Distribution of nitric oxide synthase immunoreactivity in the nervous system and peripheral tissues of *Schistosoma mansoni*

A. B. KOHN[1], L. L. MOROZ[1,2], J. M. LEA[1] *and* R. M. GREENBERG*[1,2]

[1]*Whitney Laboratory and* [2]*Department of Neuroscience, University of Florida, 9505 Ocean Shore Blvd, St Augustine, FL 32086, USA*

(*Received 7 May 2000; revised 31 July 2000; accepted 1 August 2000*)

SUMMARY

The distribution of nitric oxide synthase (NOS) immunoreactivity and putative NOS activity in adult *Schistosoma mansoni* was analysed using 3 different types of NOS antibodies and NADPH-diaphorase histochemistry. Although potential involvement of the gaseous radical nitric oxide (NO) in host response to infection by schistosomes has been suggested, there is little or no information available regarding the role, or even the presence, of the NO pathway in schistosomes themselves. Here, we demonstrate that antibodies against neuronal NOS (nNOS) and inducible NOS (iNOS) isoforms stain adult worms with distinctive patterns; anti-endothelial NOS (eNOS) shows no selective labelling. nNOS-like immunoreactivity is found in the main nerve cords and the peripheral nervous system. Putative sensory neurons with apical neuronal processes leading to the tegument of male worms are also immunoreactive for nNOS. Anti-iNOS labels a variety of predominantly non-neuronal tissues, showing intense labelling at or near the surface of the worm and in components of the gastrointestinal tract. The distribution of NADPH-diaphorase reactivity (a histochemical marker of NOS), is generally similar to the pattern of NOS immunoreactivity, including labelling of neuronal-like cells as well as developing eggs. These results suggest that an NOS-like enzyme is present in *S. mansoni*, and indicate potential roles for the different NOS isoforms in neuronal signalling, reproduction and development.

Key words: *Schistosoma mansoni*, nitric oxide, nitric oxide synthase, NADPH-diaphorase, platyhelminths, sensory systems.

INTRODUCTION

The nitric oxide (NO) signalling system is a metabolic pathway in parasites that may be particularly vulnerable to pharmacological intervention. NO is a gaseous intercellular signalling molecule that has been implicated in multiple physiological functions, including neuronal communication, non-immune defence responses, cell survival and multiplication, and dilation of blood vessels (for reviews, see Moncada, Palmer & Higgs, 1991; Culotta & Koshland, 1992; Lowenstein & Snyder, 1992; Peunova *et al*. 1996; Mayer & Hemmens, 1997). NO is produced enzymatically by nitric oxide synthase (NOS), using O_2 and the amino acid L-arginine as precursors. Three types of mammalian isoforms of NOS are encoded by 3 different genes (reviewed by Griffith & Stuehr, 1995). These include neuronal (nNOS; type I), inducible (iNOS; type II), and endothelial (eNOS; type III) isoforms. In mammals, nNOS and eNOS are Ca^{2+} dependent and constitutive; iNOS is Ca^{2+} independent, and is primarily involved in defence reactions and cytotoxicity (Nathan, 1992). Although the number and function of NOS isoforms in invertebrates is not entirely clear, they are implicated in both neuronal and non-neuronal functions similar to those found in mammals (see review by Moroz, 2000).

An extensive number of studies in the literature indicate that part of a host's response to parasitic infection involves production of NO, which can have cytotoxic and cytostatic activities (reviewed by Liew, Wei & Proudfoot, 1997). NO has been suggested to play a role in host response to schistosome infection (James, 1995; Oswald & James, 1996), but the significance of that role is not clear (Coulson *et al*. 1998). Evidence for NO synthesis or NOS-like activity is also found in a variety of parasites, including trypanosomes (Paveto *et al*. 1995; Pereira *et al*. 1997), parasitic flatworms (Gustafsson *et al*. 1996; Lindholm, Reuter & Gustafsson 1998; Terenina *et al*. 1999, 2000), and nematodes (Bowman *et al*. 1995; Bascal *et al*. 1995, 1996; Mupanomunda *et al*. 1997; Kaiser, Geary & Williams 1998; Pfarr & Fuhrman, 2000), and may be employed as a defence against host responses to infection.

