ORIGINAL RESEARCH COMMUNICATION

Taurine Chloramine Stimulates Efferocytosis Through Upregulation of Nrf2-Mediated Heme Oxygenase-1 Expression in Murine Macrophages: Possible Involvement of Carbon Monoxide

Wonki Kim,¹ Hoon-Ui Kim,¹ Ha-Na Lee,¹ Seung Hyeon Kim,¹ Chaekyun Kim,² Young-Nam Cha,² Yeonsoo Joe,³ Hun Taeg Chung,³ Jaebong Jang,¹ Kyeojin Kim,¹ Young-Ger Suh,¹ Hyeon-Ok Jin,⁴ Jin Kyung Lee,⁴ and Young-Joon Surh^{1,5,6}

Abstract

Aims: To examine the pro-resolving effects of taurine chloramine (TauCl). **Results:** TauCl injected into the peritoneum of mice enhanced the resolution of zymosan A-induced peritonitis. Furthermore, when the macrophages obtained from peritoneal exudates were treated with TauCl, their efferocytic ability was elevated. In the murine macrophage-like RAW264.7 cells exposed to TauCl, the proportion of macrophages engulfing the apoptotic neutrophils was also increased. In these macrophages treated with TauCl, expression of heme oxygenase-1 (HO-1) was elevated along with increased nuclear translocation of the nuclear factor E2-related factor 2 (Nrf2). TauCl binds directly to Kelch-like ECH association protein 1 (Keap1), which appears to retard the Keap1-driven degradation of Nrf2. This results in stabilization and enhanced nuclear translocation of Nrf2 and upregulation of HO-1 expression. TauCl, when treated to peritoneal macrophages isolated from either Nrf2 or HO-1 wild-type mice, stimulated efferocytosis (phagocytic engulfment of apoptotic neutrophils by macrophages), but not in the macrophages from Nrf2 or HO-1 knockout mice. Furthermore, transcriptional expression of some scavenger receptors recognizing the phosphatidylserines exposed on the surface of apoptotic cells was increased in RAW264.7 cells treated with TauCl. Pharmacologic inhibition of HO-1 activity or knockdown of HO-1 gene in RAW264.7 cells abolished the TauCl-induced efferocytosis, whereas both overexpression of HO-1 and treatment with carbon monoxide (CO), the product of HO, potentiated the efferocytic activity of macrophages. **Innovation:** This work provides the first evidence that TauCl stimulates efferocytosis by macrophages. The results of this study suggest the therapeutic potential of TauCl in the management of inflammatory disorders. **Conclusion:** TauCl can facilitate resolution of inflammation by increasing the efferocytic activity of macrophages through Nrf2-mediated HO-1 upregulation and subsequent production of CO. *Antioxid. Redox Signal.* 23, 163–177.

Introduction

ACUTE INFLAMMATION is an active host response to microbial infections and physical injuries. On encountering

inflammatory insults, neutrophils recruited to the inflamed site are activated and undergo oxidative burst, a critical event in the host defense. This leads to overproduction of reactive oxygen species with which the neutrophils kill and eliminate

¹Tumor Microenvironment Global Core Research Center and Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul, Republic of Korea.

²Department of Pharmacology and Toxicology, College of Medicine, Inha University, Incheon, Republic of Korea.

³Meta-Inflammation Basic Research Laboratory, School of Biological Sciences, University of Ulsan, Ulsan, Republic of Korea.

⁴KIRAMS Radiation Biobank, Korea Institute of Radiological & Medical Sciences, Seoul, Republic of Korea.

⁵Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science, Seoul National University, Seoul, Republic of Korea.

⁶Cancer Research Institute, Seoul National University, Seoul, Republic of Korea.

Innovation

Timely resolution of inflammatory response is important to prevent chronic inflammation that can cause many severe diseases. During inflammation, taurine chloramine (TauCl) is synthesized in tissues under oxidative stress. This study demonstrates for the first time that TauCl stimulates efferocytosis by macrophages, an essential event in the inflammation resolution. The results of this study suggest the therapeutic potential of TauCl in the management of inflammatory disorders.

the infectious pathogens. The activated neutrophils acquire markedly enhanced ability to phagocytose pathogens. Subsequently, the activated neutrophils undergo apoptosis. The apoptotic neutrophils are then removed by the macrophages, which are also recruited at sites of inflammation immediately after the neutrophil infiltration. On phagocytic removal of apoptotic neutrophils by macrophages through a process called "efferocytosis," the inflammation is resolved (26, 27, 35). If the dying neutrophils are not timely cleared, this can lead to chronic inflammation.

Chronic inflammation, resulting from the failure in efferocytosis, has emerged as a critical component in the pathogenesis of many prevalent human diseases, such as arthritis and cancer (14). The process of clearing apoptotic neutrophils is an active process controlled by the distinct set of chemical mediators that are formed endogenously during inflammation. These signaling molecules act as local autacoids that stimulate the pro-resolving action of macrophages.

Taurine, produced by decarboxylation of cysteine, is one of the most abundant free amino acids. It plays important roles in several essential biological processes, such as osmoregulation, membrane stabilization, calcium mobilization, and immunity (15). The concentration of taurine is particularly high in neutrophils that undergo oxidative burst, and hence have to cope with 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) activity of the activated neutrophils in the presence of chloride ion. This results in the generation of taurine chloramine (TauCl), a weak oxidant with mild cytotoxicity. Once the activated neutrophils undergo apoptosis, TauCl is then released and acts as a local autacoid in the inflamed tissues.

While TauCl is known to have a direct bacteriocidal effect (28), it has both anti-inflammatory and antioxidant activities. TauCl released from the activated and apoptotic neutrophils has been shown to inhibit the activation of nuclear factor- κ B (NF- κ B) in macrophages and to abolish the production of pro-inflammatory mediators, such as nitric oxide (NO), tumor necrosis factor alpha (TNF- α), interleukin (IL)-6, and IL-8, thereby exerting the anti-inflammatory effect (21). 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 (18, 19).

Nrf2 is a critical transcription factor involved in the cellular defense against oxidative stress and inflammatory tissue damage. Under normal conditions, Nrf2 is kept in the cytoplasm as an inactive complex with Kelch-like ECH associ-

ation 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 target genes.

