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Expression of nitric oxide synthase type II in the spinal cord under conditions producing thermal hyperalgesia

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Abstract

There is evidence supporting spinal cord nitric oxide (NO) production in the mechanisms underlying hyperalgesia, presumed to arise from the activity of neuronal nitric oxide synthase type I (NOS I). Intrathecal administration of interleukin-1β and interferon-γ to rats results in a thermal hyperalgesia which peaks at 2 h post-injection but which is undetectable 8 h post-injection. Expression of mRNA for nitric oxide synthase type II (NOS II) was detected by reverse transcription-polymerase chain reaction followed by Southern hybridization utilizing specific oligonucleotides in spinal cord tissue from animals 4 h and 8 h after cytokine injection, but not at longer time points. NOS II protein was detected in soluble fractions of spinal cords from animals 4 h and 8 h after cytokine injection. In situ hybridization for NOS II mRNA revealed positive cells bilaterally in the spinal cord 4 h after cytokine injection in a perivascular distribution and scattered throughout the gray and white matter. Immunohistochemistry for NOS II showed a similar distribution which could only be partially accounted for by macrophages/microglia. These results provide evidence for induction of NOS II expression under conditions producing thermal hyperalgesia and suggest a possible role in this behavior for the production of NO by a variety of cell types in the CNS.

Keywords: Nitric oxide; Cytokine; Nociception; In situ hybridization; Immunohistochemistry; Rat

1. Introduction

Peripheral tissue inflammation, damage, or nerve injury can lead to functional changes in the central nervous system, including an increased sensitivity to noxious stimulation, or hyperalgesia (Dubner and Ruda, 1992). Peripheral nociceptors innervating the area of injury exhibit increased activity, leading to neuronal plasticity and a hyperexcitable state in the relevant spinal cord dorsal horn. It is these neuronal changes which are thought to lead to hyperalgesia. The specific mechanism(s) underlying this plasticity are unclear but are thought to involve changes in chemical mediators which influence synaptic transmission in the spinal cord.

One class of chemical mediators which has been the subject of investigation in relationship to pathologic nociceptive processing is the excitatory amino acids. Significant evidence now exists supporting a role for *N*-methyl-D-aspartate (NMDA) receptor activation in spinal cord plastic changes, including spinal hyperalgesia (Meller and Gebhart, 1993). Nitric oxide (NO) is thought to be the mediator of at least some of the effects of NMDA receptor activation; therefore, a role for NO production in nociceptive mechanisms including hyperalgesia has also been the subject of recent investigations. Meller et al. (1992a) showed that i.t. administration of NMDA or L-arginine produced a thermal hyperalgesia in rats as measured by the nociceptive tail-flick reflex which could be reversed with a NOS inhibitor, L-NAME, supporting a role for endogenous NO production in the facilitation of thermal nociceptive reflexes. In addition, histologic studies reported at about the same time showed that neurons in sensory processing areas (superficial dorsal horn and lamina X) are one of the subsets of neurons in the rat spinal cord which ex-

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hibit NADPH-diaphorase activity and nitric oxide synthase type I (NOS I) immunoreactivity (Dun et al., 1992; Valtschanoff et al., 1992). Neurons anatomically positioned in the appropriate place for transmission of nociceptive information are therefore capable of releasing NO. Subsequent behavioral and histologic studies provided additional support for a role for NO in the mechanisms underlying spinal hyperalgesia (Meller et al., 1992a; Hama and Sagen, 1994; Steel et al., 1994; Yamamoto and Shimoyama, 1995).

Following the idea that hyperalgesia results from neuron plastic changes, the presumption exists in all of these studies that spinal cord NO released under hyperalgesic conditions is derived from the activity of constitutive NOS I. However, it is also possible that NO produced via another isoform, nitric oxide synthase type II (NOS II), expressed in neurons, other resident cells, or even infiltrating cells, may contribute to hyperalgesia. In support of a role for sustained NO production in hyperalgesia, Meller at al. (1992b) reported that loose ligation of the sciatic nerve in rats resulted in a marked thermal hyperalgesia on day 3 post-surgery which could be blocked by a NOS inhibitor. Suggestive evidence specifically for the role of NOS II expression in thermal hyperalgesia was reported by Meller at al. (1994). In these studies, i.t. administration of either interleukin (IL)-1β and interferon (IFN)-γ or endotoxin (LPS), compounds known to induce NOS II in resident CNS cells in vitro, produced a time-dependent hyperalgesia. Selective inhibition of glial metabolism with i.t. fluorocitrate resulted in reversible attenuation of the thermal and mechanical hyperalgesia produced by intraplantar zymosan, and selective inhibition of NOS II with i.t. aminoguanidine resulted in inhibition of the thermal hyperalgesia produced by intraplantar zymosan.

