ELSEVIER

European Journal of Pharmacology 402 (2000) 61–68

ejp

www.elsevier.nl/locate/ejphar

Activation of transcription factors of nuclear factor kappa B, activator protein-1 and octamer factors in hyperalgesia

Chin-Feng Chan [a], Wei-Zen Sun [c], Jen-Kun Lin [d], Shoei-Yn Lin-Shiau [a,b,*]

[a] *Institute of Toxicology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 10018, Taiwan*
[b] *Institute of Pharmacology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 10018, Taiwan*
[c] *Department of Anesthesiology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 10018, Taiwan*
[d] *Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 10018, Taiwan*

Received 25 October 1999; received in revised form 15 June 2000; accepted 20 June 2000

Abstract

Involvement of c-*fos* and neuronal nitric oxide synthase (nNOS) in the hyperalgesia induced by complete Freund adjuvant (CFA) has been reported. In this paper, we attempted to investigate whether the transcription factors regulating the gene expression of c-*fos* and nNOS, including activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), and octamer factors (Oct), are activated by CFA during the development of hyperalgesia. The electrophoretic mobility shift assay (EMSA) was used to determine whether there were changes in the transcription factors in the lumbar spinal cord of adult rats following subcutaneous injection of CFA in one hindpaw of the rats. Maximum binding of AP-1, NF-κB and Oct was found at 0.5, 1 and 2 h after CFA injection, respectively. These findings suggest that the activation of these transcription factors is pivotal for the expression of c-Fos and nNOS proteins, which reached a peak at 3 and 48 h after CFA injection, respectively. The behavioral testing of hyperalgesia demonstrated that CFA reduced the thresholds for mechanical and thermal algesia, reaching a minimum at 6 h. The thresholds had only partially recovered after 96 h. Based on these findings, we conclude that AP-1, NF-κB and Oct are crucial for the expression of c-Fos proteins at an early stage (at 3 h) and for the expression of nNOS at a late stage of hyperalgesia (48 h post-injection) induced by CFA.

Keywords: Hyperalgesia; Complete Freund adjuvant; NF-κB (nuclear factor kappa B); AP-1 (activator protein-1); Octamer factor; NO (nitric oxide) synthase, neuronal

1. Introduction

An experimental model to study "chronic" pain is adjuvant-induced arthritis in the rat, which resembles human rheumatoid poly-arthritis. A parallel clinical and behavioral study of adjuvant-induced arthritis in the rat showed four stages in the time course of the disease: pre-clinical (first week), acute (weeks 2–4), post-acute (weeks 5–8) and recovery (weeks 9–11) (Lantéri-Minet et al., 1993). Arthritis was induced by subcutaneous injection of Freund's adjuvant (killed *Mycobacterium butyricum* suspended in mineral oil) into one hindpaw. In the model of adjuvant-induced arthritis, an initial inflammatory response develops within hours. There is a dramatic modification in the activity of both superficial (I and II) and deeper (V and VI) laminae of the dorsal horn neurons receiving noxious inputs.

There is evidence that various nociceptive peripheral stimuli result in increased c-*fos* expression in the spinal cord (Hunt et al., 1987; Menétrey et al., 1989; Herdegen et al., 1991; Abbadie and Besson, 1992; Abbadie et al., 1994; Yashpal et al., 1998). C-Fos expression in spinal cord neurons occur not only after acute stimulation and inflammation, but also in chronic pain diseases such as poly- or mono-arthritis (Abbadie and Besson, 1992). Nitric oxide (NO) mediates the actions of glutamate and is involved in sustained changes in neuronal activity such as long-term suppression and long-term potentiation (Bredt and Snyder, 1992; Shibuki, 1993). Evidence suggests that nitric oxide synthase (NOS) activity in the spinal cord may participate

* Corresponding author. Institutes of Toxicology and Pharmacology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan. Tel.: +886-2-23123456 ext. 8606; fax: +886-2-23410217.

E-mail address: syl@ha.mc.ntu.edu.tw (S.-Y Lin-Shiau).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00431-3

in nociception and in the responses of spinal neurons to electrically evoke C-fiber input (Meller and Gebhart, 1993). These events may also lead to the establishment of persistent pain and hyperalgesia in a concerted manner. It is well known that NOS gene expression is promoted by activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB)(Kleinert et al., 1998; Todoroki et al., 1998; Marks-Konczalik et al., 1998). Recently, the octamer factor-2 (Oct-2) transcription factor has been identified which can also activate neuronal nitric oxide synthase (nNOS) gene expression in neuronal cells (Deans et al., 1996). Moreover, both aspirin and sodium salicylate inhibit the effects of glutamate through blockade of NF-κB activation (Grilli et al., 1996).

