Trypanothione efficiently intercepts nitric oxide as a harmless iron complex in trypanosomatid parasites

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ABSTRACT **Trypanosomatids are protozoan organisms that cause serious diseases, including African sleeping sickness, Chagas' disease, and leishmaniasis, affecting about 30 million people in the world. These parasites contain the unusual dithiol trypanothione [$T(SH)_2$] instead of glutathione (GSH) as the main intracellular reductant, and they have replaced the otherwise ubiquitous GSH/glutathione reductase redox couple with a $T(SH)_2$/trypanothione reductase (TR) system. The reason for the existence of $T(SH)_2$ in parasitic organisms has remained an enigma. Here, we show that $T(SH)_2$ is able to intercept nitric oxide and labile iron and form a dinitrosyl-iron complex with at least 600 times higher affinity than GSH. Accumulation of the paramagnetic dinitrosyl-trypanothionyl iron complex *in vivo* was observed in *Trypanosoma brucei* and *Leishmania infantum* exposed to nitric oxide. While the analogous dinitrosyl-diglutathionyl iron complex formed in mammalian cells is a potent irreversible inhibitor of glutathione reductase (IC_{50}=4 μM), the $T(SH)_2$ complex does not inactivate TR even at millimolar levels. The peculiar capacity of $T(SH)_2$ to sequester NO and iron in a harmless stable complex could explain the predominance of this thiol in parasites regularly exposed to NO.—Bocedi, A., Dawood, K. F., Fabrini, R., Federici, G., Gradoni, L., Pedersen, J. Z., Ricci, G. Trypanothione efficiently intercepts nitric oxide as a harmless iron complex in trypanosomatid parasites. *FASEB J.* 24, 1035–1042 (2010). www.fasebj.org**

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Almost all living organisms express millimolar cytosolic levels of glutathione (GSH) to maintain the thiol redox homeostasis in cells. Reactive radical species and improperly oxidized cell components can be reduced efficiently, either enzymatically or spontaneously, by GSH, which is regenerated from oxidized glutathione (GSSG) by the NADPH-dependent glutathione reductase (GR). Curiously, a small group of parasitic organisms do not express GR but use a different redox-buffer system based on trypanothione [$T(SH)_2$] and the corresponding trypanothione reductase (TR) (1). $T(SH)_2$ is composed of two GSH molecules linked by a spermidine bridge and is the most abundant low-mass thiol compound in these organisms, although low amounts of free GSH and unusual thiols such as ovothiol and glutathionylspermidine are also found. Because of the presence of two sulfhydryl groups in the same molecule and their low pK_a values (2), $T(SH)_2$ is a faster reagent in redox reactions than GSH. However, their standard redox potentials are similar (3), and the small advantage of $T(SH)_2$ over GSH in redox reactions seems unlikely be the sole determinant for the presence of $T(SH)_2$ in trypanosomatids. Despite the fact that most of the efforts to develop new drugs against these pathogens focus on the enzymes involved in $T(SH)_2$ metabolism, today, ~25 yr after it was discovered (4), there is still no explanation why the use of $T(SH)_2$ has evolved in these parasites.

Part of the life cycle of these parasites takes place in the blood and/or macrophages of the host, which produce considerable amounts of NO to eliminate intruding cells (5–7). We have therefore investigated whether $T(SH)_2$ is involved in the defensive mechanism of parasites against the cytotoxic activity of NO. The interaction between NO, Fe(II), and thiols is known to give rise to the spontaneous formation of dinitrosyl iron complexes (DNICs), paramagnetic compounds characterized by a typical electron paramagnetic resonance (EPR) spectrum centered at $g = 2.03$ (8, 9). In mammalian cells and tissues exposed to NO, DNICs are never observed as free species, but only bound to proteins (10). We have previously shown that glutathione transferase isoenzymes (GSTs), present in most mammalian cells at levels ranging from 0.1 to 0.8

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mM, can bind the complex formed with GSH, the dinitrosyl-diglutathionyl-iron complex (DNDGIC) (see **Fig. 2**), with very high affinity (K_d=10^{-9}–10^{-10} M) (11–13). X-ray diffraction data revealed that the complex is bound to the site competent for GSH binding (G site) and stabilized by an essential tyrosine residue that replaces one GSH molecule in the iron coordination (14). This interaction appears to be of physiological importance because DNICs show various toxic effects *in vitro* (8). In particular, the free form of DNDGIC is a strong and irreversible inhibitor of GR (IC_{50}=4 μM) (15), but this effect is prevented completely in the presence of GST (16). The significant amount of GST-DNGIC, up to 0.1 mM, detected in intact hepatocytes after exposure to NO, and the complete preservation of GR activity in the same cells (16, 17), provide strong evidence that intracellular NO can be neutralized by the combined interaction of DNDGIC and GSTs. The present study explores the possibility that $T(SH)_2$ could play a similar role in trypanosomatids and could have been selected during evolution for its peculiar modality of interaction with nitric oxide. Surprisingly, we found that this unusual dithiol displays much higher propensity than GSH to intercept NO in a dinitrosyl-trypanothionyl-iron complex (DNTIC). In addition, this complex does not need any sequestration by specific proteins, as it appears to be harmless to the parasite even in the free form.

