藥學碩士學位論文

Taurine Chloramine Stimulates Efferocytosis through Upregulation of Heme Oxygenase-1 Expression and Production of Carbon Monoxide

Heme Oxygenase-1 (HO-1) 의 발현과 Carbon Monoxide (CO) 의 생산을 통한 Taurine Chloramine 의 Efferocytosis 촉진 효과 기전

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ABSTRACT

Taurine Chloramine Stimulates Efferocytosis through Upregulation of Heme Oxygenase-1 Expression and Production of Carbon Monoxide

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During inflammation, taurine chloramine (TauCl) is produced abundantly in activated neutrophils and released to surrounding inflammatory milieu as the activated neutrophils undergo apoptosis. TauCl injected *in vivo* into the peritoneum of mice undergoing zymosan A-induced peritonitis was found to markedly decrease the number of peritoneal leukocytes and yet increase the blood monocyte infiltration, enhancing resolution of inflammation. Furthermore, when the macrophages obtained from peritoneal exudates were treated with TauCl *ex vivo*, their ability to phagocytose the apoptotic neutrophils obtained from the same peritoneal exudates

was increased. In the murine macrophage-like RAW264.7 cells treated with TauCl, efferocytic activity to phagocytose the apoptotic Jurkat T cells was also enhanced. In these macrophages treated with TauCl, expression of heme oxygenase-1 (HO-1) was increased along with increased nuclear translocation of the nuclear factor E2-related factor 2 (Nrf2). Furthermore, transcriptional expression of scavenger receptors recognizing the phosphatidylserines exposed on the surface of apoptotic cells were increased in these RAW264.7 cells treated with TauCl. Knock-down of HO-1 gene in RAW264.7 cells abolished the TauCl induced activation of efferocytosis, whereas both the overexpression of HO-1 and treatment with carbon monoxide (CO), the product of heme oxygenase, obtained from CO-releasing molecule (CORM) added exogenously increased the efferocytic ability of macrophages. When the macrophages not treated with TauCl were exposed to CORM, transcriptional expression of scavenger receptors was increased. Taken together, our results suggest that TauCl might facilitate resolution of inflammation by increasing the efferocytic activity of macrophages through Nrf2-mediated upregulation of HO-1 expression and overproduction of CO.

Keyword : TauCl, Resolution of inflammation, Effeocytosis, Heme oxygenase-1, Nrf2, Carbon monoxide, Scavenger receptor

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INTRODUCTION

Acute inflammation is an active host response to invading infectious pathogens and physical injuries. During inflammation, neutrophils recruited to the inflamed site are activated by phagocytosis of infectious pathogens and undergo oxidative burst. This leads to overproduction of reactive oxygen species (ROS) with which the neutrophils kill and eliminate the infectious pathogens. Subsequently, the activated neutrophils undergo apoptosis. These apoptotic neutrophils are removed by the macrophages which are recruited to the inflamed site immediately following the neutrophil infiltration. Upon phagocytic removal of apoptotic neutrophils by macrophages through a process called efferocytosis, the inflammation is resolved (18,19,25). If the apoptotic neutrophils with exposed phosphatidylserine on their surface are not cleared, this may cause chronic inflammation. Chronic inflammation has emerged as a critical component in many prevalent human diseases, such as arthritis and cancer (10). The process of resolving inflammation is an active process controlled in part by the chemical mediators that are formed endogenously during inflammation. These molecules act as local autacoids which stimulate the pro-resolving mechanisms of macrophages.

Taurine, a sulfur containing amino acid produced by decarboxylation of cysteine, is one of the most abundant free amino acids (not incorporated into proteins) stored in the cytoplasm of cells. It plays an important role in several essential biological processes, such as osmoregulation, membrane stabilization, calcium mobilization

and immunity (11). The concentration of taurine is particularly high in phagocytic cells like neutrophils that undergo oxidative burst, and hence have to cope with inflammatory oxidative stress. The stored taurine reacts stoichiometrically with hypochlorous acid (HOCl), a highly toxic antibacterial oxidant produced from hydrogen peroxide (H_2O_2) by the myeloperoxidase (MPO) in the activated neutrophils. This results in the generation of taurine chloramine (TauCl), a weak oxidant with mild toxicity. As the activated neutrophils undergo apoptosis, TauCl is then released and acts as a local autacoid at the inflamed tissues. While TauCl is known to have a direct antibacterial effect (20), it has both anti-inflammatory and antioxidant activities in the inflamed tissue. TauCl released from the activated and apoptotic neutrophils has been shown to inhibit the activation of Nuclear factor NF-kappa-B (NF-κB) in macrophages and to abolish the production of inflammatory mediators such as nitric oxide (NO), tumor necrosis factor (TNF-α), interleukin (IL)-6 and IL-8, and matrix metalloproteases (MMPs), thereby exerting the anti-inflammatory effect (14). Alternatively, TauCl has also been shown to activate nuclear factor E2-related factor 2 (Nrf2) and stimulate the transcriptional induction of several antioxidant enzymes and other cytoprotective proteins (12,13).

Nrf2 is a critical transcription factor involved in the cellular defense against cytotoxicity of oxidative stress. Under normal conditions, Nrf2 is kept in the cytoplasm as an inactive complex with Kelch-like ECH association protein 1 (Keap1) and undergoes constant proteasomal degradation. On the other hand, when cells are challenged with oxidative or electrophilic insults that oxidize or covalently modify critical cysteine residues in Keap1, Nrf2 dissociates from Keap1 and

translocates into the nucleus. Once in the nucleus, Nrf2 binds to the antioxidant response element (ARE) present in the promoter region of several defensive Among several antioxidant enzymes that are induced by the mild oxidant TauCl, heme oxygenase-1 (HO-1) draws particular attention. HO-1 is the rate-limiting enzyme catalyzing oxidative degradation of un-incorporated free-heme to produce carbon monoxide (CO), biliverdin/bilirubin and free iron (27). CO generated as a consequence of induction of HO-1 activity can act as a potent anti-inflammatory gas signal molecule that inhibits the expression of several pro-inflammatory cytokines and mediators and also exerts cytoprotective effects against oxidative stress (3,24). Recent findings demonstrate that the CO produced from upregulated HO-1 or provided exogenously at 250 ppm promotes resolution of inflammation (26,29). However, the molecular mechanisms underlying the pro-resolving effect of CO have yet to be established.

Efferocytosis or engulfment of apoptotic neutrophils by macrophages is essential for the resolution of inflammation. Apoptotic cells have phosphatidylserine exposed on their surface that serve as the ‘eat-me’ signal recognized by several scavenger receptors like BAI-1, Tim4, CD36 and others expressed on the surface of macrophages that initiate efferocytosis (6,9). Resident tissue macrophages first detect the signals of pathogens and tissue injuries and then release cytokines and mediators for leukocyte recruitment, first, blood polymorphonucleocytes (neutrophils) and then, blood monocytes which later differentiate into macrophages. Eventually, local autacoids and mediators in the inflammatory milieu alter genetic programming in the infiltrated macrophages and modulate endocytic functions that

promote efferocytosis. In the present study, we sought to investigate how TauCl could enhance efferocytic activity of macrophages undertaking the clearance of apoptotic neutrophils.

MATERIALS AND METHODS

Materials

TauCl was synthesized freshly on the day of use by adding equimolar amounts of NaOCl (Sigma-Aldrich, St. Louis, MO, USA) to taurine (Sigma-Aldrich). The authenticity of TauCl formation was monitored by UV absorption (200-400 nm). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 and fetal bovine serum (FBS) were obtained from GIBCO RBL (Grand Island, NY, USA). Carbon monoxide releasing molecules (CORM-2) was purchased from Sigma-Aldrich (Milwaukee, MI), and Dithiothreitol (DTT) and Hemoglobin (Hb) and anti-actin were purchased from Sigma-Aldrich. Primary antibodies against Nrf2 and lamin B1, small interfering RNAs (siRNAs) against Nrf2 and HO-1 and zinc protoporphyrin (ZnPP) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-HO-1 was the product of stressgen (Ann, Arbor, MI, USA), and anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were provided by Zymed Laboratories Inc. (San Francisco, CA, USA). Polyvinylidene difluoride (PVDF) membranes were supplied from Gelman Laboratory (Ann, Arbor, MI, USA). Enhanced chemiluminescent (ECL) detection kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Zymosan A-induced peritonitis

Institute of Cancer Research (ICR) mice (4 weeks of age) were purchased from Central Lab Animal Inc. (Seoul, South Korea). All the animals were maintained

according to the Institutional Animal Care Guidelines. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee at Seoul National University. Zymosan A (30 mg/kg) was administered intraperitoneally 12 h before giving PBS or TauCl (2 or 10 mg/kg, intraperitoneally), and mice were sacrificed 6 hours later. Peritoneal leukocytes were harvested by washing with 3 ml of phosphate-buffered saline (PBS) containing 3 mM ethylenediaminetetraacetate (EDTA).

Total and differential leukocyte counts

Total peritoneal leukocyte counts were carried out using Turk's solution (0.01% Crystal Violet in 3% acetic acid). For the differential count, peritoneal exudates were spun in a cytocentrifuge at 400 *g* for 5 min onto a slide and stained with Wright-Giemsa stain.

Cell culture

Murine macrophage RAW264.7 and human lymphoblastic Jurkat T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RAW264.7 cells and Jurkat T cells were cultured in DMEM and RPMI 1640, respectively, with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were maintained at 37 ℃ in a humidified atmosphere of 5% CO_2 and 95% air.

Efferocytosis assay

To assess the percentage of macrophages engulfing apoptotic PMNs *ex vitro*,

mouse peritoneal macrophages were incubated in six-well flat-bottomed microtiter plates for 24 h. Non-adherent cells were collected and incubated for additional 24 h to induce apoptosis. After washing with medium, adherent monolayer cells were co-incubated for 1 h with apoptotic non-adherent cells. Peritoneal macrophages were stained with the FluoroTag fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80 antibody (eBioscience, San Diego, CA, USA) for 20 min. The labeled cells were permeabilized for 10 min using 0.1% Triton X-100, and were incubated with phycoerythin (PE)-conjugated anti-mouse Gr-1 (Ly-6G) antibody (eBioscience) for 20 min. Macrophages containing neutrophils ($F4/80^{+}/Gr\text{-}1^{+}$) was detected using a confocal microscope (Nikon, Tokyo, Japan). To determine the efferocytic activity of macrophages *in vitro*, RAW264.7 cells were co-incubated for 1 h with apoptotic Jurkat T cells (stained with FITC-conjugated annexin V). To remove the non-engulfed apoptotic Jurkat T cells, RAW264.7 cells were washed three times with PBS and the proportion of RAW264.7 cells containing apoptotic Jurkat T cells (FITC-positive cells) was assessed by flow cytometry. Apoptosis of Jurkat T cells was induced by serum withdrawal and UVB (180 mJ/cm^2) irradiation, followed by incubation for 8 hours at 37 ℃ in an atmosphere of 5% CO_2.

Flow cytometry

Cells were fixed with 10% neutral-buffered formation solution for 30 min at room temperature, permeabilized with 0.2 % Triton X-100 for 5 min, and blocked with 2 % BSA in PBS for 30 min. Anti-HO-1 antibodies, diluted 1:100 in 2 % BSA in PBS, were applied overnight at 4 ℃. After washing with PBS. cells were incubated with

FITC-conjugated anti-rabbit IgG secondary antibody diluted at 1:1000 for 1 h. Cells were analyzed using FACSCalibur™ Flow Cytometer (BD, Franklin Lakes, NJ, USA).

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was isolated from RAW264.7 cells using TRizol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. To generate cDNA, 1 μg of total RNA was reverse transcribed by using murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). One microliter of cDNA was amplified in sequential reactions by using Maxime PCR PreMix Kit (iNtRON Biotechnology, South Korea). The primer pairs were as follows (forward and reverse, respectively): *Nrf2*, 5'-CTTAGAGGCTCATCTCACAC-3' and 5'-CTTTTGGGAACAAGGAACAC-3'; *HO-1*, 5'-GTCTATGCCCCACTCTATT-3' and 5'-TGGAAACGGATATCAAACGTG-3'; *BAI-1*, 5'-CAGAGCGGTCCGTTATCCTC-3' and 5'-TCTACCACACGGCACTTCAC-3'; *MerTK* 5'-AAGTGGGAAGAGACCGAGCTA-3' and 5'-TACGACCCATTGTCTGAGCG-3'; *Tim4* 5'-GGCTCCTTCTCACAAGAAAC-3' and 5'-TCAGCTGTGAACTTGGATGGG-3'; *GAPDH*, 5'-TGTGAACGGATTTGGCCGTA-3' and 5'-GGTCTCGCTCCTGGAAGATG-3'. PCR products were resolved by 2.5 % agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

Preparation of nuclear extracts and western blot analysis

Cells were suspended in 100 μl of hypotonic buffer A [10 mM HEPES (pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF] for 15 min on ice, followed by addition of 1 μl of 10 % Nondiet P-40 solution. The mixture was centrifuged at 12,000 *g* for 5 min. The pellets were washed with hypotonic buffer A and resuspended in hypertonic buffer C [20 mM HEPES (pH 7.8), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] for 30 min on ice and centrifuged at 12,000 *g* for 15 min. The supernatant containing nuclear proteins was collected and stored at -70 ℃ after determination of the protein concentration. The protein concentration of the nuclear extracts was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Whole cell extracts were prepared by suspending the cells in the RIPA lysis buffer [150 mM NaCl , 20 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, 1 mM ethylene glycol tetra-acetic acid (EGTA), 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leuptin, 1 mM PMSF] for 1 h on ice, followed by centrifugation for 15 min at 12,000 *g*. The protein concentration of the supernatant was measured by using the Bicinchonic acid (BCA) reagent. The protein samples were solubilized with SDS-polyacrylamide gel electrophoresis sample loading buffer and boiled for 5 min. Protein were electrophoresed on 7% or 9% SDS-polyacrylamide gel and transferred to PVDF membranes. The blot were then blocked with 5 % fat-free dry milk-TBST (tris-buffered saline containing 0.1% Tween-20) buffer for 1 h at room temperature and incubated with primary antibodies diluted at 1:1000 in 3% fat-free dry milk-

TBST. Following three washes with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibody diluted at 1:5000 in 3% fat-free dry milk-TBST for 1h at room temperature. The blot were rinsed again three times with TBST, and the transferred protein were incubated with the ECL according to the manufacturer's instruction and visualized with LAS400 (Fuji film, Tokyo, Japan)

Immunocytochemical analysis of Nrf2

Cells seeded at 3 x 10^4 cells per well in an 8 chamber plated and incubated for 3 h in the absence or presence of TauCl. After fixation with 10% neutral-buffered formalin solution for 30 min at room temperature, cells were permeabilized with 0.2% Triton X-100, incubated with blocking agents [0.1% Tween-20 in PBS containing 5% bovine serum albumin], washed with PBS and then incubated with a diluted (1:100) primary antibody overnight at 4 ℃. After washing with PBS, cells were incubated with a diluted (1:100) FITC-goat anti-rabbit IgG secondary antibody for 1 h and with propidium iodide for 5 min, and examined under a confocal microscope (Nikon, Tokyo, Japan).

Preparation and culturing of mouse embryonic fibroblasts

Nrf2-null mice, in which the *nrf2* gene is disrupted by targeted gene knockout, were provided by Dr. Jeffery Johnson, University of Wisconsin, Madison, WI. Male and female *nrf2*$^{+/-}$ mice were paired and the pregnancies were monitored. Embryos were obtained at the day 13.5 after pairing under aseptic conditions. The embryo

bodies were minced into small pieces and cultured in high glucose DMEM supplemented with 10% fetal bovine serum and kept at 37 ℃ with 5% CO_2.

Statistical analysis

All data were expressed as means ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t* test. The criterion for statistical significance was $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

TauCl enhances clearance of apoptotic neutrophils in the zymosan A induced murine peritonitis model

In the peritoneum of zymosan A injected mice, the total leukocyte count peaked at 12 h (1). To determine the pro-resolving effect of TauCl, the molecule was injected into the peritoneum at 12 h, the peak of zymosan A-induced peritoneal inflammation, and at 6 h later, peritoneal exudates were collected and the number of total leukocyte were counted. Leukocyte counts in the peritoneal exudates obtained from TauCl injected mice were decreased markedly in a concentration dependent manner **(Fig. 1A)**. To distinguish the proportion of polymorphonucleocytes (PMNs or neutrophils) and monocytes in the peritoneal exudates, differential cell counts were performed. Zymosan A administration resulted in dramatic reduction in the proportion of monocytes with concomitant increase in the PMN counts **(Fig. 1B)**. These changes caused by zymosan A were attenuated by TauCl treatment **(Fig. 1B)**. Compared to the mice challenged with zymosan A alone, the mice treated with zymosan A and TauCl showed increased proportion of peritoneal macrophages which had engulfed apoptotic PMNs and these macrophages had increased expression of HO-1 **(Fig. 1C)**. These initial *in vivo* observations suggest that the increased clearance of apoptotic neutrophils stimulated by TauCl injection might be associated with induction of HO-1 expression in the peritoneal macrophages.

TauCl enhances efferocytic ability of macrophages

To confirm the pro-resolving effect of TauCl more precisely, we performed an *ex vivo* experiment by collecting the macrophages from peritoneal exudates of zymosan A-treated mice and co-incubated with the PMNs separated also from the same peritoneal exudates. When the cells in peritoneal exudates were cultured on plates, macrophages were attached on the bottom and the neutrophils floated on the top. After separating the attached macrophages and floating apoptotic neutrophils, macrophages were treated with TauCl for 6 h and stained with fluorescein isothiocyanate (FITC) while the neutrophils were conjugated with phycoerythrin (PE) after standing for 6 h. Thereafter, the FITC-stained macrophages and PE-conjugated apoptotic neutrophils were co-incubated for 2 h. Immunocytochemical analysis showed that more of the TauCl-treated macrophages engulfed apoptotic PMNs **(Fig. 2A)**.

TauCl-derived induction of HO-1 expression in macrophages is responsible for the increased efferocytic activity.

