ORIGINAL RESEARCH COMMUNICATION

Protein S-nitrosylation in *Plasmodium falciparum*

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Abstract

Aims: Due to its life in different hosts and environments, the human malaria parasite *Plasmodium falciparum* is exposed to oxidative and nitrosative challenges. Nitric oxide (NO) and NO-derived reactive nitrogen species can constitute nitrosative stress and play a major role in NO-related signaling. However, the mode of action of NO and its targets in *P. falciparum* have hardly been characterized. Protein S-nitrosylation (SNO), a post-translational modification of protein cysteine thiols, has emerged as a principal mechanism by which NO exerts diverse biological effects. Despite its potential importance, SNO has hardly been studied in human malaria parasites. Using a biotin-switch approach coupled to mass spectrometry, we systemically studied SNO in *P. falciparum* cell extracts. **Results:** We identified 319 potential targets of SNO that are widely distributed throughout various cellular pathways. Glycolysis in the parasite was found to be a major target, with glyceraldehyde-3-phosphate dehydrogenase being strongly inhibited by S-nitrosylation of its active site cysteine. Furthermore, we show that *P. falciparum* thioredoxin 1 (PfTrx1) can be S-nitrosylated at its nonactive site cysteine (Cys43). Mechanistic studies indicate that PfTrx1 possesses both denitrosylating and transnitrosylating activities mediated by its active site cysteines and Cys43, respectively. **Innovation:** This work provides first insights into the S-nitrosoproteome of *P. falciparum* and suggests that the malaria parasite employs the thioredoxin system to deal with nitrosative challenges. **Conclusion:** Our results indicate that SNO may influence a variety of metabolic processes in *P. falciparum* and contribute to our understanding of NO-related signaling processes and cytotoxicity in the parasites. *Antioxid. Redox Signal.* 20, 2923–2935.

Introduction

MALARIA REMAINS A major global health issue, threatening more than 40% of the world's population. It causes more than 200 million cases and about 1 million deaths annually (56). The deadliest human malaria parasite *Plasmodium falciparum* is the major cause of the morbidity and mortality of severe malaria, especially in Africa (56). This unicellular parasite undergoes complicated life cycles in its transmitting mosquito vectors and the targeted human host (39). The required lifestyle transitioning from intracellular to extracellular environments together with the rapid proliferation and multiplication of the parasites makes *P. falciparum* inevitably exposed to oxidative and nitrosative challenges (6, 13, 28).

Nitric oxide (NO) has long been recognized as a potent antiparasitic effector molecule *in vivo* (9). NO and NO-

Innovation

Nitric oxide (NO) and NO-derived reactive nitrogen species are important *in vivo* effector molecules for controlling *Plasmodium* parasites in both mosquito vectors and humans. However, NO targets and the profound mechanism of NO actions in the parasites remain largely unexplored. In this study, we identified 319 protein targets of S-nitrosylation in *Plasmodium falciparum* and show that several central metabolic processes in malaria parasites, such as glycolysis, are potentially regulated by NO via S-nitrosylation. Additionally, we propose a redox status-based model for *P. falciparum* thioredoxin 1 in the regulation of protein S-nitrosylation in *P. falciparum* by mediating denitrosylation and transnitrosylation reactions.

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derived reactive nitrogen species (RNS) are major sources of nitrosative stress encountered by *P. falciparum* across different stages (6, 30, 31, 33, 41). Notably, the production of antiparasitic NO *via* host nitric oxide synthase (NOS) represents an important component of innate immune response against *Plasmodium* parasites in both mosquitoes and humans (13, 36, 41). In the mosquito stage, the expression of NOS in the mosquito midgut epithelial cells is elicited in response to several parasite-induced proinflammatory cytokines (49), plasmodial glycosylphosphatidylinositols (GPIs) (28, 58), and hemozoin (1, 20). Significant induction of mosquito NOS can lead to inflammatory levels of NO and RNS, which adversely impact the development of parasites in the mosquito midgut (30, 41). Similarly, in mammalian hosts including humans, NO production can be elicited in inducible NOS-expressing macrophages and monocytes upon *Plasmodium* infection to contribute to the clearance of intraerythrocytic *Plasmodium* parasites (31, 57). It was also suggested that RNS are involved in the inactivation of *Plasmodium* gametocytes mediated by tumor necrosis factor- α (TNF- α) in white blood cells (36). More recently, RNS were shown to impair the gametogenesis of male *Plasmodium* parasites and reduce the fertility of both male and female gametes (44). Accordingly, several *in vitro* studies confirmed that NO has both cytostatic and cytotoxic effects on *Plasmodium* parasites (4, 50), and some NO donors can induce apoptosis and autophagy-like cell death of *Plasmodium berghei* ookinetes and intraerythrocytic *P. falciparum*, respectively (2, 51).

Besides the NO stress derived from host-parasite interactions, *P. falciparum* appears to undergo endogenous nitrosative stress. By using a specific probe, RNS have been detected in ring and trophozoite stages of *P. falciparum* as well as in the parasite gametocyte (37). Although *P. falciparum* seems to lack an NOS ortholog, it has been suggested that it probably produces NO from a nitrate/nitrite reduction pathway (37, 38).

In recent years, protein S-nitrosylation (SNO) has emerged as a key player in the diverse biological functions of NO (18). SNO is a reversible protein posttranslational modification induced by nitrosative stress, referring to the covalent incorporation of a nitroso group (-NO) into a protein thiol to form S-nitrosothiol, which can regulate protein functions (18, 54). SNO can be generated either directly from the interaction of protein thiols with RNS (*e.g.*, N_2O_3) or from transnitrosylation, which represents the acquisition of a -NO moiety transferred from another S-nitrosothiol (18,35). Transnitrosylation is of more biological relevance, as it is an important mechanism of NO signaling in specific pathways (35). For example, it has been revealed that transnitrosylation by human thioredoxin (Trx) plays key roles in the regulation of cell apoptosis (7). Additionally, intracellular transnitrosylation is mediated predominantly by S-nitrosoglutathione (GSNO), a naturally occurring small-molecular-weight S-nitrosothiol that spontaneously releases NO and transnitrosylates proteins (10, 18). GSNO can be generated *in vivo* from glutathione (GSH) by reacting with RNS. Thus, GSNO serves as an NO pool that can effectively transduce NO stress and mediate NO signaling (10).

