Recombinant protein glutathione S-transferases P1 attenuates inflammation in mice

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ABSTRACT
We have reported that intracellular glutathione S-transferases P1 (GSTP1) suppresses LPS (lipopolysaccharide)-induced excessive production of pro-inflammatory factors by inhibiting LPS-stimulated MAPKs (mitogen-activated protein kinases) as well as NF-κB activation. But under pathogenic circumstances, physiologic levels of GSTP1 are insufficient to stem pro-inflammatory signaling. Here we show that LPS-induced up-regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW264.7 cells is significantly reduced by incubating cells with recombinant GSTP1 protein. In vivo study demonstrates that treatment of mice (i.p.) with recombinant GSTP1 protein effectively suppresses the devastating effects of acute inflammation, which includes reduction of mortality rate of endotoxic shock, alleviation of LPS-induced acute lung injury and abrogation of thioglycolate (TG)-induced peritoneal deposition of leukocytes and polymorphonuclear cells (PMNs). Meanwhile, GSTP1 prevented LPS-induced TNF-α, IL-1β, MCP-1 and NO production. Further investigation by using confocal microscopy and flow cytometry shows that recombinant GSTP1 protein can be delivered into RAW264.7 cells, mouse peritoneal macrophages and HEK 293 cells suggesting that extracellular GSTP1 protein could be transported across plasma membrane and act as a cytosolic protein. In conclusion our research demonstrates a new finding that increasing cellular GSTP1 level by supplement of recombinant GSTP1 effectively suppresses the devastating effects of acute inflammation.

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1. Introduction
The glutathione S-transferases (GSTs) are identified as a multi-gene family of isozymes that catalyze the nucleophilic attack of the sulfur atom of glutathione (GSH) on electrophilic groups of substrate molecules (Tew, 1994). On the basis of the amino acid sequence, the mammalian GSTs are divided into six classes: α, μ, θ, π, ξ, and ζ (Townsend and Tew, 2003). Among these isozymes, glutathione S-transferase P1 (GSTP1, GSTπ) is the most prevalent one in mammalian cells (Tew, 1994). Previous studies show that GSTP1 is determinant in cellular response to oxidative stress and protects tumor cells from apoptosis elicited by a variety of cytotoxic agents, such as H2O2, UV, cisplatin and arsenic trioxide (Gate and Tew, 2001; Gilot et al., 2002; Townsend et al., 2002; Lu et al., 2004; Zhou et al., 2004). GSTP1 also participates in the regulation of stress signaling and protects cells against apoptosis by the mechanisms related with its non-catalytic and ligand-binding activities. GSTP1 interacts with the C-terminal of JNK and functions as an endogenous inhibitor of that kinase (Adler et al., 1999; Yin et al., 2000; Wang et al., 2001). More recently, we found that GSTP1 prevents LPS-induced excessive production of pro-inflammatory factors and plays an anti-inflammatory role in response to LPS. GSTP1 expression, both at the transcription and translation levels, was up-regulated by the LPS stimulation in RAW264.7 macrophage-like cells. GSTP1 inhibited LPS-induced phosphorylation of MAPKs including ERK, JNK and p38 and activation of NF-κB dose- and time-dependently in GSTP1 transiently and stably transfected cells. Moreover, this inhibition of the signaling pathways resulted in the decrease of tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) synthesis (Xue et al., 2005). However, despite the

Abbreviations: GSTP1, glutathione S-transferases P1; SHHR, signal sequence hydrophobic region; HEK293-cell, human embryonic kidney 293 cell; IPTG, isopropyl-β-D-thiogalactopyranoside; 4M, four cystein residues mutation; V7F, tyrosine-7; GSH, glutathione; BSO, buthionine-(S,R)-sulfoximine; PGE2, prostaglandin E2; MPO, myeloperoxidase; TG, thioglycolate; PVDF, polyvinylidene difluoride; HTAB, hexadecyltrimethylammonium bromide; BAL, bronchoalveolar lavage.

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presence of physiologic regulators, such as GSTP1, the host defense systems can still pathologically perpetuate inflammation by overproducing host mediators that cause collateral damage to multiple organs.

