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RESEARCH ARTICLE

PTR1-dependent synthesis of tetrahydrobiopterin contributes to oxidant susceptibility in the trypanosomatid protozoan parasite *Leishmania major*

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Abstract *Leishmania* must survive oxidative stress, but lack many classical antioxidant enzymes and rely heavily on trypanothione-dependent pathways. We used forward genetic screens to recover loci mediating oxidant resistance via overexpression in *Leishmania major*, which identified pteridine reductase 1 (PTR1). Comparisons of isogenic lines showed *ptr1*$^-$ null mutants were 18-fold more sensitive to H_2O_2 than PTR1-overproducing lines, and significant three- to fivefold differences were seen with a broad panel of oxidant-inducing agents. The toxicities of simple nitric oxide generators and other drug classes (except antifolates) were unaffected by PTR1 levels. H_2O_2 susceptibility could be modulated by exogenous biopterin but not folate, in a PTR1- but not dihydrofolate reductase-dependent manner, implicating H_4B metabolism specifically. Neither H_2O_2 consumption nor the level of intracellular oxidative stress was affected by PTR1 levels. Coupled with the fact that reduced pteridines are at least 100-fold less abundant than cellular thiols, these data argue strongly that reduced pteridines act through a mechanism other than scavenging. The ability of unconjugated pteridines to counter oxidative stress has implications to infectivity and response to chemotherapy. Since the intracellular pteridine levels of *Leishmania* can be readily manipulated, these organisms offer a powerful setting for the dissection of pteridine-dependent oxidant susceptibility in higher eukaryotes.

Keywords Folates · Hydrogen peroxide · Pteridines · Forward genetics · Trypanothione · Nitric oxide

Abbreviations

PTR1	Pteridine reductase 1
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
DCFH-DA	2′7′-Dichlorofluorescein diacetate
DCF	Dichlorofluorescin
GSH	Glutathione
$T[SH]_2$	Trypanothione
SIN-1	3-Morpholino-sydnonimine
H_2B	Dihydrobiopterin
H_4B	Tetrahydrobiopterin
WT	Wild-type

Introduction

Trypanosomatid protozoan parasites of the genus *Leishmania* include a number of species causing diseases affecting

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more than 12 million people worldwide (Desjeux 2004). These parasites multiply as two forms: flagellated extracellular promastigotes that reside within the digestive system of the sand fly vector, and as non-flagellated intracellular amastigotes living in the phagolysosome of the vertebrate macrophage. Phagocytosis of *Leishmania* by macrophages can stimulate a respiratory burst, generating reactive oxygen species (ROS) including O_2^- and H_2O_2, and parasites must suppress, survive, or recover from this oxidative attack (Denkers and Butcher 2005). The induction of oxidant stress is an important action of clinical pentavalent antimonials, and antioxidant systems contribute to antimonial resistance (Ashutosh et al. 2007; Wyllie et al. 2004).

In dealing with oxidative stresses organisms have two basic options: 'upstream' methods involving direct detoxification of the various ROS, or 'downstream' pathways repairing oxidatively damaged molecules (Davies 1995). The balance between and the consequences of disruptions in upstream versus downstream oxidant susceptibility pathways varies among organisms (Fang 2004). Notably, *Leishmania* lack many classical systems that detoxify H_2O_2 in other organisms, such as catalase and the selenoprotein glutathione peroxidase (Krauth-Siegel et al. 2007). Instead, their antioxidant metabolism relies on the thiol N_1,N_8-bis(glutathionyl)spermidine (trypanothione, $T[SH]_2$), and a host of antioxidant systems that depend on reducing equivalents passed to $T[SH]_2$ by trypanothione reductase (Fairlamb and Cerami 1992). These include redox intermediates, such as ascorbate, tryparedoxin and ovothiol (Ariyanayagam and Fairlamb 2001; Jaeger and Flohe 2006; Krauth-Siegel and Ludemann 1996) and antioxidant enzymes such as ascorbate peroxidase, trypanothione *S*-transferase, peroxiredoxins, and cysteine homologs of classical glutathione peroxidase (Krauth-Siegel et al. 2007). In contrast to these 'upstream' pathways, 'downstream' pathways have received less attention in parasites.

Another set of redox-active metabolites in *Leishmania* are the unconjugated pteridines, whose roles in oxidative stress are less well understood. For example, while many studies have shown or assumed that reduced pteridines such as tetrahydrobiopterin (H_4B; the most common eukaryotic pteridine) can scavenge oxidants, others show that H_4B may instead promote oxidative stress through the Fenton reaction (Kirsch et al. 2003). For pteridines as a group, the biological situation is complex and highly dependent on the specific circumstances, as non-reduced pteridines can act to promote oxidative damage, and pteridines can act as both substrates and inhibitors of enzymes that both generate and protect from oxidants, such as the nitric oxide synthases (reviewed in Oettl and Reibnegger 2002; Werner-Felmayer et al. 2002). This complexity has led some to conclude that 'it is impossible to unequivocally predict a physiological net effect of pterins of different oxidation states…in real biological systems" (Oettl and Reibnegger 2002). One factor that may have contributed to these paradoxical findings is the manner of testing, typically involving simultaneous external application of both pteridines and/or oxidative stresses. This leads to difficulty in distinguishing between the intracellular and extracellular effects of the pteridines. Here, we use the unparalleled ability to manipulate pteridine metabolism internally in *Leishmania* to further our understanding of how pteridines can modulate in vivo oxidant susceptibility.

Leishmania are pteridine auxotrophs, and use an array of transporters and reductases to acquire and generate reduced pteridines (reviewed in Nare et al. 1997b; Ouellette et al. 2002). While dihydrofolate reductases (DHFRs) often can reduce both folates and unconjugated pteridines, *Leishmania* DHFR can only reduce folates (Nare et al. 1997a). Thus *Leishmania* and other trypanosomatids express a novel pteridine reductase (pteridine reductase 1, PTR1), which is broadly active and can reduce a variety of unconjugated pteridines, as well as folates (Bello et al. 1994; Nare et al. 1997a; Wang et al. 1997). Being relatively insensitive to classic antifolates such as methotrexate, PTR1 acts as a metabolic 'bypass' of DHFR inhibition, accounting for the relative inactivity of antifolates against trypanosomatids (Bello et al. 1994). The known functions of pteridines in mammalian cells include hydroxylation of aromatic amino acids, lipid cleavages and nitric oxide biosynthesis (Kaufman 1963; Kosar-Hashemi and Armarego 1993; Tayeh and Marletta 1989). However, only an NADPH-dependent ether lipid cleavage activity has been described in *Leishmania* (Ma et al. 1996), the *L. major* genome lacks an identifiable nitric oxide synthase, despite reports of NO synthesis (Wanasen and Soong 2008), and while possessing a phenylalanine hydroxylase, null mutants lacking this enzyme have normal growth, differentiation and virulence (L.-F. Lye and SMB, in preparation). Thus the precise role(s) of H_4B in *Leishmania* metabolism remains uncertain.

