Nitric oxide synthase activity in *Fasciola hepatica*: a radiometric study

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SUMMARY

The activity of neuronal nitric oxide synthase (nNOS) in homogenates of adult *Fasciola hepatica* was measured by the direct radiometric assay of the production of L-[^{3}H]citrulline. This is the first radiometric study of the activity of nNOS in a fluke. The effect of arginase was tested. In the presence of L-valine, which is an inhibitor of arginase, the formation of L-[^{3}H]citrulline decreased from 12% to 38%, depending on the time of incubation. This means that the arginase activity in the worm is high, and has to be taken into consideration when measuring the activity of nNOS. When co-factors, such as H4B, and NADPH, were omitted the formation of L-[^{3}H]citrulline decreased significantly (29%). The effects of several nNOS inhibitors were tested. N(ω)-nitro-L-arginine (L-NAME), aminoguanidine and S-methyl-L-thiocitrulline added at a concentration of 1 mM inhibited the L-[^{3}H]citrulline formation by 28%, 15% and 14%, respectively. Chelation of Ca^{2+} with 1 mM EGTA resulted in a 40% decrease in the formation of L-[^{3}H]citrulline. These results indicate the presence of nNOS activity in homogenates of *F. hepatica*.

Key words: *Fasciola hepatica*, nitric oxide, nitric oxide synthase, nitric oxide synthase inhibitors.

INTRODUCTION

The free radical nitric oxide (NO) is a water-soluble gas with diverse biological functions, including the ability to serve as a neuronal messenger (for references see Woodside & Amir, 2000; Young, Anderson & Furness, 2000). NO is generated from L-arginine and molecular oxygen by a family of highly regulated enzymes, the nitric oxide synthases (NOSs) (for references see Ignarro & Jacobs, 2000). Three main forms of NOS occur, each produced by different genes (Sessa, 1994): neuronal (type I, nNOS), inducible (type II or macrophage, iNOS), and endothelial (type III, eNOS). All NOSs require reduced nicotinamide adenine dinucleotide phosphate (NADPH) to be enzymatically active and possess NADPH diaphorase (NADPH-d) activity (for references see Vincent, 2000). nNOS and eNOS are Ca^{2+} dependent, whereas iNOS in most tissues is Ca^{2+} independent.

The production of NO can be detected either directly by NO chemiluminiscence (Marletta *et al.* 1988), or indirectly by the determination of L-citrulline, the second product of the NOS reaction, using high pressure liquid chromatography (HPLC), radiometric assay (Bredt & Snyder, 1990), or other methods. The NADPH-d staining is commonly used to localize NOS (for references see Vincent, 2000).

The occurrence of nNOS in invertebrates has been documented since 1993 (for references see Gustafsson *et al.* 2001). The first observation of NADPH-d positive nerve cells and fibres in a parasitic flatworm was made by Gustafsson *et al.* (1996) in adult *Hymenolepis diminuta*. A radiometric analysis of the nNOS activity confirmed the occurrence of nNOS in *H. diminuta* (Terenina *et al.* 2000). NADPH-d positive nerve cells and fibres have also been observed in the tapeworms, *Diphyllobothrium dendriticum* (Lindholm *et al.* 1998) and *Eubothrium rugosum* (Terenina *et al.* 1998), in the flukes, *Azygia lucii*, *Haplometra cylindracea* (Terenina *et al.* 1998), *Fasciola hepatica* (Gustafsson *et al.* 2001) and *Fasciolopsis buski* (Tandon, Kar & Saka, 2001; Kar, Tandon & Saha, 2002), and in cercaria of *Diplostomum chromatophorum* (Terenina & Gustafsson, 2002). In the free-living flatworm *Girardia tigrina*, NADPH-d positive fibres were detected in the pharyngeal musculature and the presence of NOS was proved by HPLC analysis of the L-citrulline production (Eriksson, 1996). NADPH-d staining has also been reported in the nervous system of the nematode *Ascaris suum* (Bascal *et al.* 1995, 1996). Interesting results were recently obtained by Kar *et al.* (2002) in their study of the genistein-induced effect on the activity of nNOS in *F. buski*. Treatment

