0026-895X/01/5904-939–947$3.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 59:939–947, 2001

Vol. 59, No. 4
595/895366
Printed in U.S.A.

Down-Regulation of Inducible Nitric-Oxide Synthase (NOS-2) During Parasite-Induced Gut Inflammation: A Path to Identify a Selective NOS-2 Inhibitor

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Received September 21, 2000; accepted January 5, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Nitric oxide (NO) possesses potent anti-inflammatory properties; however, an over-production of NO will promote inflammation and induce cell and tissue dysfunction. Thus, the ability to precisely regulate NO production could prove beneficial in controlling damage. In this study, advantage was taken of the well characterized inflammatory response caused by an intestinal parasite, *Trichinella spiralis*, to study the relationship between intestinal inflammation and the regulation of nitric oxide synthase-type 2 (NOS-2) expression. Our study revealed that a specific gut inflammatory reaction results in inhibition of NOS-2 expression. Characteristics of this inhibition are: 1) local jejunal inflammation induced by *T. spiralis* systemically inhibits NOS-2 gene transcription, protein expression, and enzyme activity; 2) the inhibition blunts endotoxin-stimulated NOS-2 expression; 3) the inhibition does not extend to the expression of other isoforms of NOS, to paxillin, a housekeeper protein, or to cyclooxygenase-2, another protein induced by proinflammatory cytokines; 4) the inhibition is unlikely related to the formation of specific anti-parasite antibodies; and 5) the inhibition may involve substances other than stress-induced corticosteroids. Elucidation of such potent endogenous NOS-2 down-regulatory mechanisms could lead to the development of new strategies for the therapy of inflammatory conditions characterized by the overproduction of NO.

Over the past two decades, nitric oxide signaling has been one of the most rapidly growing areas in biomedicine. Nitric oxide (NO) mediates regulatory events that play key roles in cellular function (Murad, 1999). NO is produced through the action of nitric oxide synthase (NOS), three isoforms of which have been cloned. Among those isoforms, inducible NOS (iNOS or NOS-2) is unique because 1) it requires de novo synthesis in most cells; 2) upon exposure to stimuli such as endotoxin (LPS) and proinflammatory cytokines, it is rapidly expressed and results in the production of much larger quantities of NO relative to the two other isoforms; and 3) it is widely distributed in various cell types (Xie et al., 1992; Weisbrodt et al., 1996).

The role of NO in inflammation represents an intensely studied yet controversial subject in physiology and pathology. During inflammation and sepsis, there is an increased production of various mediators including proinflammatory cytokines, eicosanoids, and endotoxin that directly or indirectly induce the activity and/or expression of NOSs, especially NOS-2. The NO that is produced, often working through NO-stimulated increases in cyclic GMP, influences a wide variety of physiological and pathophysiological processes (Murad, 1999). Indeed, NO has been credited with being both a physiological and pathophysiological messenger. Two factors that are most likely to have a major impact on the final consequence of NO production are the concentration of NO produced and the internal environment of the tissues. For example, the high levels of NO synthesized by NOS-2 triggers a set of reactions that do not occur during the low-level NO synthesis, which is due mainly to the activity of NOS-1 or NOS-3. Furthermore, NO reacts at a near diffusion-controlled rate with superoxide to form the cytotoxic species peroxynitrite (ONOO). The formation of ONOO is thought to be responsible, at least in part, for the toxicity associated with NO. For instance, ONOO can react readily with phenolic compounds to form nitrated, hydroxylated, and dimerized products. Nitration of free tyrosine, or tyrosine in

Financial support was partially provided by the John S. Dunn, G. Harold and Leila Mathers, and Robert A. Welch Foundations as well as The University of Texas.

ABBREVIATIONS: NO, nitric oxide; NOS, NO synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; ONOO, peroxynitrite; MPO, myeloperoxidase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Q-PCR, quantitative-PCR; RT-PCR, reverse transcriptase-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; IFN, interferon; FAM, carboxyfluorescein; TAMRA, carboxytetramethylthrolamine.

proteins, serves as a "marker" of ONOO formation in vivo (Bian et al., 1999a). Thus, a precise regulation of NO production is required to maintain homeostasis (Bian et al., 1999b).

In the gastrointestinal tract, NO is produced at many sites and has been shown to take part in physiological and pathological events (Salzman, 1995). For example, there is an increasing body of evidence indicating that NO is involved in the pathogenesis and pathophysiology of inflammatory bowel diseases, which includes Crohn's disease and ulcerative colitis. Increased mucosal NOS-2 activity and NO production have been reported in patients with active inflammatory bowel disease (Dijkstra et al., 1998; Guslandi, 1998) and in animals with a variety of induced or spontaneous intestinal inflammation (Miller et al., 1995; Matsumoto et al., 1998). An overall understanding of the role of NO in intestinal inflammation would help clarify the etiology and pathogenesis of many disease processes.

In this study, we report that infection with the parasite *Trichinella spiralis* down-regulates NOS-2 expression in the ileum and lung from control mice, and in the small intestine, colon, kidney, and uterus from endotoxin-treated mice. The findings suggest that a specific set of inflammatory reactions can induce down-regulation of NOS-2 expression through mechanisms that are expressed systemically. Elucidation of such potent endogenous NOS-2 down-regulatory mechanisms could lead to the development of new strategies for the therapy of inflammatory conditions characterized by the overproduction of NO.

Materials and Methods

Animals and *T. spiralis* Infection. Male CF-1 mice (b.wt. 25–30 g) were from Harlan. The experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and approved by the University of Texas Medical School Animal Care and Use Committee. *T. spiralis* was maintained by passage in CF-1 mice. Naive mice were inoculated orally with 600 *T. spiralis* larvae obtained by enzymatic digestion of skeletal muscle from infected mice (Harari, 1991). For the time course experiments, animals were sacrificed 1, 3, 7, and 10 or 20 days after oral inoculation. Mice were infected in the mid-afternoon, and tissues were harvested in mid-morning on subsequent days. For the experiments involving endotoxin (lipopolysaccharide or LPS), *T. spiralis*-infected or uninfected mice were divided into LPS- and vehicle-treated groups. LPS (serotype 0111:B4; Sigma, St. Louis, MO) was administered (1 mg/kg b.wt.) intraperitoneally 12 h before scheduled sacrifice. Then, the proximal jejunum and distal ileum, as well as other organs were quickly isolated. Intestinal segments were divided in two by cutting longitudinally, and the halves were processed separately for the isolation of RNA or protein. All organs were immediately frozen in liquid nitrogen, then stored at −135°C until further processing.