The homeostasis of cellular SNO is further regulated by denitrosylation, which degrades SNO *via* several nonenzymatic factors (*e.g.*, GSH and transition metals) and deni-

trotylases (8). In mammalian and plant cells, two major denitrosylase systems, the glutathione/GSNO reductase (GSH/GSNOR) system and the Trx system, have been identified (8). The importance of denitrosylation is emphasized by the fact that aberrant denitrosylation of proteins is closely associated with some human diseases, such as neurodegenerative diseases and cancers (8, 15, 34).

SNO has been extensively studied in *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, and *Arabidopsis thaliana*, and more than 1700 potential S-nitrosylation targets have been identified (dbSNO, <http://dbsno.mbc.nctu.edu.tw>). Despite its importance, SNO has not yet been systematically studied in human pathogenic parasites, including *P. falciparum*. In this study, we performed a large-scale proteomic analysis of SNO and identified potential S-nitrosylation targets in *P. falciparum*. SNO on selected target proteins was studied in detail *via* protein immunoblotting and enzymatic assays. Furthermore, the potential role of *P. falciparum* thioredoxin 1 (PfTrx1) in the regulation of SNO was investigated.

Results

Detection and isolation of S-nitrosylated proteins in *P. falciparum* cell extracts

The large-scale analysis of S-nitrosylated proteins in *P. falciparum* was performed by using a biotin-switch assay-based proteomic approach (14). The biotin-switch assay is an efficient and sensitive method to detect S-nitrosylated proteins based on the blockage of free protein thiols, specific reduction of S-nitrosothiols, and subsequent biotinylation of previously S-nitrosylated thiols (Fig. 1A). The sufficiency of thiol blockage and the specificity of the assay were demonstrated for *P. falciparum* samples (Fig. 1B, lanes 1–3, 8), ensuring the unbiased identification of S-nitrosylated proteins in *P. falciparum*.

For identifying S-nitrosylated proteins in unstressed and intact parasites, trophozoite stage *P. falciparum* parasites were isolated, directly lysed, and the cell extracts were immediately subjected to the biotin-switch assay. A control sample without the reduction of S-nitrosothiols by ascorbate was produced in parallel. Western blots indicated a rather low abundance of endogenous S-nitrosylated proteins in *P. falciparum* (Fig. 1B, lanes 6 and 7), which is consistent with observations in other organisms (27, 29, 32). Incubation of parasitized red blood cells with GSNO, a naturally occurring NO donor that promotes SNO (10), did not significantly enhance the signals, which is probably due to the presence of high hemoglobin concentrations and multiple cell membranes surrounding the parasite (47). Therefore, we decided to determine the targets of S-nitrosylation in *P. falciparum* cell extracts treated with GSNO (40 and 100 μ M). As a result, anti-biotin immunoblotting signals were clearly observed (Fig. 1B, lanes 4 and 5), indicating that a group of S-nitrosylated proteins were specifically detected.

By taking advantage of the biotin moiety, biotinylated proteins were further isolated *via* affinity purification with avidin and visualized in gels *via* silver staining. In parallel, a stringent control sample that omitted the reduction of S-nitrosothiols by ascorbate was set, representing biotinylated proteins derived from blocking-resistant thiols and nonspecific avidin-binding proteins. As shown in the silver-stained gel, a number of bands belonging to isolated S-nitrosylated

FIG. 1. Analysis of S-nitrosylated proteins in *Plasmodium falciparum* cell extracts. (A) Schematic diagram of the biotin-switch assay used for the analysis of S-nitrosylated proteins. In the presence of urea, free protein thiols were first blocked with methyl methanethiosulfonate (MMTS), and then, S-nitrosothiols were specifically reduced by sodium ascorbate (NaAsc) and further irreversibly biotinylated by iodoacetyl-PEG2-biotin. In the end, previously S-nitrosylated proteins were converted to biotinylated proteins that can either be detected by anti-biotin western blots or isolated by affinity purification with avidin. (B) Evaluation of the biotin-switch assay and the detection of S-nitrosylated proteins in *P. falciparum* cell extracts. *P. falciparum* cell extracts were incubated with 5 mM DTT to reduce and expose all protein thiols, which were then blocked with or without 200 mM MMTS before they were exposed to the biotinylating agent. The minimal anti-biotin immunoreactivity in MMTS-blocked cell extracts (lane 8) compared to the strong immunoreactivity in unblocked cell extracts (lane 1) indicated the minimization of false positives by efficient blockage of free thiols. The biotin-switch assay was unable to recognize protein disulfides and protein S-glutathionylation, two major types of thiol-based redox modifications induced by hydrogen peroxide (H₂O₂) and oxidized glutathione (GSSG), respectively (lanes 2 and 3). Minimal endogenous S-nitrosylated proteins in *P. falciparum* were revealed by the very weak anti-biotin immunoblotting signals in the freshly prepared, untreated cell extracts (lane 6) compared to a control sample in which the reduction of S-nitrosothiols by NaAsc was omitted (lane 7). S-nitrosylated proteins in *P. falciparum* were detected in the cell extracts treated with two different concentrations of GSNO (lanes 4 and 5). (C) A representative silver-stained gel shows the avidin-purified S-nitrosylated proteins from *P. falciparum* cell extracts treated with 100 μM GSNO. A control sample without reduction of S-nitrosothiols by NaAsc was included in parallel. DTT, 1,4-dithiothreitol; GSNO, S-nitrosoglutathione.

proteins clearly emerged in the GSNO-treated cell extracts compared to the control sample, indicating that S-nitrosylated proteins were specifically isolated (Fig. 1C).