The plasma membrane imposes tight control on the access of extracellular peptides and proteins to the cell interior. It provides a boundary for the 10,000–15,000 proteins expressed in a typical mammalian cell. Despite this barrier, transfer of information across the membrane is essential for cell development, function, and survival. Membrane receptors and transporters sense extracellular ligands, ions and nutrients to allow the cellular uptake through specific receptor-mediated endocytosis or transporter-based translocation. In addition, functional cargoes including proteins and peptides can be delivered into cells by signal sequence hydrophobic region (SSHR) (Veatch et al., 2004). Although GSTP1 is known to be a cytosolic phase II detoxification enzyme, it is considered to be present on the extracellular side of the plasma membrane in some reports (Rajmakers et al., 2003; Nijhoff et al., 1995). Thus, we reasoned that if GSTP1 protein that is replenished extracellularly can be transported into cells it represents a powerful approach to treatment of inflammatory disorders. To test this hypothesis, we developed recombinant human GSTP1 and conducted experiments to determine whether exogenously administered GSTP1 proteins can increase intracellular GSTP1 level and inhibit excess inflammatory response. Our study demonstrated that exogenous GSTP1 protein could be delivered into macrophages and suppressed iNOS and COX-2 expression in cells. Furthermore, intraperitoneally administered GSTP1 protein to mice significantly decreased mortality of endotoxic shock and inhibited acute lung injury and peritonitis.

2. Materials and methods

2.1. Antibodies and reagents

Anti-iNOS monoclonal antibody and anti-COX-2 monoclonal antibody were purchased from BD Biosciences (San Diego, CA, USA). Anti-GAPDH antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). All secondary antibodies used for Western blotting were purchased from Calbiochem (La Jolla, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Invitrogen (Carlsbad, CA, USA). LPS (from Escherichia coli 0111B4) and sodium thioglycolate (TG) were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of GSTP1 proteins

The full-length cDNA encoding hGSTP1-1 as described previously (Adler et al., 1999) was amplified by PCR for ligation into pET-28a (Novagen) which contains a hexahistidine N-terminal tag. Wild type fusion proteins comprising a full-length GSTP1 (WT) and its mutants which in alanine replaced all four cystein residues (C14A/C47A/C101A/C169A) (4M) and phenylalanine replaced the tyrosine-7 (Y7F) were engineered as described previously (Wu et al., 2007). Wild type His6-GSTP1 and its mutants were purified by metal-affinity chromatography by using IDA-Ni²⁺ affinity column (Novagen). Contained LPS in purified recombinant proteins were removed by using the Detoxi-Gel™ Endotoxin Removing Gel (Pierce) and were detected by Limulus chromogenic assay (Associates of Cape Cod). This method produced recombinant proteins with minimal LPS content (0.283 μg LPS/mg protein). Unless otherwise indicated, all purification procedures were carried out either at 4 °C or on ice. Glycerol was added to a final concentration of 20%. The purified proteins were stored at −80 °C until use.

2.3. Protein labeling and intracellular detection

The proteins including purified GSTP1 and its mutants, and BSA were labeled with fluorescein isothiocyanate (FITC, Pierce Chemical) according to the manufacturer’s instructions. After extensive dialysis against PBS to remove free FITC, the conjugate added with sodium azide at 1:80 was stored at −20 °C and protected from light. The labeled proteins were detected by using Thin Layer Chromatography scanning (TLC-scanning). After being denatured in SDS, the proteins were electrophoresed on a 12% SDS-polyacrylamide (SDS-PAGE). The green fluorescence of labeled proteins were measured in Dual-wavelength flying-spot scanner (Shimadzu, CS-900) at 495 nm.

The intracellular presence of GSTP1 in the murine macrophage-like cell line, RAW264.7 cells, was demonstrated by confocal laser scanning microscopy using direct fluorescence. Briefly, the cells were cultured in DMEM with 10% fetal bovine serum containing 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2 for 24 h. The medium was then replaced with serum-free DMEM and 1 μM indicated FITC-labeled proteins including FITC-GSTP1 (WT), FITC-GSTP1 (4M), FITC-GSTP1 (Y7F) or FITC-BSA, or an equimolar concentration of unconjugated FITC (FITC only), respectively. After 30 min incubation, the cells were observed without fixation under a fluorescence confocal laser scanning microscope (Bio-Rad, MRC–1024).

2.4. Flow cytometry-based protease accessibility assay

In order to determine FITC-GSTP1 that was translocated across the plasma membrane to reach the cytoplasm, RAW264.7 cells were incubated with FITC-GSTP1, FITC-GSTP1 (4M), FITC-GSTP1 (Y7F) and FITC-BSA or an equimolar concentration of unconjugated FITC, respectively, at 37 °C in 5% CO2 for 30 min. One of each was then treated with proteinase K (1 μM) for 10 min. As a control, proteins were incubated with proteinase K (1 μM) for 10 min at 37 °C before added to cells. After all incubations, cells were washed in PBS (pH 7.4) two times. Cell fluorescence was measured in FAC-Scalibur (BD Biosciences) using forward versus side light scatter, and green fluorescence was collected with a 520 nm band pass filter.