The purpose of this study was to determine whether expression of NOS II messenger RNA and protein occurs in the spinal cord of rats under conditions known to produce thermal hyperalgesia, and if so, to determine which cell type(s) express NOS II under these conditions.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were prepared with a sterile i.t. catheter (8.25 cm) at least three days prior to testing. An i.t. dose of either sterile preservative-free saline (15 ml) or the combination of cytokines hrIL-1β (10 ng, R&D Systems, Minneapolis, MN) plus rat IFN-γ (1000 U, GibcoBRL Grand Island, NY) was administered. Rats were sacrificed by decapitation at 4, 8, 24, 48, and 72 h after cytokine administration, and the lumbosacral enlargements of spinal cords were harvested and either frozen immediately at −70°C until future processing or placed in Histochoice MB fixative overnight prior to paraffin embedding. Control animals were sacrificed at 8 h and 72 h after saline administration. Cord tissue from one animal sacrificed after 24-h placement of a catheter only and one animal immediately sacrificed after cytokine injection as well as cervical enlargements of two animals sacrificed 4 h after cytokine injection were also obtained. It has previously been shown that administration of these cytokines in these concentrations produces a time-dependent thermal hyperalgesia compared with rats treated with saline which is maximal by 2 h post-injection and which is undetectable by 8 h post-injection (Meller et al., 1994).

2.2. Cell culture

RAW 264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's MEM with 10% (v/v) FCS and 50 μg/ml gentamicin at 37°C in 5% CO_2/95% air. Cells exposed 5 h to 50 U/ml murine IFN-γ (GibcoBRL, Grand Island, NY) and 500 ng/ml LPS (Sigma, St. Louis, MO) were used as a source of positive control total RNA for RT-PCR. Cells exposed 8–12 h to IFN-γ and LPS were used as a source of positive control protein homogenate for Western blot analysis.

2.3. RNA extraction

Total RNA was extracted from 100–200 mg (wet weight) rat lumbosacral spinal cord using TRISOLV (Biotecx Laboratories, Houston, TX) isolation of RNA according to manufacturer's instructions. Total RNA was quantitated by absorbance at 260 nm. Total RNA used for RT-PCR was first treated with 2 U of RNase-free DNase (Promega Corp., Madison, WI) for 1 h at 37°C to remove possible genomic DNA contamination.

2.4. cDNA synthesis

Eppendorf tubes containing 1 μg of total RNA and 0.5 μg oligo dT/18-mer were incubated for 2 min at 65°C and chilled on ice for 2 min. Each sample was then incubated for 2 h at 42°C after adding 20 U RNasin (Promega Corp., Madison, WI), 10 mM deoxynucleoside triphosphates (dNTPs; BMB Indianapolis, IN), 400 U reverse transcriptase (murine molony leukemia virus, GibcoBRL, Grand Island, NY), 4 μl of 5X reverse transcriptase buffer, and 1 μl of RNase-free water. The cDNA was diluted to a final volume of 80 μl with water and frozen at −20°C until use.

2.5. PCR

Ten microliters of cDNA was added to a reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol each

primer, and 1.25 U Taq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT). Each 50-μl sample was overlayed with 50 μl of mineral oil (Fisher Scientific, Pittsburgh, PA) and incubated in a DNA thermal cycler (MJ Research Inc., Watertown, MA) for a total of 35 cycles. Each cycle consisted of 1.5 min at 94°C, 1 min at 57°C, and 1.5 min at 72°C with a final elongation time of 7.0 min at 72°C. 'No target' negative controls consisted of tubes containing all the mixture components except target cDNA.

Sense and antisense NOS II primers were synthesized using the published cDNA sequence for mouse macrophage NOS II (Xie et al., 1992) and span nucleotides 258–288 (sense) and 675–698 (antisense). They produce expected products of 441 bp (murine cells) and 450 bp (rat tissue). β-Actin control primer sequences were synthesized using published sequences (Montgomery and Dallman, 1991) and produce an expected product of 571-bp length.

The sequences of the NOS II oligonucleotide primers are: NOS II sense 31-mer, 5′ GGC TTG CCC CTG GAA GTT TCT CTT CAA AGT C 3′ and NOS II antisense 24-mer, 5′ AAG GAG CCA TAA TAC TGG TTG ATG 3′.

