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Is neuronal nitric oxide involved in adjuvant-induced joint inflammation?

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Abstract

Several reports have described a role of macrophagic, endothelial and synoviocytal nitric oxide (NO) in inflammation, immunity and sensory processes in joint diseases. In view of the role of the peripheral nervous system in arthritis and owing to the presence of NO-producing neurons in primary sensory neurons, we have investigated the possible role of neuronal NO during adjuvant-induced joint inflammation in rats. Neural nitric oxide synthase production in sensory ganglia and the spinal cord was investigated by in situ hybridization and immunocytochemistry. Neuronal NO synthase mRNA expression and neuronal NO synthase immunoreactivity increased in lumbar dorsal root ganglia in arthritic rats compared to those of normal rats, whereas neuronal NO synthase mRNA expression decreased in lamina X and lamina I–II of the lumbar spinal cord. The administration of the selective neuronal NO synthase inhibitor 7-nitro indazole, reduced the joint inflammation, whereas the administration of the inducible NO synthase selective inhibitor, aminoguanidine, had no effect on inflammation when administered daily from the third day after adjuvant. These findings could indicate a role for neural NO in adjuvant arthritis.

Keywords: Nitric oxide NO synthase; Adjuvant arthritis; Aminoguanidine; 7-nitro indazole; (Rat); Dorsal root ganglia; Spinal cord

1. Introduction

Nitric oxide (NO), a free radical gas which is produced by the action of the NO synthase enzyme on L-arginine, is an important physiological mediator. NO synthase isoforms have been characterized and distinguished in a constitutive form, which is normally expressed for instance in endothelial and neural cells, and an inducible form, expressed by different cell types such as macrophages, following exposure to appropriate stimuli (Nathan and Xie, 1994a,b). There is an evidence now that NO may mediate local inflammation and tissue damage under various conditions (Laskin et al., 1994, Laskin and Pendino, 1995; Clancy and Abramson, 1995; Moilanen and Vapaatalo, 1995; Cochran et al., 1996; Calzà et al., 1997). After adjuvant and carrageenin injection in rats, resulting in arthritis, NO production increases in inflamed joints (McCartney-Francis et al., 1993; Cannon et al., 1996) and a higher urinary nitrate excretion has been described (Stichtenoth et al., 1994). Moreover, NO synthase inhibitors administered on the same day as or prior to induction of acute or chronic inflammation considerably reduces arthritis (Ialenti et al., 1992, 1993; Oyanagui, 1994; Connor et al., 1995; Medeiros et al., 1995), whereas the administration of the NO precursor, L-arginine, exacerbates this condition (Ialenti et al., 1993). Studies of patients affected by rheumatoid arthritis also confirmed the increased production of NO (Grabowski et al., 1996; McInnes et al., 1996; Ueki et al., 1996). Macrophages, synoviocytes, endothelial cells and condrocytes seem to be the natural source of NO involved in the development and maintenance of arthritis (Grabowski et al., 1997). NO can be generated either via the constitutive NO synthase in endothelial cells stimulated by some inflammatory mediators, or via the inducible enzyme carried to the inflammatory site by the infiltration of activated polymorphonuclear cells or by local tissue (Grabowski et al., 1996).

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NO also participates in the development and maintenance of the hyperalgesia induced by peripheral inflammation (Meller and Gebhart, 1993). NO acts as a peripheral mediator of chemically induced nociception, i.e., bradykinin-induced nociception, but results of recent behavioural (Malmberg and Yaksh, 1993; Meller et al., 1994) and electrophysiological (Haley et al., 1992; Stanfa et al., 1996) experiments have also suggested that the neuronal synthesis of NO in dorsal root ganglia, the spinal cord and supraspinal regions, supports nociceptive transmission.

In view of the above, and of the role played by neuroactive products released from primary sensory neurons via axon reflex as neurogenic mediators of inflammation (Iversen, 1985; Levine et al., 1985; Kidd et al., 1990), we investigated the time course modulation of neuronal NO synthase synthesis in dorsal root ganglia and spinal cord in rats with arthritis induced by the injection of complete Freund's adjuvant. We also investigated the development of arthritis in CFA-injected rats treated with aminoguanidine, an inhibitor of inducible NO synthase, and with 7-nitro indazole, an inhibitor of neuronal NO synthase. Treatments were started as soon as signs of inflammation appeared.

