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Dinitrosyl-Dithiol-Iron Complexes, Nitric Oxide (NO) Carriers *In Vivo*, as Potent Inhibitors of Human Glutathione Reductase and Glutathione-*S*-Transferase

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ABSTRACT. Human glutathione reductase (GR) and rat liver glutathione-*S*-transferases (GSTs) had been shown to be inhibited by the nitric oxide (NO) carrier *S*-nitroso-glutathione (GSNO). We have now extended these studies by measuring the effects of dinitrosyl-iron complexed thiols (DNIC-$[RSH]_2$) on human GR, GST and glutathione peroxidase. DNIC-$[RSH]_2$ represent important transport forms of NO but also of iron ions and glutathione *in vivo*. Human GR was found to be inhibited by dinitrosyl-iron-di-glutathione (DNIC-$[GSH]_2$) and dinitrosyl-iron-di-L-cysteine (DNIC-Cys_2) in two ways: both compounds were competitive with glutathione disulfide (GSSG), the inhibition constant (K_i) for *reversible* competition of DNIC-$[GSH]_2$ with GSSG being approximately 5 μM; preincubating GR for 10 min with 4 μM DNIC-$[GSH]_2$ and 40 μM DNIC-Cys_2, respectively, led to 50% *irreversible* enzyme inactivation. More than 95% GR inactivation was achieved by incubation with 36 μM DNIC-$[GSH]_2$ for 30 min. This inhibition depended on the presence of NADPH. Absorption spectra of inhibited GR showed that the charge-transfer interaction between the isoalloxazine moiety of the prosthetic group flavin adenine dinucleotide (FAD) and the active site thiol Cys63 is disturbed by the modification. Cys_2 and FAD could be ruled out as sites of the modification. Isolated human placenta glutathione-S-transferase and GST activity measured in hemolysates were also inhibited by DNIC-$[GSH]_2$. This inhibition, however, was reversible and competitive with reduced glutathione, the K_i being 20 nM. The inhibition of GST induced by GSNO was competitive with reduced glutathione (GSH) (K_i = 180 μM) and with the second substrate of the reaction, 1-chloro-2,4,-dinitrobenzene (K_i = 170 μM). An inhibition of human glutathione peroxidase by GSNO or DNIC-$[RSH]_2$ was not detectable. Inactivation of GR by DNIC-$[GSH]_2$ is by two orders of magnitude more effective than modification by GSNO; this result and the very efficient inhibition of GST point to a role of DNIC-$[RSH]_2$ in glutathione metabolism. BIOCHEM PHARMACOL **54**;12: 1307–1313, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. dinitrosyl-iron(II)-dithiolate; erythrocytes; glutathione reductase; glutathione-S-transferase; nitric oxide; *Plasmodium falciparum*

The important role of nitric oxide (NO)§ in signal transduction has been established in recent years, with NO-mediated physiologic effects ranging from cell-cell communication to the regulation of enzymatic activity [1, 2]. Considering the high reactivity of NO derivatives, their actions are diverse but surprisingly selective. NO can either be oxidized to the nitrosonium ion (NO^+) or reduced to the nitroxyl anion (NO^-) [3]. Thus, depending on the redox milieu at a given site, nitric oxide reacts with different partners producing different physiologic effects [4].

There has been increasing evidence that NO and glutathione metabolism are closely linked. The tripeptide glutathione (GSH) represents a major low molecular mass antioxidant in biological systems [5]. It has been shown that the formation of NO^- is forwarded by reduced glutathione [3] and that major effector molecules of NO are redox-regulated; for example, Niroomand *et al.* (1989) demonstrated an 80-fold increase in guanylyl cyclase activity induced by reduced glutathione [6]. Clancy (1994) proved that *S*-nitroso-glutathione (GSNO) formation from GSH and NO in activated neutrophils leads to a dramatic decrease in GSH concentration [7]. As described by Meyer

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§ *Abbreviations:* CDNB, 1-chloro-2,4-dinitrobenzene; DNIC, dinitrosyl iron complex; DNIC-Cys_2, dinitrosyl-di-L-cysteine iron complex; DNIC-$[GSH]_2$, dinitrosyl-di-glutathione iron complex; DNIC-$[RSH]_2$, dinitrosyl-iron complexed thiols; DTT, dithiothreitol; EDRF, endothelium-derived relaxing factor; EH_2, 2-electron reduced glutathione reductase containing an active site dithiol; E_{ox}, oxidized form of glutathione reductase containing an active site disulfide; FAD, flavin adenine dinucleotide; GPX, glutathione peroxidase (EC 1.11.1.9); GR, glutathione reductase (EC 1.6.4.2); GSH, reduced glutathione; GSNO, *S*-nitroso-glutathione; GSSG, glutathione disulfide; GST, π-type glutathione-*S*-transferase (EC 2.5.1.18); K_i, inhibition constant; NO, nitric oxide.