As was demonstrated before (13), HO-1 expression in TauCl treated macrophages was increased both in its mRNA (maximum at 3-6 h) and enzyme protein (maximum at 9 h) **(Fig. 2B and 2C)**. In contrast to TauCl, its precursors (taurine and chlorine in the form of NaCl) failed to induce HO-1 expression **(Fig. 3A and 3B)**. After confirming that the TauCl treated peritoneal macrophages had an increased engulfing activity (Fig. 2A), we then examined whether TauCl can also stimulate the RAW264.7 murine macrophages engulfing the apoptotic Jurkat T-cells

in an *in vitro* study. In previous studies using RAW264.7 cells, lipopolysaccharide (LPS) was shown to inhibit macrophage phagocytosis of apoptotic neutrophils by increasing the production of tumor necrosis factor alpha (TNF-α) through activation of NF-κB (7) and TauCl was shown to inhibit the LPS-derived overproduction of TNF-α as well as the activation of NF-κB (14). In addition, TauCl was also shown to induce the expression of HO-1 and several other antioxidant enzymes via activation of Nrf2 in murine macrophage cells including the RAW264.7 cells (13). To measure the efferocytic activity of RAW264.7 cells, they were co-incubated with FITC-annexin V stained-apoptotic Jurkat T cells for 1 h and were subjected to flow cytometric analysis. Prior to co-incubation with apoptotic Jurkat T cells, RAW264.7 macrophage cells were treated either with NaCl, taurine, or TauCl. Consistent with inability of taurine and chlorine to induce HO-1 expression (**Fig. 3A and B,**), only the TauCl-treated macrophages had increased efferocytic activity (**Fig. 3C**).

Although not presented, the HO activity measured using the cytosol fraction of macrophages was also increased and the peak activity was observed at 9-10 h after the TauCl treatment. In order to determine whether the TauCl-induced upregulation of HO-1 could account for its enhancement of efferocytosis in macrophages, zinc protoporphyrin (ZnPP), a well-known inhibitor of HO activity, was utilized. When the macrophages were pre-treated with ZnPP at 1 h before exposure to TauCl, increased HO activity could not be observed even while the HO-1 protein expression was markedly induced (data not shown). As the result shown in **Fig. 4A**, TauCl failed to enhance efferocytosis when the HO activity was inhibited by pretreatment with ZnPP. Next, to prove that the increased HO-1 expression induced

by TauCl is indeed responsible for the increased efferocytic activity, we treated the macrophages with TauCl in which the expression of HO-1 was knocked-down by transfecting the RAW264.7 cells with small interfering RNA (siRNA) of HO-1 (data not shown) and measured their efferocytic activitiy. Result shown in **Fig. 4B** indicated that siRNA knock down of HO-1 abolished the TauCl-induced efferocytic activity of macrophages HO-1 overexpression in the *ho-1* gene transfected macrophages was increased and the efferocytic activity (**Fig. 4C**). These results suggest that increased HO-1 expression/activity is indeed responsible for the enhanced efferocytic activity of TauCl treated macrophages.

TauCl increases expression and nuclear translocation of Nrf2.

As was observed earlier, the induction of HO-1 expression in macrophages treated with TauCl depends on the activation of Nrf2 (13). In the TauCl treated macrophages undergoing oxidative stress, the level of Nrf2 protein in cytoplasm was increased between 3 to 9 h after exposure to TauCl **(Fig. 5A),** while the expression of its mRNA transcript barely changed **(Fig. 5B)**. The effects of TauCl and its precursor molecules on Nrf2 expression was compared. In contrast to TauCl, neither taurine nor chlorine induced Nrf2 protein expression (**Fig. 5C**) while there was no change induced by each compound in the levels of Nrf2 mRNA (**Fig. 5D**). Therefore TauCl appears to have an effect on the stability of Nrf2 protein. We also noted that Nrf2 translocated into nucleus following TauCl treatment as determined by Western blot **(Fig. 5E)** and immunocytochemical analysis **(Fig. 5F)**.

Tau-Cl-induced Nrf2 activation is important for HO-1 expression.

when the transcriptional expression of Nrf2 was knocked down in RAW264.7 cells by transfecting them with siRNA against Nrf2, treatment of these macrophages with TauCl was unable to activate Nrf2 and induction of HO-1 expression was prohibited **(Fig. 6A)**. Likewise, the mouse embryonic fibroblasts (MEF) obtained from the Nrf2 gene knock-out mice showed lack of Nrf2 and HO-1 expression when treated with TauCl for 9 h **(Fig. 6B)**. When the peritoneal macrophages collected from peritoneal exudates of Nrf2 gene knock-out mice were treated with TauCl, they were unable to induce expression of HO as well as Nrf2 **(Fig. 6C)**. Furthermore, in the peritoneal macrophages isolated from Nrf2 knock-out mice neither cytoplasmic Nrf2 nor the nuclear translocation of Nrf2 was detected **(Fig. 6D)**.

Cysteine thiol residues of Keap1 may be putative targets of TauCl for its induction of Nrf2-mediated expression of HO-1.

As was mentioned earlier, TauCl is a mild oxidant with only minimal cytotoxicity. In a previous study with RAW264.7 macrophages, TauCl did not cause cell death even at 1 mM and 0.5 mM TauCl caused significant early depletion of cellular glutathione (GSH) concentration that could be restored within 6 h. The TauCl-derived depletion of GSH could be prevented by pretreatment of the macrophages with 0.5 mM dithiothreitol (DTT), a well-known reducing agent that prevents oxidation of cysteine residues in proteins exposed to oxidants or oxidative stress (13). In the present study, RAW264.7 macrophages were pretreated with 0.5 mM DTT for 1 h prior to exposure to 0.5 mM TauCl. The pretreatment of macrophages

with DTT prevented the TauCl from enhancing nuclear translocation of Nrf2 **(Fig. 7A)** and HO-1 expression **(Fig. 7B)**. DTT pretreatment also abrogated expression of mRNA transcripts of both Nrf2 and HO-1 **(Fig. 7C)**. Presumably, the pretreated DTT prevented the TauCl in oxidizing critical cysteine residues in Keap1 and preserved its ability to tether Nrf2

TauCl enhances efferocytic ability of macrophages by increasing the expression of scavenger receptors recognizing phosphatidylserine on the surface of apoptotic neutrophils.

When neutrophils undergo apoptosis, phosphatidylserine at the inner leaflet of plasma membrane flip-flops and is exposed on the surface and also undergoes oxidation. These exposed phosphatidylserine and its oxidized metabolites are recognized by several scavenger receptors, such as BAI-1, MerTK, and Tim4 expressed on the surface of macrophages and efferocytosis is initiated (6,9). We thus examined whether the transcriptional expression of these scavenger receptor mRNAs are increased in RAW264.7 macrophages following treatment with TauCl. Transcription of these scavenger receptors in the TauCl treated macrophages was transiently increased **(Fig. 8A)**. This prompted us to examine whether the TauCl-induced upregulation of HO-1 is responsible for the upregulated transcriptional expression of scavenger receptors in the TauCl-treated macrophages. Thus, RAW264.7 cells were transfected with siRNA against HO-1 and then treated with TauCl. As shown in **Fig. 8B**, TauCl failed to upregulate the transcription of scavenger receptors in the HO-1 gene knocked-down macrophages. After

confirming that TauCl-derived induction of HO-1 expression is involved in the upregulation of scavenger receptor transcription, we examined whether the CO overproduced by the elevated HO activity is involved. For this purpose, we utilized the CO arising from 100 uM CORM. Thus, macrophages were exposed to CORM for 9 h and were examined whether the mRNA levels of scavenger receptors are increased by employing the RT-PCR analysis. Results shown in **Fig. 8C** indicate that the CO arising from CORM could increase the transcription of these scavenger receptor mRNAs at a comparable level obtained with TauCl treatment. Furthermore, when we examined the efferocytic activity of these macrophages exposed to CORM alone, their ability to engulf apoptotic Jurkat T cells was increased by 2-fold **(Fig. 8D)**. Alternatively, after confirming that CO arising from CORM could enhance the efferocytic activity, we tested whether the increased efferocytic activity stimulated by the overproduced CO in the TauCl treated and HO-1 induced cells could be abolished by eliminating the CO overproduced by the upregulated HO-1 by exposing them to hemoglobin, a well-known scavenger of CO gas. As shown in **Fig. 8E**, the increased efferocytic activity of the TauCl treated macrophages is inhibited by exposure to hemoglobin in a dose dependent manner. This effect of Hb was not attributable to suppression of HO-1 but its binding to CO with high affinity. These results demonstrated that the CO overproduced in macrophages which have been treated with TauCl is responsible for the upregulated expression of scavenger receptors recognizing the apoptotic neutrophils with surface exposed phosphatidylserine, thus, enhancing the efferocytic activity.

The successful synthesis of the crystalline sodium salt (synthetic) is equivalent to TauCl.

Although aqueous solutions of TauCl can be obtained easily by reaction of taurine with chlorinating agents such as hypochlorite, the isolation of the pure substance was not successful (8). Alternatively, a more stable sodium salt form of TauCl was synthesized. As the result of **Sup. Figs. A-D,** TauCl and synthetic have equivalent effects.

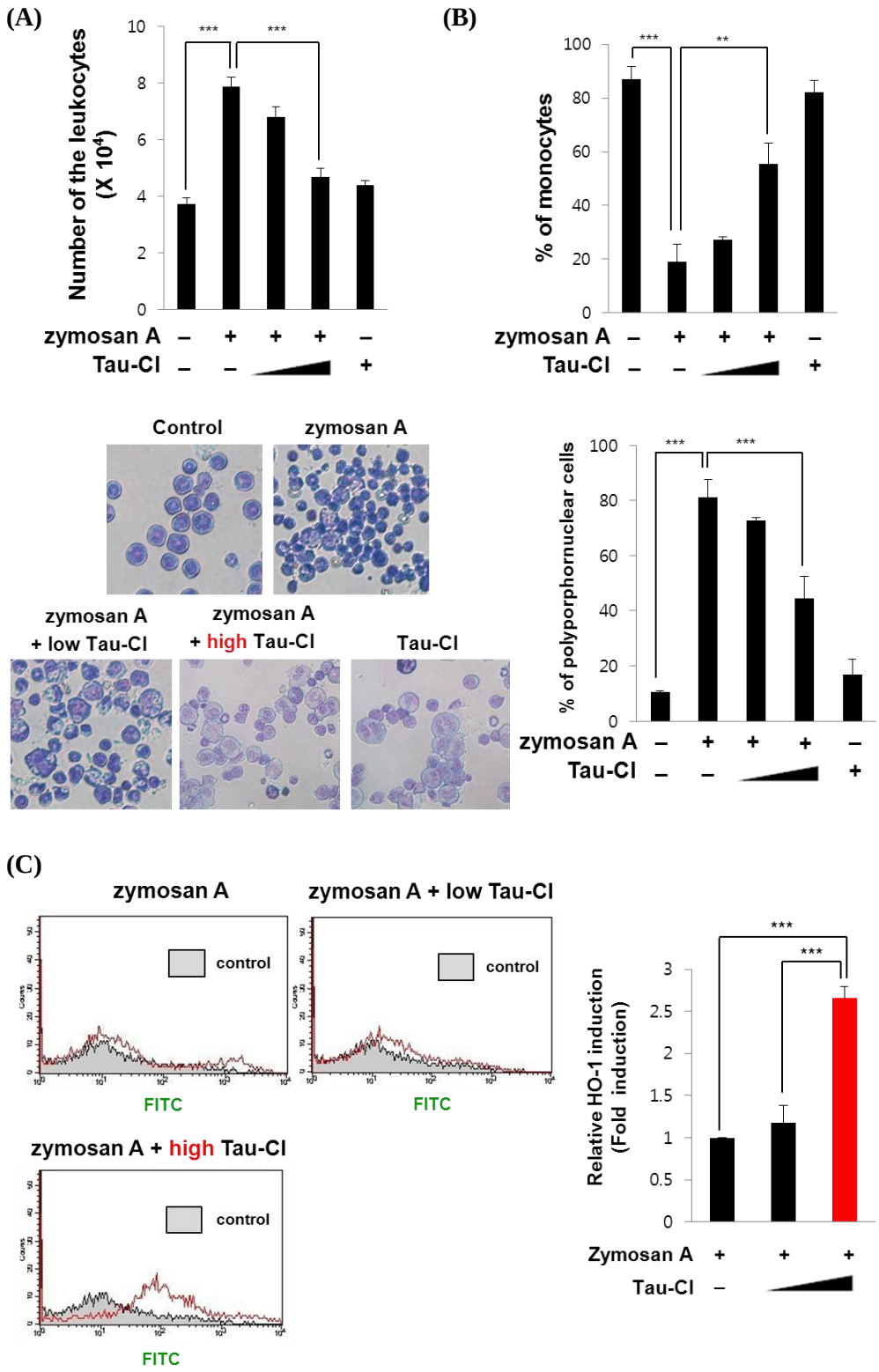

(A)
Number of the leukocytes (X 10⁴)
10
8
6
4
2
0
***
***
zymosan A – + + + –
Tau-Cl – – +
(B)
% of monocytes
100
80
60
40
20
0
***
**
zymosan A – + + + –
Tau-Cl – – +
Control
zymosan A
zymosan A + low Tau-Cl
zymosan A + high Tau-Cl
Tau-Cl
% of polyporphornuclear cells
100
80
60
40
20
0
***
***
zymosan A – + + + –
Tau-Cl – – +
(C)
zymosan A
zymosan A + low Tau-Cl
control
control
FITC
FITC
zymosan A + high Tau-Cl
control
FITC
Relative HO-1 induction (Fold induction)
3
2.5
2
1.5
1
0.5
0
***
***
Zymosan A + + +
Tau-Cl –

Fig 1. TauCl facilitates resolution of inflammation in zymosan A-induced peritonitis. Mice administered with zymosan A (30 mg/kg) for 12 h were treated with vehicle or TauCl (2 or 10 mg/kg) intraperitoneally (ip). Six hours later, peritoneal exudates were collected. (A) The number of total leukocytes in peritoneal exudates was counted. (B) The proportion of mononuclear cells and PMNs in collected peritoneal exudates was determined by differential cell counts. (C) HO-1 expression in peritoneal macrophages with ingested PMNs was analyzed by flow cytometry. All data represent mean ± S.D. (n=3), $^{**}p<0.01$, $^{***}p<0.001$.

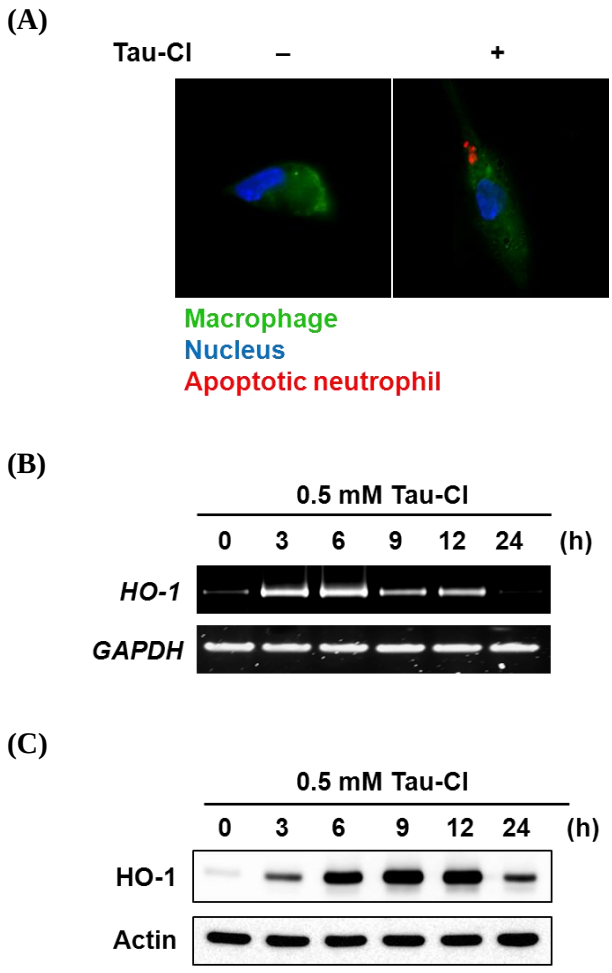


Fig 2. TauCl increases efferocytic ability and HO-1 expression of macrophages. (A) For measuring *ex vivo* efferocytosis, peritoneal macrophages treated with TauCl (0.5 mM) were co-incubated with apoptotic peritoneal neutrophils for 2 h. The engulfment of apoptotic neutrophils by macrophages was detected by immunocytochemistry using anti-F4/80 (green; macrophage marker) and anti-Gr-1 (red; neutrophil marker) antibodies. (B, C) RAW264.7 cells were treated with TauCl (0.5 mM) for indicated time periods. mRNA and protein levels of HO-1 were determined by RT-PCR and Western blot analysis, respectively.

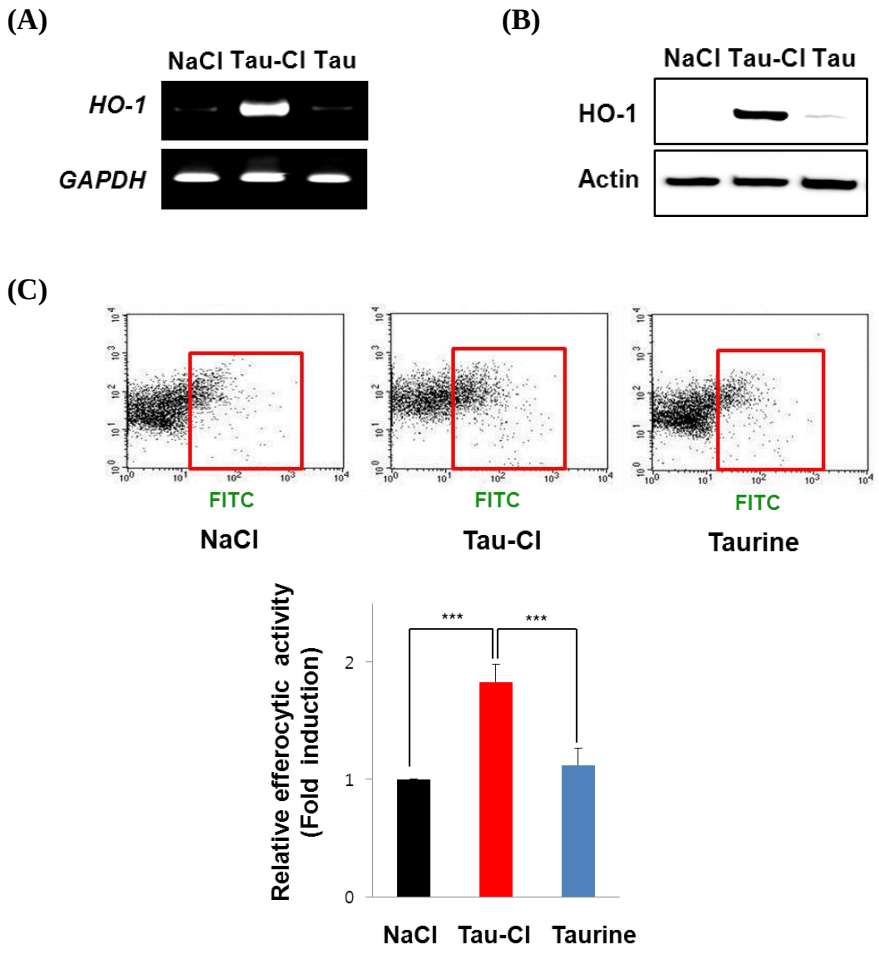


Fig 3. TauCl has higher effect than taurine and NaCl. (A, B) RAW264.7 cells were treated with TauCl, taurine or NaCl for 6 h (A), 9 h (B). mRNA and protein levels of HO-1 were determined by RT-PCR and Western blot analysis, respectively. (C) RAW264.7 cells treated with NaCl (0.5 mM), TauCl or taurine (0.5 mM) were co-incubated with FITC-annexin V stained-apoptotic Jurkat T cells for 1 h. Representative flow cytometric dot plots demonstrate changes in the percentage of macrophages engulfing FITC-annexin V stained-apoptotic Jurkat T cells. All data represent mean ± S.D. (n=3), $^{***}p<0.001$.