2. Materials and methods

2.1. Animals

Male, pathogen-free Sprague–Dawley rats (OFA strain, IffaCredò, Italy) were used. The animals were housed under standard light/dark conditions (on: 0700 h, off: 1900 h) in polypropylene cages. Adjuvant arthritis was induced by a single intradermal injection (150 μl) of heat-killed *Mycobacterium butyricum* (DIFCO) on the upper side of the tail under brief fentanyl/midazolam anesthesia. Inflammation was assessed by clinical examination performed blind every other day by two independent evaluators. A numerical score was attributed for edema and reddening observed in anterior and posterior paws and scrotum (0 = absence; 1 = low; 2 = moderate; 3 = severe). A total score (sum of the score attributed to the different areas for the evaluated symptoms) was then assigned to the animals during each observation. Only those animals with signs of inflammation three days after CFA injection were included in the study. Rats developing skin lesions were excluded from the study. The animal experiments have been carried out according to Italian regulations.

2.2. Treatments

Aminoguanidine and 7-nitro indazole were purchased from Sigma (St. Louis, MO, USA). Aminoguanidine was given at the dose of 1 mg/ml added to drinking water. 7-Nitro indazole was given at 2.5 mg/rat, i.p., dissolved in peanut oil. Controls were injected with vehicle alone. All treatments were started three days after CFA injection.

2.3. Immunocytochemical studies

The rats were killed 5, 13 and 21 days after CFA injection. The rats were anaesthetized and perfused through the ascending aorta with saline solution followed by 4% paraformaldehyde. Lumbar dorsal root ganglia (L4 and L5) were then removed and immersed for 2 h in the same ice-cold fixative. The tissues were then rinsed for 48 h in ice-cold 0.1 M Sörensen's buffer containing 5% sucrose. After rinsing, dorsal root ganglia were quickly frozen in CO_2 and cut at -20°C on a cryostat (section thickness = 14 μm). Sections were collected and immediately processed for immunofluorescence. Slides from all animals were run in the same assay. The sections were first incubated in 0.1 M phosphate-buffered saline (PBS), followed by incubation at 4°C overnight in a humid atmosphere with the primary antisera diluted 1:300 (neuronal NO synthase, EuroDiagnostic, Tema Ricerca, Bologna, Italy). Staining specificity was assessed by the overnight preincubation of the antiserum with the respective antigen (10–100 μg/ml diluted antiserum). This treatment abolished all staining patterns described in the present study. The primary antibodies were diluted in PBS containing 0.3% Triton X-100, v/v. After rinsing in PBS, the sections were incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin (Dako, Copenhagen, Denmark) containing 0.3% Triton X-100, rinsed in PBS (as above), mounted in glycerol and PBS (3:1, v/v) containing 0.1% 1,4-phenylenediamine and examined using a Nikon Microphot FXA microscope.

2.4. In situ hybridization

The rats were killed 5, 13 and 21 days after CFA injection. Lumbar dorsal root ganglia (L4–L5) and lumbar spinal cord were quickly removed and frozen on dry ice. Twenty micron-thick sections were cut in a cryostat at -20°C and thaw-mounted onto precleaned microscope glass slides (ProbeOn, Fisher Scientific, Pittsburgh, USA). The oligonucleotide probe complementary to mRNA encoding neuronal NO synthase (AA 151–164) was labelled at the 3′-end with [$\alpha-{}^{35}$S]dATP (New England Nuclear, Boston) using terminal deoxynucleotidyltransferase (NEN) in a buffer containing 10 mM $CoCl_2$, 1 mM dithiothreitol, 300 mM Tris base, and 1.4 M potassium cacodylate (pH 7.2). Afterwards, the labelled probe was purified through Nensorb-20 columns (NEN), and dithiothreitol was added to a final concentration of 10 mM. The specific activity obtained ranged from $1–4 \times 10^6$ dpm/ng oligonucleotide.

The hybridization procedure has been described previously (Dagerlind et al., 1992). Briefly, the sections were brought to room temperature, air-dried, covered with a hybridization buffer containing 50% formamide, 4 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 1 × Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin and 0.02% Ficoll), 1% sarcosyl,

0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate, 500 μg/ml heat-denatured salmon testis DNA, 200 mM dithiothreitol and 40 ng/μl of the labelled probe. Control hybridization was carried out with labelled probe plus a 100-fold excess of unlabelled probe. This treatment abolished the mRNA signals described in the present study. The slides were placed in a humid chamber and incubated for 15–20 h at 50°C. Afterwards, the sections were rinsed in 1 × SSC at 55°C for 1 h with six changes and washed in the same buffer for 1 h at room temperature. Finally, the slides were rinsed in distilled water, dehydrated and air-dried. The sections were dipped in NTB2 nuclear track emulsion (Kodak) and then exposed for four weeks before being developed. The tissue was counterstained with toluidine blue.

