Cl in the presence of LPS, and iNOS expression (D, $n = 3$) and NO production was measured (E, $n = 4$). $^{*}p < 0.05$, control vs. LPS; $^{**}p < 0.01$, LPS vs. Tau-Cl.

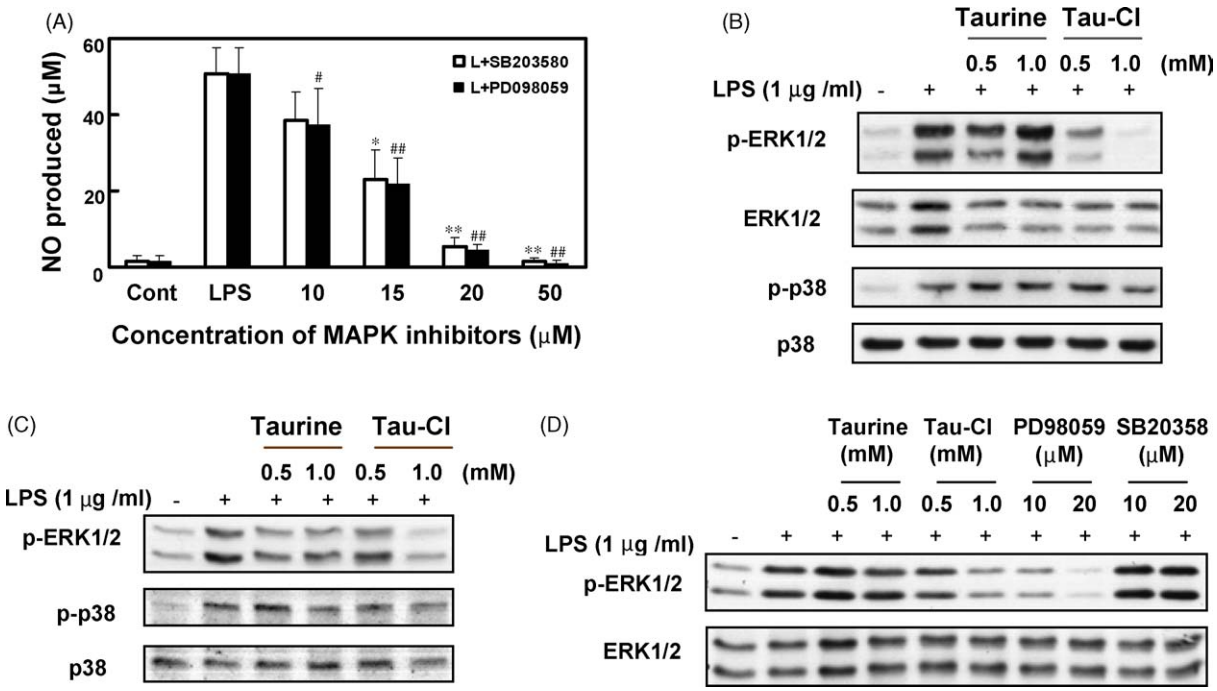

Fig. 2. Tau-Cl inhibits the activation of ERK1/2, but not p38. (A) The effect of MAPK inhibitors on LPS-induced NO production. Data are expressed as the mean ± S.D. ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$, LPS vs. SB203580; $^{\#}p < 0.01$ and $^{\#\#}p < 0.005$, LPS vs. PD98059. (B) RAW 264.7 cells (1×10^7) were pretreated with taurine and Tau-Cl for 20 min before stimulation with LPS (1 μg/ml) for 15 min. Cells were lysed and Western blotting was performed with the antibodies for phospho-ERK1/2 or phospho-p38, and reprobed with ERK1/2 or p38 antibody ($n = 5$). (C) The effect of taurine and Tau-Cl on ERK1/2 and p38 phosphorylation was determined in murine peritoneal macrophages ($n = 2$). (D) The effect of Tau-Cl on ERK and p38 phosphorylation was compared with MEK inhibitor, PD98059 and p38 inhibitor, SB203580 ($n = 3$).

100 μM ranges induces ERK activation in human vein endothelial cells. Under our experimental conditions, we did not detect any activation of ERK in RAW 264.7 cells treated with 10–1000 μM Tau-Cl alone (data not shown).