MATERIALS AND METHODS

Materials

$T(SH)_2$ was bought as the disulfide (Bachem, Bubendorf, Switzerland). Quantitative reduction of the disulfide was obtained by incubating 10 mg $T(SH)_2$ with 10 mg $NaBH_4$ in 0.1 ml H_2O for 30 min. The excess of this reagent was then destroyed by addition of a few drops of acetic acid (to a final pH of 5.0). The extent of reduction (>98%) was determined with a standard thiol assay based on Ellman's reagent (18). DNDGIC and GSNO were prepared as described previously (11). Diethylamine NONOate sodium salt hydrate (DEANO) was from Sigma-Aldrich (St. Louis, MO, USA).

In vitro synthesis of DNTIC

The $T(SH)_2$ iron complex was typically prepared by reacting 5 mM $T(SH)_2$ with 2 mM GSNO and 10 μM ferrous ions in 0.1 M potassium phosphate buffer (pH 7.4). Titration of DNTIC was performed on the basis of EPR measurements (see below).

Relative propensity of $T(SH)_2$ and GSH to form DNICs

Displacement of GSH from DNDGIC by $T(SH)_2$ to form DNTIC was studied by incubating variable amounts of $T(SH)_2$ (from 0.2 μM to 1 mM) with 0.01 mM of DNDGIC in the presence of 1 mM of GSH in 0.1 M potassium phosphate buffer (pH 7.4). After 30 s, 5-μl aliquots (~50 pmol of the complex) were reacted with 100 pmol of purified GSTA1-1. Then 0.1 M potassium phosphate buffer (pH 6.5), 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mM of GSH were added to a final volume of 1 ml for activity determinations. GST activity was measured spectrophotometrically at 340 nm (11). The extent of GSH displacement by $T(SH)_2$ was evaluated on the basis of GST inhibition, given that DNDGIC, but not DNTIC, strongly inhibits GSTA1-1 (13). Displacement of GSH from DNDGIC by cysteine, cysteamine, or dithiothreitol, in the concentration range from 0.1 mM to 200 mM, were studied by the same procedure described for $T(SH)_2$. Data were fitted to a sigmoidal dose-response (variable slope) equation:

$$y = \frac{100\%}{1 + 10^{(\log EC50 - \log[\text{thiol}]) \cdot nH}}$$

where y is the percentage of GSH displacement from DNDGIC, EC_{50} is the thiol concentration giving 50% displacement, and n_H is the Hill coefficient. Analysis was performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Parasite cultures and samples

T. brucei (MHOM/NG/73/IBADAN 73) procyclic trypomastigotes and *L. infantum* (MHOM/TN/80/IPT1) promastigotes were grown in Evans's modified Tobie's medium at 25°C and harvested during late logarithmic phase. Samples for *in vivo* EPR measurements, enzyme purification, parasite extracts, and motility inhibition assays were prepared as described previously (19, 20). Contamination by medium components (*e.g.*, intact or degraded red blood cells) were carefully avoided by mechanical manipulation and extensive washings of concentrated parasite material. The absence of exogenous cells in samples was confirmed by microscopy.

Parasite extracts

Cell extracts were made from concentrated samples containing 10^{10} cells/ml that were sonicated and centrifuged. In cell lysates, the $T(SH)_2$ concentration was diluted ~10-fold. During the extraction process, $T(SH)_2$ was rapidly oxidized and partially degraded; therefore, 0.9 mM of both $T(SH)_2$ and GSH were added to reach ~1 mM final concentration to approach the *in vivo* reducing conditions (1).

Enzyme purifications

TR and trypanothione S-transferase (TST; as the ribosomal elongation factor 1B) were partially purified from *L. infantum* (21, 22). TR and TST displayed specific activities of 0.3 U/mg and 28 mU/mg, respectively. Activities of GR, GST, TR, and TST were determined by the procedures described previously (16, 21, 22).

DNTIC formation in intact cells

T. brucei or *L. infantum* (2×10^9 cells/ml) were incubated with 2 mM GSNO in growth medium. After 30 min, the amount of DNTIC was calculated on the basis of the EPR spectra (see below).

Effect of GSNO on cell motility

Samples containing 10^7 parasites/ml were incubated with GSNO (1 to 35 mM) in Evans' modified Tobie's medium liquid phase, at 25°C. After 120 min, the motility of parasites was assessed by microscopic evaluation, in comparison with control cultures. In preliminary experiments, we observed

that the rate of decrease in motility reached a plateau at 90–120 min after exposure to NO. Treated cultures were monitored during the following 6 h, but the motility inhibition rate did not increase significantly as compared to control cultures.

TR inhibition by DNTIC

To check the effect of DNTIC on TR in intact cells, 2×10^9 cells/ml of *T. brucei* or *L. infantum* were incubated with 2 mM GSNO in growth medium. Thirty minutes after DNTIC reached the maximum level in intact cells (~90 min), parasite extracts were prepared as described above, and TR activity was measured.

