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## mRNA expression and immunohistochemical localization of inducible nitric oxide synthase (NOS-2) in the muscular niche of *Trichinella spiralis*

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**Abstract:** The aim of this study was to demonstrate iNOS mRNA expression in muscular phase of experimental trichinellosis and to localize iNOS protein in *T. spiralis*-infected muscles using specific anti-iNOS monoclonal antibodies. The expression of iNOS mRNA in skeletal muscles from *Trichinella spiralis*-infected mice was examined using the reverse transcription PCR assay. Fragments of skeletal muscles were also subjected to the immunohistochemical reaction using specific anti-iNOS monoclonal antibodies followed by Dako-Ark test. mRNA for iNOS measured on day 21 after infection was expressed in the muscular phase of trichinellosis. Positive immunostaining for iNOS occurred in infiltrating mononuclear cells around the encapsulated larvae. iNOS-positive cells could be traced from the 21st day post infection (dpi); on 42 dpi and 90 dpi most cells expressed iNOS. By assessing expression of protein and its mRNA it can be concluded that iNOS is active in the pathology of skeletal muscle tissue in experimental trichinellosis.

**Key words:** iNOS - mRNA expression - Immunocytochemistry - *Trichinella spiralis* infection - Muscular phase - Mouse

### Introduction

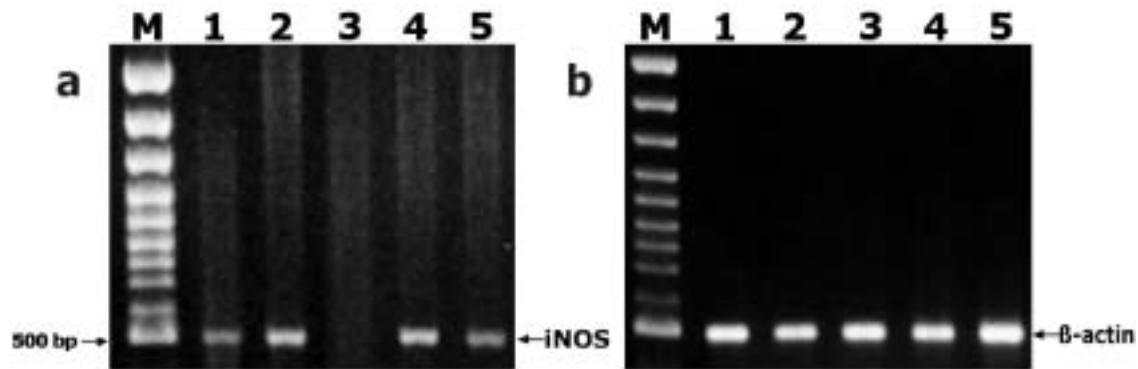
Despite the fact that trichinellosis has been a subject of scientific interest for over 150 years, the oxygen and nitrogen free radical-based host defence became the field of interest as late as in the last decade. The protective role of the following antioxidant enzymes: superoxide dismutase (SOD) and peroxidase as well as prostaglandin synthase in host muscle tissue in trichinellosis was proven in a series of histochemical studies by Hadaś *et al.* [11-13] and of biochemical studies by Boczoń *et al.* [3, 5].

Nitric oxide (NO) generated by an inducible form of nitric oxide synthase (NOS-2) is considered to be the main factor able to kill or at least to suppress various pathogens, including such intracellular parasites as *Plasmodium* [28], *Leishmania major* [9,10,21,26] *Trypanosoma cruzi* [24,29,16] and *Toxoplasma gondii* [1,31]. Trichinellosis is another example of tissue parasitosis

which has only recently attracted scientific interest in the context of reactive nitrogen intermediate-based host defence. The complex life cycle of *Trichinella spiralis* includes an intestinal phase, when the adult forms are responsible for the inflammatory changes in the intestine and a muscular phase, when the larvae cause a whole set of immunological, biochemical and physiological changes in skeletal muscles. In 1996, Hogaboam *et al.* [15] suggested that NO participated in the modulation of enteritis during the intestinal phase of *T. spiralis* infection, as appeared from the observation that enteritis was eliminated using a NOS-specific inhibitor, L-NAME. In their fundamental paper, Lawrence *et al.* [20] using iNOS-deficient mice concluded that NO might contribute substantially to the *T. spiralis*-induced enteropathy.