Tissue Processing and Sample Preparation. Frozen tissues were pulverized with a pestle and mortar that contained liquid nitrogen. For protein extraction, the tissues were homogenized at 4°C in 20 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (final concentration: 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamadine, 5 m trypsin inhibition unit/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 5 μg/ml antipain, 0.2 mM phenylmethane sulfonate fluoride, and 0.1 mM EDTA). Each sample was homogenized using a polytron at 4°C then sonicated on ice using a cell disruptor with 5 pulses at duty cycle of 40% and output of 3. The homogenate was centrifuged at 3,000*g* for 15 min at 4°C, and supernatant fractions were used for SDS-polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting. The NOS-2 enzyme can be further separated into membrane (particulate) and cytosolic fractions according to its subcellular location. To further separate those two fractions, the supernatant fraction from 3,000*g* centrifugation was centrifuged again at 100,000*g* for 60 min at 4°C. The supernatant was used for detecting the cytosolic fraction of NOS-2, whereas the pellet was dissolved in homogenate buffer in a volume equal to the supernatant and used for the particulate NOS-2 detection. For total RNA isolation, the tissue powder was placed in RNAzol B (Biotect Lab, Houston, TX), then 0.3 ml of chloroform was added. After centrifugation (12,000*g*, 15 min at 4°C), the aqueous phase was transferred to a fresh tube, an equal volume of isopropanol was added, and the mixture was stored at −20°C for 1 h. The mixture then was centrifuged to obtain the RNA precipitate.

Western Blot Analysis. The supernatant and particulate fractions prepared from proximal jejunum, distal ileum, and other organs were separated by 7.5% SDS-PAGE. Equal amounts of proteins (50 or 100 μg/well) were loaded onto the gel for each experimental sample. Separated proteins were transferred to nitrocellulose membranes for 60 min at 4°C, and the membranes were treated with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, 130 mM NaCl, pH 7.6 + 0.1% Tween 20), then incubated at 4°C overnight with the anti-NOS-2 antibody. The membranes were washed with TBS-T and incubated with peroxidase-conjugated goat anti-rabbit antibody. Chemiluminescence was used to identify NOS-2 protein according to the ECL Western blotting detection system (Amersham Pharmacia Biotech, Arlington Heights, IL). We performed immunoblot analyses of NOS-2 with both polyclonal (Weisbrodt et al., 1996) and monoclonal (Transduction Laboratories; BD, Franklin Lakes, NJ) antibodies. A monoclonal antibody to paxillin (Transduction Laboratories; BD) and polyclonal antibodies to myeloperoxidase (MPO), COX-2 (Upstate Biotechnology, Lake Placid, NY), and NOS-1 (Sigma) were also used.

Analysis of NOS-2 mRNA by Northern Blot. Total RNA (30 μg/lane) from each tissue was separated on a 1% agarose gel containing 2% formaldehyde and transferred to nitrocellulose. Ethidium bromide staining was used to confirm the amounts of loaded RNA and to assess the efficiency of transfer. The cDNA probes for NOS-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were kindly provided by Dr. Bruce Kone of our institution (Kone et al., 1995), and radiolabeled with [^{32}P]dCTP according to the recommended procedures of the manufacturer (random primed DNA labeling kit from Roche Molecular Biochemicals, Indianapolis, IN). After hybridization with the probe overnight at 42°C, the membrane was washed with 2.0× SSPE [1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA (pH 7.7)]/0.1% SDS, 1.0× SSPE/0.1% SDS, and 0.2× SSPE/0.1% SDS at 42°C, and exposed to Kodak XAR film at −70°C. The membrane was reprobed for use in another hybridization with the GAPDH probe serving as an internal control.

NOS-2 mRNA Quantitation with Real-Time Q-PCR. Real-time quantitative RT-PCR employs a fluorescent probe to monitor continuously the progress of a PCR reaction (Heid et al., 1996). This system is based on the ability of the endonuclease activity of *Taq* polymerase to hydrolyze oligonucleotides hybridized to a template undergoing replication. The Quantitative Genomics Core Laboratory in our department houses TaqMan integrated thermocycler/fluorometers (ABI/Perkin-Elmer 7700 Sequence Detector) containing a multiplexed laser and CCD detector that allows for the continuous measurement of the fluorescent signal present in PCR reactions. The amplification reaction includes a template, conventional PCR primers for mouse NOS-2 (sense: 5′-CAG CTG GGC TGT ACA AAC CTT-3′; position of cDNA 2176–2197. Antisense: 5′-ATG TGA TGT TTG CTT CGG ACA-3′; position of cDNA 2220–2241), and a specific hybridization probe derivatized with two dyes [reporter-FAM and quencher-TAMRA; mouse NOS-2 mRNA FAM(+): 5′-CGG GCA GCC TGT GAG ACC TTT G-3′; position of cDNA 2198–2220) that are quenched by fluorescence resonance energy transfer]. During the PCR, *Taq* hydrolyzes the reporter dye from the quencher and results in an increase in fluorescent signal that is directly proportional to

the number of probe molecules being hydrolyzed, which, in turn, is a direct measure of the number of template amplicons synthesized in the reaction during that cycle. The Ct (number of PCR cycles required for the fluorescent signal to reach an arbitrary threshold) is directly proportional to the amount of input template, so that with the use of a plasmid standard, the relative number of template molecules introduced into the reaction are determined. By using the same mRNA samples, control real-time Q-PCR for mouse acidic ribosomal phosphoprotein P0 (36B4; housekeeping gene) (Laborda, 1991) were performed in parallel with the experimental reactions. PCR primers for 36B4 (sense: 5′-AGA TGC AGC AGA TCC GCA T-3′; position of cDNA 189–208. Antisense: 5′-GTT CTT GCC CAT CAG CAC C-3′; position of cDNA 228–247), and a specific hybridization probe [reporter-FAM and quencher-TAMRA; mouse 36B4 mRNA FAM(+): 5′-CGC TCC GAG GGA AGG CCG-3′; position of cDNA 210–228] were used for the reactions.

Assay of NOS-2 Activity. NOS-2 activity was determined by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline using the method previously described (Bredt and Snyder, 1990) with modifications. The production of L-[^{3}H]citrulline from L-[^{3}H]arginine was measured in the presence of NADPH (1 mM), tetrahydrobiopterin (10 μM), L-valine (120 mM), L-arginine (50 μM), L-[^{3}H]arginine, and HEPES buffer (pH 7.5, containing dithiothreitol, 0.2 mg/ml) in a total volume of 200 μl for 60 min at 37°C. Reactions were stopped by dilution with 0.4 ml of ice-cold stop buffer (sodium acetate buffer, pH 5.5) containing 2 mM EDTA. Reaction mixtures were applied to a Dowex AG 50 W-X8 column (Na+ form, prepared from the H+ form); L-[^{3}H]citrulline was eluted and measured by liquid scintillation counting.

Data Analysis. Results are expressed as means ± S.E.M. A one-way analysis of variance was performed for multiple comparisons, and if there was significant variation between treatment groups, the mean values for a treated group were compared with those for the control by using Student's t test; p values of less than 0.05 were considered statistically significant. The n values indicate the numbers of animals used in the experiment.

