Role of the Endogenous Antioxidant System in the Protection of *Schistosoma mansoni* Primary Sporocysts against Exogenous Oxidative Stress

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Abstract

Antioxidants produced by the parasite *Schistosoma mansoni* are believed to be involved in the maintenance of cellular redox balance, thus contributing to larval survival in their intermediate snail host, *Biomphalaria glabrata*. Here, we focused on specific antioxidant enzymes, including glutathione-S-transferases 26 and 28 (GST26 and 28), glutathione peroxidase (GPx), peroxiredoxin 1 and 2 (Prx1 and 2) and Cu/Zn superoxide dismutase (SOD), known to be involved in cellular redox reactions, in an attempt to evaluate their endogenous antioxidant function in the early-developing primary sporocyst stage of *S. mansoni*. Previously we demonstrated a specific and consistent RNA interference (RNAi)-mediated knockdown of GST26 and 28, Prx1 and 2, and GPx transcripts, and an unexpected elevation of SOD transcripts in sporocysts treated with gene-specific double-stranded (ds)RNA. In the present follow-up study, *in vitro* transforming sporocysts were exposed to dsRNAs for GST26 and 28, combined Prx1/2, GPx, SOD or green-fluorescent protein (GFP, control) for 7 days in culture, followed by assessment of the effects of specific dsRNA treatments on protein levels using semi-quantitative Western blot analysis (GST26, Prx1/2 only, and larval susceptibility to exogenous oxidative stress in *in vitro* killing assays. Significant decreases (80% and 50%) in immunoreactive GST26 and Prx1/2, respectively, were observed in sporocysts treated with specific dsRNA, compared to control larvae treated with GFP dsRNA. Sporocysts cultured with dsRNAs for GST26, GST28, Prx1/2 and GPx, but not SOD dsRNA, were significantly increased in their susceptibility to H2O2 oxidative stress (60–80% mortalities at 48 hr) compared to GFP dsRNA controls (~18% mortality). H2O2-mediated killing was abrogated by bovine catalase, further supporting a protective role for endogenous sporocyst antioxidants. Finally, *in vitro* killing of *S. mansoni* sporocysts by hemocytes of susceptible NMRI *B. glabrata* snails was increased in larvae treated with Prx1/2, GST26 and GST28 dsRNA, compared to those treated with GFP or SOD dsRNAs. Results of these experiments strongly support the hypothesis that endogenous expression and regulation of larval antioxidant enzymes serve a direct role in protection against external oxidative stress, including immune-mediated cytotoxic reactions. Moreover, these findings illustrate the efficacy of a RNAi-type approach in investigating gene function in larval schistosomes.

Introduction

Miracidial penetration and entry into the molluscan intermediate host represent a critical transition period in which the previously free-living larval stage is now confronted with a potentially hostile environment as it attempts to establish a viable infection [1,2]. Miracidia of the human blood fluke *Schistosoma mansoni* shed their ciliated epidermal plates soon after entry into the host snail *Biomphalaria* spp., transforming to primary or mother sporocysts. It is during this time of transition and early sporocyst development that larvae are especially vulnerable to oxidative stress generated from products of oxidized plasma hemoglobin [3], or reactive oxygen or nitrogen species (ROS and RNS, respectively) resulting from hemocyte-mediated immune responses [4–7]. In such a potentially damaging environment, it is vital that parasites possess the capability of maintaining a redox equilibrium in order to counteract the effects of ROS/RNS generated both internally (products of endogenous metabolic oxidative reactions) and externally (environmental insults) [1,8].

Recent studies have shown that *S. mansoni* larvae possess numerous enzymes involved in ROS metabolism and detoxification of oxidative products [9–14], and, like their adult stage counterparts [15–18], appear to complement each other to maintain the redox balance in the parasite. Included among these enzymes are the following: (i) glutathione-S-transferases 26 and 28 (GST26 and GST28) that function to neutralize potential membrane damage by the linked catalysis of glutathione (GSH) reduction with detoxification reactions involving thiol-conjugation to xenobiotics [19], (ii) peroxiredoxin (Prx1 and Prx2) that are involved in maintaining redox balance, by reducing hydrogen...
Author Summary