Among several antioxidant enzymes and cytoprotective proteins whose expression is regulated by Nrf2, heme oxygenase-1 (HO-1) draws particular attention. HO-1 is the rate-limiting enzyme catalyzing oxidative degradation of free heme to produce carbon monoxide (CO), biliverdin/bilirubin, and free iron (38). CO generated as a consequence of induction of HO-1 expression and increased HO activity can act as a potent anti-inflammatory signaling molecule that inhibits the expression of several pro-inflammatory cytokines and mediators and also exerts antioxidant effects (5, 34). CO produced by upregulated HO-1 or provided exogenously promotes resolution of inflammation (36, 40). However, the molecular mechanisms underlying the pro-resolving effect of CO have yet to be established.

Efferocytosis, which is engulfment of apoptotic neutrophils by macrophages, is essential for the resolution of inflammation. Apoptotic cells have phosphatidylserine exposed on their surface, which serves as the "eat-me" signal and are recognized by several scavenger receptors present on the surface of macrophages (9, 13). Resident tissue macrophages first detect the signals of pathogens and tissue injuries and then release cytokines and mediators for leukocyte recruitment, first, polymorphonucleocytes (PMNs)/neutrophils and then monocytes, which later differentiate into macrophages. Eventually, local autacoids and mediators present in the inflammatory milieu alter genetic programming in the infiltrated macrophages and modulate endocytic functions that promote efferocytosis. In this study, we found that TauCl released from apoptotic neutrophils induced elevated HO-1 expression through Nrf2 activation in macrophages, thereby facilitating the efferocytosis.

Results

TauCl facilitates resolution of inflammation in a 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 *in vivo*, we used the sodium salt form of TauCl prepared according to the method previously reported by Gottardi and Nagl (11). The chemical stability of Na-TauCl is higher than that of TauCl (11, 12). Na-TauCl was injected into the peritoneum at 12 h, when the zymosan A-induced peritoneal inflammation is known to peak. Six hour later, peritoneal exudates were collected, and the number of total leukocytes was counted. The leukocyte counts in the peritoneal exudates obtained from zymosan A plus TauCl-treated mice were decreased markedly compared with those from mice given zymosan A alone (Fig. 1A).

To measure the proportion of PMNs (neutrophils) and monocytes in the peritoneal exudates, differential cell counting was performed. While the total number of peritoneal monocytes was decreased in the mice challenged with zymosan A, the proportion of PMNs was increased (Fig. 1B). TauCl administration restored the monocyte count,

FIG. 1. TauCl stimulates efferocytic activity of apoptotic cells in the zymosan A-induced murine peritonitis model. Mice administered with zymosan A (30 mg/kg) for 12 h were treated with vehicle or a sodium salt form of TauCl (2 or 20 mg/kg) intraperitoneally. 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 counting. **(C)** In a spontaneous resolving zymosan A-induced peritonitis model, the proportion of macrophages engulfing PMNs (F4/80⁺Gr-1⁺) was measured by flow cytometry. **(D)** For measuring efferocytosis *in vivo*, the macrophages from peritoneal exudates of zymosan A-treated mice were collected and co-incubated with the PMNs separated from the same peritoneal exudates. We found that macrophages were attached to the bottom of the culture plate and the neutrophils were present in suspension. After separation of the attached macrophages and nonadherent apoptotic neutrophils, an increased activity of macrophages engulfing apoptotic neutrophils was measured as described in the Materials and Methods section. Peritoneal macrophages treated with TauCl (0.5 mM) were co-incubated with apoptotic peritoneal neutrophils for 1 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. All data represent mean $\pm$ SD ($n=3$), * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. PMNs, polymorphonucleocytes; SD, standard deviation; TauCl, taurine chloramine.

but it attenuated the zymosan A-induced increases in PMN counts (Fig. 1B). Therefore, an intraperitoneal injection of TauCl is considered to facilitate the resolution of zymosan A-induced peritonitis in mice.

In the next experiment, peritoneal macrophages were labeled with the allophycocyanin (APC)-conjugated F4/80 antibody, followed by permeabilization to label the PMNs with the fluorescein isothiocyanate (FITC)-conjugated Gr-1 antibody. Cells with positive staining of both F4/80 and Gr-1 were then selectively determined by flow cytometry. Compared with the mice challenged with zymosan A alone, the mice treated with zymosan A plus TauCl (20 mg/kg) showed a higher proportion of peritoneal macrophages carrying engulfed apoptotic PMNs (F4/80⁺Gr-1⁺) (Fig. 1C). After confirming an increased efferocytic activity of macrophages *in vivo*, we performed an *ex vivo* experiment to more precisely assess the pro-resolving effect of TauCl. For this experiment, we collected the macrophages from peritoneal exudates of zymosan A-treated mice. The isolated peritoneal

macrophages were then treated with TauCl for 18 h followed by co-incubation with apoptotic neutrophils for additional 1 h. The peritoneal macrophages engulfing apoptotic neutrophils (F4/80⁺Gr-1⁺) were stained with FITC-conjugated F4/80 antibody and PE-conjugated Gr-1 antibody, and they were then subjected to immunocytochemical analysis. As illustrated in Figure 1D, TauCl treatment of peritoneal macrophages isolated from zymosan A-challenged mice also engulfed apoptotic PMNs separated from the same peritoneal exudates.

TauCl-induced Nrf2 activation is important for HO-1 expression in RAW264.7 cells and their efferocytic ability

Our previous studies have demonstrated that TauCl induces activation of Nrf2 and expression of its target protein HO-1 in murine peritoneal macrophages *in vivo* (17) as well as in the murine macrophage-like RAW264.7 cell line (19).

