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Nitric oxide-dependent changes in *Schistosoma mansoni* gene expression

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Nitric oxide (NO)-related pathways potentially play at least two critical roles in schistosomes, the causative agents of schistosomiasis. First, these pathways may represent essential signaling cascades required for normal parasite physiology and survival. Second, NO-related pathways may also play an important role in parasite–host interactions. Several reports have demonstrated that platyhelminths have nitric oxide synthase (NOS) activity and that NO is likely acting as a signaling molecule in these organisms [1–4]. Furthermore, the host NO pathway may be involved in host defense against schistosome infection, though its precise role *in vivo* is not clear [5–7].

Here we examine changes in parasite gene expression in response to exposure to exogenous NO *in vitro*. Prior studies have provided evidence of NO-related pathways in adult schistosomes [1,2,8]. However, the physiological role and downstream targets of NO have not been elucidated in adult worms. To assay NO-dependent changes in gene expression, we have used Long-SAGE (serial analysis of gene expression) [9], which provides both the identity of expressed genes and the relative levels of their expression.

In Long-SAGE, a short 21 bp sequence tag from the most polyA proximal *Nla*III restriction site of an mRNA molecule is used to uniquely identify the source gene from within the genome. Short sequence tags are sampled from all *Nla*III-positive transcripts in a mRNA sample and are linked together to form long concatenated molecules that are cloned and sequenced. Quantification of all tags provides a relative measure of gene expression (i.e., mRNA abundance). Using SAGE, we have identified genes which respond to NO by changing expression levels, and we also show by RT-PCR that RNA encoding extracellular superoxide dismutase (EC-SOD, also referred to as signal peptide-SOD [10–11]) is upregulated in response to exposure to an NO donor. These results provide insights into NO

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signaling pathways in schistosomes and might help better define the role of NO in host–parasite interactions.

Adult *Schistosoma mansoni* perfused from infected Swiss-Webster female mice (obtained from the NIAID Schistosomiasis Resource Center) 42–49 days post-infection were maintained in culture (RPMI medium) overnight and then exposed for 3 h to either 1 mM sodium nitroprusside (SNP), a well-characterized NO donor [12], or to RPMI alone. Worms remained viable and motile following treatment. Total RNA was extracted with Trizol (Invitrogen) and treated with DNase 1 (Ambion) to remove contaminating genomic DNA, and Long-SAGE libraries constructed.

After correcting for sequencing error, we sampled 26,072 SAGE tags for the control library and 26,815 tags for the SNP-exposed library. We used log-likelihood statistics ($R > 1.5$) to reduce the effects of sampling error and identified 13 tags that were upregulated two-fold or greater in the SNP-treated library and one tag downregulated two-fold or greater in the SNP-exposed library. In addition, eight tags were uniquely present in the 3 h. SNP-treated library and 21 tags uniquely present in the control library. Approximately 84% of the differentially expressed tags could be mapped to *S. mansoni* genome, cDNA, or EST sequences. Data for tags that map to genes with a putative function are presented in Table 1.

Several tags showed differential expression between the control and SNP treated library, but had R values less than 1.5. One of these, Tag ID #10395, maps to the sequence for extracellular superoxide dismutase (EC-SOD; Sm00301) [13], which showed a 1.2-fold increase in the SNP-treated library as compared to the control library, with an R value of 0.56. SOD is potentially an interesting gene for further study, as NO is known to interact with reactive oxygen intermediates such as superoxide [14,15]. Hence, we examined the expression pattern of this transcript using RT-PCR. Expression of EC-SOD RNA showed upregulation following exposure to SNP (Fig. 1). Measurement of relative band intensity for three separate experiments indicates a 2.66 ± 0.22 increase in expression of the EC-SOD transcript in SNP-treated schistosomes compared to the control ($p < 0.01$, paired two-tailed t -test).

EC-SOD is normally expressed at a far higher level in females than in males [16]. In order to examine whether NO-dependent upregulation of EC-SOD differs in males and females, we isolated male and female worms, and exposed them separately to SNP for 3 h. As in the mixed population of worms, EC-SOD is upregulated in both females and males following exposure to NO (Fig. 2). Most notably, amplification of EC-SOD was found only in males that had been exposed to SNP (Fig. 2B), but was not detectable in control males. Expression levels of 18S ribosomal RNA were essentially constant (Fig. 2E–H).

We used quantitative real-time RT-PCR to confirm these results. Real-time RT-PCR was performed using the Brilliant SYBR Green QRT PCR master mix kit (Stratagene) on an Opticon 2 Real-Time PCR Detection System (Biorad). Using the Pfaffl method, the normalized expression ratio of EC-SOD in a mixed-sex population of adult worms exposed to SNP for 3 h was 1.5 compared to the corresponding control. The normalized expression ratio of EC-SOD in SNP-exposed females to control was 3.3, and 2.4 in SNP-exposed males compared to control. Longer exposure of adult worms to SNP did not increase the relative expression of EC-SOD. The normalized expression ratio of EC-SOD in a mixed population of adult worms exposed to SNP for 24 h was 1.1.

