TAURINE CHLORAMINE INHIBITS THE PRODUCTION OF NITRIC OXIDE AND SUPEROXIDE ANION BY MODULATING SPECIFIC MITOGEN-ACTIVATED PROTEIN KINASES

Chaekyun Kim*, Hyung Sim Choi, and Jun Woo Kim

1. INTRODUCTION

Taurine is an abundant free amino acid in inflammatory cells (Fukuda and Usui, 1983; Vinton *et al*., 1986), and it has been claimed to protect cells from inflammatory injury. Taurine readily reacts with hypochlorous acid ($HOCl/OCl^-$) produced by the myeloperoxidase system in neutrophils (Thomas *et al*., 1985) and forms more stable and less toxic taurine chloramine (Tau-Cl). Tau-Cl protects inflammatory cells by attenuating the toxicity of $HOCl/OCl^-$. Tau-Cl also protects cells by regulating the production of many pro-inflammatory mediators, such as nitric oxide (NO), superoxide anion (O_2^-), tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and prostaglandin (Park *et al*., 1995; Kim *et al*., 1996; Marcinkiewicz *et al*., 1998).

Mitogen-activated protein kinases (MAPK), which include extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase, regulate many cellular functions including production of pro-inflammatory mediators. In particular, MAPK regulate signaling pathways involved in NO and O_2^- production (Chan and Riches, 2001; Kim and Dinauer, 2001). It is not much known about the role of taurine on MAPK activation. Midwinter *et al*., (2004) reported that Tau-Cl alone induced ERK activation in human vein endothelial cells. However, Tau-Cl showed no effect on ERK activation in Jurkat cells (Kontny *et al*., 2003). In addition, the activation of MAPK leads to activation of transcription factors (Angel and Karin, 1991; Baeuerle and Baichwal, 1997) which are strongly involved in the expression of pro-inflammatory genes such as nuclear transcription factor (NF-κB) and activator protein 1 (AP-1). Recently, it has been shown that Tau-Cl inhibits the activation of NF-κB in inflammatory cells (Barua *et al*., 2001; Kanayama *et al*., 2002).

* Chaekyun Kim, Center for Advanced Medical Education by BK21 Project, Inha University University College of Medicine, Incheon, 400-712, Korea.

In this study, we investigated the effect of Tau-Cl on the production of NO and O_2^-, and the activation of MAPK (ERK and p38) that lead to NO and O_2^- production. In consistent with previous results, Tau-Cl inhibited NO and O_2^- production in a dose-related manner. Tau-Cl inhibited LPS-stimulated phosphorylation of ERK1/2 in RAW 264.7 cells, while it had no effect on p38. However, Tau-Cl inhibited PMA-elicited phosphorylation of p38 in PLB 985 cells but not ERKs. These results suggest that Tau-Cl regulates pro-inflammatory mediators in a stimulus- and signaling-pathway-specific manner.

2. MATERIALS AND METHODS

2.1. Antibodies and Reagents

Rabbit polyclonal antibodies against ERK1/2, phospho-ERK1/2, and phospho-p38 were purchased from New England Biolabs (Beverly, MA). Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Fetal bovine serum (FBS) was from HyClone (Logan, UT), and penicillin, streptomycin, and HBSS were from GibcoBRL (Grand Island, NY). 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) (Thomas *et al*., 1986). Endotoxin-free or low endotoxin grade water and buffers were used.

2.2. Cell Culture and Murine Peritoneal Neutrophils

Murine macrophage cell line, RAW 264.7 cells (ATCC, Manassas, VA) were grown in DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% FBS at 37ºC in 5% CO_2. The human myeloid cell line PLB-985 was a gift from M. Dinauer (Indiana University). PLB-985 cells were grown in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37ºC in 5% CO_2. To induce granulocytic differentiation, PLB-985 cells were exposed to 0.5% *N,N*-dimethylformamide for 5 days.

To elicit peritoneal neutrophils, C57BL6/J mice (Jackson Lab, Bar Harbor, MA) were injected intraperitoneally with 1 ml of 3% thioglycollate. Peritoneal cells were harvested with HBSS after 18 h as previously described (Kim *et al*., 1996).

2.3. Measurement of Nitric Oxide

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).