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et al. (1994), glutathione transferases might be involved in the *in vivo* production of GSNO [8]. GSNO itself has been shown to modulate the activity of various enzymes including glutathione-S-transferase [9], glyceraldehyde-phosphate-dehydrogenase [10], ribonucleotide reductase [11], alcohol dehydrogenase [12], cytochrome c-oxidase [13], and glutathione reductase [14]. The function of the homodimeric flavoenzyme glutathione reductase (GR; NADPH + H^+ + GSSG → $NADP^+$ + 2 GSH) is to maintain high intracellular concentrations of reduced glutathione [15].

Free NO released from NO synthase was shown to bind to iron-sulfide groups of intracytoplasmic proteins [16] or to thiolate groups of proteins in the presence of free iron [17]. Via thiolate exchange, free thiols can displace the proteins in the complex, thus creating low-molecular dinitrosyl-iron complexes (DNIC-$[RSH]_2$). Since glutathione and cysteine are suitable intracellular thiols, dinitrosyl-iron-di-L-cysteine (DNIC-Cys_2) and dinitrosyl-iron-di-glutathione (DNIC-$[GSH]_2$) are readily formed [18, 19]. Occurrence of these compounds has been demonstrated in cells and tissues after exposure to endogenous or exogenous NO [20]. For DNIC-Cys_2, properties very similar to those of endothelium-derived relaxing factor (EDRF) have been established; they include induction of hypotension [21], relaxation of vascular smooth muscle [22], and inhibition of platelet aggregation [23]. Here we report on the effects of DNIC-$[RSH]_2$ on human glutathione reductase, glutathione peroxidase, and glutathione-*S*-transferase, all representing central enzymes of redox metabolism.

MATERIALS AND METHODS

DNIC-Cys_2 and DNIC-$[GSH]_2$ (3.6 mM, stabilized with a 20-fold excess of L-cysteine and GSH, respectively) were prepared as described previously [20] and kept on dry ice until use. GSH, GSSG, and NADPH were purchased from Boehringer, BSA and digitonin from Serva. Human placenta glutathione-*S*-transferase and human erythrocyte glutathione peroxidase were obtained from Sigma. All reagents were of the highest purity available.

Human recombinant GR and a GR mutant lacking the first 15 *N*-terminal amino acids were isolated according to refs. 24 and 25. One enzyme unit of these preparations corresponds to 5.2 μg or 100 pmol GR subunits. The apoenzyme of GR was prepared from the holoenzyme as described by Fritsch *et al.*, 1979 [26]. GR samples were diluted and assayed in GR assay buffer (50 mM potassium phosphate, 200 mM potassium chloride, 1 mM EDTA, pH 6.9). Enzyme activity was measured spectrophotometrically at 340 nm and 25° in the presence of 0.1 mM NADPH and 1 mM GSSG; the total assay volume was 1 mL [27, 28]. Solutions of GSSG (20 mM), NADPH (4 mM), and inhibitors were prepared directly before use in assay buffer.

Incubations with DNIC-dithiols were carried out in closed plastic vials at 25° in a water bath. Modified GR samples to be employed for absorption spectra were dialysed exhaustively against GR assay buffer.

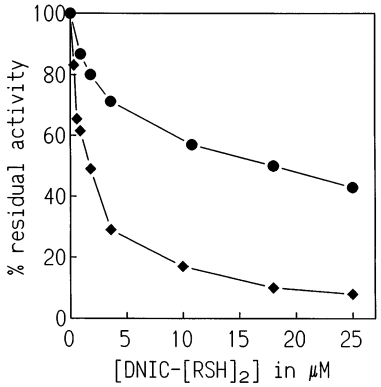

FIG. 1. Inhibition of human crystalline glutathione reductase by different concentrations of DNIC-$[GSH]_2$ (◆) and DNIC-Cys_2 (●) 3 min after addition to the standard GR assay. The inhibition comprises both reversible and irreversible components.