DNIC formation using DEANO as a NO donor

A 10 mM stock solution of DEANO was prepared by dissolving the compound in 10 mM NaOH at 0°C. This solution is stable for a few hours and was prepared fresh every day. At neutral pH values, 1 mol of the compound spontaneously releases 1.5 mol of NO at 25°C ($t_{1/2}$=16 min). DEANO was used as a NO source in *L. infantum* and *T. brucei* extracts to form DNTIC. In other experiments, NO was added directly, using a saturated NO solution (~2 mM). Briefly, deionized water was bubbled with argon for 20 min to remove oxygen. Then, the solution was bubbled with pure NO gas (passed through KOH solution) for 20 min and kept under NO atmosphere until use.

EPR measurements

DNDGIC and DNTIC titrations were performed on the basis of EPR and enzyme activity procedures (13). The extent of EPR hyperfine structure was used to quantify DNDGIC and DNTIC when present in the same mixture. All EPR measurements were made at room temperature, as described previously (12). An alternative procedure for DNDGIC quantitation is based on the strong inhibition of GSTA1-1 by this complex (13). The *in vivo* EPR measurements were made with 2×10^8 cells/100 μl for both *T. brucei* and *L. infantum*.

RESULTS

$T(SH)_2$ efficiently forms DNTIC

When $T(SH)_2$ was incubated with ferrous ions and GSNO, a paramagnetic species with an isotropic EPR spectrum centered at g = 2.03 was rapidly formed (**Fig. 1*A***). This symmetrical single-line spectrum is typical for low-mass dithiol-dinitrosyl iron complexes and is almost identical to the spectrum of the analogous complex formed with GSH (DNDGIC, Fig. 1*A*), except for a slightly larger line width, 8.4 G for DNTIC compared to 8.0 G for DNDGIC. This shows that the two complexes have very similar isotropic motion correlation times, which means that they must have approximately the same size. At high resolution, the DNDGIC spectrum shows a characteristic hyperfine splitting pattern (Fig. 1*B*), due to the coupling of an unpaired electron with the N and H nuclei of the ligands (12). Interestingly, this pattern was drastically reduced in the spectrum of DNTIC, indicating a different geometry of the $T(SH)_2$ complex (Fig. 1*B*). Binding of DNDGIC to GST isoform A1-1 not only inhibited the enzyme but also changed its EPR signal drastically (Fig. 1*C*); the wide anisotropic spectrum corresponds to an immobilized complex and is typical for all DNICs bound to proteins (8, 10, 16). In contrast, DNTIC did not cause any loss of GST activity, and no anisotropic component could be detected in the spectrum (Fig. 1*C*), demonstrating that this complex did not bind to GST at all.

The spectral differences, together with the strong inhibition of GSTA1-1 by DNDGIC but not by DNTIC, were successfully used to quantify the amounts of these two complexes when they coexisted in the same environment. Surprisingly, the competition experiments demonstrated that $T(SH)_2$ formed the complex with ~600 times higher affinity than GSH (Fig. 2). This was unexpected, because other thiols and dithiols showed affinities similar to GSH or lower (Fig. 2). While the paramagnetic dinitrosyl-iron complexes formed with monothiols have a defined and unambiguous chemical composition, a dithiol compound like $T(SH)_2$ could generate either a monotrypanothionyl complex, with the dithiol acting as a bidentate ligand, or a ditrypanothionyl complex, with the iron coordinated by two different $T(SH)_2$ molecules. The displacement behav-

