Schistosoma mansoni: Use of a fluorescent indicator to detect nitric oxide and related species in living parasites

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Running Title: NO in *S. mansoni*

ABSTRACT

Nitric oxide (NO) is synthesized enzymatically by nitric oxide synthase (NOS). Several groups have previously presented evidence for NOS activity and immunoreactivity in several parasitic platyhelminths, including schistosomes. Here, we use 4,5-diaminofluorescein-2 diacetate (DAF-2 DA), a fluorescent indicator of NO, to detect NO in living schistosomes. In adult worms, DAF-2 fluorescence is found selectively in epithelial-like cells. Fluorescence increases when worms are incubated in L-arginine, the precursor of NO synthesis, and decreases dramatically in the presence of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), indicating that predicted NO release may be NOS-dependent, and that enzymatic NO signaling pathways may play an important role in schistosome physiology.

Index descriptors: Nitric oxide, schistosomiasis, trematode.

Abbreviations:

Nitric oxide (NO)

4,5-diaminofluorescein-2 diacetate (DAF-2 DA)

N^G-nitro-L-arginine methyl ester (L-NAME)

Nitric oxide synthase (NOS)

Neuronal nitric oxide synthase (nNOS)

1. Introduction

NO is a widespread gaseous messenger molecule that has been implicated in multiple physiological functions, including neuronal communication, non-immune defense responses, cell survival and multiplication, and dilation of blood vessels in a wide variety of organisms (for reviews, see Moncada et al., 1991; Lowenstein and Snyder, 1992; Peunova et al., 1996; Mayer and Hemmens, 1997; Moroz, 2001; Torreilles, 2001; Bruckdorfer, 2005). On the other hand, NO and related radicals are very toxic species which physiologically contribute to defense against pathogens or other organisms. NO-related pathways therefore have the potential to play at least two critical roles in the schistosome life cycle. First, these pathways may represent essential signaling cascades that are required for normal physiological functioning of the parasite. Second, these pathways may also be important in parasite-host interactions, as NO cytotoxicity might be used by the parasite for non-immune defense against host responses to infection. If true, interference with these pathways by pharmacological agents could have significant effects on parasite life cycles.

An initial step in defining the potential role of NO in schistosomes is to establish the presence and distribution of components of the pathway in these parasites. In both vertebrate and invertebrate species, a significant fraction of NO is produced enzymatically by nitric oxide synthase (NOS), using O_2 and the amino acid L-arginine as precursors. Evidence for NO synthesis or NOS-like activity (*eg*, using the NADPH-diaphorase histochemical assay) has been found in a variety of parasitic platyhelminths, including *Diphyllobothrium dentriticum* (Lindholm *et al*. 1998; Gustafsson *et al*. 1998), *Hymenolepis diminuta* (Gustafsson *et al*. 1996, 1998; Terenina *et al*. 2000), *Mesocestoides vogae* (Terenina *et al*. 1999), *Fasciola hepatica* (Gustafsson *et al*. 2001), and *Fasciolopsi buski* (Tandon *et al*. 2001). In addition, we and others

have described the distribution of NOS immunoreactivity and NADPH-diaphorase activity in the human blood fluke, *Schistosoma mansoni* (Kohn *et al*. 2001; Long *et al.*, 2004). Radiometric and immunohistochemical studies in platyhelminths have demonstrated an NO-dependent rise in cGMP in specific target cells (Gustafsson *et al.*, 2003a, b; Terenina and Gustafsson, 2003; Onufriev *et al*., 2005), and Onufriev *et al*. (2005) used a spectrophotometric assay to provide evidence for NO release in *Hymenolepis diminuta*.