In this study, we have demonstrated the presence of NOS immunoreactivity in *S. mansoni*, using

* Corresponding author: Whitney Laboratory, 9505 Ocean Shore Blvd, St Augustine, FL 32086, USA. Tel: +904 461 4026. Fax: +904 461 4008. E-mail: rmg@whitney.ufl.edu

 © 2001 Cambridge University Press
https://doi.org/10.1017/S0031182000007022 Published online by Cambridge University Press

antibodies specific to each of the 3 mammalian NOS isoforms to immunolabel tissues in adult parasites. Two of these antibodies stained the worms with characteristic distributions, indicating the presence of 2 structurally and functionally distinct NOS-like isoforms in the worms. Furthermore, NADPH-diaphorase staining, a distinct histochemical marker of NOS enzyme activity, correlated in general with the labelling pattern of the anti-NOS antibodies. NADPH-diaphorase (NADPH-d) staining is a standard histochemical method for detecting NOS enzymatic activity under defined fixation conditions (Dawson *et al*. 1991; Hope *et al*. 1991). The NADPH-d activity of NOS, unlike other NADPH-d enzyme activities, is resistant to fixation with aldehydes (Matsumoto *et al*. 1993; Blottner, Grozdanovic & Gossrau, 1995).

These results indicate that NO is a potential signalling molecule in *S. mansoni*, and is likely to be playing an important role in realization of the schistosome life-cycle.

MATERIALS AND METHODS

Adult *S. mansoni* were collected from freshly perfused Swiss Webster female mice. Schistosome-infected mice were obtained from Dr Fred A. Lewis via the NIH extramural contract from the NIH repository for *S. mansoni*, the Biomedical Research Institute in Rockville, MD.

Immunocytochemistry

Adult worms were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7·0. The tissue was rinsed with PBS 3 times for 10 min each, then incubated in 10% sucrose in PBS at 4 °C overnight. Whole worms were embedded in Tissue Tek O.C.T. compound, and 12 μm thick sections were cut and placed onto gelatin-coated slides. Slides were air dried and stored at −80 °C until ready for use. After thawing, the tissue was blocked with PBS+0·25% Triton X-100 (PBST)+0·25% normal goat serum for 30 min at 37 °C in a humid chamber. The tissue was then incubated with 1 of 3 different polyclonal antibodies raised against the 3 mammalian NOS isoforms (Nitric Oxide Synthase Antibody Set, Calbiochem). This set includes anti-brain NOS (anti-bNOS, here called anti-nNOS), anti-endothelial NOS (anti-eNOS), and anti-inducible NOS (anti-iNOS). The dilutions for the polyclonal rabbit antibodies were: 1:800 for the anti-nNOS; 1:250 for the anti-eNOS; and 1:500 for the anti-iNOS. Tissue was incubated in the appropriate dilution of antibody in PBST+0·25% normal goat serum overnight at 4 °C, then rinsed 3 times with PBST for 10 min each rinse. The tissue was then incubated in the secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, Jackson Immunoresearch Laboratories) at a 1:50 dilution in PBST+0·25% normal goat serum for 1 h at room temperature and subsequently rinsed 3 times with PBS for 10 min each rinse. Finally the tissue was counterstained with 1% Evans Blue in PBS for 20 sec, rinsed twice with PBS for 20 min each rinse, and mounted with 90% glycerol in PBS+*para*-phenylenediamine (PPD).

Approximately 20 individual adult worms were stained and examined. Controls included incubation in secondary antibody only, which resulted in no signal, and incubation in the presence of excess antigenic peptide (8 μg for nNOS, 16 μg for iNOS), which resulted in a 3-fold (anti-nNOS) or 2-fold (anti-iNOS) reduction in median pixel intensity ($n = 2$).

NADPH-diaphorase histochemistry

Tissue was treated as for the antibody staining, with the exception that worms were fixed in 4% paraformaldehyde for varying times ranging from 2 to 16 h. Cryostat sections (12 μm) were incubated in a solution of 50 mM Tris (pH 8·0), 0·5 mM nitroblue tetrazolium (NBT), 1 mM β-NADPH, and 0·3% Triton X-100 for 1·5 h at 4 °C. Serial sections from approximately 8 different animals were examined. The effect of different fixation times was examined, with 10–16 h fixations proving to be optimal. Controls included reactions containing no NBT or no β-NADPH.