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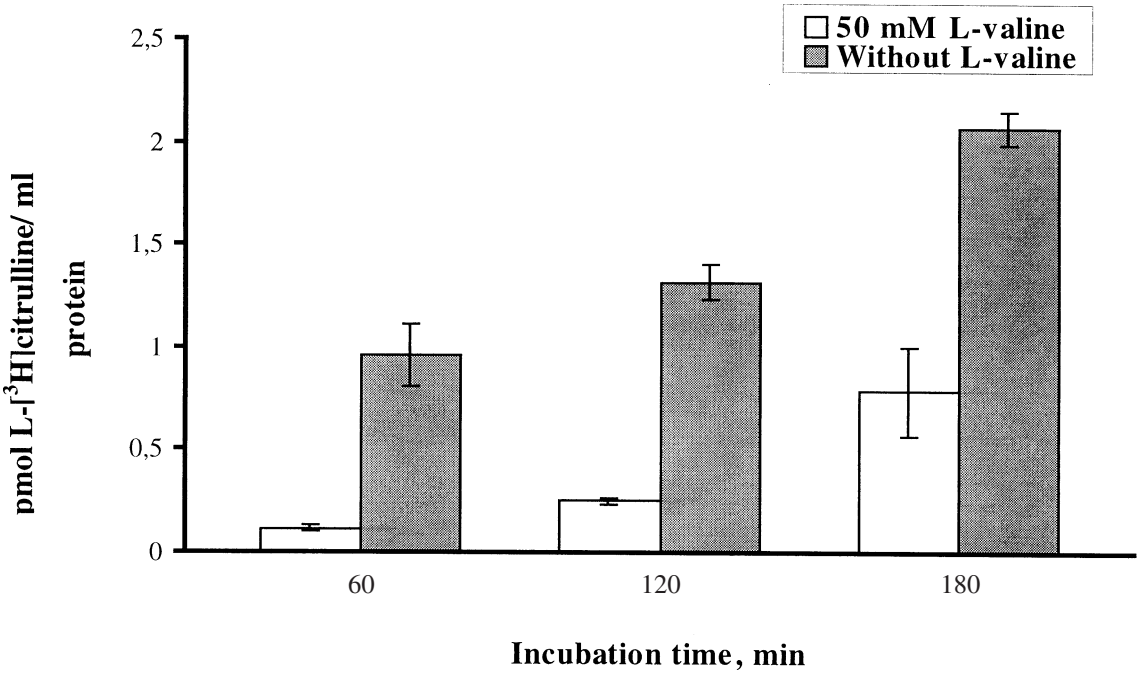

Fig. 1. The L-[^{3}H]citrulline biosynthesis rate in samples of *Fasciola hepatica* supernatants in the presence and absence L-valine in the incubation medium. Protein concentration 4 mg/ml. Incubation time 180 min. $n=5$ in every series.

with genistein lead to an enhanced nNOS activity and an increased NO release in the worm.

In the present study, the activity of nNOS was measured in homogenates of adult *F. hepatica* by the direct radiometric assay of the production of L-[^{3}H]citrulline.

MATERIALS AND METHODS

Specimens of adult *F. hepatica* Linnaeus, 1758 (Trematoda, Fasciolidea) were obtained from the bile ducts of infected cattle in the slaughterhouse of Moscow. The methods of Bredt & Snyder (1989) and Bush *et al.* (1992) were used. The worms were homogenized in 20 mM Tris-HCl buffer (pH 7·4) containing 0·5 mM EGTA and 1 mM dithiothreitol (buffer A) and centrifuged at 15 000 ***g*** for 15 min at 4 °C. Endogenous arginine was removed by adding 500 μl of Dowex AG50W-X8 (Na^+ form) in buffer A (11 g Dowex in 14 ml of buffer A). Samples containing 125–375 μg of protein were incubated for different periods at 37 °C in buffer (pH 7·4) containing 20 mM HEPES, 0·45 mM $CaCl_2$, 0·2 mM NADPH, 50 μM (6R)-5,6,7,8-tetrahydropterin, 5 μM FMN, 5 μM FAD, 1 mM L-citrulline, and 0,4-1,6 μM purified L-[^{3}H]-arginine (Amersham 2,11 TBq/mmol; 57·0 Ci/mmol). In order to inhibit the arginase activity in *F. hepatica*, 50 mM L-valine was introduced into the incubation medium. The reaction was stopped by the addition of 300 μl of ice-cold Dowex AG50W-X8 (Na^+ form) in 20 mM HEPES buffer (pH 7·4) (11 g Dowex in 14 ml of buffer) containing 2 mM EGTA to remove L-[^{3}H]arginine. L-[^{3}H]citrulline was quantified using a scintillation counter (Intertechnique, SL-4000) and Universal LSC cocktail for aqueous samples (Sigma). The formation of L-[^{3}H]citrulline, with or without the co-factors, 0·2 mM NADPH and 50 μM (6R)-5,6,7,8-tetrahydrobipterin (H4B) present and with one of four NOS inhibitors present, was studied. The following inhibitors purchased from Sigma were used (a) 1 mM methyl ester of N(ω)-nitro-L-arginine (L-NAME), (b) 1 mM EGTA, (c) 1 mM aminoguanidine, (d) 1 mM S-methyl-L-thiocitrulline. The protein concentration in the supernatant of *F. hepatica* was measured by the Bradford method (1976). The data are expressed as means $\pm$ S.E.M. Student's *t*-test was used for the comparison of the groups.