3.3. Ras activation was inhibited by Tau-Cl

To assess the mechanism by which Tau-Cl treatment suppresses LPS-induced ERK1/2 activation, we evaluated the influence of Tau-Cl on the small molecular weight G protein Ras, which is a known to be an upstream activator of ERK1/2. Cells were treated with LPS; the changes in active GTP-bound Ras in cellular lysates were determined by precipitating active Ras-GTP with a GST fusion of the Ras-binding domain of Raf-1 kinase. LPS potently activates Ras as early as 2 min after treatment, an effect that persists for at least 10 min (Fig. 3). This observation contrasts to the results by Büscher et al. [28] that showed that LPS did not induce formation of Ras-GTP complex in macrophage, BAC-1.2F5 cells. However, it has been reported by others [29,30] that BAC-1.2F5 cells show a deficient response to LPS, which may explain the differences between macrophage strains. As shown in Fig. 3, treatment of Tau-Cl reduced the LPS-induced Ras-GTP level, suggesting that Tau-Cl regulates LPS-induced ERK activation by interfering with Ras activation.

3.4. Activation of NF-κB but not AP-1was inhibited by Tau-Cl

Among several transcription factors that are phosphorylated by MAPK, NF-κB and AP-1 mediate expression of pro-inflammatory genes. Thus, we examined the effect of Tau-Cl on LPS-induced DNA binding activity of NF-κB and AP-1. The LPS-dependent increase of NF-κB activity was detected within 30 min, and increased up to 4 h (Fig. 4A), after which it remained elevated through 16 h (data not shown); the increase of AP-1 activity began at 30 min, peaked at 2 h and then decreased (Fig. 4B).

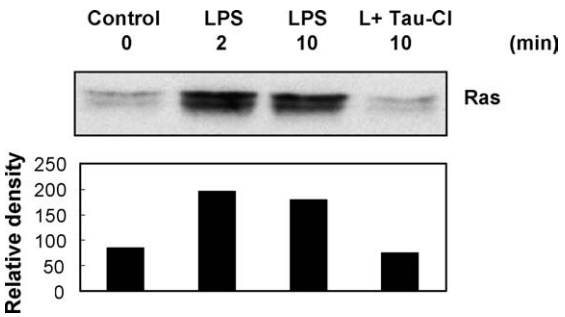

Fig. 3. Tau-Cl inhibits LPS-induced Ras activation in RAW 264.7 cells. The serum-starved cells were treated with 1.0 mM Tau-Cl for 20 min and stimulated with LPS (1 μg/ml). Cell lysates containing activated Ras were affinity precipitated and separated by SDS-PAGE, followed by immunoblotting with anti-Ras antibody. These data are representative of three independent experiments.