These observations prompted us to determine whether the TauCl-induced efferocytic activity of macrophages was attributable to the activation of Nrf2 and subsequent HO-1 expression. Nrf2 and HO-1 levels in peritoneal macrophages were measured by flow cytometry with an FITC-conjugated anti-Nrf2 antibody and a PE-conjugated anti-HO-1 antibody. Mice treated with zymosan A plus TauCl showed an increase in the proportion of peritoneal macrophages with elevated accumulation of Nrf2 (Fig. 2A) and HO-1 (Fig. 2B). These findings suggest that TauCl may facilitate macrophage elimination of the apoptotic neutrophils *via* the Nrf2-HO-1

axis. We also measured the levels of Nrf2 and HO-1 in RAW264.7 macrophages with and without TauCl exposure. TauCl-treated RAW264.7 macrophages exhibited a rapid and transient increase in HO-1 mRNA transcript as determined by real-time polymerase chain reaction (PCR) (Fig. 2C). Consistent with our previous observation (18, 19), HO-1 protein expression was also elevated in the same cell line on TauCl treatment (data not shown). In the TauCl-treated RAW264.7 macrophages, the expression of Nrf2 mRNA barely changed (Fig. 2D), but there was an increased accumulation of Nrf2 protein in the nucleus as measured by immunoblot (Fig. 2E) and nuclear translocation of Nrf2 was examined by immunoblot analysis. Lamin B was used as a specific nuclear protein marker. (F) To verify nuclear translocation of Nrf2, immunocytochemical analysis was conducted using an antibody against Nrf2 after the treatment of RAW264.7 cells or peritoneal macrophages with TauCl for 3 h. Cells were stained with PI and DAPI. Data represent mean $\pm$ SD ($n = 3$), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. All the experiments were conducted with the sodium salt form of TauCl except that for the data in the *left panel* of (F) in which RAW264.7 cells were treated with the free form of TauCl. DAPI, 4',6-diamidino-2-phenylindole; HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor 2; PCR, polymerase chain reaction; PI, propidium iodide.

FIG. 2. TauCl increases expression of Nrf2 and HO-1.

(A, B) The proportions of peritoneal macrophages expressing Nrf2 (A) and HO-1 (B) were determined by flow cytometry. (C, D) RAW264.7 cells were treated with TauCl (0.5 mM) for the indicated time periods. mRNA levels of HO-1 (C) and Nrf2 (D) were determined by real-time PCR. (E) RAW264.7 cells were treated with TauCl for 3 h, and nuclear translocation of Nrf2 was examined by immunoblot analysis. Lamin B was used as a specific nuclear protein marker. (F) To verify nuclear translocation of Nrf2, immunocytochemical analysis was conducted using an antibody against Nrf2 after the treatment of RAW264.7 cells or peritoneal macrophages with TauCl for 3 h. Cells were stained with PI and DAPI. Data represent mean $\pm$ SD ($n = 3$), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. All the experiments were conducted with the sodium salt form of TauCl except that for the data in the *left panel* of (F) in which RAW264.7 cells were treated with the free form of TauCl. DAPI, 4',6-diamidino-2-phenylindole; HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor 2; PCR, polymerase chain reaction; PI, propidium iodide.

and immunocytochemical (Fig. 2F, left panel) analyses. Based on these findings, it is likely that TauCl stabilizes Nrf2 protein. Nuclear translocation of Nrf2 was also verified in macrophages isolated from mouse peritoneal exudates (Fig. 2F, right panel). The increased levels of both Nrf2 and HO-1 were evident not only in murine macrophages but also in human macrophages derived from peripheral blood monocytes after 0.5 mM TauCl treatment (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars). TauCl itself at this concentration did

not cause apoptotic death of human neutrophils (Supplementary Fig. S1B).

It has been previously reported that TauCl-induced HO-1 expression in RAW264.7 cells is mediated through activation of Nrf2 (18, 19). When the transcriptional expression of Nrf2 was knocked down in RAW264.7 cells by transfecting them with small interfering RNA (siRNA) against this transcription factor, TauCl treatment was unable to induce expression of HO-1 as well as Nrf2 (Fig. 3A). We attempted to verify the role of Nrf2 in TauCl-induced HO-1 expression and

FIG. 3. TauCl-induced Nrf2 activation is important for HO-1 expression in macrophages and their efferocytic activity. (A) RAW264.7 cells were transfected with scrambled or *Nrf2* siRNA and then incubated in the absence or presence of 0.5 mM TauCl (sodium salt) for 9 h. (B) Peritoneal macrophages were collected from *Nrf2* wild-type and *Nrf2* knockout mice, and they were treated with TauCl (sodium salt) for 3 h. Nuclear translocation of Nrf2 was determined by immunocytochemical analysis. (C, D) Peritoneal macrophages or MEF obtained from *Nrf2* wild-type and *Nrf2* knockout mice were treated with TauCl (sodium salt) for 9 h. Protein levels of HO-1 and Nrf2 were measured by Western blot analysis. Actin was used as an equal loading control for normalization. Data represent mean $\pm$ SD ($n=3$), * $p<0.05$, ** $p<0.01$. (E) For an *ex vivo* efferocytosis assay, peritoneal macrophages were collected in *Nrf2* wild-type and knockout mice treated with TauCl (sodium salt) for 18 h, and they were co-incubated with apoptotic neutrophils for an additional 1 h. Macrophage ingestion of apoptotic PMNs was determined by immunostaining using anti-F4/80 (green; macrophage marker) and anti-Gr-1 (red; neutrophil marker). A representative fluorescence micrograph shows macrophages (green) and engulfed apoptotic neutrophils (red). Macrophages engulfing apoptotic neutrophils are shown inside the dotted square. MEF, mouse embryonic fibroblasts; siRNA, small interfering RNA.

stimulation of efferocytosis by use of Nrf2 knockout mice. In the peritoneal macrophages isolated from Nrf2 knockout mice, neither cytoplasmic localization nor the nuclear translocation of Nrf2 was detected (Fig. 3B). When the macrophages collected from peritoneal exudates of Nrf2-deficient mice were treated with TauCl, they were unable to induce expression of HO-1 as well as Nrf2 (Fig. 3C). Likewise, the mouse embryonic fibroblasts (MEF) obtained from the Nrf2 gene knockout mice showed abrogation of HO-1 expression when treated with TauCl (Fig. 3D). Notably, peritoneal macrophages from Nrf2-deficient mice exhibited markedly reduced efferocytic activity in terms of engulfment of apoptotic neutrophils, compared with those from Nrf2 wild-type mice (Fig. 3E). These results suggest that TauCl facilitates resolution of inflammation through Nrf2-mediated expression of HO-1.