We attempted to investigate whether complete Freund adjuvant (CFA) could activate the transcription factors AP-1, NF-κB and octamer factors (Oct) and what their relation is to the induction of c-Fos, nNOS and hyperalgesia. Using electrophoretic DNA mobility shift assays (EMSA), we examined the activity of nuclear protein binding to the AP-1 site or the promoter regions of nNOS after CFA injection. Our results demonstrated that the transcription factors AP-1, NF-κB and Oct were immediately activated by CFA prior to the expression of c-*fos* and nNOS. This may contribute to persistent hyperalgesia following CFA injection.

2. Materials and methods

2.1. Injection of CFA

CFA (0.1%, 50 μl, Sigma, St. Louis, MO, USA) was injected subcutaneously into the plantar surface of the proximal portion of the foot in awake male Wistar rats (250–350 g) with a 29-gauge needle ($n = 45$). A vehicle containing saline was injected into the proximal portion of the plantar surface of the foot of three additional control animals. All experiments were approved by the Institutional Animal Care Subcommittee of National Taiwan University Hospital and are in accordance with guidelines for the Care and Use of Laboratory Animals.

2.2. Behavioral testing of hyperalgesia

2.2.1. Mechanical threshold

Animals were tested for responses to Von Frey filaments applied to the plantar surface of the foot as a measurement of the threshold for mechanical stimuli (Choi et al., 1996). Animals were placed in cages on an elevated screen. Von Frey filaments with different bending forces were tested (three trials/filaments). The lowest force that caused foot withdrawal was recorded for each animal.

2.2.2. Thermal threshold

Measurement of thermal nociception was performed by a modification of a previously reported method (Hargreaves et al., 1988). Rats were placed in a plastic chamber ($18 \times 29 \times 12.5$ cm) with a glass floor and allowed to acclimate to their environment for 5 min before testing. During this time, rats initially demonstrated exploratory behavior but subsequently stopped exploring and stood quietly with occasional bouts of grooming. After the acclimation period, the radiant heat source was positioned under the glass floor directly beneath the hind paw. A trial was commenced by a switch, which activated the radiant heat source and started an electronic timer. The radiant heat source consisted of a high-intensity projector lamp bulb (8 V, 50 W) (UGO, Basile; 7371 plantar test, Varese, Italy), located 40 mm below the glass floor and projecting through a 5×10 mm aperture in the top of a moveable case. A light reflected from the paw and turned off the lamp and the electronic clock when paw movement interrupted the reflected light.

The time course of these responses to thermal stimuli was determined in rats, which had received a subcutaneous injection of CFA (0.1%, 50 μl) into the plantar surface of one hind paw and vehicle into the other paw. The response to hyperalgesia in both the CFA and vehicle control hind paws was periodically measured and expressed as paw withdrawal latency during a 96-h period. Both mechanical and thermal hyperalgesia were calculated as the ratio of latency of the CFA-injected paw over that of the non-CFA-injected paw.

2.3. Immunohistochemistry

Prior to sacrifice, the animals were deeply anesthetized with an intraperitoneal injection of pentobarbital (35 mg/kg body weight) and then the animals were perfused with phosphate saline buffer. The lumbar spinal cord was quickly removed, post-fixation in 4% paraformaldehyde overnight and cryoprotected in 30% sucrose. Coronal sections of 40 μm were processed for cytochemistry as free-floating sections. For NADPH-diaphorase reaction, sections were incubated for 45 min at 37°C with a solution containing 1 mM β-NADPH, 0.1 mM nitro blue tetrazolium (Sigma) and 0.3% Triton X-100 in 0.1 M phosphate buffer (pH 7.4) (Norris et al., 1996; Gu et al., 1997). For immunohistochemistry, sections were incubated with primary rabbit polyclonal anti-Fos antiserum (Oncogene, Cambridge, MA, USA), diluted 1:750 with phosphate-buffered saline (PBS) 0.1 mol l^{-1}, containing 3% normal goat serum and 0.3% Triton X-100 for 48 h at 4°C (Sun et al., 1996). After being washed in PBS, the sections were incubated with biotinylated second antibody (goat anti-rabbit antiserum; Vector, Burlingame, CA, USA) diluted 1:200 in PBS for 1 h at room temperature. Sections were reacted with elite ABC (Vectastain, Vector) diluted 1:50 for 1 h and then with 0.1% diaminobenzidine solution containing 0.6% nickel ammonium sulphate and 0.2% H_2O_2 as substrate was added. All sections were mounted on gelatin-

coated slides, air-dried and protected with a coverslip for light microscopic inspection.