2.5. Immunoblot analysis

Cells were rinsed twice with ice-cold PBS, and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min. Lysates were centrifuged (15,000 × g) at 4 °C for 15 min. Equal amounts of the soluble protein were denatured in sodium dodecyl sulfate (SDS), electrophoresed on a 12% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane. The immunoblotting were performed as described (Adler et al., 1999). The horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG antibodies were used against respective primary antibody. The proteins were visualized using Lumi–Light Western Blotting Substrate (Roche Molecular Biochemicals).

2.6. Measurement of intracellular GSH level

The GSH levels were measured as described previously (Fan et al., 2005) using Glutathione Assay kit (Calbiochem).
2.7. Animals

BALB/c mice (7 weeks old) were purchased from Shanghai Experimental Animal Center, China Academy of Science. Laboratory animal handling and experimental procedures were performed in accordance with the requirements of Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by Science and Technology Department of Jiangsu Province.

2.8. Endotoxin shock model

Each mouse was injected intraperitoneally (i.p.) with single dose of LPS (37.5 mg/kg) in saline. Alternatively, the same dose of GSTP1 (WT), GSTP1 (4M), GSTP1 (Y7F) or BSA was given i.p. 0.5 h after the LPS challenge. The survival rates of mice were monitored continuously for 7 days (Bochkov et al., 2002), but no more mice died after 72 h. Plasma TNF-α and IL-1β contents were measured 1, 3, and 6 h after LPS treatment. Serum NO level was detected 1, 8 and 12 h after LPS treatment.

2.9. Acute lung injury models

The general procedure was modified in a manner similar to previous reports (Rowe et al., 2002; Asti et al., 2000). Briefly, mice were anaesthetized with pentobarbital sodium (30 mg/kg) and fixed on a board at angle of 50° in a supine position. 50 μl sterile saline or sterile saline containing 10 μg LPS was instilled into the mouse trachea with a 3-gauge needle, respectively. After intratracheal instillation, the mouse was placed in a vertical position and rotated for 0.5–1 min to distribute the instillate evenly within the lungs. GSTP1 (WT) or GSTP1 (4M) was given i.p. 0.5 h after the LPS challenge. Lung tissue myeloperoxidase activity was detected 4, 8, 12 and 16 h after LPS treatment. MCP-1 in bronchoalveolar lavage fluids (BALF) was measured at indicated time points. The lungs were prepared for histological analysis by hematoxylin eosin staining 12 h after LPS treatment.

2.10. Acute peritonitis models

Each mouse received a single intraperitoneal injection of 0.5 ml of 3% sodium thioglycolate (TG) or sterile pyrogen-free saline. Ten minutes later, mice were intravenously injected with saline alone or saline containing GSTP1 (WT) or GSTP1 (4M). Mice were sacrificed 2 or 4 h after TG treatment and the peritoneal cavities were lavaged with 6 ml of ice-cold PBS containing 10 U/ml of heparin to prevent clotting. The total peritoneal leukocytes were counted double-blindly using a hemocytometer and they were followed by the differential counting of leukocytes (Giemsas staining; two counts per slide, 300 cells per count) and/or a clinical blood analyzer, Cell-Dyn 1700 (Abbott Laboratories, IL).

2.11. Serum nitrite analysis

Mice were anaesthetized and the blood samples were taken from retro-orbital veins of the eyes. NO synthesis was spectrophotometrically determined by assaying the serum for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylene diamine dihydrochloride, 5% phosphoric acid). Absorbance was measured at 550 nm and nitrite concentration was determined using sodium nitrite as a standard.

2.12. Plasma tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) measurement

Mouse blood was taken under anaesthesia from the retro-orbital plexus at indicated time points after treatment. The plasma level of TNF-α and IL-1β were evaluated by an enzyme-linked immunosorbent assay (ELISA) using the Mouse TNF-α and IL-1β ELISA Kit (BD Biosciences) according to the manufacturer’s instructions.