Glutathione-*S*-transferase activity was determined according to Beutler [29] by measuring the reaction of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH at 340 nm. Glutathione peroxidase activity was determined using *t*-butyl-hydroperoxide and GSH as substrates and detecting the formation of GSSG by the glutathione reductase catalyzed reaction [5]. Each experiment was reproduced twice.

Cells and Parasites

For experiments with intact erythrocytes, red cells from fresh EDTA-treated blood were washed twice with PBS. For determination of enzyme activities in erythrocytes or full blood, cell lysis was achieved by adding 40 mg/L digitonin to the respective assay buffer. The malaria parasite *Plasmodium falciparum* (FCBR strain) was cultured according to Trager and Jensen, 1976 [30]. The facilities to carry out inhibitor studies on *P. falciparum* were kindly provided by Prof. Klaus Lingelbach, Marburg University.

RESULTS

Competitive Inhibition of Glutathione Reductase by DNIC-$[RSH]_2$

DNIC-$[GSH]_2$ and DNIC-Cys_2 strongly inhibited human glutathione reductase when added directly to a standard GR assay. DNIC-$[GSH]_2$ was nine times more potent than DNIC-Cys_2, the IC_{50} values being 2 μM and 18 μM, respectively (Fig. 1). Reversible inhibition paralleling a rapid irreversible modification, which is described in the next paragraph, was found to be competitive with GSSG; the K_i value for DNIC-$[GSH]_2$ was estimated to be 5 μM (data not shown). Neither inhibitor was competitive with NADPH. As indicated by the stable absorption at 340 nm in the absence of enzyme, NADPH did not react with the DNIC-dithiols. In another control experiment, slow spontaneous formation of GSSG from DNIC-$[GSH]_2$ was observed. 36 μM DNIC-$[GSH]_2$ containing 720 μM excess

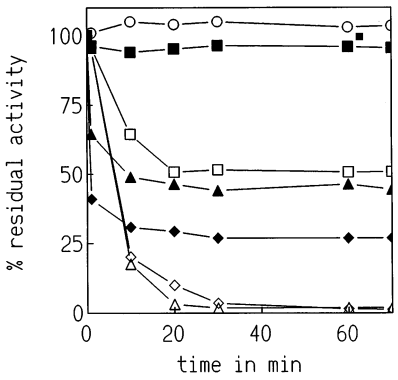

FIG. 2. Irreversible modification of human glutathione reductase by DNIC-$[GSH]_2$ and DNIC-Cys_2. GR (2 U/mL) was incubated in GR assay buffer with 1 mM NADPH at 25° and different inhibitor concentrations: DNIC-$[GSH]_2$ 3.6 μM (□), DNIC-$[GSH]_2$ 36 μM (△), DNIC-$[GSH]_2$ 72 μM (◇), DNIC-Cys_2 3.6 μM (■), DNIC-Cys_2 36 μM (▲), DNIC-Cys_2 72 μM (◆), control without DNIC-$[RSH]_2$ (○). Samples were assayed after different incubation times.

GSH released GSSG at a rate of ~3 μM per min. Under standard assay conditions this reaction can be neglected.

Irreversible Inhibition of GR by DNIC-$[RSH]_2$

When preincubating GR (2 U/mL) with DNIC-$[RSH]_2$ in the presence of NADPH, a time- and dose-dependent inhibition was detected (Fig. 2). Again, DNIC-$[GSH]_2$ proved to be the more potent inhibitor. More than 95% inactivation was achieved by incubating the enzyme with 36 μM DNIC-$[GSH]_2$ for 30 min; the same experiment carried out with DNIC-Cys_2 led to 55% enzyme modification. Furthermore, the inhibition depended on enzyme and NADPH levels: high NADPH and low GR concentrations promoted the modification by DNIC-$[RSH]_2$ (Fig. 3). For successful enzyme inhibition, the presence of NADPH was particularly important at higher inhibitor concentrations. This constellation is demonstrated for $[DNIC\text{-}GSH]_2$ in Figs. 3a and b. To interpret these results it is helpful to consider the first half reaction of GR: E_{ox} + NADPH + $H^+ \rightarrow NADP^+ + EH_2$ [31]. In E_{ox} (the oxidized enzyme), the catalytic Cys residues 58 and 63 form a disulfide, whereas in EH_2 (the two-electron reduced enzyme) these residues are present as thiols. Apparently, only EH_2 is susceptible to inactivating modification by DNIC-dithiols. The requirement of NADPH in relatively high excess is due to enzyme-catalyzed side reactions which depend on the DNIC-$[RSH]_2$ concentration. It should be emphasized that under conditions occurring *in vivo* (2 U/mL GR, 100 μM NADPH, μmolar DNIC-$[GSH]_2$, 1 mM GSH), the inactivation of GR is maximal.