The diaminofluoresceins are a group of fluorescent indicators for NO (Kojima *et al.* 1998; Suzuki *et al*. 2002). DAF-2 DA is a membrane-permeable derivative. Once inside cells, the diacetate group is cleaved by intracellular esterases, and DAF-2 remains in the cell. DAF-2 emits green fluorescence (max = 515 nm) upon binding of NO and excitation by 495 nm light. Hence, DAF-2 DA measures intracellular NO, with a reported sensitivity of 5 nM (Kojima *et al.* 1998; though see Zhang *et al.*, 2002). In this report, we show that living adult schistosomes show prominent fluorescence in specific cells when incubated in DAF-2 DA. This DAF-2 fluorescence is NOS substrate- and inhibitor-sensitive, indicating that production of NO in schistosomes may be dependent on a NOS-like enzymatic activity.

2. Materials and methods

Female Swiss Webster mice infected with *S. mansoni* (NMRI strain) were obtained from the NIAID Schistosomiasis Resource Center at the Biomedical Research Institute in Rockville, MD. Adult *S. mansoni* were collected by perfusion, as described (Lewis 1998), and incubated in RPMI media (Invitrogen) for up to 24 h at 37°C in 5% CO_2 in the presence or absence of different NOS substrates and inhibitors (Sigma; see figure legends). Following addition of 2 μM DAF-2 DA (Calbiochem), the worms were incubated an additional hour at 37°C in the dark.

Living worms incubated in DAF-2 DA were examined for green fluorescence using a Leica fluorescent microscope, and digital images saved. In Figure 2, luminosity levels of the digital images captured from the microscope were adjusted (without user input) using the "automatic levels adjust" algorithm in Photoshop v. 6.0 (Adobe). For quantitation, fluorescent cells were counted in real-time.

3. Results

As shown in Figs. 1A and 2A, DAF-2 fluorescence is prominent and found in specific sites in live male worms (females have interfering autofluorescence, and were therefore not used). Since the fluorescence is being measured in living, moving worms, it is difficult to define the cell types showing DAF-2 fluorescence rigorously. However, some of the fluorescing cells appear to be in the area of the gut, which also show immunoreactivity against anti-iNOS in *S. mansoni* (Kohn et al., 2001, Figure 3), while others appear to be neuronal-like in their morphology and appear to be distributed in a pattern reminiscent of anti-nNOS immunoreactivity and NADPH-diaphorase activity (Kohn *et al*. 2001, Figures 1, 2, and 4). Structures that appear to be the termini of transepithelial processes at the surface of the worm are often visible. Indeed, in some cases, fluorescence in these sensory-like cell processes themselves can be distinguished in the live worms, consistent with our earlier morphological observations.

Incubation of adult male worms overnight in medium supplemented with 5 mM L-arginine, the substrate for NOS, results in an increase in fluorescence (see Figs. 1B, 3), particularly near the tegument of the worm. Interestingly, addition of 50 mM KCl to the media, which would be expected to depolarize cells and thus allow influx of Ca^{2+}, results in the rapid appearance of new fluorescing cells (data not shown). In contrast, worms incubated with N^{G}-

nitro-L-arginine methyl ester (L-NAME; 10 μM - 10 mM), an arginine analog that is a specific inhibitor of NOS, show a dramatic reduction in fluorescence (Figs. 1D, 2B, 3). Incubation in 10 mM of the D-isomer (D-NAME), which is a far less potent NOS inhibitor, produces no apparent reduction in fluorescence (Figs. 1C, 3).

We have quantitated these results by counting fluorescent cells in worms. As shown in Fig. 3, incubation of worms in L-arginine significantly increases the number of fluorescent cells. In contrast, incubation of worms in L-NAME results in a significant decrease in fluorescent cells. The number of fluorescent cells counted in worms incubated in D-NAME is not significantly different from the control.