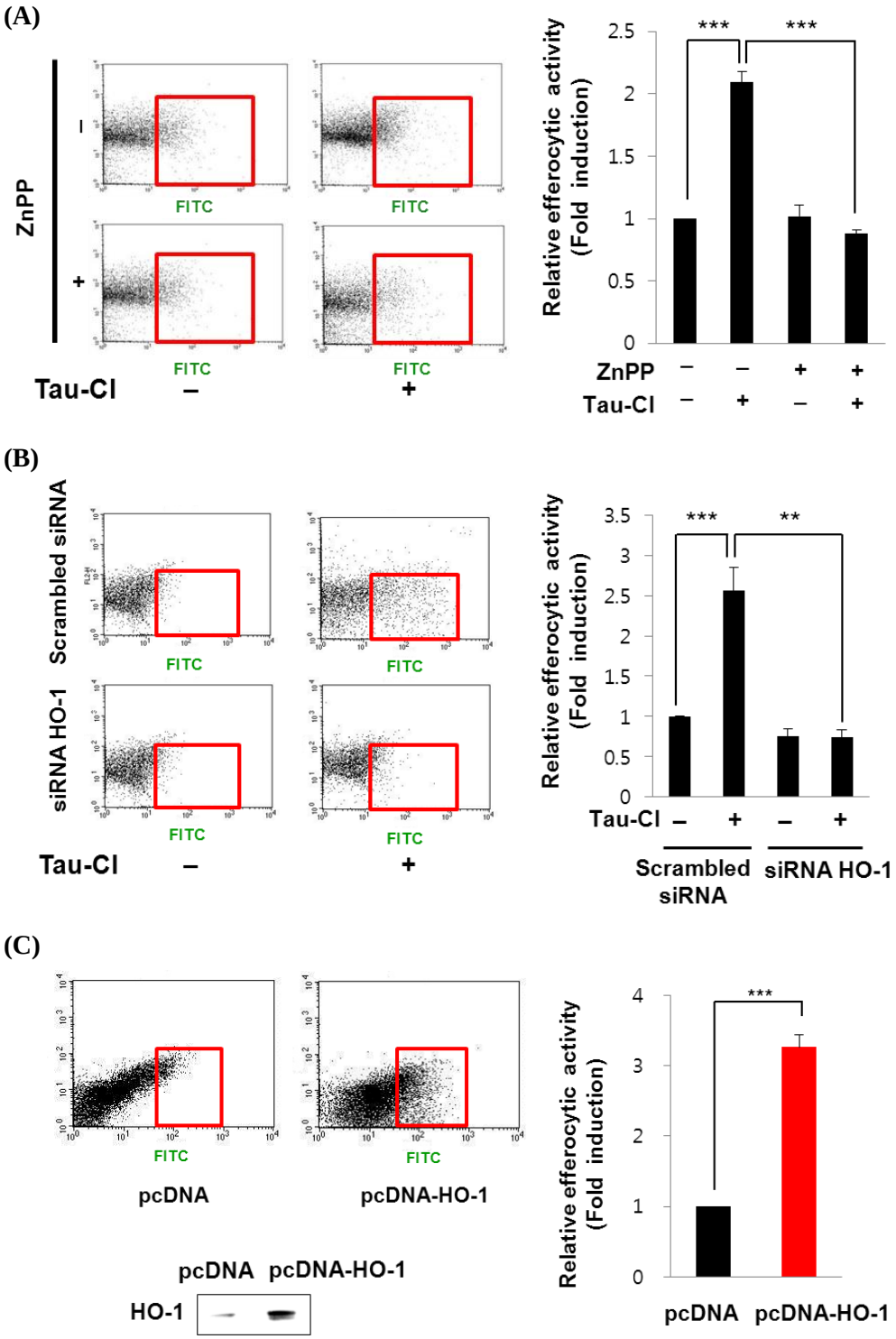

(A)
ZnPP
–
+
FITC
Tau-Cl
–
+
Relative efferocytic activity (Fold induction)
***
***
ZnPP
Tau-Cl
(B)
Scrambled siRNA
siRNA HO-1
FITC
Tau-Cl
**
Scrambled siRNA
siRNA HO-1
(C)
pcDNA
pcDNA-HO-1
HO-1
Actin

Fig 4. HO-1 is critical for stimulating efferocytosis by Tau-Cl. (A-C) RAW264.7 cells were treated with ZnPP (10 μM) for 1 h prior to incubation with TauCl for additional 12 h (A). RAW264.7 cells were transfected with scrambled or *HO-1* siRNA for 16 h, and then treated with TauCl for additional 12 h (B). RAW264.7 cells were transfected with pcDNA-mock or pcDNA-HO-1 for 24 h, followed by incubation with TauCl for additional 12 h (C). The assays for efferocytosis were performed by incubating cells with FITC-annexin V stained-apoptotic Jurkat T cells for 2 h. Representative flow cytometric dot plots demonstrate changes in the percentage of macrophages engulfing FITC-annexin V stained-apoptotic Jurkat T cells. All data represent mean ± S.D. (n=3), $^{**}p<0.01$, $^{***}p<0.001$

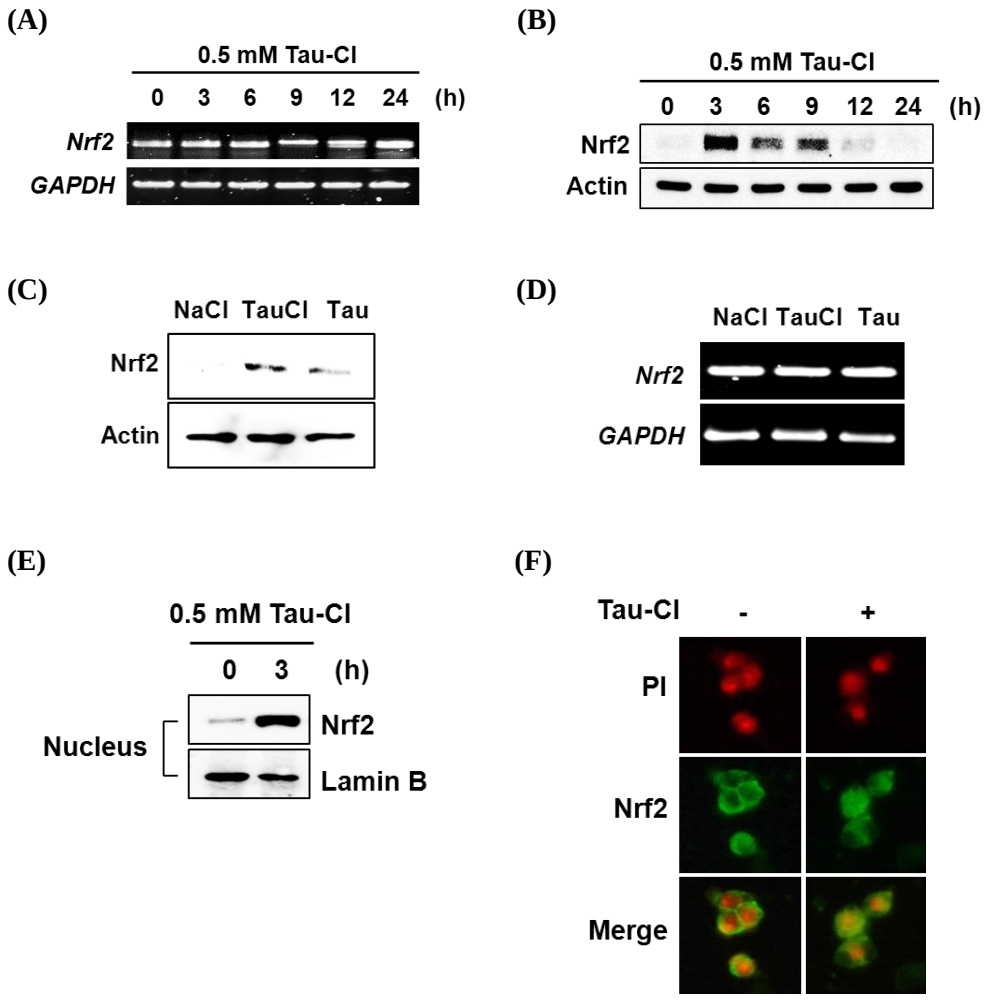


Fig 5. TauCl upregulates nuclear translocation of Nrf2. (A, B) RAW264.7 cells were treated with TauCl (0.5 mM) for indicated time periods. mRNA and protein levels of Nrf2 were determined by RT-PCR and Western blot analysis, respectively. (C, D) RAW264.7 cells were treated with TauCl, taurine or NaCl for 3 h. Protein and mRNA levels of RAW264.7 cells were determined by Western blot and RT-PCR analysis, respectively. (E) RAW264.7 cells were treated with TauCl for 3 h and nuclear translocation of Nrf2 was verified by immunoblot analysis. Lamin B was

used as a loading control for nuclear extracts. (F) Immunocytochemical analysis was performed using anti-Nrf2 after the treatment of RAW264.7 cells with TauCl for 3 h.

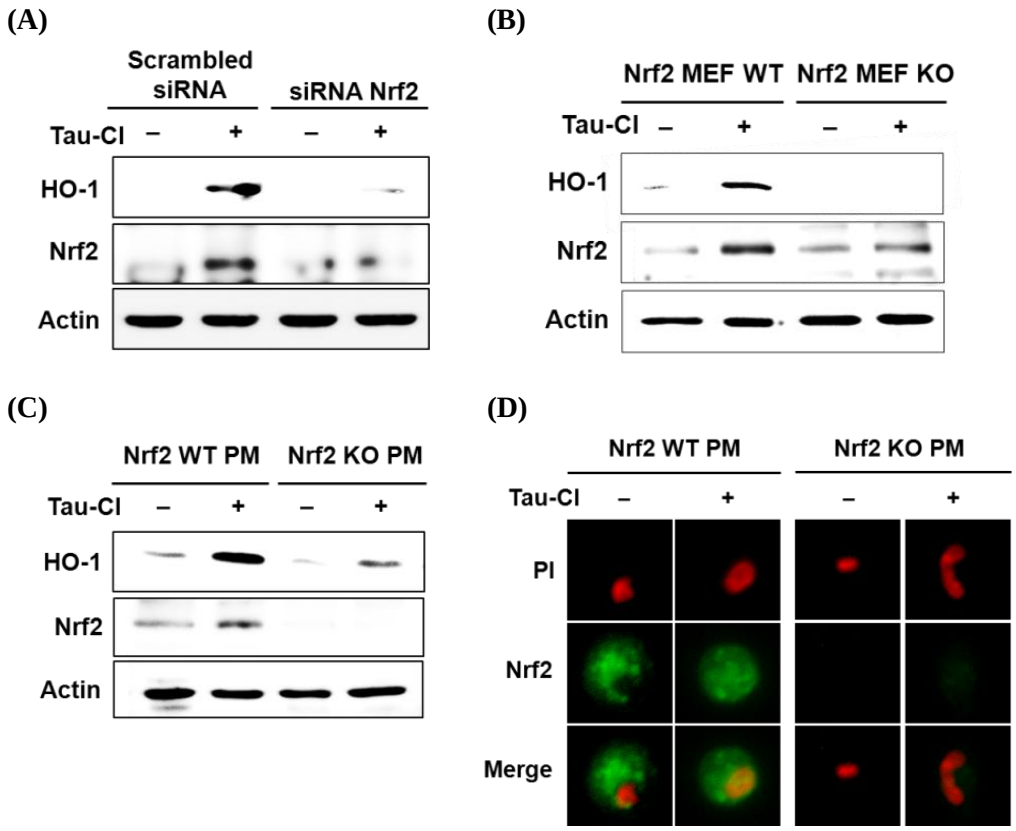


Fig 6. TauCl-induced Nrf2 activation is important for HO-1 expression. (A-C) RAW264.7 cells were transfected with scrambled or *Nrf2* siRNA, and then incubated in the absence or presence of TauCl (0.5 mM) for additional 9 h (A). MEF cells were isolated from Nrf2 wild type and knockout mice, and were treated with TauCl for 9 h (B). Peritoneal macrophages obtained from Nrf2 wild-type and Nrf2 knockout mice were treated with TauCl for 9 h (C). Protein levels of HO-1 and Nrf2 measured by Western blot analysis. Actin was used as an equal loading control for normalization. (D) Peritoneal macrophages were collected from Nrf2 wild-type and Nrf2 knockout mice, and were treated with TauCl for 3 h. Nuclear translocation of Nrf2 was determined by immunocytochemical analysis..

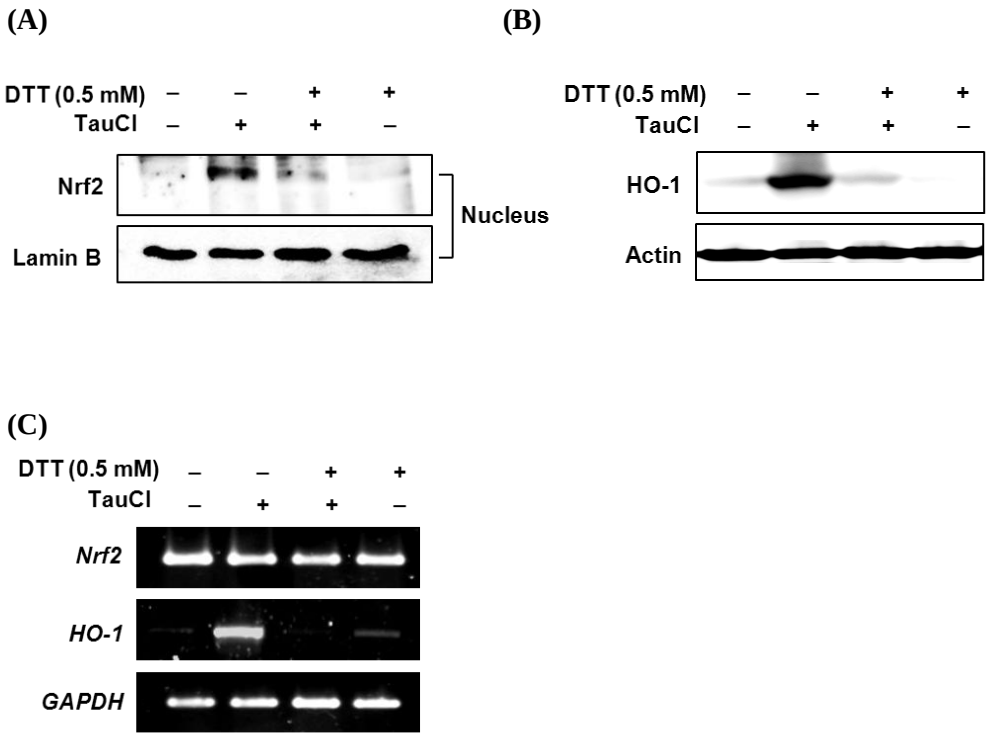


Fig 7. The possible involvement of Keap1 cysteine thiol in TauCl-induced HO-1 expression and Nrf2 translocation. (A, B, C) RAW264.7 cells were treated with DTT (0.5 mM) for 1 h prior to incubation with TauCl (0.5 mM) for additional 3 h (A), 9 h (B), or 6 h (C). The protein levels of Nrf2 (A) and HO-1 (B) were verified by Western blot analysis. Actin and lamin B were included as loading controls for normalization. mRNA level of *Nrf2* and *HO-1* were determined by RT-PCR (C).