2.6. Southern blot

Eighteen microliters of PCR amplification product was electrophoresed through 1.0% agarose (BMB, Indianapolis, IN) and transferred in 0.4 M NaOH to a nylon membrane (Scheicher and Schuell, Keene, NH). Southern hybridization was performed to verify product identity and to increase the sensitivity of detection by probing the blots in 6X standard saline citrate (SSC), 5X Denhardt's solution, 0.5% SDS, 50% formamide (BMB, Indianapolis, IN), and 100 μg/ml salmon sperm (Sigma, St. Louis, MO) DNA with a (α-^{32}P) dCTP (Dupont NEN, Boston, MA) end-labeled internal 30-mer oligonucleotide with sequence 5′ GGG GTT TTC TTC ACG TTG TTG TTA ATG TCC 3′ corresponding to nucleotides 321–350 of the murine macrophage NOS II cDNA (Xie et al., 1992) at 37°C overnight. The membranes were subjected to increasing stringency washes at room temperature in 0.1% SDS and 2–0.1X SSC followed by a final wash at 37°C in 0.1X SSC/1.0% SDS for 30 min. Membranes were then exposed to Fuji (Fisher Scientific, Pittsburgh, PA) film with two intensifying screens for 24 h at −70°C and an autoradiogram was produced.

2.7. In situ hybridization analysis

Cytokine-injected and saline-injected rats were decapitated and the lumbosacral enlargement of spinal cords were harvested and placed in Histochoice MB fixative overnight. Cords were then dehydrated and embedded in paraffin. Fifteen-micrometer horizontal sections were cut and mounted onto silanized (Sigma, St. Louis, MO) Superfrost Plus (Fisher Scientific, Pittsburgh, PA) slides, dried overnight, and then stored at −20°C until use.

For the probe, a 665-bp fragment of the murine NOS II cDNA which spans nucleotides 1–665 and is flanked by 5′ *Hin*dIII and 3′ *Bam*HI restriction sequences was subcloned from pUC 19 to Bluescript II SK- (Stratagene, La Jolla, CA). The orientation of the fragment allows antisense and sense RNA synthesis from the flanking T3 and T7 RNA polymerase promoters, respectively. One microgram of *Hin*dII linearized Bluescript II SK- was used as template for the synthesis of NOS II antisense probe in an in vitro transcription reaction containing 4 μl T3 10X transcription buffer, 0.5 μl each 20 mM cold rATP, rGTP, rCTP (BMB, Indianapolis, IN), 0.5 μl 0.4 mM cold rUTP (BMB, Indianapolis, IN), 1 μl (40 U) RNasin, 5 μl ^{35}S-rUTP (Dupont NEN, Boston, MA), 1 μl (20 U) T3 RNA polymerase (BMB, Indianapolis, IN), and RNase-free water to bring the total volume of the reaction mixture to 20 μl. After incubation for 1 h at 37°C, DNA template was removed by incubation with 1 U of RNase-free DNase for an additional 30 min at 37°C. Sense RNA probe was prepared after template linearization with *Bam*HI (BMB, Indianapolis, IN) utilizing the T7 promoter. Labeled RNA probes were separated from unincorporated nucleotides by G-50 Sephadex column (BMB, Indianapolis, IN) purification and gel purified in 5% acrylamide/8 M urea gels. The antisense NOS II cRNA was utilized in Northern blot experiments and binds to one species of mRNA of the correct size (4 kb, data not shown).

In situ hybridization was performed on sections which had first been de-waxed in xylenes and rehydrated through graded ethanols. In situ hybridization was performed as described by Perlman et al. (Perlman et al., 1988) except that sections were treated with Proteinase K (Gibco BRL, Grand Island, NY) for 8 min at 37°C and post-fixed in 4% paraformaldehyde followed by a PBS wash before acetic anhydride treatment. Sections were dipped in Kodak NTB2 autoradiographic emulsion and exposed for 12–14 days at 4°C before development.

2.8. Western blot analysis

Two hundred milligrams of concentrated soluble protein fractions of spinal cords were separated on 8.0% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. The membrane was blocked with 1% bovine serum albumin and subsequently incubated with (750-fold diluted) monoclonal antibody against NOS II (anti-mac-NOS, Transduction Laboratories, Lexington, KY). The specific protein was detected by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL).

2.9. Immunohistochemical staining

Cytokine-injected and saline-injected rats were decapitated and the lumbosacral enlargement of spinal cords was harvested and processed as described for in situ hybridization. Sections were equilibrated at room temperature and then incubated sequentially in PBS, 1.0% Triton X-100 (Fisher Scientific, Pittsburgh, PA) and 0.1% H_2O_2 before blocking in 10% goat serum (Sigma, St. Louis, MO) for 1 h. The detection of NOS II was achieved by using an immunoperoxidase technique. Briefly, sections were incubated with rabbit anti-murine NOS II Ab (diluted 1:100; Transduction Laboratories, Lexington, KY) which then was detected with a goat anti-rabbit biotinylated IgG secondary Ab (1:200; Sigma, St. Louis, MO) and an avidin-biotinylated horseradish peroxidase complex (ABC) kit used according to the manufacturer's instructions (Vector, Burlingame, CA). Staining reactions were performed with 3,3′ diaminobenzidine as substrate and enhanced in the presence of nickel chloride. Before being mounted, sections were counterstained in methyl green (0.25% aqueous). This commercial polyclonal antibody was used for immunohistochemistry because binding in histologic sections was never achieved in our hands using the commercial monoclonal antibody used for Western blotting. This polyclonal antibody has been previously demonstrated to bind specifically to spinal cord glial cells expressing NOS II in mice chronically infected with a neurotropic coronavirus (Sun et al., 1995). GFAP detection was achieved using a murine monoclonal anti-GFAP Ab (1:200; Sigma, St. Louis, MO) and a goat anti-mouse biotinylated polyvalent secondary Ab (1:200; Sigma, St. Louis, MO). ED-1 detection was achieved using a murine monoclonal anti-rat primary Ab (1:100; Harlan Bioproducts for Science, Indianapolis, IN) and a goat anti-rat IgG biotinylated secondary Ab (1:200; Sigma, St. Louis, MO). Control sections were stained in parallel, and did not receive primary antibody.