RESULTS

Immunohistochemistry of different NOS isoforms in S. mansoni

Antibodies against each of 3 mammalian isoforms of NOS were used to stain sectioned adult tissue. For these studies, only males were used because females exhibit significant autofluorescence. As summarized in Fig. 1, 2 of the 3 anti-NOS antibodies (nNOS and iNOS) selectively stained adult *S. mansoni* males, and they did so with characteristic distribution patterns. Staining of both isoforms was specifically reduced by pre-absorption with antigenic peptide. Both antibodies also reproducibly stained bands of ~200 kDa, and sometimes also faint bands of ~110 kDa, on Western blots of adult worm proteins (data not shown), a size range consistent with that found for other invertebrate preparations (Hurst *et al*. 1999).

The antibody against nNOS showed staining of cells throughout the nervous system of the worm (Figs 1 and 2). Prominently stained cells were found in the main nerve cords (MCs; Halton & Gustafsson, 1996) as well as in the peripheral nerve net. Many of

https://doi.org/10.1017/S0031182000007022 Published online by Cambridge University Press

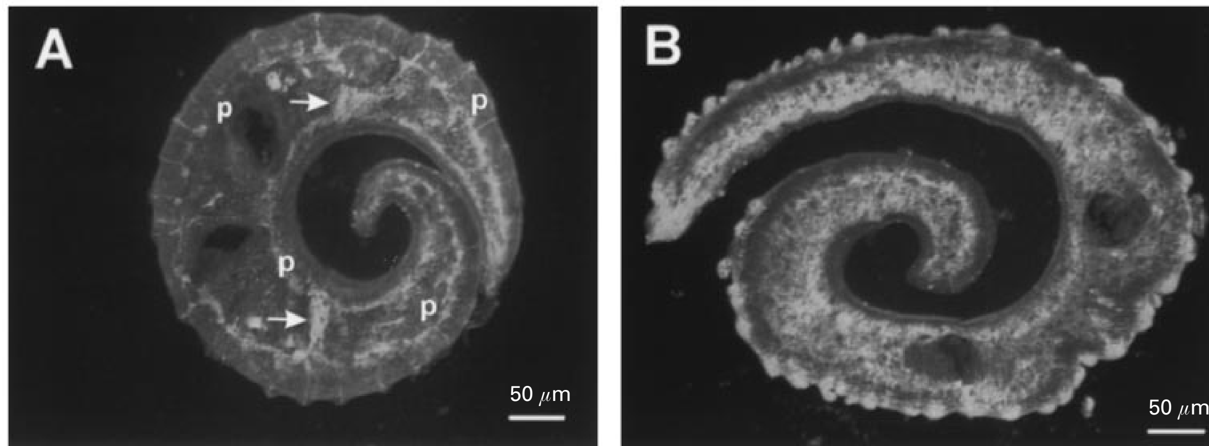

Fig. 1. NOS immunoreactivity in adult *Schistosoma mansoni*. Staining of mid-body cross-sections of adult male worms with antibodies against nNOS (A) and iNOS (B). Note the different staining patterns between A and B. Prominent labelling with anti-nNOS is found throughout the nervous system (A), including the main nerve cords (arrows) and the peripheral nervous system (p). Staining with eNOS appeared to be non-selective, and is not shown.