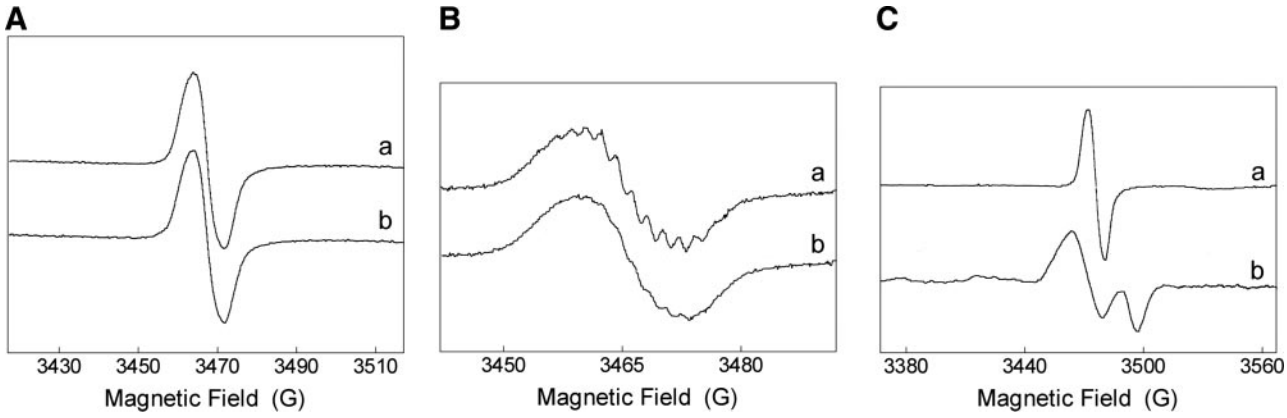

Figure 1. EPR spectra of DNTIC and DNDGIC. Room-temperature EPR spectra of the complexes obtained after 30-min incubation of 2 mM GSNO, 10 μM $FeSO_4$ and 10 mM GSH or 5 mM $T(SH)_2$, in 0.1 M potassium phosphate buffer (pH 7.4). *A*) DNDGIC (*a*) and DNTIC (*b*). *B*) High-resolution spectra of the samples shown in *A*. *C*) DNTIC (10 μM) mixed with 20 μM GSTA1-1 (*a*); DNDGIC (10 μM) mixed with 20 μM GSTA1-1 (*b*).

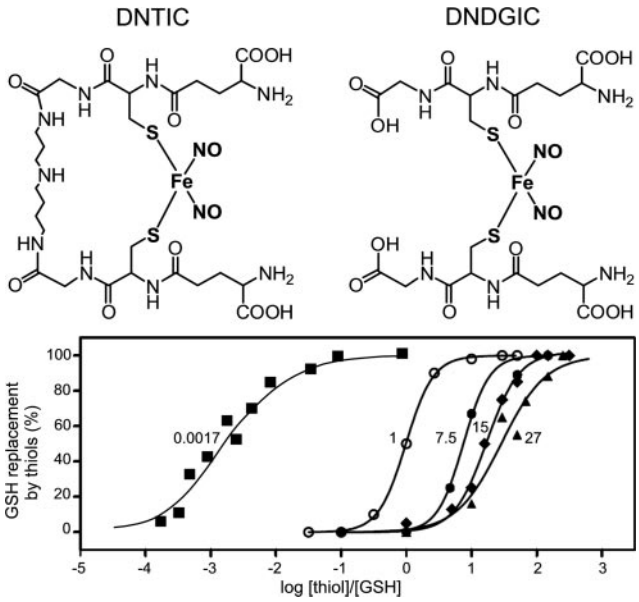

Figure 2. Chemical structures of DNTIC and DNDGIC and competitive replacement of GSH in DNDGIC by TSH and other thiols. Replacement experiments were performed as described in Materials and Methods. ○, hypothetical thiol with the same propensity as GSH to generate the complex; ●, cysteine; ■, $T(SH)_2$; ▲, dithiothreitol (DTT); ◆, cysteamine. Numbers on each curve indicate the [thiol]/[GSH] ratio at 50% replacement of GSH from DNDGIC. This ratio is inversely proportional to the relative propensity to form the complex. Experimental points were fitted to a sigmoid dose-response (variable slope) equation reported in Materials and Methods. Hill slopes were ~1 for $T(SH)_2$ and DTT, and ~2 for cysteine and cysteamine.

ior of $T(SH)_2$ and of other different thiols toward GSH from DNDGIC is diagnostic in this context. In the case of a monothiol compound, the displacement should depend on the thiol concentration squared, according to the following equilibrium equation:

$$K_d = \frac{[Fe][NO]^2[RSH]^2}{[DND\text{-}thiol\text{-}IC]}$$

Accordingly, the Hill coefficient observed for the displacement curves shown in Fig. 2 was ~2 for cysteine and cysteamine. Conversely, the Hill coefficient was ~1 for $T(SH)_2$, corresponding to the following equilibrium equation:

$$K_d = \frac{[Fe][NO]^2[T(SH)_2]}{[DNTIC]}$$

This result strongly indicates that $T(SH)_2$ acts as a bidentate ligand. As a control, the displacement by the dithiol dithiothreitol (DTT) also displayed a Hill coefficient of 1 as expected, although the propensity of this compound to form the complex was 10^4 times lower than $T(SH)_2$. We therefore conclude that DNTIC has the structure shown in Fig. 2. The bidentate ligand behavior provides a favorable entropic contribution, which explains the highly increased affinity compared to GSH. The formation of larger polymeric complexes can be excluded because of the very similar EPR line widths measured for DNTIC and DNDGIC. These two complexes have approximately the same size, but the ring structure in DNTIC imposes a constraint on the ligand arrangement, resulting in the loss of EPR hyperfine structure. These results suggest that DNTIC will be the predominant complex produced in trypanosomatids exposed to NO. Keeping in the mind that in these parasites the GSH concentration is always lower than that of $T(SH)_2$, our data indicate that DNTIC should be formed preferentially even if GSH were 100 times more concentrated than $T(SH)_2$.