Results

Suppression of NOS-2 Expression in Intestine by *T. spiralis* Infection. The distribution of worms along the small intestine of mice during a primary infection has been well studied, and for a given infection, the majority of adult worms are found in the anterior part (proximal jejunum) of the small intestine where they induce an inflammatory response (Dick and Silver, 1980). As a part of the inflammatory reaction, MPO activity (reflecting the presence of myeloid cells) is maximally elevated in the jejunum at 7 to 10 days postinfection (2- to 3-fold), while there are no elevations of MPO in the ileum (Harari and Castro, 1991). In the current study, Western blot analysis indicated that MPO (38 and 60 kDa) expression in the jejunum was increased by 3 days postinfection and reached a peak by 7 to 10 days postinfection. The 38-kDa band of MPO was markedly decayed by 20 days postinfection, whereas the 60-kDa band still exhibited significant intensity (data not shown).

In the current study, the first 3 days of *T. spiralis* infection caused an increase in NOS-2 expression in the jejunum where the worms are embedded. By day 7 postinfection, jejunal NOS-2 was abolished and was suppressed for up to 20 days (Fig. 1). Jejunal inflammation to the *T. spiralis* infection was also associated with a down-regulation of NOS-2 expression in the ileum. As reported previously (Weisbrodt et al., 1996; Hoffman et al., 1997), NOS-2 was constitutively ex-

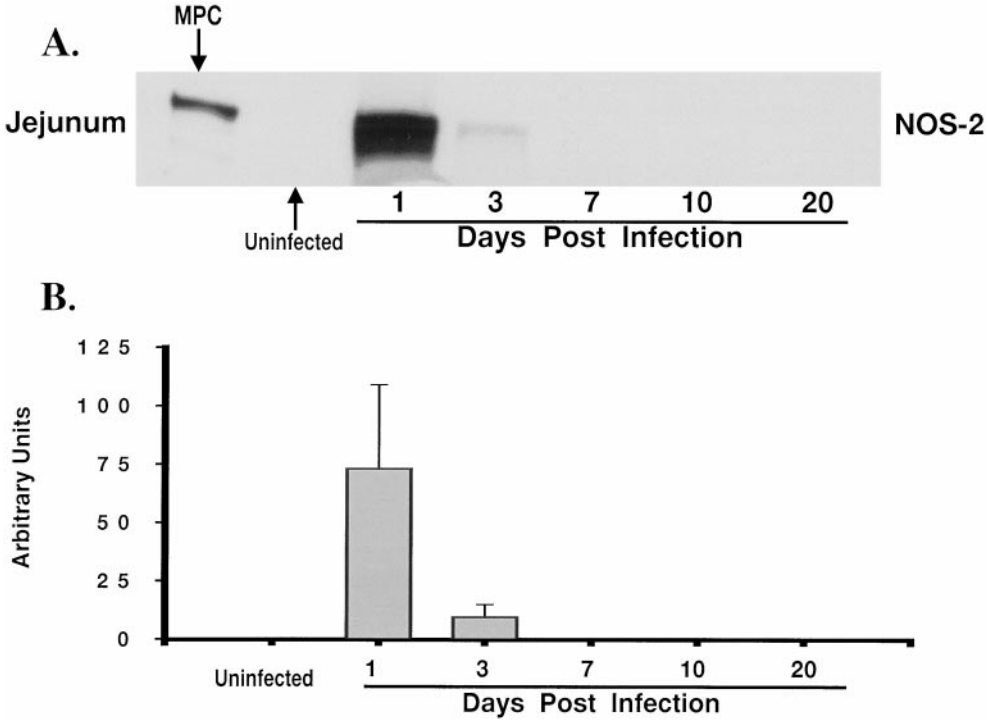

Fig. 1. Immunoblots of NOS-2 in full thickness segments of proximal jejunum following infection with *T. spiralis*. Protein extractions (100 μg/well) from normal jejunum (uninfected) and from parasitized jejunum between day 1 and day 20 post-*T. spiralis* infection were separated by size with SDS-PAGE followed by Western blot analysis. NOS-2 (130 kDa) was detected with an anti-NOS-2 polyclonal antibody developed in rabbits by our group. Protein extraction from LPS-stimulated macrophage cell line (RAW 264.7) served as a positive control (MPC). A, NOS-2 was undetectable in normal jejunum but was induced during the first 3 days of *T. spiralis* infection. By day 7 postinfection, jejunal NOS-2 was abolished and remained suppressed for up to 20 days. B, quantitative analysis of NOS-2 protein from uninfected and parasitized jejunum. Values of arbitrary unit are expressed as means and S.E.M. (n = 4).

pressed in the ileum of control animals. Following infection with *T. spiralis*, NOS-2 expression in the ileum tended to increase at day 1 after infection, but started to decrease from 3 days and was almost abolished by day 10 postinfection (Fig. 2). To evaluate our observation more carefully, we performed immunoblot analyses with both polyclonal and monoclonal antibodies specific for NOS-2. Both antibodies produced similar results: suppression of NOS-2 occurred in the intestine of *T. spiralis*-infected mice. To test whether NOS-2 is expressed in the parasite itself, we performed Western blot analysis with as much as 50 μg/well protein extracted from *T. spiralis* larvae. Even using such a large amount of protein, which is several thousand times higher than the larval protein contained in infected intestinal samples used for Western blot analysis, no NOS-2 expression was detected in *T. spiralis* (data not shown).

Suppression of NOS-2 mRNA by *T. spiralis* Infection. *Northern Blot analysis.* NOS-2 mRNA expression was detected in ilea from control mice. The level of expression was not changed at 1 day after *T. spiralis* infection. A statistically significant decrease was attained after 3 days of infection. At day 7 postinfection, levels of NOS-2 mRNA were markedly suppressed. At day 10 postinfection, no NOS-2 mRNA could be detected in the distal intestine by Northern blot analysis (Fig. 3).

Real-time Q-PCR. NOS-2 mRNA, normalized to 36B4 (a housekeeping gene) mRNA, tended to be suppressed at 3 days postinfection. The suppression of NOS-2 transcript levels was statistically significant at 7 days postinfection. At 7 and 10 days postinfection, NOS-2 mRNA transcript exhibited a 10-fold decrease compared with the levels in uninfected mice (Fig. 4).

Suppression of LPS-Induced NOS-2 Expression in *T. spiralis*-Infected Animals. The above observation (Figs. 2, 3, and 4) that constitutively expressed NOS-2 in the ileum, the region with extremely few parasites inoculated, is markedly suppressed by *T. spiralis* infection, suggests that *T. spiralis*-induced gut inflammation may initiate NOS-2 down-regulation through mechanisms that are expressed systemically. To test whether the parasite infection could inhibit NOS-2 in other organs, we performed an experiment with LPS administration, a well established model for studying systemic inflammatory events and injury. Induction of NOS-2 in response to LPS has been demonstrated in a variety of tissues including intestine, liver, lung, heart, kidney, spleen, and uterus by our group and other laboratories. As expected, LPS-treatment elicited a marked increase of NOS-2 expression in both the jejunum (Fig. 5A) and ileum (Figs. 7 and 9) in the uninfected mice. However, NOS-2 expression in the small intestine in response to LPS was significantly suppressed in *T. spiralis*-infected animals (Figs. 5A, 7, and 9). Furthermore, LPS-induced NOS-2 expression in the proximal and distal colon was abolished by the infection (Fig. 5B) and was markedly attenuated in the kidney (Fig. 6A) and uterus (Fig. 6B) post *T. spiralis* infection. NOS-2 expression also was detected in the lung of control animals (Fig. 6C), and was significantly suppressed by *T. spiralis* infection. However, in contrast to the other organs tested, LPS-induced NOS-2 expression in lung tended to resist the inhibitory effect of the intestinal parasite. The LPS