We and others have previously used genetic selections for drug resistance to identify loci active when overexpressed, either naturally by gene amplification (Beverley 1991; Ouellette et al. 2004), or engineered after transfection with random gene segments borne on multicopy episomal cosmid vectors (reviewed in Beverley 2003; Clos and Choudhury 2006). Here, we applied this method to the discovery of novel oxidant resistance genes, using treatment with H_2O_2 as the selective pressure. This screen identified *PTR1* as a mediator of susceptibility to oxidative stress, a finding corroborated by studies of isogenic lines lacking or overexpressing PTR1. Further studies point to a novel mode of oxidant resistance dependent upon reduced unconjugated pteridines, distinct from the $T[SH]_2$-dependent antioxidants cited earlier. We advanced and tested several potential

mechanisms through which PTR1-derived pteridines could modulate oxidant susceptibility. Lastly, we discuss the implications of our findings on parasite survival, virulence and chemotherapy.

Materials and methods

Chemicals and reagents

Reagents were obtained as follows: biopterin and H_2B (Schircks Laboratories, Jona, Switzerland); H_4B (RBI); methotrexate, thymidine, sodium nitrite, allopurinol, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, glutathione, folate, dihydro- and tetrahydrofolate, hydrogen peroxide, bovine catalase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), pentamidine (Sigma); 2′7′-dichlorofluorescein diacetate (Molecular Probes); *S*-nitroso-acetyl-penicillamine; 3-morpholino-sydnonimine; *S*-nitrosoglutathione (Alexis Corporation); paraquat (Aldrich); horseradish peroxidase (Boehringer Mannheim); bovine serum albumin (US Biochemicals); terbinafine (Sandoz Research Institute).

Cell lines and culture conditions

The *L. major* strains LT252 (MHOM/IR/83/IR) clone CC-1, Friedlin (MHOM/IL/81/Friedlin) clones FA1 (avirulent) and FV1 (virulent), were routinely propagated at 26°C as promastigotes in M199 containing 10% (v/v) heat-inactivated fetal bovine serum, adenine (100 μM), hemin (5 μg/ml) and biopterin (2 μg/ml) (Kapler et al. 1990). Transfection of *Leishmania* by electroporation was performed as described (Kapler et al. 1990; Ryan et al. 1993). fdM199 medium is standard M199 lacking folate and thymidine. FDLS-M199 medium is standard M199 lacking folate and thymidine, supplemented with 0.66% (w/v) bovine serum albumin, 1% (v/v) fetal bovine serum and 10 ng/ml H_2B. *L. major* CC-1 null mutants lacking DHFR-TS *(dhfr-ts*$^-$) or PTR1 (*ptr1*$^-$) by targeted gene replacement of both alleles were described previously (Bello et al. 1994; Cruz et al. 1991), and maintained in media supplemented with 10 μg/ml thymidine or 2-4 μg/ml H_2B, respectively. The CC-1 lines *ptr1*$^-$*/+PTR1* and *dhfr-ts*$^-$*/+DHFR-TS* represent the respective null-mutants transfected with episomal expression plasmids pX63NEO-PTR1 (Bello et al. 1994) or pK300 (Kapler et al. 1990), and overexpress PTR1 and DHFR-TS, respectively. Similarly, the FV1 *ptr1*$^-$/SSU::*PTR1* overexpressor is a chromosomal null *ptr1*$^-$ mutant complemented with a copy of the *PTR1* gene integrated in the small subunit ribosomal locus, which also confers high level overexpression (D. Scott et. al., manuscript in preparation).

Multi-copy selection for hydrogen peroxide resistance

We previously described the construction of cosmid libraries in the vector cLHYG containing ~30 kb inserts of *L. major* strain FV1 DNA, generated by either partial digestion with *Sau*3A or random shear, and their mass transfection into the *L. major* strain FA1 (Cotrim et al. 1999; Ryan et al. 1993). Strain FA1 and the cosmid library transfected FA1 pool were inoculated into M199 media containing 600 μM H_2O_2 at a final concentration of 1×10^6 parasites/ml, and allowed to incubate at 26°C for several weeks.

Characterization of the gene responsible for H_2O_2 resistance

Insertional mutagenesis was performed using a modified Ty1-based in vitro transposition system (Devine and Boeke 1994; Garraway et al. 1997). Transposition reactions were carried out using 2 μg of cHP DNA as target, using conditions previously described (Garraway et al. 1997). A set of single insertions was obtained and the general location of the TyK artificial transposon was mapped by enzymatic digestion with *Hind*III. TyK insertions were further analyzed by polymerase chain reaction with the following primers: SMB-392 (5′-cgcggatcccatATGACTGCTCCGACC) and SMB-393 (5′-ggcggatccTCAGGCCCGGGTAAGGCTGTA).

Determination of *Leishmania* survival

For growth inhibition, log phase promastigotes (2×10^5 cells/ml) were incubated with H_2O_2 or other test drugs in 10 ml FDLS-M199. *Leishmania* were irradiated (0–250 mJ/cm^2) in the GS Gene Linker UV chamber (Biorad) and allowed to recover in the presence or absence of visible light. Cell numbers were estimated with a Coulter Counter (Model Zf). The EC_{50} is defined as the condition decreasing growth by 50%, measured at a time when controls had reached late log phase (<1×10^7/ml).

Measurement of H_2O_2 removal by *Leishmania*

Log phase *Leishmania* were harvested and resuspended in a buffer containing 5 mM KCl, 80 mM NaCl, 2 mM $MgCl_2$, 16.2 mM Na_2HPO_4, 3.8 mM NaH_2PO_4, 50 mM D-glucose, 100 μg/ml phenol red, pH 7.5 and 20 μM H_2O_2. 1 ml aliquots were removed periodically and H_2O_2 levels determined by measuring absorbance at 610 nm as described (Penketh 1986; Pick and Keisari 1980). The concentration of H_2O_2 stocks was determined spectrophotometrically at 230 nm assuming an extinction coefficient of 81 M^{-1} cm^{-1} at 230 nm (Homan-Muller et al. 1975).

Measurement of intracellular oxidative stress

Logarithmic phase *Leishmania* were maintained in fdM199 media without biopterin for 24 h to deplete the internal pteridine pools, harvested, washed and resuspended (1×10^7 cells/ml) in Hanks balanced salt solution without phenol red, and loaded with 10 μM DCFH-DA for 15 min. Aliquots (1 ml) were removed at various time points, centrifuged and DCF fluorescence was measured spectrofluorimetrically (excitation 485 nm; emission 530 nm) (Keston and Brandt 1965). To test the involvement of peroxides on DCF-DA oxidation, catalase was added (5,000 U/ml) for 5 min and washed off the cells prior to loading with DCF-DA.

Determination of parasite viability by MTT assay

Parasites were harvested from mid-log cultures and resuspended at 8×10^6/ml in M199 media containing 2 μg/ml biopterin. H_2O_2 was then added and the cells incubated at 26°C for 2 h. Triplicate 1 ml samples were removed, the cells harvested by centrifugation and then resuspended in 1 ml fresh M199 containing 400 μg/ml MTT. These samples were incubated for a further 2 h at 26°C and the cells pelleted again. The cell pellets were lysed and the formazan product dissolved by addition of 2% (w/v) sodium dodecyl sulphate in DMSO. The reduced product was quantified by measuring its absorbance at 570 nm versus a media blank. Standard curves showed that this assay gave a linear response from samples containing 1×10^6 to 8×10^6 viable parasites.