DNTIC is formed in trypanosomatid extracts in the presence of GSNO

The influence of intracellular components on the formation of DNTIC was evaluated using crude extracts of the trypanosomatids *L. infantum* and *T. brucei* (**Fig. 3**) incubated with GSNO as NO-generating system. To compensate the dilution of endogenous thiols due to extract preparation, the content of $T(SH)_2$ and GSH was supplemented up to 1 mM final concentration in both extracts to approach the intracellular concentration of these compounds (1). The generation of a DNIC in the extracts could be detected within a few minutes (Fig. 3*A*), and a stable level was reached within 30 min of incubation, followed by a very slow decay. The complex formed could be identified as DNTIC on the basis of its EPR spectrum, and, in particular, through the diagnostic absence of the strong hyperfine pattern (Fig. 3*B*). These findings indicate that also in the parasite extracts, $T(SH)_2$ displays higher propensity than GSH to form dinitrosyl complexes and that no specific inhibitors are present that preclude the DNTIC formation. Interestingly DNTIC appeared almost entirely as the free species and not as a complex bound to TST or other proteins present in the extracts. DNICs bound to proteins are characterized by an enlarged anisotropic spectrum with a broad main band at g = 2.04 and a diagnostic g_x = 2.01 component (see Fig. 1*C*) (16) that is completely absent in the spectra shown in Fig. 3.

Influence of iron and GSNO concentrations on DNTIC formation in the parasite extracts

In trypanosomatid extracts, iron availability limited the final level of DNTIC. In fact, iron implementation (up to 20 μM) both in *L. infanum* (**Fig. 4*A***) and in *T. brucei* (not shown), extracts incubated with 2 mM GSNO gave rise to a proportional and almost stoichiometric increase of the final level of DNTIC. On the contrary, different concentrations of GSNO (from 0.5 mM to 5 mM) did not modify the final DNTIC concentration but did influence the kinetics of DNTIC formation (Fig. 4*B*). The concentration of iron in the extracts mobilized for the DNTIC formation was ~10 μM.

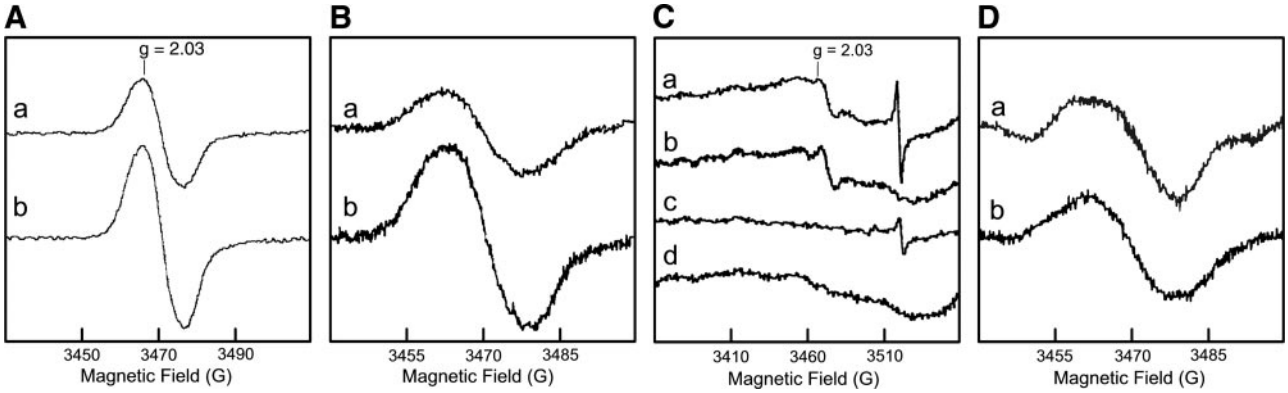

Figure 3. DNTIC formation in *T. brucei* and *L. infantum* cell extracts and intact cells. *A*) EPR spectra of *T. brucei* (*a*) and *L. infantum* (*b*) extracts incubated with 2 mM GSNO for 30 min. Extracts were supplemented with $T(SH)_2$ and GSH up to 1 mM final concentration. *B*) High-resolution EPR spectra of the same samples as in *A*. Lack of hyperfine structure demonstrates the presence of DNTIC. *C*) EPR spectra of *T. brucei* (2×10^8 cells in 100 μl of growth medium) incubated at 25°C for 30 min with 2 mM GSNO (*a*); same experiment as in the trace *a* with *L. infantum* at the same cellular concentration (*b*); and same experiments as in traces *a* and *b*, respectively, without GSNO (*c, d*). *D*) High-resolution EPR spectra of the samples reported in *C*; controls without GSNO are not shown.

DNTIC is equally formed in trypanosomatid extracts in the presence of DEANO

GSNO has been used in this study as the NO-generating system. This compound is a NO donor that shows the advantage to enter rapidly in living cells and to be a natural compound that releases free NO in the presence of metal ions and thiol compounds like GSH (23, 24). It has been shown directly that in trypanosomatids, GSNO is readily decomposed by $T(SH)_2$ releasing NO (25). In addition, it has recently been documented that nitrosothiols are not direct donors of nitrosyl to iron for DNICs, whereas DNICs can be formed from free NO generated by nitrosothiols (26). Therefore, it is most likely that DNTIC is formed as a result of interception of free NO by $T(SH)_2$, and not through direct transfer of NO from GSNO to the iron. Results reported in Fig. 4*C* confirm this idea. In fact, *L. infantum* extracts incubated with 0.33 mM DEANO (a different NO-releasing compound with a short $t_{1/2}$ of ~20 min) or 0.5 mM GSNO produced similar amounts of DNICs. Similar results have been obtained with *T. brucei* extracts (data not shown). In a second series of experiments also, the direct addition of pure NO (0.5 mM from a NO-saturated aqueous solution) caused the formation of practically the same levels of DNTIC in the extracts (data not shown). The use of pure nitric oxide allows us to exclude potential artifacts due to the donor molecules, but high initial NO concentrations are required, and it is not possible to simulate the more physiologically relevant continuous exposure to lower NO levels.