2.4. Cytochrome c Reduction Assay

Extracellular O_2^- production was measured based on superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c (Kim and Dinauer, 2001). Briefly, 2.5 x 10^5 cells were suspended in 250 μl PBSG (PBS with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose) containing 75 μM ferricytochrome c and activated by the addition of 200 ng/ml PMA. 180 units/ml SOD was added to parallel samples to measure the SOD-inhibitable values. After incubation for 30 min at 37ºC, the absorbance at 550 nm with a 490 nm reference filter was measured on a Power Wavex 340 ELISA reader.

2.5. MAPK Activation and Western Blotting

To examine the extent of the phosphorylation of MAPK, cells (1 x 10^7) treated with taurine or Tau-Cl were lysed in 200 μl of 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)benzenesulfonylfluoride, 50 mM NaF and 2 mM Na_3VO_4 for 30 min at 4ºC. Cell lysates were clarified by centrifuging at 18000 x g for 2 min at 4ºC. Protein was quantified using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Thereafter, samples (10 μg) were heated with 5X Laemmli sample buffer for 5 min at 95ºC and then resolved on 10% SDS-PAGE. Separated proteins were transferred onto nitrocellulose membrane (MSI, Westborough, MA), and the non-specific bindings were blocked with 6% non-fat milk dissolved in TBST buffer (10 mM Tri-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Phosphorylation was detected using phospho-specific ERK1/2 and p38 antibodies and the total contents of ERK1/2 were detected using antibodies against ERK1/2.

2.6. Statistical Analysis

The two-tailed Student's t-test (paired) was performed using Microsoft Excel software (Redmond, WA). Data are expressed as mean ± SD and a p value < 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. Tau-Cl Inhibited LPS-Induced NO Production in RAW 264.7 Cells

It has been previously shown that Tau-Cl inhibits the LPS and interferon (IFN)-γ-dependent production of pro-inflammatory mediators in murine macrophages. In particular, the inhibitory effect of Tau-Cl on NO production was intensively studied, and possible mechanisms were suggested (Park *et al*., 1993, 1995; Marcinkiewicz *et al*., 1995; Barua *et al*., 2001). In this study, LPS (1 μg/ml) enhanced the NO production in RAW 264.7 cells, and LPS-induced NO production was inhibited by Tau-Cl in a dose-dependent manner (Fig. 1A). However, taurine did not show significant inhibitory effect. The concentrations used in this study (0.5 and 1 mM) did not cause significant non-specific cell death when measured by trypan blue exclusion or MTT (thiazolyl blue tetrazolium bromide) assay (Fig. 1B).

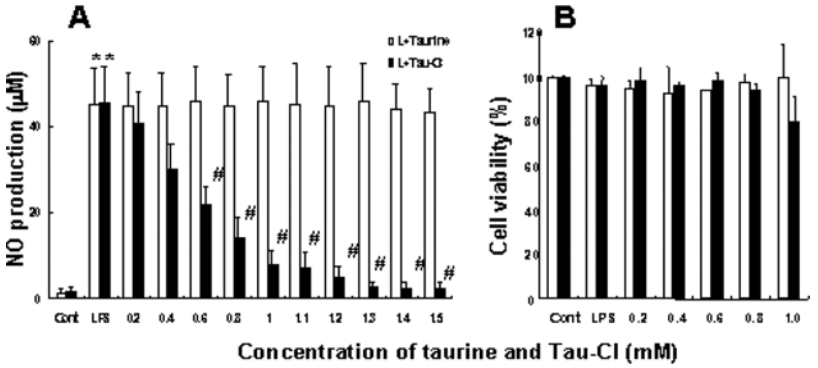

Figure 1. Tau-Cl inhibits LPS-induced NO production in macrophages. *A.* RAW 264.7 cells were treated with taurine or Tau-Cl in the presence of LPS, NO production was measured by Griess reaction (n=4). Data were expressed as mean values ± SD, * $p<0.05$, control *vs.* LPS, # $p<0.05$, LPS *vs.* Tau-Cl. *B.* Cell viability after treatment of taurine and Tau-Cl was measured by MTT assay (n=5).