Results

Genetic screening for novel H_2O_2 resistance genes

Previously we generated a library of 18,000 independent *L. major* transfectants, each bearing a ~30 kb segment of WT genomic DNA inserted in the multicopy episomal shuttle vector cLHYG (Cotrim et al. 1999). Cultures of the WT parent and the pooled transfectant library were propagated in the presence of 600 μM H_2O_2 for several weeks. While selections of untransfected WT parasites did not yield survivors, parasites grew out from the H_2O_2 treated transfectant library. This population showed a tenfold increase in its EC_{50} for H_2O_2, compared to the parent line (not shown). DNA was recovered from the H_2O_2-resistant population and transformed into *E. coli*, and analysis of 24 colonies showed that all contained independently the same cosmid, which was termed cHP. Re-transfection of the WT line with cHP conferred up to sevenfold resistance to H_2O_2 (Fig. 1b), confirming its activity.

Molecular analysis of cHP showed it contained a segment of the *L. major* genome termed the H-region (Beverley et al. 1984; Fig. 1b), a locus frequently amplified in lines selected for resistance to a variety of drugs (Beverley 1991; Ouellette et al. 2004). The H region contains at least 14 genes, most of whose functions are unknown, and includes *PTR1, PGPA* and *HTFB,* which mediate resistance to antifolates, arsenicals/antimonials, and the sterol synthetic inhibitor terbinafine, respectively. To identify the H_2O_2 resistance gene(s), we used insertional mutagenesis with a TyK artificial transposon to generate a library of

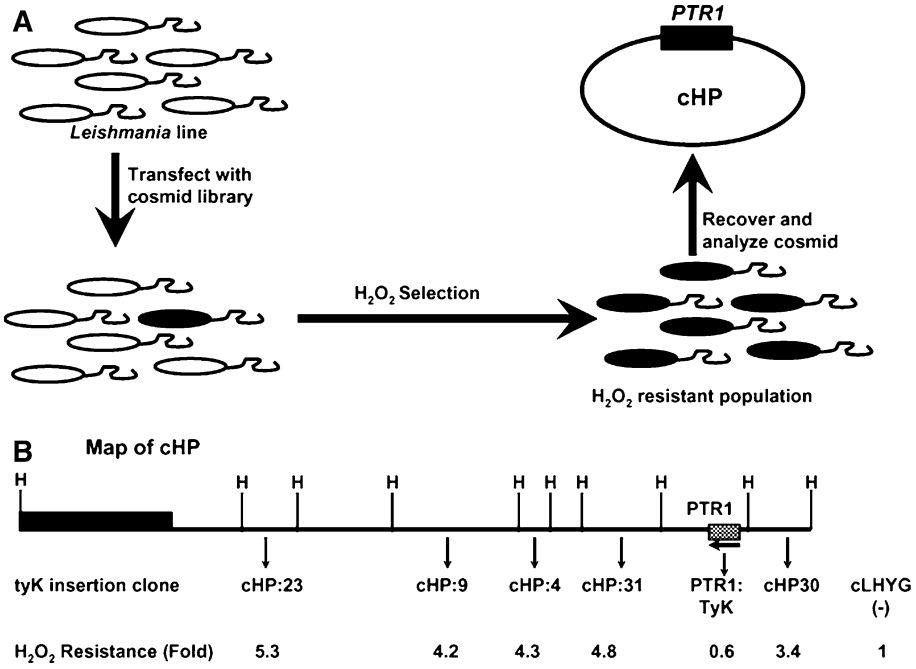

Fig. 1 Multi-copy selection scheme and TyK transposition into cHP. **a** A cosmid library-transfected parasite population was incubated in the presence of H_2O_2 to amplify a population containing cosmid-inserts that confer H_2O_2 resistance. Cosmid DNA was recovered from this population and, and genetic analysis identified the resistance gene. **b** Insertional mutagenesis was performed using the TyK artificial transposon and cHP insertion mutants were characterized by *Hin*dIII digestion or amplification of the *PTR1* coding region by polymerase chain reaction. Selected single insertions were transfected into *Leishmania* and H_2O_2 resistance was determined as described in "Methods"

independent insertions, which were mapped and tested following transfection into WT *L. major* (Fig. 1b). While most insertions had no effect, insertions disrupting *PTR1* (cHP-PTR1::TyK) abrogated H_2O_2 resistance (Fig. 1b). Consistent with the established role of *PTR1* in antifolate resistance, cHP transfectants were methotrexate resistant, while cHP-PTR1::TyK transfectants had sensitivities similar to controls (data not shown). These data identified *PTR1* specifically as the sole cHP oxidant resistance gene.

PTR1 protects against oxidative stress

To probe the role of PTR1 in oxidant susceptibility, we employed isogenic lines in the *L. major* CC-1 strain, where *PTR1* had been specifically deleted by homologous gene replacement (*ptr1*$^-$), or overproduced using an episomal expression vector (*ptr1*$^-$*/+PTR1*; (Bello et al. 1994; Nare et al. 1997a). Focusing first on agents known to generate oxidative stress, the *ptr1*$^-$ line was nearly fourfold more sensitive to H_2O_2, glucose oxidase (which generates H_2O_2 in the presence of glucose), and primaquine, a redox-cycling drug (which produces O_2^- and H_2O_2) (Table 1; $P < 0.01$). Modest sensitivity was seen to a second redox-cycling agent, paraquat (1.3-fold), although this was not statistically significant, and no change was seen in susceptibility to *tert*-butylhydroperoxide, which forms alkoxyl, peroxyl and hydroxyl radicals (·OH) (Table 1). Conversely, overexpression of PTR1 led to significant increases in oxidant resistance with all agents tested, ranging from 1.5-fold for primaquine to 4.8-fold for H_2O_2 (Table 1; Fig. 2). A small effect was seen for glucose oxidase/glucose stress, although this was not statistically significant (Table 1).

We found in these studies that the ratio of the EC_{50} of the PTR1 overexpressor to that of the *ptr1*$^-$ mutant provided a convenient parameter to assess the impact of PTR1 on oxidant susceptibility. Thus the differential sensitivity between the PTR1-overproducer and the *ptr1*$^-$ null mutant was 18-fold with H_2O_2, and ranged from three to fivefold with all other oxidant generating systems, with strong statistical significance for all (Table 1). These data revealed a general role for PTR1 in determining the susceptibility of *Leishmania* to oxidative stress.

