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Further evidence that rat liver microsomal glutathione transferase 1 is not a cellular protein target for *S*-nitrosylation

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Abstract

By adopting biotin switch method, we recently reported that liver microsomal glutathione transferase 1 (MGST1) might not be a protein target for *S*-nitrosylation in rat microsomes or in vivo. However, alternative analytic methods are needed to confirm this observation, as a single biotin switch method in judging specific protein *S*-nitrosylation in biological samples is increasingly recognized as insufficient, or even unreliable. Besides, only MGST1 localized on endoplasmic reticulum (ER), but not mitochondria which favors protein *S*-nitrosylation was examined in the previous report. Present study was therefore carried out to address these issues. Primary cultured hepatocytes were used. A physiological existing nitric oxide (NO) donor *S*-nitrosoglutathione (GSNO) was adopted to trigger protein *S*-nitrosylation. MGST1 was immunoprecipitated and its *S*-nitrosothiol content was measured by the NO probe 2,3-diaminonaphthalene. In parallel, *S*-nitrosylated proteins were immunoprecipitated by a monoclonal anti-*S*-nitrosocysteine antibody and probed with an anti-MGST1 antibody. In hepatocytes, neither ER nor mitochondria were found to contain *S*-nitrosylated MGST1 after GSNO treatment, showing that differently distributed MGST1 was consistently un-nitrosylable in the cellular environment. But under broken cell conditions, when samples were incubated directly with GSNO, MGST1 *S*-nitrosylation was indeed detectable in both the microsomal and mitochondrial proteins, indicating that previous failure in detecting MGST1 *S*-nitrosylation in microsomes is due to the limitations of biotin switch method. These results clearly, if not definitely, demonstrate that MGST1 is not a ready candidate for *S*-nitrosylation in the cellular content, despite its susceptibility to *S*-nitrosylation under broken cell conditions.
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1. Introduction

Microsomal glutathione transferase 1 (MGST1) is a constitutively expressed enzyme displaying both glutathione *S*-transferase and glutathione-dependent peroxidase activities [1,2]. It is a homo-trimeric enzyme with each sub-unit containing a single cysteine (Cys49) and seven tyrosines, which are sensitive to various modifications including alkylation [3], nitration (on Tyr92) [4,5], *S*-nitrosylation [6], *S*-glutathionylation [7], dimerization [8], proteolysis [9] etc. These modifications usually lead to its enhanced catalytic activities, promoting its detoxifying functions and anti-lipid peroxidation capabilities. Cys49 and Tyr92 are therefore proposed as sensors detecting pathological and toxicological changes in the

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cellular environments. In response to nitric oxide (NO) overproduction, covalent attachment of a NO group to Cys49 forming *S*-nitrosothiol (SNO) structure, i.e. *S*-nitrosylation, has been recently suggested to be a novel mechanism for activation of purified MGST1 in vitro [6]. However, by using biotin switch method, we found no detectable *S*-nitrosylated MGST1 either in microsomes treated with NO donor *S*-nitrosoglutathione (GSNO), or in rats subjected to nitrosative stress [10]. This casted some doubts on the in vivo significance of MGST1 *S*-nitrosylation.

More recently, biotin switch method, first introduced by Jaffrey et al. in 2001 [11,12], has received intense criticism in detecting specific *S*-nitrosylated proteins in biological samples. First, its sensitivity is far from satisfactory in measuring endogenously *S*-nitrosylated proteins [13,14], which is generally presented at very low concentrations (1–200 pmol/mg protein). It was found that only when the protein *S*-nitrosothiol levels are beyond 6 nmol/mg protein could biotin switch method present detectable signals on Western blot [14]. Sample loss and spontaneously de-nitrosylation would inevitably lead to false-negative results. Secondly, its specificity is not as high as assumed, as a recently study demonstrated that certain *S*-glutathionylated proteins, in addition to *S*-nitrosylated ones, would also present a positive signal [15,16]. In the third place, some modifications of the original procedures are necessary to achieve optimal results [14]. Awareness of these newly discovered limitations prompted us to re-examine whether MGST1 is indeed *S*-nitrosylabe in cellular content.