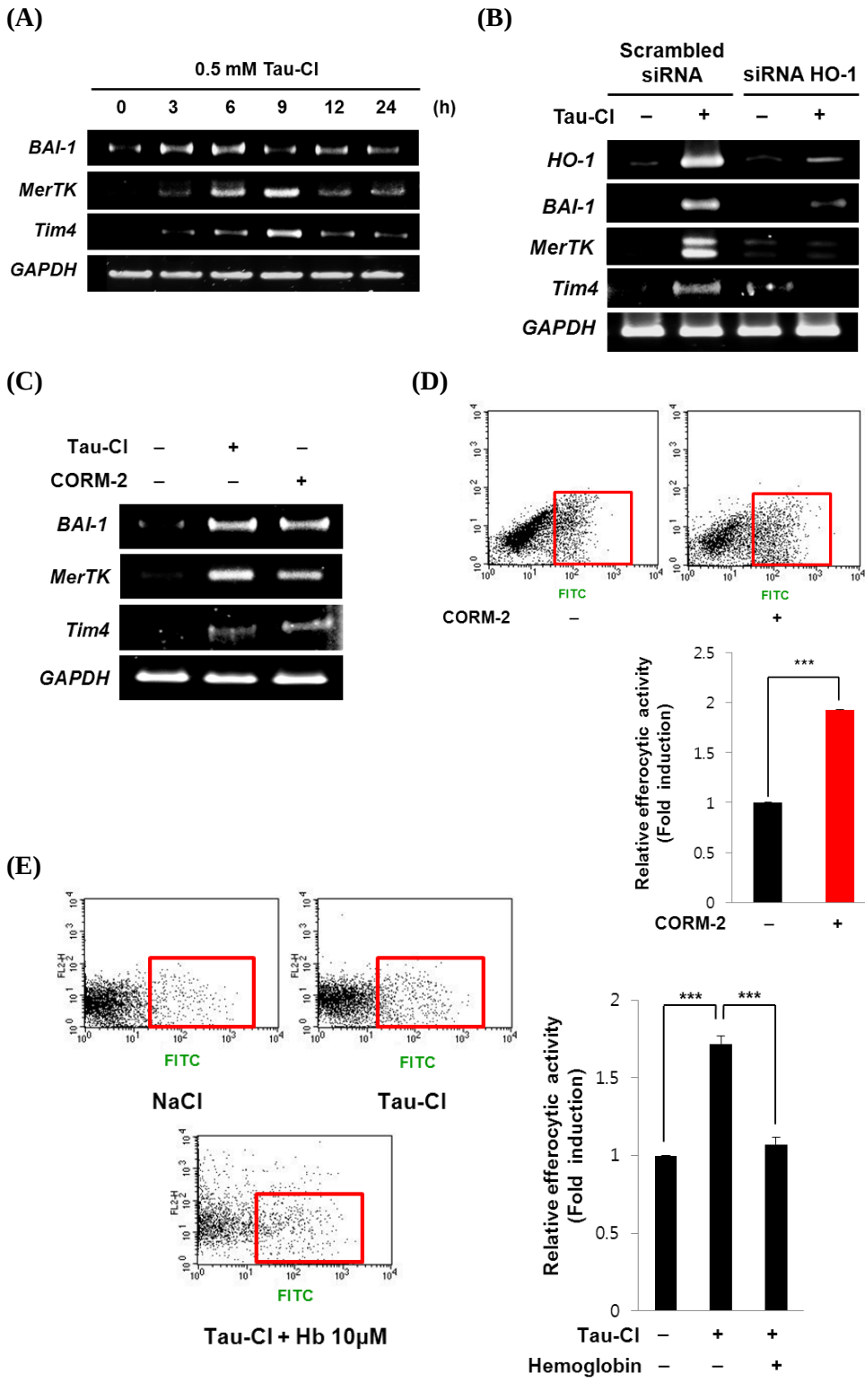

(A)
0.5 mM Tau-Cl
0
3
6
9
12
24
(h)
BAI-1
MerTK
Tim4
GAPDH
(B)
Scrambled siRNA
siRNA HO-1
Tau-Cl – + – +
HO-1
BAI-1
MerTK
Tim4
GAPDH
(C)
Tau-Cl – + –
CORM-2 – – +
BAI-1
MerTK
Tim4
GAPDH
(D)
FITC
FITC
CORM-2 – +
Relative efferocytic activity (Fold induction)
2.5
2
1.5
1
0.5
0
***
CORM-2 – +
(E)
FITC
FITC
NaCl
Tau-Cl
FITC
Tau-Cl + Hb 10μM
Relative efferocytic activity (Fold induction)
2
1.5
1
0.5
0
***
***
Tau-Cl – + +
Hemoglobin – – +

Fig 8. TauCl increases the efferocytic ability of macrophage by upregulating the expression of engulfment receptors for apoptotic cells. RAW264.7 cells were treated with TauCl (0.5 mM) for indicated time periods (A). RAW264.7 cells were transfected with scrambled or *HO*-1 siRNA, and then incubated in the absence or presence of TauCl for additional 9 h (B). RAW264.7 cells were treated with TauCl or CORM-2 (100 μM) for 9 h. The mRNA levels of *BAI-1*, *MerTK*, *Tim4* and *GAPDH* were determined by RT-PCR (C). RAW264.7 cells were treated with CORM-2 for 24 h, were co-incubated with FITC-annexin V stained-apoptotic Jurkat T cells for 2 h (D). RAW264.7 cells containing FITC-annexin V stained-apoptotic Jurkat T cells were assessed by using flow cytometry. RAW264.7 cells, treated with NaCl, TauCl, and TauCl plus hemoglobin (Hb; 10 μM) for 24 h, were co-incubated with FITC-annexin V stained-apoptotic Jurkat T cells for 2 h (E). All data represent mean ± S.D. (n=3), $^{***}p<0.001$.

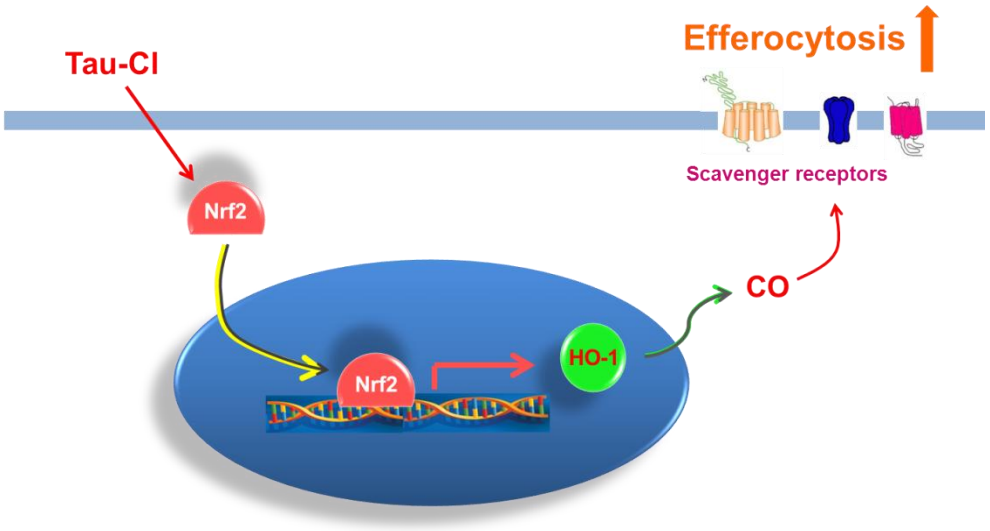
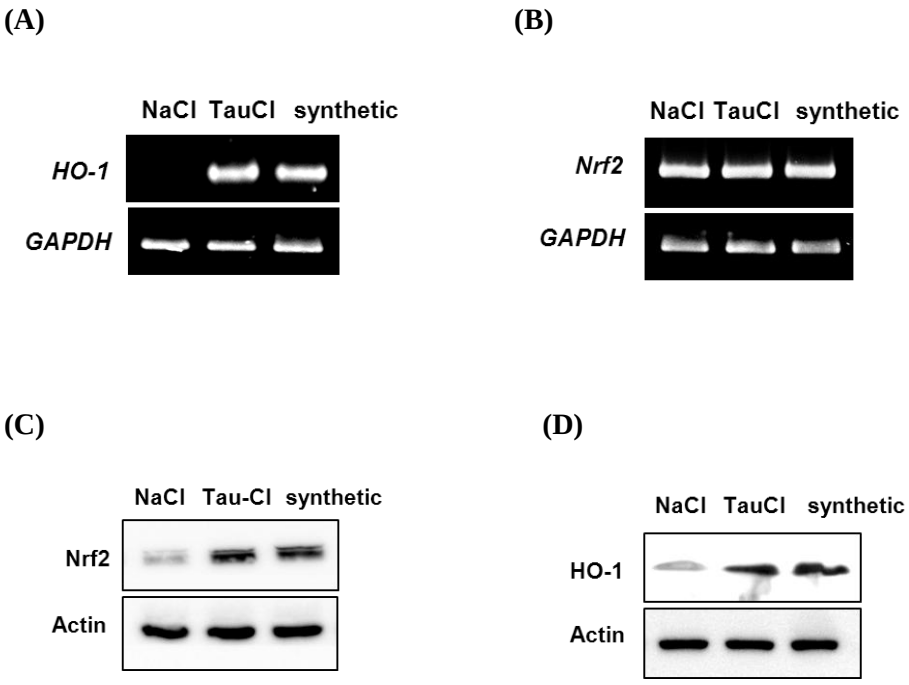


Fig 9. A proposed mechanism underlying the effects of TauCl on resolution of inflammation. TauCl is endogenously produced in neutrophils under inflammatory conditions. During resolution of inflammation, the generation and release of TauCl by activated neutrophils increase efferocytic ability in macrophages through upregulation of HO-1 expression and production of CO.



Supplementary Fig . Equivalent effects of TauCl and its salt form on macrophage. (A-D) RAW 264.7 cells were treated with TauCl (0.5 mM), synthetic TauCl (0.5 mM) and NaCl for 3 h, 6 h or 9 h. mRNA and protein levels of RAW 264.7 cells were determined by RT-PCR and Western blot analysis.

DISCUSSION

Results shown in the present study demonstrate for the first time that TauCl enhances the efferocytic activity of macrophages. Although some amount of TauCl may have been produced and released by the peritoneal neutrophils that have infiltrated into the peritoneum of zymosan-A injected mice, peritoneal injection of additional TauCl potentiated the efferocytic activity of peritoneal macrophages engulfing the apoptotic neutrophils and accelerated the rate of resolving the zymosan-A induced peritonitis. The TauCl-derived enhancement of efferocytic activity of macrophages appeared to result from induction of HO-1 expression, stimulation of HO activity and subsequent overproduction of CO gas as a byproduct. This overproduced CO appeared to serve as a signal molecule that induce reprogramming of genes in macrophages so that expression of scavenger receptors recognizing the surface exposed phosphatidylserine of apoptotic neutrophils is increased, thus enhancing the efferocytic activity of macrophages exposed to TauCl.

TauCl is produced endogenously by the activated neutrophils and is released into the inflammatory milieu as the activated neutrophils undergo apoptosis. The macrophages co-existing with apoptotic neutrophils at the inflammatory milieu are exposed to TauCl. Previously, TauCl has been considered as an end-product of taurine-derived detoxification of hypochlorous acid, a strong antibacterial oxidant produced in activated neutrophils by the myeloperoxidase from the overproduced H_2O_2, which is simply discarded after protecting the cells at the inflammation site

from the toxicity of hypochlorous acid. In previous studies, TauCl has been shown to inhibit the production of pro-inflammatory cytokines in activated macrophages through inhibition of NF-κB pathway, thus providing anti-inflammatory activity. Alternatively, TauCl has also been shown to provide antioxidant and cytoprotective activity in macrophages and other cells by inducing the expression of several antioxidant enzymes (12). The pro-resolving and efferocytic activity of TauCl has been overlooked.

Efferocytosis by macrophages is an essential process in resolving inflammation by engulfing apoptotic neutrophils. Through effective efferocytosis of dying neutrophils, macrophages prevent the release of inflammatory contents from dying neutrophils to the inflammatory milieu and avoid additional pro-inflammatory disruption of other cells at the inflammation site. For effective efferocytosis, the macrophages at the inflammation site undergo genetic reprogramming by activation of nuclear receptors like PPARγ, PPARδ, LXR, and RXRα which promote the expression of surface scavenger receptors that recognize the exposed phosphatidylserine and its oxidized metabolites on the surface of apoptotic neutrophils. As the activated neutrophils undergo apoptosis, the phospholipid phosphatidylserine at the inner leaflet of neutrophil plasma membrane flip-flops and is exposed to the surface and becomes oxidized. The surface scavenger receptors expressed on the macrophages recognize the exposed phosphatidylserine and its oxidized metabolites now exposed on the surface of apoptotic neutrophils and begin the process of efferocytosis. Recognition of apoptotic neutrophils and efferocytosis by macrophages then promotes secretion of anti-inflammatory cytokines like TGF-β

and IL-10 that inhibit the production of inflammatory mediators by the macrophages (5,28). As the TauCl has now been shown to enhance efferocytic activity of macrophages in the present study, it would be highly interesting to examine whether TauCl could also enhance the secretion of these anti-inflammatory cytokines.

It has been reported that induction of HO-1 expression with PPARγ ligands is involved in the resolution of inflammation on *in vitro* and *in vivo* models of chronic obstructive pulmonary disease (15). As the macrophages treated with TauCl were found to induce HO-1 expression (13) and present study confirmed that TauCl increases the efferocytic activity of macrophages through induction of HO-1 expression and overproduction of CO, it is expected that CO may stimulate efferocytosis by increasing the expression of scavenger receptors in the TauCl treated macrophages. In support of this speculation, transcriptional expression of scavenger receptors like BAI-1, MerTK, and Tim4 which are the product resulting from activation of PPARγ circuit (2,17,23) were increased in the macrophages that have been treated with TauCl but exposed to the CO gas being released from CORM. Presumably, the TauCl treated macrophages increased their efferocytic activity through this induction of scavenger receptors promoted by the CO gas overproduced from HO-1 induction. In support of this observation, inhaled CO gas has been shown to accelerate the resolution of inflammation via HO-1 circuits (4). While the expression of aforementioned scavenger receptors that are known to be upregulated by PPARγ activation in macrophages exposed to CO gas, the molecular events associated with CO on the activation of nuclear receptors such as PPARγ,

PPARδ, and LXR have not been examined. Thus, it would be highly interesting to examine whether the CO gas arising from CORM can activate these nuclear receptors in macrophages and this is currently being investigated in association with activation of Rac1 or inhibition of RhoA, the two small Rho GTPases that have opposing roles in regulating efferocytosis. Rac1 is known to enhance but RhoA is known to inhibit efferocytosis (16,21), and thus, the relative balance between these small GTPases play a key role in determining the efferocytic activity of macrophages. In this connection, while preliminary, TauCl appears to activate Rac1 (unpublished observation).

It is well known that expression of HO-1 is regulated by activation of Nrf2, a key redox sensitive transcription factor which is released from Keap1 in the cytoplasm and translocates into nucleus to bind the AREs localized in the promoter region of many antioxidant enzyme genes and stimulates their transcriptional expression, among which the induction of HO-1 expression is most pronounced. Our present study demonstrates that Nrf2 is essential for the TauCl-derived upregulation of HO-1 expression in macrophages. Interestingly, the Nrf2 mRNA level remained constant even while the Nrf2 protein level in the cytoplasm increased upon TauCl treatment. This suggested that TauCl has influence on the stability of Nrf2. It has been demonstrated that increase of Nrf2 level in the cytoplasm requires both the oxidation of critical cysteine thiol residues in Keap1 to release Nrf2 and prevent the Nrf2 from being degraded by proteasomes. In the present study, we found that stabilization of Nrf2 induced by TauCl treatment was abolished when the macrophages were pretreated with DTT, a well-known reducing

agent that prevents oxidation of cysteine thiol residues. This suggested that the cysteine thiols in Keap1 are considered to be targets of oxidation by TauCl. In support of this speculation, TauCl has been shown to target critical cysteines and inactivated creatine kinase and glyceraldehyde-3-phhosphate dehydrogenase more selectively than the highly thiol reactive hypochlorous acid (22). As the chloride ion of TauCl is an excellent leaving electrophile, it may play a decisive role in covalent modification of the cysteine thiol residues in Keap1 which serve as nucleophile. Alternatively, as TauCl is an oxidant that readily oxidizes the reduced glutathione chemically in test tubes (12), it may simply oxidize cysteine thiols in Keap1. Whether TauCl binds covalently to critical cysteine residues in Keap1 or simply oxidizing the cysteine thiol to induce structural modification in Keap1 and thus prevents tethering Nrf2 needs to be clarified.

In summary, unresolved inflammation caused by un-cleared apoptotic neutrophils remaining in the inflammatory environment promotes chronic inflammation that results in many human diseases like arthritis and cancer. Efferocytosis, engulfment and clearing of apoptotic neutrophils by macrophages, is an essential process in resolving inflammation, thus preventing the development of chronic inflammatory diseases. Our study demonstrates that TauCl, produced and released to the inflammatory milieu as the activated neutrophils undergo apoptosis, stimulates the efferocytic activity of the macrophages co-existing with apoptotic neutrophils at the inflammatory site and enhances resolution of inflammation. Our study demonstrates also that the increased efferocytic activity induced by TauCl is accompanied by induction of HO-1 and overproduction of CO gas. In turn, this CO gas

overproduced in the TauCl treated macrophages induces transcriptional expression of scavenger receptors that recognizes the phosphatidylserines that have been exposed as the activated neutrophils undergo apoptosis. This may promote the efferocytic activity of TauCl treated macrophages promoting the resolution of inflammation. As proposed in the schematic diagram (Fig. 8), TauCl released into the inflammatory milieu from apoptotic neutrophils stimulates efferocytosis through Nrf2-mediated upregulation of HO-1 expression. It is evident that the upregulated HO-1 overproducing CO gas is one of the key events required for the increased efferocytic activity enhancing the resolution of inflammation. Thus, TauCl may have therapeutic potential in the management of chronic inflammatory disorders.

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국문초록

염증반응 동안, Taurine Chloramine (TauCl) 은 활성화된 neutrophil 에서 대량 생산되고, 활성화된 neutrophil 은 세포자살을 통해 주변 염증 부위로 방출된다. zymogen A 에 의해 유도된 복막염이 생긴 마우스의 복막으로 TauCl 의 주입은 복강 내 leukocyte 의 수를 상당히 감소시키고 염증 해소를 강화시키며 monocyte 의 침투를 증가시킨다. 또한, 복강 내 삼출물에서 얻은 macrophage 에 TauCl 처리 시, 동일한 삼출물에서 얻은 apoptotic neutrophil 를 식균하는 능력이 증가하였다. 게다가, TauCl 를 처리한 마우스 대식세포 세포주인 RAW264.7 에서 apoptotic Jurkat T 세포를 식균하는 efferocytosis 능력 또한 향상되었고, TauCl 처리한 macrophage 에서 heme oxygenase-1 (HO-1) 의 발현은 Nrf2 의 핵 내 이동이 증가함에 따라 증가하였다. 또한, apoptotic 세포 표면에 노출되는 phosphatidylserince 를 인지하는 scavenger receptor 의 mRNA 발현은 TauCl 를 처리한 RAW264.7 cell 에서 증가하였다. RAW264.7 cell 에서 HO-1 유전자의 knock-down 은 TauCl 에 의해 유도된 efferocytosis 의 활성화를 abolish 했다. 반면, HO-1 유전자의 과발현과 외부로부터 주입된 CO-releasing molecule (CORM) 으로부터 얻어진 HO 의 부산물인 carbon monoxide (CO) 의 처리는 macrophage 의 efferocytosis 능력을 상승시켰다. 또한, TauCl 를 처리

하지 않은 macrophage 를 CORM 에 노출시켰을 때, scavenger receptor 의 mRNA 의 발현이 증가하였다. 따라서, Nrf2 에 의해 조절되는 HO-1 발현 상승과 CO 의 과발현을 통해 TauCl 이 macrophage 의 efferocytosis 능력을 증가시킴으로써 염증 해소를 촉진시킬 것으로 사료된다.