Species of the human blood fluke Schistosoma are estimated to infect approximately 200 million people worldwide, resulting in loss of health, vitality and productivity mainly among the world’s poorest inhabitants. Since snail intermediate hosts represent an essential part of the flukes’ life cycle, an understanding of the strategies used by the intramolluscan schistosome larvae to survive within this host may provide novel approaches for disrupting larval development and thus transmission to humans. Anti-oxidant enzymes produced by the parasite Schistosoma mansoni are believed to play a critical role in the maintenance of cellular redox balance, contributing to larval survival in their snail host, Biomphalaria glabrata. In this study, we have incorporated a RNA interference approach attempting to knock down specific anti-oxidant enzymes, including glutathione-S-transferases 26 and 28 (GST26 and 28), glutathione peroxidase (GPx), peroxidoxins 1 and 2 (Prx1/2) and superoxide dismutase (SOD), and to evaluate their endogenous anti-oxidant function in the sporocyst stage of S. mansoni. Results clearly demonstrated a significantly higher susceptibility of antioxidant double-stranded (ds)RNA-treated larvae to in vitro H2O2 treatment or hemocytic encapsulation compared to GFP dsRNA controls. Taken together, our findings support the hypothesis that endogenous expression and regulation of larval antioxidant enzymes serve a direct role in protection against external oxidative stress, including immune-mediated cytotoxic reactions.

Materials and Methods

Ethics statement

Research procedures involving mice used in the course of this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Madison under assurance no. A3368-01.

In vitro cultivation of larval Schistosoma mansoni

The NMRI strain of S. mansoni was used for all experiments. S. mansoni eggs were isolated from livers obtained from mice harboring 7-week old infections, and miracidia hatched in an artificial “pond water” supplemented with antibiotics (50 μg/mL streptomycin and 60 μg/mL penicillin) [27]. Larvae were washed twice in ice-cold, sterile pond water by centrifugation, before being resuspended in Chemini’s Balanced Saline Solution (CBS; [28]), containing glucose and trehalose (1 g/L each) streptomycin and penicillin (50 μg/mL and 60 μg/mL, respectively). Miracidia were then counted and distributed into 48- or 96-well polystyrene tissue culture plates (Costar, Corning Incorporated, NY), at concentrations of ~500, 1000 or 8000 miracidia/well for oxidative stress experiments, immunocytochemistry or Western blot analyses, respectively. Finally, double-stranded RNAs were synthesized from isolated sporocyst cdNA using T7 RiboMAX Express RNAi Kit (Promega, Madison, WI), according to manufacturer protocol. Briefly, dsRNAs synthesis reactions were allowed to incubate for 16 hr at 37°C prior to DNase treatment. dsRNA products were then extracted by phenol/chloroform and purified by precipitation with isopropanol. dsRNAs (50 nM final concentration) for specific antioxidant genes or green-fluorescent protein (GFP; specificity control dsRNA) were added to cultures containing 100 μg/mL CBSS for the oxidative stress assays and immunocytochemistry and 400 μL for the Western blot experiments. Because of the sequence and functional similarities of Prx1 and 2, dsRNAs for these transcripts were combined as a single treatment, designated hereafter as Prx1/2. Larvae were incubated for 7 days as previously detailed [25], after which time the functional consequences of dsRNA treatments were determined in functional assays described below. It should be noted that in a previous series of RNAi experiments conducted in parallel with the present study [25], a consistent significant knockdown of steady-state transcript levels for each of the antioxidant genes currently under study was well documented. The only exception was the Cu/Zn superoxide dismutase (SOD) gene, in which larval exposure to SOD dsRNA resulted in a consistent increase, not knockdown, of SOD transcripts.

Western blot analysis

To assess the effects of antioxidant dsRNA on the expression of specific proteins in sporocytes, we analyzed protein extracts of dsRNA-exposed sporocytes by Western immunoblot analysis [29] incorporating specific antibodies to two antioxidant species; namely SmGST26 (Cell Signaling Technology, Danvers, MA) and SmPrx1/2 (gift from Dr. D. Williams). Briefly, protein samples (~8 μg) and Precision Plus Dual Color Marker (Bio-Rad, Bio-Rad Laboratories, Inc., Hercules, CA) were separated on 12.5% SDS-PAGE gels and transferred by semi-dry electroblotting (Amersham Biosciences) to nitrocellulose membranes (Bio-Rad). After blocking overnight in TBS (2.42 g Tris base, 8 g NaCl, pH 7.6) containing 5% bovine serum albumin (BSA), membranes were incubated in specific antibodies or a mouse anti-α tubulin

Changes, especially their relevance to S. mansoni sporocyst interactions with the intermediate snail host B. glabrata.