2.4. Electrophoretic mobility shift assay

The lumbar spinal cords (L3 to L5) were removed from rats and homogenized with homogenization buffer and centrifuged at 11,000 × *g* for 30 s. Then 1 ml of hypotonic lysis buffer (*N*-[2-hydroxyethyl]piperazine-*n*′-[4-butanesulfonic acid] (HEPES) 10 mM, KCl 10 mM, $MgCl_2$ 2 mM, DL-dithiothreitol 1 mM, ethylenediaminetetraacetic acid (EDTA), 1 mM, phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and leupeptin, pH 7.8) was added to the pellets, mixed well and then placed on ice for 15 min. After centrifugation at 12,000 rpm for 30 s, the supernatant was discarded and the pellets were mixed with 50 μl hypertonic lysis buffer (HEPES 10 mM, KCl 10 mM, NaCl 300 mM, DTT 1 mM, EDTA 0.1 mM, PMSF 0.1 mM, 10% glycerol, pH 7.8) (Paliogianni et al., 1993) at 4°C for 20 min, and then centrifuged at 11,000 × *g* for 5 min. The suspension was stored at −70°C freezer for determination of protein concentration.

Nuclear extracts were reacted with 10,000 cpm ^{32}P-labeled probes in binding buffer (HEPES 10 mM, NaCl 50 mM, DTT 1 mM, EDTA 1 mM, PMSF 0.1 mM, 10% glycerol, 1% Ficoll, 0.2 mol/ml, pH 7.9) also containing 2 μg poly (dI-dC) poly (dI-dC) (Sigma) and incubated at room temperature for 30 min. The DNA–protein complex was electrophoresed on 4.5% non-denatured polyacryamide gels in 0.5 × Tris–Borate–EDTA (TBE) buffer. Radioactive bands were detected by autoradiography with X-ray film.

2.5. Immunoblotting

The lumbar spinal cords (L3 to L5) were homogenized in a buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 1 mM NaF, 1 mM Na_1VO_4, 1 mM PMSF, 10 μg/ml aprotinin and leupeptin, pH 7.4 and placed on ice for 20 min. The lysates were centrifuged at 14,500 rpm for 20 min at 4°C, and the concentration of protein in each lysate was measured with a commercial BCA kit (Pierce, Rockford, IL, USA). A 50-μg sample of each lysate was subjected to electrophoresis on discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel. Electrophoresis was carried out on 4% stacking gel (40 V for 40 min) and 10% separating gel (100 V for 3 h). Proteins were then transferred to nitrocellulose and immunoblotted with anti-nNOS antibody (Transduction Laboratory, Lexington, KY, USA) and anti-alpha tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection was performed with the blotting reagent ECL (Amersham, Arlington Heights, IL, USA), and chemiluminescence was detected by exposure of filters to Fuji medical X-ray films.

2.6. Statistical analysis

All data are given as means ± standard error (S.E.M.). Statistical significance ($^*P < 0.01$) was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's tests.

3. Results

3.1. The behavioral profiles of hyperalgesia

The time course of two behavioral correlates of CFA-induced hyperalgesia was determined with Von Frey filaments and radiant heat. As seen in Fig. 1, the mechanical threshold of CFA-injected paws had decreased significantly ($P < 0.01$) 3 h after CFA injection, to reach its minimal value at 6 h (12.7 ± 5.6% of control). At 24 h, the mechanical threshold of the paw started increasing, and hyperalgesia was still detected even at 96 h (40.4 ± 16.6% of control, $P < 0.01$). The thermal threshold of CFA-treated paws had the same tendency to decrease as the mechanical threshold. However, as compared to mechanical stimulation, CFA-treated paws were less sensitive to thermal stimulation and reached a minimal threshold, 54.2 ± 9.0%

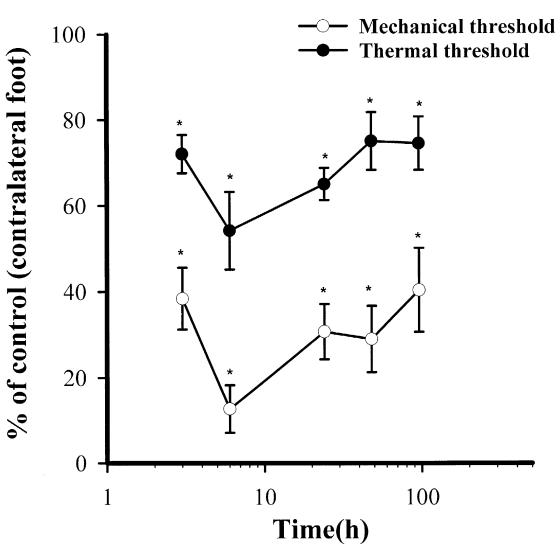

Fig. 1. The behavioral profile of hyperalgesia in rats. Hyperalgesia induced by CFA was assayed by paw withdrawal in response to either mechanical pressure (○), using the Von Frey filaments as described in Section 2, or exposure to radiant heat (●). CFA was injected into a hind paw of rats and the thresholds of responses of the inflamed paw were calculated as percentages of those of the contralateral vehicle-injected paw. Data are presented as means ± S.E.M. ($n = 7$), $^*P < 0.01$ as compared with the control.