To determine whether a specific protein is *S*-nitrosylated in certain biological samples, two approaches, besides the biotin switch method, have received universal acceptance. The first one involves immunoprecipitation of a candidate protein prior to decomposition of protein *S*-nitrosothiols by mercuric ion ($HgCl_2$) to release NO, followed by detection of fluorescence generated from the NO probe 2,3-diaminonaphthalene (referred herein as DAN method) [17,18]. The second approach utilized an anti-*S*-nitrosocysteine antibody, which recognizes the proteins that contain the *S*-nitrosothiol moiety [19], to immunoprecipitate the *S*-nitrosylated protein, which is then probed with antibodies against the protein of interest [20] (referred herein as anti-SNO method). Both strategies are adopted in the present study to determine whether MGST1 is a cellular target for *S*-nitrosylation.

As MGST1 is found in abundance in either endoplasmic reticulum (ER) or mitochondria outer membrane, accounting for 3% and 5% of the total proteins in each organelle [21], and protein *S*-nitrosylation appears to be mitochondria-favorable (if not mitochondria-specific) [22,23], both the two organelles were examined with respect to MGST1 *S*-nitrosylation.

2. Materials and methods

2.1. Materials

Sephadex G-25 (fine) and CM-Sepharose CL-6B were purchased from Pharmacia. Dulbecco's modified eagle's medium and Percoll were from Sigma. Peroxidase-conjugated goat anti-rabbit IgG were from Santa Cruz Biotechnology. Seize × Protein G Immunoprecipitation Kit, enhanced chemiluminescence reagents were from Pierce, and 2,3-diaminonaphthalene (DAN) was from Acros. Anti-*S*-nitrosocysteine monoclonal antibody was from A.G. scientific. Hydroxyapatite was the product of Calbiochem. GSNO was synthesized by combining equimolar (200 mM) $NaNO_2$ and glutathione in 0.5N HCl containing 50 μM diethylene triaminepentaacetic acid (DTPA) in the dark at room temperature for 10 min as described previously [23]. Before use, the GSNO solution was neutralized to pH 7.0 with 0.1N NaOH. This solution was prepared daily to avoid storage derived decomposition.

2.2. Purification of MGST1 and preparation of anti-MGST1 antibody

Rat MGST1 was purified from the liver microsomal fractions by one hydroxyapatite chromatography in combination with two CM-Sepharose chromatography as detailed before [24]. Anti MGST1 serum was raised by the method presented in our previous report [10]. The antibody was purified by DEAE-Sepharose CL-4B.

2.3. Primary hepatocyte culture

Hepatocytes from male Sprague–Dawley rats (250–300 g) were isolated by two-step collagenase perfusion followed by Percoll gradient centrifugation [25]. Animal protocols were approved by the Experimental Animal Care and Use Committee of Zhejiang University. Briefly, rats were anesthetized by an i.p. injection of 40 mg/kg sodium pentobarbital. The liver was perfused with a buffer containing collagenase (100 U/ml) via the portal vein for 30 min. Digested liver was then excised and filtered through a sterile 100-mesh nylon net. The resulting suspension was centrifuged at 50 × *g* for 3 min. Cell pellet was re-suspended in DMEM and Percoll was added to a

final concentration of 45%. After centrifuging at room temperature for 3 min at $60 \times g$, cell pellets were suspended in DMEM and washed once. Then cell viability was tested with trypan blue exclusion method. Cells with the viability of more than 85% were suspended in DMEM, which was supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.31 ml/l insulin, 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Collagen-coated culture dishes (60 mm) were used and cell density was 2×10^6 cells per dish. Hepatocytes were allowed to attach for 4 h for establishing of mono-layers.

2.4. S-nitrosylation of proteins in hepatocytes

After establishing of hepatocyte mono-layers, the medium was removed and replaced with fresh one containing GSNO at the final concentrations of 0, 40, 100, and 250 μM, L-cystine (200 μM) was also included to maximize cellular protein *S*-nitrosylation. The treatment period lasted for 1 h and then all medium was removed. The cells were washed three times with phosphate buffered saline (PBS) and microsomal and mitochondria fractions were prepared immediately as described below.