2.13. Measurement of lung tissue myeloperoxidase (MPO) activity

The mice were anaesthetized with 1% pentobarbital sodium. Lungs were perfused via the right ventricle with 2 ml of sterile PBS and then whole lungs were homogenized and sonicated in 0.5% hexadecyltrimethylammonium bromide (HTAB) buffer. After centrifugation at 12,000 × g for 10 min at 4 °C, the supernatant fluids containing MPO were incubated in a 50 mM KPO4 buffer containing the substrate H2O2 (1.5M) and o-dianisidine dihydrochloride (167 mg/ml; Sigma–Aldrich) for 30 min. The enzymatic activity was determined spectrophotometrically by measuring the change in absorbance at 460 nm using a 96-well plate reader.

2.14. Measurement of monocyte chemoattractant protein-1 (MCP-1) in bronchoalveolar lavage fluids (BALF)

Mice were anaesthetized and a plastic cannula was inserted into the trachea. BAL was performed three times with 0.8 ml of sterile saline. The recovered BAL fluid was centrifuged at 300 × g for 10 min at 4 °C. The cell-free supernatant fluids were prepared for MCP-1 determination by ELISA using the mouse MCP-1 ELISA Kit (BD Biosciences).

2.15. Histological analysis

Mice were anaesthetized and lungs were perfused in situ, collected and immersed in 4% paraformaldehyde for 24 h at 4 °C. Fixed lungs were rinsed in phosphate-buffered saline, dehydrated and embedded in paraffin using standard procedures. Five-micrometer tissue sections were stained with hematoxylin eosin and observed through light microscopy.

2.16. Statistical analysis

All experimental data obtained from cultured macrophages and mice were expressed as mean ± S.D. A two-way repeated measure analysis of variance and a log-rank test were used to determine the significance of the differences in cytokine production, enzyme activity, cell counts and survival, respectively.

3. Results

3.1. Expression, purification, and characterization of wild type human GSTP1 and its mutants

We designed and developed recombinant hexahistidine-tagged human GSTP1 proteins and its mutants. The primers for the QuickChange site-directed mutagenesis is listed in Table 1. Expression of GSTP1 resulted in 30–50% of total cell protein after 6 h induction. GSTP1 expression in E. coli before and after induction with IPTG were monitored by SDS-PAGE and stained with Coomassie blue (Fig. 1A). After IDA-Ni2+ resin column purification, the purified mutants, GSTP1 (4M) and GSTP1 (Y7F), gave a single band on SDS-PAGE with an apparent Mr of 28 kDa equivalent to that of the wild type GSTP1 (Fig. 1B).
Cys169 stained with Coomassie blue after IDA-Ni²⁺ resin column purification. was performed under denaturing conditions in 12% SDS-PAGE and the proteins were coupled with a protease accessibility test. The broad spectrum pro-

Table 1

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer</th>
<th>Sense</th>
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<tbody>
<tr>
<td>Tyr-7 → Phe</td>
<td>5'-ACCCTGGCTCTTCCAGTT-3'</td>
<td>Forward</td>
</tr>
<tr>
<td>Cys14 → Ala</td>
<td>5'-TCGAGCCGCCGCGCCGCGCTGC-3'</td>
<td>Forward</td>
</tr>
<tr>
<td>Cys47 → Ala</td>
<td>5'-CAAGGCTTGCCTATACGCGGC-3'</td>
<td>Forward</td>
</tr>
<tr>
<td>Cys101 → Ala</td>
<td>5'-GGACCTGGCGCAATACATCT-3'</td>
<td>Forward</td>
</tr>
<tr>
<td>Cys169 → Ala</td>
<td>5'-AGCCCTGGCCTTGATACGT-3'</td>
<td>Forward</td>
</tr>
</tbody>
</table>

Changed bases are shown by underlines.

3.2 Intracellular delivery of recombinant GSTP1 protein and its mutants

In order to analyze if exogenous GSTP1 protein could transported into cells, we detected the intracellular delivery of recombinant GSTP1 proteins in RAW264.7 cells and mouse peritoneal macrophages by confocal laser scanning microscopy. GSTP1 and BSA proteins were labeled with fluorescein isothiocyanate (FITC), and the labeling efficiency was detected by TLC-scanning (Fig. 2A). The confocal laser scanning microscopy showed that FITC-labeled GSTP1 proteins including wild type, 4M and Y7F were abundantly present in RAW264.7 cells (Fig. 2B) and mouse peritoneal macrophages (Fig. 2C), and in contrast, FITC-labeled BSA un conjugated FITC (FITC only) were not detected (Fig. 2B and C). To eliminate the interferne of non-specific binding of proteins to the cell surface and provide further definitive proof for intracellular location of the proteins, we analyzed protein translocation across the plasma membrane in RAW264.7 cells, mouse peritoneal macrophages and HEK293 cells using flow cytometry coupled with a protease accessibility test. The broad spectrum pro-