Taking into consideration the data obtained in *T. spiralis*-infected intestine of the host, it seemed to us that NO might play such role also in muscle pathology during trichinellosis. The activity of iNOS in the muscles of *Trichinella*-infected mice was measured quantitatively by some of us [5]. A two-stage phenomenon of up-regulation of iNOS was observed on days 21 and 70 after *T.*

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**Fig. 1. a:** Inducible nitric oxide synthase (iNOS) gene expression in uninfected and *T. spiralis*-infected mice on the 21st dpi. RT-PCR products were electrophoresed through 1% agarose gel and visualized by ethidium bromide staining. Lane M: size marker (Gibco BRL DNA Ladder). Lane 3: muscle isolate from uninfected mouse (negative results). Lanes 1, 2, 4 and 5: muscle isolates from *T. spiralis*-infected mice (positive results). **b:**  $\beta$ -actin gene expression in the same mice. Lane M: size marker. Lane 3: muscle isolate from uninfected mouse (positive result). Lanes 1, 2, 4 and 5: muscle isolates from *T. spiralis*-infected mice (positive results).

*spiralis* infection. It is worthy of note that the first increase was abolished by glucocorticoid treatment of the experimental animals. Our parallel investigations showed that in the case of *T. pseudospiralis* larvae which did not encapsulate in host muscles, their continuous migration (as the result of the lack of capsules) caused much greater damage to host muscles accompanied by intensive and long lasting generation of nitrogen free radicals as compared to *T. spiralis* [5].

To solve the problem of iNOS participation in host biochemical defence against *Trichinella* larvae in muscles, the timing of iNOS mRNA synthesis and the localization of iNOS in the cell have to be determined. Nitric oxide is an extremely labile, readily diffusible substance and direct measurements of NO formation and its localization in tissue have proven difficult. In parallel to quantitative measurements, attempts to localize NOS in mice muscles during experimental trichinellosis had been undertaken by our group some years ago [14]. At that time our report seemed to be the first presentation of NOS participation in host biochemical defence during muscular phase of trichinellosis, however, the use of NADPH for the purpose of staining for NOS activity (observation of formazan-nitroblue tetrazolium product) did not provide accurate information on the specific iNOS localization.

The aim of the current study was: (1) to demonstrate iNOS mRNA expression in the muscular phase of experimental trichinellosis, and (2) to localize iNOS protein in *T. spiralis*-infected muscles using specific anti-iNOS monoclonal antibodies. Hence, iNOS was traced in *T. spiralis*-infected muscles both at mRNA and at protein levels.

## Materials and methods

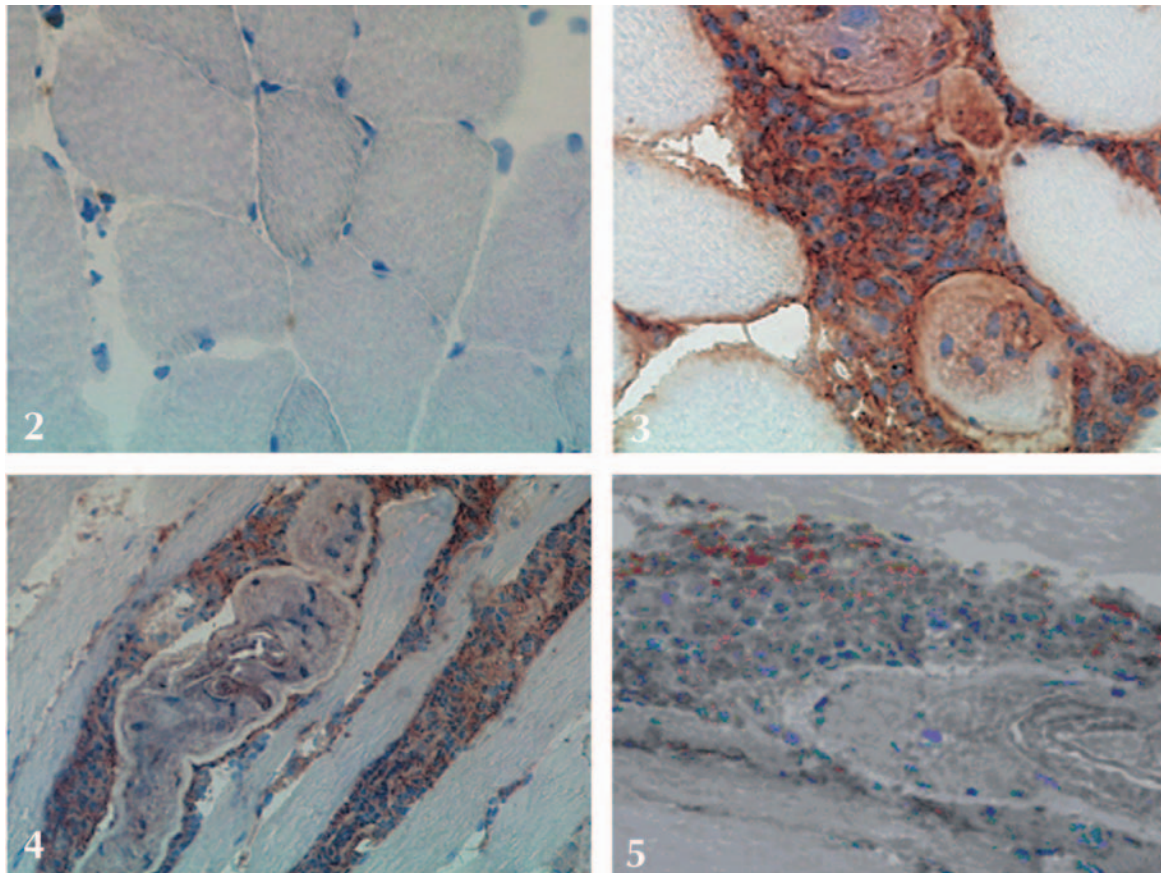
Biological material for molecular and immunohistochemical investigations consisted of skeletal muscles collected from mice (strain