Identification of S-nitrosylated proteins in *P. falciparum*

S-nitrosylated proteins isolated from fresh *P. falciparum* cell extracts treated with GSNO (100 μM) were trypsin-digested and identified using a linear trap quadrupole-Orbitrap mass spectrometer (MS). Only proteins for which at least two peptides were detected in at least two independent experiments were included in the target list. Proteins that were also abundantly detected in control samples (without reduction of S-nitrosothiols) were subtracted. By setting these strict criteria, we reproducibly identified 267 S-nitrosylated annotated proteins (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/ars) and 52 S-nitrosylated hypothetical proteins (Supplementary Table S2) in *P. falciparum*. Concomitantly, the MS/MS analysis revealed 37 S-nitrosylation sites from 34 proteins (Supplementary Tables S1 and S2). The identified proteins represent potential targets of SNO in *P. falciparum*. We further ana-

lyzed the characteristics of identified proteins in more detail by categorizing the proteins according to their gene ontology annotated in PlasmoDB (version 9.2). This analysis revealed that S-nitrosylated proteins participate in a wide range of biological processes in *P. falciparum*, mainly in DNA and RNA metabolism/processing, protein folding, translation, ubiquitin-dependent protein catabolism, pathogenesis, hemoglobin degradation, glycolysis, and transcription (Fig. 2A). The S-nitrosylated proteins also differ in molecular functions, among which nucleotide binding, hydrolase activity, protein binding activity, and peptidase activity appear to be predominant (Fig. 2B). S-nitrosylation of several recombinantly produced proteins was confirmed by western blot analysis (see Supplementary Fig. S1 and Fig. 3A).

Glycolytic enzymes as targets for S-nitrosylation in *P. falciparum*

Among various cellular processes possibly affected by S-nitrosylation in *P. falciparum*, glycolysis appears to be a major target because all 11 enzymes contributing to glycolysis were identified as potential SNO targets (Supplementary

FIG. 2. Functional classification of identified *S*-nitrosylated proteins in *P. falciparum*. The identified *S*-nitrosylated proteins were clustered according to their gene ontology (GO) annotations in biological processes (A) and molecular functions (B). Only annotated proteins were used in the analysis, and the most predominant GO terms were shown using the program PatternLab for Proteomics (24). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Table S1). This prompted us to investigate *S*-nitrosylation on these enzymes in more detail. We focused on four proteins, namely *P. falciparum* glyceraldehyde-3-phosphate dehydrogenase (PfGAPDH), phosphoglycerate mutase (PfPGM), pyruvate kinase (PfPK), and lactate dehydrogenase (PfLDH).

These enzymes were heterologously overexpressed in *Escherichia coli* and purified to homogeneity. The pure proteins were incubated with different concentrations of GSNO and then subjected to the biotin-switch assay followed by anti-biotin western blot analysis. *P. falciparum* thioredoxin

FIG. 3. *S*-nitrosylation of glycolytic enzymes of *P. falciparum*. (A) Analysis of *S*-nitrosylation on PfGAPDH, PfPGM, PfPK, and PfLDH. Prereduced recombinant enzymes were incubated with different concentrations of GSNO at 25°C for 1 h and subjected to a biotin-switch assay with or without NaAsc (as a control). *S*-nitrosylated enzymes were detected by the anti-biotin western blot analysis. Ponceau S staining shows equal amounts of protein loaded into each lane. *P. falciparum* glutathione *S*-transferase (PfGST) and thioredoxin reductase (PfTrxR), which were not identified as SNO targets, served as negative controls, were indeed not *S*-nitrosylated under the conditions chosen; these results further underline the modification specificity of the assay. (B) Concentration- and time-dependent inhibition of the PfGAPDH activity by *S*-nitrosylation induced by GSNO. Enzymatic activity is given as the percentage of PfGAPDH incubated without GSNO. (C) Protection of the PfGAPDH activity from GSNO by the substrate glyceraldehyde 3-phosphate (GAP) and the cofactor nicotinamide adenine dinucleotide (NAD⁺). PfGAPDH was incubated with 1 mM GAP or 2 mM NAD for 5 min before the treatment with 0.2 mM GSNO. Data are represented as mean ± standard deviation from at least three independent determinations. (D) Protection of PfGAPDH from GSNO-induced *S*-nitrosylation by preincubation with GAP and NAD⁺ as revealed by anti-biotin western blot analysis. PfGAPDH, *P. falciparum* glyceraldehyde-3-phosphate dehydrogenase; PfLDH, *P. falciparum* lactate dehydrogenase; PfPGM, *P. falciparum* phosphoglycerate mutase; PfPK, *P. falciparum* pyruvate kinase; SNO, *S*-nitrosylation.

reductase (PfTrxR) and glutathione *S*-transferase (PfGST), which were not identified as SNO targets in the study, were included as negative controls to show the modification specificity. Consistent with the large-scale study, we individually validated that the glycolytic enzymes were all susceptible to *in vitro* *S*-nitrosylation by GSNO in a concentration-dependent manner (Fig. 3A). Furthermore, we tested the effect of *S*-nitrosylation on the enzymatic activities of PfGAPDH, PfPK, and PfLDH. Interestingly, *S*-nitrosylation by GSNO resulted in a concentration and time-dependent inhibition of the PfGAPDH activity (Fig. 3B), whereas incubation with GSNO (up to 1 mM) did not significantly influence PfPK and PfLDH activities (data not shown).

It is known that PfGAPDH uses its active site cysteine (Cys153) to metabolize its substrate glyceraldehyde 3-phosphate (GAP) in the presence of the adjacently bound cofactor, nicotinamide adenine dinucleotide (NAD⁺) (45). To examine whether Cys153 is the target for *S*-nitrosylation, PfGAPDH was preincubated with GAP or NAD⁺ for 5 min before it was exposed to GSNO treatment. Indeed, preincubation with either substrate or cofactor markedly protected PfGAPDH from inhibition by GSNO (Fig. 3C). In parallel, western blot analysis of *S*-nitrosylated PfGAPDH also showed a reduced level of *S*-nitrosylation of PfGAPDH, which had been preincubated with GAP or NAD⁺ (Fig. 3D). The results indicate that GAP and NAD⁺ competed with GSNO at the active site, thus protecting PfGAPDH from *S*-nitrosylation and strongly suggesting that Cys153 is the target site for *S*-nitrosylation in PfGAPDH.