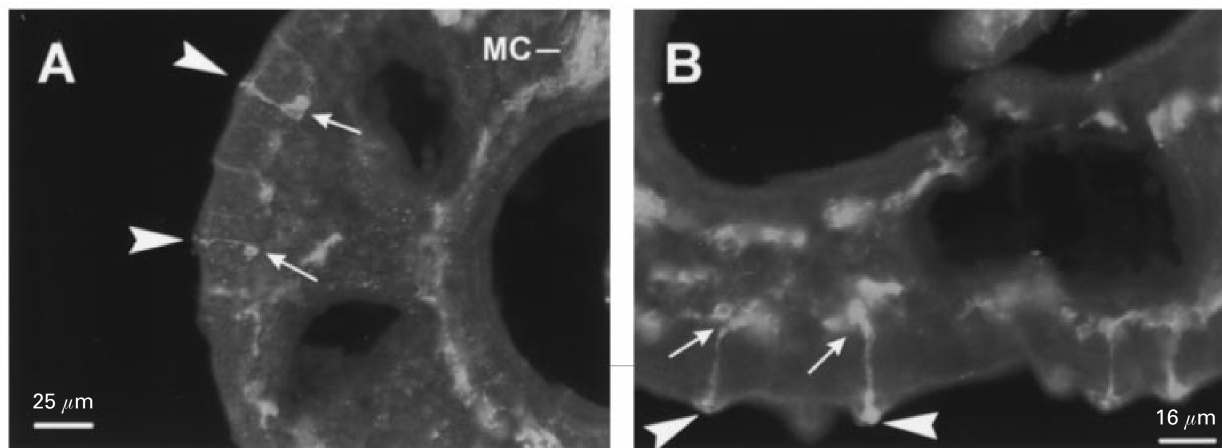

Fig. 2. Neuronal NOS immunolabelling in adult *Schistosoma mansoni*. (A) Adult male section. Labelling of the peripheral nervous system is apparent, and one of the main nerve cords is noted (MC). Note the neuronal processes (arrows) terminating at sensory structures at or near the surface (arrowheads). (B) Higher power close-up of labelling, focusing on the plexus surrounding the longitudinal muscles. Note the bipolar cells (arrows), and the apical processes leading to the surface (arrowheads).

the labelled cells in the peripheral nerve net were bipolar, with prominent morphological characteristics of sensory cells. Apical processes from these cells (20–30 μm) extended to the dorsal tegument and ended in a sensory receptor (Fig. 2). In many cases, we detected ciliated transepidermal processes, characteristic of the primary receptor-like cells on the surface of the worm (Fig. 2B). These putative sensory receptors were far more abundant along the dorsal surface than along the ventral surface, and the processes leading to the dorsal surface often terminated in tegumental protuberances (Fig. 2). Most of the stained cell bodies had an estimated diameter ranging from 5 to 15 μm.

The antibody against iNOS showed the majority of staining around the outer surface of the worm (Figs 1B and 3), and most particularly on protuberances such as the tubercles and papillae (Fig. 3) on the dorsal surface of male worms. This pattern of iNOS immunoreactivity may indicate that this NOS isoform is involved in generating NO targeted towards the outside of the worm. In addition, anti-iNOS prominently labelled dorsal subtegumental tissue, but was specifically excluded from the layer of muscle fibres just below the tegument. iNOS immunoreactivity was also found in cell layers of the gut (Fig. 3).

NADPH-diaphorase staining of S. mansoni

We used this histochemical marker to determine the distribution of putative NOS activity in adult worms. Staining in male adult worms was similar to that for the combined nNOS and iNOS antibodies. Labelling of neuronal-like cells, dorsal subtegumental regions, and other areas of the worm was apparent

https://doi.org/10.1017/S0031182000007022 Published online by Cambridge University Press

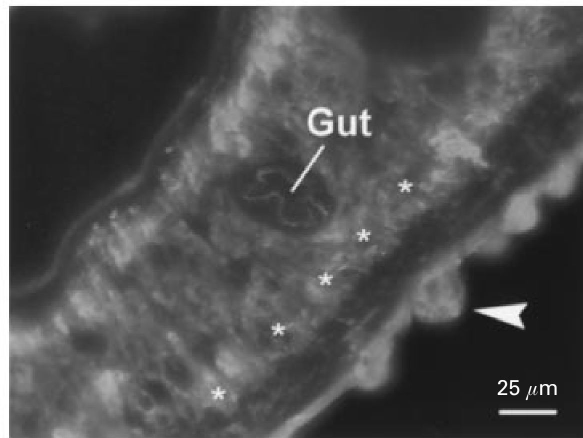

Fig. 3. Inducible NOS immunolabelling in *Schistosoma mansoni*. Staining of adult male worm section with anti-iNOS. Note intense staining of the worm's dorsal surface, particularly surface protuberances (arrowhead). Note also labelling of a subtegumental layer and staining in cell layers of the gut. Asterisks indicate areas in which labelling represents a combination of specific, green fluorescence and yellow, non-specific autofluorescence.