3.2. Tau-Cl Inhibited PMA-Elicited O_2^- Production in PLB-985 Cells

We previously reported that that Tau-Cl inhibits the PMA-elicited O_2^- production in murine peritoneal neutrophils (Kim *et al*., 1996). In this study, we examined the effect of Tau-Cl on O_2^- production in PLB-985 cells which differentiated to granulocytes. Tau-Cl inhibited PMA-elicited O_2^- production, while taurine did not have any significant inhibitory effect (Fig. 2A). This result does not agree with our previous result which showed that taurine inhibited O_2^- production in murine peritoneal neutrophils (Kim *et al*., 1996). Thus, we measured O_2^- production using murine peritoneal neutophils. In murine neutophils, Tau-Cl inhibited O_2^- production similarly as in PLB-985 granulocytes, while taurine slightly inhibited O_2^- production (Fig. 2B). These combined results suggest that

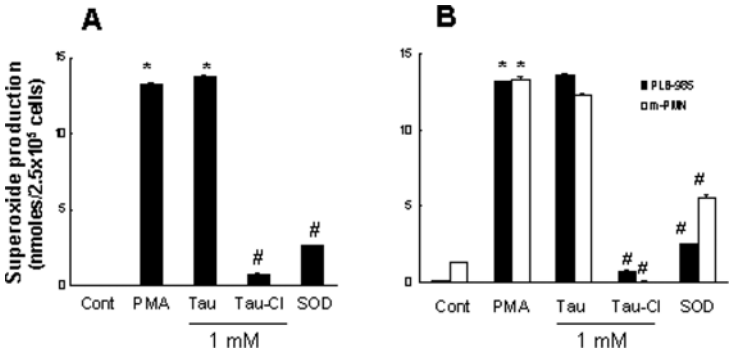

Figure 2. Tau-Cl inhibits PMA-elicited superoxide anion production in neutrophils. (A) Superoxide production following the stimulation with 200 ng/ml PMA in PLB-985 granulocytes was monitored by the reduction of cytochrome c (n=5). (B) Comparison of superoxide production between PLB-985 granulocytes and murine peritoneal neutrophils (n=5). Data were expressed as mean values ± SD, * $p<0.05$, control *vs.* PMA, # $p<0.05$, PMA *vs.* Tau-Cl or SOD.

the inhibition of NO and O_2^- production by Tau-Cl protects cells from inflammatory injury caused by overproduction of reactive nitrogen/oxygen species.

3.3. Tau-Cl Selectively Inhibited MAPK Activation

Although it is controversial, the involvement of ERK and p38 in LPS-stimulated NO production or PMA-stimulated O_2^- production has been reported (Bhat *et al*., 1998; Ajizian *et al*., 1999; Karlsson *et al*., 2000; Kim and Dinauer, 2001; Watters *et al*., 2002). As a possible mechanism which Tau-Cl inhibits NO and O_2^- production, we hypothesized that Tau-Cl may interfere with MAPK signaling pathway. We examined the effect of Tau-Cl on either LPS- or PMA-induced phosphorylation of ERK and p38. Tau-Cl inhibited LPS-stimulated phosphorylation of ERK1/2 in RAW 264.7 cells without affecting the phosphorylation of p38 (Fig. 3A). However, Tau-Cl did not inhibit PMA-stimulated phosphorylation of ERK in PLB-985 cells (Fig. 3B).

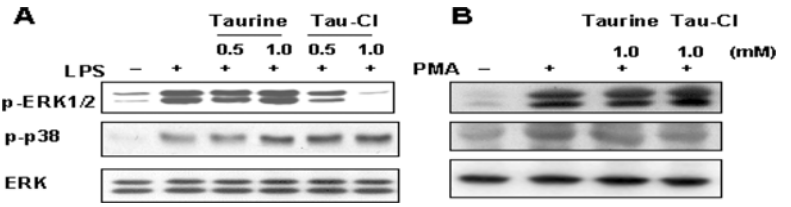

Figure 3. Tau-Cl inhibits the activation of specific MAPK. Cells (1 x 10^7) were pretreated with taurine and Tau-Cl for 20 min before stimulation with LPS (1 μg/ml) for 15 min. Cells were lysed and western blotting was performed with the antibodies for phospho-ERK1/2 or phospho-p38, and same blot was reprobed with ERK1/2 antibody (n=5).

In summary, we show that Tau-Cl inhibits LPS-induced NO production by inhibiting ERK phosphorylation and PMA-stimulated O_2^- production by inhibiting p38 phosphorylation, suggesting stimulus-specific regulation of reactive nitrogen/oxygen species by Tau-Cl.

4. ACKNOWLEDGMENTS

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