Attempts to Reverse the Modification

To test reversibility of the stable GR modification, GR was inhibited with DNIC-$[GSH]_2$ and DNIC-Cys_2 by <95% and 50%, respectively. This inhibition was not reversible by 1000-fold dilution, by dialysis, nor by incubating the dialyzed enzyme for 60 min with different reducing agents (2–100 mM DTT, or 2 mM each of 2-mercaptoethanol, GSH or NADPH). This irreversibility of the modification is different from the result obtained for other enzymes modified by NO or NO derivatives where the activity can be recovered using thiol-containing reductants [12, 32]. It remains to be tested if modification of GR with DNIC-$[RSH]_2$ leads to short-lived intermediates which can be reactivated by dithiothreitol.

Absorption Spectra of DNIC-$[GSH]_2$-Modified GR

The absorption spectrum of DNIC-$[GSH]_2$-modified GR in the absence of NADPH was found to be similar to that of oxidized GR. After addition of 0.2 mM NADPH, which completely reduces wild-type GR to the EH_2 form, the typical EH_2 absorption with a shoulder at 530 nm [31] was not observed. This indicates that the charge-transfer interaction between Cys63 and the flavin does not occur in the modified enzyme. Therefore, a modification of one or more active site residues by DNIC-$[GSH]_2$ is very likely.

Modification of apoGR by DNIC-$[RSH]_2$

FAD as the prosthetic group of GR can be reversibly removed from the protein. ApoGR is inactive but can be reconstituted by adding FAD to the enzyme solution. Reconstituted holoGR is largely identical to native holoGR [26, 27, 33]. To test the influence of FAD on the DNIC-$[RSH]_2$ modification of GR, we incubated apoGR (2 cryptic U/mL) with 3.6, 36, and 72 μM of DNIC-$[GSH]_2$ and DNIC-Cys_2, respectively. At given time points, samples were taken and their activity was measured in assay mixtures containing 10 μM FAD. Inhibition patterns of apoGR (with the active site cysteines being reduced [33]) were very similar to those of the holoenzyme in the presence of 1 mM NADPH (data not shown). A major involvement of FAD in the protein's modification can therefore be ruled out. This finding may also be of interest for the *in vivo* situation. In malnutrition, which is often accompanied by riboflavin deficiency, or in other pathologic conditions, the percentage of unsaturated inactive apoGR can be 50% or higher [34, 35].

Inhibition of the Delta(1–15)hGR-Mutant by DNIC-$[RSH]_2$

The *N*-terminal 15 amino acids of GR form a flexible extension of still unknown function. To rule out that this part of the protein, and particularly Cys2, is involved in enzyme inactivation by DNIC-$[GSH]_2$, we tested the effects of DNIC-$[GSH]_2$ on the delta(1–15) mutant of hGR. The results obtained (for competitive and irreversible inhibition) were almost identical to the inhibition pattern observed for the wild-type enzyme.

