Taurine chloramine protects RAW 264.7 macrophages against hydrogen peroxide-induced apoptosis by increasing antioxidants

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Taurine chloramine is the major chloramine generated in activated neutrophils via the reaction between the overproduced hypochlorous acid and the stored taurine. Taurine chloramine has anti-inflammatory and cytoprotective effects in inflamed tissues by inhibiting the production of inflammatory mediators. Taurine chloramine increases heme oxygenase activity and also protects against hydrogen peroxide (H_2O_2)-derived necrosis in macrophages. In this study, we examined further whether taurine chloramine could protect RAW 264.7 macrophages from apoptosis caused by H_2O_2. Macrophages treated with 0.4 mM H_2O_2 underwent apoptosis without showing immediate signs of necrosis, and the cells pre-treated with taurine chloramine were protected from the H_2O_2-derived apoptosis. Taurine chloramine increased heme oxygenase-1 expression and heme oxygenase activity. The taurine chloramine-derived upregulation of heme oxygenase-1 expression was blocked by inhibition of ERK phosphorylation. Taurine chloramine decreased cellular glutathione (GSH) levels initially, but the GSH level increased above the control level by 10 h. Taurine chloramine also increased catalase expression and protected macrophages from the apoptotic effect of H_2O_2. Combined, these results indicate that the taurine chloramine, produced and released endogenously by the activated neutrophils, can protect the macrophages in inflamed tissues from the H_2O_2-derived apoptosis not only by increasing the expression of cytoprotective enzymes like heme oxygenase-1 and catalase, but also by increasing the intracellular antioxidant GSH level.

Key Words: taurine chloramine, hydrogen peroxide, apoptosis, heme oxygenase 1, antioxidant

R eactive oxygen species (ROS) like superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are generated in all aerobic cells, moderately under physiological condition and excessively under pathological conditions such as during inflammation. Low levels of H_2O_2 are reduced efficiently to H_2O by cytosolic glutathione peroxidase (GPx). In contrast, high level H_2O_2 produced during inflammation causes extensive oxidation of glutathione (GSH) to GSSG and limits cytosolic GPx activity, shifting the reduction process to peroxisomal catalase activity. Under such oxidative stress condition, thiol residues in heme-containing proteins are oxidized and heme is released free. The released free heme then catalyzes Fenton reaction to produce highly toxic hydroxyl radical from H_2O_2. The hydroxyl radical then causes cytotoxic degradation of most biological macromolecules i.e., peroxidation of lipids, oxidation of thiols in proteins, and oxidative damage of DNA.[1] Thus, the overproduced H_2O_2 and the free heme released by inflammatory oxidative stress must be cleared quickly by increasing GSH, GPx, catalase and heme oxygenase (HO) activity for cells to survive from the inflammation-derived cytotoxicity.

Taurine is generated from cysteine and is one of the most abundant free amino acids stored in all mammalian monocytes and tissues.[2,3] Taurine protects activated neutrophils from inflammatory injury by detoxifying the highly oxidizing hypochlorous acid (HOCl/OCl⁻) produced from H_2O_2 by its myeloperoxidase (MPO) system.[4] Taurine removes excessive HOCl by reacting readily with HOCl/OCl⁻ and forms taurine chloramine (TauCl), which is much more stable and weaker oxidant. After the neutrophils have cleared the invaded bacteria, they undergo apoptosis and release TauCl to the surrounding macrophages and inflammatory tissues. Neutrophil apoptosomes and debris are then phagocytosed by the macrophages. The macrophages in turn become activated and produce inflammatory mediators that prolong the inflammatory process. However, the released TauCl from apoptotic neutrophils appears to enhance the resolution of inflammation by suppressing the macrophage-derived overproduction of inflammatory mediators.[5–7] In particular, TauCl inhibits the macrophage-derived overproduction of $O_2^{\bullet-}$ and NO in a dose dependent manner without causing significant cytotoxicity to macrophages.[7–9] While TauCl inhibits the macrophage-derived overproduction of oxidants and pro-inflammatory mediators, it can also protect the macrophages against necrosis caused by the high-dose H_2O_2 produced abundantly in inflamed tissues. TauCl does this by inducing anti-oxidant enzymes like peroxiredoxin (Prx)-1, thioredoxin (Trx)-1, and HO-1 via activation of the redox-sensitive transcription factor, Nrf 2.[10] To extend this anti-necrotic effect of TauCl, we investigated whether TauCl could also protect RAW 264.7 cells from H_2O_2-derived apoptotic cell death.