DNTIC is formed in intact *T. brucei* and *L. infantum* cells

Since EPR is a noninvasive technique, it was possible to carry out *in vivo* studies on living parasites. On incubation of cultured *L. infantum* or *T. brucei* with 2 mM GSNO, the time-dependent accumulation of DNTIC could be observed directly in the intact cells. Even though the EPR spectra were less defined than those of the corresponding cell extracts, the formation of

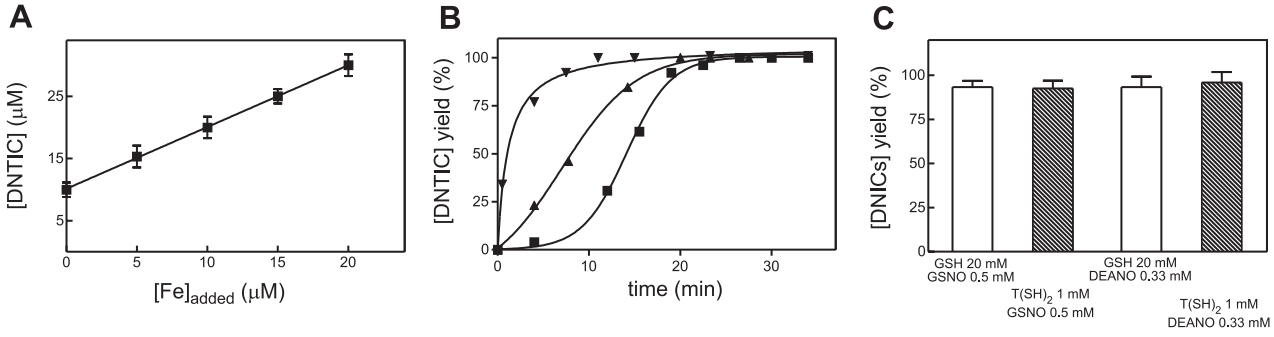

Figure 4. DNIC formation in the presence of different amounts of iron and NO-donors in *L. infantum* extracts. *A*) *L. infantum* extracts were implemented by variable amounts of ferrous ions (up to 20 μM) and incubated at 25°C with 1 mM $T(SH)_2$ and 2 mM GSNO as NO source. Level of DNTIC formed was evaluated on the basis of EPR spectrum. *B*) Kinetics of DNTIC formation in *L. infantum* extracts incubated with different amounts of GSNO; 0.5 mM (■), 2 mM (▲), and 5 mM (▼). *C*) DNDGIC and DNTIC formation in *L. infantum* extracts using GSNO and DEANO as NO donors. One hundred percent of DNTIC yield (*B, C*) corresponds to ~10 μM (see *A*).

DNTIC in the living parasites was also verified here by the characteristic peak at $g = 2.03$ and by the absence of hyperfine splitting (Fig. 3*C, D*). As in the extracts, most of the dinitrosyl complex appears as a free species. In these experiments, as well as in the experiments made with cell extracts, we never observed the characteristic three-line signals of mononitrosylated complexes or iron proteins, such as NO hemoglobin. It should be emphasized that all EPR measurements were made at room temperature, at which signals due to Fe(III) normally cannot be detected.

The cytosolic volume of 10^8 cells is ~2 μl for *L. infantum* (27), and ~6 μl for *T. brucei*; based on these values, it can be calculated that the concentration of DNTIC formed in intact cells was ~0.07 mM for both parasites. This value is close to the total content of 0.1 mM intracellular iron found in *Trypanosoma* (28). As observed for the parasite extracts, iron seems to be the limiting factor for DNTIC formation; in fact, the incubation of intact parasites with higher GSNO concentrations (up to 5 mM) did not yield any increase in the final level of DNTIC (data not shown). The mobilization of such conspicuous amounts of iron is not surprising, since it has been shown that as little as 0.01 mM iron, corresponding to 40,000 atoms, is sufficient for *Trypanosoma* survival (28). This parsimonious behavior allows these organisms to sequester the astonishing quantity of 0.14 mM intracellular NO in the form of DNTIC. The precise amount of iron available in the *L. infantum* cells used here is not known. *Leishmania* has developed an active iron import system (LIT1) to extract the metal from the host cells (29), and large variations in the iron levels of different *L. enriettii* strains have been reported (30). Therefore, it seems possible that DNTIC may reach even higher concentrations in *Leishmania* under physiological conditions, under which additional iron will be available.