tease, proteinase K, was used to distinguish between proteins not internalized and those translocated to the interior of the cell. As shown in Fig. 3A, this analysis revealed that GSTP1 was susceptible to proteinase K, but it escaped proteolytic attack after being transported into RAW264.7 cells where it remained inaccessible to proteinase K. The additional gain in fluorescence in control cells that were not treated with proteinase K likely reflects a pool of GSTP1 adsorbed on the cell surface and accessible to proteinase K. Thus, the protease-resistant fluorescence indicated that GSTP1 and its mutants, GSTP1 (4M) and GSTP1 (Y7F), were able to penetrate cells (Fig. 3B–D).

3.3 Effects of GSTP1 on intracellular GSH levels

GSTs catalyze the nucleophilic attack of the sulfur atom of glutathione (GSH) on electrophilic groups of substrate molecules (Tew, 1994), besides, GSH level has been reported to relate with the response of cells to inflammatory stimuli (Song et al., 2004). Therefore, we measured the intracellular GSH level after GSTP1 treatment. RAW264.7 cells were incubated with 5 μg/ml GSTP1 for 6 h or 50 mM buthionine-(S,R)-sulfoximine (BSO) for 16 h, or transfected with 2 μg Xpress-GSTP1 24 h before cells were harvested. pcDNA3.1-His-Xpress-GSTP1 was generated from pcDNA3-HA-GSTP1 described previously by Adler et al. (1999) by inserting hGSTP1 cDNA into pcDNA3.1-His vector (Invitrogen). Fig. 4 displays that both exogenous GSTP1 protein treatment and GSTP1 transfection did not alter the GSH level in RAW246.7 cells, and as the control, BSO, an inhibitor of γ-glutamylcysteine synthase obviously decreased the GSH level in RAW246.7 cells.

3.4 Inhibitory effects of GSTP1 on LPS-induced iNOS and COX-2 expression in RAW264.7 cells

Two of the most prominent molecular mechanisms mediating inflammatory processes are the production of NO by inducible iNOS and the formation of prostaglandins by COX-2 (prostaglandin H2 synthase). We have found that GSTP1 over-expressed in RAW264.7 cells inhibited LPS-induced activation of MAPKs and NF-κB, and as the consequence inhibited TNF-α and iNOS expression and NO release (Xue et al., 2005). In the present experiment, we detected LPS-induced COX-2 and iNOS expression in RAW264.7 cells which were incubated with GSTP1 protein. RAW264.7 cells were treated with 500 ng/ml LPS and 30 min later, cells were incubated with indicated concentration of GSTP1, respectively. Eight hours after LPS challenge, the cells were subjected for Western blot analysis. The data showed that GSTP1 inhibited LPS-induced COX-2 and iNOS expression in a dose dependent manner (Fig. 5A). When it was added into cell culture 0, 30, 60 or 120 min after LPS treatment, GSTP1 (5 μg/ml) significantly inhibited LPS-induced COX-2 expression at 30 and 60 time points while inhibited LPS-induced iNOS expression at 0, 30, 60 and 120 time points. Besides, GSTP1 showed maximum efficiency at 60 min time point in inhibition of LPS-induced COX-2 and iNOS expression (Fig. 5B). GSTP1 itself did not influence the basal level of COX-2 and iNOS protein (Fig. 5A and B).

3.5 Anti-inflammatory activities of GSTP1 in the mouse models of endotoxin shock, acute lung injury and acute peritonitis