TauCl appears to directly bind to Keap1, thereby diminishing the association between Nrf2 with Keap1

One of the well-defined mechanisms responsible for an increase of the Nrf2 level in the cytoplasm involves modification of critical cysteine thiol residues in Keap1, which prevents the Nrf2 from ubiquitination and proteasomal degradation. Treat-

ment of RAW264.7 macrophages with dithiothreitol (DTT), a well-known reducing agent, for 1 h before exposure to TauCl abrogated the nuclear translocation of Nrf2 (Fig. 4A) and expression of the HO-1 protein (Fig. 4B) and its mRNA transcript (Fig. 4C). To determine whether TauCl could directly bind to Keap1, we performed a pull-down assay using TauCl-conjugated Sepharose 4B beads with lysates of RAW264.7 macrophages. The data in Figure 4D indicate that TauCl is bound to Keap1 precipitated from RAW264.7 murine macrophages. To further investigate whether the direct binding of TauCl with Keap1 could attenuate the association of the Keap1–Nrf2 transcriptional complex, Nrf2 in lysates from TauCl-treated RAW264.7 cells was co-immunoprecipitated with Keap1 antibody. We found that TauCl suppressed the association of Keap1 with Nrf2 (Fig. 4E). Furthermore, ubiquitination of Nrf2 in TauCl-treated cells was markedly decreased compared with that in the untreated control cells (Fig. 4F).

Pharmacologic inhibition of HO-1 attenuates pro-resolving effects of TauCl

To determine whether the TauCl-induced upregulation of HO-1 could account for its enhancement of efferocytosis by RAW264.7 macrophages, we utilized zinc protoporphyrin

FIG. 4. TauCl appears to directly bind to Keap1, thereby diminishing the association between Nrf2 with Keap1. (A, B) RAW264.7 cells were treated with DTT (0.5 mM) for 1 h before incubation with 0.5 mM TauCl (sodium salt) for additional 3 h (A) or 9 h (B). The protein levels of Nrf2 (A) and HO-1 (B) were measured by Western blot analysis. Data represent mean $\pm$ SD ($n=3$), * $p<0.05$, ** $p<0.01$. (C) The mRNA level of HO-1 was determined by RT-PCR. (D) To determine whether TauCl could directly bind to Keap1, RAW264.7 macrophage cell lysates were incubated with Sepharose 4B beads alone or TauCl-conjugated Sepharose 4B beads, and the pulled-down proteins were analyzed by immunoblotting analysis. (E) To determine whether TauCl could disrupt the Keap1–Nrf2 complex, cell lysates derived from TauCl-treated RAW264.7 macrophages were subjected to an immunoprecipitation assay. (F) To assess the proteasomal degradation of Nrf2 in TauCl-treated RAW264.7 macrophages, the cell lysates were subjected to the ubiquitination assay. DTT, dithiothreitol; Keap1, Kelch-like ECH association protein 1; RT-PCR, reverse transcription–polymerase chain reaction.

IX (ZnPP), a commonly used HO-1 inhibitor (4). To address whether pharmacologic inhibition of HO-1 blocks the pro-resolving effects of TauCl in a zymosan A-induced mouse peritonitis model, ZnPP (25 mg/kg) was administered intraperitoneally at 2 h before the TauCl treatment. TauCl increased the proportion of monocytes while it decreased the proportion of PMNs in the peritoneal exudates during resolution of zymosan A-induced peritonitis, but these alterations induced by TauCl were abrogated by administration of ZnPP (Fig. 5A). The proportion of cells with positive staining of both F4/80 (macrophage marker) and Gr-1 (neutrophil marker) was then determined by flow cytometry. ZnPP pretreatment abolished TauCl-induced stimulation of efferocytic activity of mouse peritoneal macrophages *in vivo* (Fig. 5B).

We then examined whether HO-1 inhibition could suppress the capability of cultured macrophages to carry out

efferocytosis on TauCl treatment. For this purpose, RAW264.7 cells were treated with ZnPP (10 μ M) or vehicle for 1 h before incubation with TauCl (0.5 mM) for an additional 18 h. RAW264.7 cells treated with TauCl were then co-incubated with apoptotic Jurkat T cells labeled with FITC-annexin V for an additional 1 h. Representative flow cytometric dot plots demonstrate changes in the percentage of macrophages engulfing FITC-annexin-V-stained-apoptotic Jurkat T cells. Again, inhibition of the HO-1 activity with ZnPP attenuated the efferocytosis (Fig. 5C) by cultured macrophages as measured by engulfment of apoptotic Jurkat T cells.

To further ensure that the increased HO-1 expression induced by TauCl is responsible for the increased efferocytic activity of macrophages, we transfected the RAW264.7 cells with siRNA against HO-1. siRNA knockdown of HO-1 gene abolished the TauCl-induced efferocytosis (Fig. 6A). In

FIG. 5. Inhibition of HO by ZnPP attenuates pro-resolving effects of Tau-Cl. Mice administered with zymosan A (30 mg/kg) for 12 h were given a single intraperitoneal injection of ZnPP (25 mg/kg) or a vehicle 2 h before the 20 mg/kg of TauCl (sodium salt) treatment. Six hours later, peritoneal exudates were collected. (A) The proportions of mononuclear cells and PMNs in collected peritoneal exudates were determined by differential cell counts. (B) In a zymosan-initiated peritonitis model, the proportion of macrophages ingesting PMNs (F4/80⁺Gr-1⁺) was measured by flow cytometry. (C) RAW264.7 cells were treated with ZnPP (10 μ M) for 1 h before incubation with 0.5 mM TauCl (sodium salt) for an additional 18 h. Cells were then 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. Data represent mean $\pm$ SD ($n = 3$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. FITC, fluorescein isothiocyanate; ZnPP, zinc protoporphyrin IX.

FIG. 6. TauCl-induced HO-1 is critical for stimulating efferocytosis by macrophages. (A) RAW264.7 cells were transfected with scrambled or *HO-1* siRNA for 16 h and then treated with TauCl (sodium salt) for an additional 18 h. (B) RAW264.7 cells were transfected with pcDNA-mock or pcDNA-HO-1 for 24 h. (C) For measuring *ex vivo* efferocytosis, 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. The experimental procedure and other details are described in the Materials and Methods section. Data represent mean $\pm$ SD ($n = 3$), $**p < 0.01$, $***p < 0.001$. pcDNA, plasmid construct DNA; PE, phycoerythrin.

contrast, overexpression of HO-1 further enhanced the efferocytosis mediated by RAW264.7 cells (Fig. 6B). We also verified the involvement of HO-1 in resolution of inflammation by using HO-1 wild-type and knockout mice. Compared with peritoneal macrophages from HO-1 wild-type mice, those from HO-1-deficient mice failed to engulf apoptotic neutrophils (Fig. 6C).