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antibody (serving as loading control, 1:1000 dilution; Upstate Biotechnology Inc., Lake Placid, NY) for 16 hr at 4°C with gentle rocking. Membranes were then washed for 30 min in TBS-T (1×) and incubated for 1 hr in TBS-BSA (5%) containing either alkaline phosphate (AP-)conjugated goat anti-rabbit IgG or AP-rabbit anti-mouse IgG at dilutions of 1:1000 and 1:5000, respectively (Promega, Madison, WI). The colorimetric immunoactivity was detected with the chromogen 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) diluted in AP buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5).

To quantify the observed immunoreactivity of each target protein in sporocysts treated with specific dsRNA and control GFP dsRNA, the intensities of reactive target bands were measured using Ultraviolet Transilluminator BioImaging Systems (UVP, Inc., Upland, CA) and normalized to the tubulin band with LabWorks Image Acquisition and Analysis Software (version 4.6) in order to quantitatively evaluate the effects of antioxidant dsRNA treatment on specific protein levels. Three independent experimental replicates were performed and analyzed by Student’s t-test, with significance set at P≤0.05.

Immunocytochemistry

In order to compare in situ GST26 and Prx protein levels in antioxidant dsRNA-treated parasites, we prepared whole, intact sporocysts for immunofluorescent observations. All washing steps, in eppendorf tubes, were performed by centrifugation at 1600 rpm for 2 min and repeated 5 times, or as otherwise mentioned. Following transformation and in vitro cultivation (24 hr), sporocysts were washed 3 times in CBSS, to remove detached ciliated plates, prior transfer to siliconized-tubes containing 2% paraformaldehyde and 1% Triton-X100/sPBS. Larvae were fixed overnight at 4°C under gentle agitation, then washed in snail phosphate-buffered saline (sPBS; [30]) and resuspended in blocking buffer (5% normal goat serum + 0.02% sodium azide in sPBS) for 16 hr at 4°C. Rabbit-anti-GST26 or mouse anti-Prx1/2 primary antibodies, diluted at 1:2000, and 1:1000, respectively, were then added to the larvae in fresh blocking buffer for 16 hr at 4°C under gentle agitation. This was followed by 5 washes, 10 min each, in sPBS, and resuspension in blocking buffer containing 4 μg/mL Alexa-Flour 488-conjugated anti-rabbit/mouse antibody, 7 units/mL phalloidin-Alexa 546 and 10 μg/mL Hoechst 33258 dye (Invitrogen). Tubes containing samples were incubated for 16 hr at 4°C under constant rotation, followed by washing in sPBS, resuspension in 40 μl of sPBS and mounting on coverslips. A Nikon Eclipse TE300 (Nikon Instrument Inc., Melville, NY) inverted epifluorescent inverted microscope Nikon Eclipse TE 300 (Nikon Instruments Inc.). Data were represented as mean percentage dead sporocysts: \[ % \text{dead sporocysts} = \frac{\text{number of dead larvae (PI+)} \times 100}{\text{total number of larvae}} \]

Larval mortality in ≤50 μM H₂O₂ was comparable to untreated sporocysts (CBSS alone) at both time intervals (data not shown), and therefore 50 μM was chosen as our sublethal H₂O₂ concentration.

Effect of antioxidant gene knockdown on sublethal H₂O₂-mediated sporocyst killing. To evaluate the functional relevance of the antioxidant enzymes GPx, GST26, GST28, Prx1/2 and SOD on parasite survival under oxidative stress conditions, 7-day dsRNA-treated sporocysts were exposed to 50 μM H₂O₂ for 4, 24 and 48 hr prior to evaluating larval viability. Freshly-hatched miracidia were axenically isolated and soaked in CBSS containing 50 nM of GPx, GST26, GST28, Prx1/2, SOD or control GFP dsRNAs and cultured for 7 days in 24-well plate as previously described [25]. Each treatment group was then divided into 3 wells of a 96-well plate: 2 of the wells were exposed to 50 μM of H₂O₂, and the third well was used as a no-treatment control (no H₂O₂). Cultures were incubated at 26°C and dead parasites (PI+) were counted at 4 hr, 24 hr and 48 hr using an epifluorescent inverted microscope Nikon Eclipse TE 300 (Nikon Instruments Inc.).