Site-Specific *S*-nitrosylation of PfTrx1

PfTrx1 (the major cytosolic Trx), a ubiquitous protein disulfide oxidoreductase, was identified as a target of *S*-nitrosylation. Similar to human Trx1 (hTrx1), PfTrx1 contains a pair of active site cysteines in the conserved sequence Cys-Gly-Pro-Cys and a third cysteine (Cys43) (26). It has been shown for hTrx1 that distinct responses of its different cysteine residues to nitrosylating stress direct denitrosylating and transnitrosylating activities of hTrx1 in the regulation of SNO (5, 57). To explore possible regulatory roles of PfTrx1 in SNO, it is thus of particular interest to investigate susceptibilities of different cysteines of PfTrx1 to *S*-nitrosylation. Recombinant wild-type and different cysteine-mutated PfTrx1s were prereduced with 1,4-dithiothreitol (DTT) followed by GSNO treatment. The *S*-nitrosylation of these proteins was detected by using the biotin-switch assay coupled to western blot analysis. As shown in Figure 4, wild-type PfTrx1 (PfTrx1-WT) was *S*-nitrosylated by high concentrations of GSNO (more than 200 μ M). Mutation of the resolving active site cysteine (Cys33) to serine in PfTrx1 (PfTrx1^{C33S}) showed an increased susceptibility to *S*-nitrosylation, as reflected by the *S*-nitrosylation of the mutant upon relatively low concentrations of GSNO (100 μ M). Interestingly, PfTrx1 with both active site cysteines mutated to serine (PfTrx1^{C30S/C33S}) was concentration-dependently *S*-nitrosylated by GSNO, implying that Cys43 is an important site for *S*-nitrosylation. This was further evidenced by the finding that a single mutation of Cys43 in PfTrx1 (PfTrx1^{C43S}) completely abrogated *S*-nitrosylation of the protein, despite the fact that high concentrations of GSNO were used. Taken together, our results suggested that Cys43

FIG. 4. Characterization of the *S*-nitrosylation site in *P. falciparum* thioredoxin 1 (PfTrx1). Recombinant wild-type PfTrx1 (PfTrx1-WT) and different PfTrx1 variants with cysteine mutated to serine were prereduced and incubated with different concentrations of GSNO for *S*-nitrosylation at 25°C for 1 h. Proteins were then subjected to a biotin-switch assay with or without NaAsc (as a control) followed by anti-biotin western blot analysis. Ponceau S staining shows equal amounts of protein loaded into each lane.

of PfTrx1 is site-specifically targeted by *S*-nitrosylation, whereas the active site cysteines are not major sites of *S*-nitrosylation. The site-specific *S*-nitrosylation of PfTrx1 clued a potential of this protein in the regulation of SNO in *P. falciparum*.

Denitrosylation by PfTrx1

To better understand how SNO is regulated in *P. falciparum*, we began to study denitrosylation by searching for a plasmodial homolog of GSNOR, which governs cellular

concentrations of GSNO and regulates SNO levels in other organisms (8). However, bioinformatic analysis using the Basic Local Alignment Search Tool (BLAST) revealed that *Plasmodium* genomes appear to lack the sequence encoding a putative GSNOR (data not shown). Besides GSNOR, Trx is a key denitrosylase that directly denitrosylates *S*-nitrosylated proteins (8, 57). Considering the fact that *P. falciparum* also possesses a functional Trx system and having learned about the site-specific response of PfTrx1 to *S*-nitrosylating stress, we hypothesized that PfTrx1 functions as a general denitrosylase. To test this, we examined whether PfTrx1 is able to denitrosylate proteins at a biochemical level. A large variety of *S*-nitrosylated proteins was generated in *P. falciparum* cell extracts treated with GSNO and incubated with recombinant PfTrx1-WT for denitrosylation. The residual *S*-nitrosylated proteins were then detected by the biotin-switch assay followed by antibiotin western blot analysis (Fig. 5A). We found that PfTrx1 dramatically reduced the levels of *S*-nitrosylated plasmodial proteins. Incubation of PfTrx1 along with a

backup system containing PfTrxR and NADPH nearly eliminated all *S*-nitrosylation signals. This treatment was approximately equipotent to treatment with 1 mM GSH. On the contrary, incubation only with PfTrxR and NADPH showed no obvious denitrosylating effect, implying that PfTrxR has—by reducing Trx—a catalytic effect in the denitrosylation by PfTrx1.

To investigate this denitrosylation mechanism in more detail, we assessed activities of PfTrx1 and its cysteine-mutated variants in the denitrosylation of PfGAPDH by using both protein immunoblotting and enzymatic assays. Recombinant PfGAPDH was incubated with GSNO to generate *S*-nitrosylated PfGAPDH, which was then incubated with prereduced PfTrx1-WT and different cysteine-mutated PfTrx1 variants for denitrosylation. *S*-nitrosylation levels of PfGAPDH were further analyzed with a biotin-switch assay followed by antibiotin western blots (Fig. 5B). We found that PfTrx1 retaining intact active site cysteines (PfTrx1-WT and PfTrx1^{C43S}) significantly denitrosylated PfGAPDH with an

FIG. 5. *In vitro* denitrosylation of plasmodial proteins by PfTrx1. (A) Denitrosylation of a large variety of *S*-nitrosylated proteins in *P. falciparum* cell extracts by PfTrx1 together with or without a backup system containing PfTrxR and NADPH. Incubation of GSH and PfTrxR/NADPH alone was set as positive and negative controls, respectively. *S*-nitrosylated proteins were detected with a biotin-switch assay followed by antibiotin western blot analysis. Immunoblotting of PfGST in cell extracts was used as a loading control. (B) Denitrosylation of PfGAPDH by PfTrx1. *S*-nitrosylated PfGAPDH was generated by the incubation of PfGAPDH with 1 mM GSNO. For denitrosylation, *S*-nitrosylated PfGAPDH was incubated with 5 mM DTT, PfTrx1-WT, and different cysteine-mutated PfTrx1 variants at 37°C for 30 min. The molar ratio of PfTrx1 proteins to PfGAPDH in the denitrosylation assay system was 2:1 or 5:1. *S*-nitrosylated PfGAPDH without denitrosylation was set as a control. After denitrosylation, the level of residual *S*-nitrosylated PfGAPDH was analyzed with a biotin-switch assay coupled with antibiotin western blot analysis. Ponceau S staining shows an equal amount of PfGAPDH loaded into each lane. (C) The enzymatic activity of *S*-nitrosylated PfGAPDH was restored by PfTrx1-WT in both concentration- and time-dependent manners but not by active site-mutated PfTrx1 variants (PfTrx1^{C33S} and PfTrx1^{C30S/C33S}). (D) PfTrxR accelerated the denitrosylation of PfGAPDH which is by PfTrx1. The molar ratio of PfTrx1-WT to PfGAPDH in the denitrosylation assay system was 1:1 or 2:1. Data are represented as mean $\pm$ standard deviation from at least three independent determinations. Incubation with PfTrxR/NADPH alone did not recover the PfGAPDH activity.