4. Discussion

NO signaling pathways in schistosomes likely play important physiological roles in the parasite's life cycle. One way of delineating the functional significance of these pathways is to measure levels and distribution of authentic NO within the parasite. In this report, we have used DAF-2 fluorescence as an indicator of NO in living adult *S. mansoni*. The specificity and selectivity of this assay have been confirmed for various conditions both *in vivo* and *in vitro* (*eg*, Suzuki *et al*. 2002; Kasim *et al*. 2001; Leikert *et al*. 2001; Nakatsubo *et al*. 1998), although there is also evidence that DAF-2 reacts with dehydroascorbic acid and ascorbic acid (Zhang *et al.*, 2002).

Several groups, including ours, have previously described the distribution of NOS-like histochemical activity or immunoreactivity in a variety of parasitic flatworms. The assumption of these studies is that measurements of enzymatic NOS activity and presence of putative NOS will correlate with NO production. However, these approaches are indirect, and, furthermore, NO can

be produced non-enzymatically in cells from nitrites under acidic and reducing conditions (see Zweier *et al*. 1999 for review). Here, we provide a direct estimate of the presence and distribution of authentic NO in living schistosomes, and the effect of pharmacological agents known to specifically modify enzymatic NO production in mammals and insects.

DAF-2 fluorescence is prominent and found in restricted sites in adult schistosomes. Since the worms are alive and moving, it is difficult to precisely localize and identify DAF-2 fluorescent cell types reliably. However, many of the cells showing DAF-2-dependent fluorescence resemble the sensory-like cells identified as immunoreactive against mammalian nNOS antibodies (Kohn *et al*. 2001). Immunoreactivity against mammalian nNOS in schistosomes appears prominently in cells of the adult nervous system, including the main nerve cords and sensory-like cells in the periphery. A similar distribution of NOS activity, as determined from NADPH-diaphorase staining, is also found in *S. mansoni* adults.

DAF-2-dependent fluorescence is significantly increased when worms are incubated in media containing L-arginine, the substrate for NOS, and significantly decreased in the presence of the mammalian NOS inhibitor L-NAME. Inhibition is stereoselective, as no loss of fluorescence occurs in the presence of D-NAME, which is a far less potent inhibitor of all biochemically characterized NOSs. Other NOS inhibitors, such as L-N^5-(1-iminoethyl)-L-orinithine (L-NIO) and diphenyleneiodonium chloride (DPI) also affect levels of fluorescence, though not as dramatically (data not shown). These pharmacological effects on DAF-2 fluorescence in *S. mansoni*, along with the coincidence of DAF-2 fluorescence with NOS immunoreactivity and activity, support the notion that DAF-2 is indeed acting as an NO indicator in these cells. These results indicate that adult schistosomes might produce NO, likely via an L-arginine-dependent NOS activity, and also provide further support that NO signaling may be

playing important physiological roles in the parasite.

Acknowledgements

We thank Jessica Roberts-Misterly for excellent technical support. This work was supported by NIH grant NS 39103 and NSF grants 0304569 (LLM), and NIH grant AI 40522 and the Neal Cornell Research Fund at the Marine Biological Laboratory (RMG).

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FIGURE LEGENDS

Figure 1. DAF-2 fluorescence in live male schistosomes. Adult worms were incubated in RPMI media for up to 24 h at 37°C in a CO_2 incubator under different drug conditions. 2 μM DAF-2 DA was added, and incubation continued for 1 h at 37°C in the dark. The conditions were: **A**. medium alone; **B**. medium supplemented with the NO precursor, L-arginine (5 mM); **C**. medium supplemented with D-NAME (10 mM); and **D**. medium supplemented with the NOS inhibitor, L-NAME (10 mM). Scale bar = 50 μm.

Figure 2. Effects of 1 mM L-NAME on DAF-2 fluorescence in live adult schistosomes. Experiments were as in Fig. 1, except that the concentration of L-NAME was 1 mM. **A**. medium alone; **B**. medium supplemented with 1 mM L-NAME. L-NAME concentrations as low as 10 μM also result in virtual elimination of DAF-2 fluorescence. Scale bars = 50 μm.