PTR1 does not affect resistance to nitrosative stress or other agents

Nitric oxide (NO) is an important microbicidal product of macrophages and is cytotoxic to *Leishmania* in vitro (Liew et al. 1990), so we tested whether *PTR1* also mediated resistance to reactive nitrogen species (RNS). All three lines displayed equivalent sensitivity to the NO-generating compounds sodium nitrite and *S*-nitrosoglutathione (Table 1), suggesting that *PTR1* was not involved in protection against nitrosative stress. *S*-Nitrosopenicillamine

Table 1 Relationship between the PTR1 status of *Leishmania* and their resistance to stress conditions

Stress treatment	Mode	WT EC_{50}	*ptr1*$^-$ (KO) EC_{50}	*ptr1*$^-$/WT	*ptr1*$^-$*/+PTR1* (OE) EC_{50}	OE/WT	OE/KO
H_2O_2 (μM)	ROS	32.8 ± 5.2	8.7 ± 1.9	0.27**	157 ± 2	4.8**	18**
Glucose oxidase (mU)	ROS	0.4 ± 0.1	0.11 ± 0.02	0.28**	0.5 ± 0.1	1.3 *ns*	5.0**
t-Butyl hydroperoxide (μM)	ROS	0.13 ± 0.02	0.13 ± 0.02	1.0 *ns*	0.4 ± 0.04	3.1**	3.1**
Primaquine (μM)	ROS	22 ± 3	5.9 ± 0.5	0.27**	32 ± 2	1.5**	5.4**
Paraquat (mM)	ROS	1.6 ± 0.1	1.3 ± 0.2	0.81 *ns*	4.1 ± 0.9	2.6**	3.2**
3-Morpholinosydninimine (μM)	RNS + ROS	110 ± 20	32 ± 5	0.29**	170 ± 70	1.5 *ns*	5.3*
Na-nitrite (pH 7.2, mM)	RNS	2.4 ± 0.2	2.1 ± 0.5	0.88 *ns*	3.1 ± 0.4	1.3 *ns*	1.5 *ns*
Na-nitrite (pH 6.8, mM)	RNS	1.5 ± 0.4	1.2 ± 0.4	0.80 *ns*	1.5 ± 0.2	1.0 *ns*	1.3 *ns*
S-Nitrosoacetylpenicillamine (μM)	RNS	49 ± 2	58 ± 3	1.18*	37 ± 2	0.8**	0.6**
S-Nitrosoglutathione (μM)	RNS	1.3 ± 0.2	1.2 ± 0.1	0.92 *ns*	1.4 ± 0.1	1.1 *ns*	1.2 *ns*
Allopurinol (μM)	Other	1.1 ± 0.5	1.9 ± 1.0	1.73 *ns*	1.4 ± 0.1	1.3 *ns*	0.7 *ns*
Terbinafine (μM)	Other	2.7 ± 0.3	3 ± 1	1.11 *ns*	3 ± 1	1.1 *ns*	1 *ns*
Pentamidine (μM)	Other	2.3 ± 0.7	2.6 ± 0.5	1.13 *ns*	2.6 ± 0.5	1.1 *ns*	1 *ns*
Nitrosoguanidine (μM)	Other	10 ± 3	11 ± 4	1.10 *ns*	11 ± 4	1.1 *ns*	1 *ns*
UV light (mJ cm^{-1})	Other	7 ± 2	6 ± 3	0.86 *ns*	6 ± 3	0.9 *ns*	1 *ns*

Results are EC_{50}s of 3–5 determinations in independent experiments, shown ± standard deviations. EC_{50}s are defined as the condition that decreases *Leishmania major* growth by 50%. *KO* null mutant (*ptr1*$^-$); *OE* overexpressor (*ptr1*$^-$*/+PTR1*). Statistical tests were calculated first as between *ptr1*$^-$ versus WT, then the overexpressor (OE; *ptr1*$^-$*/+PTR1* vs. WT), and then of the ratio of the EC_{50}s of the OE/KO

** $P < 0.01$; * $P < 0.05$

ns not significant

Student's unpaired two tailed *t* test; calculated by algorithm implemented in GraphPad Software

showed opposite effects to that seen with oxidants, with *ptr1*$^-$ showing slight resistance and the *PTR1* overexpressor showing sensitivity; however, the magnitude of these effects were small and not significant (~1.2-fold; Table 1). An apparent exception was the finding that the *ptr1*$^-$ line was 5.3-fold more sensitive than the *PTR1* overproducer to morpholinosydnonimine (SIN-1), a generator of both NO and O_2^-. This could suggest a role of PTR1 in protection against peroxynitrite, a product of the reaction between NO and O_2^-; however, an alternative explanation is that resistance arose solely to the O_2^- formed by SIN-1.

Tests of drugs not thought to act through oxidative stress such as allopurinol, terbinafine or pentamidine showed no significant differences between *ptr1*$^-$, WT and *PTR1* overexpressing lines (Table 1). The genomes of *L. major* and other trypanosomatids encode numerous DNA repair pathway activities, including DNA photolyase (LmjF33.0470) and cryptochrome DASH (LmjF09.0360), which in some species bear 5,10-methenyltetrahydrofolate chromophores (Brudler et al. 2003; Sancar 2004). Previously we obtained preliminary data suggesting the presence of a DNA photolyase repair activity in *L. major*, with UV-treated parasites showing a twofold enhancement in survival when allowed to recover while illuminated by visible light, compared to in darkness (data not shown). Thus we tested sensitivity to mutagens such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanadine (MNNG) or UV light toward *L. major*; however, these were unaffected by PTR1 levels (Table 1). Consistent with this, studies described in following section did not implicate folates in oxidant susceptibility in *Leishmania*.

Taken together, the results in Table 1 strongly suggest that the role of PTR1 is limited to protection against ROS or the repair of oxidative damage.

PTR1-dependent modulation of H_2O_2 toxicity with exogenous biopterin

Several studies suggest exogenous pteridines can act oppositely in either mitigating or elevating oxidative stress depending upon circumstances (Oettl and Reibnegger 2002). The availability of the *ptr1*$^-$ null mutant and *PTR1* overexpressor, coupled with the ability to control intracellular pteridine levels through supplementation and uptake via the *BT1* biopterin transporter (Cunningham and Beverley 2001; Ouellette et al. 2002), allowed us to stringently assess the intracellular role of pteridines in *Leishmania*. In these experiments, parasites were tested in folate-deficient media (fdM199) containing various amounts of biopterin or H_2B; under all conditions parasites grew normally.

Since *ptr1*$^-$ *L. major* requires H_2B for growth, biopterin-dependency was tested only with WT and PTR1 overexpressors. WT parasites cultured without biopterin were highly susceptible to H_2O_2, with an EC_{50} of ~2 μM

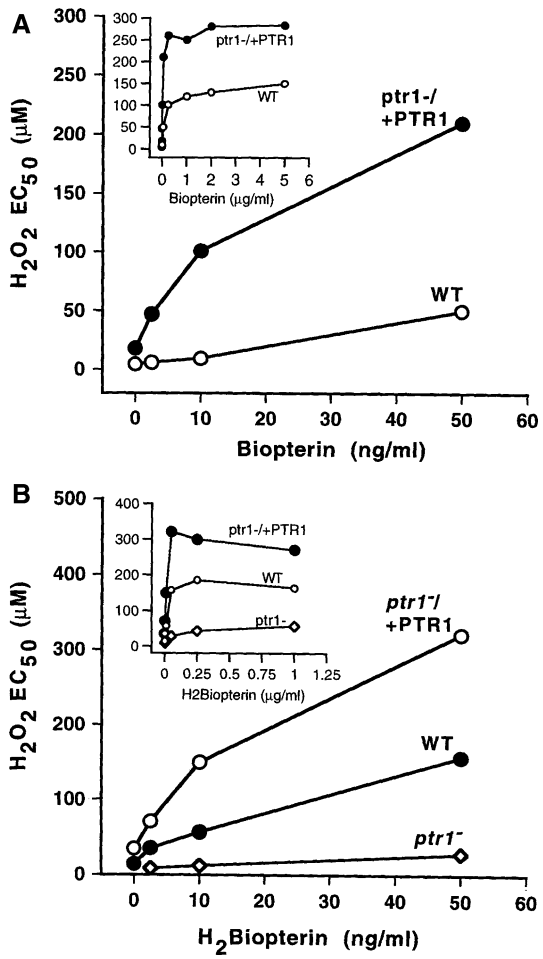

Fig. 2 Growth inhibition by H_2O_2 and modulation with biopterin. *Leishmania* promastigotes (2×10^5 ml^{-1}) were incubated in fdM199 containing H_2O_2 (0–450 μM) in the presence of increasing concentrations of biopterin (**a**) or H_2B (**b**). The *inset* in each *panel* shows the effects at higher concentrations. Growth inhibition was determined as described in "Methods". WT (*open circle*); *ptr1*$^-$/+PTR1 (*filled circle*); and *ptr1*$^-$ (*open triangle*). This experiment was repeated three times, with similar results to the one shown

