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The effect of a nitric oxide donor on the synthesis of cGMP in *Hymenolepis diminuta*: a radiometric study

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Abstract The formation of cGMP in homogenates of the adult rat-tapeworm *Hymenolepis diminuta* was followed with a radiometric assay during 3 h after stimulation with the nitric oxide donor sodium nitroprusside (SNP) and in the presence of isobutylmethylxanthine (IBMX). The level of cGMP was stable in worms incubated with IBMX during the first hour. After 3 h of incubation, the level of cGMP had declined by 27%. Addition of SNP stimulated the formation of cGMP during the first hour of incubation. After 3 h of incubation, a two-fold decline in cGMP formation was observed. The rate of nitric oxide (NO) release by the worm was determined by a spectrophotometric assay for the accumulation of nitrites and nitrates, the stable degradation products of NO, using the Griess reaction. The results are discussed from the perspective of the current concept on the role of the nitrergic mechanisms in the flatworm nervous system.

Introduction

Nitric oxide (NO) represents a new category of neuronal signal substances: a transmitter gas. Cellular signalling mediated by NO involves the highly regulated synthesis of NO by neuronal nitric oxide synthase (nNOS), the diffusion of NO into adjacent target cells, the activation of the soluble isoform of guanylyl cyclase in the target cells and the synthesis of the second messenger, cGMP.

The first indication of the presence of nitrergic mechanisms in the nervous system of parasitic flatworms was the observation of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd)-positive neurones in the adult rat tapeworm *Hymenolepis diminuta* (Gustafsson et al. 1996). Since then, NADPHd-positive neurones have been detected in eight parasitic and two free-living flatworms (see Gustafsson et al. 2003a, 2003b). The activity of nNOS has been analysed in homogenates of adult *H. diminuta* and *Fasciola hepatica* by measuring the formation of L-[^{3}H]citrulline after incubation with L-[^{3}H]arginine (Terenina et al. 2000, 2003). In order to localise the target cells for NO in flatworms, the pattern of cGMP-immunostaining (cGMP-IS) was investigated in adult *H. diminuta* (Gustafsson et al. 2003a), plerocercoid larvae of *Diphyllobothrium dendriticum*, adult *F. hepatica* (Gustafsson et al. 2003b) and cercaria of *Diplostomum chromatophorum* (Terenina and Gustafsson 2003).

In this study, we followed the formation of cGMP in adult *H. diminuta* with a radiometric assay after stimulation with sodium nitroprusside (SNP) and in the presence of a phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). The rate of NO release was also determined by a spectrophotometric assay for the accumulation of the stable degradation products of NO, nitrites and nitrates (NO_x), using the Griess reaction (Miranda et al. 2001).

Materials and methods

Specimens of *Hymenolepis diminuta* (Rudolphi, 1819) (Cestoda, Cyclophyllidea) were obtained from experimentally infected rats at the Institute of Parasitology (Russian Academy of Sciences, Moscow). Living tapeworms were incubated in Ringer's solution at 37 °C for

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30, 60 and 180 min with 10 m*M* SNP and 0.1 m*M* IBMX. Control worms were incubated in Ringer's solution with 0.1 m*M* IBMX only.

Radioimmunoassay

The level of cGMP in *H. diminuta* was determined using a radioimmunoassay kit for cGMP (TRK 500: Amersham, UK). The method is based on the competitive binding of unlabelled and tritium (^{3}H)-labelled cGMP with a specific antibody towards cGMP. Samples of *H. diminuta* were homogenised in 50 m*M* Tris-HCl buffer (pH 7.5), containing 4 m*M* EDTA to prevent the enzymatic degradation of cGMP. In order to separate the proteins, the homogenates were boiled for 5 min and centrifuged at 15,000 *g* for 10 min at 4 °C. The supernatants were then mixed with ^{3}H-cGMP and the antibody and incubated for 60 min at 4 °C. The antibody was precipitated by the addition of ammonium sulfate (60%) for 5 min and the samples were centrifuged at 15,000 *g* for 2 min at 4 °C. The pellet was dissolved in distilled water. The radioactivity was measured using a scintillation counter (1414 LSC; Wallac, Finland). The level of cGMP was calculated using a calibration curve of radioactive samples containing known quantities of labelled and unlabelled cGMP.

Quantitative detection of NO_x

The spectrophotometric method for the simultaneous detection of NO_x in VCl_3-reduced samples with Griess reagent was used (Miranda et al. 2001). Equal volumes of: (a) a solution of 8% VCl_3 in 1 *N* HCl, (b) the Griess reagent and (c) the supernatant of *H. diminuta* were mixed and incubated for 45 min at 37 °C. The optical density in the samples was measured at 540 nm. The quantity of NO_x was evaluated using a calibration curve for $NaNO_3$.

Results

Table 1 shows the level of cGMP in *Hymenolepis diminuta* during incubation either with IBMX alone or with IBMX and SNP. The levels of cGMP in homogenates of *H. diminuta* incubated with IBMX were quite stable during the first 60 min of incubation (5.46–5.54 pmol/g wet weight). However, 2 h later, the level of cGMP dropped to 3.98 pmol/g wet weight. The addition of SNP stimulated the synthesis of cGMP in the homogenates of *H. diminuta* during the first 60 min of incubation (up to 6.63–7.67 pmol/g wet weight). At 60 min, the level of cGMP was significantly higher than in the control. Incubation for a further 2 h resulted in a cGMP level slightly below the control level (3.78 pmol/g wet weight).