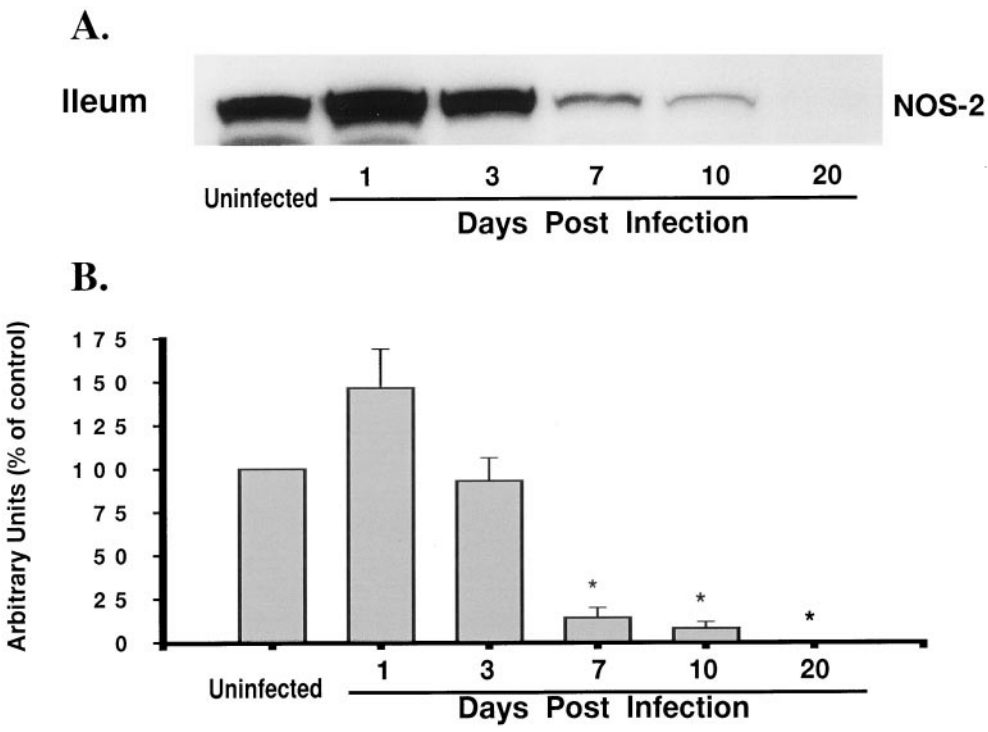

Fig. 2. Immunoblots of NOS-2 in full thickness segment of distal ileum following infection with *T. spiralis.* Protein extractions (100 μg/well) from normal (uninfected) and *T. spiralis*-infected mice at indicated times were separated by size with SDS-PAGE followed by Western analysis. NOS-2 (130 kDa) was detected with an anti-NOS-2 antibody developed by our group. A, NOS-2 was detected in normal (uninfected) ileum, and the level of NOS-2 expression was greatly attenuated as the infection in the jejunum progresses. B, quantitative analysis of NOS-2 protein level between day 1 and day 20 post-*T. spiralis* infection. Data were normalized and expressed as percentage of control (100%). Values are expressed as means and S.E.M. (n = 7). *$p < 0.05$ compared with control.

treatment markedly induced NOS-2 expression in spleen, and it is notable that the induction of NOS-2 was not significantly affected by *T. spiralis* infection (Fig. 6D) in this organ.

Suppression of NOS-2 Expression by *T. spiralis* Infection in Mucosal and Nonmucosal Layers of the Ileum. NOS-2 immunoreactivity was distributed both in the mucosa and non-mucosal tissues of ileum from control animals (Fig. 7A). In LPS-treated animals, there was a marked increase of NOS-2 expression in both mucosa and non-mucosa, which was attenuated to a similar degree in both compartments in *T. spiralis*-infected animals (Fig. 7A). The NOS-2 enzyme can be further separated into membrane (particulate) and cytosolic fractions according to its subcellular location. LPS injection elevated NOS-2 expression in both subcellular compartments of the ileum in uninfected animals. The suppression of LPS-induced NOS-2 expression in *T. spiralis*-infected animals also was evident in both the particulate and cytosolic compartments (Fig. 7B).

Suppression of NOS-2 Activity by *T. spiralis* Infection. To determine whether *T. spiralis* infection inhibits NOS-2 activity as it did NOS-2 protein expression, the enzyme activity was measured in homogenates of ileum. NOS-2 activity was significantly increased by LPS-treatment of uninfected mice. However, the same dose of endotoxin failed to elevate NOS-2 activity of the ileum from *T. spiralis*-infected mice (Fig. 8)

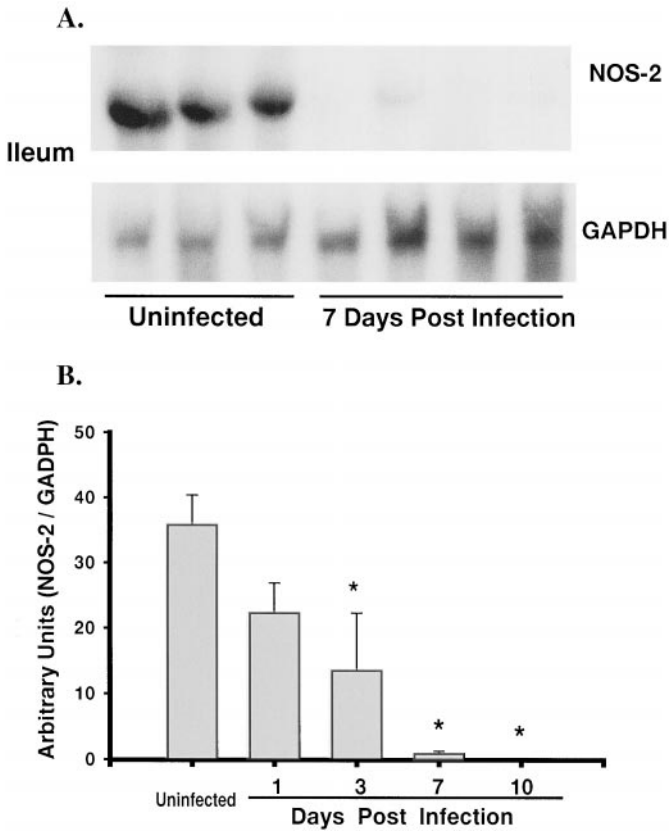

Fig. 3. Northern analysis of NOS-2 mRNA expression in the ileum after *T. spiralis* infection. A, aliquots of 30 μg of RNA isolated from normal and 7 days infected mice were used for Northern analysis. The same sample was blotted with each of ^{32}P-labeled NOS-2 or GAPDH probes. NOS-2 mRNA was abolished following infection with *T. spiralis* whereas no change was noticed in GADPH mRNA expression. B, changes in ileal NOS-2 mRNA levels at different times post-*T. spiralis* infection were quantitated after normalizing to GADPH mRNA. Data are expressed as means ± S.E.M. (n = 5 for control, n = 3 for day 1 and day 3, and n = 4 for day 7 and day 10). *$p < 0.05$ compared with control.