Figure 3. Effects of NOS substrates and inhibitors on DAF-2 fluorescence. Live worms (n = 3 for each condition, except n = 4 for control) were incubated in media as described (Fig. 1), and the number of fluorescent cells was counted. Statistically significant differences from control (unpaired *t*-test, $P<0.01$) are denoted by an asterisk.

Figure1-grayscale

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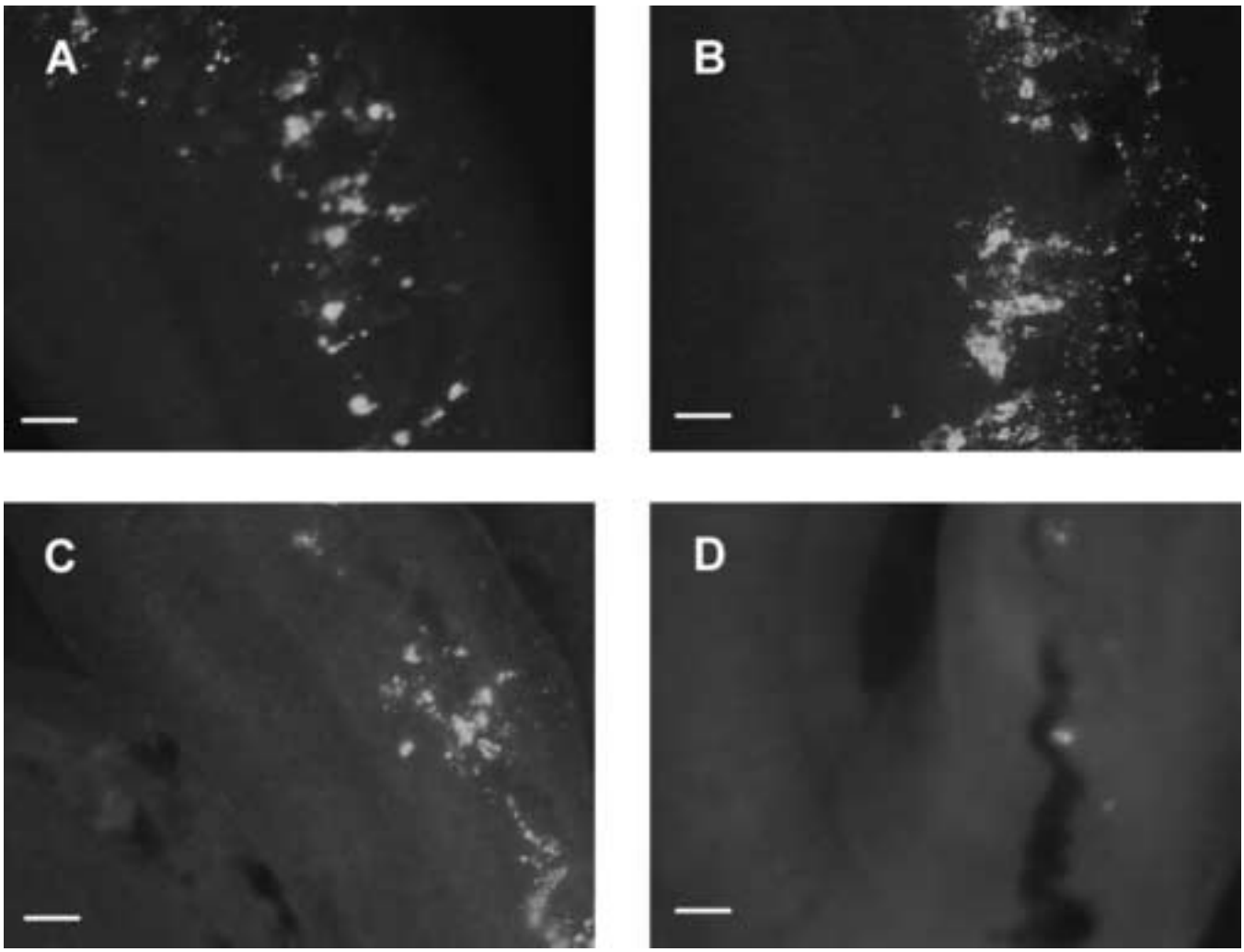