주요어 : TauCl, Resolution of inflammation, Effeocytosis, Heme oxygenase-1, Nrf2, Carbon monoxide, Scavenger receptor

학번 : 2012-21765

藥學碩士學位論文

Taurine Chloramine Stimulates Efferocytosis through Upregulation of Heme Oxygenase-1 Expression and Production of Carbon Monoxide

Heme Oxygenase-1 (HO-1) 의 발현과 Carbon Monoxide (CO) 의 생산을 통한 Taurine Chloramine 의 Efferocytosis 촉진 효과 기전

2014 年 2 月

서울大學校 大學院

醫學科 腫瘍生物學科

金 勳 義

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지도 교수 서 영 준

이 논문을 약학석사 학위논문으로 제출함

2013년 10월

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ABSTRACT

Taurine Chloramine Stimulates Efferocytosis through Upregulation of Heme Oxygenase-1 Expression and Production of Carbon Monoxide

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During inflammation, taurine chloramine (TauCl) is produced abundantly in activated neutrophils and released to surrounding inflammatory milieu as the activated neutrophils undergo apoptosis. TauCl injected *in vivo* into the peritoneum of mice undergoing zymosan A-induced peritonitis was found to markedly decrease the number of peritoneal leukocytes and yet increase the blood monocyte infiltration, enhancing resolution of inflammation. Furthermore, when the macrophages obtained from peritoneal exudates were treated with TauCl *ex vivo*, their ability to phagocytose the apoptotic neutrophils obtained from the same peritoneal exudates

was increased. In the murine macrophage-like RAW264.7 cells treated with TauCl, efferocytic activity to phagocytose the apoptotic Jurkat T cells was also enhanced. In these macrophages treated with TauCl, expression of heme oxygenase-1 (HO-1) was increased along with increased nuclear translocation of the nuclear factor E2-related factor 2 (Nrf2). Furthermore, transcriptional expression of scavenger receptors recognizing the phosphatidylserines exposed on the surface of apoptotic cells were increased in these RAW264.7 cells treated with TauCl. Knock-down of HO-1 gene in RAW264.7 cells abolished the TauCl induced activation of efferocytosis, whereas both the overexpression of HO-1 and treatment with carbon monoxide (CO), the product of heme oxygenase, obtained from CO-releasing molecule (CORM) added exogenously increased the efferocytic ability of macrophages. When the macrophages not treated with TauCl were exposed to CORM, transcriptional expression of scavenger receptors was increased. Taken together, our results suggest that TauCl might facilitate resolution of inflammation by increasing the efferocytic activity of macrophages through Nrf2-mediated upregulation of HO-1 expression and overproduction of CO.

Keyword : TauCl, Resolution of inflammation, Effeocytosis, Heme oxygenase-1, Nrf2, Carbon monoxide, Scavenger receptor

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INTRODUCTION

Acute inflammation is an active host response to invading infectious pathogens and physical injuries. During inflammation, neutrophils recruited to the inflamed site are activated by phagocytosis of infectious pathogens and undergo oxidative burst. This leads to overproduction of reactive oxygen species (ROS) with which the neutrophils kill and eliminate the infectious pathogens. Subsequently, the activated neutrophils undergo apoptosis. These apoptotic neutrophils are removed by the macrophages which are recruited to the inflamed site immediately following the neutrophil infiltration. Upon phagocytic removal of apoptotic neutrophils by macrophages through a process called efferocytosis, the inflammation is resolved (18,19,25). If the apoptotic neutrophils with exposed phosphatidylserine on their surface are not cleared, this may cause chronic inflammation. Chronic inflammation has emerged as a critical component in many prevalent human diseases, such as arthritis and cancer (10). The process of resolving inflammation is an active process controlled in part by the chemical mediators that are formed endogenously during inflammation. These molecules act as local autacoids which stimulate the pro-resolving mechanisms of macrophages.

Taurine, a sulfur containing amino acid produced by decarboxylation of cysteine, is one of the most abundant free amino acids (not incorporated into proteins) stored in the cytoplasm of cells. It plays an important role in several essential biological processes, such as osmoregulation, membrane stabilization, calcium mobilization

and immunity (11). The concentration of taurine is particularly high in phagocytic cells like neutrophils that undergo oxidative burst, and hence have to cope with inflammatory oxidative stress. The stored taurine reacts stoichiometrically with hypochlorous acid (HOCl), a highly toxic antibacterial oxidant produced from hydrogen peroxide (H_2O_2) by the myeloperoxidase (MPO) in the activated neutrophils. This results in the generation of taurine chloramine (TauCl), a weak oxidant with mild toxicity. As the activated neutrophils undergo apoptosis, TauCl is then released and acts as a local autacoid at the inflamed tissues. While TauCl is known to have a direct antibacterial effect (20), it has both anti-inflammatory and antioxidant activities in the inflamed tissue. TauCl released from the activated and apoptotic neutrophils has been shown to inhibit the activation of Nuclear factor NF-kappa-B (NF-κB) in macrophages and to abolish the production of inflammatory mediators such as nitric oxide (NO), tumor necrosis factor (TNF-α), interleukin (IL)-6 and IL-8, and matrix metalloproteases (MMPs), thereby exerting the anti-inflammatory effect (14). Alternatively, TauCl has also been shown to activate nuclear factor E2-related factor 2 (Nrf2) and stimulate the transcriptional induction of several antioxidant enzymes and other cytoprotective proteins (12,13).

Nrf2 is a critical transcription factor involved in the cellular defense against cytotoxicity of oxidative stress. Under normal conditions, Nrf2 is kept in the cytoplasm as an inactive complex with Kelch-like ECH association protein 1 (Keap1) and undergoes constant proteasomal degradation. On the other hand, when cells are challenged with oxidative or electrophilic insults that oxidize or covalently modify critical cysteine residues in Keap1, Nrf2 dissociates from Keap1 and

translocates into the nucleus. Once in the nucleus, Nrf2 binds to the antioxidant response element (ARE) present in the promoter region of several defensive Among several antioxidant enzymes that are induced by the mild oxidant TauCl, heme oxygenase-1 (HO-1) draws particular attention. HO-1 is the rate-limiting enzyme catalyzing oxidative degradation of un-incorporated free-heme to produce carbon monoxide (CO), biliverdin/bilirubin and free iron (27). CO generated as a consequence of induction of HO-1 activity can act as a potent anti-inflammatory gas signal molecule that inhibits the expression of several pro-inflammatory cytokines and mediators and also exerts cytoprotective effects against oxidative stress (3,24). Recent findings demonstrate that the CO produced from upregulated HO-1 or provided exogenously at 250 ppm promotes resolution of inflammation (26,29). However, the molecular mechanisms underlying the pro-resolving effect of CO have yet to be established.

Efferocytosis or engulfment of apoptotic neutrophils by macrophages is essential for the resolution of inflammation. Apoptotic cells have phosphatidylserine exposed on their surface that serve as the ‘eat-me’ signal recognized by several scavenger receptors like BAI-1, Tim4, CD36 and others expressed on the surface of macrophages that initiate efferocytosis (6,9). Resident tissue macrophages first detect the signals of pathogens and tissue injuries and then release cytokines and mediators for leukocyte recruitment, first, blood polymorphonucleocytes (neutrophils) and then, blood monocytes which later differentiate into macrophages. Eventually, local autacoids and mediators in the inflammatory milieu alter genetic programming in the infiltrated macrophages and modulate endocytic functions that

promote efferocytosis. In the present study, we sought to investigate how TauCl could enhance efferocytic activity of macrophages undertaking the clearance of apoptotic neutrophils.

MATERIALS AND METHODS

Materials

TauCl was synthesized freshly on the day of use by adding equimolar amounts of NaOCl (Sigma-Aldrich, St. Louis, MO, USA) to taurine (Sigma-Aldrich). The authenticity of TauCl formation was monitored by UV absorption (200-400 nm). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 and fetal bovine serum (FBS) were obtained from GIBCO RBL (Grand Island, NY, USA). Carbon monoxide releasing molecules (CORM-2) was purchased from Sigma-Aldrich (Milwaukee, MI), and Dithiothreitol (DTT) and Hemoglobin (Hb) and anti-actin were purchased from Sigma-Aldrich. Primary antibodies against Nrf2 and lamin B1, small interfering RNAs (siRNAs) against Nrf2 and HO-1 and zinc protoporphyrin (ZnPP) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-HO-1 was the product of stressgen (Ann, Arbor, MI, USA), and anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were provided by Zymed Laboratories Inc. (San Francisco, CA, USA). Polyvinylidene difluoride (PVDF) membranes were supplied from Gelman Laboratory (Ann, Arbor, MI, USA). Enhanced chemiluminescent (ECL) detection kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Zymosan A-induced peritonitis

Institute of Cancer Research (ICR) mice (4 weeks of age) were purchased from Central Lab Animal Inc. (Seoul, South Korea). All the animals were maintained

according to the Institutional Animal Care Guidelines. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee at Seoul National University. Zymosan A (30 mg/kg) was administered intraperitoneally 12 h before giving PBS or TauCl (2 or 10 mg/kg, intraperitoneally), and mice were sacrificed 6 hours later. Peritoneal leukocytes were harvested by washing with 3 ml of phosphate-buffered saline (PBS) containing 3 mM ethylenediaminetetraacetate (EDTA).

Total and differential leukocyte counts

Total peritoneal leukocyte counts were carried out using Turk's solution (0.01% Crystal Violet in 3% acetic acid). For the differential count, peritoneal exudates were spun in a cytocentrifuge at 400 *g* for 5 min onto a slide and stained with Wright-Giemsa stain.

Cell culture

Murine macrophage RAW264.7 and human lymphoblastic Jurkat T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RAW264.7 cells and Jurkat T cells were cultured in DMEM and RPMI 1640, respectively, with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were maintained at 37 ℃ in a humidified atmosphere of 5% CO_2 and 95% air.

Efferocytosis assay

To assess the percentage of macrophages engulfing apoptotic PMNs *ex vitro*,

mouse peritoneal macrophages were incubated in six-well flat-bottomed microtiter plates for 24 h. Non-adherent cells were collected and incubated for additional 24 h to induce apoptosis. After washing with medium, adherent monolayer cells were co-incubated for 1 h with apoptotic non-adherent cells. Peritoneal macrophages were stained with the FluoroTag fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80 antibody (eBioscience, San Diego, CA, USA) for 20 min. The labeled cells were permeabilized for 10 min using 0.1% Triton X-100, and were incubated with phycoerythin (PE)-conjugated anti-mouse Gr-1 (Ly-6G) antibody (eBioscience) for 20 min. Macrophages containing neutrophils ($F4/80^{+}/Gr\text{-}1^{+}$) was detected using a confocal microscope (Nikon, Tokyo, Japan). To determine the efferocytic activity of macrophages *in vitro*, RAW264.7 cells were co-incubated for 1 h with apoptotic Jurkat T cells (stained with FITC-conjugated annexin V). To remove the non-engulfed apoptotic Jurkat T cells, RAW264.7 cells were washed three times with PBS and the proportion of RAW264.7 cells containing apoptotic Jurkat T cells (FITC-positive cells) was assessed by flow cytometry. Apoptosis of Jurkat T cells was induced by serum withdrawal and UVB (180 mJ/cm^2) irradiation, followed by incubation for 8 hours at 37 ℃ in an atmosphere of 5% CO_2.

Flow cytometry

Cells were fixed with 10% neutral-buffered formation solution for 30 min at room temperature, permeabilized with 0.2 % Triton X-100 for 5 min, and blocked with 2 % BSA in PBS for 30 min. Anti-HO-1 antibodies, diluted 1:100 in 2 % BSA in PBS, were applied overnight at 4 ℃. After washing with PBS. cells were incubated with

FITC-conjugated anti-rabbit IgG secondary antibody diluted at 1:1000 for 1 h. Cells were analyzed using FACSCalibur™ Flow Cytometer (BD, Franklin Lakes, NJ, USA).

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was isolated from RAW264.7 cells using TRizol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. To generate cDNA, 1 μg of total RNA was reverse transcribed by using murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). One microliter of cDNA was amplified in sequential reactions by using Maxime PCR PreMix Kit (iNtRON Biotechnology, South Korea). The primer pairs were as follows (forward and reverse, respectively): *Nrf2*, 5'-CTTAGAGGCTCATCTCACAC-3' and 5'-CTTTTGGGAACAAGGAACAC-3'; *HO-1*, 5'-GTCTATGCCCCACTCTATT-3' and 5'-TGGAAACGGATATCAAACGTG-3'; *BAI-1*, 5'-CAGAGCGGTCCGTTATCCTC-3' and 5'-TCTACCACACGGCACTTCAC-3'; *MerTK* 5'-AAGTGGGAAGAGACCGAGCTA-3' and 5'-TACGACCCATTGTCTGAGCG-3'; *Tim4* 5'-GGCTCCTTCTCACAAGAAAC-3' and 5'-TCAGCTGTGAACTTGGATGGG-3'; *GAPDH*, 5'-TGTGAACGGATTTGGCCGTA-3' and 5'-GGTCTCGCTCCTGGAAGATG-3'. PCR products were resolved by 2.5 % agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

Preparation of nuclear extracts and western blot analysis

Cells were suspended in 100 μl of hypotonic buffer A [10 mM HEPES (pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF] for 15 min on ice, followed by addition of 1 μl of 10 % Nondiet P-40 solution. The mixture was centrifuged at 12,000 *g* for 5 min. The pellets were washed with hypotonic buffer A and resuspended in hypertonic buffer C [20 mM HEPES (pH 7.8), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] for 30 min on ice and centrifuged at 12,000 *g* for 15 min. The supernatant containing nuclear proteins was collected and stored at -70 ℃ after determination of the protein concentration. The protein concentration of the nuclear extracts was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Whole cell extracts were prepared by suspending the cells in the RIPA lysis buffer [150 mM NaCl , 20 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, 1 mM ethylene glycol tetra-acetic acid (EGTA), 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leuptin, 1 mM PMSF] for 1 h on ice, followed by centrifugation for 15 min at 12,000 *g*. The protein concentration of the supernatant was measured by using the Bicinchonic acid (BCA) reagent. The protein samples were solubilized with SDS-polyacrylamide gel electrophoresis sample loading buffer and boiled for 5 min. Protein were electrophoresed on 7% or 9% SDS-polyacrylamide gel and transferred to PVDF membranes. The blot were then blocked with 5 % fat-free dry milk-TBST (tris-buffered saline containing 0.1% Tween-20) buffer for 1 h at room temperature and incubated with primary antibodies diluted at 1:1000 in 3% fat-free dry milk-

TBST. Following three washes with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibody diluted at 1:5000 in 3% fat-free dry milk-TBST for 1h at room temperature. The blot were rinsed again three times with TBST, and the transferred protein were incubated with the ECL according to the manufacturer's instruction and visualized with LAS400 (Fuji film, Tokyo, Japan)

Immunocytochemical analysis of Nrf2

Cells seeded at 3 x 10^4 cells per well in an 8 chamber plated and incubated for 3 h in the absence or presence of TauCl. After fixation with 10% neutral-buffered formalin solution for 30 min at room temperature, cells were permeabilized with 0.2% Triton X-100, incubated with blocking agents [0.1% Tween-20 in PBS containing 5% bovine serum albumin], washed with PBS and then incubated with a diluted (1:100) primary antibody overnight at 4 ℃. After washing with PBS, cells were incubated with a diluted (1:100) FITC-goat anti-rabbit IgG secondary antibody for 1 h and with propidium iodide for 5 min, and examined under a confocal microscope (Nikon, Tokyo, Japan).

Preparation and culturing of mouse embryonic fibroblasts

Nrf2-null mice, in which the *nrf2* gene is disrupted by targeted gene knockout, were provided by Dr. Jeffery Johnson, University of Wisconsin, Madison, WI. Male and female *nrf2*$^{+/-}$ mice were paired and the pregnancies were monitored. Embryos were obtained at the day 13.5 after pairing under aseptic conditions. The embryo

bodies were minced into small pieces and cultured in high glucose DMEM supplemented with 10% fetal bovine serum and kept at 37 ℃ with 5% CO_2.

Statistical analysis

All data were expressed as means ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t* test. The criterion for statistical significance was $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

TauCl enhances clearance of apoptotic neutrophils in the zymosan A induced murine peritonitis model

In the peritoneum of zymosan A injected mice, the total leukocyte count peaked at 12 h (1). To determine the pro-resolving effect of TauCl, the molecule was injected into the peritoneum at 12 h, the peak of zymosan A-induced peritoneal inflammation, and at 6 h later, peritoneal exudates were collected and the number of total leukocyte were counted. Leukocyte counts in the peritoneal exudates obtained from TauCl injected mice were decreased markedly in a concentration dependent manner **(Fig. 1A)**. To distinguish the proportion of polymorphonucleocytes (PMNs or neutrophils) and monocytes in the peritoneal exudates, differential cell counts were performed. Zymosan A administration resulted in dramatic reduction in the proportion of monocytes with concomitant increase in the PMN counts **(Fig. 1B)**. These changes caused by zymosan A were attenuated by TauCl treatment **(Fig. 1B)**. Compared to the mice challenged with zymosan A alone, the mice treated with zymosan A and TauCl showed increased proportion of peritoneal macrophages which had engulfed apoptotic PMNs and these macrophages had increased expression of HO-1 **(Fig. 1C)**. These initial *in vivo* observations suggest that the increased clearance of apoptotic neutrophils stimulated by TauCl injection might be associated with induction of HO-1 expression in the peritoneal macrophages.

TauCl enhances efferocytic ability of macrophages

To confirm the pro-resolving effect of TauCl more precisely, we performed an *ex vivo* experiment by collecting the macrophages from peritoneal exudates of zymosan A-treated mice and co-incubated with the PMNs separated also from the same peritoneal exudates. When the cells in peritoneal exudates were cultured on plates, macrophages were attached on the bottom and the neutrophils floated on the top. After separating the attached macrophages and floating apoptotic neutrophils, macrophages were treated with TauCl for 6 h and stained with fluorescein isothiocyanate (FITC) while the neutrophils were conjugated with phycoerythrin (PE) after standing for 6 h. Thereafter, the FITC-stained macrophages and PE-conjugated apoptotic neutrophils were co-incubated for 2 h. Immunocytochemical analysis showed that more of the TauCl-treated macrophages engulfed apoptotic PMNs **(Fig. 2A)**.

TauCl-derived induction of HO-1 expression in macrophages is responsible for the increased efferocytic activity.