RESULTS

Four series of experiments were performed.

(1) First the effect of L-valine on the formation of L-[^{3}H]citrulline in *F. hepatica* was studied. Fig. 1 shows that the formation of L-[^{3}H]citrulline is much lower in the presence of L-valine. The effect increased with time, from 12% after 60 min incubation, to 38% after 180 min incubation. In all the following experiments, 50 mM L-valine was included in the incubation medium.

(2) To evaluate the optimal conditions for the assay of the nNOS activity in *F. hepatica*, the dependence of the formation of L-[^{3}H]-citrulline in the supernatants on (a) the incubation time and (b) the protein concentration was studied. In Fig. 2 the linearity of the reaction at protein concentrations between 1·25 and 5·0 mg/ml and at the incubation

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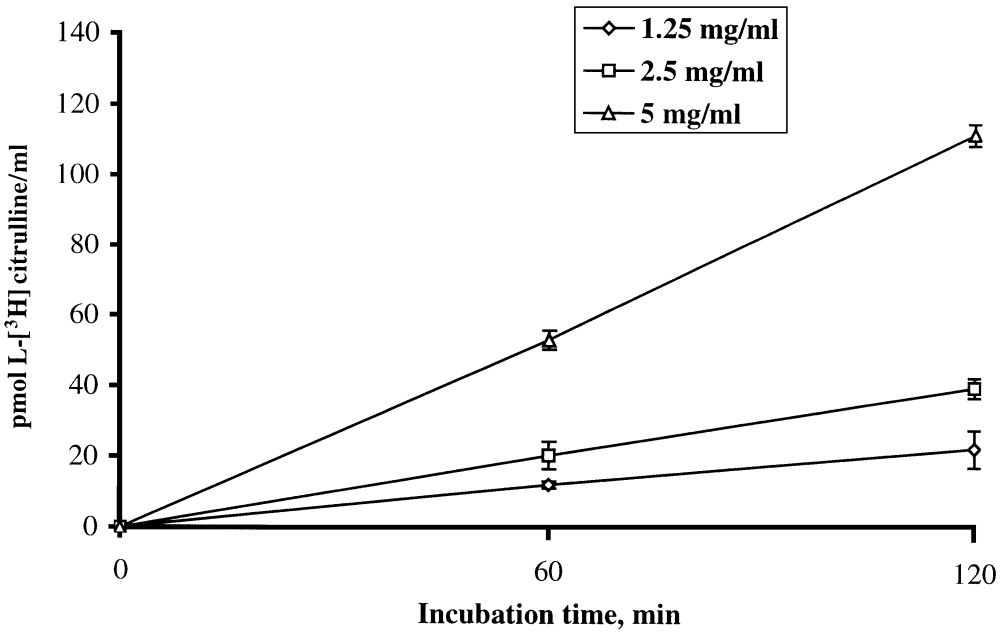

Fig. 2. The dependence of L-[^{3}H]citrulline biosynthesis rate on protein concentration in samples of *Fasciola hepatica* supernatants. $n=4$ in every series.