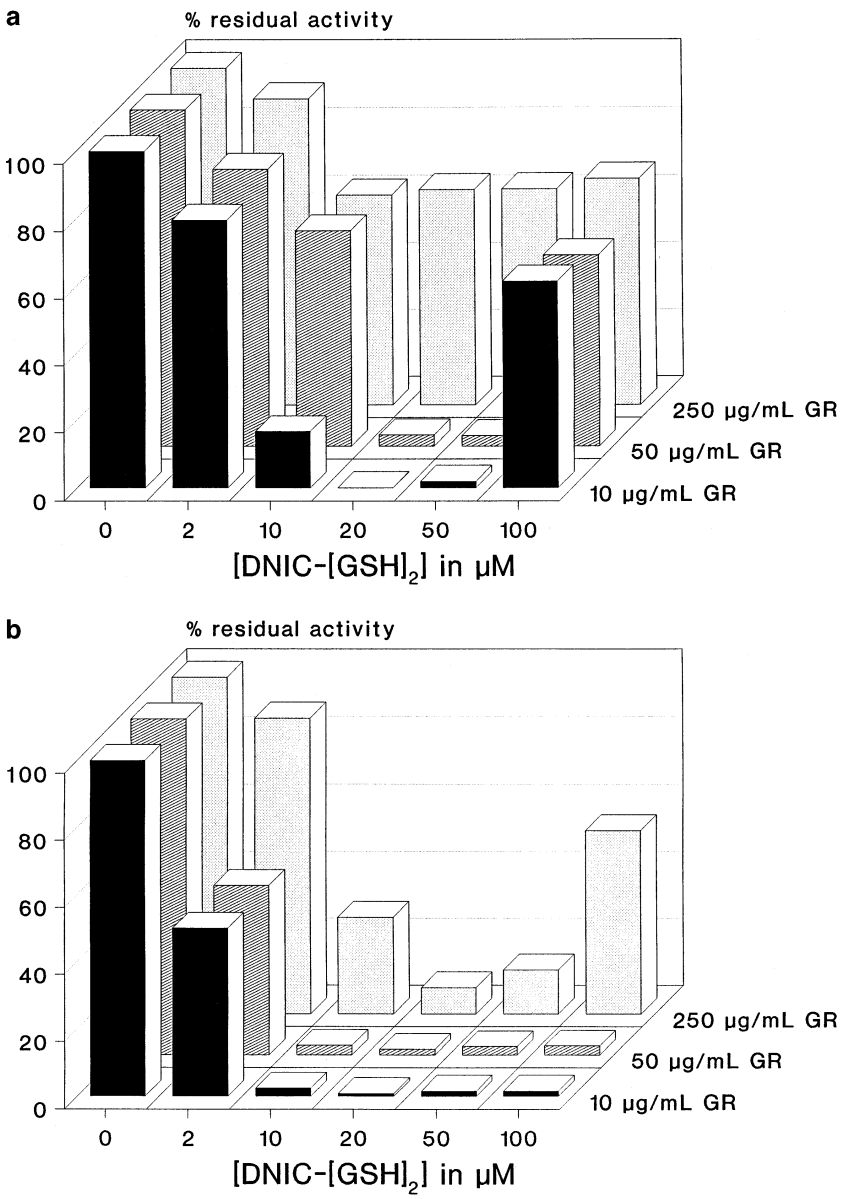

FIG. 3. Inhibition of different GR concentrations by DNIC-$[GSH]_2$ at 100 µM NADPH (a) and 1 mM NADPH (b). The samples were incubated for 1 hr at 25°. No inactivation was observed in the absence of NADPH. Note that at higher inhibitor concentrations the percentage of inhibition decreases (see text).

Inhibition of Human π-Type Glutathione-S-Transferase (GST) by NO Carriers

DNIC-$[GSH]_2$ and DNIC-Cys_2 were found to be very potent inhibitors of human GST; as indicated in Fig. 4 for the enzyme of fresh hemolysates, the IC_{50} values were 60 nM for DNIC-$[GSH]_2$ and 180 nM for DNIC-Cys_2. This inhibition was competitive with GSH and fully reversible by dilution. With GST isolated from human placenta, a K_i value of 20 nM was determined for competition of DNIC-$[GSH]_2$ with GSH (Fig. 5). Competition with the other substrate of the reaction, 1-chloro-2,4-dinitrobenzene (CDNB), was not observed.

GST was also inhibited by the NO carrier *S*-nitrosoglutathione. This inhibition was also fully reversible and competitive with GSH (K_i = 180 µM; see Fig. 6a) and with CDNB (K_i = 170 µM; see Fig. 6b). These data are consistent with the results of Clark and Debnam (1988), who described the reversible inhibition of glutathione-S-transferase from rat liver by GSNO [9].

Inhibitor Studies on Glutathione Peroxidase

When preincubating human erythrocyte GPX with DNIC-$[RSH]_2$ or GSNO there was no inhibitory effect. Interestingly, these data contrast with the work of Asahi et al. (1995), who observed a clear inhibition of purified bovine GPX by the nitric oxide donor *S*-nitroso-*N*-acetyl-DL-penicillamine [36]. When adding the inhibitors directly to the GR-coupled GPX assay, an apparent increase in activity was detected at lower micromolar concentrations. This increase did not depend on the presence of GPX and is likely to be due to the nonenzymatic formation of GSSG from DNIC-$[GSH]_2$ under aerobic conditions in the presence of 2 mM GSH in the assay mixture. The nascent GSSG is then reduced by GR at the expense of NADPH.