Chlorination of taurine is critical in its generation of the pro-resolving mediator TauCl

Taurine, which accumulates in abundance in the inflammatory cells, especially in neutrophils, protects host cells from self-destruction, which may occur as a consequence of oxidative burst. Taurine reacts with highly reactive and cytotoxic HOCl generated in activated neutrophils by MPO-mediated peroxidation of chloride ions to produce TauCl. We treated RAW264.7 macrophage cells with either taurine or TauCl. In contrast to TauCl, the parent compound taurine failed to induce efferocytosis

(Fig. 7A) and also expression of HO-1 at both transcriptional (Fig. 7B) and translational (Fig. 7C) levels.

We have also examined whether pretreatment with a taurine-enriched diet can ameliorate zymosan A-induced peritonitis. Dietary supplementation of taurine till 4 weeks resulted in slight, but measurable, decreases in the number of total leukocytes and the proportion of PMNs with a small but significant increase in the proportion of monocytes in the zymosan A-induced peritonitis model (Supplementary Fig. S2). As taurine is present at high concentrations in the 5–50 mM range in most tissues, it is speculated that the extra dietary supplementation may provide a marginal contribution to the intracellular pool of this sulfur-containing β -amino acid that is constantly synthesized from cysteine *via* the cysteine sulfinic acid pathway.

TauCl enhances efferocytic activity of macrophages by increasing the expression of scavenger receptors

When neutrophils undergo apoptosis, phosphatidylserine at the inner leaflet of plasma membrane flip-flops is exposed

FIG. 7. Comparison of TauCl and its parent compound taurine for their capabilities to induce efferocytosis and HO-1 expression. (A) RAW264.7 cells treated with 0.5 mM of TauCl or the same concentration of taurine for 18 h 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. (B, C) RAW264.7 cells were treated with TauCl or Taurine for 6 h (B) or 9 h (C). mRNA and protein levels of HO-1 were determined by quantitative real-time PCR and Western blot analysis, respectively. Data represent mean $\pm$ SD ($n = 3$), $*p < 0.05$, $***p < 0.001$. All the experiments were conducted with the free form of TauCl except for the data in Figure 2C in which cells were treated with the sodium salt form of TauCl. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

on the surface, and is also subjected to oxidation. This exposed phosphatidylserine and its oxidized products are recognized by distinct scavenger receptors expressed on the surface of macrophages, and efferocytosis is initiated (9, 13).

Annexin V, commonly labeled with FITC, is used in identifying the apoptotic cells by binding to cell surface expressing phosphatidylserine, an early apoptotic marker. As annexin V can interact with phosphatidylserine, its use in measuring efferocytosis may hamper uptake of apoptotic Jurkat T cells by macrophages. To avoid such a complication, we utilized carboxyfluoresceinsuccinimidyl ester (CFSE) in place of annexin V in labeling the apoptotic Jurkat T cells. The TauCl-stimulated efferocytic activity of RAW264.7 cells engulfing CFSE-labeled apoptotic Jurkat T cells was dampened in the presence of annexin V (Fig. 8A). However, blockade of phosphatidylserine by annexin V could not completely abolish the efferocytic activity. These findings suggest that TauCl-treated macrophages exert the efferocytic activity through a mechanism other than recognition of phosphatidylserine on the surface of apoptotic neutrophils.

The recognition of phosphatidylserine-expressing apoptotic cells by macrophages is effected by phagocytic receptors. In RAW264.7 cells treated with TauCl, the mRNA levels of some representative scavenger receptors, such as brain-specific angiogenesis inhibitor 1 (BAI-1), *c-mer* proto-oncogene tyrosine kinase (MerTK), and T-cell immunoglobulin and mucin-domain-containing molecule 4 (Tim4), were increased (Fig. 8B). This prompted us to examine whether the TauCl-induced HO-1 expression is responsible for the upregulated transcriptional expression of scavenger receptors in RAW264.7 macrophages. TauCl failed to upregulate the transcription of BAI-1, in the HO-1 gene knocked-down macrophages (Fig. 8C). After confirming that TauCl-induced HO-1 expression is associated with the upregulation of BAI-1 transcription, we examined the possible involvement of CO, a byproduct of the HO-1-catalyzed reaction. As shown in Figure 8D, the increased efferocytotic capability of the TauCl-treated RAW264.7 macrophages was inhibited by exposure to hemoglobin (Hb), a well-known scavenger of NO as well as CO. However, we noticed that TauCl treatment did not increase the

production of NO in these cells (Supplementary Fig. S3). Moreover, Hb failed to inhibit the TauCl-induced HO-1 expression (Fig. 8D). Thus, the inhibitory effect of Hb on TauCl-induced efferocytosis is attributable primarily to its binding to CO with a high affinity. Next, we utilized the CO-releasing molecule-2 (CORM-2). When we examined the efferocytosis by RAW264.7 cells exposed to CORM-2, their ability to engulf apoptotic Jurkat T cells was elevated (Fig. 8E). These results show that CO produced as a byproduct of HO-1 in RAW264.7 macrophages is likely to be involved in mediating efferocytosis induced by TauCl.

Discussion

This study demonstrates that TauCl enhances the efferocytosis by macrophages. Although some amount of TauCl may have been produced and released by the neutrophils that infiltrate into the peritoneum of zymosan A-injected mice, a peritoneal injection of exogenous TauCl potentiated the efferocytic activity of peritoneal macrophages engulfing the apoptotic neutrophils. This leads to acceleration of resolution of the zymosan A-induced peritonitis. The TauCl-induced efferocytic activity of macrophages was associated with Nrf2-mediated induction of HO-1 expression, stimulation of HO activity, and subsequent overproduction of CO gas as a byproduct. CO might serve as a signaling molecule that induces reprogramming of genes in macrophages in a way that expression of scavenger receptors recognizing the surface-exposed phosphatidylserine of apoptotic neutrophils is increased.

