Glutathione S-transferase P1 suppresses iNOS protein stability in RAW264.7 macrophage-like cells after LPS stimulation

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Abstract
Glutathione S-transferase P1 (GSTP1) is a ubiquitous expressed protein which plays an important role in the detoxification and xenobiotics metabolism. Previous studies showed that GSTP1 was upregulated by the LPS stimulation in RAW264.7 macrophage-like cells and GSTP1 overexpression downregulated lipopolysaccharide (LPS) induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression. Here we show that GSTP1 physically associates with the oxygenase domain of iNOS by the G-site domain and decreases the protein level of iNOS dimer. Both overexpression and RNA interference (RNAi) experiments indicate that GSTP1 downregulates iNOS protein level and increases S-nitrosylation and ubiquitination of iNOS. The Y7F mutant type of GSTP1 physically associates with iNOS, but shows no effect on iNOS protein content, INOS S-nitrosylation, and changes in iNOS from dimer to monomer, suggesting the importance of enzyme activity of GSTP1 in regulating iNOS S-nitrosylation and stability. GSTM1, another member of GSTs shows no significant effect on regulation of iNOS. In conclusion, our study reveals the novel role of GSTP1 in regulation of iNOS by affecting S-nitrosylation, dimerization, and stability, which provides a new insight for analyzing the regulation of iNOS and the anti-inflammatory effects of GSTP1.

Keywords: inducible nitric oxide synthase (iNOS); glutathione S-transferase P1 (GSTP1); S-nitrosylation; ubiquitination; dimerization

Introduction
Glutathione S-transferases (GSTs) are a well-studied superfamily of detoxification enzymes and catalyze the nucleophilic attack of the sulfur atom of glutathione (GSH) on electrophilic groups of substrate molecules [1]. According to their biochemical, immunologic, and structural properties, mammalian cytosolic GSTs are divided into six major classes: Alpha, Mu, Pi, Omega, Theta, and Zeta [2,3]. Among them, GSTP1 (GSTπ) is the most ubiquitous in mammalian cells. There has been considerable interest in the properties of GSTP1, because it plays an important role in susceptibility to cancer and other diseases [4,5]. Adler et al. first demonstrated that GSTP1 participates in the regulation of stress signaling and protects cells against apoptosis by mechanisms via its noncatalytic, ligand-binding activity. GSTP1 acts as an endogenous inhibitor of c-Jun N-terminal kinase (JNK) by interacting with the C-terminal of this kinase; oxidative stress could cause the dissociations of GSTP1-JNK complex and then, enhance the JNK activity [6]. Our previous research demonstrated that GSTP1 can regulate tumor necrosis factor (TNF)-α-induced signaling and inhibit cell apoptosis by forming ligand-binding interactions with TNF receptor-associated factor 2 (TRAF2) [7]. It has been reported that GSTP1 may act as a nitric oxide (NO) carrier under different cellular conditions, while maintaining its detoxifying activity toward dangerous compounds [8]. We have reported that GSTP1 could be upregulated in response to lipopolysaccharide (LPS) stimulation in RAW264.7 macrophage-like cells and overexpression of GSTP1 prevented LPS-induced excessive production of pro-inflammatory factors in RAW264.7 cells and release of NO [9]. We also found that the recombinant GSTP1 protein attenuated inflammation in mice [10]. We noticed that in both the above researches, GSTP1 could reduce inducible NO synthase (iNOS) protein level. NO is considered as a mediator of inflammatory responses. In inflammation, overproduction of NO was mainly by aberrant expression of iNOS which could be upregulated in macrophages by LPS stimulation [11–13]. Excessive or aberrant expression of iNOS has been implicated in the pathogenesis of many disease processes [14–16]. These results allow us to consider how GSTP1 regulates iNOS protein level in response to LPS stimulation.

In our present study, we found that GSTP1 significantly decreased the content of iNOS in the protein level. Our further study demonstrated that GSTP1 did not reduce iNOS expression but increased S-nitrosylation and ubiquitination of iNOS suggesting that GSTP1 influenced iNOS protein level through regulating post-translational modification. The enzyme activity of GSTP1 was necessary.