Selective Down-Regulation of NOS-2 Protein by *T. spiralis*-Induced Gut Inflammation. To determine whether the expression of other proteins is also down-regulated by *T. spiralis* infection, we measured both paxillin and COX-2 immunoreactivity in control and infected animals. Paxillin is a cytoskeletal component that localizes to the focal adhesion sites at the ends of actin stress fibers and is often used as a "housekeeping protein" in Western blot analysis. Figure 9 depicts blots of samples derived from ilea of three groups of mice: 1) saline-treated uninfected; 2) LPS-treated (1 mg/kg b.wt., i.p.); and 3) 7 day *T. spiralis*-infected + LPS-treated. NOS-2 immunoreactivity was markedly decreased in the samples from *T. spiralis*-infected animals, whereas paxillin immunoreactivity was not significantly altered. COX-2 is another protein induced by proinflammatory cytokines and it shares similar transcriptional regulation mechanisms with NOS-2 (Hauck et al., 1999). Figure 10 illustrates two blots with the same samples from ileal tissue. Following infection with *T. spiralis*, NOS-2 immunoreactivity started to decrease from 3 days and was almost abolished by day 10 postinfection, whereas no significant alteration was observed in COX-2 immunoreactivity. Furthermore, the expression of nNOS (NOS-1) in ileal tissue was not affected by *T. spiralis* infection (data not shown).

Discussion

Our current study reveals that during the first 3 days of *T. spiralis* infection, an increased NOS-2 expression is detected in the jejunum where the worms are embedded and are inducing an inflammatory reaction. On the other hand, by day 7 postinfection, despite severe inflammation that is reflected in histological changes as well as by increased MPO levels, jejunal NOS-2 expression is abolished and is suppressed for up to 20 days of infection. *T. spiralis* infection resulted not only in local suppression of jejunal NOS-2, but also in inhibition of NOS-2 expression in other non-inflamed organs such as ileum and lung. By using an LPS-induced systemic inflammatory model we also found that endotoxin induced up-regulation of NOS-2 was markedly inhibited by the nematode infection in several tissues. Thus, our study clearly indicates that an ongoing specific gut inflammatory reaction can set into motion systemically operated mechanisms, which down-regulate NOS-2 expression. Such down-regulation may prove to be important for preventing inflammatory injury associated with endotoxin exposure (Laszlo et al., 1994).

The down-regulation of NOS-2 expression by *T. spiralis* may reflect an adaptive mechanism of the parasitic invader. It has long been recognized that macrophages activated by immune responses acquire potent microbicidal mechanisms to destroy a variety of pathogens, several of which are linked to the production of NO and/or subsequent oxidation products (Oswald and James, 1996). The requirement for L-arginine, the substrate for NOS, has also been identified in macrophage-mediated killing or cytostasis of various extracellular and intracellular parasites such as *Schistosoma mansoni* (James and Glaven, 1989), *Trypanosoma musculi, brucei*, and *cruzi* (Gazzinelli et al., 1992; Vincendeau et al., 1992), *Toxoplasma gondii* (Adams et al., 1990), *Leishmania*

enriettii (Mauel et al., 1991), and *Leishmania major* (Liew et al., 1990). Thus, prevention of the generation of NO by the host, may be required for *T. spiralis* to complete its life cycle. It may be that secretion products from both adult and newborn larvae contain regulatory substances that directly or indirectly inhibit NOS-2 expression. Newborn larvae begin to penetrate the intestinal wall, circulate in the blood stream, and embed and encyst in skeletal muscles starting at about day 5 postinfection (Castro et al., 1980). Suppression of NOS-2 in organs other than the intestine may facilitate this process.

Only a few studies so far offer information regarding the regulation of NOS-2 expression by *T. spiralis* infection. Using cultured human colonic epithelial cell lines, Li et al. (1998) compared the epithelial responses to the invasion by *T. spiralis* with the responses elicited by the invasive bacteria *Listeria monocytogenes* and found that only bacteria elicited increased NOS-2 mRNA. The authors concluded that epithelial NO may not be important in innate defense mechanisms against multicellular parasites. The other NOS-2 related *T. spiralis* study (Hogaboam et al., 1996) focused mainly on whether the damaging effects of NO contributed to pathological changes in rat small intestine during *T. spiralis* infection. Using a non-quantitative PCR method, they detected NOS-2 mRNA in jejunum from rats 6 days postinfection but not from control rats. At the same time point, they reported a significant decrease of NOS activity in infected jejunum although MPO activity remained elevated. The authors proposed that NOS-2 activity in the parasitized jejunum is regulated at a stage after mRNA synthesis. Our Northern blot analysis revealed that NOS-2 mRNA expression was statistically decreased after 3 days. Although our results using real-time Q-PCR did not indicate a statistically significant decrease of NOS-2 mRNA at 3 days postinfection, data showing that the levels of both NOS-2 mRNA and protein were markedly suppressed at days 7 and 10 postinfection strongly support the conclusion that the *T. spiralis*-derived NOS-2