2.5. Preparation of microsomal and mitochondria proteins

Microsomal and mitochondria fractions were prepared from hepatocytes by the established method with minor modifications [26]. Briefly, the cells were scraped by use of a rubber policeman and re-suspended cells were disrupted by homogenization. Mitochondria were prepared by first removing debris and nuclei via centrifugation at $800 \times g$ for 10 min. The supernatant was decanted and the mitochondria were pelleted at $5000 \times g$ for 20 min. For preparation of total microsomes, the suspension of the disrupted cells was centrifuged at $10{,}000 \times g$ for 20 min and the supernatant was further centrifuged at $105{,}000 \times g$ for 1 h to pellet the total microsomal fraction. Both mitochondria and microsomal proteins were suspended in PBS containing 0.1% Triton-X-100, 0.1% SDS and 50 μM DTPA. All procedures were carried out at 0–4 °C and samples were prevented from light to reduce de-nitrosylation. Protein concentrations were determined by the Bradford method using bovine serum albumin (BSA) as standards. Ensuing detection of MGST1 *S*-nitrosylation was performed immediately after sub-cellular fractionation.

2.6. In vitro S-nitrosylation of microsomal and mitochondrial proteins

Microsomal or mitochondrial proteins were adjusted to 1 mg/ml, and incubated with freshly prepared GSNO at a final concentration of 0, 40, 100, and 250 μM. Samples were kept in the dark at room temperature for 20 min. Excessive GSNO was removed by passing a Sephadex G-25 (fine) column prior to the detection of MGST1 *S*-nitrosylation.

2.7. DAN method for the detection of MGST1 S-nitrosylation

Briefly, 10 μg anti-MGST1 antibody, or an equal amount of unimmune rabbit IgG was incubated with the microsomal or mitochondrial proteins in PBS (pH 7.2) containing 0.1% Triton-X-100, 0.1% SDS and 50 μM DTPA at 4 °C for 2 h. Then 80 μl Protein-G-Sepharose was added and allowed to shake for 1 h. After washed by 0.5 M NaCl in PBS for three times, the Sepharose bead was incubated with glycine buffer pH 3 for 15 min to elute the bound antigen. Elution was repeated once. In order to normalize the MGST1 contents among each immunoprecipitation, a small volume was taken to perform Western blot, together with purified MGST1 of known concentrations. The remaining samples were divided into two equal aliquots, one is added DAN to a final concentration of 100 μM, another is added the same amount of DAN plus $HgCl_2$ at a final concentration of 100 μM. Samples were kept in the dark for 30 min prior to the addition of 1N NaOH to adjust pH above 10. Then the fluorescence was measured with an excitation wavelength of 375 nm and an emission wavelength of 450 nm. The difference of fluorescence intensity between samples containing $HgCl_2$ and free of $HgCl_2$ represents the *S*-nitrosothiol specific signal. Standard curves were generated using freshly prepared GSNO.

2.8. Anti-SNO method for the detection of MGST1 S-nitrosylation

Briefly, 8 μg anti-*S*-nitrosocysteine antibody, or an equal amount of unimmune mice IgG was incubated with the microsomal or mitochondria proteins (1 mg/ml) at 4 °C with gentle shaking for 24 h. Then 80 μl Protein-G-Sepharose was added and allowed to shake for 2 h. After washed by 0.5 M NaCl in PBS for three times, the Sepharose bead was boiled at 95 °C for 3 min. The immunoprecipitated proteins were separated by SDS-PAGE. Some gels were used to perform silver staining,

while others were used for transferring of the proteins to nitrocellulose membranes. Anti-MGST1 antibody (1:1000) was added to probe with the membranes for 2 h. Peroxidase-conjugated goat anti-rabbit IgG (1:5000) were used as secondary antibody. Immunoreactive bands were visualized using enhanced chemiluminescence reagents and analyzed by the QuantityOne Program (Bio-Rad).

2.9. DAN method for the detection of total protein S-nitrosothiols in hepatocytes fractions

Microsomal or mitochondria proteins, prepared from hepatocytes treated with GSNO (0, 40, 100, 250 μM), were passed through a Sephadex G-25 column to remove low molecular *S*-nitrosothiols. Samples were then adjusted to 1 mg/ml and added DAN (100 μM) with or without $HgCl_2$. After kept in the dark for 30 min, proteins were removed by sulfosalylic acid precipitation. Then the fluorescence was measured and *S*-nitrosothiols content calculated from standard curves generated from GSNO.