(Fig. 2a). With increasing biopterin, the H_2O_2 EC_{50} rose nearly 100-fold, plateauing at 150 μM H_2O_2 at concentrations above 840 nM biopterin (200 ng/ml; Fig. 2a, inset). In contrast, the *PTR1* overexpressor was much less sensitive to H_2O_2 at all biopterin concentrations tested, with an EC_{50} of 15 μM H_2O_2 in the absence of biopterin supplementation, increasing to 250 μM above 211 nM biopterin (50 ng/ml; Fig. 2a). At 42 nM biopterin (10 ng/ml), a concentration which can support growth indefinitely, the difference in H_2O_2 EC_{50}S between WT and the PTR1 overexpressor was tenfold (Fig. 2a). Conversely, an EC_{50} of 50 μM H_2O_2 was obtained with only 10 nM biopterin with the PTR1 overexpressor versus 211 nM for the WT.

Similar results were obtained in tests with H_2B supplementation (Fig. 2b). WT parasites showed a dose-dependent increase in H_2O_2 resistance, plateauing above 210 nM

H_2B at an EC_{50} of 150 μM H_2O_2. In contrast, *ptr1*$^-$ parasites were much more sensitive to H_2O_2 at all concentrations tested, plateauing at 1.05 μM H_2B with an EC_{50} of only 40 μM H_2O_2. Correspondingly, the PTR1 overexpressor was more resistant at all concentrations, plateauing at 210 nM H_2B with an EC_{50} of 300 μM H_2O_2 (Fig. 2b). At 42 nM H_2B (10 ng/ml), WT showed an EC_{50} of 40 μM H_2O_2, *ptr1*$^-$ an EC_{50} of 5 μM, and the *PTR1* overexpressor an EC_{50} of 150 μM. Conversely, an EC_{50} of 50 μM was obtained at a concentration of 1 μM H_2B for the *ptr1*$^-$ line, 42 nM for WT, and less than 4 nM for the PTR1 overexpressor (Fig. 2b).

Thus, biopterin supplementation invariably increased H_2O_2 resistance—an effect that was dramatically enhanced by *PTR1* overexpression and abolished in its absence. These data suggest that PTR1 protects against oxidative stress by providing *Leishmania* with reduced biopterin and specifically H_4B intracellularly.

Alterations in DHFR-TS or folate levels do not affect H_2O_2 susceptibility

While PTR1 can reduce both biopterin and folate, DHFR is only able to reduce folates (Bello et al. 1994; Nare et al. 1997a). Unlike the PTR1 null or overexpressors, the H_2O_2 sensitivities a *dhfr-ts*$^-$ null mutant or a *DHFR-TS* overexpressor were similar to that of WT (Fig. 3). Furthermore, *Leishmania* killing by H_2O_2 was unaffected by varying external folate levels (not shown). Thus, neither reduced folates nor DHFR-TS activity could be implicated in H_2O_2 resistance.

PTR1-dependent oxidant susceptibility does not arise through increased detoxification of H_2O_2

Pteridines can react directly with ROS, suggesting the possibility that reduced pteridines formed by PTR1 might mediate susceptibility directly by detoxification, or 'interception' (Oettl and Reibnegger 2002). Thus we asked whether oxidant consumption was altered in a *PTR1*-dependent manner. The rate of extracellular H_2O_2 consumption by WT *Leishmania* was rapid (initial rates of 4–5 nmol/min/10^8 cells), but no differences between WT, *ptr1*$^-$ and the PTR1 overproducer were observed (Fig. 4a). Controls showed that H_2O_2 consumption was eliminated by boiling or sonicating the parasites prior to assay (data not shown), ruling out media effects and showing that H_2O_2 was only consumed by viable organisms. H_2O_2 was also stable in the absence of cells, and pre-incubation of *Leishmania* with high concentrations of biopterin (5–10 μg/ml) had no effect on the rate of H_2O_2 consumption (not shown). As a positive control, WT *L. major* pre-loaded with bovine catalase showed elevated rates of H_2O_2 consumption (Fig. 4a).

These results on the rate of H_2O_2 consumption were not unexpected, since intracellular H_4B levels are far below

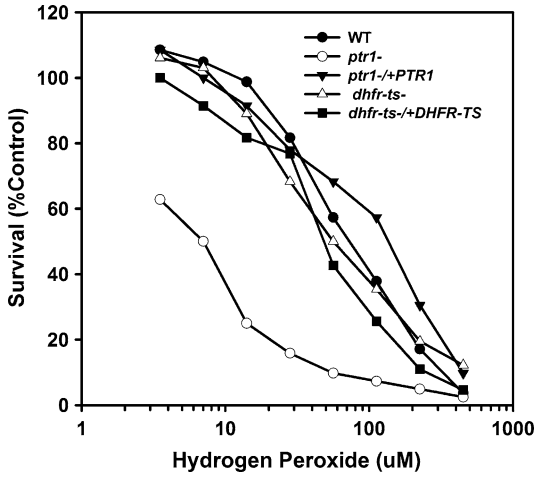

Fig. 3 Comparison of PTR1 and DHFR-TS in protection against oxidative damage *Leishmania* (2×10^5 ml^{-1}) were incubated in fdM199 containing H_2O_2 (0–450 μM) in the presence of 10 ng/ml H_2B and 10 μg/ml thymidine. Growth inhibition was determined as described in "Methods". WT (*filled circle*); *ptr1*$^-$ (*open circle*); *ptr1*$^-$*/+PTR1* (*filled inverted triangle*); *dhfr-ts*$^-$ (*open triangle*); *dhfr-ts*$^-$*/+DHFR-TS* (*filled square*). For comparison with data included in Table 1, the EC_{50}s calculated for WT, *ptr1*$^-$ and *ptr1*$^-$*/+PTR1* in this experiment were 62, 7 and 140 μM, respectively. This experiment was repeated three times, with similar results to the one shown

that of cellular thiols such as trypanothione (<10 μM vs. 0.4–2 mM; Cunningham and Beverley 2001; Fairlamb and Cerami 1992; Moutiez et al. 1994). Potentially, reduced pteridines could play a role in scavenging H_2O_2 if they were significantly more reactive than thiols. However, no data support this idea (Oettl and Reibnegger 2002; Werner-Felmayer et al. 2002) and we did not find significant differences in the reactivity of H_2O_2 with various pteridines and thiols (Fig. 5).