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for iNOS S-nitrosylation and ubiquitination. GSTM1, another member of GSTs, did not work in the same manner as GSTP1 in regulation of iNOS. Our study indicates the novel mechanism by which GSTP1 regulates iNOS protein stability.

Materials and methods

Antibodies and reagents

Anti-Xpress tag monoclonal antibody was purchased from Invitrogen (Carlsbad, CA, USA). Monoclonal antibody against Myc tag was purchased from Roche Applied Science (Indianapolis, Indiana). Monoclonal antibodies against iNOS and GSTP1 were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Monoclonal antibody against GAPDH was purchased from Bioworld Biotechnology (Minneapolis, MN, USA). Secondary antibodies coupled to IRDye800 fluorophore for use with the Odyssey Infrared Imaging System were purchased from Rockland (Gilbertsville, PA, USA). G418 sulfate was purchased from Calbiochem (La Jolla, CA, USA). Arbobenzoxylleucinyl-lleucinyl-leucinal-H (MG-132) was from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Amresco (Solon, OH). Tetrahydrobiopterin was purchased from Biomol (Plymouth Meeting, PA). Biotin-HPDP, methyl methanethiosulfonate (MMTS), and Streptavidin-agarose were purchased from Pierce (Rockford, IL). LPS (from Escherichia coli 0111B4) was purchased from Sigma.

DNA constructs

pcDNA3-HA-GSTP1 has been described previously [6]. pcDNA3-His was a generous gift from Dr. Zhenguo Wu (University of Science & Technology, HK), pFLAG-CMV5x-GSTM1 was a gift from Kwang Je Kim (Korea University), and pcDNA3-iNOS was from Dr. Solomon H. Snyder (the Johns Hopkins University School of Medicine, USA). pET-28a-His6-hGSTP1 (Y7F) plasmid was constructed as previously described [10] and Xpress-tagged GSTP1 (Y7F) were made by PCR from His6-hGSTP1 and cloned into pcDNA3 vectors. The His-Xpress-tagged GSTP1 and truncated GSTP1 constructs were made by PCR from pcDNA3-HA-GSTP1 and cloned into pcDNA3 vectors. The Myc-tagged iNOS and truncated iNOS constructs were made by PCR from pcDNA3-iNOS and cloned into pcDNA3 vectors.

Human GSTP1 (hGSTP1) are encoded by a single gene, hGSTP1, while mice have two such genes, mGstp1 and mGstp2. Since the degree of protein homology is striking, monoclonal antibodies against GSTP1 can recognize hGSTP1 and mGSTP1. However, in this experiment, we designed two different GSTP1 RNA interference (RNAi) to interfere hGSTP1 and mGSTP1. In this experiment, we designed two different GSTP1 RNA interference (RNAi) to interfere hGSTP1 and mGSTP1. pRNA-U6.1/neo-hGSTP1 RNAi and pRNA-U6.1/neo-mGSTP1 RNAi were constructed into pRNA-U6. All expression vectors were sequenced for confirmation and purified using the Endofree Plasmid Preparation Kit (Qiagen).

Cells were lysed on ice in a lysis buffer containing 20 mM Tris (pH: 7.5), 135 mM NaCl, 2 mM ethylenediaminetetraacetic acid or EDTA, 2 mM dithiothreitol (DTT), 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, and complete protease inhibitor cocktail for 20 min. Nonreducing sample buffer lacking DTT but SDS was added. Lysates were centrifuged (12,500 × g) at 4°C for 15 min. Proteins were immunoprecipitated for 2 h with the indicated antibodies. The precleared Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) were incubated with immunocomplexes for another 2 h and washed four times with the lysis buffer. The immunoprecipitates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on polyvinylidene difluoride or PVDF, or nitrocellulose membrane at 350 mA for 100 min on ice. After transferring, the membranes were blocked in Tris-buffered saline and Tween 20 or TBST-5% skim milk for 45 min and then were incubated overnight at 4°C with primary antibodies (1:1000). The membranes were washed and incubated with infrared dye coupled secondary antibodies (1:5000) for another 1 h. The antibody–antigen
complexes were visualized by the LI-COR Odyssey Infrared Imaging System according to the manufacturer’s instruction (LI-COR Biosciences, Lincoln, NE). Quantification was directly performed on the blot using the LI-COR Odyssey analysis software. Aliquots of whole cell lysates were subjected to immunoblotting to confirm the appropriate expression of proteins.