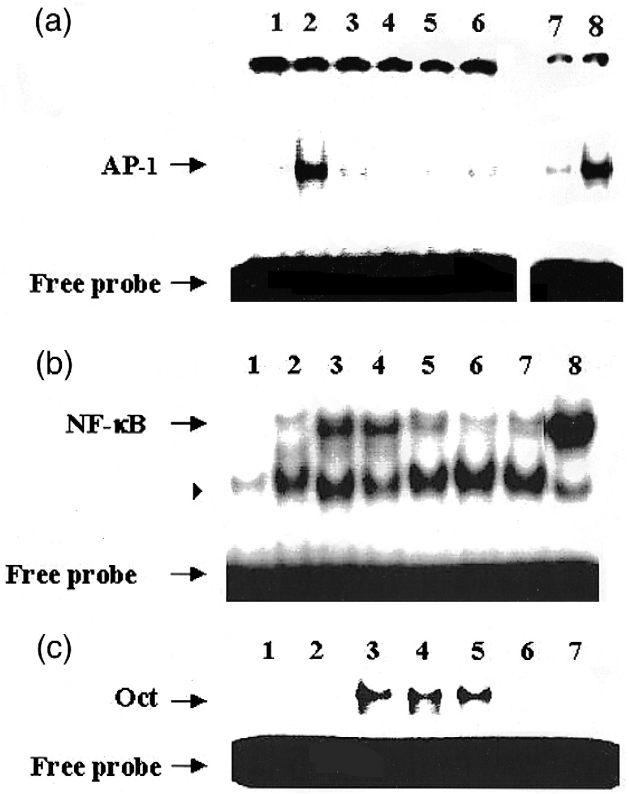

Fig. 2. EMSA of transcription factors following paw injection of CFA in rats. (a) AP-1 binding ability. Lane 1: Control, lanes 2, 3, 4, 5 and 6 represent AP-1 binding 0.5, 1, 2, 4 and 8 h after CFA injection, respectively. The competition study by using 50-fold concentration of cold probe (lane 7) was performed as compared with that without cold probe (lane 8) 0.5 h after CFA injection. (b) NF-κB binding ability — Lane 1: negative control; using cold probe (50-fold of concentration) to compete with ^{32}P-labeled probe in sample obtained 0.5 h after CFA injection. Lanes 2, 3, 4, 5, 6 and 7 represent samples collected 0, 0.5, 1, 2, 4 and 8 h after CFA injection, respectively. Lane 8: 1 μM PMA incubated with NIH 3T3 cells for 1 h and served as positive control. Arrow indicates p50/p65 heterodimer and arrowhead represents p50/p50 homodimer. (c) Oct binding ability, lane 1: negative control; cold probe (50-fold of concentration) competes with ^{32}P-labeled probe in a sample collected 0.5 h after CFA injection. Lanes 2 to 7 is the same order as (b).

of control, at 6 h. As compared to contralateral vehicle-injected paws, CFA-treated paws had a significantly lower mechanical and thermal threshold during exposure to mechanical and thermal stimulation (Fig. 1).

3.2. Assay of EMSA following paw injection of CFA

Blockade of the expression of genes, which mediate the signaling pathway of hyperalgesia, may be the best policy to resolve the issue of clinical pain. Thus, we further investigated gene regulation in hyperalgesia. C-Fos is already recognized as an AP-1 mediator and NF-κB and Oct have been reported as being involved in the regulation of nNOS. Gene regulation was detected in the EMSA and this may be the first report of gene regulation in hyperalgesia. As shown in Fig. 2, AP-1 binding dramatically increased at 0.5 h following CFA injection and quickly declined to basal level at 1–8 h (Fig. 2a). The p50/p65 heterodimer of NF-κB immediately increased at 0.5 h and started to decrease 2 h following injection, where there was no significant change in p50/p50 homodimer (Fig. 2b). Cold NF-κB probe (50-fold, Fig. 2b, lane 1) was used to compete both p50/p50 and p50/p65 with hot probe in the 0.5 h CFA treatment condition, and NIH 3T3 incubated with 1 μM phorbal 12-myristate 13-acetate (PMA) for 1 h served as positive control for the dominant p50/p65 heterodimer (Fig. 2b, lane 8). Oct quickly increased at 0.5 h and returned to a basal level at 4 h following CFA injection (Fig. 2c). Cold Oct probe (50-fold, Fig. 2c, lane 1) was used to compete with hot probe in the 0.5-h CFA treatment condition. The binding of the three probes was still slightly higher than the basal level 8 h after CFA injection. The data may provide an explanation for c-Fos and nNOS expression at different times after CFA injection.