PTR1-dependent oxidant susceptibility is not associated with decreased levels of intracellular oxidative stress

The H_2O_2 consumption assay does not directly measure the level of intracellular oxidative stress. Thus we employed the fluorescent dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to measure oxidants within the cell; following uptake this dye is metabolized and develops elevated fluorescence when exposed to ROS (Robinson et al. 1988). *L. major* were loaded with 5–10 μM DCFH-DA, exposed to H_2O_2 and then oxidative stress assessed by this method. Under these conditions, H_2O_2 exposure increased DCF fluorescence in a dose-dependent manner (Fig. 4b). Remarkably, the *ptr1*$^-$ mutant and *PTR1*-overexpressor behaved identically to WT (Fig. 4b). This was not changed in the presence of high levels of biopterin (5 μg/ml) that enhanced PTR1-dependent survival after H_2O_2 exposure (Fig. 2; data not shown). Flow

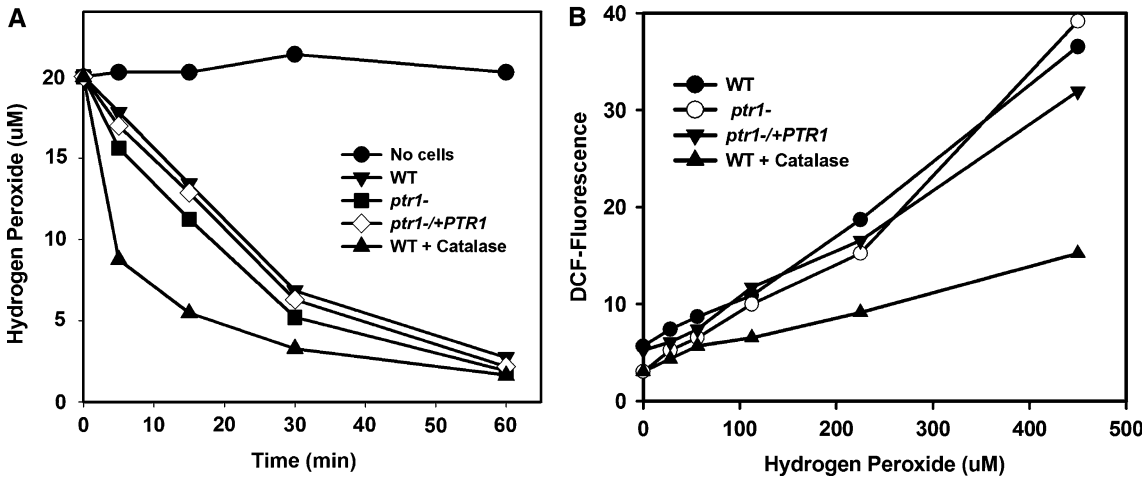

Fig. 4 Metabolism of H_2O_2 and measurement of intracellular oxidative stress. **a** Cells (1×10^7/ml) were incubated in buffer containing 20 μM H_2O_2 at zero time. Aliquots (1 ml) were withdrawn at indicated time intervals and extracellular H_2O_2 assayed as described in "Methods". No cells (*filled circle*), WT (*filled inverted triangle*); *ptr1*$^-$ (*filled square*); *ptr1*$^-$*/+PTR1* (*open triangle*); WT (*filled triangle*) pre-loaded with catalase. **b** Cells were rinsed, resuspended in Hanks balanced salt solution (1×10^7 ml^{-1}) and loaded with 10 μM DCF-DA for 15 min. Intracellular oxidant stress was determined by monitoring fluorescence due to oxidation of DCF-DA to DCF, in the presence of indicated amounts of H_2O_2 as described in "Methods". WT (*filled circle*); *ptr1*$^-$ (*open circle*); *ptr1*$^-$*/+PTR1* (*filled inverted triangle*); WT (*filled triangle*) pre-loaded with catalase. This experiment was repeated three times, with similar results to the one shown

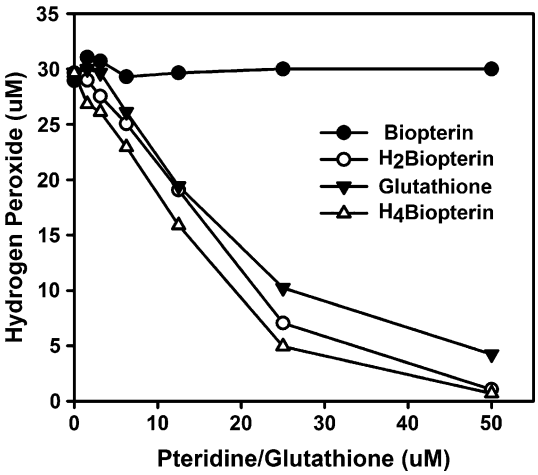

Fig. 5 Comparison of the reactivity of H_2O_2 with pteridines and thiols. H_2O_2 (30 μM) was added to 1 ml buffer containing increasing concentration of freshly prepared pteridine or glutathione and allowed to react for 5 min at 30°C. Remaining H_2O_2 was quantified as described in "Methods". Biopterin (*filled circle*); H_2B (*open circle*); H_4B (*open triangle*); GSH (*filled inverted triangle*). This experiment was repeated twice, with similar results to the one shown

cytometry of DCFH-DA loaded and H_2O_2 treated *Leishmania* revealed a homogeneous fluorescence distribution for each cell population, with no difference between *ptr1*$^-$ and *PTR1*-overexpressing lines (not shown). As a negative control, WT parasites were loaded with catalase and DCFH-DA; as expected these parasites showed greatly reduced fluorescence in response to H_2O_2 treatment (Fig. 4b).

These data showed that despite having a 20-fold difference in H_2O_2 susceptibility, *ptr1*$^-$ and *PTR1*-overexpressing lines show similar levels of intracellular oxidative stress across a wide range of external H_2O_2 concentrations.

PTR1-mediated resistance confers increased H_2O_2 survival

Since H_4B depletion can slow parasite growth under some circumstances (Bello et al. 1994), oxidation of this pteridine might suppress parasite growth without impairing parasite survival. Short-term cellular viability was therefore monitored using an MTT reduction assay. Exposure of WT *L. major* to 250 or 500 μM H_2O_2 reduced the viability of cells in a dose-dependent manner, with 500 μM peroxide killing 70 ± 3% of the WT parasites (Fig. 6). In contrast, PTR1 overexpressors showed increased survival, with 500 μM H_2O_2 killing only 41 ± 4% of the parasites. This indicates that although PTR1 overexpression does not reduce the intracellular level of ROS, it nonetheless enhances short-term survival of oxidative stress.