BALB/c) infected *per os* with *T. spiralis* (strain MSUS/PO/60/ISS3, 450 larvae/mouse).

For molecular investigations, in one series of experiments conducted in parallel to immunohistochemical studies, the muscle tissue was excised from hind legs of 2 control mice and 3-4 infected mice at early stage of muscular phase of infection *i.e.* on the 21st day post infection (dpi). Earlier stages of *T. spiralis* infection were not studied because our previous biochemical investigations showed that the first increase in total activity of iNOS occurred on day 21. This is the time when so-called "newborn" larvae start to settle down in muscles.

The expression of iNOS mRNA in the skeletal muscles was measured using RT-PCR assay. The total cellular RNA was isolated from muscles by using a TRI Reagent (Sigma) according to the manufacturer's instructions. RNA was then denatured by heat and reverse transcribed to yield cDNA. The synthesis of cDNA and the subsequent PCR were performed by using an Enhanced Avian RT-PCR Kit (Promega). The primer sequences for the PCR were as follows: sense iNOS: 5' CCC TTC CGA AGT TTC TGG CAG CAG C 3', antisense iNOS: 5' GGT TGT CAG AGC CTG GTG GCT TTG G 3'. Amplification of cDNA was carried out in a thermal cycler for 35 cycles using the following cycle parameters: denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec and extension at 72°C for 75 sec. The PCR products were then resolved by 1% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed. Expression of gene for beta-actin was used as control for RT-PCR reaction.

For immunohistochemical studies, muscle samples (from hind legs and diaphragm) were collected from mice (n=30) on the 7th (n=2), 21st (n=8), 50th (n=4), 70th (n=2) and 90th (n=5) dpi. In each time interval of the infection, 2 uninfected mice were checked as controls. Tissue blocks were rapidly frozen in precooled petroleum aether and dry ice slurry and stored at -70°C in deep freezer. Six  $\mu$ m sections (n=5-8) were cut in cryostat, air dried, fixed in cold acetone and subjected to the immunohistochemical procedure. Briefly, mouse ascites fluid primary monoclonal anti-iNOS antibody (Sigma, N-9657, clone NOS-IN, lot 28H4826) was used. In addition, subsequent sections were incubated with anti-CD68 (anti-macrophage) and anti-CD3 (anti-T cell) monoclonal MoAbs. Overnight incubation at 4°C with the primary antibody was followed by DAKO ARK™ (Animal Research Kit) test based on the application of biotinylated F(ab) anti-mouse Ig, peroxidase-conjugated streptavidin and diaminobenzidine (DAB) as chromogen. In control sections, the primary antibody was replaced by normal mouse serum and by PBS. In order to facilitate the penetration of the reagents into cells, the primary antibody solution and all the subsequent solutions including washing fluid were supplemented with 0.1% saponin. This