2.5. Quantitative analysis and presentation of the results

Quantitative procedures used in this study have been described in detail (Calzà et al., 1997, 1998). Briefly, quantification of the hybridization signal was carried out on single cells after light toluidine-blue counterstaining using an automatic grain counting procedure (Image analyzer AIS Imaging Research, Ontario, Canada). This software uses a processed image and can count silver grains in situations where clusters of grains are formed due to high mRNA expression, by dividing the total area of a cluster by the mean grain size. Only cells in which the nucleus was evident were analyzed. For each animal, four lumbar ganglia were analyzed by measuring five to eight cells/section in three nonconsecutive sections. In the spinal cord, five to eight cells/area/side/section in three nonconsecutive sections were measured for each animal. Mean NO synthase mRNA expression in individual neurons was then calculated for each animal, and these data were subsequently used to calculate the mean expression for each group of animals. All sections to be compared were processed and analyzed at the same time. Comparison were made only between sections mounted on the same slide. One-way analysis of variance (ANOVA) followed by Dunnett's test and Student's *t*-test were used for statistical analysis of the data (JMP software package Macintosh, Cupertino, CA, USA).

3. Results

Joint inflammation was scored for visual signs of inflammation. Although an objective evaluation (i.e., paw perimeter, edema volume, etc.) would have been preferable to this subjective one, we decided to avoid animal manipulation to minimize pain and alteration of gene expression in dorsal root ganglia and the spinal cord due to evoked pain (Uhl and Nishimori, 1990). Signs of bilateral inflammation appeared in the hind paws three days after CFA injection and gradually increased until the 21st day, when severe edema and reddening including the forelimbs, and the scrotum in some cases, was observed. NO synthase inhibitors were administered as soon as signs of inflammation appeared (e.g., three days after CFA injection). Treatment with the inducible NO synthase selective inhibitor, aminoguanidine, failed to reduce arthritis development, whereas treatment with the neuronal NO synthase selective inhibitor, 7-nitro indazole, significantly reduced arthritis severity from 15 days after CFA injection (Fig. 1).

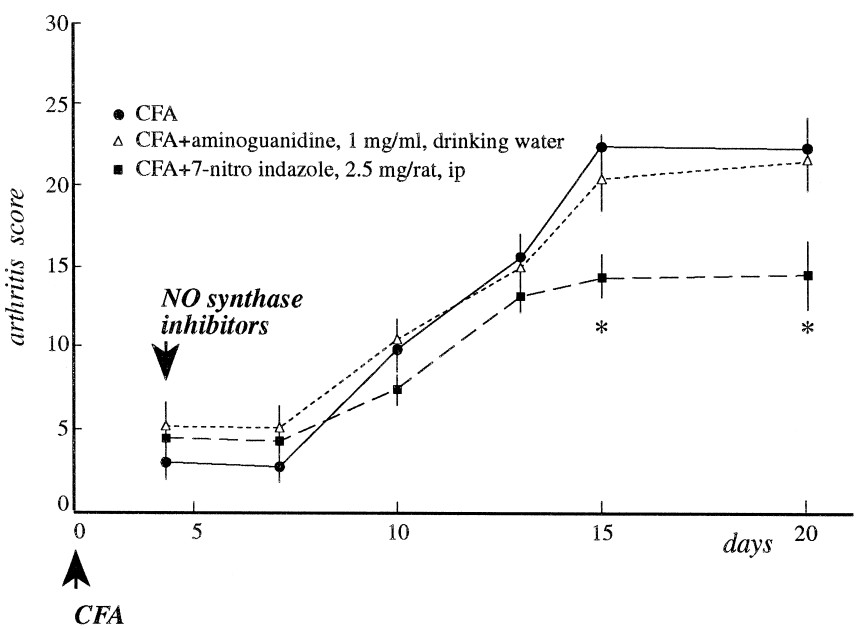

Fig. 1. Time-course of behavioral signs of paw inflammation in adjuvant-injected rats and adjuvant-injected rats treated with NO synthase inhibitors, aminoguanidine and 7-nitro indazole. Statistical analysis—Wilcoxon signed-rank test: $* = P < 0.05$.