TauCl is produced endogenously in the activated neutrophils and is released into the inflamed site as the activated neutrophils undergo apoptosis. The macrophages co-existing with apoptotic neutrophils at the inflammatory milieu are exposed to TauCl. Previously, TauCl had been considered an end-product of taurine-mediated detoxification of HOCl, a strong antibacterial oxidant formed in activated neutrophils by the MPO activity. This will protect the cells at the inflammation site from the toxicity of HOCl. However, recent studies suggest that at the site of inflammation, TauCl

FIG. 8. TauCl increases the efferocytic activity of macrophages by upregulating the expression of some scavenger receptors recognizing apoptotic cells. (A) RAW264.7 cells were co-incubated with CFSE-labeled apoptotic Jurkat T cells in the absence or presence of annexin V. After 1 h, the cells were determined by flow cytometry. (B) RAW264.7 cells were treated with 0.5 mM TauCl (sodium salt) for indicated time periods. The mRNA levels of *BAI-1*, *MerTK*, and *Tim4* were determined by real-time PCR. (C) RAW264.7 cells were transfected with scrambled or *HO-1* siRNA and then incubated in the absence or presence of TauCl. The mRNA levels of *BAI-1* were determined by RT-PCR (left panel) and quantitative real-time PCR (right panel). (D) RAW264.7 cells containing FITC-annexin-V-stained-apoptotic Jurkat T cells were detected by using flow cytometry. (E) RAW264.7 cells, treated with TauCl (sodium salt) or TauCl (sodium salt) plus Hb (10 μ M) for 18 h, were co-incubated with FITC-annexin-V-stained-apoptotic Jurkat T cells for an additional 1 h. Data represent mean $\pm$ SD ($n=3$), * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. *BAI-1*, brain-specific angiogenesis inhibitor 1; CFSE, carboxyfluoresceinsuccinimidyl ester; CORM-2, CO-releasing molecule-2; Hb, hemoglobin; MerTK, *c-mer* proto-oncogene tyrosine kinase; *Tim4*, T-cell immunoglobulin and mucin-domain-containing molecule 4. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

functions as a specific signaling molecule that coordinates the generation of inflammatory mediators in macrophages (24). Thus, TauCl has been shown to suppress the production of pro-inflammatory cytokines in activated macrophages through inhibition of the NF- κ B pathway (2, 16).

In addition to having anti-inflammatory activity, TauCl rescues macrophages and other cells from oxidative stress by inducing the expression of several antioxidant enzymes (18). However, the pro-resolving activity of TauCl has been overlooked.

Efferocytosis mediated by macrophages is an essential process in resolving inflammation by engulfing apoptotic neutrophils. Through effective efferocytosis, macrophages prevent the release of cytotoxic waste from dying neutrophils to the inflammatory microenvironment, thereby hampering additional pro-inflammatory disruption of other cells. In mediating efferocytosis, the macrophages at the inflammation site undergo genetic reprogramming by activation of nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR) γ , PPAR δ , LXR, and RXR α (6, 10, 32).

It has been reported that induction of HO-1 expression with PPAR γ ligands is involved in the resolution of inflammation in experimental models of chronic obstructive pulmonary disease (22). We speculate that TauCl potentiates the efferocytic activity of macrophages through activation of PPAR γ , which, in turn, induces expression of scavenger receptors, such as BAI-1, MerTK, and Tim4 (3, 33). Recognition of apoptotic neutrophils and efferocytosis by macrophages then trigger secretion of anti-inflammatory cytokines such as TGF- β and IL-10 that inhibit the production of inflammatory mediators by the macrophages (8, 39). In the context of enhancement of efferocytic activity of macrophages by TauCl demonstrated in this study, it would be worthwhile determining whether TauCl could also enhance the secretion of anti-inflammatory cytokines.

The majority of reports on HO-1 have focused on its cytoprotective role and anti-inflammatory activities (25, 30). It has been reported that M2 macrophages increase HO-1 expression (37). Moreover, CO, the byproduct of HO-1, has been shown to accelerate the resolution of inflammation (7). Thus, it would be highly interesting to examine whether the CO can activate PPAR γ 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. It is considered that Rac1 enhances efferocytosis whereas RhoA suppresses it (23, 29). Therefore, the relative balance between these small GTPases can be an important determinant of the efferocytic activity of macrophages.

It is well known that expression of HO-1 is regulated by activation of Nrf2, a key redox-sensitive transcription factor that is liberated from Keap1 in the cytoplasm and translocates into the nucleus to bind the ARE localized in the promoter region of many cytoprotective genes, including HO-1. This study demonstrates that Nrf2 is essential for the TauCl-induced HO-1 expression in macrophages. Interestingly, the Nrf2 mRNA level remained constant even while the Nrf2 protein level in the cytoplasm increased on TauCl treatment, suggesting that TauCl may stabilize Nrf2.

Under physiologic conditions, Nrf2 is repressed by Keap1, which is a substrate adaptor for the Cullin-3 (Cul3)-dependent E3 ubiquitin ligase machinery. The modification of key sensor cysteine residues present in Keap1 by TauCl may disrupt the Keap1 interaction with the Nrf2 and Cul3 ubiquitin ligase complex. This will hamper degradation of Nrf2 via the Keap1-Cul3-dependent ubiquitination. We found that accumulation of Nrf2 protein induced by TauCl treatment was abolished when the macrophages were pretreated with DTT, a well-known reducing agent that prevents oxidation or covalent modification of cysteine thiol residues.

As a mild oxidant, TauCl is considered to oxidize cysteine containing cellular proteins, including Keap1, which may alter their three-dimensional structures, and consequently alter bio-

logical activities. Thus, TauCl was shown to inactivate creatine kinase and glyceraldehyde-3-phosphate dehydrogenase by oxidizing their cysteine residues, and glutathione (GSH) competed directly with the enzyme thiols for TauCl, thereby protecting against oxidative inactivation (31). In line with this observation, TauCl oxidizes GSH in murine macrophage cells and also in a test tube reaction (18). Besides possible oxidation of Keap1 cysteine thiols, TauCl may directly bind to Keap1. The result of the pull-down assay using TauCl-conjugated Sepharose 4B beads with lysates of RAW264.7 macrophages supports the possibility of Keap1 modification by TauCl. However, additional studies are needed to identify the specific residue(s) of Keap1 as a *bona fide* target of TauCl in its activating Nrf2.