HO is a microsomal enzyme that catalyzes the first and rate-limiting step in the oxidative degradation of the released free heme and yields equimolar amounts of carbon monoxide (CO), iron, and biliverdin.[11] In most aerobic cells, the HO activity is derived from two isozymes, the constitutively expressed HO-2 and the stress inducible HO-1. Increased HO-1 expression occurs in aerobic cells in response to oxidative stimuli and also to the increased level of free heme. HO activity is elevated upon induction of HO-1 expression and this contributes to the adaptive survival against excessive production of hydroxyl radical by removal of free heme. Thus, increased HO-1 expression and elevated HO activity can accelerate the elimination of potentially toxic free heme, and at the same time, provide the antioxidants (bile pigments) and CO to counteract the ROS-derived cytotoxicity. Thus, cells with high level HO-1 expression had markedly reduced cytotoxicity

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when exposed to oxidants that cause severe oxidative stress.[12,13]

H_2O_2 is an oxidant generated continuously in all aerobic cells and is overproduced during inflammation. As H_2O_2 has no charge, it can pass through membrane easily and thus extracellular H_2O_2 is detected easily in inflammatory tissues. During membrane passage, the hydrophobic phospholipids within the lipid bilayer undergo peroxidation to generate negatively charged hydrophilic hydroperoxides, which then flip to face the hydrophilic outer environment. Overproduction of these hydroperoxides occurs during inflammation and apoptosis is initiated.[14–16] Macrophages near the activated neutrophils in the inflamed tissues are exposed to high levels of both H_2O_2 and TauCl. Thus, we examined the effect of TauCl on H_2O_2-decomposing systems in macrophages exposed to H_2O_2.

In this study, we have examined whether TauCl could enhance the rate of heme oxidation and H_2O_2 decomposition through elevation of HO-1 and catalase levels and thus protect macrophages from H_2O_2-derived apoptosis. We found that TauCl protects RAW 264.7 macrophages against the H_2O_2-induced apoptosis by up-regulating HO-1 and catalase expression together with elevation of GSH level.

Materials and Methods

Antibodies and reagents. Dulbecco's modified eagle medium (DMEM) was purchased from Welgene (Seoul, Korea). Characterized fetal bovine serum (FBS) was bought from Hyclone (Logan, UT). MTT reagent 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Calbiochem (Darmstadt, Germany). Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) was purchased from Roche (Mannheim, Germany). Anti-mouse HO-1 antibody was purchased from Stressgen (Victoria, Canada). Horseradish peroxidase-conjugated mouse-IgG antibody was obtained from BD Pharmingen (San Diego, CA). Other chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise stated.

TauCl was synthesized freshly on the day of use by adding equimolar amounts of NaOCl (Aldrich Chemical, Milwaukee, MI) to taurine. The authenticity of TauCl formation was monitored by its UV absorption (200–400 nm).[17]

RAW 264.7 cell culture. RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% heat-inactivated FBS in an incubator maintained at 37°C in humidified air containing 5% CO_2.

Cell viability test by MTT assay. Cells (1×10^4/well) were plated into 96-well plate and incubated for 24 h to assess cytotoxicity caused by H_2O_2. MTT (0.25 mg/ml) dissolved in DMEM was added to each well, and after 2 h incubation, media were aspirated and 100 μl DMSO was added. The absorbance of DMSO-dissolved MTT crystals was read at 550 nm with a VersaMax microplate reader and analyzed by Softmax software (Molecular Devices, Sunnyvale, CA).

Apoptosis detection by staining with propidium iodide (PI) and annexin V-FITC. The proportion of cells undergoing necrosis or apoptosis was determined by flow cytometry using PI and annexin V staining. Cells were diluted to 5×10^6/ml in PBSG (PBS with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose) and then stained with 3 μl of PI (250 ng/ml) and 10 μl of annexin V-FITC (BD Pharmingen, Cat No. 556420). After shaking for 15 min at room temperature in the dark, cells were centrifuged and washed twice with PBSG and then resuspended in 500 μl of PBS. Samples were analyzed on FACScan cytometer (BD Bioscience, San Jose, CA) using CellQuest software (BD Bioscience).

Western blot analysis. RAW 264.7 cells were treated with TauCl and washed with ice-cold PBS. Cells were lysed in the presence of ice-cold lysis buffer containing 20 mM Tris-Cl,

pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μM leupeptin, 20 μg/ml chymostatin, and 2 mM phenylmethanesulphonyl fluoride (PMSF). Protein concentrations in the lysates were quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Equal amounts of lysate proteins were mixed with 5× Laemmli sample buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane immersed in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20) containing 6% non-fat dried milk to block the nonspecific binding. Protein bands were developed using the ECL method (Amersham, Arlington Heights, IL).