Above results showed that recombinant GSTP1 protein suppressed inflammatory mediators in vitro. We further evaluated the anti-inflammatory activity of GSTP1 in the mouse models. The i.p. injection of LPS at dose of 37.5 mg/kg bodyweight caused 75% mice to die from endotoxin shock. Administration of GSTP1 (WT) (2 mg/kg bodyweight, i.p.) 30 min after LPS treatment reduced mor-
Fig. 2. Intracellular detection of GSTP1 in RAW264.7 cells and mouse peritoneal macrophages by fluorescence confocal laser scanning microscopy. (A) Prestained protein Mark/FITC-GSTP1/FITC-BSA were denatured in SDS, electrophoresed on a 12% SDS-PAGE. The fluorescence was measured in TLC and green fluorescence was collected with a 495 nm band pass filter. (B) RAW264.7 cells were incubated with 1 μM FITC-GSTP1 (WT)/Y7F/4M or an equimolar concentration of FITC-BSA/unconjugated FITC at 37 °C in 5% CO2 for 30 min. The intracellular localization of GSTP1 in RAW264.7 cells was observed under a fluorescence confocal laser scanning microscope (bottom). The same cell was shown by Nomarski image of (top). (C) The mouse peritoneal macrophages were treated as same as RAW264.7 cells. The intracellular localization of GSTP1 in mouse peritoneal macrophages was observed under a fluorescence confocal laser scanning microscope (bottom). The same cell was shown by Nomarski image of (top). Pictures are representative of multiple unfixed cells from three independent experiments in mouse peritoneal macrophages.
Fig. 3. Flow cytometry-based protease accessibility assay for GSTP1 translocation across cells. (A) RAW264.7 cells were incubated at 37 °C in 5% CO2 for 30 min with 1 μM FITC-labeled GSTP1 without proteinase K treatment (green) or subsequently treated with 1 μM proteinase K for 10 min at 37 °C (blue). Alternatively, FITC-labeled GSTP1 was pretreated with 1 μM proteinase K for 10 min at 37 °C before incubation with cells at 37 °C for 30 min (purple). Control cells were not exposed to any GSTP1 protein (black) or incubated with unconjugated FITC (red). (B) RAW264.7 cells were incubated at 37 °C in 5% CO2 for 30 min with 1 μM FITC-labeled GSTP1 (WT)/red, GSTP1 (Y7F)/blue or GSTP1 (4M)/orange. Control cells not exposed to any GSTP1 protein (black) or incubated with unconjugated FITC (green). (C) The similar results as B were obtained from HEK293 cells. (D) The similar results as (B) were obtained from mouse peritoneal macrophages. Each panel is representative of three independent experiments (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).

Furthermore, we examined the effects of GSTP1 on the mouse model of acute peritonitis (Melnicoff et al., 1989). Zero point five ml of 3% sodium thioglycolate (TG) was administrated (i.p.) to each mouse to induce peritoneal infiltration of leukocytes. GSTP1 was injected intravenously 10 min after thioglycolate treatment. The data in Fig. 8 demonstrates that GSTP1 (WT) inhibited the peritoneal deposition of the total amount of leukocytes and PMNs and was more effective than GSTP1 (4M) suggesting that GSTP1 also alleviated acute peritonitis.

4. Discussion

Our previous research has demonstrated that GSTP1 overexpressed in RAW264.7 cells prevents LPS-induced excessive production of pro-inflammatory factors and plays an anti-inflammatory role in response to LPS (Xue et al., 2005). Our study also shows that GSTP1 regulates TNF-α-induced signaling by forming ligand-binding interactions with TRAF2 (Wu et al., 2006). These findings strongly suggested the anti-inflammatory activity of GSTP1. We supposed that if extracellular replenishment of GSTP1 proteins may increase its intracellular level, it will provide a new way for powerful anti-inflammatory therapy. In fact, cellular uptake of proteins and peptides occurs through specific receptor-mediated endocytosis or transporter-based translocation. Besides, proteins and peptides can be delivered into cells via SSHR-directed translocation (Veach et al., 2004). GSTP1 is a dimeric protein due to a non-covalent association of identical subunits with a molecular mass of 22.5 kDa, and each subunit is characterized by two distinct domains and an active side that acts independently of the other subunit. The N-terminal domain consists of beta sheets and alpha helices which function as the G site, the binding site for GSH. The C-terminal domain is an all-helical domain which provides structural elements for the recognition of hydrophobic substrates, the H site, and helps to define the substrate selectivity.
Fig. 5. GSTP1 inhibits LPS-induced COX-2 and iNOS expression in RAW264.7 cells. (A) RAW264.7 cells were pretreated with LPS (500 ng/ml) 30 min before they were incubated with GSTP1 at dose of 1, 3, 5 or 10 μg/ml, respectively. Eight hours after LPS administration, iNOS and COX-2 level in cell lysates were detected by Western blotting. GAPDH expression was measured to confirm the equal amount of protein. (B) RAW264.7 cells were treated with LPS (500 ng/ml) and 0, 30, 60 or 120 min later, GSTP1 protein (5 μg/ml) was added into cultural medium. Eight hours after cells were exposed to LPS, iNOS and COX-2 protein level in cells were detected by Western blotting. GAPDH expression was measured to confirm the equal amount of protein. The results were representative of three independent experiments.