2.10. Histology

Spinal cord sections from one animal at each time point and condition analyzed for NOS II expression adjacent to those used for in situ hybridization analysis or immunohistochemical staining were stained with H & E in order to identify possible microscopic pathologic changes in spinal cord tissues.

3. Results

3.1. NOS II gene expression in the spinal cord

A combination of IL-1β and IFN-γ or saline was injected into a total of 21 rats. A sterile catheter was placed i.t. in one rat which received no injection but which was sacrificed 24 h after catheter placement. Rats were sacrificed and spinal cord tissues were analyzed for NOS II mRNA at 4 h, 8 h, 24 h, 48 h, and 72 h after cytokine injection and at 8 h and 72 h after saline injection. RT-PCR followed by Southern analysis revealed the presence of NOS II mRNA in tissues of 3/4 rats at 4 h after cytokine injection and 4/4 rats 8 h after cytokine injection (Fig. 1). NOS II mRNA was also expressed by 2/4 rat spinal cords from animals receiving saline injections and sacrificed 8 h after injection. Tissue from one rat immediately sacrificed after cytokine injection and from one rat with i.t. catheter placement for 24 h alone did not express NOS II mRNA. Tissues from rats sacrificed at time points greater than 8 h after injections also did not express NOS II mRNA.

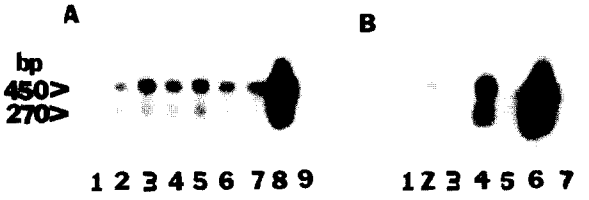

Fig. 1. Expression of NOS II mRNA after i.t. injection of cytokines. Rats were sacrificed at different time points after lumbosacral i.t. injection of either hrIL-1β (10 ng) + IFN-γ (1000 U) or saline. Lumbosacral spinal cord was harvested and RNA extraction followed by RT-PCR was performed. The expected size cDNA product is 450 bp; additionally, a minor 270-bp NOS II amplification product resulting from upstream antisense primer hybridization is also present in positive samples. (A) Lane 1, no target (negative) control; 2–4, three separate 4-h animals; 5–7, three separate 8-h animals; 8, LPS + IFN-γ induced RAW cells (positive control); 9, 24-h animal. (B) Lane 1, 48-h animal; 2–4, three separate 8-h saline-injected animals; 5, catheter only animal; 6, LPS + IFN-γ induced RAW cells; 7, 72-h animal.

3.2. NOS II protein expression in the spinal cord

A total of 14 rats received either i.t. saline or IL-1β plus IFN-γ. Saline-injected rats were sacrificed at either 8 h or 72 h after injection; cytokine-injected animals were sacrificed at 4 h, 8 h, 24 h, 48 h, or 72 h after injec-

Fig. 2. Expression of NOS II protein after i.t. injection of cytokines. Rats were sacrificed at different times after i.t. injection of either hrIL-1β (10 ng) + IFN-γ (1000 U) or saline. Concentrated soluble protein fractions from lumbosacral spinal cord were prepared and separated by 8% SDS-PAGE. Immunoblots were incubated with a monoclonal antibody against NOS II (130 kDa) and detected by enhanced chemiluminescence. Lane 1, induced RAW cells (positive control); 2 and 3, uninduced cells (negative control); 4 and 5, 4-h animals; 6, 8-h animal; 7, 8-h saline-injected animal; 8, 24-h animal; 9, 48-h animal.

tion. Western blot analysis of concentrated soluble protein fractions of spinal cord tissues utilizing a monoclonal antibody to NOS II protein revealed the presence of NOS II protein (130 kDa) in 2/2 animals at 4 h after cytokine injection, 1/2 animals at 8 h after cytokine injection, and in 1/2 animals receiving saline and sacrificed 8 h after injection (Fig. 2). Tissues from animals sacrificed at longer time points after injections did not exhibit a 130-kDa band. The monoclonal antibody used in immunoblotting reportedly recognizes all three isoforms of human NOS (Nakane et al., 1995). A protein band of higher molecular weight was detected in all spinal cord tissues irrespective of treatment and this may represent NOS I.