**Real-time PCR**

Total RNA was extracted with TRIzol reagent (Takara, China) and was reverse-transcribed into cDNA using a PrimeScript RT reagent Kit (Takara, China). Quantitative PCR (ABI 7500, USA) was performed using SYBR green kit (Takara, China). Primer sequences are as follows:

- iNOS sense primer, CAGCTGGGCTGTACAAAACCTT,
- iNOS antisense primer, CATTGGAAGTGAAGCGTTTCG;
- GAPDH sense primer, GCCAAGGCTGTGGGCAAGGT;
- GAPDH antisense primer, TCTCCAGGCGGCAAGTCGAGA.

**Docking**

The crystal structure of GSTP1 and iNOS used for molecular modeling was obtained from Protein Data Bank or PDB. Docking of GSTP1 to iNOS was performed with ZDOCK3.0 [17]. With rigid-body docking, 2000 poses were generated. All complexes were analyzed and scored by Zrank [18]. The complex with the lowest Z-rank score was regarded as the most possible native-like model.

**Biotin switch**

The biotin switch assay was carried out according to the strategy developed by Jaffrey and Snyder [19]. Briefly, the protein samples were treated with 20 mM MMTS and 2.5% SDS at 50°C for 20 min with frequent vortexing. MMTS was then removed by precipitation with two volumes of −20°C acetone. 1 mM sodium ascorbate solution and 100 μM biotin-N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (HPDP) were added after resuspending the proteins in HENS buffer. The samples were incubated for 2 h at 25°C in the dark and then S-nitrosylated nitrosothiols were precipitated with two volumes of −20°C acetone. The proteins were centrifuged, and resuspended in HENS buffer. After that, two volumes of neutralization buffer and Streptavidin-agarose were added and the mixtures were incubated for 1 h at room temperature. The agarose was washed 3–4 times with HENS buffer. Bound proteins were then eluted in SDS loading buffer.

**Statistical analysis**

Data were represented as mean ± SD. We performed statistical comparison by Student’s t-test. A value of $P < 0.05$ was considered statistically significant. Statistical calculations were performed by SPSS 13.0 software.

**Results**

**Overexpression of GSTP1, but not GSTM1 and GSTP1 (Y7F) mutant, reduces endogenous iNOS protein level**

Our previous studies have demonstrated that intraperitoneal administration of GSTP1 protein to mice significantly decreased mortality of endotoxic shock and inhibited acute lung injury and peritonitis[10]. We also found that GSTP1 could inhibit LPS-induced iNOS increase and NO production in RAW264.7 cells. In the present study, overexpression of GSTP1 in RAW264.7 cells inhibited elevation of LPS-induced iNOS protein level in a dose-dependent manner (Figure 1A). Similarly, iNOS protein levels in stable GSTP1-overexpressing cells were lower than those in the control cells after LPS stimulation (Figure 1B). In order to confirm the effect of GSTP1 on iNOS, HEK293 cells were cotransfected with pcDNA3-iNOS and various concentrations of Xpress-GSTP1 or pFLAG-CMV5α-GSTM1, and 36 h after transfection, the cells were subjected to immunoblotting analysis. Results showed that overexpression of GSTP1, but not GSTM1, another member of GSTs, reduced iNOS protein level in a dose-dependent manner (Figure 1C). The same result was obtained from G418–selected, GSTP1-stable transfected HEK293 clones (Figure 1B to be found at online http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1085978 and Figure 1C). The same result was obtained from G418–selected, GSTP1-stable transfected HEK293 clones (Figure S1B to be found at online http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1085978). These results suggested the specificity of GSTP1 in regulation of iNOS protein level. In order to evaluate if the enzyme activity of GSTP1 was involved in iNOS regulation, we transfected pcDNA3-iNOS and various concentrations of Xpress-GSTP1 (Y7F) plasmids into HEK293 cells and observed the effect of overexpressive GSTP1 (Y7F) on iNOS protein level reduction. Western blot results showed that GSTP1 Y7F mutant did not affect iNOS protein level, suggesting that the enzyme activity of GSTP1 is very important for such iNOS regulation (Figure 1D).