Discussion

PTR1 is an oxidant resistance gene in vitro

Here, we showed by several independent approaches that *Leishmania* pteridine reductase PTR1 plays an important role in mediating resistance to oxidative stress. First, genetic selections for loci mediating resistance when

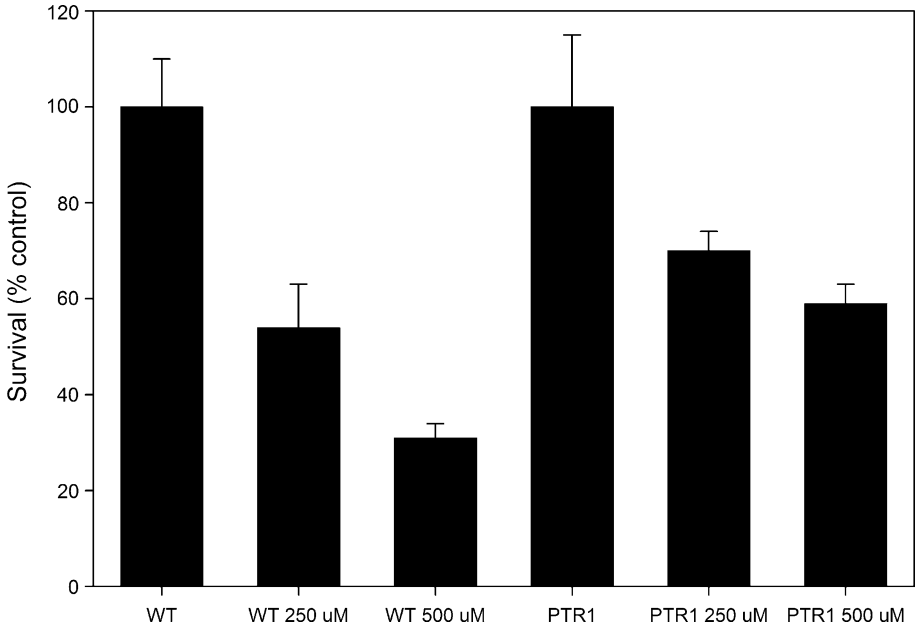

Fig. 6 Effects of H_2O_2 on short-term cell viability in WT versus PTR1 overexpressors. Triplicate samples of *L. major* strain FV1 WT or *ptr1*$^-$/SSU::*PTR1* cells were incubated at 8×10^6 cells ml^{-1} in FDSL-M199 medium alone, or containing 250 or 500 μM H_2O_2, for 2 h. Samples were then removed and the number of viable cells quantified using the MTT assay, as described in "Methods". Data are expressed as a percentage of the amount of MTT reduced by the respective negative control for each cell line

over-expressed led to the recovery of episomal cosmids bearing PTR1, embedded within a region of the *Leishmania* chromosome designated the H region (Beverley et al. 1984). The H region is notable because it has been found to be amplified independently in a number of *Leishmania* strains and species, in response to selection for resistance to an array of diverse agents including antifolates, sterol synthesis inhibitors, primaquine, and arsenicals and antimonial derivatives (Beverley 1991; Ouellette et al. 2004). This initially suggested to some workers that the H region amplification represents one kind of non-specific 'stress' response. However, subsequent studies have shown this model to be incorrect, as resistance to each agent arises from specific elements encoded within the H region, e.g., PTR1 and antifolates, metals and the P-glycoprotein *PGPA*/*MRP* (Callahan and Beverley 1991; Ouellette et al. 1994), and terbinafine with *HTBF* (Marchini et al. 2003). We extended this model here to show that resistance to H_2O_2 and a wide array of compounds that produce oxidative stress (including primaquine) is attributable specifically to PTR1. This was shown by in vitro generated transposon inactivation of PTR1 within the cHP/H region cosmid (Fig. 1), as well as studies of isogenic *L. major* specifically lacking or over-expressing PTR1 (Table 1).

One advantage of this selection strategy is that it allows the parasite to select the critical gene(s) without assumptions about the identity of these genes. Potentially a number of loci could mediate H_2O_2 resistance via overexpression, although here only the *PTR1* locus was recovered. Interestingly, overproduction of trypanothione reductase, the enzyme that maintains the $T[SH]_2$ pool, failed to protect *Leishmania* against H_2O_2-mediated oxidative stress (Kelly et al. 1993). This suggests that unlike the generation of H_4B, the rate of trypanothione reduction is not generally limiting in these parasites, even under oxidative stress, perhaps explaining why this key antioxidant enzyme was not recovered in our over-expression based screen. It is possible that other methods for placing and/or selecting *Leishmania* under oxidative stress would yield other loci, particularly since our screen was not exhaustive and did not yield evidence of genetic saturation.

PTR1 mediates oxidant resistance through provision of H_4B intracellularly

Our studies on parasite lines lacking or overexpressing *PTR1* showed a strong correlation between the level of this enzyme and resistance to a range of oxidative stresses. The diversity of the agents used renders unlikely the possibility that the resistance arises through 'off-target' effects unrelated to oxidative stress. Our data show that PTR1 mediates oxidant resistance through provision of H_4B, as the H_2O_2 susceptibility of *Leishmania* could be strongly modulated by varying external biopterin or H_2B. Importantly, this effect was strongly *PTR1*-dependent, establishing that it occurs intracellularly, unlike many studies of oxidative stress where pteridines were provided externally. In contrast, folates, although good PTR1 substrates, failed to modulate the susceptibility of *Leishmania* to H_2O_2, nor did the loss or overexpression of *DHFR-TS* (Nare et al. 1997a). Previous studies of the phenotypes of *PTR1* and *DHFR-TS* knockouts and overexpressors (Bello et al. 1994; Cruz et al. 1991; Nare et al. 1997a) indicated that unconjugated pteridines have uncharacterized functions in *Leishmania*, one of which we now assign as oxidant resistance. Interestingly, amplification of the H region in response to primaquine, a

compound thought to act via the production of ROS (Augusto et al. 1986), led to the suggestion that one of the H region proteins and possibly *PTR1* were implicated in oxidant resistance (Bello et al. 1994; Ellenberger and Beverley 1989).

In contrast, damage mediated by agents solely generating RNS (acidified nitrite, nitrosoglutathione) was not modulated by *PTR1*, suggesting that reduced unconjugated pteridines are not involved in defense against nitric oxide and its metabolites in *Leishmania*. This contrasts to the action of the thiol-based antioxidants in *Leishmania*, such as ovothiol and the peroxiredoxins, which are active against both ROS and RNS (Iyer et al. 2008; Vogt and Steenkamp 2003). This is another argument against a detoxification model for PTR1-mediated ROS resistance, as discussed below. Interestingly, PTR1 did provide some protection against killing by SIN-1, a generator of both of NO and O_2^- (Feelisch et al. 1989). The differential toxicity of SIN-1 between *ptr1*$^-$ and the PTR1-overproducer most parsimoniously reflects the effects of O_2^-, which will be converted to H_2O_2 by the *Leishmania* superoxide dismutase (Plewes et al. 2003). Alternatively, PTR1 could mediate resistance to peroxynitrite formed from the reaction of SIN-1 generated NO and O_2^- (Stamler 1994), as peroxynitrite is toxic to *Leishmania* in vitro (Denicola et al. 1993; Gatti et al. 1995), and H_4B has been shown to react with peroxynitrite (Milstien and Katusic 1999). Future studies will be required to establish whether PTR1 and H_4B affect the peroxynitrite sensitivity of *Leishmania*.