The incubation with 2 mM GSNO over a period of 2 h at room temperature did not affect the viability of the cells, as estimated by their relative motility (see **Fig. 5*A***).

DNTIC is a harmless complex

The fact that most DNTIC in trypanosomatids appeared as the free species, whereas the corresponding complex in mammalian cells, DNDGIC, has only been found bound to GST (16), indicated that free DNTIC cannot be very cytotoxic. The free form of DNDGIC has been reported to be a strong competitive inhibitor of GR ($K_i = 5$ μM) (30), but it also irreversibly inactivates this enzyme by oxidizing the essential thiol group of Cys63 to sulfinic level (IC_{50}=4 μM) in the presence of 1 mM NADPH (15). If free DNTIC had a similar effect on TR, the redox status of trypanosomatids would be drastically compromised. However, measurements of TR activity in *Leishmania* extract showed that DNTIC was a very weak competitive inhibitor of TR, with a K_i value of 1.2 mM (see Fig. 5*B*). More important, a concentration of 0.1 mM DNTIC did not cause any irreversible inactivation of TR after 30 min incubation, in the presence or absence of 1 mM NADPH (Fig. 5*C*); in comparison, GR is completely inactivated by 36 μM of DNDGIC after 20 min of incubation in the presence of 1 mM NADPH (Fig. 5*C*) (31). The complete absence of TR inactivation was confirmed also in living parasites exposed to NO and measured 30 min after reaching the maximum concentration of DNTIC (Fig. 5*D*). In cultured *L. infantum*, an increase in the basal activity of TR was actually observed, possibly due to up-regulation of the enzyme triggered by NO (Fig. 5*D*). Finally, we also found that DNTIC was not a substrate for TR in the presence of 1 mM NADPH or 1 mM $NADP^+$ (data not shown).

Mammalian GSTs bind DNDGIC with high affinity, and as a consequence, they are also strongly inhibited

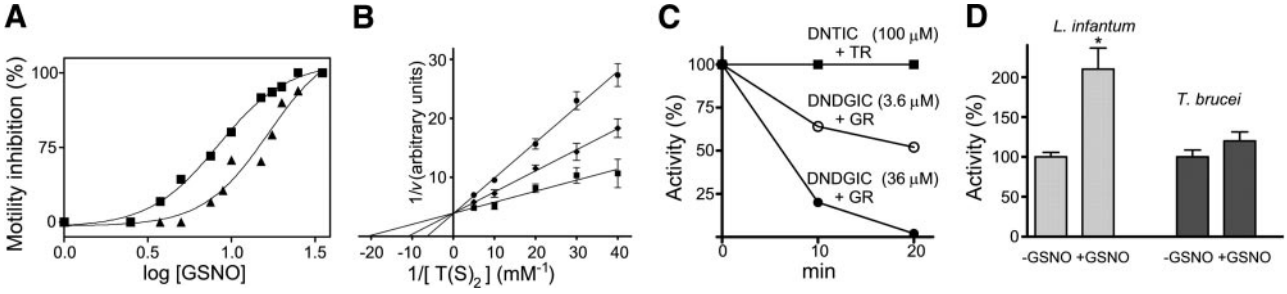

Figure 5. Inhibitory effects of GSNO and DNTIC. *A*) Effect of different concentrations of GSNO (1–35 mM) on the motility of *L. infantum* promastigotes (■) and *T. brucei* procyclic trypomastigotes (▲), assayed at 25°C after incubation with GSNO for both parasites in growth medium. Motility inhibition was assessed by microscopic evaluation as compared to controls, on ~100 organisms. *B*) Double reciprocal plot showing competitive inhibition of TR by DNTIC. Enzyme activity was determined at 25°C as reported previously (21), with minor modifications (100 mM potassium phosphate buffer, pH 7.0), using 0.1 mM NADPH and TS_2 as substrates, without inhibitor (■) and in the presence of 1.2 mM (◆) or 2.4 mM DNTIC (●). From the abscissae intercepts, an inhibition constant K_i of 1.2 mM was calculated. Data are means ± SD of 3 independent measurements. *C*) Time-dependent inhibition of partially purified TR (0.1 U) incubated with 100 μM DNTIC in 0.1 M potassium phosphate buffer, pH 7.4 (■); for comparison, data from Keese *et al.* (31) are reported, showing the inhibition of GR by 3.6 μM (○) or 36 μM DNDGIC (●). *D*) Inhibition of TR in *L. infantum* and *T. brucei* exposed to NO. Cells (2×10^8/0.1 ml) were incubated at 25°C in growth medium containing 2 mM GSNO. TR activity was determined in the extracts obtained 30 min after reaching the maximum level of DNTIC (~90 min). Bars represent means ± SE of 3 independent experiments. $^*P < 0.01$; Student's *t* test.

by this complex (K_i=10^{-9}–10^{-10} M). In contrast, DNTIC was found to be only a weak competitive inhibitor of TST, the analogous transferase in trypanosomatid organisms (22, 32), showing a K_i value of 0.1 mM (see **Fig. 6**). This result is in agreement with the low amount of the complex bound to proteins in intact trypanosomatid cells. The unique properties of $T(SH)_2$ are supported by the fact that the intracellular DNTIC in trypanosomatids, as monitored here by *in vivo* EPR spectroscopy, is the only free dinitrosyl-iron species ever found in a biological system.