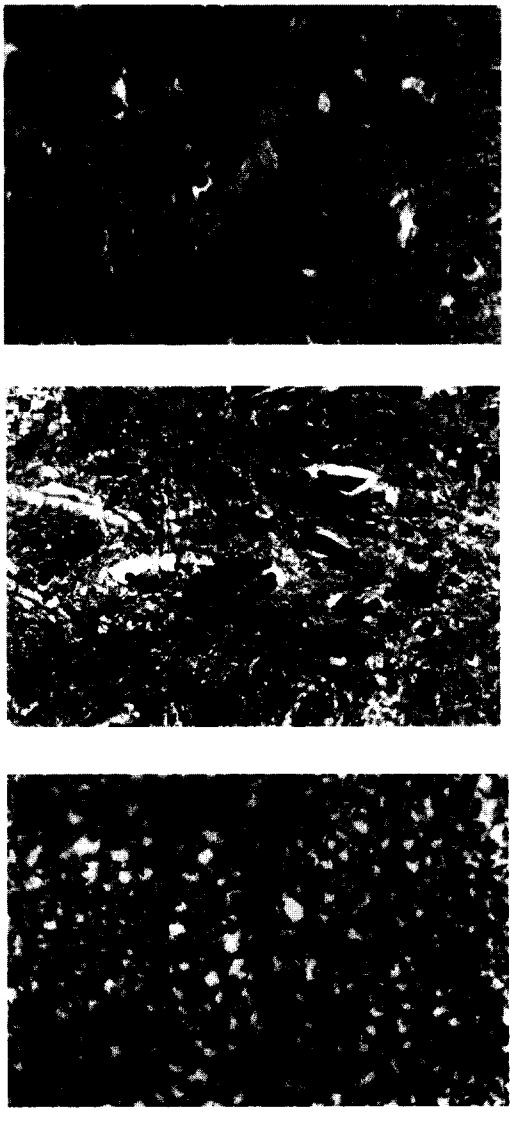

Fig. 3. Pathologic changes in the spinal cord of rats after i.t. cytokine injection. (A) Leukocytes (arrowheads) within, and migrating out of a vessel in the gray matter. (B) Leukocytes scattered throughout the gray matter. (C) Leukocytes in a vessel in the white matter. Scale bar = 50 μm.

3.3. Microscopic pathologic changes in spinal cords

H & E staining of spinal cord sections from one rat at each time point and condition analyzed for NOS II expression revealed the presence in some samples of a mild inflammatory reaction characterized by the presence of leukocytes inside and adjacent to scattered gray and white matter parenchymal vessels, as well as occasional individual leukocytes scattered in the cord parenchyma (Fig. 3A, B, and C). This mild inflammatory reaction was noted in lumbosacral sections from rats sacrificed 4, 8, and 24 h after cytokine injection as well as in cervical sections from rats sacrificed 4 h after cytokine injection. No reaction was noted in sections from rats sacrificed at 48 or 72 h after cytokine injection, or in sections from the rat with catheter placement alone. Occasional leukocytes were seen in the cord of a rat sacrificed 8 h after saline injection. No reaction was noted in the cord of a rat sacrificed 72 h after saline injection. No well-defined perivascular infiltrates or parenchymal infiltrates were evident in any of the cords examined.

3.4. In situ localization of NOS II RNA

In situ hybridization analysis utilizing a ^{35}S-labeled NOS II antisense cRNA probe was performed on spinal cord sections from a rat sacrificed 4 h after cytokine injection and from a rat sacrificed at 8 h after saline injection. NOS II mRNA evidenced by silver grain clustering was present in cells bilaterally in both gray and white matter areas of the cord 4 h after cytokine injection (Fig. 4B). Many of these cells were small and round in shape, resembling macrophages or other leukocytes. They were scattered in the parenchyma and in a perivascular distribution. These cells did not appear only in the dorsal horn but were scattered diffusely throughout the cord. Hybridization with sense NOS II cRNA probe revealed only a random background scattering of silver grains (Fig. 4A). NOS II antisense probe hybridizations performed on spinal cord sections from a rat 8 h after saline injection revealed no evident clustering of silver grains in any spinal cord area (data not shown).