(Fig. 4A). In females, developing eggs in the vitellariae also stained very prominently (Fig. 4B), indicating that, at least while in the adults, eggs and developing eggs have NADPH-d activity.

DISCUSSION

NO is involved in a multitude of functions, including neuronal signalling, feeding, and non-immune defence mechanisms (for reviews, see Moncada *et al*. 1991; Lowenstein & Snyder, 1992; Peunova *et al*. 1996; Mayer & Hemmens, 1997). In this report, we present evidence for the NOS-dependent pathway in adult *S. mansoni* using both immunolabelling and NADPH-diaphorase staining. Antibodies against nNOS and iNOS stain adult male worms selectively, and with strikingly different distributions, indicating the possibility of functionally distinct NOS isoforms in schistosomes.

The antibody against nNOS stains mainly neuronal structures, including the main nerve cords and what appear to be sensory neurons with processes innervating surface structures of male worms. The pattern of staining with anti-nNOS is very similar to that found for substance-P immunoreactive cells in adult worms, although the distribution has similarities as well with that found for other neuropeptides and serotonin (Gustafsson, 1987). It will be interesting to determine by double immunolabelling how closely these patterns overlap. Interestingly, comparative analysis of the distribution of putative nitrergic (NO-producing) neurons reveals a similar pattern of NOS distribution in various sensory systems of invertebrates, again suggesting a sensory (chemosensory) role for neuronal NO in adult worms (reviewed by Moroz, 2000).

In contrast, the antibody against iNOS, the isoform most associated with defence mechanisms (Nathan, 1992), prominently labels predominantly non-neuronal cells, including structures at or near the surface of the worm. Labelling is also apparent in the gut region, perhaps indicating a role for iNOS in feeding and digestion in adult worms.

These antibody staining results are confirmed and extended using fixative-resistant NAPDH-diaphorase reactivity, which is widely used as a histochemical marker of NOS activity. Positive NADPH-d staining has been used to detect catalytic activity associated with both nNOS (Dawson *et al*. 1991; Hope *et al*. 1991) and iNOS (see, for example, Ding *et al*. 1997 and Reichner *et al*. 1999). The

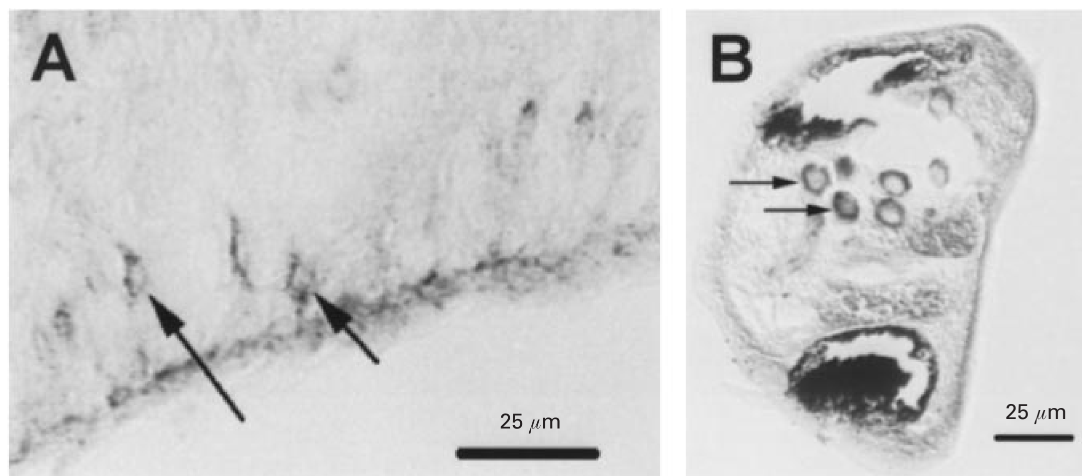

Fig. 4. NADPH-diaphorase reactivity in *Schistosoma mansoni*. (A) Section of adult male worm. Arrows denote cell structures resembling the bipolar neurons stained by anti-nNOS (Fig. 2). Prominent staining is also apparent just beneath the tegument. (B) Cross-section of female adult worm. Arrows show high level of NADPH-d reactivity on the surface of developing eggs in the vitelline gland.

https://doi.org/10.1017/S0031182000007022 Published online by Cambridge University Press

pattern of NADPH-d staining in *S. mansoni* adults is similar to that seen with the antibodies, particularly the anti-nNOS antibody. In addition, developing eggs in females stain very prominently, indicating a potential role for NOS in schistosome reproduction and development.