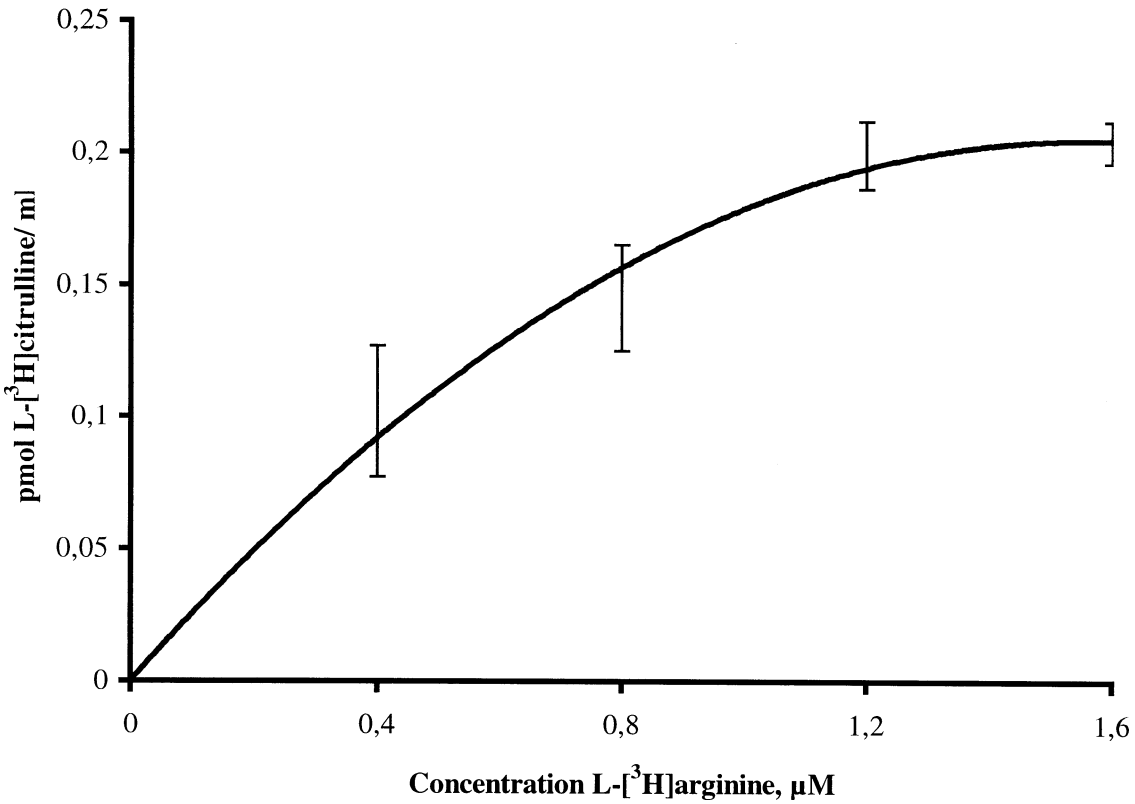

Fig. 3. Dependence of L-[^{3}H]citrulline biosynthesis rate on the concentration of L-[^{3}H]arginine in samples of *Fasciola hepatica* supernatants. $n=5$ in every series.

time of 60–120 min is shown. In the following experiments, a standard protein concentration of 4·0 mg/ml and an incubation time of 120 min were used.

(3) The dependence of the formation of L-[^{3}H]-citrulline on the concentration of L-[^{3}H]arginine was studied. Fig. 3 shows that at a concentration of 1·6 μM L-[^{3}H]arginine the formation of L-[^{3}H]citrulline reaches a peak. In the following experiments, we used 1·6 μM L-[^{3}H]arginine as working concentration.

(4) The effects of the presence or the absence of cofactors and the presence of different inhibitors