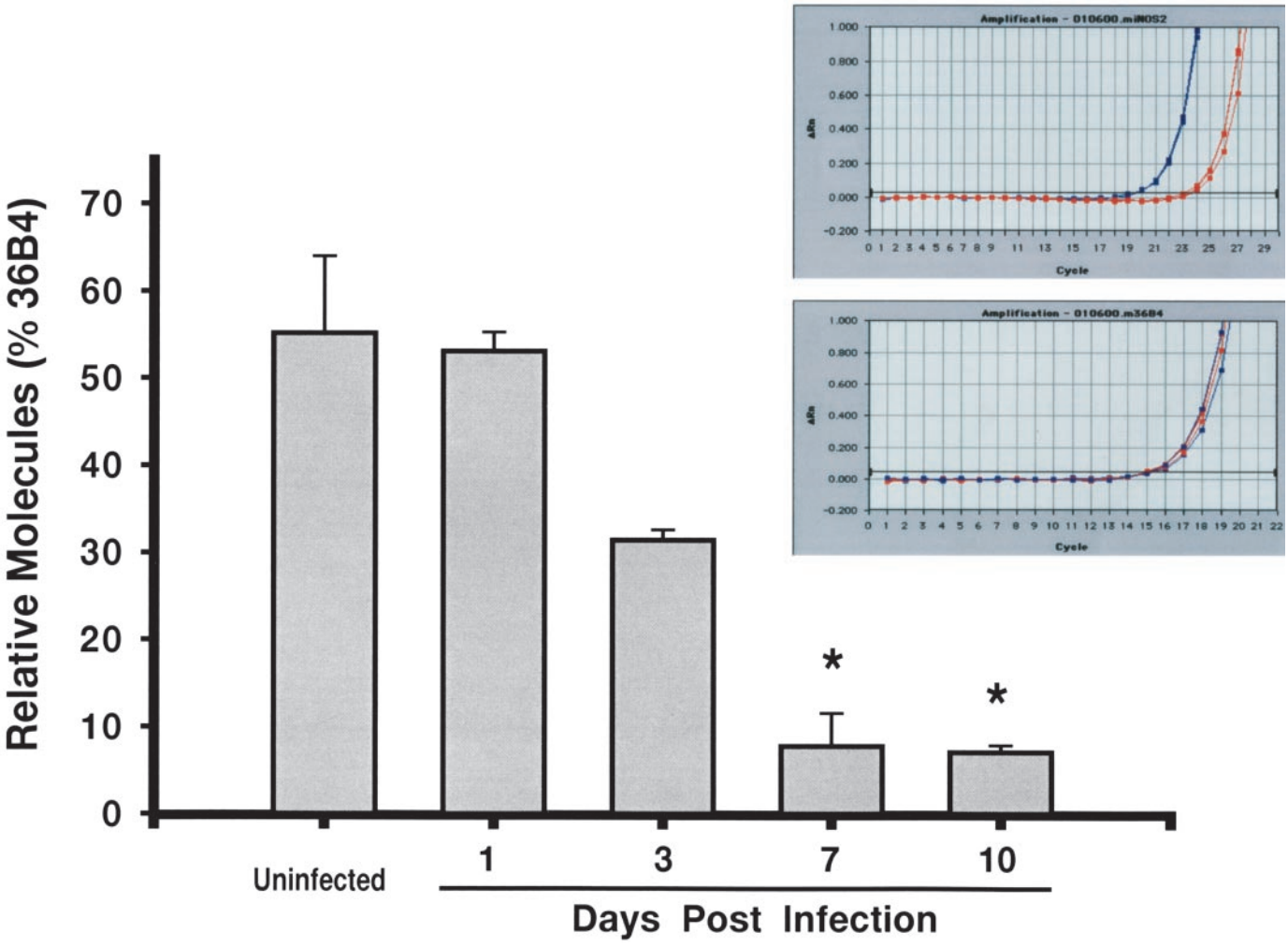

Fig. 4. Real-time quantitative RT-PCR analysis of NOS-2 mRNA in the ileum following *T. spiralis* infection. Changes in ileal NOS-2 mRNA between day 1 and day 10 post-*T. spiralis* infection were quantitated after normalizing to 36B4 (a housekeeping gene) mRNA. Data are expressed as means ± S.E.M. ($n = 5$ for control, $n = 3$ for day 1 and day 3, $n = 4$ for day 7, and $n = 5$ for day 10). *$p < 0.05$ compared with control. Inset: top panel, amplification plots show a significant difference between the accumulation of ileal NOS-2 cDNA of uninfected (blue: composed of 3 separate lines each presenting one reaction) and 7 days postinfected (red: composed of 3 separate lines each presenting one reaction) animals detected by ABI/Perkin-Elmer 7700 Sequence Detector. Bottom panel, amplification plots indicate the similar accumulation of ileal 36B4 cDNA in both uninfected (blue: composed of 3 separate lines each presenting one reaction) and 7 days postinfected (red: composed of 3 separate lines each presenting one reaction) animals. The *y*-axis (ΔRn) represents the normalized fluorescent signal of the reporter dye minus the baseline signal established in the first few cycles of PCR.

inhibition involves both gene transcription and protein expression.

The mechanisms responsible for the down-regulation of NOS-2 seen from 3 to 20 days postinfection are not known. As described above, trichinosis is characterized by an initial intestinal phase involving development of the parasite through larval stages to sexually mature worms, and a subsequent extra intestinal phase that involves the migration of second generation larvae from the gut and their eventual encystment in skeletal muscle. Although the intestinal stages of the parasite cause histological and functional changes that underlie symptoms of early infection, major antibodies (both IgM and IgG) against all tested antigenic components of *T. spiralis* are not detectable during this period (Takahashi et al., 1990). Our studies demonstrate that NOS-2 down-regulation became significant 3 days postinfection. Thus, the systemic NOS-2 regulation mechanism is activated or initiated at a time that may not correspond directly with specific immune reactions such as the formation of anti-*T. spiralis* antibodies. On the other hand, a systemic anti-inflammatory response does take place concomitantly with inflammation of the intestinal mucosa induced by *T. spiralis*. Castro et al. (1980) demonstrated a potent systemic anti-inflammatory effect during the intestinal phase of *T. spiralis* infection and compared it with the effects of dexamethasone, a synthetic adrenocortical steroid. Their study indicated that the magnitude of immunosuppression caused by infection with 6.6×10^3 larvae/kg body weight of rat was equivalent to that produced by 1.0 mg/kg body weight of dexamethasone. It is generally believed that the suppression of NOS-2 expression is a major contributor to the anti-inflammatory effect of the steroids (Radomski et al., 1990). Therefore, it would be easy to propose that the stress-induced production of corticosteroids plays a key role in the inhibition of NOS-2 expression associated with enteric trichinellosis.

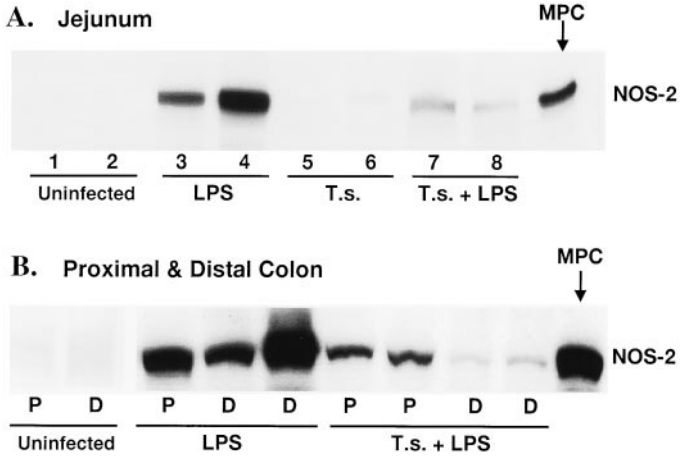

Fig. 5. Immunoblots of NOS-2 in full thickness segments of jejunum as well as proximal (P) and distal (D) colon from normal and LPS- or *T. spiralis*-treated mice. A, jejunum: LPS treatment markedly up-regulated NOS-2 expression, which was significantly inhibited by 7 days of parasite infection (T.s.+LPS). Each numbered lane represents an individual animal used in the experiment. Study was replicated to four animals in each experimental group. B, proximal and distal colon: there was no detectable NOS-2 in proximal (P) and distal (D) colon from normal animals. LPS treatment markedly increased NOS-2 expression, which was significantly inhibited by parasite infection. Each lane represents an individual animal used in the experiment. Study was replicated to four animals in each experimental group. Protein from LPS-treated (RAW 264.7) cells served as positive control (MPC) of NOS-2 (130 kDa).