Heme oxygenase activity. HO activity was determined as described previously, with some modifications.[18] RAW 264.7 macrophages were scrape in the presence of 1 ml of cold homogenization buffer containing 30 mM Tris HCl (pH 7.5), 0.25 mM sucrose, 0.15 M NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 1 mM PMSF. Cells were sonicated on ice for 2 min at 2 watts output using the Vibracell ultrasonic processor (Sonics & Materials, Newtown, CT) and centrifuged to obtain the microsomal pellet. The microsomal pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4), containing 2 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 1 mM PMSF. This microsomal suspension was added to the reaction mixture containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 20 μM hemin, and 2 mg of rat-liver cytosol in 100 mM potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h in the dark at 37°C and then HO reaction was terminated by placing the reaction tubes on ice. The amount of bilirubin produced was determined by calculating its concentration from the difference in absorbance between 464 and 530 nm (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). HO activity was expressed as nano moles of bilirubin formed by 1.0 mg of microsomal protein per hour.

Total GSH level. RAW 264.7 cells were washed twice with cold PBS (pH 7.4) and scraped after adding 600 μl scraping solution that contained 5% 5-sulfosalicylic acid and 0.2% Triton X-100. Suspended cells were freeze-thawed three times and then sonicated on ice for 5 sec at 2 watts output using the Vibracell ultrasonic processor. After centrifugation at 20,000 × g for 15 min at 4°C, the acidic supernatant was extracted. Protein concentrations were quantified using the BCA protein assay kit. The total glutathione levels (reduced and oxidized glutathione) were determined as described.[19] The reaction mixture (200 μl) contained 0.1 M sodium phosphate buffer (pH 7.4), 5.3 mM EDTA, 0.6 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.2 mM NADPH, 0.5 unit of glutathione reductase, and 10 μl of the supernatant. The rate of 5-thio-2-nitrobenzoic acid (TNB) formation from DTNB is proportional to the total amount of glutathione. The rate of TNB production was measured at 405 nm for 6 min at 30-s intervals using a VersaMax microplate reader at 30°C.

Statistical analysis. Statistical analyses were performed using the two-tailed Student's t test. Data are expressed as the mean ± SD, and $p<0.05$ was considered statistically significant.