The above data indicated that GSTP1 overexpression could decrease both endogenous and exogenous iNOS protein level. To further confirm the effect of endogenous GSTP1 on iNOS, we utilized RNA interference (RNAi) technology to knock down endogenous mGSTP1 protein expression in RAW264.7 cells and endogenous mGSTP1 was successfully downregulated by RNAi (Figure 2A). As expected, iNOS level was obviously higher in mGSTP1 downregulated cells than in pRNA-U6.1 transfected control cells after LPS stimulation (Figure 2A). LPS stimulation also led to elevation of iNOS protein level, in
RAW264.7 cells in which mGSTP1 was stably knocked down, than that in the control cells (Figure 2B). The same results were obtained in HEK293 cells (Figure S2 to be found at online http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1085978).

**GSTP1 decreases the stability of iNOS protein**

To further investigate how GSTP1 influences iNOS level, HEK293 cells were cotransfected with pcDNA3-iNOS and Xpress-GSTP1 plasmids or empty vectors and pretreated with cycloheximide (CHX), an inhibitor of protein synthesis for the indicated times. As shown in Figure 3A, under CHX treatment iNOS protein level was lower in GSTP1-overexpressing cells than that in empty-vector-transfected cells. We then checked the change in iNOS at transcriptional level using real-time PCR in RAW264.7 cells. The result showed that overexpression of GSTP1 did not influence iNOS at mRNA level (Figure 3B). These results suggested that GSTP1 could enhance the degradation of iNOS.

In order to explore which domain of iNOS was involved in the downregulation of iNOS induced by GSTP1, HEK293 cells were cotransfected with pcDNA3-iNOS and various concentrations of Flag-GSTM1(c) or Xpress-GSTP1 (Y7F) plasmids (D) for 36 h. Cell lysates were subjected to Western blot using iNOS, Flag, or Xpress antibody. GAPDH expression was measured to confirm the equal amount of protein. A representative Western blot for each treatment from three independent experiments is shown. Results are represented by means ± S.E. (n = 3), *p < 0.05 compared with the cells transfected with iNOS only.

**GSTP1 physically interacts with iNOS**

As shown above, GSTP1 was able to downregulate the level of iNOS protein; we thus probed whether GSTP1 could be directly targeting on iNOS. RAW264.7 cells stably expressing GSTP1 and control pcDNA3 were treated with LPS for 12 h and harvested. Cell lysates were subjected to immunoprecipitation with anti-Xpress or anti-iNOS antibody followed by immunoblotting analysis. Results showed that endogenous GSTP1 specifically associated with iNOS (Figure 4A). We further investigated the
interaction of GSTP1 with iNOS in HEK293 cells under overexpression conditions and obtained the same results (Figure 4B and C). In order to determine if the enzyme activity of GSTP1 was related with this process, HEK293 cells were cotransfected with Xpress-GSTP1 (Y7F) and Myc-iNOS. Figure 4D showed that GSTP1 (Y7F) also interacted with iNOS in HEK293 cells suggesting that the enzyme activity was not necessary for GSTP1–iNOS interaction. Figure 4E showed that GSTM1 did not interact with iNOS in HEK293 cells.