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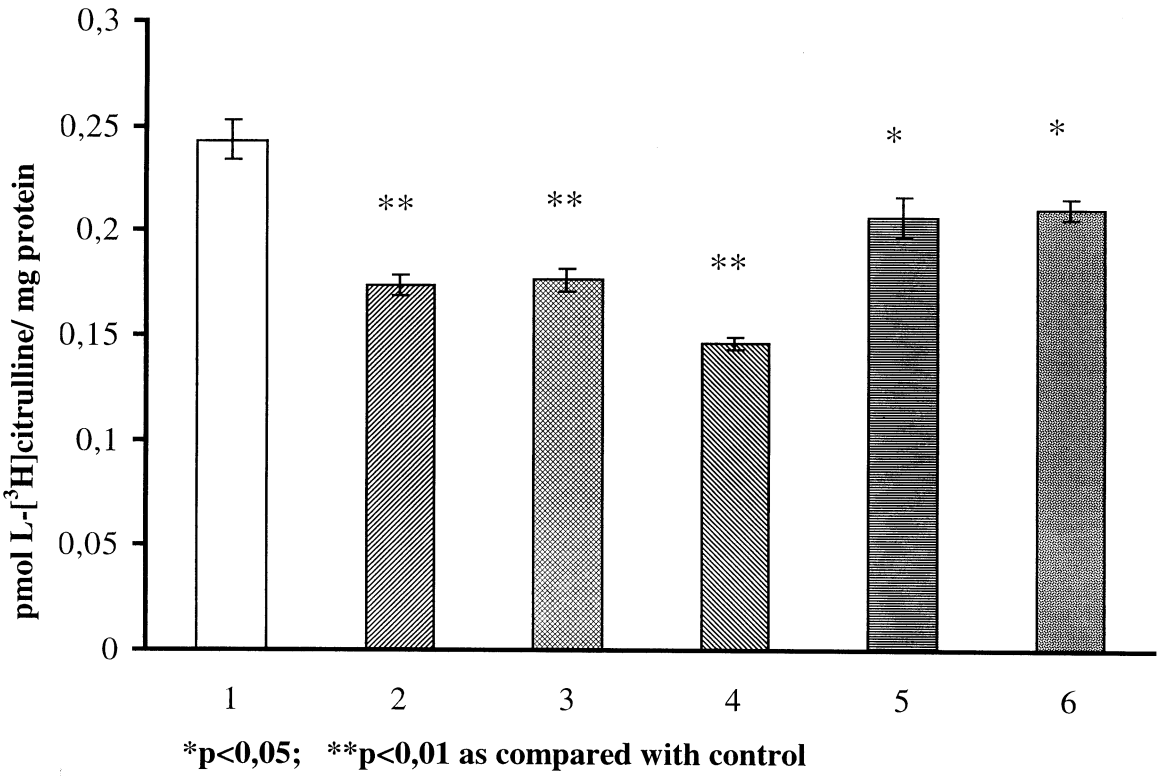

Fig. 4. The effects of cofactors and NOS inhibitors on L-[^{3}H]citrulline biosynthesis rate in samples of *Fasciola hepatica* supernatants. (1) Control; (2) without cofactors B4H and NADPH; (3) +1 mM methyl ester of N(ω)-nitro-L-arginine (L-NAME); (4) +1 mM EGTA; (5) +1 mM aminoguanidine; (6) +1 mM S-methyl-L-thiocitrulline.

were studied. Fig. 4 shows a reduction of 29% in the formation of L-[^{3}H]citrulline if H4B and NADPH were omitted from the incubation medium. This result is highly significant. The specific NOS activity in *F. hepatica*, (i.e. 0·07 pmol/mg/protein/min), is the difference between the control value (0·243 pmol/mg/protein/min) and the value obtained when H4B and NADPH are lacking from the incubation medium (0·173 pmol/mg/protein/min). In the presence of L-NAME or EGTA the formation of L-[^{3}H]citrulline was also significantly reduced by 28% and 40%, respectively. A smaller reduction in the formation of L-[^{3}H]citrulline was caused by aminoguanidine (15%) and S-methyl-L-thiocitrulline (14%).