Catalase neutralization of H₂O₂-mediated oxidative stress. To verify that H₂O₂ is the major oxidizing agent responsible for initiating sporocyst death, dsRNA-treated larval parasites were prepared as described above and exposed to 50 μM H₂O₂ containing 0.1%, 0.05%, 0.025%, 0.0125% of bovine catalase (Sigma-Aldrich). Additional control cultures containing H₂O₂ alone (positive killing control) and catalase alone (catalase control) were run concurrently. All parasites were incubated at 26°C, and larval death at 0, 4, and 48 hr post-treatment, using propidium iodide (Invitrogen). For each dsRNA treatment, the mean percentage mortality between H₂O₂-exposed, H₂O₂ + catalase-exposed and unexposed parasites was used to compare the % mortality of antioxidant dsRNA-treated groups over exposure time interval to the GFP dsRNA-treated control group. Significance was set at P≤0.05; N = 5.

Protective effect of antioxidants against in vitro hemocyte-mediated killing. To investigate the larval protective role of endogenous antioxidant molecules, sporocysts were treated with GPx-, Prx1/2-, GST26-, GST28-, SOD- and GFP-(control) dsRNAs for 7 days, followed by co-incubation with hemocytes of the susceptible NMRI strain of Biomphalaria glabrata in an in vitro cell-mediated cytotoxicity assay [5]. Because we wished to test for an effect of antioxidant knockdown on the efficacy of hemocyte-mediated killing, cells of the susceptible NMRI strain were used to determine if their basal level of killing efficiency could be significantly altered due to antioxidant knockdown. Eighteen snails (14–18 mm shell diameter) were used for each assay, in which the shell of each animal was dried, swabbed with 70% ethanol, and soaked in filter-sterilized “pond” water containing 60 μg/mL penicillin G, 50 μg/mL streptomycin and 25 μg/mL amphotericin B for 30 min. Snail shells were again

% dead sporocysts = \left[ \frac{\text{number of dead larvae (PI+)} \times 100}{\text{total number of larvae}} \right]
dried and swabbed with 70% ethanol before headfoot bleeding as described in Sminia and Barendsen [31]. Whole hemolymph was pooled in a Petri dish on ice to facilitate removal of any extraneous shell debris or mucus, and then transferred to sterile 15-mL centrifuge tubes containing an equal volume of ice-cold CBSS. The cytotoxicity assay described by Hahn et al. [5] was used with some modifications. Approximately 500 μL of hemolymph was gently aliquoted in siliconized-epandorf tubes containing an agarose plug (0.2% agarose) and 50 μL of 5% Ficoll (Sigma-Aldrich Inc.) in incomplete or I-Bge (24% Schneider’s Drosophila medium, Invitrogen; 0.5% lactalbumin hydrolysate, Sigma-Aldrich; 7.2 mM galactose). Tubes were centrifuged at 20 × g for 17 min to isolate and concentrate hemocytes. Cell-free plasma and Ficoll were removed and discarded, followed by resuspension of hemocytes in sterile CBSS and redistribution in equal aliquots to wells of a 16 CultureWell™ Chambered Coverglass slide (Invitrogen) containing approximately 100 dsRNA-treated sporocysts in I-Bge medium. After 1 hr of co-cultivation at 26°C, 3 μL of propidium iodide (PI; 5 μg/mL) (Invitrogen) were added to a subset of wells and the total number of sporocysts (Nomarski DIC optics) and number of dead sporocysts, those exhibiting positive PI staining (+), per treatment were counted in order to establish an initial mortality rate. Enumeration of total and dead sporocysts was again determined at 24 hr post-cultivation. The percentage of sporocysts killed after 24 hr of co-culture was calculated for groups of larvae treated with antioxidant dsRNAs and control GFP dsRNA, and compared according to the following formula [5]:

\[
\text{\% sporocysts killed} = \left[ \left( \frac{d_{24 \text{ hr}} - d_{1 \text{ hr}}}{T - d_{1 \text{ hr}}} \right) \times 100 \right],
\]

where “d” = # dead sporocysts at the indicated time interval (1 or 24 hr) and “T” = total # sporocysts. Statistical analyses were performed using Student’s t-test in which the % sporocyst death at 24 hr was compared between antioxidant dsRNA-treated and control GFP dsRNA-treated groups. Significance was set at \( P < 0.05 \) (\( N = 4 \)).