3.3. Time course of c-Fos following CFA injection

Fos-like immunoreactive neurons are present in the entire lumbar enlargement and are most numerous in the L3–L4 segments (Abbadie and Besson, 1992). Thus, the quantification was only made in these segments. As shown in Fig. 3, CFA induced c-Fos expression (all laminae) and this also reached a peak at 3 h. C-Fos expression of CFA-treated paws started to decrease at 6 h (95.1 ± 4.7% of CFA 3 h) and was significantly lower at 24–96 h

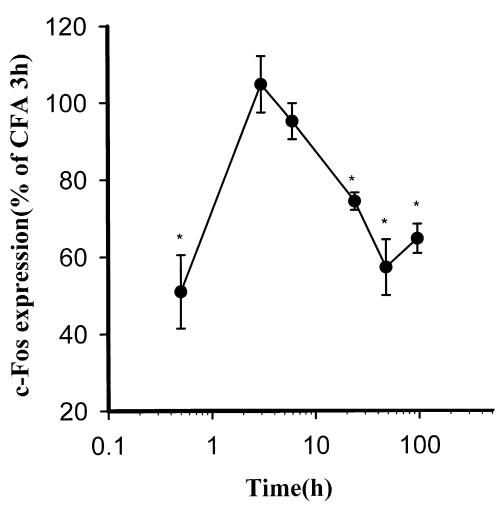

Fig. 3. c-Fos expression following paw injection of CFA in rats. Total c-Fos expression on lumbar 4 of spinal cords was analyzed by c-Fos antibody as described in Section 2. Expression of c-Fos 3 h after CFA injection was determined as 100%. Control level of c-Fos was almost undetectable. Data are presented as means ± S.E.M. ($n = 5$), $^{*}P < 0.01$ as compared with that of CFA 3 h.

(74.3 ± 2.2% and 64.6 ± 3.8% of CFA 3 h, respectively). The basal level of the control paw without CFA treatment was almost undetectable. Fos-like immunoreactivity was mainly distributed in the neck of the dorsal horn (laminae V and VI) and in the ventral horn, whereas very few Fos-like immunoreactive neurons were observed in the superficial dorsal horn (laminae I and II) and in the nucleus proprius (laminae III and IV).

3.4. NADHP-diaphorase histochemistry following CFA injection

In addition to c-Fos, NOS has been reported to contribute to the signaling pathway of hyperalgesia. As seen in Fig. 4, nNOS activity (detected by NADPH-diaphorase activity) gradually reached a peak at 48 h, and NADPH-diaphorase reactive neurons (NDP) were mainly distributed