3. Results

We first used DAN method to detect MGST1 *S*-nitrosylation in microsomes or mitochondria, which were incubated directly with GSNO in vitro. GSNO concentration ranged from 40 to 250 μM, as these are believed to be of physiological and pathological relevance [11,12]. Fig. 1A shows that when anti-MGST1 antibody was used in the immunoprecipitation experiments starting from microsomal proteins, an apparent band at 17 kDa was detected on Western blot using the anti-MGST1 antibody (lane 2), while the unimmune IgG produced no visible signal (lane 1). This demonstrated that MGST1 could be successfully enriched from microsomal proteins by immunoprecipitation method. Lane 3 in Fig. 1A shows the Western blot signal of 0.5 μg purified MGST1. Similar results were obtained from the mitochondrial proteins (Fig. 1B). Fig. 1C shows that in samples incubated without GSNO, no detectable *S*-nitrosothiols were observed in the immunoprecipitated MGST1 samples, suggesting that MGST1 does not exist in the *S*-nitrosylated form within microsomes or mitochondria under physiological conditions. After incubation with GSNO, a well-recognized *S*-nitrosylating reagent, *S*-nitrosylated MGST1 began to appear, which showed a does dependence on GSNO concentrations (Fig. 1C), suggesting that MGST1 in microsomes or mitochondria is susceptible to *S*-nitrosylation under broken cell conditions. These results raised the possibility that MGST1 in the biological samples are indeed prone to be *S*-nitrosylated when treated directly with NO donors.

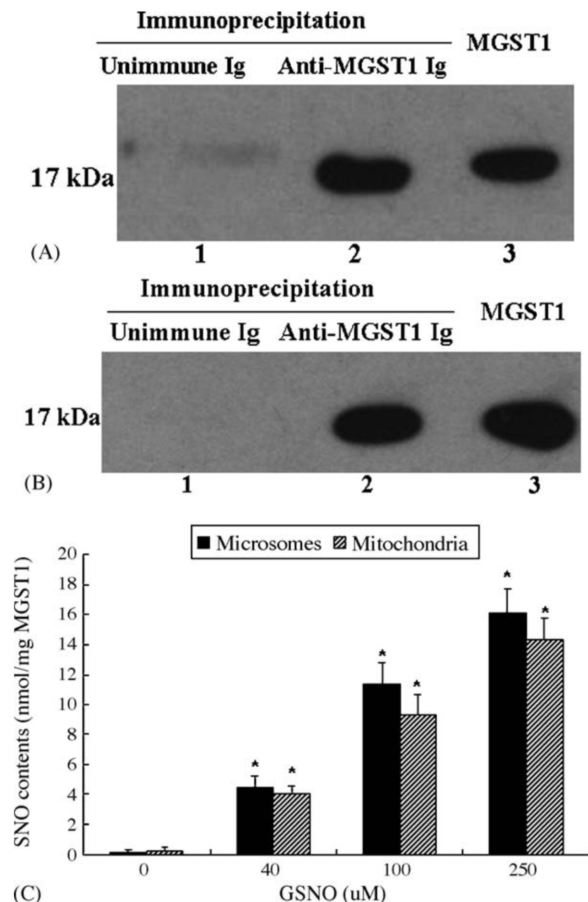

Fig. 1. MGST1 *S*-nitrosylation under broken cell conditions measured by DAN method. Microsomal or mitochondrial proteins, after incubated directly with GSNO at a final concentration of 0, 40, 100, 250 μM, were added 10 μg anti-MGST1 antibody (or unimmune rabbit IgG) and 80 μl Protein-G-Sepharose to immunoprecipitate MGST1. One fourth of the resulting samples were loaded on SDS-PAGE gels to perform Western blot using anti-MGST1 antibody. (A) and (B) showed a representative results from untreated microsomal or mitochondrial samples, respectively. On lane 3, 0.5 μg purified MGST1 was loaded. The remaining samples were subjected to *S*-nitrosothiol measurement by DAN fluorescence (C). Standard curves were generated using freshly prepared GSNO. Data represent means ± S.D. ($n = 5$). $^{*}P < 0.05$ compared with 0 μM GSNO (one-way ANOVA, Neuman–Keul post hoc test).