As was demonstrated before (13), HO-1 expression in TauCl treated macrophages was increased both in its mRNA (maximum at 3-6 h) and enzyme protein (maximum at 9 h) **(Fig. 2B and 2C)**. In contrast to TauCl, its precursors (taurine and chlorine in the form of NaCl) failed to induce HO-1 expression **(Fig. 3A and 3B)**. After confirming that the TauCl treated peritoneal macrophages had an increased engulfing activity (Fig. 2A), we then examined whether TauCl can also stimulate the RAW264.7 murine macrophages engulfing the apoptotic Jurkat T-cells

in an *in vitro* study. In previous studies using RAW264.7 cells, lipopolysaccharide (LPS) was shown to inhibit macrophage phagocytosis of apoptotic neutrophils by increasing the production of tumor necrosis factor alpha (TNF-α) through activation of NF-κB (7) and TauCl was shown to inhibit the LPS-derived overproduction of TNF-α as well as the activation of NF-κB (14). In addition, TauCl was also shown to induce the expression of HO-1 and several other antioxidant enzymes via activation of Nrf2 in murine macrophage cells including the RAW264.7 cells (13). To measure the efferocytic activity of RAW264.7 cells, they were co-incubated with FITC-annexin V stained-apoptotic Jurkat T cells for 1 h and were subjected to flow cytometric analysis. Prior to co-incubation with apoptotic Jurkat T cells, RAW264.7 macrophage cells were treated either with NaCl, taurine, or TauCl. Consistent with inability of taurine and chlorine to induce HO-1 expression (**Fig. 3A and B,**), only the TauCl-treated macrophages had increased efferocytic activity (**Fig. 3C**).

Although not presented, the HO activity measured using the cytosol fraction of macrophages was also increased and the peak activity was observed at 9-10 h after the TauCl treatment. In order to determine whether the TauCl-induced upregulation of HO-1 could account for its enhancement of efferocytosis in macrophages, zinc protoporphyrin (ZnPP), a well-known inhibitor of HO activity, was utilized. When the macrophages were pre-treated with ZnPP at 1 h before exposure to TauCl, increased HO activity could not be observed even while the HO-1 protein expression was markedly induced (data not shown). As the result shown in **Fig. 4A**, TauCl failed to enhance efferocytosis when the HO activity was inhibited by pretreatment with ZnPP. Next, to prove that the increased HO-1 expression induced

by TauCl is indeed responsible for the increased efferocytic activity, we treated the macrophages with TauCl in which the expression of HO-1 was knocked-down by transfecting the RAW264.7 cells with small interfering RNA (siRNA) of HO-1 (data not shown) and measured their efferocytic activitiy. Result shown in **Fig. 4B** indicated that siRNA knock down of HO-1 abolished the TauCl-induced efferocytic activity of macrophages HO-1 overexpression in the *ho-1* gene transfected macrophages was increased and the efferocytic activity (**Fig. 4C**). These results suggest that increased HO-1 expression/activity is indeed responsible for the enhanced efferocytic activity of TauCl treated macrophages.

TauCl increases expression and nuclear translocation of Nrf2.

As was observed earlier, the induction of HO-1 expression in macrophages treated with TauCl depends on the activation of Nrf2 (13). In the TauCl treated macrophages undergoing oxidative stress, the level of Nrf2 protein in cytoplasm was increased between 3 to 9 h after exposure to TauCl **(Fig. 5A),** while the expression of its mRNA transcript barely changed **(Fig. 5B)**. The effects of TauCl and its precursor molecules on Nrf2 expression was compared. In contrast to TauCl, neither taurine nor chlorine induced Nrf2 protein expression (**Fig. 5C**) while there was no change induced by each compound in the levels of Nrf2 mRNA (**Fig. 5D**). Therefore TauCl appears to have an effect on the stability of Nrf2 protein. We also noted that Nrf2 translocated into nucleus following TauCl treatment as determined by Western blot **(Fig. 5E)** and immunocytochemical analysis **(Fig. 5F)**.

Tau-Cl-induced Nrf2 activation is important for HO-1 expression.

when the transcriptional expression of Nrf2 was knocked down in RAW264.7 cells by transfecting them with siRNA against Nrf2, treatment of these macrophages with TauCl was unable to activate Nrf2 and induction of HO-1 expression was prohibited **(Fig. 6A)**. Likewise, the mouse embryonic fibroblasts (MEF) obtained from the Nrf2 gene knock-out mice showed lack of Nrf2 and HO-1 expression when treated with TauCl for 9 h **(Fig. 6B)**. When the peritoneal macrophages collected from peritoneal exudates of Nrf2 gene knock-out mice were treated with TauCl, they were unable to induce expression of HO as well as Nrf2 **(Fig. 6C)**. Furthermore, in the peritoneal macrophages isolated from Nrf2 knock-out mice neither cytoplasmic Nrf2 nor the nuclear translocation of Nrf2 was detected **(Fig. 6D)**.

Cysteine thiol residues of Keap1 may be putative targets of TauCl for its induction of Nrf2-mediated expression of HO-1.

As was mentioned earlier, TauCl is a mild oxidant with only minimal cytotoxicity. In a previous study with RAW264.7 macrophages, TauCl did not cause cell death even at 1 mM and 0.5 mM TauCl caused significant early depletion of cellular glutathione (GSH) concentration that could be restored within 6 h. The TauCl-derived depletion of GSH could be prevented by pretreatment of the macrophages with 0.5 mM dithiothreitol (DTT), a well-known reducing agent that prevents oxidation of cysteine residues in proteins exposed to oxidants or oxidative stress (13). In the present study, RAW264.7 macrophages were pretreated with 0.5 mM DTT for 1 h prior to exposure to 0.5 mM TauCl. The pretreatment of macrophages

with DTT prevented the TauCl from enhancing nuclear translocation of Nrf2 **(Fig. 7A)** and HO-1 expression **(Fig. 7B)**. DTT pretreatment also abrogated expression of mRNA transcripts of both Nrf2 and HO-1 **(Fig. 7C)**. Presumably, the pretreated DTT prevented the TauCl in oxidizing critical cysteine residues in Keap1 and preserved its ability to tether Nrf2

TauCl enhances efferocytic ability of macrophages by increasing the expression of scavenger receptors recognizing phosphatidylserine on the surface of apoptotic neutrophils.

When neutrophils undergo apoptosis, phosphatidylserine at the inner leaflet of plasma membrane flip-flops and is exposed on the surface and also undergoes oxidation. These exposed phosphatidylserine and its oxidized metabolites are recognized by several scavenger receptors, such as BAI-1, MerTK, and Tim4 expressed on the surface of macrophages and efferocytosis is initiated (6,9). We thus examined whether the transcriptional expression of these scavenger receptor mRNAs are increased in RAW264.7 macrophages following treatment with TauCl. Transcription of these scavenger receptors in the TauCl treated macrophages was transiently increased **(Fig. 8A)**. This prompted us to examine whether the TauCl-induced upregulation of HO-1 is responsible for the upregulated transcriptional expression of scavenger receptors in the TauCl-treated macrophages. Thus, RAW264.7 cells were transfected with siRNA against HO-1 and then treated with TauCl. As shown in **Fig. 8B**, TauCl failed to upregulate the transcription of scavenger receptors in the HO-1 gene knocked-down macrophages. After

confirming that TauCl-derived induction of HO-1 expression is involved in the upregulation of scavenger receptor transcription, we examined whether the CO overproduced by the elevated HO activity is involved. For this purpose, we utilized the CO arising from 100 uM CORM. Thus, macrophages were exposed to CORM for 9 h and were examined whether the mRNA levels of scavenger receptors are increased by employing the RT-PCR analysis. Results shown in **Fig. 8C** indicate that the CO arising from CORM could increase the transcription of these scavenger receptor mRNAs at a comparable level obtained with TauCl treatment. Furthermore, when we examined the efferocytic activity of these macrophages exposed to CORM alone, their ability to engulf apoptotic Jurkat T cells was increased by 2-fold **(Fig. 8D)**. Alternatively, after confirming that CO arising from CORM could enhance the efferocytic activity, we tested whether the increased efferocytic activity stimulated by the overproduced CO in the TauCl treated and HO-1 induced cells could be abolished by eliminating the CO overproduced by the upregulated HO-1 by exposing them to hemoglobin, a well-known scavenger of CO gas. As shown in **Fig. 8E**, the increased efferocytic activity of the TauCl treated macrophages is inhibited by exposure to hemoglobin in a dose dependent manner. This effect of Hb was not attributable to suppression of HO-1 but its binding to CO with high affinity. These results demonstrated that the CO overproduced in macrophages which have been treated with TauCl is responsible for the upregulated expression of scavenger receptors recognizing the apoptotic neutrophils with surface exposed phosphatidylserine, thus, enhancing the efferocytic activity.

The successful synthesis of the crystalline sodium salt (synthetic) is equivalent to TauCl.

Although aqueous solutions of TauCl can be obtained easily by reaction of taurine with chlorinating agents such as hypochlorite, the isolation of the pure substance was not successful (8). Alternatively, a more stable sodium salt form of TauCl was synthesized. As the result of **Sup. Figs. A-D,** TauCl and synthetic have equivalent effects.

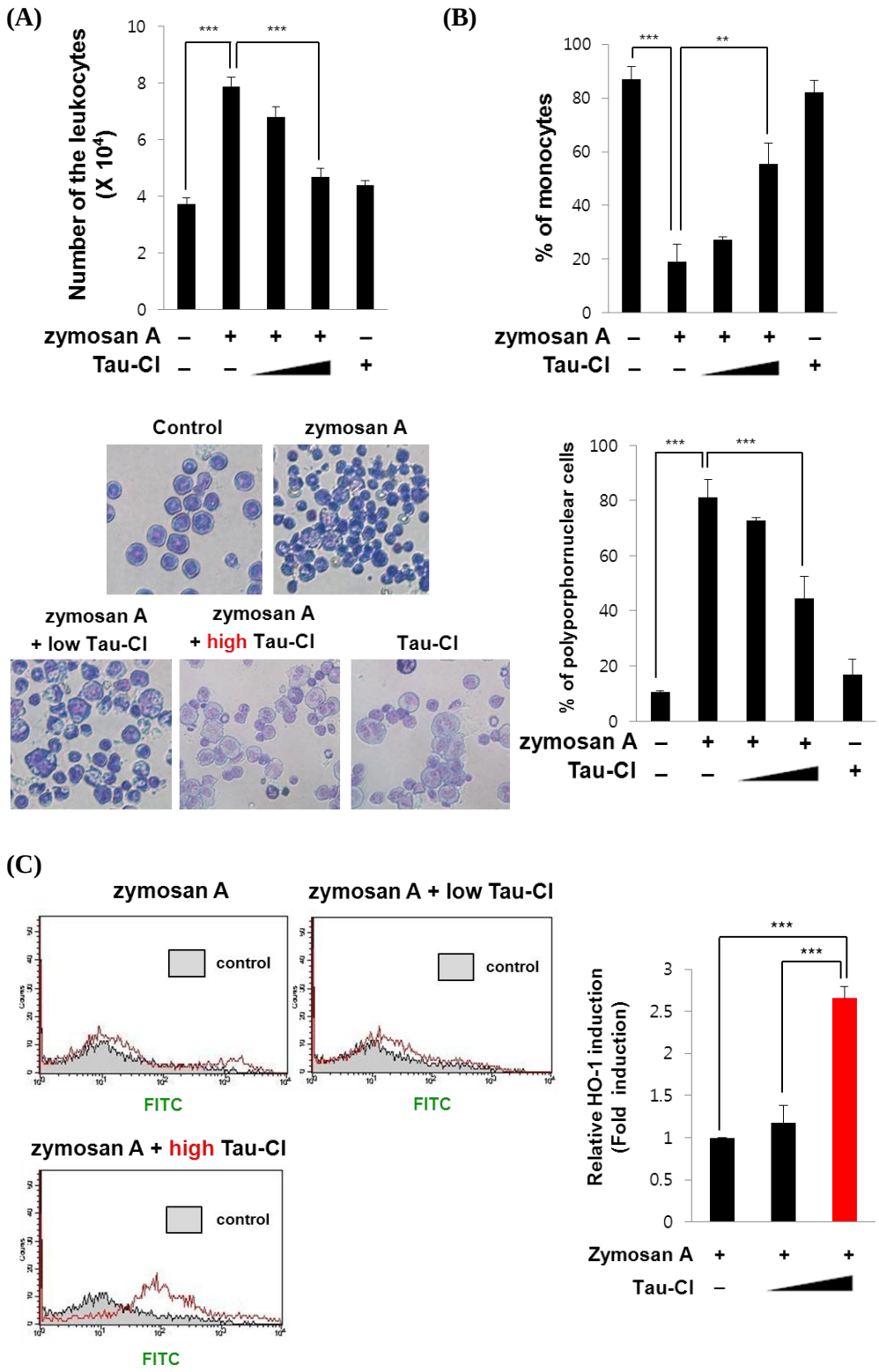

(A)
Number of the leukocytes (X 10⁴)
10
8
6
4
2
0
***
***
zymosan A – + + + –
Tau-Cl – – +
(B)
% of monocytes
100
80
60
40
20
0
***
**
zymosan A – + + + –
Tau-Cl – – +
Control
zymosan A
zymosan A + low Tau-Cl
zymosan A + high Tau-Cl
Tau-Cl
% of polyporphornuclear cells
100
80
60
40
20
0
***
***
zymosan A – + + + –
Tau-Cl – – +
(C)
zymosan A
zymosan A + low Tau-Cl
control
control
FITC
FITC
zymosan A + high Tau-Cl
control
FITC
Relative HO-1 induction (Fold induction)
3
2.5
2
1.5
1
0.5
0
***
***
Zymosan A + + +
Tau-Cl –

Fig 1. TauCl facilitates resolution of inflammation in zymosan A-induced peritonitis. Mice administered with zymosan A (30 mg/kg) for 12 h were treated with vehicle or TauCl (2 or 10 mg/kg) intraperitoneally (ip). Six hours later, peritoneal exudates were collected. (A) The number of total leukocytes in peritoneal exudates was counted. (B) The proportion of mononuclear cells and PMNs in collected peritoneal exudates was determined by differential cell counts. (C) HO-1 expression in peritoneal macrophages with ingested PMNs was analyzed by flow cytometry. All data represent mean ± S.D. (n=3), $^{**}p<0.01$, $^{***}p<0.001$.

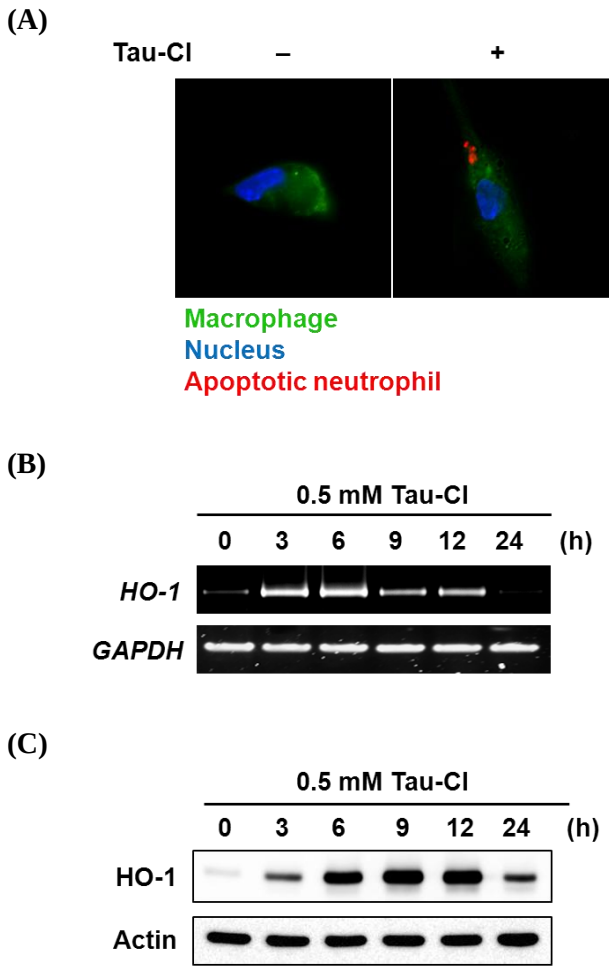


Fig 2. TauCl increases efferocytic ability and HO-1 expression of macrophages. (A) For measuring *ex vivo* efferocytosis, peritoneal macrophages treated with TauCl (0.5 mM) were co-incubated with apoptotic peritoneal neutrophils for 2 h. The engulfment of apoptotic neutrophils by macrophages was detected by immunocytochemistry using anti-F4/80 (green; macrophage marker) and anti-Gr-1 (red; neutrophil marker) antibodies. (B, C) RAW264.7 cells were treated with TauCl (0.5 mM) for indicated time periods. mRNA and protein levels of HO-1 were determined by RT-PCR and Western blot analysis, respectively.

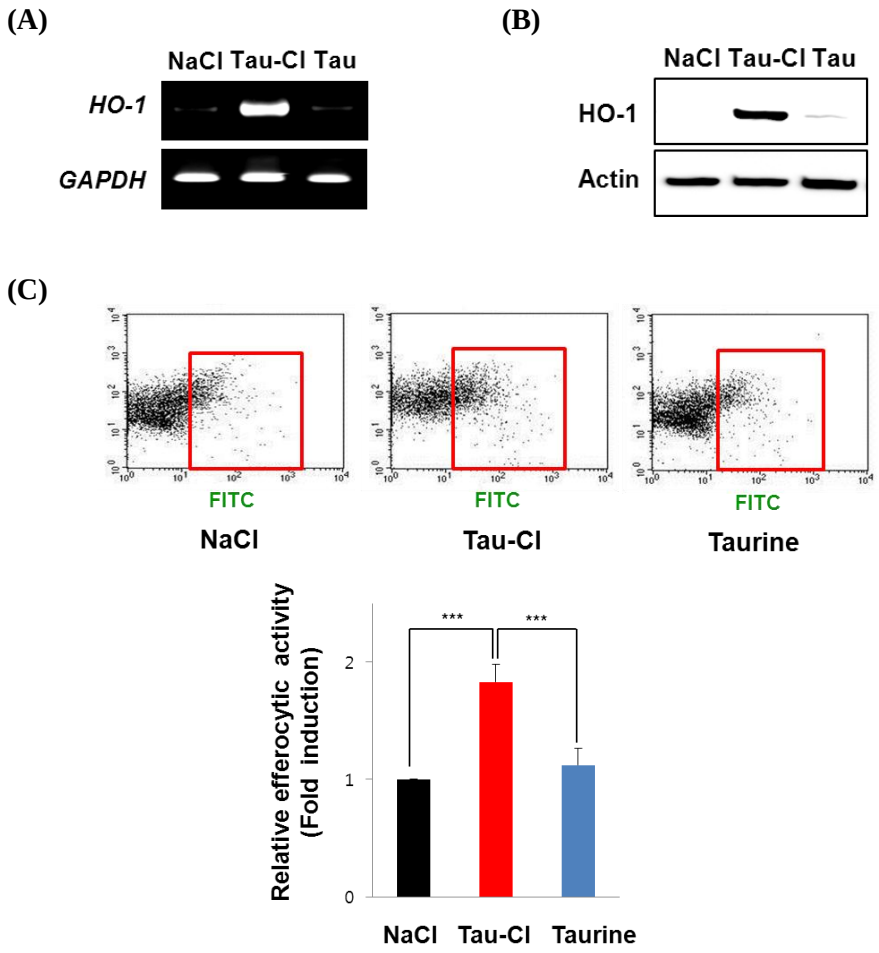


Fig 3. TauCl has higher effect than taurine and NaCl. (A, B) RAW264.7 cells were treated with TauCl, taurine or NaCl for 6 h (A), 9 h (B). mRNA and protein levels of HO-1 were determined by RT-PCR and Western blot analysis, respectively. (C) RAW264.7 cells treated with NaCl (0.5 mM), TauCl or taurine (0.5 mM) were co-incubated with FITC-annexin V stained-apoptotic Jurkat T cells for 1 h. Representative flow cytometric dot plots demonstrate changes in the percentage of macrophages engulfing FITC-annexin V stained-apoptotic Jurkat T cells. All data represent mean ± S.D. (n=3), $^{***}p<0.001$.