**Results**

Previous work in our lab has established a consistent and specific pattern of altered antioxidant transcript expression in primary sporocysts after 7 days of double-stranded (ds) RNA exposure [25]. Specifically, statistically significant knockdown of *S. mansoni* GST26, GST28, GPx, and Prx1/Prx2 transcript levels, and an unexpected robust increase in those of SOD were observed in dsRNA-treated larval populations. To further explore the functional relevance of these enzymes in this parasite model, we conducted experiments to determine how antioxidant dsRNA exposure affected gene expression at the protein level (for selected enzymes), and whether a functional association could be established between antioxidant gene knockdown and parasite survival in presence of stressors such as reactive oxygen species (H₂O₂) or encapsulating hemocytes.

To verify that specific dsRNA treatments had a predicted downregulating effect on sporocysts at the protein levels, Western blot analyses were performed on sporocysts treated with dsRNA for GST26, Prx1/2 and GFP (control) using antibodies specifically against *S. mansoni* GST26 and Prx1/2 [20]. In all experiments a crossreactive anti-α tubulin antibody served as a loading control. As shown in Figure 1, proteins extracted from GFP dsRNA-treated sporocysts (specificity control) presented two distinctive bands at ~26 and 55 kDa, corresponding to GST26 and α tubulin, respectively. However, although larvae treated with GST26 dsRNA also exhibited the 55 kDa α tubulin protein, little immunoreactivity was observed at 26 kDa, suggesting an RNAi-induced GST26 protein knockdown (Fig. 1A). Quantification of band intensities by scanning densitometry was used to quantify immunoreactive GST26 (Fig. 1C) and Prx1 (Fig. 1D) intensities in specific dsRNA-treated vs. GFP dsRNA control sporocyst groups. Both GST26 and Prx protein levels were significantly knocked down by 80% and 50%, respectively, when compared to the GFP dsRNA treatment. **\( P < 0.01 \); *** \( P < 0.001 \); \( N = 3 \).”

![Figure 1. Western blot analyses of SDS-PAGE separated extracts of Schistosoma mansoni sporocysts. Larvae were cultured for 7 days in CBSS containing GFP, GST26, or Prx1/2 dsRNA, followed by probing with specific anti-GST26 (Fig. 1A), anti-Prx1 (Fig. 1B) or sample loading control anti-α tubulin antibodies. Using anti-α tubulin reactivity to normalize sample loads, scanning densitometry was used to quantify immunoreactive GST26 (Fig. 1C) and Prx1 (Fig. 1D) intensities in specific dsRNA-treated vs. GFP dsRNA control sporocyst groups. Both GST26 and Prx protein levels were significantly knocked down by 80% and 50%, respectively, when compared to the GFP dsRNA treatment. ** \( P < 0.01 \); *** \( P < 0.001 \); \( N = 3 \). doi:10.1371/journal.pntd.0000550.g001](http://www.plosntds.org/4-November-2009/-Volume-3/-Issue-11/-e550).
In order to evaluate the effects of a potential loss of antioxidant activity in sporocysts due to dsRNA-induced antioxidant knockdown, we exposed groups of treated parasites to a range of hydrogen peroxide (H$_2$O$_2$) concentrations. In these preliminary tests 50 μM H$_2$O$_2$ was determined to represent a sublethal dosage under our experimental conditions (% larval death was not significantly different from control groups), whereas mortality rates significantly increased at 100 μM and higher H$_2$O$_2$ concentrations (data not shown). As shown in Figure 3, none of the dsRNA-treated sporocysts exhibited significant increases in H$_2$O$_2$-mediated mortality when compared to the GFP control treatments after 4 hr of exposure. However, at 24 and 48 hr sporocysts in all dsRNA-treatments, except the SOD dsRNA-exposed group, displayed significant increases in mortality with an average of 25% average sporocyst death in the catalase treatment groups (see Fig. 4 for Bonferroni’s post-test comparisons). As previously observed, SOD dsRNA-treated larvae, again showed no difference in mortality rates between the different treatments, nor when compared to the control GFP dsRNA group.