Fig. 6. GSTP1 protects against LPS-induced lethal shock. (A) Mice were treated with 2 mg/kg of GSTP1 (WT), GSTP1 (4M), GSTP1 (Y7F), BSA or LPS-free saline, respectively, 30 min after LPS (37.5 mg/kg, i.p.) challenge, and survival rate of mice was observed continuously. (B and C) TNF-α and IL-1β were measured by an ELISA in blood plasma obtained from retroorbital sinus of mice at indicated intervals after LPS administration. (D) NO levels in serum from retroorbital sinus of mice were assayed as the content of nitrite at the indicated time point after LPS administration. Error bars indicate mean ± S.D. derived from each assay done in at least eight mice. *P<0.05 compared with LPS challenged mice treated with LPS-free saline.

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Fig. 7. GSTP1 prevents LPS-induced acute lung injury. Mice were instilled intratracheally with 50 μl LPS-free saline containing 10 μg LPS and were then administered intraperitoneally with 2 mg/kg of GSTP1 (WT) or GSTP1 (4M), or LPS-free saline, respectively, 30 min after LPS treatment. The mice instilled intratracheally with 50 μl LPS-free saline only were used as control. (A) The lungs of mice were prepared for histological analysis 12 h after LPS challenge. (B) MCP-1 in BALF was measured by ELISA at indicated intervals after the mice were exposed to LPS. (C) Myeloperoxidase (MPO) activity in lung tissue was detected 4, 8, 12 and 16 h after LPS treatment. Error bars indicate mean ± S.D. derived from each assay done in at least eight mice. **P < 0.01, *P < 0.05 compared with LPS challenged mice treated with LPS-free saline.

Fig. 8. GSTP1 prevents thioglycollate-induced acute peritonitis. (A and B) Each mouse was received a single intraperitoneal injection of 0.5 ml of 3% sodium thioglycollate (TG). Ten minutes later, mice were intravenously injected with saline alone or saline containing GSTP1 (WT) or GSTP1 (4M) (2 mg/kg). Two and 4 h after TG treatment, total peritoneal leukocytes (A) were counted double-blindly using a hemocytometer followed by the differential counting of leukocytes (Giemsa staining) for PMNs counting (B). Error bars indicate mean ± S.D. derived from each assay done in at least eight mice. ***P < 0.001, **P < 0.01, *P < 0.05 compared with TG-induced peritonitis model mice.

Activated macrophages are among the prime organizers of inflammatory response and are responsible for most of the cellular and molecular pathophysiology of sepsis by producing cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-8 and IL-12, and other pro-inflammatory molecules including platelet-activating factor, prostaglandins, enzymes and free radicals, such as nitric oxide (NO) (Hurley, 1995; Victor et al., 2003; Noguchi et al., 2003). By using confocal microscopy and flow cytometry, we found the translocation of recombinant GSTP1 protein across the plasma membrane of living RAW246.7 cells and mouse peritoneal macrophages. Furthermore, GSTP1 protein was also transported into HEK293 cells in the present experiment.