In summary, 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 is one of the key events required for the increased efferocytic activity of macrophages. CO, a byproduct of the HO-1 reaction, is speculated to play a role in mediating TauCl-induced efferocytosis and resolution of inflammation (Fig. 9). Unresolved inflammation caused by uncleared apoptotic cells remaining in the inflammatory environment can result in chronic inflammation implicated in many human diseases such as arthritis and cancer. Efferocytosis,

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 subsequent release of TauCl by activated neutrophils potentiate efferocytic activity of macrophages through Nrf2-mediated upregulation of HO-1 expression. HO-1-derived CO production may mediate efferocytosis during TauCl-induced resolution of inflammation. CO, carbon monoxide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

engulfment and clearing of apoptotic cells by macrophages, is an essential process in resolving inflammation, thus preventing the development of chronic inflammatory diseases. Thus, TauCl that has pronounced pro-resolving as well as anti-inflammatory activity may have a therapeutic potential in the management of chronic inflammatory disorders.

Materials and Methods

Materials

Taurine was purchased from Sigma-Aldrich (St. Louis, MO). TauCl was synthesized freshly on the day of use by adding equimolar amounts of NaOCl (Sigma-Aldrich) to taurine (Sigma-Aldrich). The authenticity of TauCl formation was monitored by UV absorption (200–400 nm). Its sodium salt form (molecular weight 181.57) was prepared, and its purity was checked according to a previously published method (11). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and fetal bovine serum (FBS) were obtained from Gibco RBL (Grand Island, NY). CORM-2 was purchased from Sigma-Aldrich (Milwaukee, MI). DTT, Hb, and anti-actin were purchased from Sigma-Aldrich. Primary antibodies against Nrf2 and lamin B1, siRNAs against Nrf2 and HO-1, and ZnPP were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HO-1 was the product of Stressgen (Ann Arbor, MI), and anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were provided by Zymed Laboratories, Inc. (San Francisco, CA). Polyvinylidene difluoride (PVDF) membranes were supplied from Gelman Laboratory (Ann Arbor, MI). The enhanced chemiluminescent (ECL) detection kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

Cell culture

Murine macrophage RAW264.7 and human lymphoblastic Jurkat T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). RAW264.7 cells and Jurkat T cells were cultured in DMEM and RPMI 1640, respectively, with 10% FBS, 100 μ g/ml streptomycin and 100 U/ml penicillin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Zymosan A-induced peritonitis

Male 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 at 12 h before giving phosphate-buffered saline (PBS) or TauCl (2 or 10 mg/kg, intraperitoneally), and mice were sacrificed 6 h later. Peritoneal leukocytes were harvested by washing with 3 ml of PBS containing 3 mM ethylenediaminetetraacetic acid (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.

Efferocytosis assay

For measuring efferocytosis *in vivo*, exudate cells were labeled with APC-conjugated anti-F4/80-antibody (eBioscience, San Diego, CA), permeabilized with 0.1% Triton X-100, and then labeled with FITC-anti-Gr-1 antibody (eBioscience, San Diego, CA). The macrophages engulfing apoptotic PMNs were analyzed by flow cytometry. To assess the percentage of macrophages engulfing apoptotic PMNs *ex vivo*, mouse peritoneal macrophages were incubated in six-well flat-bottomed microtiter plates for 24 h. Nonadherent cells were collected and incubated for an additional 24 h to induce apoptosis. After washing with medium, adherent monolayer cells were co-incubated for 1 h with apoptotic nonadherent cells. Peritoneal macrophages were stained with the FITC-conjugated anti-mouse F4/80 antibody for 20 min. The labeled cells were permeabilized for 10 min using 0.1% Triton X-100 and were incubated with PE-conjugated anti-mouse Gr-1 (Ly-6G) antibody for 20 min. Macrophages containing neutrophils (F4/80⁺/Gr-1⁺) were visualized under 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 nonengulfed apoptotic Jurkat T cells, RAW264.7 cells were washed thrice 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/cm²) irradiation, followed by incubation for 8 h at 37°C in an atmosphere of 5% CO₂.

Flow cytometry

Cells were fixed with 10% neutral-buffered formalin solution for 30 min at room temperature, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 2% bovine serum albumin (BSA) in PBS for 30 min. Anti-HO-1 antibody, diluted 1:100 in 2% BSA in PBS, was applied overnight at 4°C. After washing with PBS, cells were incubated with FITC-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody diluted at 1:1000 for 1 h. Cells were analyzed using an FACSCalibur™ Flow Cytometer (BD, Franklin Lakes, NJ).

Real-time RT-PCR

Total RNA was isolated from RAW264.7 cells using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To generate complementary DNA (cDNA), 1 μ g of total RNA was reverse transcribed by using murine leukemia virus reverse transcriptase (Promega, Madison, WI). A TaqMan quantitative real-time PCR was used for amplification of cDNA. The primers were prepared from (Taqman probe, respectively): *Nrf2* (Cat 4453320); *HO-1* (Cat 4453320); *BAI-1* (Cat 4448892); *MerTK* (Cat 4453320); *Tim4* (Cat 4448892); and *GAPDH* (Cat 4453320). PCR products were measured by Applied Biosystems (Foster City, CA), and the results were analyzed.

Preparation of nuclear extracts and Western blot analysis

Cells were suspended in 100 μ l of hypotonic buffer A (10 mM HEPES [pH 7.8], 1.5 mM magnesium chloride

[MgCl₂], 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride [PMSF] for 15 min on ice, followed by addition of 1 μ l 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 MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 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°C after determination of the protein concentration using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). 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 Na₂EDTA, 1 mM ethylene glycol tetra-acetic acid [EGTA], 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 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 sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis sample loading buffer and boiled for 5 min. Protein samples were electrophoresed on 7% or 9% SDS–polyacrylamide gel and transferred to PVDF membranes. The blots 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. After three washes with TBST, the blots were incubated with HRP-conjugated secondary antibody diluted at 1:5000 in 3% fat-free dry milk-TBST for 1 h at room temperature. The blots were rinsed again thrice with TBST, and the transferred protein was incubated with the ECL according to the manufacturer's instructions and visualized with LAS400 (Fuji film, Tokyo, Japan).