Results

H_2O_2 induces apoptosis of RAW 264.7 cells. RAW 264.7 cells were exposed to H_2O_2 and the resulting apoptosis was measured using annexin V staining. The extracellular annexin V binds preferentially to the negatively charged and peroxidized phospholipids. RAW 264.7 cells were treated with H_2O_2 (0, 0.05, 0.1, 0.2, 0.5, 1.0 mM) for 12 h and they showed signs of apoptosis at doses higher than 0.2 mM (Fig. 1A, annexin V staining). Cells exposed to 0.5 mM and 1.0 mM H_2O_2 for 12 h showed 14% and 55% cell death, respectively. Most of the cell death caused by 12 h exposure to 0.5 mM H_2O_2 was due to apoptosis rather than
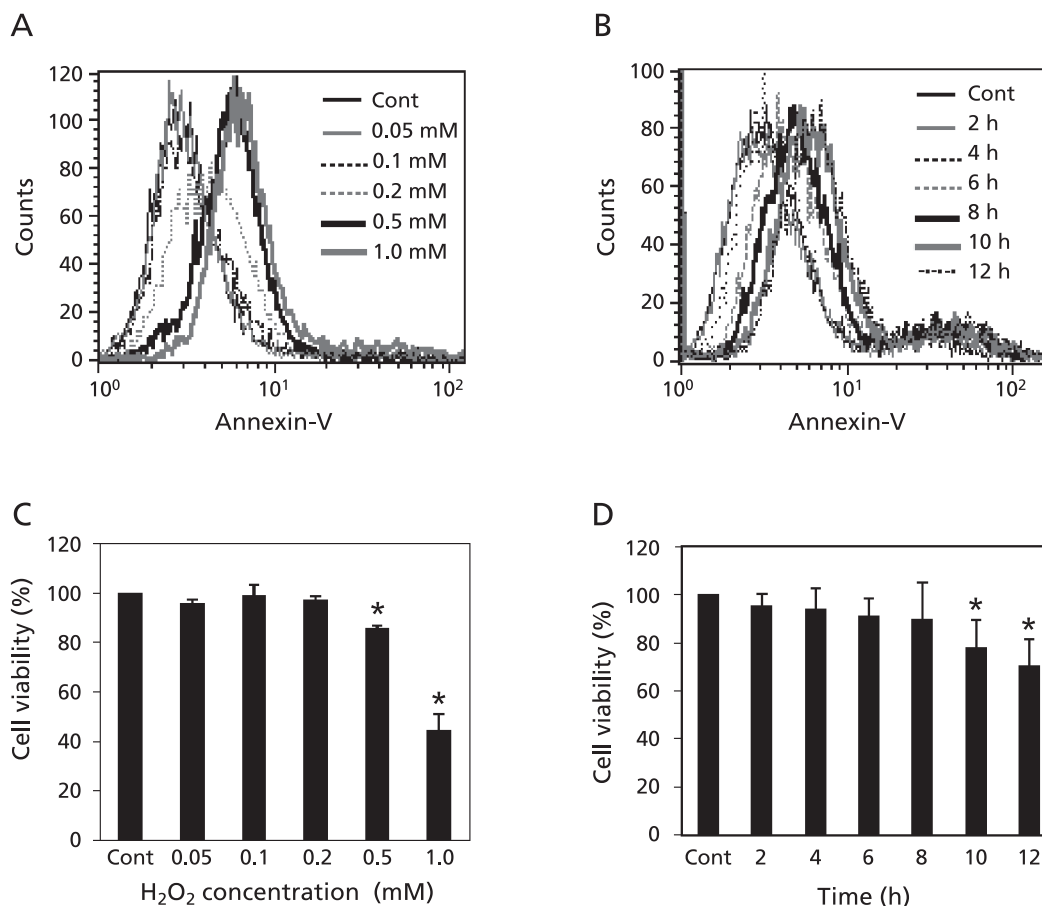
Fig. 1. H_2O_2-induced apoptosis and necrosis of RAW 264.7 cells detected by annexin V stain and MTT assay. (A) RAW 264.7 cells were treated with various concentrations (0, 0.05, 0.1, 0.2, 0.5, 1.0 mM) of H_2O_2 for 12 h and (B) cells were treated with 0.5 mM H_2O_2 for the indicated time points (0, 2, 4, 6, 8, 10, 12 h), and annexin V stained cells were examined by flow cytometry (n = 3). (C) Cells were treated as described in (A), and cell viability was measured using the MTT assay (n = 5). (D) Cells were treated as described in (B), and cell viability was measured using MTT assay (n = 5). Data are expressed as mean $\pm$ SD, *p<0.01 compared to control.

necrosis. We previously reported that 50% of cells treated with 1 mM H_2O_2 died within 1 h, and most of this cell death was due to immediate necrosis.[10] At 0.5 mM H_2O_2, apoptosis was observed first at 6 h and peaked at 10–12 h (Fig. 1B). While negligible at 6 h, the proportion of necrotic cell death increased to 22–30% at 10–12 h (Fig. 1D).

Pretreatment with TauCl protects RAW 264.7 cells from H_2O_2-induced apoptosis. To explore the anti-apoptotic effect of TauCl, RAW 264.7 cells were incubated with 0.5 mM TauCl for 12 h before exposing them to 0.4 mM H_2O_2 for 10 h and cell death caused either by apoptosis or necrosis was measured by using PI-annexin V staining and MTT assay, respectively. As shown in Fig. 2A (annexin V staining), TauCl pretreatment decreased H_2O_2-derived apoptotic cell death from 67% to 34% indicating obvious protective effect of TauCl. The percentages of PI-negative /annexin V-positive cells were 6.5 ± 2.0 (control), 6.7 ± 1.7 (TauCl), 13.5 ± 1.1 (H_2O_2) and 9.4 ± 0.4 (TauCl + H_2O_2), and the percentages of PI-positive/annexin V-negative cells were 4.2 ± 1.2 (control), 4.8 ± 1.3 (TauCl), 12.9 ± 1.8 (H_2O_2) and 6.9 ± 2.3 (TauCl + H_2O_2). Viability of H_2O_2 treated cells were increased from 82% to 95% in the TauCl treated cells, while TauCl treatment alone had no effect on cell viability (Fig. 2B, MTT assay). These data suggest that TauCl protects the RAW 264.7 cells from H_2O_2-induced apoptosis as well as necrotic death.