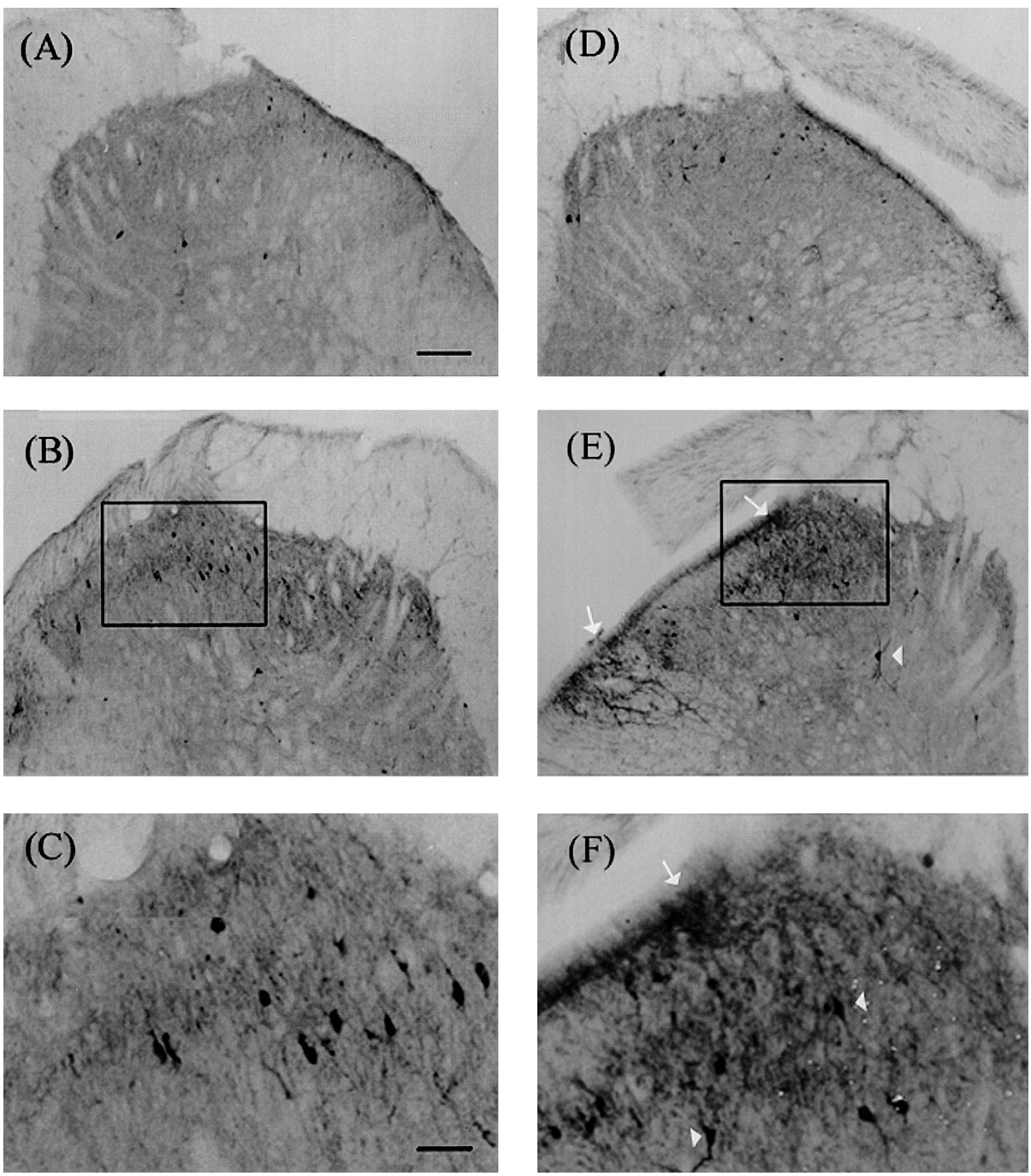

Fig. 4. Photomicrographs showing cells of LD spinal cord stained histochemically with NADPH-diaphorase at 24 h (A, B, C) and 48 h (D, E, F) after CFA injection into rats. (A) and (D) are controls. (C) and (F) are the amplification of selective areas in (B) and (E), respectively. Arrows in (E) and (F) show the great numbers of NADPH-diaphorase cells in the superficial laminae and arrowheads represent larger neurons with extended neurites. Bar = 60 μm in (A), (B), (D) and (E) and 20 μm in (C) and (F).

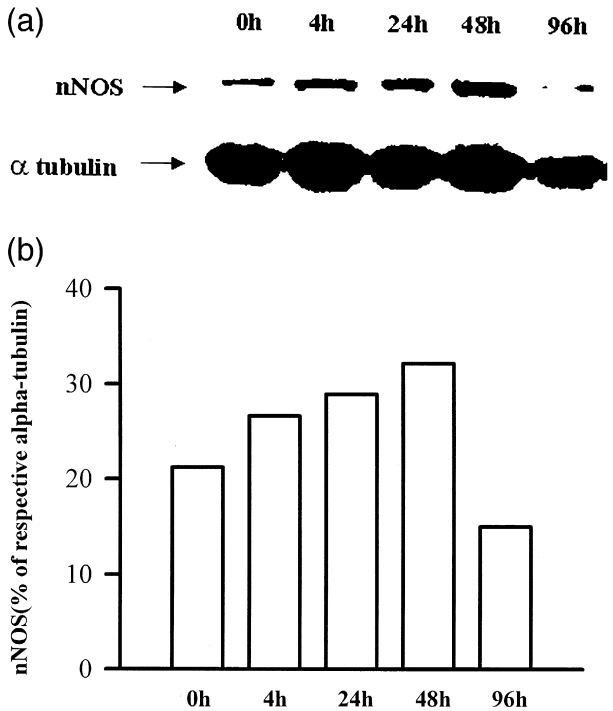

Fig. 5. Determination of nNOS protein expression after CFA injection by immunoblotting. (a) The expression of nNOS was determined at various time intervals after CFA injection by using nNOS immunoblotting as described in Section 2. The expression of alpha tubulin was used as internal control. Data shown are the representative result of three separate experiments. (b) The result was quantified by densitometry and expressed as a ratio with respect to the expression of alpha tubulin.

in the superficial dorsal horn. Our results demonstrate that c-Fos and nNOS contributed to hyperalgesia induced by CFA at different time intervals.

3.5. Increased nNOS protein expression after CFA injection

Induction of nNOS expression following CFA injection was detected by Western blot. As seen in Fig. 5a, nNOS proteins were gradually induced until 48 h and reached a maximum at 48 h after CFA injection. Thereafter, the expression of nNOS decreased and was slightly less than basal level 96 h after CFA injection. The level of alpha tubulin was used as internal standard control. The expression of nNOS was increased 10% compared to that of alpha tubulin 48 h after CFA injection (from 22% to 32%) and then decreased to 15% of that of alpha tubulin.