efficiency comparable to 5 mM DTT. Once, one or both active site cysteines were mutated, PfTrx1 markedly lost the denitrosylating activity on PfGAPDH, although a slight denitrosylation was observed, which might be due to protein-protein transnitrosylation from S-nitrosylated PfGAPDH to PfTrx1. In the enzymatic assay, PfTrx1-WT consequently recovered the activity of S-nitrosylated PfGAPDH in a dose- and time-dependent manner, whereas active site cysteine-mutated PfTrx1 variants did not (Fig. 5C). These results suggest that active site cysteines are mainly responsible for the denitrosylating activity of PfTrx1. Furthermore, we found that the presence of PfTrxR substantially promoted the PfGAPDH-denitrosylating activity of PfTrx1, reflected by the accelerated recovery of the PfGAPDH activity (Fig. 5D). This implied that an active site disulfide in PfTrx1 was

formed in the catalysis of PfGAPDH denitrosylation and in turn reduced by PfTrxR. This result indicates that denitrosylation by PfTrx1 is based on a dithiol-disulfide exchange mechanism (26). Collectively, our results clearly suggest a denitrosylating activity of PfTrx1 mediated by its active site cysteines.

Transnitrosylation by plasmodial Trx1

Because PfTrx1 can be S-nitrosylated at Cys43, we further tested whether PfTrx1 has a transnitrosylating activity mediated by the S-nitrosylated Cys43 residue. PfTrx1-WT and the PfTrx1 variant with double-mutated active site cysteines (PfTrx1^{C30S/C33S}) were S-nitrosylated by GSNO and then incubated with *P. falciparum* cell extracts. After analysis via the biotin-switch assay coupled with an anti-biotin western blot, a number of S-nitrosylated proteins in cell extracts emerged upon the incubation of S-nitrosylated PfTrx1-WT, indicating that numerous plasmodial proteins were transnitrosylated by PfTrx1 (Fig. 6A). Compared to PfTrx1-WT, the similar immunoblotting pattern of S-nitrosylated proteins induced by S-nitrosylated PfTrx1^{C30S/C33S} indicated that Cys43 is responsible for the transnitrosylating activity of PfTrx1. Additionally, by using PfGAPDH as a transnitrosylation substrate, we found that both PfTrx1-WT and PfTrx1^{C30S/C33S} were able to transnitrosylate PfGAPDH in a dose-dependent manner (Fig. 6B). Furthermore, we found that the PfGAPDH activity was more rapidly inhibited by the S-nitrosylated PfTrx1^{C30S/C33S} than by the same molar amount of GSNO, indicating a higher efficiency of PfTrx1 in the protein transnitrosylation compared to the *in vivo* NO donor GSNO. Taken together, these results strongly indicate a transnitrosylating activity of PfTrx1, which is mediated by its Cys43 residue.

FIG. 6. *In vitro* transnitrosylation of plasmodial proteins by PfTrx1. (A) Protein transnitrosylation in *P. falciparum* cell extracts by S-nitrosylated PfTrx1-WT and active site cysteine-mutated PfTrx1 (PfTrx1^{C30S/C33S}). PfTrx1 and PfTrx1^{C30S/C33S} were first S-nitrosylated by GSNO and then incubated with freshly prepared *P. falciparum* cell extracts for transnitrosylation. S-nitrosylated proteins were detected with a biotin-switch assay followed by anti-biotin western blot analysis. Immunoblotting of PfGST in cell extracts was used as a loading control. (B) Transnitrosylation of PfGAPDH by S-nitrosylated PfTrx1 and PfTrx1^{C30S/C33S}. The molar ratio of PfTrx1 proteins to PfGAPDH in the transnitrosylation assay system was 2:1 or 5:1. Incubation of 100 μ M GSNO with PfGAPDH was used as a positive control. Ponceau S staining shows an equal amount of PfGAPDH loaded into each lane. (C) Comparison of the transnitrosylation efficiency of PfTrx1^{C30S/C33S} with GSNO using PfGAPDH as a substrate. Prereduced PfTrx1^{C30S/C33S} was S-nitrosylated by the incubation with a 50-fold molar excess of GSNO, and then, the residual GSNO was completely removed by a desalting step. In the assay, the same molar amounts of S-nitrosylated PfTrx1^{C30S/C33S} and GSNO were individually incubated with PfGAPDH (molar ratio of both agents to PfGAPDH were 40:1, 52.5:1, and 100:1). A time-dependent inactivation of PfGAPDH was monitored to reflect the transnitrosylation efficiency. PfTrx1-WT-SNO, S-nitrosylated PfTrx1-WT; PfTrx1^{C30S/C33S}-SNO, S-nitrosylated PfTrx1^{C30S/C33S}; *Statistically significant difference $p < 0.05$ (Student's *t*-test).

Discussion

Although NO is known to be a key player in the redox regulatory processes of eukaryotic cells and it can impair the development of malaria parasites in both mosquito vectors and humans, the mode of action and molecular targets of NO in *Plasmodium* have hardly been characterized. As a highly membrane-permeable molecule, NO diffuses easily across cell membranes and enters parasite cells, where it is quickly oxidized to RNS, especially in the presence of a high flux of reactive oxygen species (ROS) within the parasites (6, 21). A compelling feature of RNS is to induce SNO (18). Thus, SNO could serve as an important mechanism for NO to exert biological functions in *P. falciparum*. To shed light on how NO acts in malaria parasites, we present here the first comprehensive analysis of SNO induced by the NO donor GSNO in the human protozoan parasite *P. falciparum*. Based on a biotin-switch assay coupled to proteomic analysis, 319 potential protein targets and 37 sites for *S*-nitrosylation could be identified. When interpreting this number, one should keep in mind that there might be additional targets that have not been detected due to possible thiol oxidation during protein extraction from the parasites. However, the proteins identified give an initial insight into the *S*-nitrosoproteome of *P. falciparum*.