Immunocytochemical analysis of Nrf2

Cells seeded at 3×10^4 cells per well in an eight chamber were 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% BSA), washed with PBS, and incubated with a diluted (1:100) primary antibody overnight at 4°C . 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 (PI) for 5 min, and examined under a confocal microscope (Nikon).

Preparation and culturing of MEF

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 day 13.5 after pairing under aseptic conditions. The embryo bodies were minced into small pieces, cultured in high-glucose DMEM supplemented with 10% FBS, and maintained at 37°C with 5% CO₂.

Preparation of esculetin-Sepharose 4B beads

To activate Sepharose 4B beads, TauCl and Sepharose 4B powder (0.3 g) were suspended in 1 mM HCl. Then, the coupled solution (0.1 M NaHCO₃, pH 8.3, and 0.5 M NaCl) was added and rotated overnight at 4°C . The mixture was washed with coupling buffer and transferred to 0.1 M Tris-HCl buffer (pH 8.3). The excess of uncoupled TauCl was removed by washing with 0.1 M acetate buffer (pH 4.0) and 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl.

Cell-based pull-down assay

Proteins (500 mg) of RAW264.7 cells extracted with reaction buffer were mixed with Sepharose 4B beads (as a negative control) or TauCl-Sepharose 4B beads (100%) in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 mg/ml BSA, 0.2 mM PMSF, and 1 $\times$ protease inhibitor mixture). After incubation with gentle rocking overnight at 4°C , the beads were washed five times with buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF), and binding was detected by Western blotting.

Immunoprecipitation assay

RAW264.7 cells were treated with the indicated concentration of TauCl for 3 h and then disrupted with lysis buffer (50 mM Tris-HCl, pH 8, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 10% glycerol, and 1 $\times$ protease inhibitor cocktail). Total protein (500 mg) was subjected to immunoprecipitation by shaking with Keap1 primary antibody at 4°C for 2 h followed by the addition of protein G-agarose bead suspension (25% slurry, 20 ml) and shaking for another 2 h at 4°C . After centrifugation at 3000 rpm for 30 s, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. The immunoprecipitate was then resuspended in 40 ml of 2 $\times$ SDS electrophoresis sample buffer and boiled for 3 min. Supernatant (20 ml) from each sample was collected by centrifugation and loaded on SDS–polyacrylamide gel.

Ubiquitination assay

For ubiquitination of Nrf2 *in vitro*, RAW264.7 cells were treated with the indicated concentration of TauCl for 3 h, and then the cells were disrupted with lysis buffer (50 mM Tris-HCl, pH 8, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 10% glycerol, and 1 $\times$ protease inhibitor cocktail). Total protein (500 mg) was subjected to immunoprecipitation with Nrf2 primary antibody at 4°C for 2 h followed by the addition of protein G-agarose bead suspension (25% slurry, 20 ml) and shaking for another 2 h at 4°C . After centrifugation at 3000 rpm for 30 s, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. The immunoprecipitate was then resuspended in 40 ml of 2 $\times$ SDS electrophoresis sample buffer and boiled for 3 min. Supernatant (20 ml) from each sample was collected by centrifugation and loaded on SDS–polyacrylamide gel.

Statistical analysis

All data were expressed as means $\pm$ 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 $***p < 0.001$.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Prof. Young-Joon Surh
Tumor Microenvironment Global Core Research Center
College of Pharmacy
Seoul National University
1 Gwanak-Ro
Gwanak-Gu
Seoul 151-742
Republic of Korea

E-mail: surh@snu.ac.kr
surhyoungjoon@yahoo.co.kr

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Abbreviations Used

APC = allophycocyanin
ARE = antioxidant response element
BAI-1 = brain-specific angiogenesis inhibitor 1
BCA = bicinchonic acid
BSA = bovine serum albumin
cDNA = complementary DNA
CFSE = carboxyfluoresceinsuccinimidyl ester
CO = carbon monoxide
CORM-2 = CO-releasing molecule-2
Cul3 = cullin-3
DAPI = 4',6-diamidino-2-phenylindole
DMEM = Dulbecco's modified Eagle's medium
DTT = dithiothreitol
ECL = enhanced chemiluminescent
EDTA = ethylenediaminetetraacetic acid
EGTA = ethylene glycol tetra-acetic acid
FACS = fluorescence activated cell sorting
FBS = fetal bovine serum
FITC = fluorescein isothiocyanate
GSH = glutathione
Hb = hemoglobin
HEPES = 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HO-1 = heme oxygenase-1
HOCl = hypochlorous acid
HRP = horseradish peroxidase
H₂O₂ = hydrogen peroxide
IgG = immunoglobulin G
IL = interleukin
KCl = potassium chloride
Keap1 = Kelch-like ECH association protein 1
LPS = lipopolysaccharide
LXR = liver X receptor
MEF = mouse embryonic fibroblasts
MerTK = c-mer proto-oncogene tyrosine kinase
MgCl₂ = magnesium chloride
MPO = myeloperoxidase
NF- κ B = nuclear factor-kappa B
NO = nitric oxide
Nrf2 = nuclear factor E2-related factor 2
PBMC = peripheral blood monocytes
PBS = phosphate-buffered saline
pcDNA = plasmid construct DNA
PCR = polymerase chain reaction
PE = phycoerythrin
PI = propidium iodide
PMNs = polymorphonucleocytes
PMSF = phenylmethylsulfonyl fluoride
PPARs = peroxisome proliferator-activated receptors
RT-PCR = reverse transcription-polymerase chain reaction
RXR = retinoid X receptor
SD = standard deviation
SDS = sodium dodecyl sulfate
siRNA = small interfering RNA
TauCl = taurine chloramine
TGF- β = transforming growth factor-beta
Tim4 = T-cell immunoglobulin and mucin-domain-containing molecule 4
TNF- α = tumor necrosis factor alpha
ZnPP = zinc protoporphyrin IX