Finally, in order to evaluate the effect of dsRNA antioxidant knockdown on snail hemocyte-sporocyst interactions in vitro, dsRNA-treated sporocysts were co-cultured with isolated hemocytes from the susceptible NMRI strain of Biomphalaria glabrata. After 24 hr of sporocyst-hemocytes incubation in an in vitro cell-mediated cytotoxicity assay [5], we observed that dsRNA knockdown of GST26 ($t=2.50$, $P=0.01$), GST28 ($P=0.0461$) and Prx1/2 ($t=3.17$, $P=0.04$) resulted in small, but statistically significant increases in larval death, averaging ~20% compared to ~8% mortality in the GFP dsRNA control group (Fig. 5). Note that sporocysts treated with GPx dsRNA also showed an increase in mean mortality rate, but was not statistically significant when compared to the GFP control parasites. As observed in previous experiments, sporocysts treated with SOD dsRNA exhibited no difference in mortality compared to the GFP-treated control sample.

**Discussion**

Enzymes involved in cellular redox pathways, which include proteins with antioxidant activities, are believed to be essential components regulating *B. glabrata/S. mansoni* molecular interaction [1,2]. It is now well recognized that certain strains of *B. glabrata* snail immune cells or hemocytes produce substantial amounts of reactive oxygen [4,5] and nitrogen [6] species as a consequence of stimulation by known activators of ROS/RNS or when encountering *S. mansoni* sporocysts, and that sporocysts are exquisitely sensitive to ROS-mediated killing, especially to H$_2$O$_2$. Moreover, in a series of followup studies, Bayne and co-workers have implicated a Cu/Zn-superoxide dismutase (SOD1) as a key enzyme involved in oxidative killing activity by hemocytes of resistant (R) strains of *B. glabrata* snails. Their studies demonstrated that (1) SOD transcript expression and enzyme activity are higher in certain R vs. susceptible (S) snail hemocytes [32] and this correlates with greater H$_2$O$_2$ production in the R strain [33], (2) *B. glabrata* SOD1 is comprised of 3 alleles, of which one (B allele) is significantly associated with R snails [34], and (3) SOD1 B allelic expression is higher in R hemocytes than those of the S strain [35]. Based on their findings it is suggested that snail strain differences in SOD hemocyte expression may be causally linked to the observed S and R strain phenotypes. Because SOD catalyzes the conversion of superoxide to cytotoxic H$_2$O$_2$, it is reasoned that upregulation of the SOD1 gene and its resultant heightening of SOD enzymatic activity in R hemocytes may represent a possible mechanism for the differential larval killing response by R vs. S snail hemocytes [2].

While snail hemocytes produce H$_2$O$_2$ as an anti-parasite effector molecule, evidence also strongly supports the presence of an active antioxidant system in early developing *S. mansoni* sporocysts [11–13]. Catalase gene homologues were not found in exposure ($F_{\text{dsRNA}} = 7.44$, $P=0.001$; $F_{\text{GFP}} = 15.33$, $P=0.0001$, $N = 6$). Within each treatment group, the percent mortalities for sporocysts exposed to GPx, GST26, GST28 and Prx1/2 dsRNAs were very similar when incubated in H$_2$O$_2$-catalase or catalase only ($t$ values ranging from 0.23–1.74; all nonsignificant) (Fig. 4). These results are in contrast to the effects of exposure to H$_2$O$_2$ alone (positive killing control), in which mortality rates for sporocysts treated with the same antioxidant dsRNAs were significantly higher (ranging from 50–75%) when compared to 25% average sporocyst death in the catalase treatment groups (see Fig. 4 for Bonferroni’s post-test comparisons). As previously observed, SOD dsRNA-treated larvae, again showed no difference in mortality rates between the different treatments, nor when compared to the control GFP dsRNA group.

Figure 2. Representative confocal epifluorescent photomicrographs of *Schistosoma mansoni* sporocysts showing immunolocalization of anti-GST26 and anti-Prx1 antibodies after culation in medium containing GST26, Prx or control GFP dsRNA. Fluorescence specific to anti-GST26 reactivity (green) observed in GST26 dsRNA-treated larvae (Fig. 2B) was noticeably reduced compared to the nonspecific GFP dsRNA control sporocysts (Fig. 2A), consistent with the high protein knockdown (~80%) seen in Western blot analysis. By contrast, little difference in fluorescence levels was observed between the nonspecific GFP dsRNA control-treated (Fig. 2C) and Prx1/2 dsRNA-treated (Fig. 2D) sporocysts, reflecting the relatively small decrease (~50%) observed in immunoblot protein levels. N = 3. doi:10.1371/journal.pntd.0000550.g002
Figure 3. Graphic representation of the effect of exogenous H_{2}O_{2} exposure on Schistosoma mansoni sporocysts following treatment with dsRNAs for GFP (specificity control), SOD, GPx, GST26, GST28 and Prx1/2. Double-stranded RNA-treated sporocysts were exposed to 50 μM H_{2}O_{2} for 4, 24 and 48 hrs (stippled, grey and black bars, respectively). Knockdown of larval GPx, GST26, GST28 and Prx1/2 antioxidants increased sporocyst mortality after 24 and 48 hr under oxidative stress conditions when compared to GFP dsRNA-treated or no treatment controls. Note that sporocysts treated with SOD dsRNA showed no difference in susceptibility to H_{2}O_{2} oxidation at any of the time points compared to controls. **P<0.001; ***P<0.0001; N=4.