Mapping of iNOS and GSTP1 domains required for interaction with each other

The above data indicated that GSTP1 could bind to iNOS. Besides, iNOS comprises two catalytic units: a C-terminal reductase domain and an N-terminal oxygenase domain. In order to determine the region of iNOS responsible for the binding with GSTP1, Xpress-GSTP1 and Myc-iNOS (full), Myc-iNOS (1-500), or Myc-iNOS (501-1145) were cotransfected into HEK293 cells and then the cell lysates

Figure 3. GSTP1 decreases the stability of iNOS and does not influence iNOS expression. A, HEK293 cells were transfected with pcDNA3-iNOS (1 μg) and pcDNA3-Xpress-GSTP1 (0.5 μg) or empty plasmid and 36 h after transfection, the cells were treated with CHX (2.0 μg/ml) for the indicated time. Cell lysates were immunoblotted with anti-iNOS and anti-Xpress antibodies. B, RAW264.7 cells were transiently transfected with 0.5, 1, and 2 μg of Xpress-GSTP1 or control vectors by Lipofectamine 2000 and 18 h after transfection, the cells were treated with or without 100 ng/ml of LPS for 8 h. Then the mRNA level of iNOS was determined by real-time PCR. C, HEK293 cells were cotransfected with Myc-iNOS (full) (1.0 μg), Myc-iNOS (1-500) (1.0 μg), or Myc-iNOS (501-1145) (1.0 μg) as indicated. The lysates were immunoblotted with anti-Myc and anti-Xpress antibodies. GAPDH level was used as the control. A representative Western blot for each treatment from three independent experiments is shown. Results are represented by means ± S.E. (n = 3), *p < 0.05 compared with the cells transfected with iNOS only.

Figure 2. Knockdown of endogenous GSTP1 by shRNA increases iNOS level. A, RAW264.7 cells were transfected with pRNA-U6.1-mGSTP1 constructs or pRNA-U6.1 as control by Lipofectamine 2000 and after 36 h, the cells were treated with 100 ng/ml of LPS for 12 hours. B, The clones of control and RAW264.7 cells stably expressing mGSTP1 RNAi were treated with 100 ng/ml of LPS for 12 hours. The immunoblotting was performed using anti-iNOS, anti-GSTP1, or anti-GAPDH antibody as indicated. A representative Western blot for each treatment from three independent experiments is shown. Results are represented by means ± S.E. (n = 3), *p < 0.05 compared with the cells transfected with iNOS only.
were immunoprecipitated with Xpress-specific antibody followed by Western blot analysis. The results revealed that the oxygenase domain (1-500), but not the reductase domain (501-1145), of iNOS was required for the binding with GSTP1 (Figure 5A).

Both the GSH-binding site (G-site) and the xenobiotic substrate-binding site (H-site) exist in all GST family members [20]. To find the region of GSTP1 responsible for binding with iNOS, different Xpress-tagged GSTP1-truncated fragments were constructed. HEK293 cells were cotransfected with indicated Xpress-tagged GSTP1 fragments and Myc-tagged iNOS, respectively, followed by co-immunoprecipitation with Myc-specific antibody and Western blotting with anti-Xpress antibody. The results showed that the G-site, but not the H-site, domain of GSTP1 was required for the specific association of GSTP1 with iNOS (Figure 5B). Taken together, GSTP1 was physically associated with the oxygenase domain of iNOS by the G-site domain. The above results coincided with the prediction of protein docking software ZDOCK3.0 [17]. As shown in Figure 5C-E, the amino acids involved in the interaction between GSTP1 and iNOS might be Tyr 7, Phe 8, Val 32, Val 33, Glu 36, Lys 190, and Asn 200 in GSTP1 and Tyr 78, Arg 80, Lys 97, and Glu 154 in iNOS.

GSTP1 regulates iNOS monomer/dimer level

The excessive production of NO by dimeric form of iNOS has been well documented [21,22]. We thus investigated whether GSTP1 regulated iNOS monomer/dimer level. RAW264.7 cells were transiently transfected with different concentrations of Xpress-GSTP1 or control vectors and 18 h after transfection, the cells were treated with 100 ng/ml of LPS. Monomer/dimer iNOS were detected by nonreducing Western blotting. As shown in Figure 6A, the dimerized iNOS decreased under GSTP1 overexpression condition. Similar results were also obtained in RAW264.7 cells stably overexpressing GSTP1 (Figure 6B). On the contrary, in RAW264.7 cells in which