The functional classification of the *S*-nitrosylated proteins identified highlights the regulatory potential of NO in a broad scope of cellular processes and metabolic pathways in *P. falciparum* (Fig. 2 and Supplementary Table S1). In accordance with *S*-nitrosoproteomes of higher eukaryotic organisms (dbSNO, <http://dbsno.mbc.nctu.edu.tw>), the *P. falciparum* *S*-nitrosoproteome possesses many conserved proteins whose mammalian and plant counterparts have been reported as canonical *S*-nitrosylation targets. These proteins are mainly gene transcription and translation factors, protein chaperones, ribosome proteins, proteasome subunits, cytoskeletal and antioxidant proteins, and miscellaneous metabolic enzymes. The specificity of *S*-nitrosylation on these proteins is most likely due to the evolutionary conserved structure of these proteins in *P. falciparum* and higher eukaryotes, indicating that SNO may play similar regulatory roles in corresponding pathways. For example, *S*-adenosylmethionine synthetase (SAMS), a key enzyme involved in methionine and polyamine metabolism, was identified to be *S*-nitrosylated at a cysteine residue conserved in both *P. falciparum* (Cys113; see Supplementary Table S1) and rats (Cys121) (11, 40). It has been shown for rat SAMS that the acidic (Asp355) and basic (Arg357, Arg363) amino acids in the vicinity of Cys121 are critically involved in the deprotonation of the sulfur group of the cysteine and concertedly facilitate the GSNO-induced *S*-nitrosylation at Cys121 (18, 40). Intriguingly, *P. falciparum* SAMS (PfSAMS) shares a sequence identity of 57% with rat SAMS and preserves the surrounding acidic/basic amino acids required for *S*-nitrosylation: Asp352, Arg354, and Gln360 (a conservative substitution of Arg363 in rat SAMS) (11). Thus, *S*-nitrosylation may occur at Cys113 of PfSAMS due to the coordination of surrounding acidic/basic amino acids in the same manner. More importantly, *S*-nitrosylation of Cys121 has been shown to markedly inactivate rat SAMS and result in a reduction of the *S*-adenosylmethionine (SAM) level in rat hepatocytes (40). Regarding the similar metabolic function

and the presence of the same *S*-nitrosylation site in PfSAMS compared to rat SAMS, it is reasonable to speculate that SNO may affect the SAM biosynthesis in *P. falciparum* by influencing the PfSAMS activity. Because SAMS is a key precursor for polyamine biosynthesis, which is closely related to the proliferation of *P. falciparum* (12), SNO on PfSAMS may correlate with the growth arrest of *Plasmodium* parasites caused by NO, which has been observed in several studies (4, 50). Of course, this hypothesis needs to be substantiated by further experiments.

Of particular interest, a series of essential proteases involved in the cascade of hemoglobin digestion were identified as potential SNO targets (Supplementary Table S1). It is well known that hemoglobin digestion in the parasites' food vacuole is essential for *Plasmodium* parasites, providing a main source of amino acids during parasite growth (16). Given that intraparasitic RNS are abundantly present around the food vacuole where many of these hemoglobins reside (37), nitrosative stress-derived *S*-nitrosylation has a high likelihood of occurrence on these hemoglobins. Indeed, the cysteine protease falcipain has been demonstrated to be substantially inactivated by 10 μ M of NO donors due to *S*-nitrosylation of its active site cysteine (55). The susceptibility of falcipain to NO has been linked to the growth inhibition of parasites under NO donor treatments (33, 55). Thus, it is likely that SNO influences hemoglobin digestion *via* modulating hemoglobins, thereby being relevant to the inhibitory effects of NO on the proliferation of the parasites.

Glycolytic enzymes of *P. falciparum* appear to undergo multiple thiol-based redox regulations. We have shown previously that nine enzymes in the glycolysis pathway can be modified by GSH *via S*-glutathionylation *in vivo* (24). Moreover, PfPK, PfLDH, PfGAPDH, and PfHK (hexokinase) are regulated by the Trx superfamily in *P. falciparum* (48). Strikingly, in this study, we identified all 11 glycolytic enzymes of *P. falciparum* as potential SNO targets, and we found that the PfGAPDH activity is specifically inhibited by *S*-nitrosylation at its active-site cysteine (Fig. 3B, C), suggesting that the glycolysis pathway is highly susceptible to redox modulation by NO. The importance of glycolysis to *P. falciparum* is highlighted by the fact that the uptake and consumption of glucose by parasitized erythrocytes is increased by a factor of ~ 100 compared to normal erythrocytes (46). Additionally, because of the absence of a pyruvate dehydrogenase complex in mitochondria, *P. falciparum* becomes critically dependent on glycolysis for energy production (42). Hence, perturbation of glycolysis may render detrimental effects on the proliferation of parasites as suggested before (53).

It has been reported that reversible inactivation of GAPDH under oxidative stress may protect the enzyme from over-oxidation and re-route the metabolic flux from glycolysis to the pentose phosphate pathway (PPP), which is a critical process for generating reducing equivalents in the form of NADPH (43). Similarly, the reversible inactivation of PfGAPDH by *S*-nitrosylation is presumably also an early signaling event that redirects the carbohydrate flux to the PPP to generate more NADPH, which can be used to cope with oxidative and nitrosative stress in the parasites. For example, the plasmodial TrxR-Trx system may utilize NADPH to recover the PfGAPDH activity *via* denitrosylation (Fig. 5). In this way, *S*-nitrosylation of PfGAPDH may

serve as a signaling switch to contribute to the parasitic tolerance to nitrosative stress.