3.5. Distribution and identity of cells expressing NOS II protein

Immunohistochemical staining of spinal cord sections utilizing a NOS II polyclonal antibody revealed the presence of cells positive for NOS II protein in the lumbosacral portion of the cord of 3/3 rats sacrificed 4 h after cytokine injection. Similar to the distribution pattern of cells positive for NOS II mRNA described above, cells positive for NOS II protein were scattered diffusely and bilaterally in both gray and white matter regions of the spinal cord; they were not confined to the

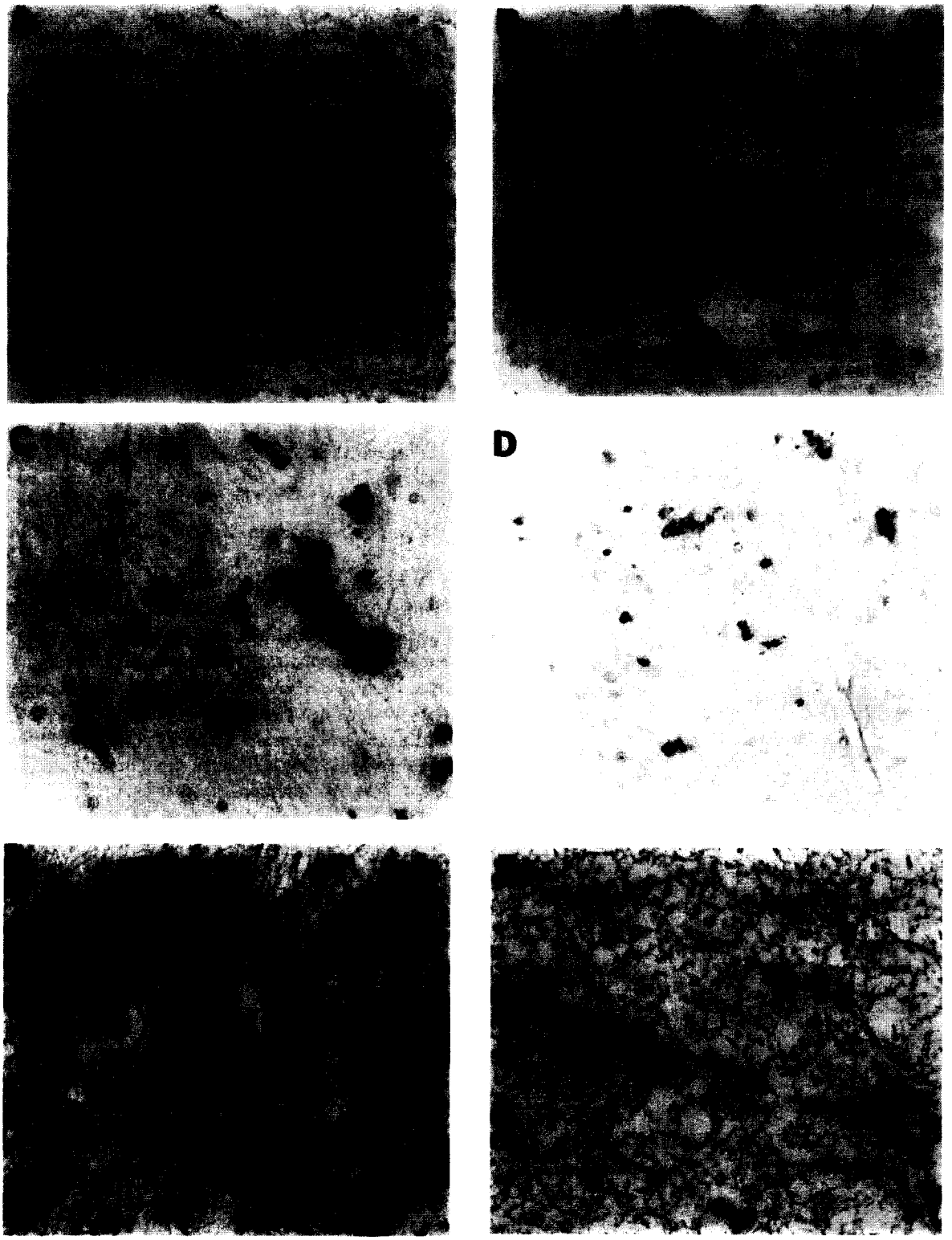

Fig. 4. Cellular localization of NOS II mRNA and protein in the spinal cord of rats 4 h after i.t. injection of cytokines. In situ hybridization with ^{35}S-labeled NOS II sense (A) and antisense (B) riboprobes. Note silver grains over cells adjacent to vessels in B. Immunohistochemical staining for NOS II (C) and (D), for GFAP (E), and for ED-1 (F). Note positive cells within and around vessels (C and D) and their morphological similarity with cells expressing ED-1 (arrowheads in F). A, B, D are the same magnification. C, E, F are the same magnification. Scale bar = 50μm.

dorsal horn. Many of these cells appeared small and round and resembled macrophages, other leukocytes, or microglia (Fig. 4D); some were located around vessels and may represent perivascular macrophages (Fig. 4C). No positive cells were seen in sections from rats receiving saline injections, in tissue from a rat with catheter placement only, or in sections not receiving primary antibody (data not shown). Labelling of cells morphologically resembling vascular endothelial cells or neurons was not seen in any sections.