Evidence for NOS activity has been reported for other platyhelminths. Gustafsson *et al.* (1996), Lindholm *et al.* (1998) and Terenina *et al.* (1999) have examined the pattern of NADPH-d reactivity in, respectively, *Hymenolepis diminuta* adults, *Diphyllobothrium dendriticum* adults and *Mesocestoides vogae* tetrahyridia. Similar to what we find in *S. mansoni* adults, extensive NADPH-d reactivity was found in the nervous systems of both *H. diminuta* (Gustafsson *et al.* 1996) and *D. dendriticum* (Lindholm *et al.* 1998) adults, including prominent labelling of the nerve cords and of sensory-like neuronal processes extending to the tegument. More recently, Terenina *et al.* (2000) have demonstrated NOS activity in homogenates of *H. diminuta* using conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as their assay. This activity was partially sensitive to Ca^{2+}, and partially inhibited by 1 mM N^G-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS.

The function of NOS in platyhelminths is not clear, though enzymatically produced NO is presumably acting as an intercellular signalling molecule, as it does in mammals. Terenina *et al.* (1999) have described behavioural effects of L-arginine and the NOS inhibitor N^G-nitro-L-arginine on cluster formation in *M. vogae* tetrahyridia. Interestingly, Mupanomunda *et al.* (1997) have shown that the filarial parasite *Dirofilaria immitis* alters the relaxation behaviour of endothelial cells of the pulmonary artery, and that NO is implicated in this effect. It will be interesting to determine whether schistosome NO is used to mediate these or other types of changes in host physiology.

The role of NO production in the host response to schistosome infection is unclear. A large body of work indicates that NO plays a role in protective immunity, with apparent involvement in the elimination of lung-stage schistosomulae from hosts (reviewed by Oswald, 1999). According to these models, IFN-γ-activated macrophages up-regulate iNOS and produce higher levels of NO, which has cytotoxic effects on the parasites (reviewed by Oswald, 1999). However, although administration of NOS inhibitors (Wynn *et al.* 1994; Coulson *et al.* 1998) or infection of iNOS-deficient mice (Coulson *et al.* 1998; James *et al.* 1998) results in somewhat lowered resistance, that decrease is only slight, and is far less than would be expected if the NO pathway played a dominant role in worm elimination. Although the data presented here do not speak directly to the role of host NO in schistosome infections, the fact that schistosomes themselves appear to be using NO as a signalling molecule adds another complicating factor to this issue. Analysis of the schistosome targets of NO action, as well as a more complete biochemical and molecular characterization of schistosome NOS isoforms, may help resolve some of these questions.

We thank Lynn Milstead for assistance with manuscript preparation and Fred A. Lewis, Biomedical Research Institute, for providing schistosome-infected mice. We also thank Michael J. Greenberg for helpful discussions. This work was supported by NIH grant no. AI 40522 to R.M.G. and NIH grant no. NS 39103 to L.L.M.

REFERENCES

BASCAL, Z. A., MONTGOMERY, A., HOLDEN-DYE, L., WILLIAMS, R. G., THORNDYKE, M. C. & WALKER, R. J. (1996). NADPH diaphorase activity in peptidergic neurones of the parasitic nematode, *Ascaris suum*. *Parasitology* **112**, 125–134.

BASCAL, Z. A., MONTGOMERY, A., HOLDEN-DYE, L., WILLIAMS, R. G. & WALKER, R. J. (1995). Histochemical mapping of NADPH diaphorase in the nervous system of the parasitic nematode *Ascaris suum*. *Parasitology* **110**, 635–637.

BLOTTNER, D., GROZDANOVIC, Z. & GOSSRAU, R. (1995). Histochemistry of nitric oxide synthase in the nervous system. *Histochemical Journal* **27**, 785–811.