TauCl induces HO-1 expression and elevates HO activity. Upregulation of HO-1 expression protects against H_2O_2-derived

oxidative cytotoxicity by decreasing content of free heme thus by limiting the heme-catalyzed hydroxyl radical production.[20] TauCl induces HO-1 expression and protects cells from H_2O_2-derived necrosis.[10,17] TauCl (0.5 mM) increased HO-1 protein expression beginning from 2 h and peaking at 12 h. While H_2O_2 can also increase HO-1 level that peak at 10 h (Fig. 3A), the H_2O_2-derived HO-1 induction was only 30% of that observed in the TauCl-treated cells. Along with induction of HO-1 expression, the HO activity was increased maximally at 12 h after the TauCl treatment (Fig. 3B). This suggests that TauCl protects macrophages from H_2O_2-derived apoptosis by increasing HO-1 expression as well as elevating HO activity.

TauCl elevates intracellular glutathione and catalase levels. Elevated glutathione content and catalase activity would protect cells from H_2O_2 toxicity.[21] We therefore tested whether TauCl could elevate intracellular level of GSH and induce the expression of H_2O_2-decomposing catalase. TauCl decreased total intracellular GSH at 0.5 h, but the GSH level increased above the control level by 2 h, remained elevated above the control level for 10 h, and returned to normal level by 20 h (Fig. 4A). TauCl also increased catalase levels from 2 h to 12 h (Fig. 4B). TauCl may therefore protect against H_2O_2-derived apoptosis by increasing glutathione content along with catalase levels.

Inhibition of ERK decreases TauCl-induced HO-1 expression. Mitogen-activated protein kinase (MAPK) signaling pathways that increase the phosphorylation of extracellular
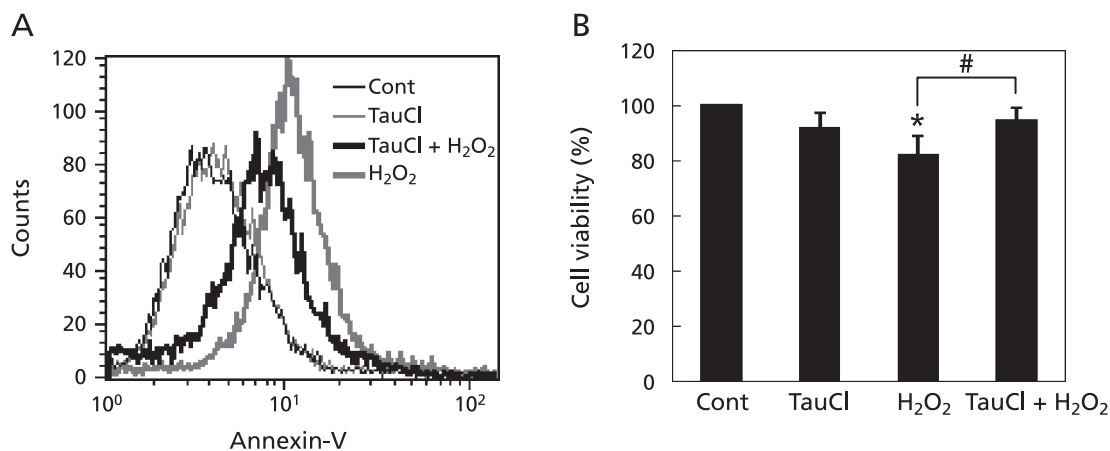
Fig. 2. Effect of TauCl pretreatment on H_2O_2-induced apoptosis in RAW 264.7 cells. (A) RAW 264.7 cells were treated with 0.5 mM TauCl for 12 h, and then exposed to 0.4 mM H_2O_2 for 10 h. Annexin V stained cells were examined by flow cytometry. Representative result of the flow cytometric analysis is presented ($n = 3$). (B) Cells were treated as described in (A), and cell viability was measured by MTT assay ($n = 5$). Data are expressed as mean ± SD, *$p<0.01$ compared to control cells and #$p<0.05$ compared to H_2O_2 treated cells.