The percentage of NO-producing neurons in the dorsal root ganglia varies according to the level. In the thoracic segments, up to 20–30% of the entire neural population express NO synthase or NADPH positivity, as well as NO synthase mRNA labelling (Fig. 2A,B), whereas only 2% of neurons in lumbar dorsal root ganglia are able to synthetize NO in normal animals (Aimi et al., 1991) (Fig. 2C). Almost all NO-producing ganglionic cells belong to the

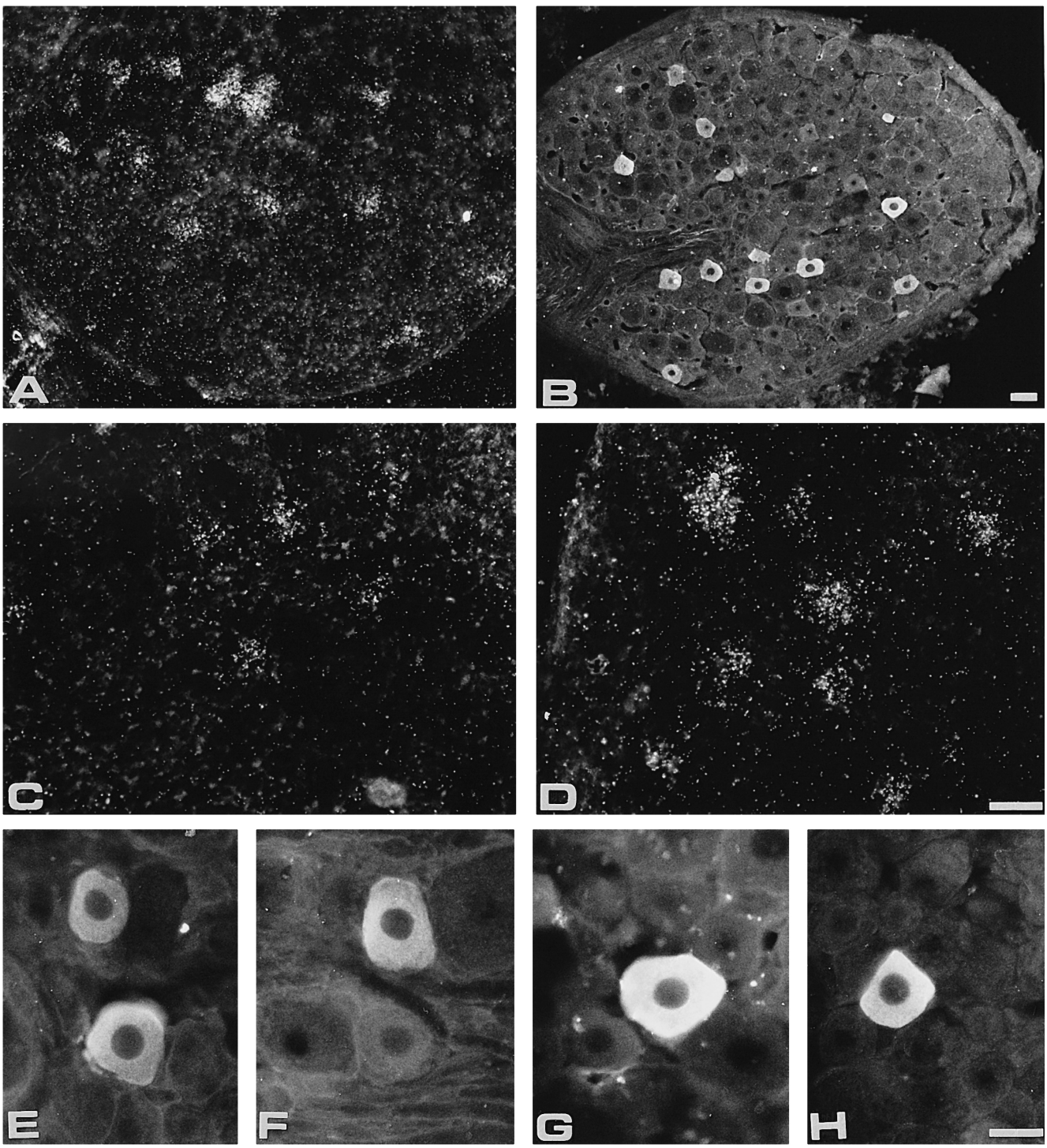

Fig. 2. NO synthase-producing neurons in dorsal root ganglia in rats. NO synthase mRNA-expressing neurons (A) and NO synthase-positive neurons (B) in dorsal root ganglia of control rats (thoracic level) as visualized by in situ hybridization and immunocytochemistry, respectively. NO synthase mRNA expression and NO synthase immunoreactivity in lumbar dorsal root ganglia increase in arthritic compared to control rats. (C,D) NO synthase mRNA-expressing neurons in dorsal root ganglia in adjuvant-injected and control rats, respectively. NO synthase immunoreactive neurons in dorsal root ganglia in control (E,F) and adjuvant injected (G,H) rats, respectively. Bars: B = 100 μm, D = 50 μm.