However, our observation indicates that LPS-induced NOS-2 expression in certain organs such as lung, liver, and spleen was less sensitive than the intestine to the suppressive effects of trichinellosis. The actions of endogenous corticosteroids would not be expected to show organ selectivity. In addition, COX-2 expression, which would be inhibited by glucocorticoid hormones (Masferrer et al., 1994), is not reduced by *T. spiralis* infection. Thus, our data do not support the glucocorticoid inhibition theory, and the possibility that other unidentified substances or pathways are involved in the systemic down-regulation of NOS-2 is strongly suggested.

Because NOS-2 expression is under the influence of various cytokines, it is reasonable to view the results of our study in light of cytokine profiles. Two very distinct cytokine secretion patterns are originally defined among a panel of T-cell clones. T-helper type-1 (Th1) cells mainly produce IL-2, IL-8,

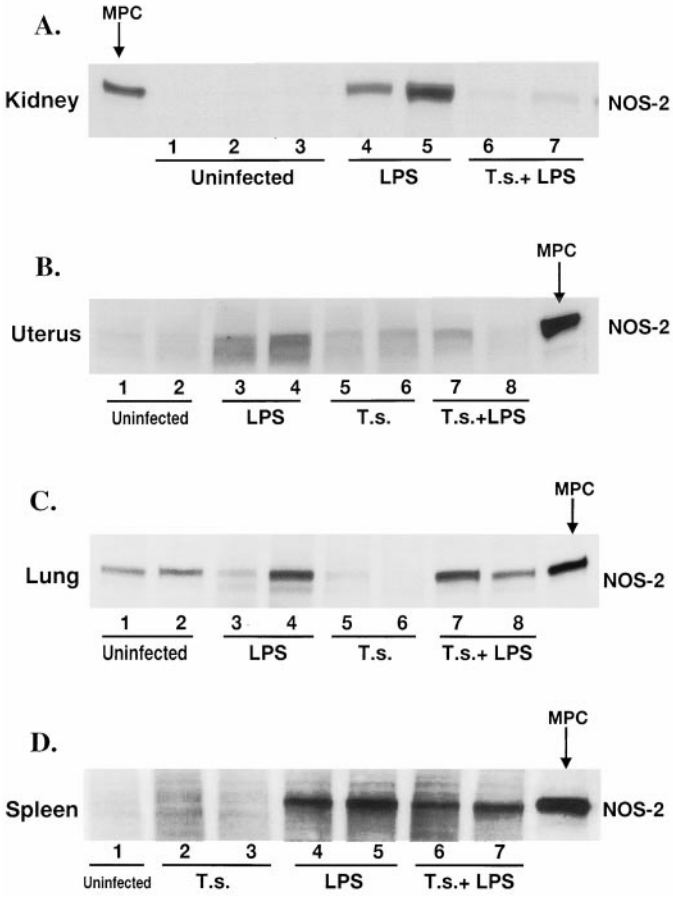

Fig. 6. Western analysis of change of NOS-2 levels in kidney (A), uterus (B), lung (C), and spleen (D) after treatment with LPS or *T. spiralis*. Protein extractions (100 μg/well) from control (uninfected), *T. spiralis*-infected (T.s.), LPS-treated normal (LPS) or *T. spiralis* 7 days infected animals (T.s.+LPS) were subjected to SDS-PAGE followed by Western analysis. LPS-induced NOS-2 expression was abolished in the kidney (A) and markedly attenuated in the uterus (B) by *T. spiralis* infection. In this study, NOS-2 expression was detected in the lung of control animals (C) but could not be detected in *T. spiralis*-infected animals. LPS-induced NOS-2 expression in lung tended to resist the inhibitory effect of the intestinal parasite. In spleen (D), the LPS treatment markedly induced NOS-2 expression that was not significantly affected by *T. spiralis* infection. Each numbered lane represents an individual animal used in the experiment. Study was replicated to a total of three to four animals in each experimental group. Protein extraction from LPS-stimulated macrophage cell line (RAW 264.7) served as a positive control (MPC) of NOS-2.

IFN-γ, and tumor necrosis factor-α, whereas Th2 express IL-4, -5, -6, -10, -13, and transforming growth factor-β (Street and Mosmann, 1991). Although the reciprocal role of Th1 and Th2 cytokines on regulation of NOS-2 expression has been demonstrated (Berkman et al., 1996), the explanation that down-regulation of NOS-2 is mainly due to parasite-induced increases of Th2 cytokines is not supported by our results. The expression of both Th1 and Th2 cytokines during the early course of infection with *T. spiralis* has been carefully monitored by several groups (Ramaswamy et al., 1996; Ishikawa et al., 1998; Stewart et al., 1999), and the profile of cytokine release suggests that although a blended Th1/Th2 response may be in effect during the first several days of infection, Th1 cells probably exert a significant influence in the enteric environment out to day 8 postinfection, then to be replaced by the Th2 subset. Despite the existence of early elevated Th1 cytokines, steady-state levels of NOS-2 mRNA are statistically decreased after 3 days of infection (Fig. 3). In addition, the early phase of IL-4 activity in intestinal lymph has been shown to decline to normal levels during day 4 to day 9 postinfection, although IFN-γ is still being expressed (Ramaswamy et al., 1996; Stewart et al., 1999). However, we found that ileal and jejunal NOS-2 expression are markedly suppressed during this period. Furthermore, we did not observe significant alterations of COX-2 expression in the ileum during the course of *T. spiralis* infection (Fig. 7B), which would be expected if there is any markedly increased release of cytokines (Endo et al., 1998). Moreover, our data, which will be published in another article, have demonstrated that 7 days following infection with *T. spiralis* (600 larvae/mouse), athymic mice also exhibit NOS-2 down-regulation in ileum.

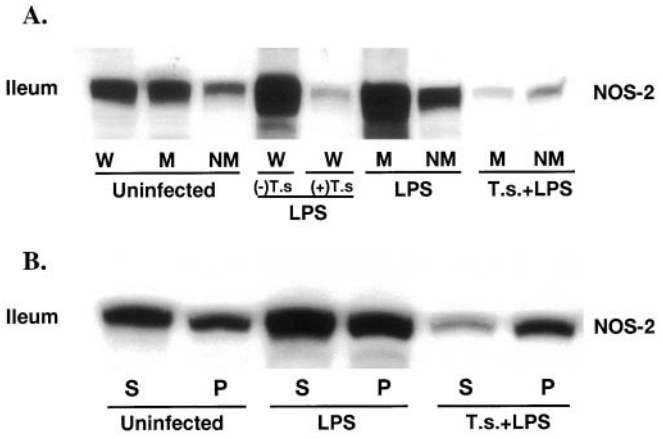

Fig. 7. Western blot analysis of NOS-2 expression in different compartments of the ileum. Protein extractions (100 μg/well) from full thickness (W), mucosal (M), and non-mucosal (NM) layers of distal ileum were separated by size with SDS-PAGE and analyzed by Western blot. A, LPS treatment markedly up-regulated NOS-2 expression in full thickness (W), mucosal (M), and non-mucosa (NM) layers of distal ileum. This up-regulation was significantly inhibited at 7 days postinfection (T.s.+LPS). B, the NOS-2 enzyme was further separated into membrane (particulate; P) and cytosolic (supernatant; S) fractions according to its subcellular location. Control (uninfected) animals had NOS-2 expressed in both soluble and particulate fractions, which was markedly increased with LPS treatment. The suppression of LPS-induced NOS-2 expression in 7 day *T. spiralis*-infected animals (T.s.+LPS) was also evident in both the membrane (P) and cytosolic (S) compartments.