Immunohistochemical staining was also performed on sections from an animal sacrificed at 4 h after cytokine injection adjacent to those found to contain NOS II positive cells utilizing ED-1 or GFAP antibodies in an effort to identify the cell type(s) expressing NOS II. The pattern and distribution of cells positive for GFAP was generally unlike the pattern and distribution of NOS II positive cells (Fig. 4E). Based on these studies, astrocytes cannot be ruled out as a cellular source of some of the possible positive fibers seen in these tissues; however, they do not appear to be a major source of NOS II under these conditions. The pattern and distribution of cells positive for ED-1 was similar to some NOS II positive cells (Fig. 4F). Based on this comparison and on morphology, it is highly likely that a subset of cells expressing NOS II protein under these conditions are perivascular and/or parenchymal macrophages and/or microglial cells.

4. Discussion

This study reveals the expression of NOS II in the spinal cords of rats under conditions producing thermal hyperalgesia. Since it is thought that behavioral hyperalgesia is one manifestation of activity-dependent functional and anatomic dorsal horn neuron plasticity, with the exception of one previous study by Meller et al. (1994), all previous studies investigating the possible involvement of NO in hyperalgesia have concentrated on the expression of the constitutively expressed NOS I. This isoform was first cloned from neurons (Bredt et al., 1991) and is known to be expressed in a subset of neurons located in sensory processing areas of the rat spinal cord (Dun et al., 1992). The small amount of NO released transiently via the activity of this form of the enzyme is involved in physiologic and perhaps pathophysiologic neuronal signalling.

One model proposed for the link between spinal cord NO production and hyperalgesia (Meller and Gebhart, 1993) is that persistent nociceptive input as a result of tissue or nerve injury leads to high frequency discharge of C fibers innervating the area of injury. This leads to the release of glutamate and other neuroactive substances from primary afferent central terminals, which results in NMDA receptor activation of neurons in the superficial and deeper dorsal horn areas. As a consequence of NMDA receptor activation, neuronal intracellular calcium increases, resulting in the activation of protein kinases, early genes, and NOS I, among other intracellular effects. Released NO diffuses to presynaptic neurons and adjacent cell bodies (neurons, glia, etc.) where it enters the cell(s) and causes an increase in cGMP due to activation of soluble guanylate cyclase. This increase in cGMP is thought to ultimately produce a potentiation of synaptic transmission or a hyperexcitable state which is manifested by hyperalgesia and expansion of receptive fields.

Given that NO is a highly diffusible molecule, it is also hypothetically possible in this model that neuron activity in the dorsal horn could be affected not only by NO released via the activity of NOS I in adjacent neurons, but also by NO released via the activity of other isoforms (including NOS II) expressed in any nearby cell.

Significant evidence exists supporting a role for spinal cord NO production in the mechanism of hyperalgesia (for review, see Meller and Gebhart, 1993). For example, NOS inhibitors or methylene blue (a NOS and sGC inhibitor) abolish the thermal hyperalgesia produced by i.t. NMDA administration (Meller et al., 1992a). NMDA hyperalgesia is also blocked by hemoglobin, an extracellular NO scavenger (Kitto et al., 1992).

Since spinal cord NO is clearly involved in hyperalgesia, if NO release due to NOS II activity could be important in hyperalgesia, then one would predict that compounds which induce NOS II activity in cells would produce hyperalgesia. Meller at al. (1994) have shown that i.t. administration of LPS or IL-1β + IFN-γ to rats does produce a time-dependent thermal hyperalgesia. They have also shown that aminoguanidine, a selective NOS II inhibitor, attenuates the thermal hyperalgesia produced with peripheral inflammation.

This study describes the induction of NOS II message and protein in the spinal cords of rats after i.t. administration of IL-1β + IFN-γ. Additionally, 2/4 rats receiving i.t. saline injections expressed NOS II message by RT-PCR and 1/3 expressed NOS II protein by Western blot. One explanation for these observations is that a low level of NOS II induction may occur in CNS cells of some animals with some component of the manipulation involved in placing an i.t. catheter and injecting fluid into the i.t. space. The fact that a control animal which received an i.t. catheter but did not receive an injection of fluid did not express NOS II suggests that i.t. injection may induce NOS II in some cells. The RT-PCR method used for these analyses was not quantitative; therefore the relative amounts of message induced with injections could not be assessed. It may be that while NOS II induction can occur with i.t. injection alone, less NOS II induction occurs with saline injection than with cytokine injection which is insufficient to contribute to behavioral changes.