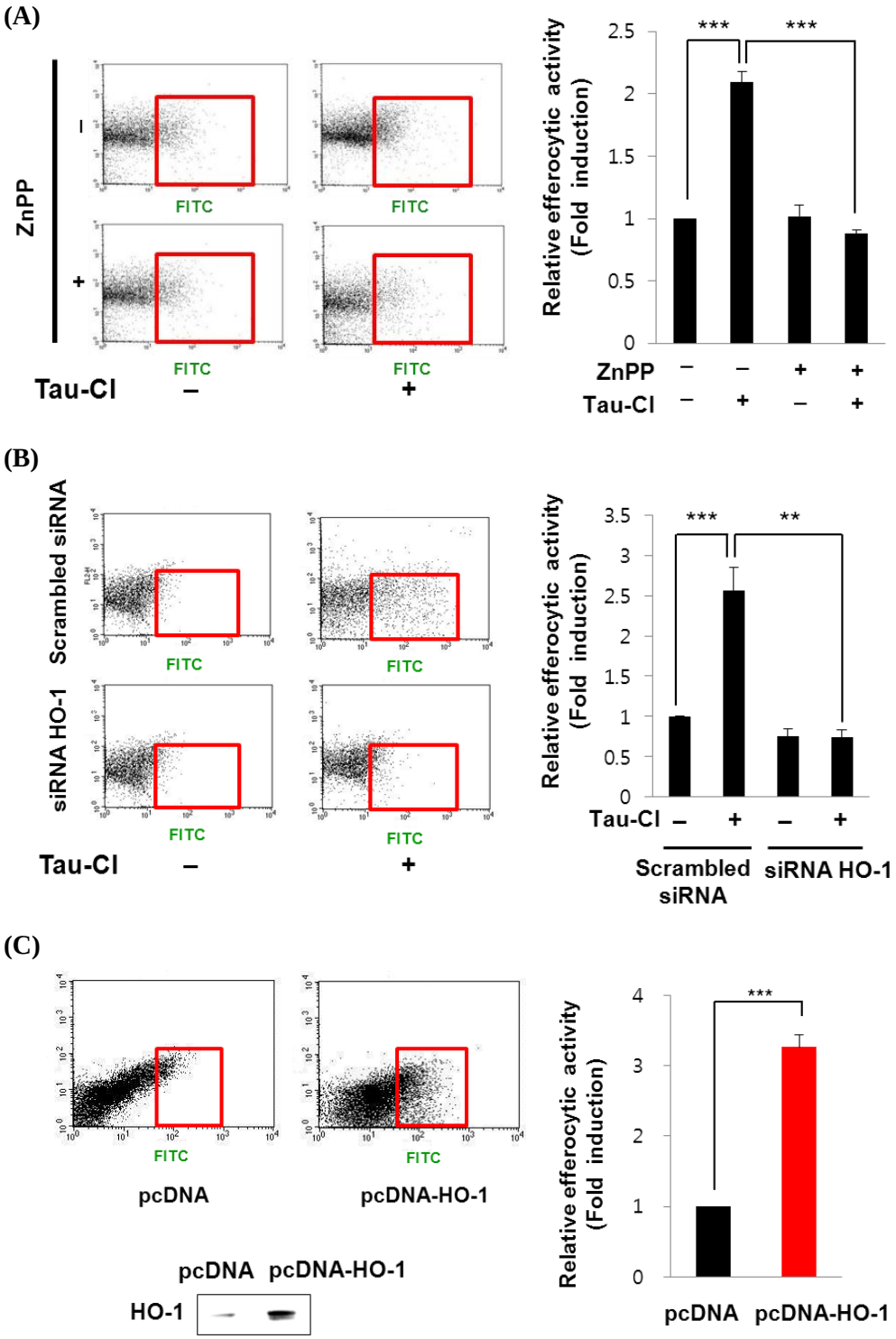

(A)
ZnPP
–
+
FITC
Tau-Cl
–
+
Relative efferocytic activity (Fold induction)
***
***
ZnPP
Tau-Cl
(B)
Scrambled siRNA
siRNA HO-1
FITC
Tau-Cl
**
Scrambled siRNA
siRNA HO-1
(C)
pcDNA
pcDNA-HO-1
HO-1
Actin

Fig 4. HO-1 is critical for stimulating efferocytosis by Tau-Cl. (A-C) RAW264.7 cells were treated with ZnPP (10 μM) for 1 h prior to incubation with TauCl for additional 12 h (A). RAW264.7 cells were transfected with scrambled or *HO-1* siRNA for 16 h, and then treated with TauCl for additional 12 h (B). RAW264.7 cells were transfected with pcDNA-mock or pcDNA-HO-1 for 24 h, followed by incubation with TauCl for additional 12 h (C). The assays for efferocytosis were performed by incubating cells with FITC-annexin V stained-apoptotic Jurkat T cells for 2 h. Representative flow cytometric dot plots demonstrate changes in the percentage of macrophages engulfing FITC-annexin V stained-apoptotic Jurkat T cells. All data represent mean ± S.D. (n=3), $^{**}p<0.01$, $^{***}p<0.001$

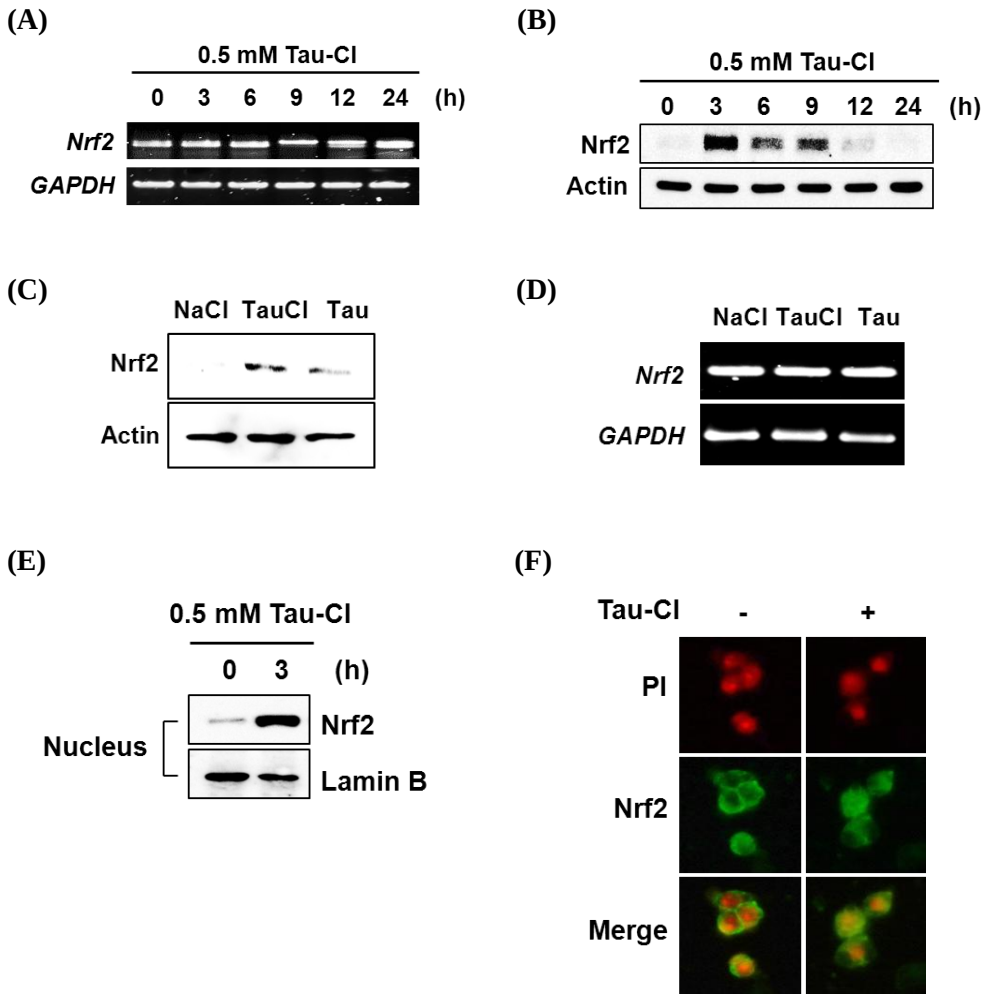


Fig 5. TauCl upregulates nuclear translocation of Nrf2. (A, B) RAW264.7 cells were treated with TauCl (0.5 mM) for indicated time periods. mRNA and protein levels of Nrf2 were determined by RT-PCR and Western blot analysis, respectively. (C, D) RAW264.7 cells were treated with TauCl, taurine or NaCl for 3 h. Protein and mRNA levels of RAW264.7 cells were determined by Western blot and RT-PCR analysis, respectively. (E) RAW264.7 cells were treated with TauCl for 3 h and nuclear translocation of Nrf2 was verified by immunoblot analysis. Lamin B was

used as a loading control for nuclear extracts. (F) Immunocytochemical analysis was performed using anti-Nrf2 after the treatment of RAW264.7 cells with TauCl for 3 h.

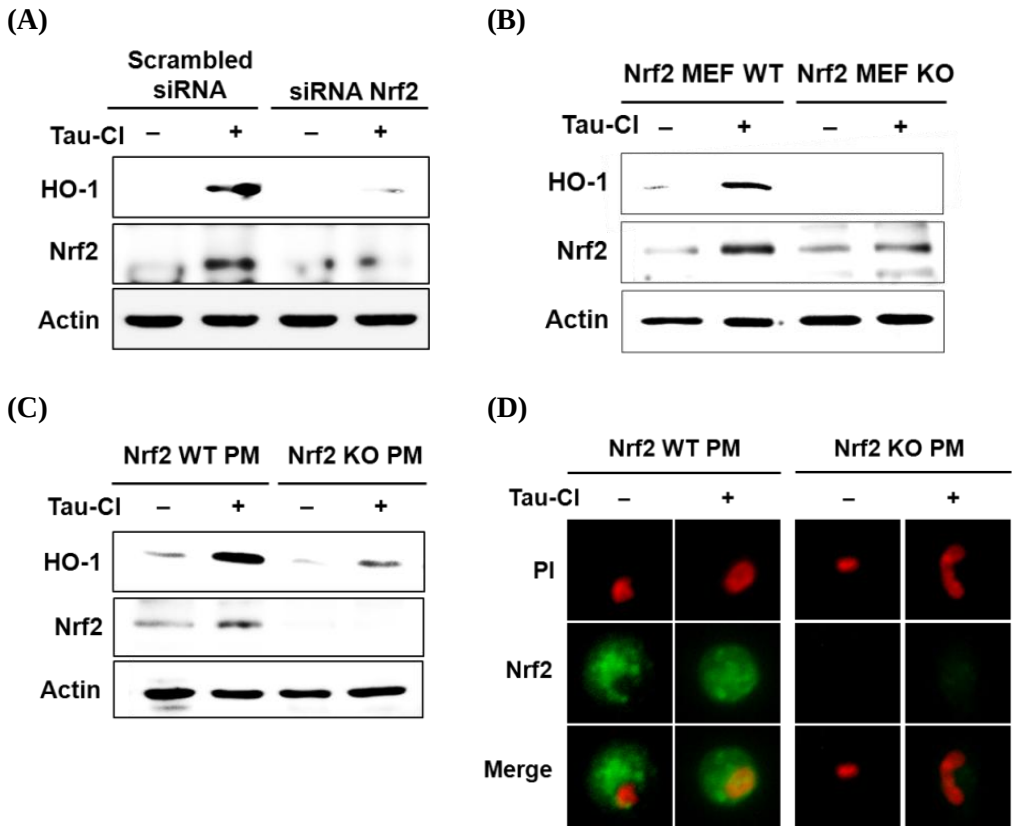


Fig 6. TauCl-induced Nrf2 activation is important for HO-1 expression. (A-C) RAW264.7 cells were transfected with scrambled or *Nrf2* siRNA, and then incubated in the absence or presence of TauCl (0.5 mM) for additional 9 h (A). MEF cells were isolated from Nrf2 wild type and knockout mice, and were treated with TauCl for 9 h (B). Peritoneal macrophages obtained from Nrf2 wild-type and Nrf2 knockout mice were treated with TauCl for 9 h (C). Protein levels of HO-1 and Nrf2 measured by Western blot analysis. Actin was used as an equal loading control for normalization. (D) Peritoneal macrophages were collected from Nrf2 wild-type and Nrf2 knockout mice, and were treated with TauCl for 3 h. Nuclear translocation of Nrf2 was determined by immunocytochemical analysis..

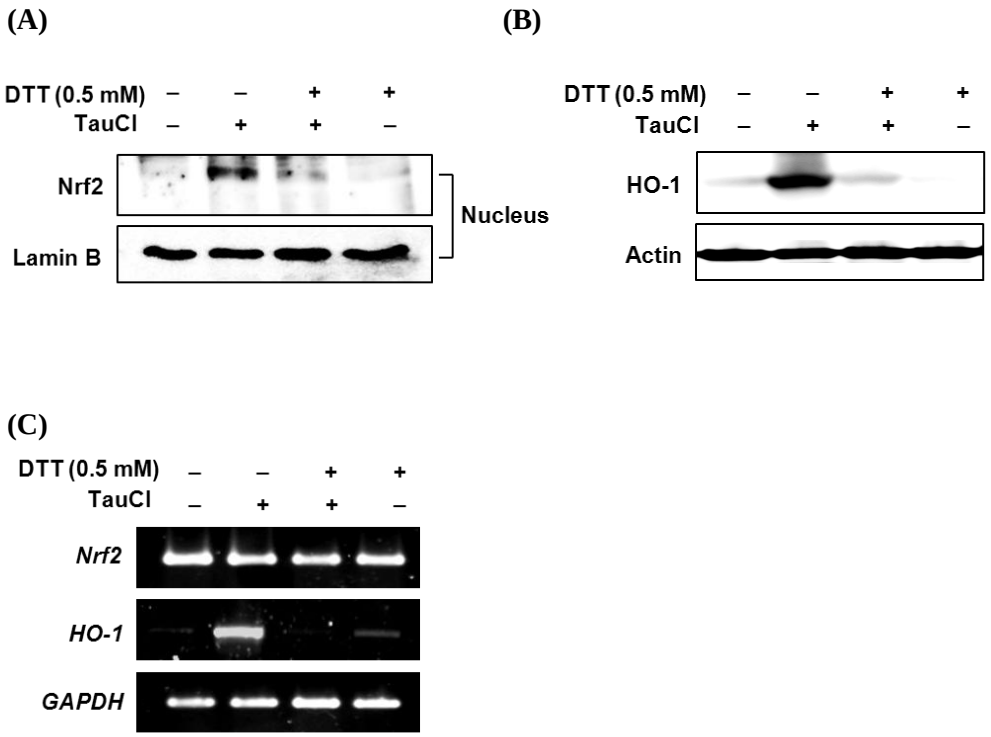


Fig 7. The possible involvement of Keap1 cysteine thiol in TauCl-induced HO-1 expression and Nrf2 translocation. (A, B, C) RAW264.7 cells were treated with DTT (0.5 mM) for 1 h prior to incubation with TauCl (0.5 mM) for additional 3 h (A), 9 h (B), or 6 h (C). The protein levels of Nrf2 (A) and HO-1 (B) were verified by Western blot analysis. Actin and lamin B were included as loading controls for normalization. mRNA level of *Nrf2* and *HO-1* were determined by RT-PCR (C).