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Figure 4. Effects of catalase on H_{2}O_{2}-mediate killing of GPx, GST26, GST28, SOD, Prx1/2 dsRNA-treated and control GFP dsRNA-treated Schistosoma mansoni sporocyst in vitro. After 7 days of dsRNA incubation sporocysts were exposed to H_{2}O_{2} alone, catalase alone or catalase combined with H_{2}O_{2} for 48 hr followed by evaluation of sporocyst death using propidium iodide staining. Significant increases in H_{2}O_{2}-mediated mortality was abrogated in the presence of bovine catalase (H_{2}O_{2}+catalase) showing that H_{2}O_{2} was the primary source of larval killing in antioxidant dsRNA-treated sporocysts, with the exception of SOD. *P<0.05; **P<0.001; ***P<0.0001; N=6.

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ed that Prx1/2 in LTP can function as scavengers of exogenous H₂O₂ and other ROS. As clearly demonstrated in mammalian systems, these parasites must possess alternative means for neutralizing exogenous oxidative stress. In this study, we provide the first evidence for a functional role of the endogenous antioxidants GPx, Prx and GSTs in the survival of intact primary sporocysts of *S. mansoni*. In a previous companion study that was run in parallel with the current experiments [25] we showed that larval treatment with double-stranded RNA (dsRNA) for all of the antioxidants, except SOD, produced a consistent, significant and specific transcript knockdown in sporocysts. In the present study, consistent with the transcript knockdown seen earlier, we demonstrated a dsRNA-associated decrease in GST26 and Prx1/2 protein levels using specific antibodies in a semi-quantitative Western blot assay. This protein knockdown effect was supported by immunocytochemistry (ICC) in the case of GST26, but not as clearly for Prx. Importantly, the dsRNA-mediated decrease in GST26 and Prx protein content correlated well with significant increases in sporocyst mortality at 24 and 48 hr post-H₂O₂ exposure compared to the dsRNA control groups, implying a functional role for endogenous GST26 and Prx in the protection of primary sporocysts against external oxidative stress. Although lack of specific antibodies to the other antioxidants precluded a complete analysis of the other RNAi targeted genes used in this study, we continued to see a consistent correlation between dsRNA-induced decrease in transcript levels [25] and sporocyst survival patterns for larvae treated with GST28 and GPx dsRNA that were similar to those treated with GST26 and Prx1/2 dsRNAs. Indeed, compared to the untreated and GFP dsRNA controls, exposure of antioxidant dsRNA-treated sporocysts to a sublethal concentration of H₂O₂ in vitro resulted in dramatic decreases in parasite survival in all treatment groups except SOD, supporting the notion that GST28 and GPx, similar to Prx and GST26, also are capable of enhancing sporocyst survival in an oxidative environment.

These new findings are consistent with the extensive and ongoing work on the redox mechanism in the adult stage of *S. mansoni*, in which an active thiol-dependent redox maintenance system revolves around a thioredoxin glutathione reductase (TGR; [36]), a single enzyme that combines the activities of two enzymes, thioredoxin reductase and glutathione reductase, present in mammals [17]. Schistosome TGR is responsible for maintaining the reduced and active states of both thioredoxin (TR) and glutathione (GSH), allowing them to activate several Prxs and GSTs, as the central enzyme driving redox reactions [36]. Similarly, early intramolluscan larval stages also express redox genes, including TGR, thioredoxin, Cu/Zn SOD, GPx, Prx and GST [7,11–13,37], and in the case of GPx [7] and Prx1 and 2 [11], sporocyst expression levels are dramatically increased in response to ROS exposure. In addition, Cu/Zn SOD, GST26 and 28 and Prx were recently identified in larval transformation proteins (LTP) released during *in vitro* transformation of miracidia to sporocysts, demonstrating not only the synthesis of these antioxidants by miracidia, but also their active release during larval infection [9,14]. Implanted in these findings is the notion that antioxidant LTPs may be playing a potential protective role during early parasite development. This prospect of larval-protective antioxidants was given further credence by Vermeire and Yoshino [11] who demonstrated that Prx1/2 in LTP can function as scavengers of exogenous H₂O₂ suggesting the potential importance of excreted antioxidants as a sporocyst defense mechanisms.