To confirm the results from DAN method, anti-SNO method was next adopted to test MGST1 *S*-nitrosylation in microsomes (Fig. 2A) or mitochondria (Fig. 2B). Results show that when the enriched *S*-nitrosylated proteins were probed with anti-MGST1 antibody, a positive protein band at 17 kDa was observed in the GSNO treated samples but not in control ones. This indicates

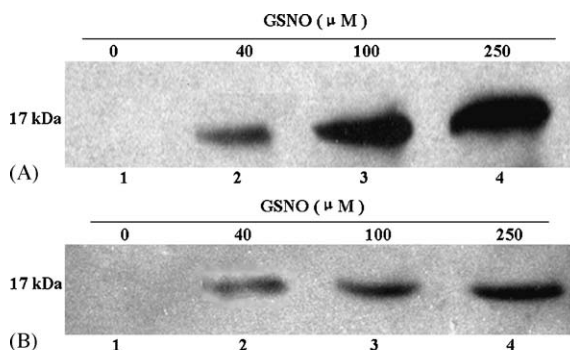

Fig. 2. MGST1 *S*-nitrosylation under broken cell conditions determined by anti-SNO method. Microsomal (A) or mitochondrial (B) proteins were incubated directly with GSNO at a final concentration of 0, 40, 100, and 250 μM for 20 min. After removing of excessive GSNO, 8 μg anti-*S*-nitrosocysteine antibody and 80 μl Protein-G-Sepharose were used to capture *S*-nitrosylated proteins, which were eluted by boiling for 5 min. One half of the resulting samples were loaded on SDS-PAGE gels. Anti-MGST1 antibody was used to perform Western blot. Immunoreactive bands were visualized by enhanced chemiluminescence reagents. Data are representative of five separate experiments.

that MGST1 was among the *S*-nitrosylated proteins after GSNO treatment. In other words, it demonstrates that MGST1 was indeed *S*-nitrosylable in both microsomal and mitochondria proteins after incubation with GSNO under broken cell conditions.

We then examined MGST1 *S*-nitrosylation in the cellular environment. Hepatocytes were incubated with GSNO plus L-cystine to trigger liver protein *S*-nitrosylation, which was assessed by both anti-SNO method and DAN method. As shown in Fig. 3A, in the microsomal proteins from untreated hepatocytes, immunoprecipitation using anti-*S*-nitrosocysteine antibody produced only two protein bands on the silver stained SDS-PAGE gels, which might represent the antibody itself and the protein-G; while GSNO plus L-cystine treatment did produce several additional protein bands, which represents the *S*-nitrosylated liver proteins. Similar results were obtained from the mitochondrial proteins (Fig. 3B). Fig. 3C shows that when the unimmune mouse IgG was used, no *S*-nitrosylated proteins could be obtained from immunoprecipitation experiments. These results demonstrate that GSNO plus L-cystine treatment of hepatocytes could indeed trigger liver protein *S*-nitrosylation. In supporting this, DAN method was also adopted to measure protein *S*-nitrosylation in hepatocytes. As shown in Fig. 3D, untreated hepatocytes were found to contain very low level of protein *S*-nitrosothiols in microsomes and mitochondria (nearly undetectable by the DAN method), while GSNO plus L-cystine treatment of cells caused a significant increase of *S*-nitrosylated proteins, suggesting that this approach is very effective in triggering cellular protein *S*-nitrosylation in the hepatocytes. However, among the anti-SNO antibody enriched proteins, MGST1 was not detected in both the microsomal (Fig. 4A, lanes 2–5) and mitochondria samples (Fig. 4B, lanes 2–5). In control experiments, protein samples were incubated directly with GSNO (100 μM) prior to *S*-nitrosothiol assay, and positive signals were indeed observed (Fig. 4A and B, lane 1). This demonstrates that MGST1 is not readily *S*-nitrosylable in the hepatocytes after GSNO plus L-cystine treatment. Further evidence comes from the DAN method (Fig. 5), in which the immunoprecipitated MGST1 was found to contain no detectable *S*-nitrosothiols in either microsomal (A) or mitochondrial (B) fractions after hepatocytes were treated with GSNO plus L-cystine, while direct incubation of samples with 100 μM GSNO (control) indeed caused MGST1 *S*-nitrosylation under broken cell conditions.

4. Discussions

Protein *S*-nitrosylation, the addition of a NO group to protein cysteine(s) forming *S*-nitrosothiol structure, is increasingly recognized as a universal regulatory mechanism for many physiological and pathological processes [27–29]. Essentially all proteins harbor cysteine(s), and NO is ubiquitously distributed, therefore it is conceivable that a large number of proteins including MGST1 would be susceptible targets for *S*-nitrosylation in vivo. However, due to its extreme instability and low abundance, a golden criterion for determining individual protein *S*-nitrosylation, especially stoichiometrically under cellular content, is still lacking [30]. It is also this reason that data from different laboratories are in conflict [31]. An emerging consensus seems to be that at least two different methods should be adopted in judging cellular *S*-nitrosylation of a specific protein.