BOWMAN, J. W., WINTERROWD, C. A., FRIEDMAN, A. R., THOMPSON, D. P., KLEIN, R. D., DAVIS, J. P., MAULE, A. G., BLAIR, K. L. & GEARY, T. G. (1995). Nitric oxide mediates the inhibitory effects of SDPNFLRFamide, a nematode FMRFamide-related neuropeptide, in *Ascaris suum*. *Journal of Neurophysiology* **74**, 1880–1888.

COULSON, P. S., SMYTHIES, L. E., BETTS, C., MABBOTT, N. A., STERNBERG, J. M., WEI, X-G., LIEW, F. Y. & WILSON, R. A. (1998). Nitric oxide produced in the lungs of mice immunized with the radiation-attenuated schistosome vaccine is not the major agent causing challenge parasite elimination. *Immunology* **93**, 55–63.

CULOTTA, E. & KOSHLAND, D. E. (1992). NO news is good news. *Science* **258**, 1862–1865.

DAWSON, T. M., BREDT, D. S., GOTUHI, M., HWANG, P. M. & SNYDER, S. H. (1991). Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proceedings of the National Academy of Sciences, USA* **88**, 7797–7801.

DING, M., ST. PIERRE, B. A., PARKINSON, J. F., MEDBERRY, P., WONG, J. L., ROGERS, N. E., IGNARRO, L. J. & MERRILL, J. E. (1997). Inducible nitric oxide synthase and nitric oxide production in human fetal astrocytes and microglia. *Journal of Biological Chemistry* **272**, 11327–11335.

GRIFFITH, O. W. & STUEHR, D. J. (1995). Nitric oxide synthases: properties and catalytic mechanism. *Annual Review of Physiology* **57**, 707–736.

GUSTAFSSON, M. K. S. (1987). Immunocytochemical demonstration of neuropeptides and serotonin in the nervous systems of adult *Schistosoma mansoni*. *Parasitology Research* **74**, 168–174.

https://doi.org/10.1017/S0031182000007022 Published online by Cambridge University Press

GUSTAFSSON, M. K. S., LINDHOLM, A. M., TERENINA, N. B. & REUTER, M. (1996). NO nerves in a tapeworm. NADPH-diaphorase histochemistry in adult *Hymenolepis diminuta*. *Parasitology* **113**, 559–565.

HALTON, D. W. & GUSTAFSSON, M. K. S. (1996). Functional morphology of the platyhelminth nervous system. *Parasitology* **113**, S47–S72.

HOPE, B. T., MICHAEL, G. J., KNIGGE, K. M. & VINCENT, S. R. (1991). Neuronal NADPH-diaphorase is a nitric oxide synthase. *Proceedings of the National Academy of Sciences, USA* **88**, 2811–2814.

HURST, W. J., MOROZ, L. L., GILLETTE, M. U. & GILLETTE, R. (1999). Nitic oxide synthase immunolabelling in the molluscan CNS and peripheral tissues. *Biochemical and Biophysical Research Communications* **262**, 545–548.

JAMES, S. L. (1995). Role of nitric oxide in parasitic infections. *Microbiological Reviews* **59**, 533–547.

JAMES, S. L., CHEEVER, A. W., CASPAR, P. & WYNN, T. A. (1998). Inducible nitric oxide synthase-deficient mice develop enhanced type 1 cytokine-associated cellular and humoral immune responses after vaccination with attenuated *Schistosoma mansoni* cercariae but display partially reduced resistance. *Infection and Immunity* **66**, 3510–3518.

KAISER, L., GEARY, T. G. & WILLIAMS, J. F. (1998). *Dirofilaria immitis* and *Brugia pahangi*: Filiarial parasites make nitric oxide. *Experimental Parasitology* **90**, 131–134.

LIEW, F. Y., WEI, X. Q. & PROUDFOOT, L. (1997). Cytokines and nitric oxide as effector molecules against parasitic infections. *Philosophical Transactions of the Royal Society of London, B* **352**, 1311–1315.

LINDHOLM, A. M., REUTER, M. & GUSTAFSSON, M. K. S. (1998). The NADPH-diaphorase staining reaction in relation to the aminergic and peptidergic nervous system and the musculature of adult *Diphyllobothrium dentriticum*. *Parasitology* **117**, 283–292.