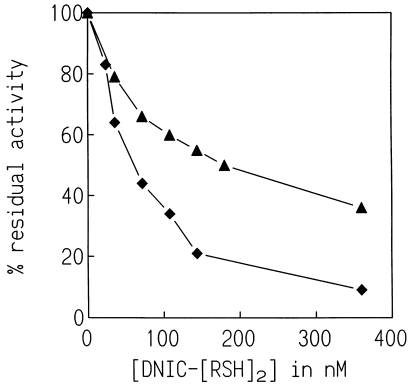

FIG. 4. Inhibition of human GST in hemolysates by DNIC-$[GSH]_2$ (◆) and DNIC-Cys_2 (▲). To measure enzyme activity, fresh hemolysates were diluted by a factor of 40 and the inhibitors were directly added to the assay.

Incubation of Erythrocytes and Plasmodium falciparum *with DNICs*

Concentrated erythrocytes were suspended in PBS to simulate a hematocrit of 45% and then incubated for 1 hr at 25° in the presence of 10 mM glucose with 3 μM to 100 μM DNIC-$[GSH]_2$ and DNIC-Cys_2, respectively. After centrifugation (500 × *g*), the cells were added to a GR assay mixture containing 40 mg/L digitonin for hemolysis. Only slight effects on GR activity were detected. The inhibition achieved by 100 μM of either inhibitor was ≤45%. High reactivity and inability of DNICs to cross the red cell membrane might explain this result. It should be noted that other extracellular NO donors do not have a major effect on erythrocyte GR either, which could be explained by the NO-trapping effect of hemoglobin [14].

Membrane permeability of red blood cells infected with the malaria parasite *Plasmodium falciparum* differs from that of normal erythrocytes [37]. Furthermore, NO donors such as S-nitroso-cysteine and S-nitroso-glutathione were shown to kill *P. falciparum in vitro* [38]. We therefore tested the effects of DNICs on malaria parasites. DNIC-$[GSH]_2$ or DNIC-Cys_2 (100 nM to 100 μM) were added to 0.2 mL

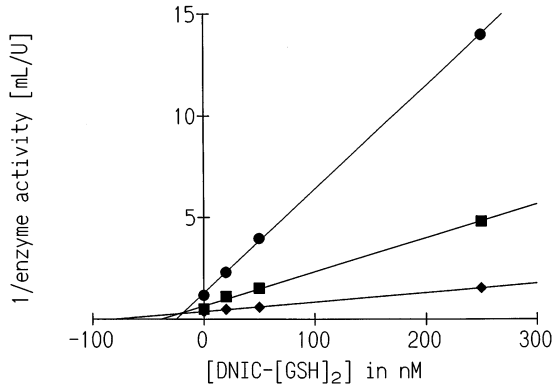

FIG. 5. Dixon plot for the inhibition of isolated human placenta GST by DNIC-$[GSH]_2$. The inhibition is competitive with GSH (●—110 μM; ■—280 μM; ◆—1085 μM), the K_i being 20 nM.