As for MGST1, *S*-nitrosylation of its purified form in vitro has been first demonstrated by Ji Y. et al. [6], and it was lately confirmed by our own study [10]. However, MGST1 *S*-nitrosylation in the biological samples, i.e., in microsomes, has only been assumed, but not proved [6]. Even in a very recent report, direct experimental evidence supporting this assumption is still not available [35]. In our efforts to solve this issue, we previously failed to present favorable evidence by biotin switch assay [10].

Currently, three approaches, including biotin switch method, DAN method, anti-SNO method, are available for identifying individual proteins *S*-nitrosylated in a biological sample. Among those, we now found that the

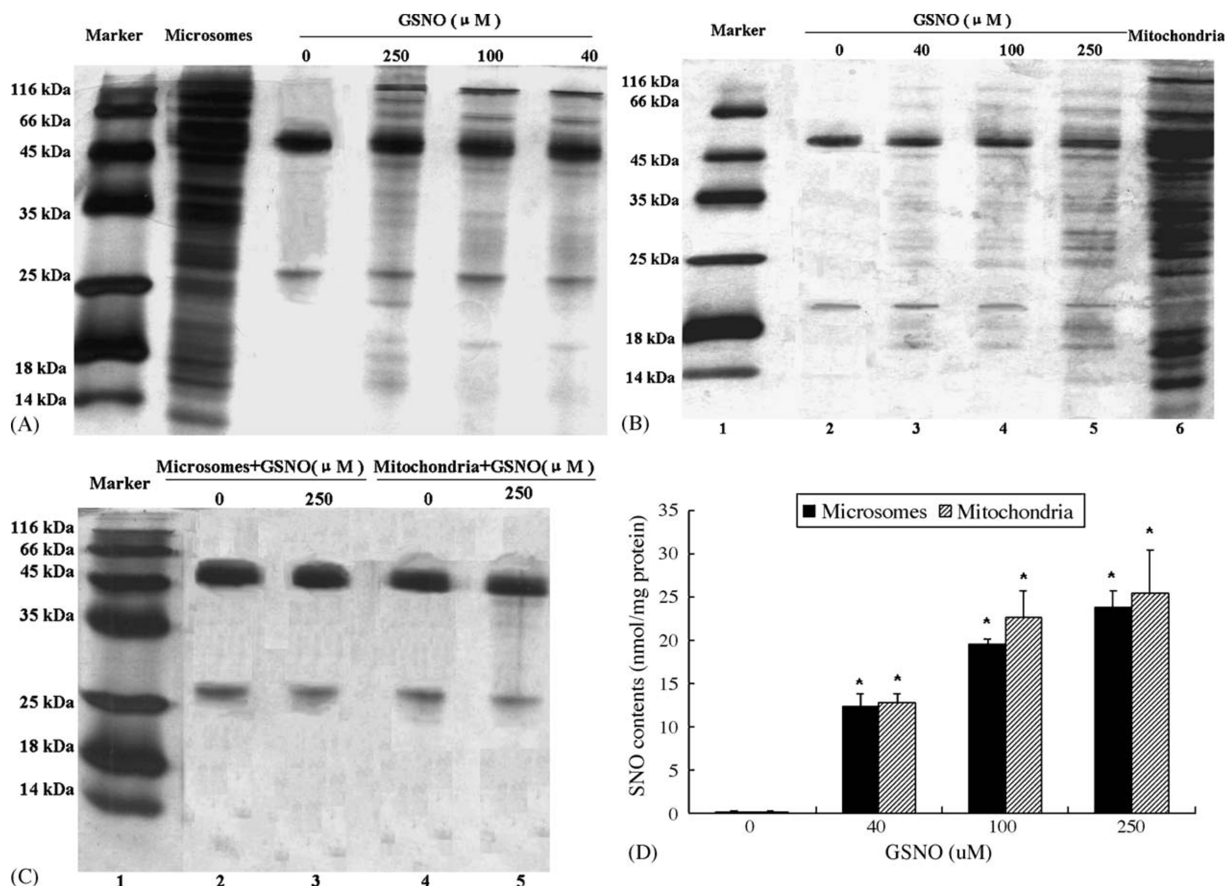

Fig. 3. Effects of GSNO on protein *S*-nitrosothiol levels in hepatocytes detected by anti-SNO method and DAN method. After treated with GSNO at a final concentration of 0, 40, 100, and 250 μM together with 200 μM L-cystine for 1 h, hepatocytes were collected to prepare microsomal and mitochondrial proteins. Then 8 μg anti-*S*-nitrosocysteine antibody (or an equal amount of unimmune mouse IgG) and 80 μl Protein-G-Sepharose were used to capture *S*-nitrosylated proteins, which were eluted by boiling for 5 min. One half of the resulting samples were loaded on SDS-PAGE gels and visualized by silver staining (A)–(C). The remaining samples were added DAN (100 μM) with or without $HgCl_2$ to measure *S*-nitrosothiol content, which were calculated from standard curves generated from GSNO. ($n=5$). $^*P<0.05$ compared with 0 μM GSNO (one-way ANOVA, Neuman–Keul post hoc test).

latter two of them indeed presented positive results in detecting MGST1 *S*-nitrosylation in microsomes. Possible reasons for failure of biotin switch method in detecting MGST1 *S*-nitrosylation among microsomal protein might lie in: acetone precipitation of samples would un-avoidably render MGST1 loss, the lengthy process of labeling protein *S*-nitrosothiols and subsequent purification process might lead to MGST1-SNO