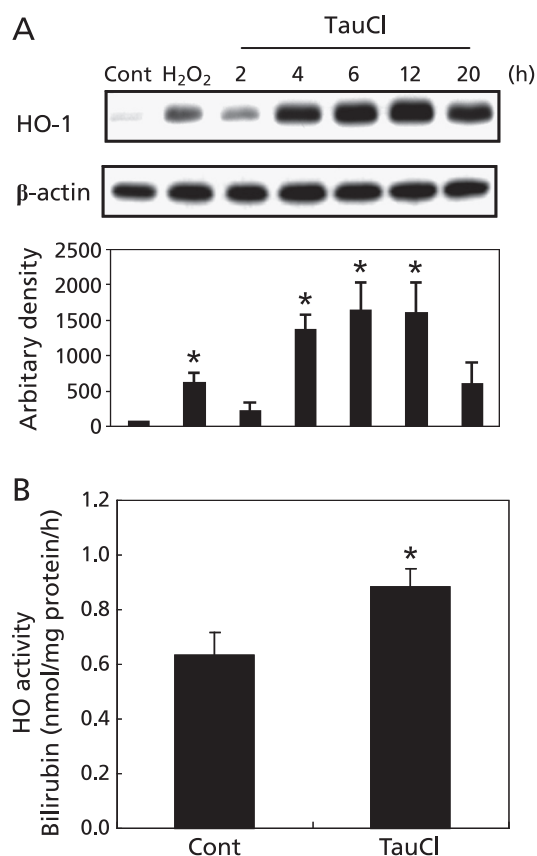**Fig. 3.** Effects of TauCl on HO-1 protein expression and HO activity in RAW 264.7 cells. (A) RAW 264.7 cells were treated either with 0.4 mM H_2O_2 alone or with 0.5 mM TauCl for 10 h. At the indicated time points (0, 2, 4, 6, 12, 20 h), cells were lysed and proteins were extracted to determine the relative amount of HO-1 using immunoblot analysis. Blots were reprobed with β-actin to show equal loading. A representative immunoblot from three independent experiments is shown and the bar graph shows the relative levels of HO-1 protein normalized to β-actin ($n = 3$), *$p<0.05$ compared to control. (B) RAW 264.7 cells were treated with 0.5 mM TauCl for 12 h and HO activity was determined by employing the bilirubin production assay ($n = 3$). Data are expressed as the mean ± SD, *$p<0.05$ compared to control.
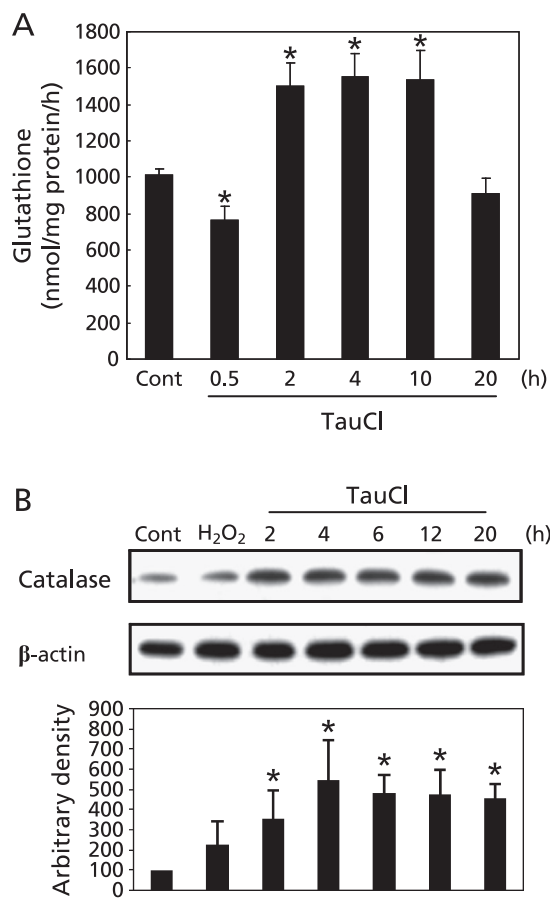
Fig. 4. Effects of TauCl on intracellular glutathione and catalase levels in RAW 264.7 cells. (A) RAW 264.7 cells were treated with 0.5 mM TauCl and harvested at the indicated times. Harvested cells were rinsed, treated with 5-SSA and solubilized with Triton X-100. Acidic supernatants were analyzed to determine the total glutathione concentration ($n = 3$). Graph shows the mean ± SD, *$p<0.05$ compared to control. (B) RAW 264.7 cells were treated with 0.4 mM H_2O_2 for 10 h and 0.5 mM TauCl and lysed at the indicated time points (0, 2, 4, 6, 12, 20 h). Total proteins were extracted and the amount of catalase in the cell lysates was determined using immunoblot analysis. Blots were reprobed with β-actin to show equal loading. Bar graphs show the relative levels of catalase normalized to β-actin ($n = 3$), mean ± SD, *$p<0.05$ compared to control.
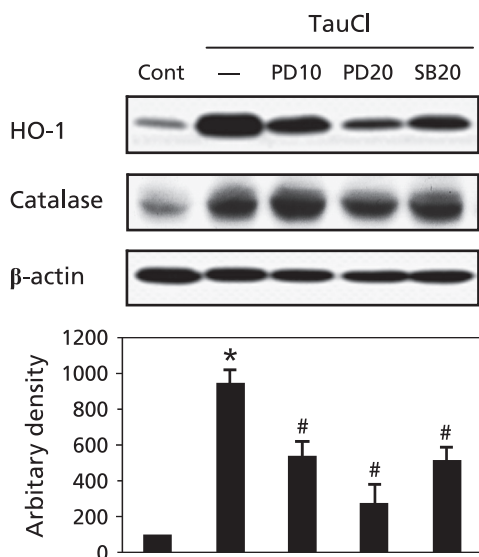
Fig. 5. Effects of inhibitors of ERK and p38 MAPK on TauCl-induced HO-1 and catalase expression. RAW 264.7 cells were treated with the MEK inhibitor, PD98059 (10, 20 µM), and p38 MAPK inhibitor, SB20385 (20 µM), together with 0.5 mM TauCl. Total cellular proteins were extracted at 12 h and the expressions of HO-1 and catalase were determined with immunoblot analysis. Blots were reprobed with β-actin to show equal loading. The bar graphs show the relative levels of HO-1 protein normalized to the β-actin (n = 3), mean ± SD, *$p<0.01$ compared to control and #$p<0.05$ compared to TauCl-treated cells.