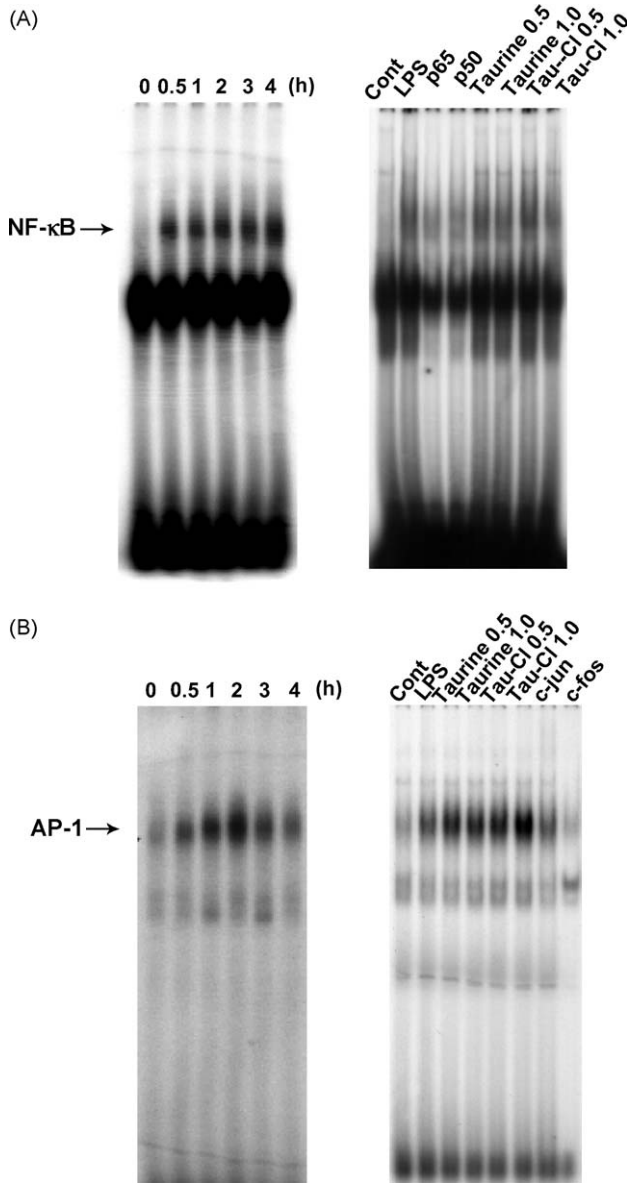

Fig. 4. Tau-Cl inhibits the activation of NF-κB, but not AP-1. Raw 264.7 cells were stimulated with LPS (1 μg/ml) for the indicated time points (left panels). To determine the DNA binding activity of NF-κB and AP-1, cells were treated with Tau-Cl for 4 and 2 h, respectively (right panels). Nuclear proteins (10–20 μg) were subjected to EMSA using the consensus binding sequence of NF-κB (A) or AP-1 (B). Supershift assay was performed using antibodies against p65 and p50 (A), or antibodies against c-fos and c-jun (B). Data are representative of at least three independent experiments.

Nuclear protein extracts obtained at 4 h from Tau-Cl-treated cells reduced LPS-induced NF-κB activity (Fig. 4A). However, the 2 h extracts from Tau-Cl-treated cells did not reduce AP-1 activity; instead, AP-1 activity was facilitated by Tau-Cl (Fig. 4B). This result contrasts to the result by Kontny et al. [31] that showed that Tau-Cl diminished IL-1β-stimulated DNA binding activity of NF-κB and AP-1 in fibroblast-like synoviocytes from rheumatoid arthritis patients. To assess the specificity of the NF-κB and AP-1, supershift experiments were carried out by adding antibodies against subunits of NF-κB or AP-1 to the nuclear extract before EMSA. Supershift EMSA analyses which were performed using nuclear extracts from LPS-stimulated cells incubated with antibodies to either p50 or p65 subunits, showed a decrease in the mobility of the DNA–protein complex of NF-κB (Fig. 4A); those which used nuclear extracts incubated with antibodies to either c-fos or c-jun subunits also showed retardation of the AP-1 band (Fig. 4B).

3.5. Cell viability and apoptosis in Tau-Cl-treated RAW 264.7 cells

Tau-Cl is a more stable and less toxic than $HOCl/OCl^-$, thus it has been suggested to be a detoxification mechanism for $HOCl/OCl^-$. However, cytotoxic effects of Tau-Cl at high concentrations have been reported [32,33]. The optimum Tau-Cl concentration is variable depending on cell type [9,11,22,34,35]. Therefore, we examined cell viability and apoptosis in Tau-Cl-treated RAW 264.7 cells. Although a high concentration of Tau-Cl has a cytotoxic effect, the concentrations used in this study (0.1–1.0 mM) did not cause non-specific cell death when measured by trypan blue exclusion (data not shown). Cell viability was also assessed by an MTT reduction assay. Although 1.0 mM Tau-Cl showed decreased MTT reduction, the amount was not significant (Fig. 5A). However, at higher concentrations (above 1.0 mM) cell death was significantly increased (data not shown). We chose time points 1, 2 (data not shown), 4 and 20 h, which correspond to those used for MAPK kinase activation (35 min), AP-1 activation (2 h), NF-κB activation (4 h), and NO release (20 h). Tau-Cl did not cause apoptosis when measured by FACScan after PI nuclear staining (Fig. 5B). In control cells, less than 1% cells were in sub-G1 phase in all tested time points, and reached only 1.2% even after 20 h Tau-Cl treatment ($n = 2$). This observation contrasts to the result observed by Klamt and Shacter [32] that showed that 0.5 mM Tau-Cl induced apoptosis in the Burkitt lymphoma cell line JLP-119, and also to the results reported by Emerson et al. that showed that concentrations higher than 1.0 mM Tau-Cl cause apoptosis in FL5.12 hematopoietic polymorphocytic B cells [33].