de-nitrosylation. Of note, even if a positive signal was indeed detected by the biotin switch assay, it cannot be viewed as direct evidence that MGST1 is *S*-nitrosylable, as *S*-glutathionylated MGST1 (which possibly exists after GSNO treatment) might also present positive signals. Complementary analytic methods are necessary to confirm results from single biotin switch detection. It appears apparent that biotin switch method is not suitable for detecting MGST1 *S*-nitrosylation among the biological samples.

Due to the inherent limitations of the biotin switch method, and the universal acceptance of other two methods, we believe it is reasonable to suggest, according data presented here, that MGST1 is indeed *S*-nitrosylable in the microsomes. Thus our previous assumption that MGST1 is not *S*-nitrosylated in microsomal proteins in vitro [10] has to be modified. In addition, not only MGST1 in microsomes, but also in mitochondria, were demonstrated in the present study to be *S*-nitrosylable in vitro after NO donor treatment. To our knowledge, data presented here are the first direct experimental evidence demonstrating that MGST1 is *S*-nitrosylable in the biological samples under broken cell conditions.

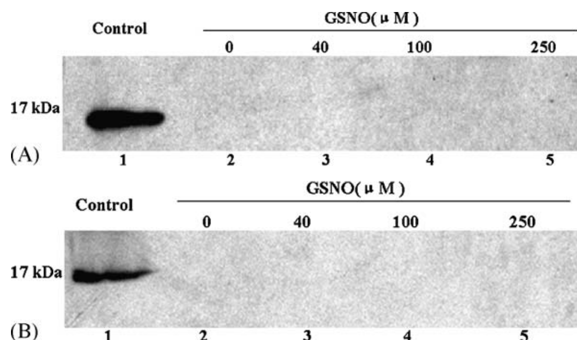

Fig. 4. MGST1 *S*-nitrosylation in hepatocytes detected by anti-SNO method. Primary cultured hepatocytes were treated with GSNO at a final concentration of 0, 40, 100, 250 μM together with 200 μM L-cystine for 1 h. Microsomal (A) or mitochondrial (B) proteins were prepared and the *S*-nitrosylated proteins were enriched by immunoprecipitation via anti-*S*-nitrosocysteine antibody (8 μg) and Protein-G-Sepharose (80 μl). One half of the resulting samples were subjected SDS-PAGE and transferred to nitrocellulose membranes. The anti-MGST1 antibody was used to probe with the membranes and Peroxidase-conjugated goat anti-rabbit IgG was used to visualize the immunoreactive band. Samples incubated directly with GSNO (100 μM) were used as a positive control (lane 1). Data are from five separate experiments.