In this study, we provide the first evidence for a functional role of the endogenous antioxidants GPx, Prx and GSTs in the survival of *S. mansoni* sporocysts confronted with exogenous oxidative stress. By successfully knocking down antioxidant transcript/protein levels using an RNAi-type approach, we were able to characterize the impact of introduced molecular H₂O₂ and presumed ROS produced during hemocyte encapsulation reactions on survival of susceptible NMRI snail hemocytes from *B. glabrata* strain. Our rationale for incorporating susceptible snail hemocytes in these experiments was to test the hypothesis that reducing the antioxidant capacity of sporocysts would increase their vulnerability to sublethal levels of ROS normally produced by NMRI snail hemocytes in *in vitro* cell-mediated cytotoxicity (CMC) assays [5,38]. In this *in vitro* biologically-relevant context, we demonstrated a significant protective role of Prx and GSTs in sporocysts during hemocyte interactions. Co-culture of plasma-free hemocytes with Prx, GST26, and GST28 dsRNA-treated sporocysts increased an increase in sporocyst mortality (to ~20%) within 24 h of initial contact, when compared...
to GFP dsRNA-treated control group (8%). GPx dsRNA-treated sporocysts also showed a comparable increase in hemocyte-mediated killing, but high variance in replicate values rendered the increase nonsignificant. Thus the protective role of GPx against hemocyte-mediated ROS attack still remains to be proven. Taken together, however, our overall results suggest that ROS production in susceptible snail hemocytes is capable of overpowering antioxidant-deficient parasites. Zeck and Janowsky [7] hypothesized that susceptible snails generate relatively small amount of ROS, which in turn may induce antioxidant production in schistosomes, effectively neutralizing snail-generated ROS. In this study, we have demonstrated that effectively reducing their antioxidant enzyme capacity, sporocyst survival, when confronted by a usually benign hemocyte challenge, is significantly reduced, thus supporting the critical importance of the endogenous antioxidant system in establishing viable larval infections within the susceptible snail host.

Finally, a major exception to our present finding of enhanced larval susceptibility to oxidative stress by redox proteins was signal peptide (SP) Cu/Zn SOD [39]. In this case Cu/Zn SOD dsRNA treatment consistently had no effect on parasite survival whether in the presence of sublethal H$_2$O$_2$ or encapsulating hemocytes. These differing effects of SOD dsRNA exposure may have been predicted as treated S. mansoni sporocysts consistently displayed extreme elevations, rather than knockdown in transcript levels [25], indicating a strong induction of SOD gene expression in these larval stages. At present, the signaling mechanisms involved in this response are not known although, as suggested by Zeck and Von Janowsky [7] and Vermeire and Yoshino [11], sporocysts may be sensing ROS levels (including H$_2$O$_2$) and responding by upregulating protective antioxidant proteins. It is speculated that larval treatment with SOD dsRNA may have caused an initial downregulation of SOD transcripts that then led to a compensatory triggering of SOD over-expression. However, as shown in other systems, small interfering dsRNA also can trigger activation of transcription [40] and, therefore, could also represent a likely mechanism [25]. Its unusual expression pattern not withstanding, results indicate that hyperexpression of the SOD gene in S. mansoni sporocysts appeared to have a “neutral” effect on dsRNA-treated larvae (i.e., an effect similar to control dsRNA treatment) (present study). This does not necessarily imply that SOD has no role to play in maintaining redox balance within sporocysts both internally or in response to exogenous ROS sources. However, the mechanisms by which this is accomplished are currently unknown and represent the subject of further followup investigations in our lab.

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Author Contributions

Conceived and designed the experiments: TPY. Performed the experiments: MMM ND. Analyzed the data: MMM ND GRF. Contributed reagents/materials/analysis tools: TPY. Wrote the paper: MMM ND TPY. Revisions of draft manuscripts: MMM ND GRF. Obtained fellowship support for MMM: GRF. Provided research funds: TPY.

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