signal-regulated kinase (ERK) and p38 MAPK play an important role in inducing HO-1 expression.[22,23] Thus, we examined whether the TauCl-derived upregulation of HO-1 expression is associated with increased phosphorylation of ERK and p38 MAPK. Specific inhibitor of ERK phosphorylation (PD98059) blocked the TauCl-derived increases in HO-1 expression in a dose dependent manner (Fig. 5), despite the fact that we could not observe any increase in ERK and p38 MAPK phosphorylation in the TauCl treated cells (data not shown). However, PD98059 had no effect on catalase expression. These data suggested that increased phosphorylation of ERK or p38 MAPK could participate in the TauCl-derived upregulation of HO-1 expression.

Discussion

TauCl, the taurine-derived end product of HOCl detoxification, is released into inflamed tissues from activated and apoptotic neutrophils. It was thought previously that the TauCl is excreted in urine without any prominent biological effect. However, TauCl inhibits the production of pro-inflammatory mediators by suppressing NF-κB activation[24,25] and at the same time, increases the expression of several antioxidant enzymes such as, HO-1, Prx, and Trx by activating the Nrf 2/ARE system in macrophages.[10,17] Particularly during the resolution phase of inflammation, TauCl-mediated inhibition of pro-inflammatory mediator production as well as the upregulation of antioxidant enzyme expression in macrophages could protect inflamed tissues against the cytotoxicity of inflammation. We previously showed that while 50% of RAW 264.7 macrophage cells died of necrosis at 1 h after exposure to 1 mM H_2O_2, pretreatment with 0.5 mM TauCl protected the cells from H_2O_2-derived necrotic cell death.[10] We show here that TauCl could protect macrophages also from the apoptosis caused by moderate dose of H_2O_2. With 0.5 mM H_2O_2, apoptosis began to be observed at 6 h and peaked at 10–12 h (Fig. 1) and this was consistent with the report of others using the same cell

line.[26,27] Since there was no apparent difference in the extent of apoptosis caused by 0.4 mM and 0.5 mM H_2O_2, we treated cells routinely with 0.4–0.5 mM H_2O_2 for 10–12 h in this study. The cells pretreated with TauCl were protected from H_2O_2-derived apoptosis and had increased viability (Fig. 2). This suggests that TauCl released from the activated and apoptosing neutrophils could protect the surrounding macrophages from inflammatory cytotoxicity and the surviving macrophages could promote resolution of inflammation by cleaning the neutrophil debris.

H_2O_2 has been used widely as a toxicant causing apoptotic cell death in the common model of exogenous oxidative stress. Thus, protecting inflamed tissues from ROS-induced apoptosis is an important therapeutic target in chronic inflammatory diseases. In this regard, induction of HO-1 expression by several phenolic plant antioxidants has been reported to protect inflamed tissues from H_2O_2-derived apoptosis. For example, quercetin prevented H_2O_2-induced apoptosis by inhibiting mitochondrial release of cytochrome C and activation of caspase 3 as a result of increased HO-1 expression.[26] Neuroblastoma cells were protected from H_2O_2-induced apoptosis upon chondroitin sulfate-derived induction of HO-1 expression that occurs via an increase of ERK phosphorylation.[28] Thus, the TauCl-derived induction of HO-1 expression (Fig. 3) may underlie the anti-apoptotic effect against H_2O_2 and this may be caused by up-regulating Bcl-2 expression and also by suppressing the activation of caspase 3. Although 0.5 mM H_2O_2 decreased Bcl-2 expression and activated caspase 3 in PC12 cells,[29] we did not detect such changes of Bcl-2 or caspase 3 in RAW 264.7 cells, but detected activation of caspase 3 with 1.0 mM H_2O_2 (data not shown).