DISCUSSION

$T(SH)_2$ seems to be used quite exclusively by a few parasitic organisms, and this represents a fascinating enigma, because the evolutionary advantage of this choice is still unknown. Here, we propose that $T(SH)_2$ could be involved in the defense system of these parasites against NO; for the first time, there is a specific physiological function in which $T(SH)_2$ cannot be substituted by GSH. The results demonstrate that $T(SH)_2$ intercepts NO as a dinitrosyl iron complex with 600 times higher affinity than GSH, probably because $T(SH)_2$ acts as a bidentate ligand to the iron. The consequence is that DNTIC is the only dinitrosyl-iron complex likely to be produced in these organisms. This fact only becomes of importance when combined with the other main finding reported in this study: the absence of deleterious effects of DNTIC on the activity of key enzymes in the cells. While the analogous dinitrosyl-iron complex with GSH formed in mammalian cells, the DNDGIC, at micromolar levels, severely impaired the activity of GR, the DNTIC is practically harmless to TR, the key parasite enzyme that regulates the redox balance in *Leishmania* (33) and in *Trypanosoma* (21). This is confirmed by the observation of the

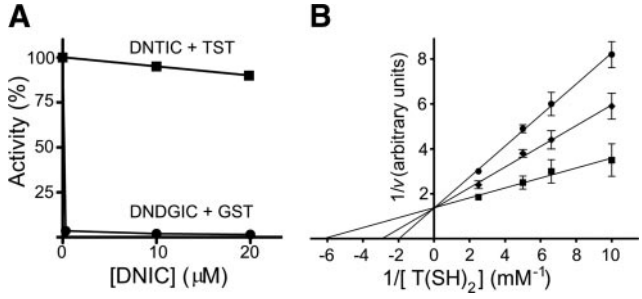

Figure 6. Effects of DNTIC on enzyme activities. *A*) Relative sensitivity of TST and GST toward DNICs. Inhibition of TST (0.02 U) by DNTIC (■) and inhibition of purified GSTA1-1 (0.02 U) by DNDGIC (●). *B*) Double reciprocal plot showing competitive inhibition of TST by DNTIC. Enzyme activity was determined as described previously (22) in 100 mM potassium phosphate buffer (pH 6.5) at 25°C, using 1 mM 1-chloro-2,4-dinitrobenzene as cosubstrate without inhibitor (■) and in the presence of 0.1 mM (◆) or 0.2 mM DNTIC (●). From the abscissae intercepts, an inhibition constant K_i of 0.1 mM was calculated. Data are means ± SD of 3 independent measurements.

formation of the complex *in vivo* when parasites are exposed to NO. DNTIC is clearly present in the cells as the free complex, the first time ever that a free form of a DNIC has been observed in cells. The cytotoxic DNDGIC is, in fact, always detected as a protein-bound species; in particular, DNDGIC has been found to be sequestered by GSTs (13, 16). The generation of DNTIC is probably limited by the iron availability, and it may engage a large amount of the total iron present in these parasites (~80–90%). This level of iron mobilization may be tolerated by the cells, because a well-known property of *Trypanosoma* parasites is their capacity to survive with very low intracellular iron levels (28), while *Leishmania* cells can actively extract iron from the host cells (29). In conclusion, our results may provide the first explanation for the development of a thiol homeostasis system based on $T(SH)_2$ instead of GSH in trypanosomatids and a few other protozoan parasites. The ability to form a nontoxic dinitrosyl-iron complex with $T(SH)_2$ could give these parasites a higher NO tolerance threshold than the host cells, which accumulate the cytotoxic GSH-based complex. The particular capacity of these parasites to make almost their entire iron pool available for binding of NO may enable them to survive in host compartments where high levels of NO are produced in response to infection (7).

Interestingly, dinitrosyl iron complexes have actually been detected in footpad lesions of mice infected by *L. amazonensis*, but the researchers did not comment on the nature of the complex (34). Although the EPR spectrum found in footpad lesions may also contain a component due to DNDGIC from host cells, this spectral evidence demonstrates that NO is actually produced in infected cells and that it is sequestered into dinitrosyl complexes. Obviously, the definitive demonstration that $T(SH)_2$ plays a fundamental role in the defense against NO requires further studies, such as the identification of the complex in the mammalian stage of trypanosomatids or studies on the effect of the down-regulation of key parasite enzymes like TR and trypanothione synthetase, all investigations clearly out of the scope of the present research. In any case, after down-regulation of TR in *L. donovani*, the parasites were still capable of maintaining a reduced intracellular environment during cell growth, but displayed a decreased ability to survive inside macrophages activated with interferon-γ and lipophosphoglycan (35). This observation is consistent with the hypothesis of the present work. FJ

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