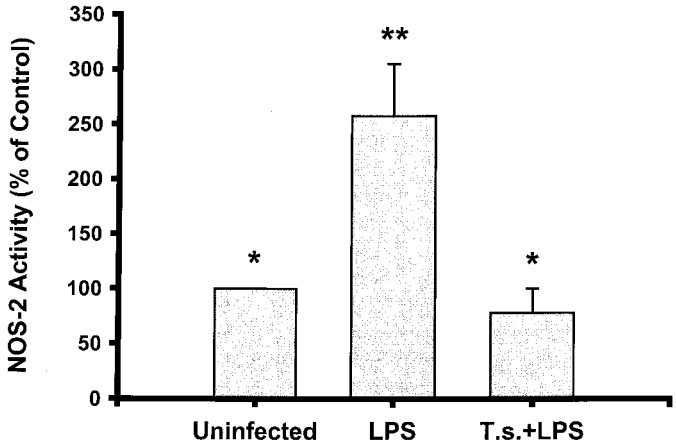

Fig. 8. Regulation of NOS-2 activity in distal ileum after treatment with LPS or *T. spiralis*. NOS-2 activity was determined by monitoring the conversion of L-arginine to citrulline. Enzyme activities associated with normal (uninfected, $n = 4$), LPS-treated normal mice (LPS, $n = 5$), and LPS-treated *T. spiralis* 7 days infected mice (T.s.+LPS, $n = 5$) were compared after normalizing data to the controls as 100%. Values are expressed as means and S.E.M. $^*p < 0.05$ compared with both control and LPS-treated *T. spiralis*-infected mice (T. S.+LPS). The specific NOS-2 activity of the control group (uninfected, $n = 4$) was 441.5 ± 84.9 pmol/mg of protein/60 min.

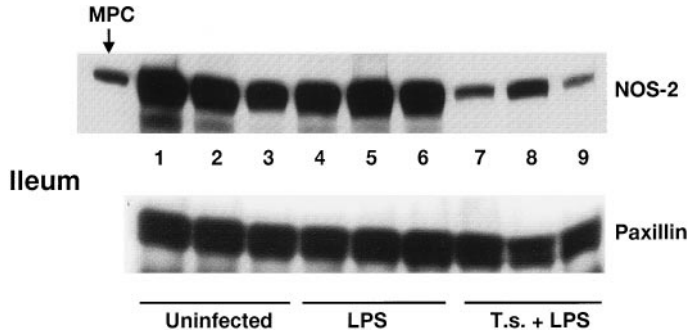

Fig. 9. Immunoblots of NOS-2 and paxillin in full thickness samples of distal ileum. Protein extractions (100 μg/well) from control (uninfected), LPS-treated normal mice (LPS), or *T. spiralis* 7 days infected mice (T.s.+LPS) were subjected to SDS-PAGE followed by Western analysis. Each indicated lane number represents an individual animal used in the experiment ($n = 3$ for each experimental group). The same sample was blotted with anti-NOS-2 (130 kDa) or anti-paxillin (68 kDa) antibodies. The up-regulation of NOS-2 immunoreactivity by LPS was almost abolished in the *T. spiralis*-infected animals, whereas paxillin was not significantly altered. The protein sample extracted from LPS-stimulated macrophage cell line served as a positive control (MPC) for NOS-2.

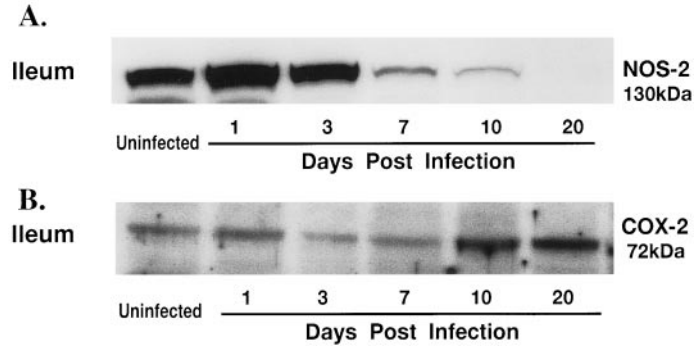

Fig. 10. Immunoblots of NOS-2 and COX-2 in full thickness samples of distal ileum. Protein extractions (100 μg/well) from normal (uninfected) and *T. spiralis*-infected mice at indicated times, were separated by size with SDS-PAGE followed by Western analysis. A, NOS-2 was detected in normal (uninfected) ileum, and the level of NOS-2 expression was greatly attenuated as the infection in the jejunum progresses. B, the same sample used in A was blotted with anti-COX-2 (72 kDa) antibody. In contrast to the change of NOS-2 expression, COX-2 immunoreactivity was not significantly altered during the infection.

Thus, at least, thymic-derived gut-associated lymphoid tissue is not involved in *T. spiralis*-initiated NOS-2 down-regulation, whereas it has been shown to be important in the development of Th2 cytokines (Scott et al., 1990; Svetic et al., 1993). In this separate article, we also demonstrate that terminating the *T. spiralis* infection (intestinal phase) at day 3 postinfection by administration of an anthelminthic resulted in no alteration of NOS-2 expression. Thus, although inoculated *T. spiralis* had sufficient time (3 days) to interact with gut mucosa, which is important for developing T-cell-mediated reactions including Th cell differentiation, the systemic NOS-2 regulation pathway had not been triggered under this condition. Further characterization of the cytokine profiles, as well as other inflammation related biological or biochemical changes, together with the down-regulation of NOS-2, in these regions are needed to completely establish the mechanisms by which *T. spiralis* infection alters the status and profile of inflammation.

In summary, we have demonstrated that infection with a nematode can markedly inhibit NOS-2 expression in both control as well as endotoxin-induced pathological conditions. These results indicate that certain ongoing gut inflammatory reactions can set into motion systemically operated mechanisms, which down-regulate NOS-2 expression. The characteristics of this NOS-2 inhibitory pathway or substance include: 1) an inhibition of NOS-2 gene transcription, protein expression, and enzyme activity; 2) an inhibition that is expressed in several different compartments where NOS-2 is located; 3) an inhibition that is probably not related to the formation of specific anti-parasite antibodies; and 4) an inhibition that may involve substances other than stress-induced corticosteroids. Elucidation of such novel and potent endogenous NOS-2 down-regulatory mechanisms could lead to the development of new strategies for the therapy of inflammatory conditions characterized by the overproduction of NO.

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