4. Discussion

Tau-Cl has been shown to inhibit production of pro-inflammatory mediators in macrophages [9–11,25,36]. Although Tau-Cl has been suggested to inhibit activation of NF-κB [21,22], its mechanism of action is not clear. In the present study, we studied the effect of Tau-Cl on LPS-induced activation of Ras and its downstream, MAPK (ERK and p38) that lead to activation of NF-κB and AP-1 resulting in NO production. Currently, it is controversial whether activation of ERK or p38 is necessary for LPS-induced NO production in macrophages. Some studies showed that activation of both ERK and p38 are

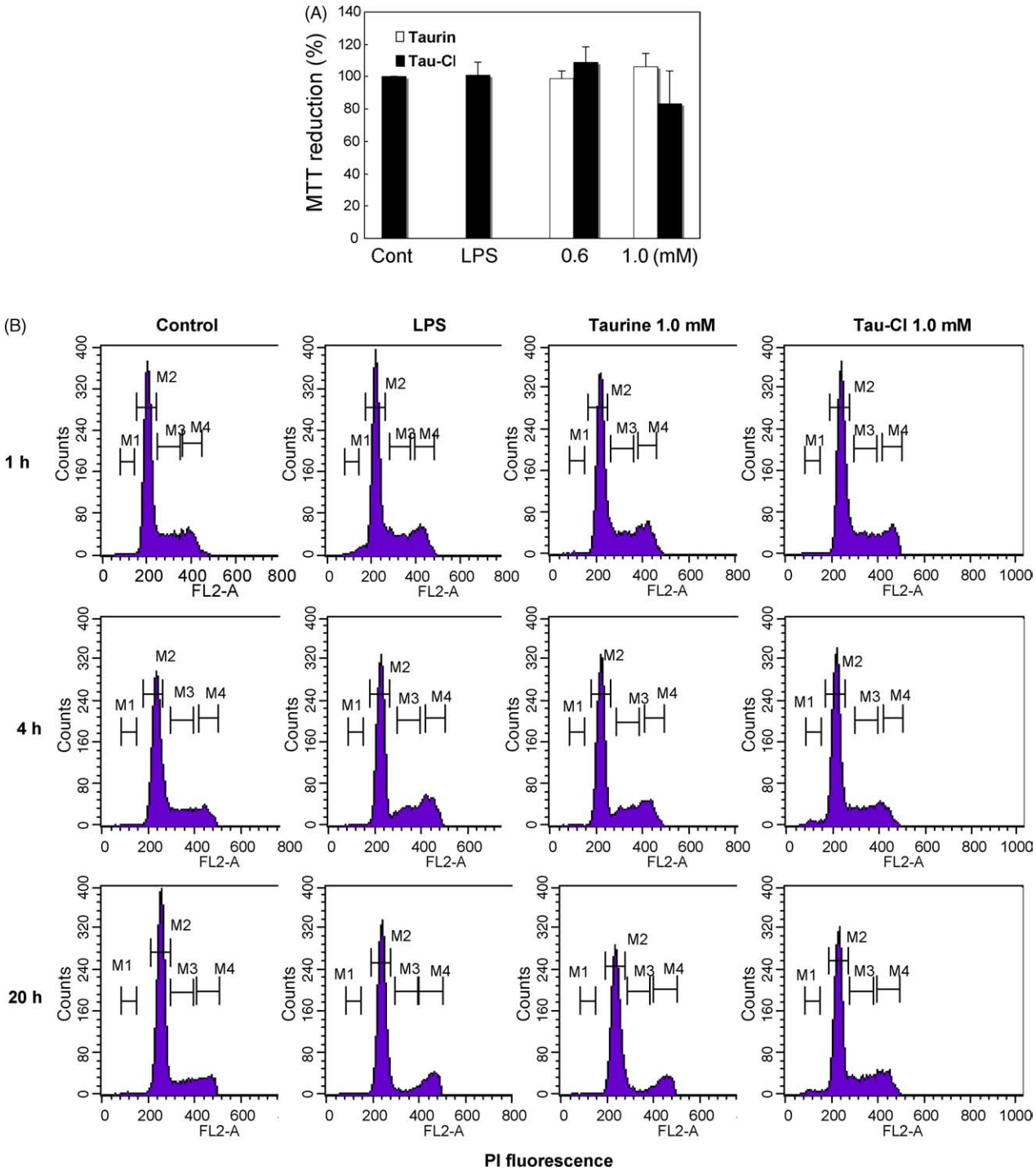

Fig. 5. Effect of Tau-Cl on cell viability and programed cell death. (A) RAW 264.7 cells were treated with 0.6 and 1.0 mM taurine or Tau-Cl, and then cell viability was determined using the MTT assay, mean ± S.D. ($n = 3$). (B) RAW 264.7 cells (1×10^6) were treated with taurine or Tau-Cl for 1, 4, and 20 h, fixed with 70% ethanol, and stained with PI for 10 min. Data show representative histograms of cell cycle analysis of two independent experiments.

involved in LPS/IFN-γ-induced NO production [37,38], but others reported that ERK activation is not required, and that only p38 activation is associated with LPS-induced NO production [39]. This controversy suggests that macrophages may utilize different MAPK signaling pathways for LPS-inducible NO production and that the involved MAPK signaling pathways are affected differently by anti-inflammatory compounds. Recently, it has been shown that Tau-Cl had no effect on ERK activation in Jurkat cells [40], suggesting that Tau-Cl has cell specificity. The present study demonstrated that Tau-Cl inhibited LPS-induced iNOS expression and NO production through selective inhibition of ERK1/2 but not p38 activation in macrophages. ERK is a downstream of Ras-mediated signaling in response to several stimuli in various cell types. Tau-Cl inhibited LPS-induced Ras activation in RAW 264.7cells.

Therefore, the present results suggest that Tau-Cl inhibits the specific signaling pathway, Ras-dependent ERK activation, to suppress LPS-induced NO production in macrophages.