Intracellular GSH, an abundant antioxidant tripeptide, detoxifies nitrosative stress by reacting with RNS (10). This results in the formation of GSNO, which can be denitrosylated back to GSH by GSNOR as shown in higher eukaryotic cells (8). Although GSNOR seems to be conserved from bacteria to humans, a plasmodial homolog of GSNOR has not been identifiable in the genome of *P. falciparum* so far. The lack of GSNOR in the parasites may render RNS detoxification more dependent on the Trx system. Indeed, we have previously reported that PfTrx1 can efficiently reduce GSNO to GSH at a biologically relevant rate (23), suggesting that PfTrx1 might compensate for GSNOR to reduce GSNO *in vivo*. More importantly, we proved in this study that PfTrx1 functions as a general denitrosylase that can efficiently denitrosylate a large variety of proteins (Fig. 5A), suggesting that PfTrx1 may protect proteins from NO stress. For example, the PfTrx1 system was demonstrated to be able to denitrosylate PfGAPDH and efficiently recover its glycolytic activity (Fig. 5B–D), indicating a role for the PfTrx1 system in protecting glycolysis from nitrosative stress. The active site cysteines were demonstrated to be responsible for the denitrosylating activity of PfTrx1, consistent with the denitrosylation mechanism of Trx in higher eukaryotic organisms (57). Considering the fact that PfTrx1 is generally located in the parasites' cytosol and ubiquitously expressed in asexual and sexual stages of *P. falciparum* and also in parasite ookinetes (25, 52), denitrosylation mediated by PfTrx1 may represent an important defense mechanism against nitrosative stress throughout the life cycle of *Plasmodium* parasites.

Whereas PfTrx1 mediates denitrosylation *via* active site cysteines, PfTrx1 itself can be site-specifically S-nitrosylated at Cys43, which in turn mediates the transnitrosylating activity (Figs. 4 and 6). In contrast to denitrosylation, transnitrosylation by Trx has been reported to transduce NO stress and mediate NO signaling in cells (35, 57). Based on these findings, we proposed a redox status-based model for PfTrx1 in protein denitrosylation and transnitrosylation (Fig. 7); under normal conditions, the reduced PfTrx1 is predominantly present in the parasites (22, 23, 26). The model of denitrosylation by reduced PfTrx1 may represent a general mechanism by which reduced PfTrx1 protects the parasites from RNS/NO stress. This protective mechanism may explain, at least in part, the parasitic tolerance to nitrosative stress generated either endogenously or exogenously (30, 37); however, upon extreme NO stress or along with an increase in ROS/RNS stress together with a gradual loss of the recycling of reduced PfTrx1, PfTrx1 could be S-nitrosylated at the nonactive-site cysteine (Cys43) to a significant extent, turning the overall role of PfTrx1 from denitrosylation to transnitrosylation for transducing NO stress/signaling. The transnitrosylation model of PfTrx1 is probably of more physiological relevance during parasite transmission between mosquito vectors and hosts, as the parasites are directly exposed to the host immune response in which inflammatory levels of NO are clearly involved (2, 3). For example, during the initial invasion of the mosquito midgut, the *Plasmodium* parasite ookinetes temporarily suffer NO stress generated by the mosquito NOS (2). Although specific denitrosylation and transnitrosylation substrates of PfTrx1 are yet to be discovered, the model may give a first insight into possible

FIG. 7. Proposed redox-based regulation of S-nitrosylation by PfTrx1. Upon mild-to-moderate nitrosative stress, the predominantly reduced PfTrx1 in cells mainly acts as a denitrosylase by catalyzing the denitrosylation of S-nitrosylated biomolecules *via* its active site cysteines. In this process, its active site cysteines are oxidized to a disulfide, which can be reduced by PfTrxR in the presence of NADPH. When parasite cells encounter severe and continuous oxidative/nitrosative stress (*e.g.*, drug treatment) or local NO stress is severely enhanced (*e.g.*, under NO burst generated from host iNOS), the reduction of oxidized PfTrx1 by PfTrxR can be compromised. Under these conditions, Cys43 of PfTrx1 can be S-nitrosylated to a significant extent and transnitrosylate other proteins, thus mediating NO stress (see text for more details). In addition to the processes shown in this scheme, S-nitrosylation of Cys43 in reduced PfTrx1 might also occur as well as transnitrosylation from S-nitrosylated proteins to PfTrx1. iNOS, inducible nitric oxide synthase; NO, nitric oxide.

regulatory potentials of PfTrx1 in the interplay of NO and the *Plasmodium* parasites within a specific redox milieu.

In conclusion, this study explored the targets of *S*-nitrosylation in *P. falciparum*, thus contributing to the understanding of profound mechanisms of NO effects on this parasite. The identified *S*-nitrosylated proteins involved in diverse cellular processes and biological pathways provide a molecular basis for further studies on how NO exerts its action in *P. falciparum* and—at high concentrations—mediates parasite killing. One should note that although the *S*-nitrosylated proteins were identified in cell extracts of asexual intra-erythrocytic *P. falciparum*, many proteins are expressed throughout the complete life cycle of the parasites, including the mosquito stages. Thus, our data may also contribute to the understanding of SNO in the mosquito stages when the parasites encounter severe oxidative/nitrosative stresses (3, 30, 41). Importantly, our results suggest that PfTrx1 may protect the parasite from nitrosative stress *via* protein denitrosylation, whereas upon intensive nitrosative stress, PfTrx1 may switch its role to transduce nitrosative stress *via* protein transnitrosylation. It will be of great interest to identify and characterize denitrosylation and transnitrosylation substrates of PfTrx1 in *P. falciparum* to reveal specific pathways regulated by PfTrx1 upon nitrosative stress.

Materials and Methods

Cultivation of P. falciparum and preparation of parasite cell extracts

Intraerythrocytic stages of *P. falciparum* (3D7) were maintained in the culture and prepared using standard procedures. Details are described in the Supplementary Materials and Methods.

Biotin-switch assay, detection of S-nitrosylated proteins, and purification of biotinylated proteins

The biotin-switch assay was performed as previously described with modifications (14, 17, 19). To detect biotinylated proteins that were previously *S*-nitrosylated, western blot analysis using anti-biotin antibodies was performed. An avidin pull-down assay was used to purify biotinylated proteins. Details are described in the Supplementary Materials and Methods.

Sample preparation for mass spectrometry

Protein samples were prepared for MS analysis using standard procedures as described in the Supplementary Materials and Methods.

Multidimensional protein identification technology and the analysis of tandem mass spectra

The trypsin-digested protein samples were analyzed using multidimensional protein identification technology, and the MS/MS spectra were analyzed following the standard software analysis protocols as described in the Supplementary Materials and Methods.

Heterologous overexpression and purification of P. falciparum proteins

Recombinant His-tagged PfGAPDH, PfPK, PfLDH, and PfPGM were overexpressed and purified as previously

described (24). The following recombinant His-tagged proteins were overexpressed and purified as described elsewhere: recombinant PfTrx1-WT, cysteine-mutated PfTrx1^{C33S}, PfTrx1^{C30S/C33S} (23), PfTrx1^{C43S} (26), and PfTrxR (22).