During inflammation development, overproduction of NO was mainly produced by inducible iNOS which could be up-regulated by LPS stimulation (Sun et al., 2006). COX-2, the rate limiting enzyme in the synthesis of prostaglandin E2 (PGE2), is responsible for the development of inflammation (Surh et al., 2001). Moreover, it has been reported that COX-2 expression is dependent on the NO lev-
els, and iNOS binds, s-nitrosylates and activates COX-2 (Park et al., 2005; Kim et al., 2005). In accordance with our previous reported results showing that intracellular GSTP1 significantly inhibits LPS-induced iNOS expression in RAW264.7 cells (Xue et al., 2005), the present study indicated that recombinant GSTP1 protein added into the culture medium decreased the LPS-induced iNOS expression in RAW264.7 cells. As expected, the LPS-induced COX-2 expression in RAW264.7 cells was also depressed by incubating cells with recombinant GSTP1 protein. In order to further investigate the anti-inflammatory effects of recombinant GSTP1 protein, three widely used animal models of inflammatory disorders were adopted for in vivo study. Administered systemically, LPS mimic septic effects by directly and indirectly inducing various inflammatory processes (Jüttler et al., 2007). LPS-induced endotoxic shock represents an adequate tool for understanding inflammatory processes (Remick (Jüttler et al., 2007). Intrastracheal administration of LPS to mice triggers an acute pulmonary inflammation, which is characterized by increased capillary permeability, interstitial and alveolar edema, and an influx of circulating inflammatory cells (Karmpaliotis et al., 2005). Thioglycollate (TG) peritonitis induces a rapid and time dependent accumulation (TG) peritonitis induces a rapid and time dependent accumulation of PMNs in the peritoneal cavity (Ajubor et al., 1999). We found that GSTP1 protein injected intraperitoneally to mice 30 min after LPS challenge significantly reduced mortality rate of endotoxin shock by inhibiting TNF-α, IL-1β and NO production. GSTP1 alleviated LPS-induced acute lung injury, which included decrement of BALF MCP-1 level, inhibition of leukocyte infiltration into lung and reduction of morphological changes of lungs. GSTP1 also prevented TG-induced acute peritonitis. Thus, our in vitro and in vivo studies strongly demonstrated the therapeutic efficacy of GSTP1 for treating various inflammatory disorders. Combined with our previous findings that the association of GSTP1 and TRAF2 regulates TNFα signaling (Wu et al., 2006) and GSTP1 inhibits LPS-induced TNFα and iNOS expression by suppressing cellular MAPKs activation (Xue et al., 2005), the present study suggests that recombinant GSTP1 protein could down-regulate LPS-induced TNF-α, IL-1β, COX-2, iNOS and MCP-1 expression by inhibiting MAPKs and NF-κB activation.

In our experiment, GSTP1 (WT) as well as its mutants, GSTP1 (4M) and GSTP1 (Y7F), can be delivered into cells, suggesting that GSTP1 (4M) and GSTP1 (Y7F) act in the same way with GSTP1 (WT) in entering living cells. The structure and function of GSTP1 has been the subject of numerous investigations. All the cysteins but Cys101 are conserved throughout the π class GSTs of human, rat, pig and mouse. The three-dimensional structure of GST suggests the possibility that cysteine residues play an essential role in substrate binding affinity or catalysis (Kong et al., 1991; Nishida et al., 1998; Vega et al., 1998). The GSTP1, as opposed to the other gene class of GSTs, is highly susceptible to oxidation due to a reactive cysteine residue (Cys47 in human, rat, and mouse GSTP1) situated near the G-site (Nicolas et al., 1996). GSTP1 (4M) is the GSTP1 mutant in which alanine replaced all four (C14A/C47A/C101A/C169A) cysteine residues. Tyr-7 is conserved in all four families of soluble GSTs. Through mutational replacement by phenylalanine, Tyr-7 has been shown to be necessary for the enzyme activity of GSTP1 (Kolm et al., 1992; Kong et al., 1992; Manoharan et al., 1992). Our previous study indicated that catalytic activity of GSTP1 (4M) and GSTP1 (Y7F) was lower compared to the same content as GSTP1 (WT) (Wu et al., 2007). In the animal model of endotoxic shock the same activity of GSTP1 (4M) and GSTP1 (Y7F) was also observed in decreasing LPS-induced mortality rate. Though showing anti-inflammatory effect, GSTP1 (4M) and GSTP1 (Y7F) were less effective than GSTP1 (WT) in protecting animals from inflammatory injury, which suggests that the anti-inflammatory effects of GSTP1 is partly dependent of the cysteine residues which are the key residues and are essential for the enzyme activity in the GSTP1 molecule. It has been reported that cellular GSH level is related to inflammatory response of cells (Song et al., 2004). GSTP1 facilitates glutathionylation of target electrophilic groups in cells, but intracellular GSH level is regulated by a feedback mechanism from GSH (Deleve and Kaplowitz, 1990) or by modulating γ-glutamylcysteine synthetase (GCS) activity or the GSH efflux (Goss et al., 1994; Lu et al., 1992, 1990). Thus, in our current study, it is not surprising that GSTP1, both by exogenous protein treatment and transfection, does not alter intracellular GSH content suggesting that the anti-inflammatory effects of GSTP1 are independent of cellular GSH levels.

In conclusion, we demonstrate the therapeutic potential of recombinant GSTP1 protein in inhibiting inflammatory injury from in vitro and in vivo studies. Besides, replenishing the intracellular stores of GSTP1 with recombinant GSTP1 protein effectively suppresses the production of cytokines and enzymes associated with acute inflammation. These findings may provide a new strategy for treating systemic and localized inflammatory syndromes.

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References