LPS-induced stimulation of NF-κB DNA binding activity is believed to be the major mechanism involved in NO production in immune cells. LPS is known to induce the MAPK-dependent phosphorylation and degradation of IκBα, thereby activating NF-κB to translocate to the nucleus facilitating NF-κB DNA binding activity, and leading to upregulation of iNOS expression [41]. Along with NF-κB activation, AP-1 activation is important for expression of the pro-inflammatory mediator gene, and activation of AP-1 is also regulated by MAPK. Recently, it was reported that Tau-Cl inhibited NF-κB activation in various cell types, including macrophages, Jurkat cells, and synoviocytes [21,22,40]. In the present study, we observed that the DNA binding activity of NF-κB and AP-1 was increased by LPS treatment and that Tau-Cl inhibited NF-κB DNA binding activity, while Tau-Cl did not inhibit AP-1 activation (Fig. 4). These observations confirm that Tau-Cl inhibits ERK1/2 selectively in the MAPK signaling pathways to specifically suppress LPS-induced activation of NF-κB.

The physiological concentrations for different cell types have not been thoroughly scrutinized. Moreover, the optimal inhibitory concentrations which appears in the literature depend on cell types used [9,11,22,31]. We did not detect any sign of apoptosis in the range of concentrations from 0 to 1.0 mM used in our experiments. Macrophages and neutrophils may tolerate higher concentrations of Tau-Cl. This may occur because macrophages and neutrophils are prone to oxidative stress, and therefore able to resist to higher oxidative potential compared to other cells. Recently, it was reported that Burkitt lymphoma cells became apoptotic when they are treated with higher than 500 μM Tau-Cl. Although apoptosis was not apparent in the cells used in our study, further investigation should be warranted to see if the cytotoxic effects of Tau-Cl are associated with change of mitochondrial membrane potential, capase-3 and -9 activation, and induction of apoptosis-related genes.

In summary, we show that Tau-Cl inhibits LPS-induced Ras activation, ERK activation, and NF-κB activation, but not p38 and AP-1 in RAW 264.7 cells. These data suggest that Tau-Cl regulates specific signaling pathways selectively in pro-inflammatory response to LPS.

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