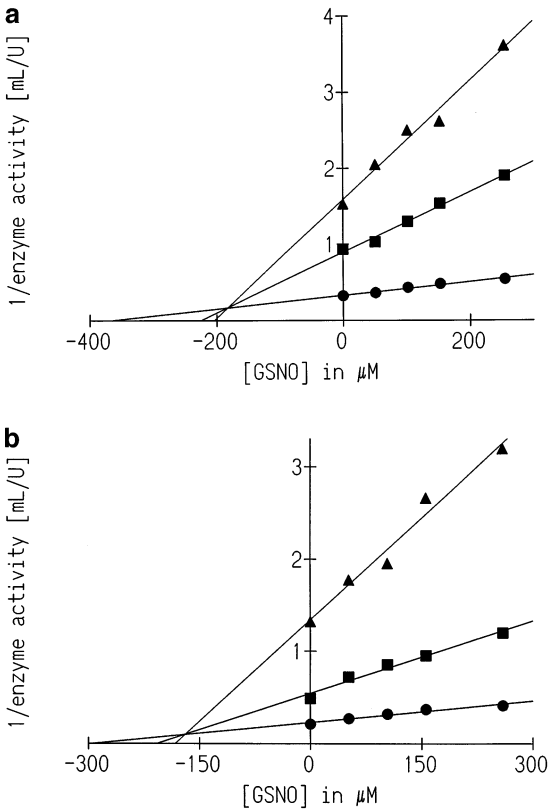

FIG. 6. Dixon plots for the inhibition of human placenta GST by GSNO. (a) The inhibition is competitive with GSH (▲—120 μM, ■—220 μM; ●—1200 μM), the K_i being 180 μM. (b) The inhibition is also competitive with CDNB (▲—60 μM; ■—210 μM; ●—840 μM), the K_i being 170 μM.

synchronized *P. falciparum* cultures in RPMI medium (hematocrit 3%, parasitemia 5%, ring stage). To avoid reactions of the inhibitors with serum proteins, the incubation was carried out for 1 hr without serum and then continued under standard conditions in RPMI medium with 10% human serum. After 48 hr neither reinvasion nor growth of the parasites was significantly affected by the inhibitors.

DISCUSSION

Dinitrosyl-dithiol-iron complexes seem to represent physiologic transport forms of nitric oxide, but also of iron and thiols such as glutathione or cysteine. *In vivo*, nitric oxide can be stabilized and stored in the form of dinitrosyl-iron complexes with proteins and is probably released from cells in the form of low molecular weight dinitrosyl-iron-dithiolates [20, 39]. Formation and release of dinitrosyl-iron-complexed thiols has been demonstrated for activated macrophages and endothelial cells [40, 41]; superoxide radicals may play an indirect role by releasing iron ions from ferritin [42] and other proteins [43, 44]. Dinitrosyl-iron-di-L-cysteine was shown to be a potent activator of purified soluble guanylyl cyclase and to have vasodilating activity [39]. Since glutathione reductase, a central enzyme in antioxidant defense, is known to be inhibited by the NO

carrier *S*-nitroso-glutathione, we investigated the effects of dinitrosyl-iron-dithiolates on GR and two other enzymes of glutathione metabolism, namely glutathione-*S*-transferase and glutathione peroxidase.

GR is irreversibly inhibited by micromolar concentrations of DNIC-$[GSH]_2$ (IC_{50} = 4 μM) and, to a lesser degree, by DNIC-Cys_2. This inhibition is by a factor of >200 more potent than the GR inhibition induced by GSNO. As summarized by Asahi *et al.* (1995), several enzymes have been shown to be inactivated or modified by NO [36]. The modifications of these enzymes can be classified into two groups: (1) NO binds to the iron of iron-cofactor-containing enzymes such as guanylyl cyclase, aconitase, cytochrome c oxidase, and cyclooxygenase; and (2) NO interacts with an SH group of enzymes containing catalytically essential thiols. The GR-modification induced by DNIC-$[RSH]_2$ could not be reversed by incubation with different reducing agents. We therefore propose a mechanism of inhibition which involves the active site thiols Cys58 and/or Cys63 but which does not necessarily imply a nitrosylation. A role of Cys2 or the prosthetic group FAD in the mode of inhibition was ruled out by studying a delta(1-15)-mutant of GR and the FAD-free apoenzyme. We have recently crystallized DNIC-$[GSH]_2$-modified GR to investigate the chemical nature of the modification in atomic detail.

Glutatione-S-transferase is very potently but reversibly inhibited by DNICs, the K_i for competition with GSH being as low as 20 nM. This result could point to a relevant role of GST-inhibition *in vivo*. Enhanced formation of DNIC-$[RSH]_2$ induced by oxidative stress or other mechanisms, which elevate concentrations of intracellular NO and free iron, could lead to significant inhibition of GST. This inhibition would become even more pronounced if GSH-levels are low. GST inhibition could therefore represent a GSH-saving mechanism providing reducing equivalents for other reactions of higher priority. One of these reactions could be the detoxification of peroxides by GPX which, as delineated above, is not significantly affected by DNIC-$[RSH]_2$. On the basis of the data discussed in this paper, the differential effects of NO carrier molecules on enzymes of the glutathione metabolism are likely to play a relevant role *in vivo*.

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