We next tested whether MGST1 is also *S*-nitrosylable in the cellular content. Primary cultured hepatocytes were chosen as MGST1 distributes predominantly in the liver. The physiologically existing NO donor GSNO was added together with L-cystine to trigger protein *S*-nitrosylation, because it is the mostly widely used and

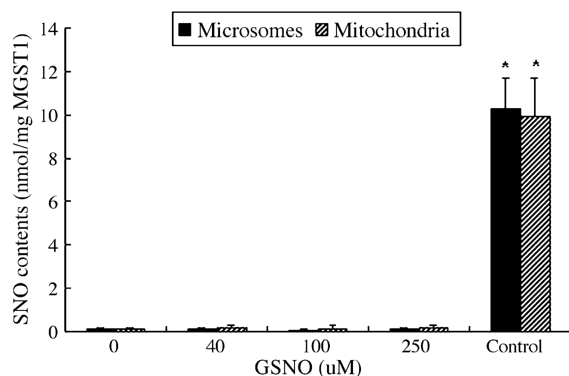

Fig. 5. MGST1 *S*-nitrosylation in hepatocytes detected by DAN method. Primary cultured hepatocytes were treated with GSNO at a final concentration of 0, 40, 100, 250 μM together with 200 μM L-cystine for 1 h. Microsomal or mitochondrial proteins were prepared, and then anti-MGST1 (10 μg) antibody and Protein-G-Sepharose were added to immunoprecipitate MGST1. *S*-Nitrosothiols were measured by DAN fluorescence. Freshly prepared GSNO was used as standards. Microsomal or mitochondrial proteins incubated directly with GSNO (100 μM) were used as a positive control. Data represent means ± S.D. ($n = 5$). $^{*}P < 0.05$ compared with 0 μM GSNO (one-way ANOVA, Neuman–Keul post hoc test).

well-established strategy to enhance *S*-nitrosylation of cellular proteins. L-Cystine is essential for transferring of *S*-nitroso group into intracellular space, as GSNO alone appears to have little intracellular effects as for protein *S*-nitrosylation. Unexpectedly, both two methods for *S*-nitrosothiol assays produced negative results in detecting MGST1 *S*-nitrosylation in the hepatocytes, suggesting that MGST1 might not be susceptible to *S*-nitrosylation under cellular conditions. Even in mitochondria, which are believed to compartment *S*-nitrosylated proteins, no detectable MGST1 in *S*-nitrosylated form was found. In additional experiments, when another physiological NO donor *S*-nitrosocysteine, which is believe to have access to the cytoplasm, was used, similar results were obtained (data not shown). These results clearly demonstrate that MGST1 is not readily *S*-nitrosylable in the hepatocytes.

In hepatocytes, two factors that contribute to the unreadiness of MGST1 *S*-nitrosylation could be expected. First, and most importantly, a liver abundant cytosolic enzyme, GSNO reductase (GSNOR), which catalyze the decomposition of low molecular *S*-nitrosothiols and thus control the homeostasis of protein *S*-nitrosothiols [32], might play a role in the regulation of MGST1 *S*-nitrosylation. Studies show that in GSNOR null mice, a significant increase of protein *S*-nitrosothiols was observed in the liver after nitrosative challenge, while the wide type mice displayed only a mild enhancement of liver protein *S*-nitrosothiols [33]. GSNOR was therefore proposed as a protective mechanism against nitrosative stress. As Cys49 of MGST1 is localized on the cytoplasmic side of the ER [3], we speculate that its proximity to the cytosolic GSNOR would prevent cellular MGST1 *S*-nitrosylation. To confirm this speculation, however, further studies using the GSNOR knockout animals are needed. Secondly, GSNO treatment of cells would also cause protein *S*-glutathionylation in addition to *S*-nitrosylation [34]. Whether the former or the latter plays a dominant role in MGST1 modification in the hepatocyte is currently unknown. Our data seem to favor the exclusion of *S*-nitrosylation, but additional evidence for *S*-glutathionylation is needed.

Reactive nitrogen species (RNS) related modifications are a recent focus on MGST1. Many novel mechanisms have been discovered, and all these modifications point to the possibility that both Cys49 and Tyr92 might serve as sensors detecting the cellular alterations of NO homeostasis, affording a novel protective mechanism against nitrosative stress [4–6,35]. However, previous studies were almost exclusively carried out using the in vitro purified enzyme or the microsomes. These approaches would sometimes produce artifact of in vitro conditions, leaving the in vivo significance uncertain.

Also, direct experimental evidence for a specific modification in the cellular environment is lacking in most cases. As for *S*-nitrosylation, data present here clearly demonstrated that MGST1 is not a susceptible target in hepatocytes, though it is indeed *S*-nitrosylable under broken cell conditions. To firmly establish the pathological and toxicological relevance of RNS related modifications on MGST1, experiments with hepatocytes or animals, especially animal models of suitable pathological conditions should be carried out in future studies.

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