PTR1-dependent susceptibility to oxidants does not correlate with increased oxidant detoxification

The data presented establish that PTR1 mediates resistance to ROS through H_4B. There are several ways that H_4B might protect against oxidative stress. First, while H_4B is known to induce oxidative stress through Fenton chemistry (Kirsch et al. 2003), the complexities of pteridine/oxidant interactions (Oettl and Reibnegger 2002) conspire to make it difficult to rule out the possibility that under some circumstances scavenging might occur. However, in tests of lines lacking or overexpressing PTR1, and showing nearly 20-fold differential H_2O_2 sensitivity, H_2O_2 metabolism was similar, as was the degree of intracellular oxidative stress elicited by H_2O_2 treatment (Fig. 4); neither result would be expected if H_4B did exert direct protective or oxidative effects. A lack of cross-resistance to pure RNS stress is a second argument against a direct detoxification model, as noted above (Table 1). Although direct oxidant-pteridine reactions undoubtedly occur within the cell, they apparently contribute little to the mechanism of oxidative resistance described here.

Thus, we conclude that PTR1/biopterin dependent oxidative resistance does not arise through a detoxification mechanism in *Leishmania*, which instead is the responsibility of the well-known trypanothione-dependent antioxidant systems of *Leishmania* and other trypanosomatids (Krauth-Siegel et al. 2007; Krauth-Siegel et al. 2003). This conclusion is also consistent with the >100-fold higher concentrations of cellular thiols relative to unconjugated pteridines, with $T[SH]_2$ ranging from at 0.4–2 mM (Fairlamb and Cerami 1992; Moutiez et al. 1994), while H_4B levels are typically less than 10 μM (Cunningham and Beverley 2001). These calculations suggest that at most the presence or absence of H_4B would alter the cellular concentration of potential oxidant scavenging species by <1%. While it is theoretically possible that the concentration differential could be balanced by hyper-reactivity of pteridines with oxidants, current data suggest this is unlikely (Fig. 5; Oettl and Reibnegger 2002; Werner-Felmayer et al. 2002).

While our manuscript was under review, Moreira et al. (2009) presented data similarly implicating *PTR1* in H_2O_2 susceptibility, albeit with some important differences. In contrast to our findings (Table 1), their *ptr1*$^-$ mutants showed about twofold increased susceptibility to SNAP, although the *PTR1*-overexpressors resembled WT. SNAP has the potential to generate peroxynitrite (Huang et al. 2005), which as noted in our studies of SIN-1 might be targeted by PTR1-dependent activities (Table 1). Notably, 'pure' RNS generators such as acidified nitrite or *S*-nitrosoglutathione revealed no resistance (Table 1). Secondly, Moreira et al. inferred a role for increased scavenging in comparisons of WT versus *ptr1*$^-$ mutants. The effect was modest (<twofold), and little effect was seen in the *PTR1* overexpressors despite their increased H_2O_2 resistance. The difference in these studies may be attributable to the experimental methods used; in our study DCFH-DA loaded parasites were challenged after 15 min, with a range of H_2O_2 concentrations and followed over time. In contrast, Moeira et al. maintained parasites 45 min in buffered saline, which were then exposed to oxidants for a further 45 min prior to addition of DHCH-DA, and a single measurement taken. This prolonged incubation, under stressful conditions of nutrient deprivation, may have perturbed cellular metabolism. We believe our data, in combination with studies showing no increase in H_2O_2 consumption and the overwhelming 100-fold excess of thiols such a trypanothione, argue strongly against a significant contribution by oxidant scavenging to PTR1-dependent oxidant resistance.

What is the mechanism of PTR1-mediated oxidant resistance?

The known role of *PTR1* is to maintain H_4B pools, as this metabolite is required for growth (Bello et al. 1994; Nare

et al. 1997a). Thus PTR1 may act to confer oxidant resistance simply by restoring essential H_4B pools. Since the reason why H_4B is required in *Leishmania* metabolism is not yet known, specific tests for the role of H_4B-dependent enzymes in oxidant resistance are not presently feasible. A major challenge in such attempts to define the functions of reduced pteridines is the relative scarcity and instability of these compounds. One model consistent with the data presented here implicates reduced unconjugated pteridines in the recovery of *Leishmania* from damage by ROS, either by directly repairing oxidative damage to cellular components, or indirectly by maintaining cellular pathways affecting oxidant susceptibility. Organisms possess many pathways for the repair of oxidative damage to proteins, lipids and nucleic acids (Davies 1995), and perhaps H_4B participates in the reversal of oxidant damage to any of these macromolecules (Oettl and Reibnegger 2002; Werner-Felmayer et al. 2002), or in other cellular protective responses.

H_4B-mediated oxidant resistance, gene amplification and *Leishmania* virulence

Since the ability of pathogens to withstand oxidative stress is often important to infectivity (Fang 2004), it seems likely that PTR1-dependent oxidant susceptibility could be important to *Leishmania* survival and virulence, especially within mammalian macrophages. For example, decreased expression of trypanothione reductase reduces survival within macrophages (Dumas et al. 1997), and studies of the abundant *Leishmania* surface glycoconjugate lipophosphoglycan have shown that even twofold alterations in oxidant sensitivity have strong effects on parasites' ability to survive within macrophages (Spath et al. 2003).

One argument against a significant role in virulence is the fact that the H_2O_2 sensitive *ptr1*$^-$ *L. major* CC-1 line studied here is able to infect susceptible mice (Cunningham et al. 2001). However, the relationship between oxidative susceptibility measured in vitro, and its relevancy to virulence of pathogens in vivo, is often complex, depending on the pathogen and specific gene tested. For example, catalase-deficient mutants of *Haemophilus influenzae* show little effect on virulence in animal models, despite increased oxidant susceptibility in vitro (Bishai et al. 1994; Vergauwen et al. 2006). A further complication is the fact that the CC-1 line shows somewhat attenuated in mouse infectivity studies relative to fully virulent lines, probably the result of prolonged cultivation in vitro (Titus et al. 1995). Lastly, the CC-1 strain, like many laboratory-adapted strains, shows significant alterations in pteridine metabolism (Cruz and Beverley 1990; Cruz et al. 1993; Roy et al. 2001). Thus, an assessment of the role(s) of reduced pteridines and oxidative stress in the *Leishmania* infectious cycle will need to be carried out in fully virulent strains, in both sand flies, mice and macrophages.

Leishmania as a platform for studying the role of pteridines and susceptibility to oxidative stress

More generally, *L. major* provides an excellent model for studies aimed at dissecting the potential role of pteridines in resistance to oxidative stress in vivo. As shown here and elsewhere, the potent salvage pathways of *Leishmania* facilitate the manipulation of intracellular pteridine levels through nutritional supplementation, as do the availability of lines lacking or overexpressing critical pteridine metabolic enzymes such as PTR1, DHFR-TS and other folate dependent pathways (Cunningham and Beverley 2001; Nare et al. 1997a; Roy et al. 2001). In combination, these features provide a unique ability to manipulate the intracellular levels of reduced pteridines. This is especially relevant to studies of pteridine-mediated oxidant resistance in mammalian cells, which has proven to be exceedingly complex (reviewed in Ref. Oettl and Reibnegger 2002). *Leishmania* thus provides a unique opportunity to probe the role of pteridines in oxidant resistance in a comparatively simple and readily manipulated experimental system.

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