HO-1 knockout mice die easily when exposed to oxidative challenge.[30] Cells treated with an inducer of HO-1 or cells derived from HO-1 overexpressing transgenic mice are relatively resistant to oxidative or nitrosative stress.[31,32] The induction of HO-1 in cardiac tissues of rats prior to transplantation prolongs the survival of cardiac allograft and inhibits the allograft apoptosis and dysfunction caused by ischemia and reperfusion involved with surgery.[33,34] Prior elevation of HO activity in kidney also protects against apoptosis caused by oxidative stress by increasing the production of biliverdin/bilirubin and CO gas.[35] As we have shown previously, TauCl induces HO-1 expression and elevates HO activity in a time- and dose-dependent manner[17] and this may have been responsible for the observed blockage of H_2O_2-induced apoptosis.

TauCl is a mild oxidant and was responsible for the initial lowering of GSH levels. This can activate the Nrf 2/ARE system that induces expression of cytoprotective antioxidant enzymes like glutathione synthase, Prx-1, Trx-1, and HO-1,[10,17] and primes the cells against the H_2O_2-induced oxidative stress and cytotoxicity. As H_2O_2 is converted to H_2O by GPx, stimulation of GSH synthesis along with increased GPx expression can protect cells against the H_2O_2-derived oxidative stress by maintaining cellular thiol proteins.[36,37] TauCl decreased GSH levels initially, but then the GSH level increased above the control level over time (Fig. 4A). This elevation of GSH level may assist the detoxification of H_2O_2, and protect the macrophages from the H_2O_2-derived apoptosis. Although exposing the cells with H_2O_2 did not increase catalase expression, TauCl increased the expression of catalase (Fig. 4B). Inducing the expression of catalase by prior exposure to NO protects the macrophages from H_2O_2-induced apoptosis.[15] In this connection, prior induction of catalase enhances the rate of H_2O_2 disposal and protected H_2O_2-exposed astroglia.[1] Because catalase is usually located in peroxisome, we determined the expression of GPx1 which is the most abundant form of GPx in the cytoplasm. TauCl increased the expression of GPx1, however, its expression level was very low in RAW 264.7 cells (data not shown). Furthermore, prior induction of glutathione reductase in human alveolar macrophages enhances rate of GSH regeneration from GSSG and protects cells from H_2O_2-derived cytotoxicity.[38]

Thus, the TauCl-derived increase of glutathione level and GPx-1 and catalase expression also explain how TauCl may protect cells from H$_2$O$_2$-induced apoptosis.

Activation of the Nrf 2/ARE system leading to upregulation of HO-1, glutathione synthase, catalase and other antioxidant enzyme expression requires both the oxidation of cysteine thiols in Keap1 that prevent Nrf 2 degradation and enhance Nrf 2 release as well as the phosphorylation of the released Nrf 2 to promote its nuclear translocation. While the oxidation of cysteine thiols in Keap1 promotes the release of Nrf 2 from Keap1, nuclear translocation of Nrf 2 is dependent on its phosphorylation catalyzed by ERK and p38 MAPK. In the rat primary hepatocytes, induction of HO-1 expression was dependent on JNK and p38 MAPK activation,[22] and the HO-1 induced by arsenite depends on the activation of ERK and p38 MAPK in human hepatoma cells. Although the effects of TauCl on the phosphorylation of MAPK are not clear,[8,39,40] we were unable to detect increased phosphorylation of ERK and p38 MAPK in TauCl treated RAW 264.7 cells (data not shown). However, the pharmacological inhibitor of ERK suppressed TauCl-derived induction of HO-1 expression (Fig. 5). These results suggest that ERK signaling pathways is somehow involved in the phosphorylation of Nrf 2 and may modulate the induction of HO-1 expression. Further studies are necessary to investigate the role of MAPKs in the activation of Nrf 2 by TauCl.

In conclusion, the TauCl, produced abundantly in the activated neutrophils and released from apoptotic neutrophils to surrounding macrophages and tissues, can protect against H$_2$O$_2$-induced apoptosis by increasing the expression of HO-1, elevating HO activity, and also by enhancing intracellular production of anti-oxidants like GSH and by increasing the expression of catalase.

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