**Fig. 2.** Immunohistochemical staining using anti-iNOS primary antibody and DAKO ARK Peroxidase. Skeletal muscle section from uninfected mice. No immunostaining can be seen.  $\times 400$ . **Fig. 3.** Immunohistochemical reaction as in Fig. 2. Encapsulated *T. spiralis* larvae and cellular infiltrates in muscles of infected mice on the 90th dpi. Distinct positive immunostaining for iNOS in cells surrounding larvae and also around some muscle fibers apparently without larvae.  $\times 400$ . **Fig. 4.** Subsequent tissue section and reaction as above, but with anti-CD68 primary antibody. Most infiltrating cells show positive staining.  $\times 100$ . **Fig. 5.** Subsequent tissue section and reaction as above but anti-CD3 primary antibody. A small portion of infiltrating cells shows positive staining.  $\times 400$ .

significantly intensified the positive staining for iNOS in the cells, but at the same time it enhanced the background staining. Sections of tissues from uninfected mice were run in parallel. Serial sections of mouse muscle tissues from each study group were stained with hematoxylin-eosin.

## Results

In the experiments where RT-PCR was used to investigate the expression of iNOS mRNA in skeletal muscles from *T. spiralis* infected mice, iNOS mRNA expression was demonstrated in the muscular phase of the infection (on the 21st dpi). The visualization of the respective bands after ethidium bromide staining is demonstrated in Figure 1; detection of  $\beta$ -actin mRNA in skeletal muscles from *T. spiralis*-infected mice was used as control for RT-PCR reaction. No iNOS mRNA bands were observed after RT-PCR of muscles from uninfected mice (Fig.1).

iNOS-positive cells surrounding the encapsulated larvae could be seen from the 21st dpi (no iNOS-positive

staining observed in pilot experiments on day 7). Infiltrates of cells showing positive brownish reaction in the cytoplasm were evident around larvae on the 50th dpi and persisted there at least to the 90th dpi (Figs. 3-5). Slight brownish coloration of larva and connective tissue around muscle fibers was considered nonspecific, because it was also seen in the control sections. Infiltrates of iNOS-positive cells could also be seen around some muscle fibers with no visible larvae. Host cells showing specific reaction in the cytoplasm apparently corresponded to tissue macrophages as evidenced by anti-CD68 immunoreactivity (Fig. 4). A small portion of the iNOS-positive cells around *T. spiralis* larvae belonged to T lymphocyte (CD3+) population (Fig. 5). No positive reaction in muscle fibers could be traced in the control (uninfected) mice (Fig. 2). There was no immunostaining of cells whatsoever, when monoclonal anti iNOS Abs were replaced by normal mouse serum or PBS; in the latter abundant cell infiltrates within infected muscles but without iNOS activity were observed (not shown).

## Discussion

Due to the fact that *T. spiralis* invasion starts with the penetration of epithelium of the small intestine, infection caused by that parasite has often served as an experimental model for the study of mucosal inflammatory responses in animals [2, 19, 23].

The intestinal phase of *T. spiralis* invasion in the animal host has been relatively well elucidated. It is clear that the expulsion of parasites from the gut depends on efficient adaptive immune response. The crucial role is played by CD4 Th2 cell-dependent cytokines such as IL-4, IL-6 and others. A subsequent release of various mediators, such as histamine, serotonin, heparin and various enzymes, stimulates the peristaltic movements and mucus release, which together facilitate the removal of the parasite from the gut. Some of the mediators released during the intestinal phase of *T. spiralis* infection do not participate in the parasite expulsion, however they cause the induction/perpetuation of the inflammation in intestinal mucosa, which leads to tissue damage. The aforementioned mediators include TNF- $\alpha$  and nitric oxide (NO). The latter, produced from arginine by inducible NO synthase (iNOS) has been shown to participate in intestinal pathology during the intestinal phase of the infection. As shown in elegant experiments of Lawrence *et al.* [20], iNOS<sup>-</sup> mice could eliminate *T. spiralis* in the same way as iNOS<sup>+</sup> mice, however, the range of pathological lesions was definitely higher in the former. This suggests that NO plays a minor role in parasite elimination, however its presence is crucial for the intestinal pathology.