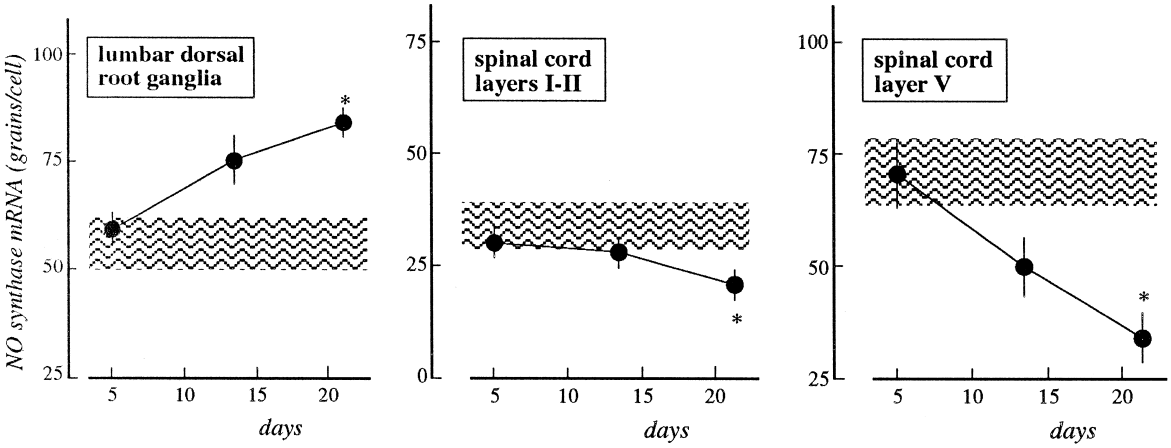

Fig. 3. Relative NO synthase mRNA expression in single cells of lumbar dorsal root ganglia, layers I–II and V of dorsal horn of the spinal cord in control ($n = 8$) and arthritic rats ($n = 8$), as analyzed 5, 13 and 21 days after adjuvant injection. Statistical analysis—ANOVA and Dunnett's test: $* = P < 0.05$.

medium-sized cell class (Aimi et al., 1991). In the spinal cord, NO synthase mRNA-expressing neurons were found among small neurons in layers I–II, in layer V and in layer X around the central canal (Dun et al., 1992). In arthritic rats, a progressive increase in NO synthase mRNA expression in dorsal root ganglia was found from days 5 to 21 following CFA injection compared to control rats (Fig. 2C, control; D arthritic rat, 21 days after CFA injection). On the contrary, neuronal NO synthase mRNA expression gradually decreased in neurons in layers I–II and V of the spinal cord. Quantitative analysis revealed that differences between control and arthritic rats were significant 21 days after CFA injection (Fig. 3). We also investigated neuronal NO synthase immunostaining in lumbar dorsal root ganglia. Concentration of the primary antiserum was tested in preliminar experiments, using a dilution curve in order to obtain a linear correlation between antiserum concentration and staining intensity, according to a previously published procedure (Calzà et al., 1990). Immunostaining for neuronal NO synthase in lumbar dorsal root ganglia also increases in arthritic rats in terms of staining intensity, as illustrated in Fig. 2E,F (control) and G,H (arthritic, 21 days after CFA injection). No variations were found in the type of neuronal NO synthase-expressing neurons, as well as in the percentage of neuronal NO synthase-positive neurons in lumbar dorsal root ganglia.

4. Discussion

In this paper, we report that neuronal NO synthase expression in lumbar dorsal root ganglia and the spinal cord is altered during CFA-induced inflammation. In particular, neuronal NO synthase expression increases in dorsal root ganglia and decreases in the spinal cord, respectively, according to a time-course that mirrors inflammation severity and joint tissue damage. We obtained a reduction of arthritis severity by administration of the neuronal NO synthase selective inhibitor, 7-nitro indazole, but not of aminoguanidine when administered starting from the third day after CFA injection.