While NOS II mRNA and protein was detected in cord tissue from saline-injected animals, no tissue sections from saline-injected animals contained cells positive for either NOS II mRNA or protein. This observation could be due to sampling error. The entire lumbosacral sample was used for RNA extraction or protein concentration when RT-PCR or Western blot was performed, in contrast to the 15-μm sections of cord used for localization studies. Alternatively, cells which may be induced to express NOS II after intrathecal saline injection may be cells not anatomically associated with those that may contribute to the behavioral hyperalgesia, for example, mononuclear cells in the meninges.

H & E staining of spinal cord sections from rats revealed the presence of a mild acute inflammatory reaction in tissues primarily from animals receiving i.t. cytokines; some leukocytes were also seen in the cord of one saline-injected animal, but no reaction was observed in tissue from an animal with catheter placement only. Based on all the observations, a mild acute inflammatory response appeared to correlate most closely with i.t. cytokine injection. Pro-inflammatory cytokine injection into the CSF may induce an inflammatory response via induction of chemokines in resident cells of the CNS (Vanguri and Farber, 1994; Glabinski et al., 1995).

Consistent expression of NOS II mRNA and protein was demonstrated in multiple animals at 4 h and 8 h after cytokine injection. The cellular pattern and distribution of both NOS II mRNA and protein in 4 h samples was the same, suggesting that at least some of the NOS II expression can be attributed to macrophages/microglia or other inflammatory cells. A mild inflammatory response was also evident in tissue from animals sacrificed 4 h after cytokine injection; inflammatory cells occurred in perivascular locations and scattered in the spinal cord parenchyma, a pattern of distribution similar to that of NOS II mRNA and protein.

Based on these results, one hypothesis that can be offered regarding the involvement of NOS II expression and NO release in this model is that injection of proinflammatory cytokines into the i.t. space induces an inflammatory response in the cord resulting in the upregulation of NOS II in exogenous inflammatory cells as well as perhaps in some resident cells (e.g., microglia). Released NO under these conditions may affect neuronal and/or glial signalling. The time-course of NOS II expression observed in this study does not correlate completely with the time course of behavioral hyperalgesia in this model. While expression of NOS II in cord tissue was observed in animals at 4 h after cytokine injection, a time during which animals express thermal hyperalgesia, NOS II message and protein were also detected 8 h after injection, when thermal hyperalgesia is no longer demonstrable. Additionally, we observed cells positive for NOS II message and protein throughout all regions of the gray and white matter of the cord, not just in regions specific for sensory processing. (However, since we did not perform functional studies, we do not know how much of the observed protein is enzymically active). These observations do not support a causal role for NOS II expression and consequent NO release in the development of thermal hyperalgesia. Previous reports by Solodkin et al. (1992) and Hama and Sagen (1994) describe bilateral expression of NADPH-d activity in dorsal horn neurons after manipulations which result in unilateral behavioral hyperalgesia. These prior observations as well as the observations described in this study suggest that spinal cord NO (released via the activity of any NOS isoform present) may be a correlate of the neuronal hyperexcitable state. NO production may be just one of several neurochemical changes in the spinal cord which ultimately result in activity-dependent neuronal plasticity expressed as behavioral hyperalgesia; however, the fact that hyperalgesia can be abolished in some models, including this model, with NOS inhibitors underscores the importance of NO in the development of this abnormal behavior.

Another possibility is that i.t. injection of proinflammatory cytokines has a more direct effect on spinal cord neurons, influencing synaptic transmission through effects on central neurotransmitters or second messenger systems, including those involved in NOS I activation (Rothwell and Hopkins, 1995). The induction of NOS II in this hypothetical scheme would be a parallel event, but one unimportant to the expression of behavioral hyperalgesia. While this hypothesis is possible, the fact that spinal cord NO is known to be important in the hyperalgesic response supports at least a modulatory role for NOS II expression and sustained NO release from any source in the synaptic events resulting in this abnormal nociceptive processing.

In summary, this study demonstrates the induction of NOS II expression in the spinal cords of rats under conditions producing thermal hyperalgesia. Macrophages and/or microglia are most likely at least one of the cellular sources of NOS II; astrocytes do not appear to express significant amounts of NOS II under these conditions. Multiple hypotheses can be formulated regarding the role of NOS II induction and consequent NO release in the expression of behavioral hyperalgesia in this model. Without specific inhibitors for individual NOS isoforms, it has been impossible to define the exact role NOS II may play in the mechanism(s) underlying the development of specific neuropathologies, including hyperalgesia. With the recent report describing NOS II deficient transgenic mice (MacMicking et al., 1995; Wei et al., 1995), the opportunity now exists to more specifically define the role of NOS II and NO production in abnormal nociceptive processing.

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