The intestinal phase of *T. spiralis* infection is followed by the muscular phase, which manifests itself by the invasion of skeletal muscles by parasite larvae. The host skeletal muscle cells in a *T. spiralis*-infected host undergo cell cycle arrest in the G2/M phase, as reviewed by Jasmer [18]. In order to identify the potential parasite and/or host involvement in the transformation of muscles in trichinellosis at the structural and biochemical levels, we have to investigate the host-parasite interplay, paying attention to the free radical-based host defence.

The concept that NO as a pluripotent molecule may be produced during prolonged periods of the disease by numerous cell types within tissues, including skeletal muscles (the main types of muscles invaded by parasite in the muscular phase of trichinellosis), was supported as early as in 1996 by Thompson *et al.* [27]. In tissue invasions, the generation of nitrogen free radicals is frequently more intense than of oxygen free radicals [8]. In skeletal muscles of *T. spiralis*-infected mice, oxygen free radicals [3, 12, 13] can combine with NO to form peroxynitrate, which leads to injury or death of the muscle fibers. Some researchers, including our team [4, 25, 27], have shown that both the oxygen and nitrogen reactive intermediates participate in muscle injury. Con-

sequently, we carried out research aimed at direct detection of iNOS mRNA and immunohistochemical localization of iNOS in *T. spiralis*-infected mice.

Although the increase in the total iNOS activity at certain stages of the muscular phase of *T. spiralis* and *T. pseudospiralis* infections was reported by us some years ago [5], no confirmation of mRNA synthesis for iNOS has been published up to now. The results of the RT-PCR analysis as well as of immunohistochemistry presented in this paper confirm the idea of iNOS up-regulation in host muscles in the muscular phase of the invasion, starting from the 21st dpi. Our previous investigations have shown that iNOS down-regulation on the 7th dpi was followed by iNOS up-regulation also on the 21st and additionally on the 70th dpi [5]. These results supplement the more recent observations of Wandurska-Nowak and Wiśniewska [30] who found a statistically significant increase in the NO serum levels and in the NO release from infected mouse peritoneal macrophages (maximal, about 3-fold on the 21st dpi and in serum (about 2-fold already on the 14th dpi).

The results of this study confirm that iNOS is active in infiltrating cells almost throughout the entire muscular phase of infection with *T. spiralis* larvae. It was recently found by one of us that the level of iNOS expression in peritoneal macrophages isolated from infected mice measured by quantitative real-time PCR assay was 18 times higher as compared to uninfected animals (unpublished). This observation confirms the localization of iNOS in macrophages, suggested by us on the basis of immunohistochemical observation using monoclonal antibodies against CD68.

These findings in connection with our previous results [14] suggest that in the case of *T. spiralis* larvae invasion into muscles, biochemical host defence includes the production of NO. It is worthy of note that the timing and intensity of the maximal positive immunostaining (70-90 dpi) was comparable to the statistically significant increase in iNOS activity in muscles measured quantitatively by Boczoń *et al.* [5]. Unlike in the case of *Trypanosoma cruzi*, another muscle tissue parasite, after immunostaining, iNOS was not observed on the surface of *T. spiralis* or inside the capsule. In the case of the latter parasite, cytoplasmic iNOS was seen in inflammatory cells infiltrating the muscular tissue and the positive immunostaining was stronger after saponin treatment. Bruschi *et al.* [7] have recently applied confocal microscopy to study c- and iNOS in the nurse cell of *T. spiralis* larvae using polyclonal antibodies. They have concluded that in the nurse cell c- and iNOS do not undergo up-regulation process. Their preliminary observations partly confirm our findings, although we have used anti-iNOS monoclonal antibodies which makes our results more convincing.

iNOS expression and its impact on the pathology of infected tissues has up to now been found not only in



muscle tissue parasitoses mentioned above but also in some other tissue parasitoses: strong staining for iNOS in macrophages was evident (as confirmed by CD68 staining) in cerebral malaria [22], localization of iNOS mainly in liver neutrophils surrounding amoebas was shown in hepatic amoebiasis [17] and iNOS localized in leukocyte infiltrations within granuloma were demonstrated in liver of animals infected by *Schistosoma mansoni* [6].

To conclude, we were able to demonstrate the activity of inducible NO synthase in skeletal muscles in the course of *T. spiralis* invasion both at the mRNA and protein level. The functional significance of this finding, and in particular the subsequent production of NO in the infected muscles, awaits further studies.

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