Protein immunoblotting analysis

S-nitrosylation of recombinant PfGAPDH, PfPK, PfLDH, PfPGM, PfTrx1-WT, PfTrx1^{C33S}, PfTrx1^{C30S/C33S}, and PfTrx1^{C43S} was analyzed *via* a protein immunoblotting assay as described in the Supplementary Materials and Methods.

Enzyme treatments and enzymatic assays

PfGAPDH, PfPK, and PfLDH were treated with GSNO, and their enzymatic activities were measured spectrophotometrically with standard methods as described in the Supplementary Materials and Methods.

Denitrosylation assays

S-nitrosylated proteins in *P. falciparum* cell extracts were generated by incubating parasite cell extracts (0.2 mg protein) with 1 mM GSNO in the buffer containing 50 mM Tris, 1 mM EDTA, and 0.2 mM neocuproine (pH 7.4) at room temperature for 30 min in the dark. The residual GSNO was removed by passing the cell extracts through a Zeba desalting column (Thermo Scientific). Sixty micrograms of prereduced recombinant PfTrx1 was added to the cell extracts and incubated at 37°C for 30 min in the dark for the denitrosylation. In another sample, 0.2 μ M PfTrxR and 200 μ M NADPH were added along with PfTrx1. For control samples, PfTrxR/NADPH alone or 1 mM reduced glutathione were added to the cell extracts. After denitrosylation, proteins were precipitated and washed with acetone and then subjected to a biotin-switch assay. The residual *S*-nitrosylated proteins were detected *via* anti-biotin western blot analysis as described previously. A western blot analysis of PfGST in cell extracts was used as a loading control.

For denitrosylating PfGAPDH, PfGAPDH was first *S*-nitrosylated by incubating the 2 mg recombinant PfGAPDH with 1 mM GSNO in 0.5 ml buffer containing 50 mM Tris, 1 mM EDTA, and 0.2 mM neocuproine (pH 7.4) at room temperature for 30 min in the dark. After removing residual GSNO *via* gel filtration, 0.2 mg PfGAPDH (11 μ M) was taken out and incubated with a twofold or fivefold molar excess of prereduced PfTrx1 and different PfTrx1 cysteine mutants at 37°C for 30 min in the dark for denitrosylation. The residual *S*-nitrosylated PfGAPDH was then analyzed *via* a biotin-switch assay followed by an anti-biotin western blot.

To enzymatically study the denitrosylation of PfGAPDH, PfGAPDH was *S*-nitrosylated *via* incubation with 1 mM GSNO for 30 min at 25°C. The residual GSNO was removed by desalting PfGAPDH using a Zeba desalting column. The GSNO-treated PfGAPDH was incubated with a different-fold molar excess of recombinant PfTrx1-WT (with or without 0.2 μ M PfTrxR and 200 μ M NADPH) and cysteine-mutated PfTrx1 variants. Aliquots were taken at different incubation time points to monitor the PfGAPDH activity as described previously.

Transnitrosylation assays

Briefly, *S*-nitrosylated PfTrx1-WT and PfTrx1^{C30S/C33S} were generated by incubating respective recombinant proteins

with 1 mM GSNO for 30 min at 25°C. After residual GSNO was removed, 20 μ M S-nitrosylated PfTrx1-WT and PfTrx1^{C30S/C33S} were incubated with *P. falciparum* cell extracts (0.2 mg) in 0.5 ml buffer containing 50 mM Tris, 1 mM EDTA, and 0.2 mM neocuproine, pH 7.4 for 1 h at 25°C in the dark. For the transnitrosylation of PfGAPDH, a twofold or fivefold molar excess of S-nitrosylated PfTrx1-WT and PfTrx1^{C30S/C33S} were incubated with 11 μ M PfGAPDH for 30 min at 25°C in the dark. After transnitrosylation, proteins were subjected to the biotin-switch assay. The resulting S-nitrosylated proteins in cell extracts and the S-nitrosylated PfGAPDH were detected by the antibiotin western blot analysis. To compare the transnitrosylation efficiency of PfTrx1 and GSNO, pre-reduced PfTrx1^{C30S/C33S} was S-nitrosylated with a 50-fold molar excess of GSNO for 30 min and then desalted to remove residual GSNO. In 300 μ l buffer system (50 mM Tris, 1 mM EDTA, and 0.2 mM neocuproine, pH 7.4), 2.4 μ g PfGAPDH was mixed with the same molar amounts of GSNO and S-nitrosylated PfTrx1^{C30S/C33S} individually; 60 μ l of mixture was taken out every 5 min to measure the PfGAPDH activity.

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Author Disclosure Statement

The authors have nothing to declare.

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Abbreviations Used

BLAST = Basic Local Alignment Search Tool
 DTT = 1,4-dithiothreitol
 GAP = glyceraldehyde 3-phosphate
 GPIs = glycosylphosphatidylinositols
 GSH = reduced glutathione

GSNO = S-nitrosoglutathione
 GSNOR = S-nitrosoglutathione reductase
 GSSG = oxidized glutathione disulfide
 HK = hexokinase
 MMTS = methyl methanethiosulfonate
 MS = mass spectrometer
 NaAsc = sodium ascorbate
 NAD⁺ = nicotinamide adenine dinucleotide
 NO = nitric oxide
 NOS = nitric oxide synthase
 PfGAPDH = *P. falciparum* glyceraldehyde-3-phosphate dehydrogenase
 PfGST = *P. falciparum* glutathione S-transferase
 PflDH = *P. falciparum* lactate dehydrogenase
 PfPGM = *P. falciparum* phosphoglycerate mutase
 PfPK = *P. falciparum* pyruvate kinase
 PfTrx1 = *P. falciparum* thioredoxin 1
 PfTrxR = *P. falciparum* thioredoxin reductase
 PfTrx1-WT = wild-type PfTrx1
 RNS = reactive nitrogen species
 ROS = reactive oxygen species
 SAM = S-adenosylmethionine
 SAMS = S-adenosylmethionine synthetase
 SNO = protein S-nitrosylation
 TNF- α = tumor necrosis factor- α
 Trx = thioredoxin
 TrxR = thioredoxin reductase