4. Discussion

4.1. Intracellular signaling induced by CFA

It may be the best strategy to diminish pain by blocking the expression of target genes in hyperalgesia. In a previous study, formalin-induced nociceptive behavior was decreased when we administered c-*fos*antisense oligodeoxynucleotide and cycloheximide to rats (Hou et al., 1997a). We further investigated the regulation of the transcription of target genes (c-*fos* and nNOS) associated with hyperalgesia. The recently found transcription factor Oct-2, a member of the POU (a 60-amino acid homeodomain and tissue-specific transcription factors including Oct-1, Oct-2, which are expressed widely in the developing nervous system) family, can activate nNOS gene expression (Deans et al., 1996; Gay et al., 1998), and another transcription factor of the POU family, Brn-3, can activate inducible nitric oxide synthase (iNOS) gene expression by binding to the octamer motif in the promoter (Gay et al., 1998). In this study, we found the maximal binding of NF-κB, and Oct occurred later than that of AP-1. This result is consistent with the time course of c-Fos protein expression followed by the increased activity of nNOS. NF-κB is an inducible transcription factor in many cell types and is constitutively expressed in many cell types, including mature B cells, thymocytes, adherent macrophages and central nervous system (CNS) neurons (Kaltschmidt et al., 1994). The activity of NF-κB correlates with the presence of the NF-κB subunits p50 and p65 (Rel A) in the nuclei of cultured cells. In our study, CFA-induced hyperalgesia was associated with the p50/p65 heterodimer rather than with the p50/p50 homodimer (Fig. 2). Activation of nNOS may cause an opposite effect on NF-κB activation in different cell types. NO derived from nNOS in glia inhibits the transcription of NF-κB activation because NOS inhibitors enhance the basal NF-κB activation (Togashi et al., 1997). The same authors also demonstrated that nNOS in astrocytes can regulate NF-κB activity and iNOS expression. Although our results strongly suggest that nNOS plays a crucial role in CFA-induced inflammatory pain, we did not exclude the effect of iNOS in the regulation of nociception.

It has been reported that the amount of c-Fos-like immunoreactivity is at least twice that of c-Jun-like immunoreactivity in rat spinal following noxious colorectal distension (Traub et al., 1993). We propose that the dramatic dropping of binding of AP-1 may be due to the poor association of c-Fos and c-Jun proteins, which is connected with the dimerization of c-Fos (AP-1), following CFA injection. The immunoreactivity of c-Fos declined faster than activity of nNOS in NADPH-diaphorase containing-neurons (Figs. 3 and 4). Moreover, immediate early genes (IEGs) are poorly expressed in NDP neurons of the deep dorsal horn following single application of CFA. In summary, neurons can co-express NOS and inducible transcription factors but this co-expression is restricted to neuronal sub-populations such as superficial dorsal horn neurons and involves the formation of specific AP-1 complexes, e.g. without the participation of c-Jun.

4.2. NADPH-diaphorase reaction

It has been reported that nNOS and the transcription factors Fos, Jun, and Krox are colocalized following for-

malin injection into one hindpaw of rats (Herdegen et al., 1994). This is a great advance in defining pain sensation at a molecular level, which is usually scored by the behavioral testing. NO is an important neurotransmitter and is also recognized as a crucial factor in the signaling pathway of hyperalgesia. Evidence suggests that NOS activity be also involved in hyperalgesia (Choi et al., 1996). Inhibition of NOS activity results in antinociception and reduces the responses of spinal neurons to noxious stimulation (Meller and Gebhart, 1993; Handy et al., 1996). It is a well-known observation that peripheral noxious stimulation generates lasting changes in spinal neurons, including enlargement of receptive fields and hyperexcitability with consequent hyperalgesia (Dubner and Ruda, 1992). This could also be mediated by an enhanced release of NO from primary afferent neurons and spinal neurons, which express NOS (Fiallos-Estrada et al., 1993). In our study, the pattern of NADPH-diaphorase reaction and also nNOS protein expression in lumbar spinal cord was very similar to that described in previous reports (Herdegen et al., 1994). There was a significant increase (above basal values) in the number of neurons labeled by NADPH-diaphorase reaction in the sDH of L3–L4 segments 24 h following CFA stimulation. This area also represents the main region of termination of sciatic nerve fibers (Swett et al., 1985).