Our data indicate that exposure of schistosomes to an NO donor induces rapid changes in gene expression (Table 1), though these changes are not large-scale. Some of the genes upregulated in SNP-treated schistosomes, such as cyclic nucleotide phosphodiesterase (Tag ID #20272) and calcineurin (Tag ID #461), share similarity to genes which have previously

been reported to be involved in NO signaling in other systems [17]. For example, NO/cGMP-dependent protein kinase appears to regulate calcineurin activity via the modulation of intracellular Ca^{2+} concentration in vascular smooth muscle cells, partially inhibiting proliferation, but not affecting cell viability [18]. Several other potentially interesting genes are also upregulated, including those encoding a protein involved in metal ion transport (Tag ID #1891) and a protein subunit of the coatamer complex (Tag ID #1188).

Our SAGE analysis also reveals potentially interesting genes that are downregulated in SNP-exposed schistosomes. One of these is Rac GTPase (Tag ID #1142), which has been shown to interact with NO in other systems [19]. Other potentially interesting downregulated genes include a subunit of vacuolar ATP synthase (Tag IDs #7301 and #6413), a putative coated vesicle membrane protein (Tag ID #7720), and a putative ligand-gated ion channel (Tag ID #602). Furthermore, the transcript for a cathepsin L-like protein (Tag ID #5905) shows reduced expression following exposure to SNP, while a sequence coding for protease inhibitor-like sequences (Tag ID #9566—AMBIP precursor) is upregulated. Whether these changes are simply coincidental or represent a general pattern to reduce proteolytic activity (perhaps in the gut) in response to NO remains to be determined.

The increased expression level of EC-SOD transcripts that we find in SNP-treated schistosomes suggests a potential interaction between the NO and oxygen free-radical pathways. Indeed, there is precedence in the literature for such an interaction. SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide and oxygen [20,21]. It is involved in the elimination of superoxide radicals produced during metabolism and has relevance for detoxification inside organisms [20,21]. NO is known to react with the free radical superoxide, which is trapped by SOD [22]. Furthermore, studies in rat fibroblasts suggest that NO-mediated apoptosis is inhibited by EC-SOD [23]. Thus, upregulation of EC-SOD in response to NO may be a protective defense mechanism of the parasite to oxidative stress.

As in mammals, schistosome SOD is a major antioxidant involved in detoxification [24]. Schistosome EC-SOD contains a signal peptide sequence, but does not appear to be localized extracellularly in *S. mansoni* [10,13]. Far higher levels of *S. mansoni* EC-SOD transcripts are found in adult females than in adult males [16]. Consistent with these findings, we detect EC-SOD transcripts by RT-PCR only in adult females. Exposure to SNP results in upregulation of EC-SOD in females, but also the appearance of EC-SOD transcripts in males. The role of this induction in both males and females remains to be determined.

The relatively modest overall response to SNP at the RNA level may reflect the exposure time we chose to evaluate or indicate limited effects of NO on the most abundant transcripts detected most readily by SAGE. Nevertheless, NO likely plays an important role in regulation of *S. mansoni* gene expression. Further studies to elucidate the function and expression levels of NO-responsive genes should provide insights into NO signaling pathways in schistosomes and schistosome compensatory mechanisms against host NO.

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Fig. 1.

Exposure of *S. mansoni* to NO donor for 3 h results in upregulation of a putative schistosome EC-SOD transcript. RT-PCR amplification of EC-SOD sequence from (A) control and (B) 3 h SNP-treated parasites. Amplification of Rho2GTPase from (C) control and (D) 3 h SNP-treated parasites. Rho2GTPase does not change in response to NO donor and is used as a control. Equivalent amounts of total RNA from each condition were reverse-transcribed to single-stranded cDNA using Superscript II Reverse Transcriptase (Invitrogen) and random hexamers and amplified using gene-specific primers. The primers used to detect EC-SOD were: 5'-TACGTTAATGGAAGCGTGGCAGGA-3' (forward), and 5'-ATTTTCGCTACACCACCTCGTCCAA-3' (reverse). Primer sequences for Rho2GTPase were as follows: 5'-CCAGAAGTGTACGTGCCAACTGT-3' (forward), and 5'-TTGGCTAATTCATGATGTGCCCCG-3' (reverse). Primers were designed to amplify a 205 bp fragment of the EC-SOD cDNA and a 302 bp fragment of the Rho2GTPase cDNA. All amplifications included appropriate controls (e.g., no template, no reverse transcriptase). Following a 1 min hot start at 94 °C, PCR conditions were as follows: 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, with the appropriate number of cycles determined empirically. Relative band intensities were quantified using Metamorph software.

Fig. 2.