DISCUSSION

The formation of L-[^{3}H]citrulline from L-[^{3}H]arginine indicates the presence of nNOS in homogenates of *F. hepatica*. This is the first radiometric study of the activity of nNOS in a fluke. Both nNOS and arginase utilize arginine as substrate, thus raising the question of whether these two enzymes directly compete for intracellular arginine. Little is known about the role of arginase in the synthesis of NO by constitutive NOS (cNOS) (for references see Li *et al.* 2001). The situation is complicated. On the one hand, arginase hydrolyses arginine to ornithine and urine, thus depleting the content of arginine that can be utilized by cNOS for the synthesis of NO. On the other hand, ornithine transcarbamyolase converts ornithine to citrulline, which means that ornithine serves as an alternative source for the formation of citrulline. This naturally complicates the results of radiometrical studies of the formation of L-[^{3}H]-citrulline. The regulative role of arginase in the synthesis of NO has been investigated in hepathocytes by Smith, Ceppi & Titheradge (1997), in activated macrophages by Chang, Liao & Kuo (1998), and in bovine endothelial cells by Li *et al.* (2001). The arginase activity in *F. hepatica* has never been tested, as far as we know. In this study we introduced L-valine, which is a specific inhibitor of arginase, in the incubation medium (Knowles *et al.* 1990*b*). In the presence of L-valine, the production of L-[^{3}H]-citrulline in *F. hepatica* was significantly lower (12%, 19%, 38%) than in the absence of L-valine. This result means that the arginase activity in the fluke is high and the transformation of ornithine to citrulline takes place. It is necessary to keep this in mind when investigating the activity of nNOS by measuring the formation of L-[^{3}H]-citrulline.

The rate of formation of L-[^{3}H]citrulline in *F. hepatica* was 0·243 pmol/min/mg protein. This value is about 50% lower but still of the same order of magnitude as that in *H. diminuta* (0·49 pmol/min/mg protein) (Terenina *et al.* 2000). However, when analysing the formation of L-[^{3}H]citrulline in *H. diminuta* no consideration was paid to the presence of arginase in the worm.

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The activity of nNOS in *F. hepatica* in the presence of H4B and NADPH was estimated to be 0·07 pmol/min/mg protein. This value is clearly lower than corresponding values for *H. diminuta* (0·2 pmol/min/mg protein) (Terenina *et al*. 2000), *G. tigrina* (0·196 pmol/min/mg protein) (Eriksson, 1996), *A. suum* (0·19 pmol/min/mg protein) and *P. redivivus* (0·47 pmol/min/mg protein) (Bowman *et al*. 1995). The lower value for nNOS in *F. hepatica* may again be due to the presence of L-valine in the incubation medium.

The study on the effects of the 4 NOS inhibitors made it possible to determine the nature of the NOS in *F. hepatica*. All inhibitors used caused a remarkable reduction in the production of L-[^{3}H]-citrulline. However, neither of them abolished it totally, indicating that only a part of the biosynthesis of L-[^{3}H]-citrulline in *F. hepatica* is due to nNOS. According to the data obtained from different groups, the activity of the invertebrate nNOS measured in biochemical assays is not completely inhibited by usually potent NOS inhibitors (Knowles *et al*. 1990*a*; Radomski, Martin & Moncada, 1991; Johansson & Carlberg, 1994; Bowman *et al*. 1995; Eriksson, 1996; Terenina *et al*. 2000).

The relation between NADPH-d staining and NOS activity has been widely discussed. In many situations, nNOS was shown to co-localize with NADPH-d, first of all in the nervous system (for references see Vincent, 2000). In *F. hepatica* strong deep blue NADPH-d staining was observed in both neuronal tissue and non-neuronal tissue (Gustafsson *et al*. 2001). Large NADPH-d stained neurones were localized in the nervous system. The oral and ventral suckers are innervated with many large NADPH-d stained neurones. In addition, the NADPH-d staining reaction occurs in nerve fibres that follow closely the muscle fibres in both suckers, in the body, and in the ducts of the reproductive organs. However, strong specific NADPH-d staining was also observed in the prostate gland (Gustafsson *et al*. 2001). Non-specific pink-purple NADPH-d staining was observed in *F. hepatica*, especially in the subtegumental tissue and in the eggs. This was regarded as an indication of the presence of other oxide reductases. Non-specific NADPH-d staining has been described from studies of the mammalian autonomous nervous system (Young *et al*. 1992; Grozdanovic *et al*. 1995), the leech (Leake *et al*. 1995), and the helminths *A. suum* (Bascal *et al*. 1995, 1996), *H. diminuta* (Gustafsson *et al*. 1996), *G. tigrina* (Eriksson, 1996), *D. dendriticum* (Lindholm *et al*. 1998), *E. rugosum*, *A. lucii* and *H. cylindracea* (Terenina *et al*. 1998).

This study presents biochemical evidence for the occurrence of nNOS activity in adult *F. hepatica* and, together with the previous data on the occurrence of NADPH-d activity in the nervous system, provides strong evidence that the worm possesses a nNOS-like enzyme.

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