CFA injection into the dorsal tail induces severe polyarthritis. Early signs of inflammation appear three days after injection and disease severity reaches its peak after 21–25 days, when severe impairment of joint tissues is also observed (Esser et al., 1995). Neuropeptide synthesis in lumbar dorsal root ganglia and the spinal cord is severely altered in the different phases of the disease (Calzà et al., 1998) and we showed that NO production is also modified in spinal nociceptive circuits. The neuronal NO synthase mRNA increases in dorsal root ganglia in arthritic rats, suggesting a higher production of NO by the primary sensory neuron (Gross and Wolin, 1995) and a wider diffusion of NO in peripheral tissues and in the spinal cord through the axon reflex, due to increased sensory neuronal firing (see Kidd et al., 1996). It has been suggested that peripheral NO plays a key role in the destruction of the cartilage and bone of the inflamed joint (Kaur and Halliwell, 1994), and that neuronal production of NO may contribute to this peripheral pool of free radical. We also found a reduced expression of neuronal NO synthase mRNA in layers I–II and V of the spinal cord, suggesting an altered production of NO by spinal neurons also. NO is thought to play a major role in NMDA-mediated hypersensitivity of spinal nociceptive neurons in hyperalgesia during inflammation. An analgesic role of NO has also been suggested (Meller and Gebhart, 1993). However, the mechanisms by which NO participates in the processing of nociceptive information are still unknown. It has been proposed that enhanced levels of immediate-early genes, which have been found in arthritic rats (Abbadie and Besson, 1994) could activate transcription of neuronal NO synthase gene, resulting in the up-regulation of spinal NO in the early phases of inflammation (Herdegen et al., 1994).

The modulation of neuronal NO synthase synthesis in the spinal cord and dorsal root ganglia that we have found in polyarthritis when the disease reaches its peak corresponds exactly to the one described for models of neuropathic pain. In fact, in acute inflammation and pain without tissue damage, both an increase (formalin injection, Lam et al., 1996) and an unchanged neuronal NO synthase immunoreactivity (carrageenin injection, Traub et al., 1994) have been described in the spinal cord. On the contrary, a reduction in spinal NO synthase activity (Choi et al., 1996) and an increase in neuronal NO synthase expression in dorsal root ganglia have been described for various models of neuropathic pain involving sciatic nerve lesion (Verge et al., 1992; Steel et al., 1994; Choi et al., 1996), and after capsaicin administration (Vizzard et al., 1995). The initial stages of our arthritic model may be characterized by an inflammatory type of pain, whereas later stages may also include a neuropathic component. In accordance with this, mRNA expression of the peptide, galanin, in dorsal root ganglia of polyarthritic rats 21 days after CFA injection is also higher (Calzà et al., 1998) as was the case in models of neuropathic pain (Hökfelt et al., 1994). This possibility is suggested in view of the severe rearrangement of articular and bone tissues presumably involving joint nerve endings which occurs 20–25 days after CFA injection (Esser et al., 1995).

7-Nitro indazole, which exhibits antinociceptive activities (Moore et al., 1993a,b), also reduces the arthritis score. It is interesting to note that this effect is obtained by treatment three days after CFA injection, i.e., as soon as inflammation develops, consistent with possible therapeutic use. Conversely, aminoguanidine does not reduce arthritis severity when administered according to the same schedule, contrary to results obtained from prophylactic treatment with nonselective inhibitors of inducible NO synthase (Ialenti et al., 1993). This finding further supports the role of a neural component, and also of neuronal NO, in the development of inflammation. Moreover, it supports the hypothesis that inducible NO synthase is mainly involved in the early phases of CFA-induced inflammation, when immunological events involving T cells and macrophages are predominant (Ialenti et al., 1993; Connor et al., 1995) rather than in maintenance. A number of questions then arise about the therapeutic or prophylactic use of inducible NO synthase inhibitors as possible intra-articular therapeutic strategy during rheumatoid arthritis, as already suggested by others (Stefanovich-Racic et al., 1995).

In conclusion, our data support the involvement of neuronal NO in the development and maintenance of arthritis after CFA injection. However, speculations about roles of NO in arthritis and resulting pain and possible therapeutic implications arising from data obtained using different models of acute and chronic inflammation should be formulated with caution. We suggest that inflammation without significant tissue lesion should be clearly distinguished from inflammation associated with severe peripheral tissue damage. Accurate time course experiments would help both to explain conflicting results and to formulate a prophylactic or therapeutic hypothesis regarding the use of different NO synthase inhibitors in arthritis.

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