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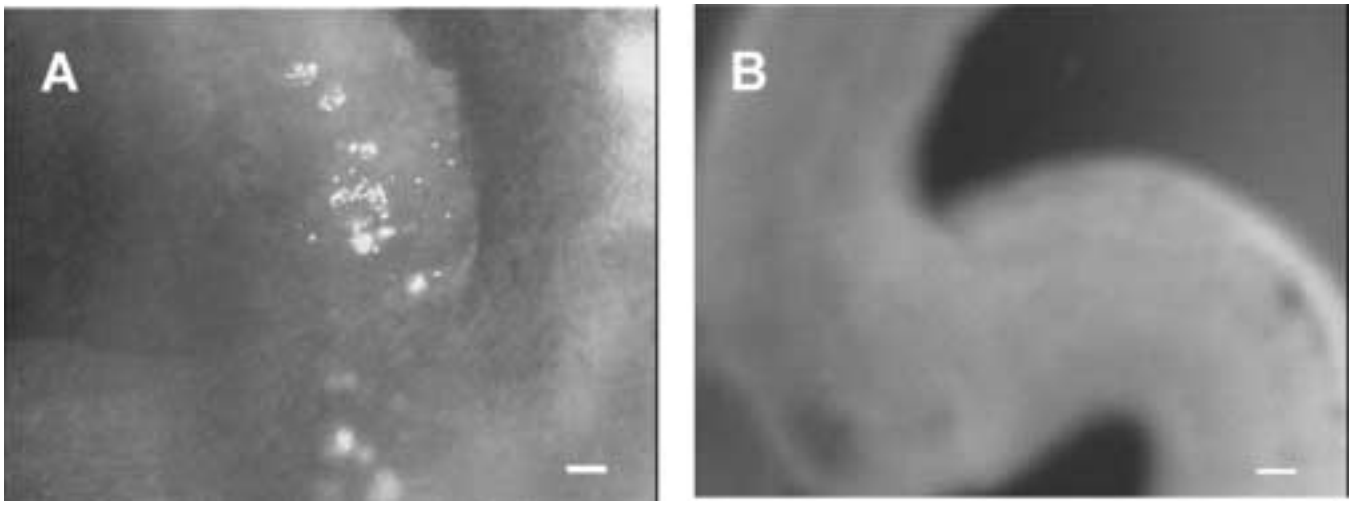

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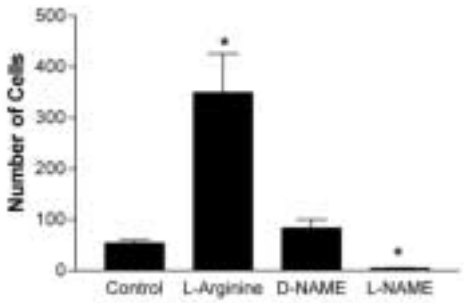

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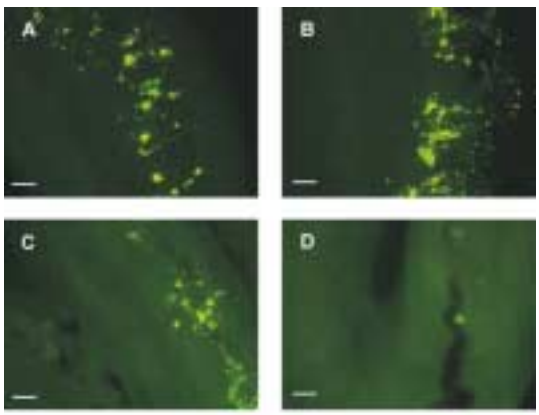

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