Figure 4. GSTP1 interacts with iNOS. A, RAW264.7 cells stably expressing GSTP1 and the control pcDNA3 were treated with LPS (100 ng/ml) for 12 h and cell lysates were subjected to immunoprecipitation with anti-Xpress or anti-iNOS antibody. B and C, HEK293 cells were transiently transfected with Xpress-GSTP1 and Myc-iNOS, and cell lysates were subjected to immunoprecipitation with anti-Xpress (B) or anti-Myc (C). D and E, HEK293 cells were transiently transfected with Xpress-GSTP1 (Y7F) (D) or pFLAG-CMV5α-GSTM1 (E) and Myc-iNOS and 36 h after transfection, the cells were harvested. Cell lysates were subjected to immunoprecipitation with anti-Xpress, and the precipitates were analyzed by immunoblotting with anti-Myc and anti-Xpress antibodies.
mGSTP1 were stably knocked down, LPS induced higher iNOS dimer level than that in pRNA-U6.1 transfected cells (Figure 6C).

To further prove the effect of GSTP1 on iNOS monomer/dimer, GSTP1 and iNOS were cotransfected in HEK293 cells and cell lysates were subjected to Western blot. The results showed that GSTP1 overexpression obviously reduced the amount of dimerized iNOS and increased monomer form of iNOS in a dose-dependent manner (Figure S3A to be found at online http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1085978). Both in GSTP1 stably overexpressed and hGSTP1 RNAi HEK293 cells, similar results were also obtained (Figure S3B to be found at online http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1085978 and C). As expected, data showed that GSTM1 and GSTP1 (Y7F) have no effect on monomer/dimer form of iNOS (Figure S3D to be found at online http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1085978). GSTP1 increases S-nitrosylation and ubiquitination of iNOS, but has no effect on Tyr phosphorylation of iNOS

A variety of post-transcriptional mechanisms regulate the activity of iNOS, which mainly include phosphorylation, S-nitrosylation, and ubiquitination [23,24]. iNOS can be phosphorylated, although its biological significance is unclear [25]. Thus, we first explored the effect of GSTP1 on Tyr phosphorylation of iNOS. HEK293 cells were transiently transfected with Myc-iNOS or cotransfected with Xpress-GSTP1 and Myc-iNOS, and after 36 h cells were incubated with 400 mM Na3VO4 for 30 min before being harvested. Cell lysates were then subjected to immunoprecipitation with anti-Myc, and the precipitates were analyzed by immunoblotting with anti-p-Tyr and anti-Myc antibodies. Results showed that the level of p-Tyr iNOS increased after Na3VO4 was added, but overexpression of GSTP1 showed no obvious effect on Tyr phosphorylation of iNOS (Figure 7A).

We next investigated the effect of GSTP1 on iNOS S-nitrosylation. RAW264.7 cells stably expressing GSTP1 were simulated with 500 ng/ml of LPS for 12 h. After using nonreducing lysate to harvest cells, we subjected the samples to the biotin switch procedure, and then affinity purified them by immobilized streptavidin. The proteins were separated by SDS-PAGE, electrophotoblot to nitrocellulose, and probed with antibody of iNOS. The results showed that GSTP1 increased iNOS S-nitrosylation level in RAW264.7 cells (Figure 7B). Furthermore, we utilized the same method to explore the effect of exogenous expression of GSTP1 on iNOS S-nitrosylation level in HEK293 cells. Our data showed that exogenous overexpression of GSTP1...
also increased iNOS S-nitrosylation level in HEK293 cells (Figure 7C). The same results were observed in HEK293 cells under stable overexpression and knockdown of GSTP1 conditions (Figure 7D). In addition, Figure 7E demonstrated that GSTM1 and GSTP1 (Y7F) have no effect on the regulation of iNOS S-nitrosylation level.