Exposure of adult *S. mansoni* to NO induces upregulation of EC-SOD in both males and females. RT-PCR amplification of EC-SOD from (A) male control; (B) 3 h SNP-treated male parasites; (C) female control; (D) 3 h SNP-treated female parasites. Levels of 18S ribosomal RNA did not change in response to NO donor, and are used as a control (E–H). Primers for EC-SOD were as described in Fig. 1. Primers for 18S ribosomal RNA gene were as follows: 5'-TTAACGAGGACCAATTGGAGGG-3' (forward) and 5'-CCCCGTCTGTCCCTCTTAACCA-3' (reverse). Size of amplified bands for EC-SOD was 205 bp and 441 bp for 18S ribosomal RNA.

Table 1Successfully annotated, differentially expressed (R 1.5) SAGE tags, in order of decreasing R value

Tag ID	Frequency in control library	Frequency in SNP-treated library	R value	Fold change	Annotation
7301	10	0	3.07	Uniquely absent	Vacuolar ATP synthase 16 kDa proteolipid
3634	10	0	3.07	Uniquely absent	Perere non-LTR retrotransposon, Pol polyprotein
92	5	24	2.85	4.76	Hypothetical protein
7428	9	0	2.76	Uniquely absent	Reinfection-related protein
1891	0	9	2.6	Uniquely present	Similar to solute carrier family 39 (metal ion transporter), member 11
903	0	9	2.6	Uniquely present	Similar to fibrillar collagen chain FAp1 alpha
10542	8	0	2.5	Uniquely absent	Putative histamine-releasing factor/translationally controlled tumor protein
2963	8	0	2.4	Uniquely absent	Hypothetical protein
9	278	213	2.3	-1.30	Mitochondrial rRNA
220	8	27	2.2	3.35	Putative granulin precursor
1629	16	3	2.2	-5.29	Mitochondrial rRNA
93	50	24	2.2	-2.07	Mitochondrial rRNA
6413	7	0	2.2	Uniquely absent	Vacuolar ATP synthase 16 kDa proteolipid
50	19	44	2.1	2.30	Putative complement binding protein
20272	0	7	2.1	Uniquely present	Weak similarity to CNP2 (cyclic nucleotide phosphodiesterase)
775	7	0	2.1	Uniquely absent	Myosin light chain
16663	0	7	2.1	Uniquely present	Similar to p105 coactivator
8046	6	0	1.84	Uniquely absent	Hypothetical protein
3704	6	0	1.84	Uniquely absent	Putative SH3 domain-containing protein
602	6	0	1.84	Uniquely absent	Possible ligand-gated ion channel
4546	6	0	1.84	Uniquely absent	Hypothetical protein
1188	9	26	1.77	2.87	Putative epsilon subunit of coatamer protein complex
10302	0	6	1.76	Uniquely present	Hypothetical protein
9566	13	32	1.68	2.44	Possible AMBP protein precursor
90	9	1	1.65	-8.93	Putative dynein light chain 3
1385	9	25	1.61	2.76	Hypothetical protein
2873	5	18	1.615	3.57	Putative eukaryotic translation initiation factor 3 subunit
461	3	14	1.610	4.63	Putative calcineurin B
965	1	9	1.55	8.93	Hypothetical protein
4043	5	0	1.54	Uniquely absent	Putative Hunchback protein
7720	5	0	1.54	Uniquely absent	Putative coated vesicle membrane protein
601	5	0	1.54	Uniquely absent	putative Mf2 protein
5905	5	0	1.54	Uniquely absent	Putative cathepsin L-like protein
14649	5	0	1.53	Uniquely absent	Hypothetical protein
3302	5	0	1.53	Uniquely absent	Hypothetical protein
1142	5	0	1.54	Uniquely absent	Rac GTPase

Raw frequencies in each SAGE library are presented for each tag. Fold change includes correction for sampling depth, with negative symbols used for downregulation. SAGE libraries corresponding to control and SNP-exposed *S. mansoni* were constructed using 10µg total RNA isolated from adult *S. mansoni* following the I-SAGE Long kit protocol (Invitrogen), except for cloning of concatemers into pGEM3Z (Promega). Recombinant clones were purified and sequenced. Sequences collected were analyzed as in [25]. SAGE tags not detected more than once in at least one SAGE library were excluded from analyses as putative sequencing error, unless the tag sequence had a perfect match to all available genomic and EST data. The unique tag sequences were mapped to available *S. mansoni* DNA sequences to determine the identity of expressed genes. These included ongoing TIGR/Sanger genome assemblies, TIGR *S. mansoni* gene index sequences (<http://www.tigr.org/tdb/tgi>), sequences from the *S. mansoni* EST project (<http://verjo18.iq.usp.br/schisto/>), and *S. mansoni* entries in GenBank. The results were then visualized by use of a Generic Model Organism Database and the Gbrowse interface [26]. SAGE tags were scored for differential expression among the two libraries using the *R*-statistic [27]. Higher scores represent a greater deviation from the null hypothesis of equal frequencies, while scores close to zero represent near-constitutive expression. Genes with significant differential expression between the SAGE libraries were annotated by BLAST against the *Schistosoma mansoni* GeneDB (<http://www.genedb.org/genedb/smanson/>).