Table 2 shows the accumulation of NO_x in *H. diminuta* during incubation with IBMX or with IBMX and SNP. In the controls incubated with IBMX only, the levels of NO_x remained constant during the first two measurements (30, 60 min). However, 2 h later, the level of NO_x almost doubled. The addition of SNP induced a significant rise in the level of NO_x after 60 min of incubation and 2 h later, when the level of NO_x rose almost 8-fold.

Discussion

This is the first radiometric analysis of the production of cGMP in a flatworm and the fourth report in a series on NO in the rat tapeworm *Hymenolepis diminuta*, completing a study on the pattern of cGMP-IS in this worm (Gustafsson et al. 1996, 2003a; Terenina et al. 2000). The results of this study show that the levels of cGMP in homogenates of *H. diminuta* incubated with IBMX are rather low, compared with the levels measured in other invertebrates. The addition of SNP slightly stimulated the synthesis of cGMP in homogenates of *H. diminuta* during the first hour of incubation. However, a clear decline in the production of cGMP later took place.

Only a few studies of the levels of cGMP in invertebrates have been performed. Huang et al. (1998) studied NO-mediated cGMP synthesis in neuronal ganglia of *Helix pomatida*. The cGMP basal level in the ganglia in *Helix* saline (control) was 22.9 ± 15.8 pmol/g wet weight. Incubation of the ganglia with IBMX elevated the cGMP concentration about 3-fold (64.7 ± 12.1 pmol/g wet weight). Stimulation of cGMP synthesis in the presence of IBMX and SNP elevated the cGMP concentration about 22-fold (509.3 ± 116.3 pmol/g wet weight). NO-mediated cGMP synthesis in brain extracts

Table 1 The formation of cGMP (pmol/g wet weight) in *Hymenolepis diminuta* during incubation with sodium nitroprusside (SNP) and isobutylmethylxanthine (IBMX). The data are presented as mean ± S.E.M., $n = 3$. . The *asterisk* indicates $P < 0.05$

Group	Incubation time		
	30 min	60 min	180 min
IBMX	5.46 ± 0.24	5.54 ± 0.69	3.98 ± 0.46
IBMX + SNP	6.63 ± 0.92	7.67 ± 0.27*	3.78 ± 0.30

Table 2 The formation of nitrites and nitrates (pmol/g wet weight) in *H. diminuta* during incubation with SNP and IBMX. The data are presented as mean ± S.E.M., $n = 3$. The *asterisk* indicates $P < 0.05$

Group	Incubation time		
	30 min	60 min	180 min
IBMX	0.17 ± 0.02	0.17 ± 0.03	0.30 ± 0.01
SNP + IBMX	0.18 ± 0.01	0.32 ± 0.02*	2.50 ± 0.18*

of *Schistocerca gregaria* was analysed by Elphick et al. (1993). The basal level of cGMP was 300 fmol/brain. The addition of three NO donor compounds (3-morpholinosydnonimine, S-nitroso-N-actylpenicillamine, or hydroxylamine) resulted in a 4- to 8-fold increase in the cGMP content in the locust brain relative to the control. Zayas et al. (2002) tested the role of neuronal nicotinic acetylcholine receptors (nAChRs) in NO-signalling in the central nervous system of *Manduca sexta*. They showed that stimulation of nAChRs activated the NO/cGMP pathway in the larval central nervous system of *M. sexta*. Since the levels of cGMP are presented as *x*-fmol/brain or *x*-fmol/nerve cord, it is impossible to compare these figures with the levels of cGMP in *Hymenolepis diminuta*.

The low levels of cGMP in *H. diminuta* are mainly due to the fact that the homogenates were made of whole worms, not isolated nervous tissue. A similar situation was met when measuring the formation of L-[^{3}H]citrulline from L-[^{3}H]arginine in *H. diminuta* and *Fasciola hepatica*. The homogenates were made of whole worms: and (per minute) the L-[^{3}H]citrulline formation was 0.49 pmol/mg protein in *H. diminuta* and 0.243 pmol/mg protein in *F. hepatica* (Terenina et al. 2000, 2003). The corresponding value (per minute) for rat cerebral cortex was 52.5 pmol/mg protein (Terenina et al. 2000).

When studying the pattern of cGMP-IS in *H. diminuta*, *Diphyllobothrium dendriticum*, *F. hepatica* and *Diplostomum chromatophorum* intense IS was detected in nerve fibres after incubation for 15–30 min in SNP and IBMX (Gustafsson et al. 2003a, 2003b; Terenina and Gustafsson 2003) Longer incubations were not tested. In the tapeworms and the liver fluke, strong cGMP-IS was observed in nerve fibres closely associated to muscle fibres, indicating that NO activates the motor neurones.

NO has an extremely short half-life, which makes it difficult to detect and study this molecule directly. However, because NO is metabolised to NO_x, quantification of these stable anions can be used to determine the NO release indirectly and quantitatively. The nitrite/nitrate ratio is variable, depending upon the pathway of oxidative inactivation. Reaction with superoxide anions predominantly yields nitrate, while the reaction with molecular oxygen yields nitrite. Nitrate can be detected after reduction to nitrite using nitrate reductase (Schmidt 1995). In this study, NO_x were detected simultaneously using the Griess reagent after the reduction of nitrates to nitrite by VCl_3 (Miranda et al. 2001). In controls incubated with IBMX, the levels of NO_x remained constant during the first hour. However, 2 h later the level of NO_x almost doubled. The addition of SNP induced a significant rise in the level of NO_x after 1 h of incubation. After 3 h of incubation, the level of NO_x rose almost 8-fold. The results show that SNP diffused into *H. diminuta* and that large amounts of NO were generated, starting after 1 h of incubation. A rise in the level of NO_x might be the cause of the decline in the level of cGMP in *H. diminuta*.

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