LOWENSTEIN, C. J. & SNYDER, S. H. (1992). Nitric oxide, a novel biological messenger. *Cell* **70**, 705–707.

MATSUMOTO, T., NAKANE, M., POLLOCK, J. S., KUK, J. E. & FORSTERMANN, U. (1993). A correlation between soluble brain nitric oxide synthase and NADPH-diaphorase activity is only seen after exposure of the tissue to fixative. *Neuroscience Letters* **155**, 61–64.

MAYER, B. & HEMMENS, B. (1997). Biosynthesis and action of nitric oxide in mammalian cells. *Trends in Biochemical Sciences* **22**, 477–481.

MONCADA, S., PALMER, R. M. & HIGGS, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* **43**, 109–142.

MOROZ, L. L. (2000). On the origin and early evolution of neuronal NO signaling: a comparative analysis. In *Nitric Oxide and Free Radicals in Peripheral Neurotransmission* (ed. Kalsner, S.), pp. 1–34. Springer-Verlag, New York.

MUPANOMUNDA, M., WILLIAMS, J. F., MACKENZIE, C. D. & KAISER, L. (1997). *Dirofilaria immitis*: heartworm infection alters pulmonary artery endothelial cell behavior. *Journal of Applied Physiology* **82**, 389–398.

NATHAN, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB Journal* **6**, 3051–3064.

OSWALD, I. P. (1999). Nitric oxide in schistosomiasis. In *Nitric Oxide and Infection* (ed. Fang, F. C.), pp. 343–360. Kluwer Academic/Plenum Publishers, New York.

OSWALD, I. P. & JAMES, S. L. (1996). Nitrogen oxide in host defense against parasites. *Methods* **10**, 8–14.

PAVETO, C., PEREIRA, C., ESPINOSA, J., MONTAGNA, A. E., FARBER, M., ESTEVA, M., FLAWIA, M. M. & TORRES, H. N. (1995). The nitric oxide transduction pathway in *Trypanosoma cruzi*. *Journal of Biological Chemistry* **14**, 16576–16579.

PEREIRA, C., PAVETO, C., ESPINOSA, J., ALONSO, G., FLAWIA, M. M. & TORRES, H. N. (1997). Control of *Trypanosoma cruzi* epimastigote motility through the nitric oxide pathway. *Journal of Eukaryotic Microbiology* **44**, 155–156.

PEUNOVA, N., KUZIN, B., ROBERTS, I., O'KANE, C. & ENIKOLOPOV, G. (1996). Nitric oxide, cell multiplication and cell survival. *Cold Spring Harbor Symposia on Quantitative Biology* **61**, 417–426.

PFARR, K. M. & FUHRMAN, J. A. (2000). *Brugia malaya*: Localization of nitric oxide synthase in a lymphatic filariid. *Experimental Parasitology* **94**, 92–98.

REICHNER, J. S., MESZAROS, A. J., LOUIS, C. A., HENRY, W. L., MASTROFRANCESCO, B., MARTIN, B-A. & ALBINA, J.E. (1999). Molecular and metabolic evidence for the restricted expression of inducible nitric oxide synthase in healing wounds. *American Journal of Pathology* **154**, 1097–1104.

TERENINA, N. B., ONUFRIEV, M. V., GULYAEVA, N. V., LINDHOLM, A. M. & GUSTAFSSON, M. K. S. (2000). A radiometric analysis of nitric oxide synthase activity in *Hymenolepis diminuta*. *Parasitology* **120**, 91–95.

TERENINA, N. B., REUTER, M. & GUSTAFSSON, M. K. S. (1999). An experimental, NADPH-diaphorase histochemical and immunocytochemical study of *Mesocestoides vogae* tetrathyridia. *International Journal for Parasitology* **29**, 787–798.

WYNN, T. A., OSWALD, I. P., ELTOUM, I., CASPAR, P., LOWENSTEIN, C. J., LEWIS, F. A., JAMES, S. L. & SHER, A. (1994). Elevated expression of Th1 cytokines and NO synthase in the lungs of vaccinated mice after challenge infection with *Schistosoma mansoni*. *Journal of Immunology* **153**, 5200–5209.

https://doi.org/10.1017/S0031182000007022 Published online by Cambridge University Press