It has been reported that the ubiquitination of iNOS is required for its degradation [24]. To further determine whether GSTP1 could also affect iNOS ubiquitination, we employed proteasome inhibitor MG132 to terminate the protein degradation through proteasome pathway. The results of co-immunoprecipitation revealed that GSTP1 facilitates iNOS ubiquitination in HEK293 cells (Figure 7F).

Discussion

NO is a neutral free radical that can react fast with several targets such as other free radicals or metal centers in proteins and plays an important role in many physiological and diverse pathophysiological conditions [11,12,26]. Findings over the last decade have implicated that NO produced by aberrant expression of iNOS may result in many diseases including Alzheimer’s disease, Parkinson’s disease, asthma, inflammatory bowel disease, arthritis, etc. [14,16,27,28]. iNOS is a Ca^{2+}-independent isoenzyme of NOS and can produce cytotoxic levels of NO from L-arginine in response to inflammatory mediators. iNOS can be regulated through its synthesis, stability, and catalytic activity [13,29,30]. The factors that affect iNOS stability have not been studied in detail. Our previous study suggests that GSTP1 plays an anti-inflammatory role in response to LPS and inhibits LPS-induced iNOS protein increase and NO release [9]. Earlier report demonstrates that GSTP1 acts as a NO carrier [8]. However, up till now the detailed mechanism utilized by GSTP1 for regulating cellular iNOS protein level remains largely unclear.

Here, we used overexpression and RNAi technique to show that GSTP1 inhibited LPS-induced iNOS protein level in RAW264.7 cells. Since HEK293 cells lack NOS expression background, they are suitable for investigating exogenous iNOS expression. In HEK293 cells, we got the same results as those in RAW264.7 cells. In GST family, both GSTmu and GSTpi have a similar structure of C-terminal domain, which implies some of their similar properties, but different protein binding features between these two enzymes have also been reported [31]. Although they share 25%–30% sequence identity, their substrate specificity and diversity have been reshaped by gene duplication, genetic recombination, and an accumulation of mutations. In the present experiment, unlike GSTP1, GSTM1 protein posed no effect on iNOS protein level. This result suggests that among GSTs, GSTP1 is specific in regulating elevation of LPS-induced iNOS protein level. Our study also suggests that the enzyme activity of GSTP1 was necessary for GSTP1 to regulate iNOS protein level.
Since real-time PCR experiments demonstrated that GSTP1 did not influence iNOS mRNA level, we focused on the effect of GSTP1 on iNOS protein stability. We found that GSTP1 reduced the full length of iNOS protein level and both oxygenase and reductase domains of iNOS were involved in regulation by GSTP1. GSTP1 participates in the regulation of stress signaling via its noncatalytic, ligand-binding activity[31]. Through analyzing the interaction of GSTP1 with iNOS, we found that G-site domain of GSTP1 physically associated with the oxygenase domain of iNOS. Although GSTP1 (Y7F) did not affect iNOS stability, it could interact with iNOS, which indicated that the interaction of GSTP1 and iNOS depended on ligand-binding activity of GSTP1 and the enzyme activity of GSTP1 was necessary for regulating iNOS stability. This result suggested that GSTP1 regulated iNOS stability through modifying iNOS at post-translational level.

Dimerization of iNOS protein is essential for NO synthetic activity [21,22]. In our following study, we discovered that GSTP1 but not GSTM1 and GSTP1 (Y7F) obviously decreased the amount of iNOS dimer level, which suggested the effect of GSTP1 on iNOS dimerization. There are very few reports about post-translational modification of iNOS. It has been reported that human Tyr1055 in iNOS might be a target for Src-mediated phosphorylation and such post-translational modification serves to stabilize iNOS half-time [32]. S-nitrosylation, a redox-based post-translational modification of proteins by NO, is recognized to regulate the activities of an increasing number of target proteins. S-nitrosylation is a ubiquitous regulatory reaction comparable to phosphorylation and is already considered a main form for NO to play its “second messenger” function [33–35]. NO could also alter iNOS dimer stability through increasing the protein S-nitrosylation. S-nitrosylation of the zinc tetrahedral cysteines in iNOS
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Supplementary material available online

Supplementary Figure 1 to 3 to be found at online http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1027199.