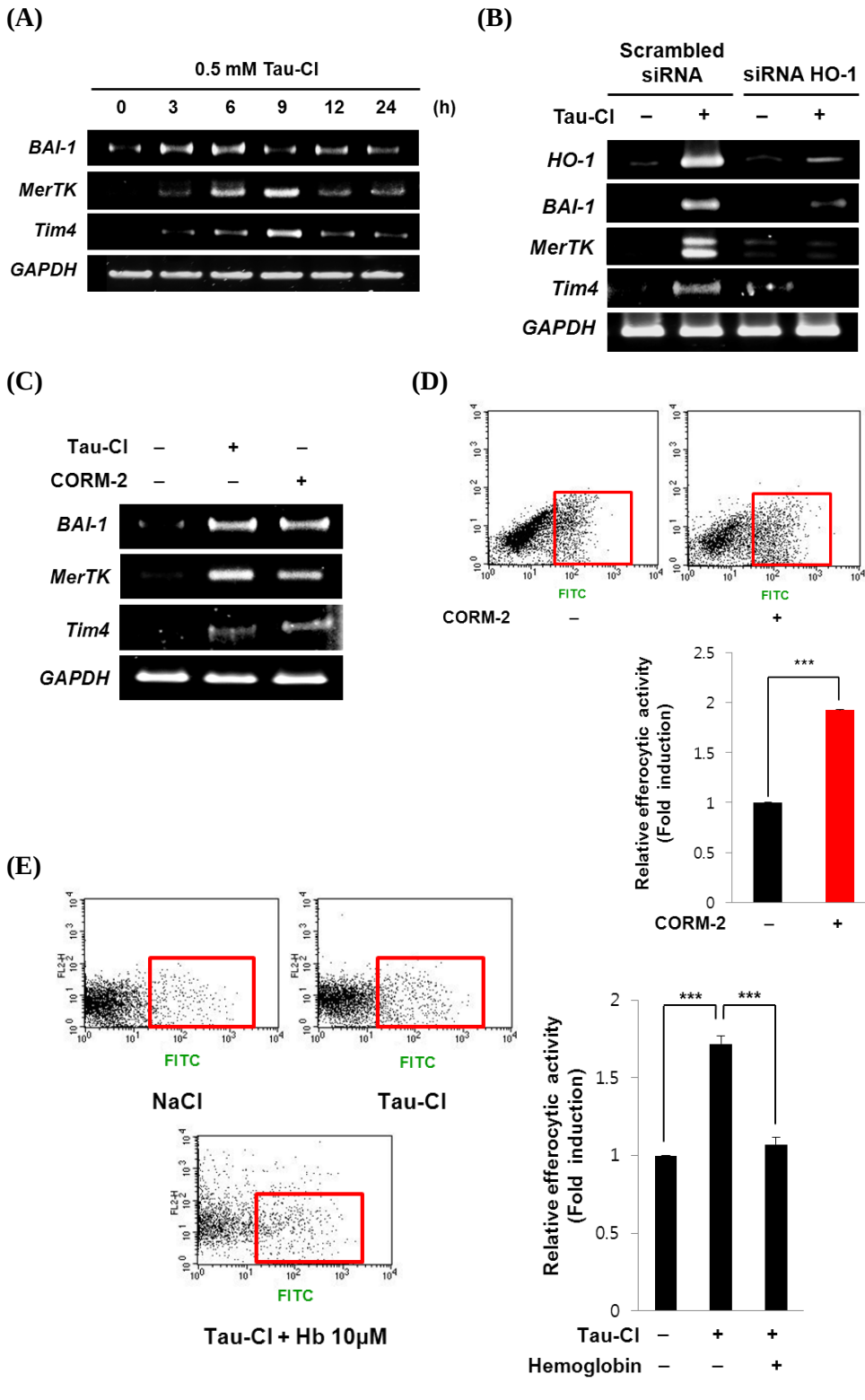

(A)
0.5 mM Tau-Cl
0
3
6
9
12
24
(h)
BAI-1
MerTK
Tim4
GAPDH
(B)
Scrambled siRNA
siRNA HO-1
Tau-Cl – + – +
HO-1
BAI-1
MerTK
Tim4
GAPDH
(C)
Tau-Cl – + –
CORM-2 – – +
BAI-1
MerTK
Tim4
GAPDH
(D)
FITC
FITC
CORM-2 – +
Relative efferocytic activity (Fold induction)
2.5
2
1.5
1
0.5
0
***
CORM-2 – +
(E)
FITC
FITC
NaCl
Tau-Cl
FITC
Tau-Cl + Hb 10μM
Relative efferocytic activity (Fold induction)
2
1.5
1
0.5
0
***
***
Tau-Cl – + +
Hemoglobin – – +

Fig 8. TauCl increases the efferocytic ability of macrophage by upregulating the expression of engulfment receptors for apoptotic cells. RAW264.7 cells were treated with TauCl (0.5 mM) for indicated time periods (A). RAW264.7 cells were transfected with scrambled or *HO*-1 siRNA, and then incubated in the absence or presence of TauCl for additional 9 h (B). RAW264.7 cells were treated with TauCl or CORM-2 (100 μM) for 9 h. The mRNA levels of *BAI-1*, *MerTK*, *Tim4* and *GAPDH* were determined by RT-PCR (C). RAW264.7 cells were treated with CORM-2 for 24 h, were co-incubated with FITC-annexin V stained-apoptotic Jurkat T cells for 2 h (D). RAW264.7 cells containing FITC-annexin V stained-apoptotic Jurkat T cells were assessed by using flow cytometry. RAW264.7 cells, treated with NaCl, TauCl, and TauCl plus hemoglobin (Hb; 10 μM) for 24 h, were co-incubated with FITC-annexin V stained-apoptotic Jurkat T cells for 2 h (E). All data represent mean ± S.D. (n=3), $^{***}p<0.001$.

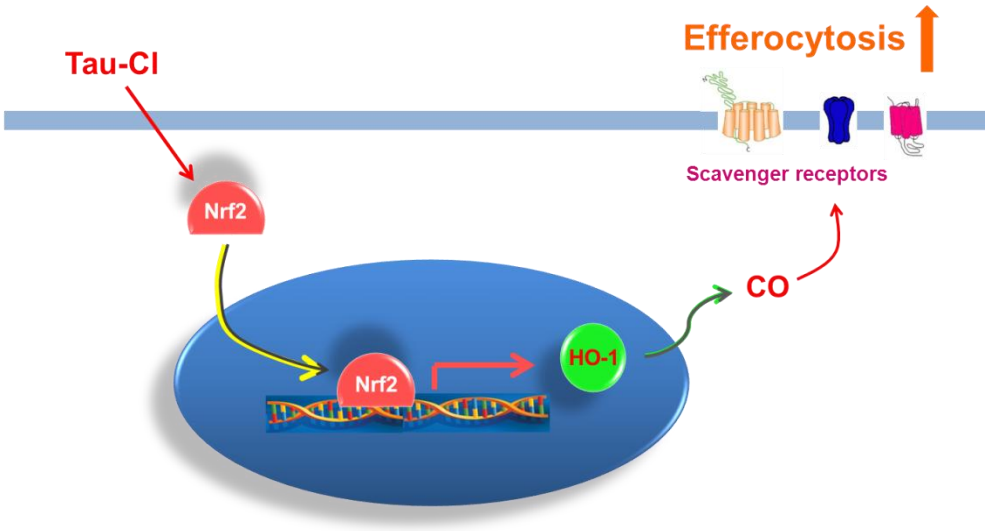
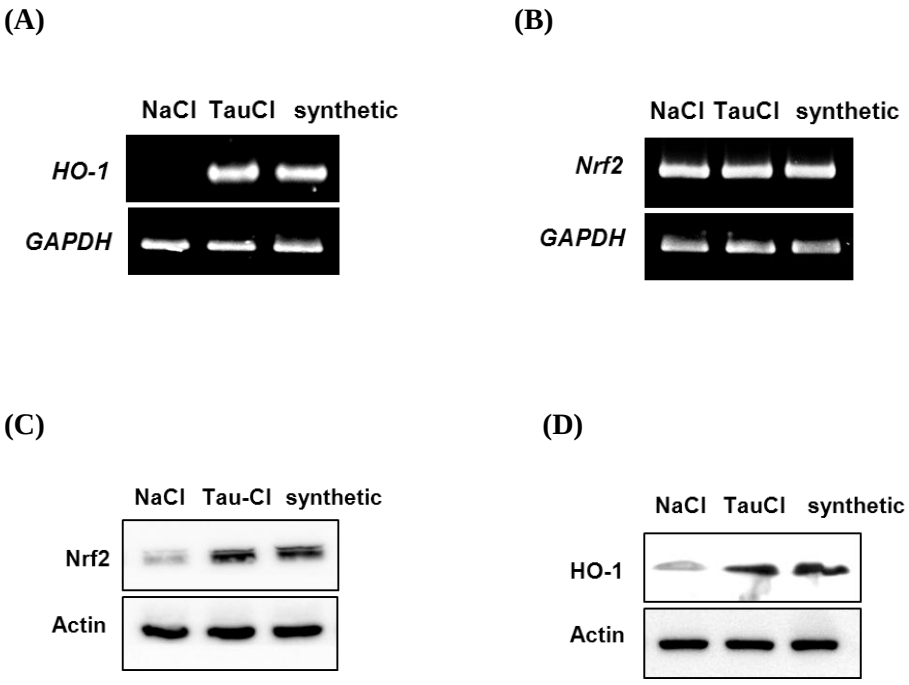


Fig 9. A proposed mechanism underlying the effects of TauCl on resolution of inflammation. TauCl is endogenously produced in neutrophils under inflammatory conditions. During resolution of inflammation, the generation and release of TauCl by activated neutrophils increase efferocytic ability in macrophages through upregulation of HO-1 expression and production of CO.



Supplementary Fig . Equivalent effects of TauCl and its salt form on macrophage. (A-D) RAW 264.7 cells were treated with TauCl (0.5 mM), synthetic TauCl (0.5 mM) and NaCl for 3 h, 6 h or 9 h. mRNA and protein levels of RAW 264.7 cells were determined by RT-PCR and Western blot analysis.

DISCUSSION

Results shown in the present study demonstrate for the first time that TauCl enhances the efferocytic activity of macrophages. Although some amount of TauCl may have been produced and released by the peritoneal neutrophils that have infiltrated into the peritoneum of zymosan-A injected mice, peritoneal injection of additional TauCl potentiated the efferocytic activity of peritoneal macrophages engulfing the apoptotic neutrophils and accelerated the rate of resolving the zymosan-A induced peritonitis. The TauCl-derived enhancement of efferocytic activity of macrophages appeared to result from induction of HO-1 expression, stimulation of HO activity and subsequent overproduction of CO gas as a byproduct. This overproduced CO appeared to serve as a signal molecule that induce reprogramming of genes in macrophages so that expression of scavenger receptors recognizing the surface exposed phosphatidylserine of apoptotic neutrophils is increased, thus enhancing the efferocytic activity of macrophages exposed to TauCl.

TauCl is produced endogenously by the activated neutrophils and is released into the inflammatory milieu as the activated neutrophils undergo apoptosis. The macrophages co-existing with apoptotic neutrophils at the inflammatory milieu are exposed to TauCl. Previously, TauCl has been considered as an end-product of taurine-derived detoxification of hypochlorous acid, a strong antibacterial oxidant produced in activated neutrophils by the myeloperoxidase from the overproduced H_2O_2, which is simply discarded after protecting the cells at the inflammation site

from the toxicity of hypochlorous acid. In previous studies, TauCl has been shown to inhibit the production of pro-inflammatory cytokines in activated macrophages through inhibition of NF-κB pathway, thus providing anti-inflammatory activity. Alternatively, TauCl has also been shown to provide antioxidant and cytoprotective activity in macrophages and other cells by inducing the expression of several antioxidant enzymes (12). The pro-resolving and efferocytic activity of TauCl has been overlooked.

Efferocytosis by macrophages is an essential process in resolving inflammation by engulfing apoptotic neutrophils. Through effective efferocytosis of dying neutrophils, macrophages prevent the release of inflammatory contents from dying neutrophils to the inflammatory milieu and avoid additional pro-inflammatory disruption of other cells at the inflammation site. For effective efferocytosis, the macrophages at the inflammation site undergo genetic reprogramming by activation of nuclear receptors like PPARγ, PPARδ, LXR, and RXRα which promote the expression of surface scavenger receptors that recognize the exposed phosphatidylserine and its oxidized metabolites on the surface of apoptotic neutrophils. As the activated neutrophils undergo apoptosis, the phospholipid phosphatidylserine at the inner leaflet of neutrophil plasma membrane flip-flops and is exposed to the surface and becomes oxidized. The surface scavenger receptors expressed on the macrophages recognize the exposed phosphatidylserine and its oxidized metabolites now exposed on the surface of apoptotic neutrophils and begin the process of efferocytosis. Recognition of apoptotic neutrophils and efferocytosis by macrophages then promotes secretion of anti-inflammatory cytokines like TGF-β

and IL-10 that inhibit the production of inflammatory mediators by the macrophages (5,28). As the TauCl has now been shown to enhance efferocytic activity of macrophages in the present study, it would be highly interesting to examine whether TauCl could also enhance the secretion of these anti-inflammatory cytokines.

It has been reported that induction of HO-1 expression with PPARγ ligands is involved in the resolution of inflammation on *in vitro* and *in vivo* models of chronic obstructive pulmonary disease (15). As the macrophages treated with TauCl were found to induce HO-1 expression (13) and present study confirmed that TauCl increases the efferocytic activity of macrophages through induction of HO-1 expression and overproduction of CO, it is expected that CO may stimulate efferocytosis by increasing the expression of scavenger receptors in the TauCl treated macrophages. In support of this speculation, transcriptional expression of scavenger receptors like BAI-1, MerTK, and Tim4 which are the product resulting from activation of PPARγ circuit (2,17,23) were increased in the macrophages that have been treated with TauCl but exposed to the CO gas being released from CORM. Presumably, the TauCl treated macrophages increased their efferocytic activity through this induction of scavenger receptors promoted by the CO gas overproduced from HO-1 induction. In support of this observation, inhaled CO gas has been shown to accelerate the resolution of inflammation via HO-1 circuits (4). While the expression of aforementioned scavenger receptors that are known to be upregulated by PPARγ activation in macrophages exposed to CO gas, the molecular events associated with CO on the activation of nuclear receptors such as PPARγ,

PPARδ, and LXR have not been examined. Thus, it would be highly interesting to examine whether the CO gas arising from CORM can activate these nuclear receptors in macrophages and this is currently being investigated in association with activation of Rac1 or inhibition of RhoA, the two small Rho GTPases that have opposing roles in regulating efferocytosis. Rac1 is known to enhance but RhoA is known to inhibit efferocytosis (16,21), and thus, the relative balance between these small GTPases play a key role in determining the efferocytic activity of macrophages. In this connection, while preliminary, TauCl appears to activate Rac1 (unpublished observation).

It is well known that expression of HO-1 is regulated by activation of Nrf2, a key redox sensitive transcription factor which is released from Keap1 in the cytoplasm and translocates into nucleus to bind the AREs localized in the promoter region of many antioxidant enzyme genes and stimulates their transcriptional expression, among which the induction of HO-1 expression is most pronounced. Our present study demonstrates that Nrf2 is essential for the TauCl-derived upregulation of HO-1 expression in macrophages. Interestingly, the Nrf2 mRNA level remained constant even while the Nrf2 protein level in the cytoplasm increased upon TauCl treatment. This suggested that TauCl has influence on the stability of Nrf2. It has been demonstrated that increase of Nrf2 level in the cytoplasm requires both the oxidation of critical cysteine thiol residues in Keap1 to release Nrf2 and prevent the Nrf2 from being degraded by proteasomes. In the present study, we found that stabilization of Nrf2 induced by TauCl treatment was abolished when the macrophages were pretreated with DTT, a well-known reducing

agent that prevents oxidation of cysteine thiol residues. This suggested that the cysteine thiols in Keap1 are considered to be targets of oxidation by TauCl. In support of this speculation, TauCl has been shown to target critical cysteines and inactivated creatine kinase and glyceraldehyde-3-phhosphate dehydrogenase more selectively than the highly thiol reactive hypochlorous acid (22). As the chloride ion of TauCl is an excellent leaving electrophile, it may play a decisive role in covalent modification of the cysteine thiol residues in Keap1 which serve as nucleophile. Alternatively, as TauCl is an oxidant that readily oxidizes the reduced glutathione chemically in test tubes (12), it may simply oxidize cysteine thiols in Keap1. Whether TauCl binds covalently to critical cysteine residues in Keap1 or simply oxidizing the cysteine thiol to induce structural modification in Keap1 and thus prevents tethering Nrf2 needs to be clarified.

In summary, unresolved inflammation caused by un-cleared apoptotic neutrophils remaining in the inflammatory environment promotes chronic inflammation that results in many human diseases like arthritis and cancer. Efferocytosis, engulfment and clearing of apoptotic neutrophils by macrophages, is an essential process in resolving inflammation, thus preventing the development of chronic inflammatory diseases. Our study demonstrates that TauCl, produced and released to the inflammatory milieu as the activated neutrophils undergo apoptosis, stimulates the efferocytic activity of the macrophages co-existing with apoptotic neutrophils at the inflammatory site and enhances resolution of inflammation. Our study demonstrates also that the increased efferocytic activity induced by TauCl is accompanied by induction of HO-1 and overproduction of CO gas. In turn, this CO gas

overproduced in the TauCl treated macrophages induces transcriptional expression of scavenger receptors that recognizes the phosphatidylserines that have been exposed as the activated neutrophils undergo apoptosis. This may promote the efferocytic activity of TauCl treated macrophages promoting the resolution of inflammation. As proposed in the schematic diagram (Fig. 8), TauCl released into the inflammatory milieu from apoptotic neutrophils stimulates efferocytosis through Nrf2-mediated upregulation of HO-1 expression. It is evident that the upregulated HO-1 overproducing CO gas is one of the key events required for the increased efferocytic activity enhancing the resolution of inflammation. Thus, TauCl may have therapeutic potential in the management of chronic inflammatory disorders.

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국문초록

염증반응 동안, Taurine Chloramine (TauCl) 은 활성화된 neutrophil 에서 대량 생산되고, 활성화된 neutrophil 은 세포자살을 통해 주변 염증 부위로 방출된다. zymogen A 에 의해 유도된 복막염이 생긴 마우스의 복막으로 TauCl 의 주입은 복강 내 leukocyte 의 수를 상당히 감소시키고 염증 해소를 강화시키며 monocyte 의 침투를 증가시킨다. 또한, 복강 내 삼출물에서 얻은 macrophage 에 TauCl 처리 시, 동일한 삼출물에서 얻은 apoptotic neutrophil 를 식균하는 능력이 증가하였다. 게다가, TauCl 를 처리한 마우스 대식세포 세포주인 RAW264.7 에서 apoptotic Jurkat T 세포를 식균하는 efferocytosis 능력 또한 향상되었고, TauCl 처리한 macrophage 에서 heme oxygenase-1 (HO-1) 의 발현은 Nrf2 의 핵 내 이동이 증가함에 따라 증가하였다. 또한, apoptotic 세포 표면에 노출되는 phosphatidylserince 를 인지하는 scavenger receptor 의 mRNA 발현은 TauCl 를 처리한 RAW264.7 cell 에서 증가하였다. RAW264.7 cell 에서 HO-1 유전자의 knock-down 은 TauCl 에 의해 유도된 efferocytosis 의 활성화를 abolish 했다. 반면, HO-1 유전자의 과발현과 외부로부터 주입된 CO-releasing molecule (CORM) 으로부터 얻어진 HO 의 부산물인 carbon monoxide (CO) 의 처리는 macrophage 의 efferocytosis 능력을 상승시켰다. 또한, TauCl 를 처리

하지 않은 macrophage 를 CORM 에 노출시켰을 때, scavenger receptor 의 mRNA 의 발현이 증가하였다. 따라서, Nrf2 에 의해 조절되는 HO-1 발현 상승과 CO 의 과발현을 통해 TauCl 이 macrophage 의 efferocytosis 능력을 증가시킴으로써 염증 해소를 촉진시킬 것으로 사료된다.

주요어 : TauCl, Resolution of inflammation, Effeocytosis, Heme oxygenase-1, Nrf2, Carbon monoxide, Scavenger receptor

학번 : 2012-21765