4.3. Correlation of c-Fos and nNOS

The expression of c-*fos* and other IEGs has been accepted as a molecular marker of hyperalgesia following noxious stimulation (Hunt et al., 1987; Herdegen et al., 1991, 1994; Abbadie and Besson, 1992; Abbadie et al., 1994; Sun et al., 1996; Hou et al., 1997b). In our experiment, c-Fos proteins were highly expressed following CFA-induced chronic inflammatory pain within 96 h. Similar to the results of Herdegen et al. (1994), we found that numerous small-diameter c-Fos cells could be seen in lamina VIII. Subsequently, c-Fos-positive cells in the superficial dorsal horn disappeared more rapidly than did c-Fos-positive and NDP-labeled cells in the deep dorsal horn (data not shown). We found that fewer NDP neurons co-expressed with c-Fos ($< 3\%$) after CFA treatment than after formalin treatment. About 8% of the total number of c-Fos-labeled neurons following were also labeled by NDP after formalin treatment (Herdegen et al., 1994). The variance may be due to the different noxious stimuli and the persistence of hyperalgesia.

4.4. Expression of different proteins during hyperalgesia

It is intriguing that our study showed that the peak expression of c-Fos and the activity of nNOS (also in nNOS protein concentration) occurred at different times interval (3 and 48 h, respectively) following CFA injection. This may provide an explanation for the poor colocalization of c-Fos and NDP neurons. Our data also suggest that different noxious stimuli may induce the expression of different hyperalgesia-associated proteins at different times and contribute to the different roles in this signaling pathway. It seems consistent with our hypothesis that the co-expression of c-Fos and NDP decreased with time, from 2 h (about 8%), 5 h (3%) to 10 h (0%) following formalin injection (Herdegen et al., 1994). Thermal hyperalgesia was less sensitive than mechanical hyperalgesia to CFA treatment (Fig. 1). However, both types of hyperalgesia reached a minimal threshold 6 h after CFA injection, which was later than the expression of the IGE. This may indicate that both thresholds of hyperalgesia are under the regulation of these transcription factors and IGEs. The regulation of the molecular mechanism of hyperalgesia can be investigated by using EMSA to monitor the transcriptional regulation of the target genes of hyperalgesia. In conclusion, we report for the first time that CFA administration immediately induces the activation of transcription factors (AP-1, NF-κB and Oct) prior to the expression of c-Fos and nNOS. Thus, inhibitors of the activation of these transcription factors may be efficient antinociceptive drugs.

References

Abbadie, C., Besson, J.-M., 1992. C-*fos* expression in rat lumbar spinal cord during the development of adjuvant-induced arthritis. Neuroscience 48, 985–993.

Abbadie, C., Besson, J.-M., Calvino, B., 1994. C-Fos expression in the spinal cord and pain-related symptoms induced by chronic arthritis in the rat are prevented by pretreatment with Freund adjuvant. J. Neurosci. 14 (10), 5865–5871.

Bredt, D.S., Snyder, S.H., 1992. Nitric oxide, a novel neuronal messenger. Neuron 8 (1), 3–11.

Choi, Y., Raja, S.N., Moore, L.C., Tobin, J.R., 1996. Neuropathic pain in rats is associated with altered nitric oxide synthase activity in neural tissue. J. Neurol. Sci. 138, 14–20.

Deans, Z., Dawson, S.J., Xie, J., Young, A.P., Wallace, D., Latchman, D.S., 1996. Differential regulation of the two neuronal nitric-oxide synthase gene promoters by the Oct-2 transcription factor. J. Biol. Chem. 271 (50), 32153–32158.

Dubner, R., Ruda, M.A., 1992. Activity-dependent neuronal plasticity following tissue injury and inflammation. Trends Neurosci., 96–103.

Fiallos-Estrada, C.E., Kummer, W., Mayer, B., Bravo, R., Zimmermann, M., Herdegen, T., 1993. Long-lasting increase of nitric oxide synthase immunoreactivity, NADPH-diaphorase reaction and c-Jun co-expression in rat dorsal root ganglion neurons following sciatic nerve transaction. Neurosci. Lett. 150 (2), 169–173.

Gay, R.D., Dawson, S.J., Murphy, W.J., Russel, S.W., Latchman, D.S., 1998. Activation of the iNOS gene promoter by Brn-3 POU family transcription factors is dependent upon the octamer motif in the promoter. Biochim. Biophys. Acta 1443 (3), 315–322.

Grilli, M., Pizzi, M., Memo, M., Spano, P., 1996. Neuroprotection by aspirin and sodium salicylate through blockade of NF-κB activation. Science 274, 1383–1385.

Gu, Y., Spasic, Z., Wu, W., 1997. The effects of remaining axons on motoneuron survival and NOS expression following axotomy in the adult rat. Dev. Neurosci. 19, 255–259.

Handy, R.L.C., Wallace, P., Moore, P.K., 1996. Inhibition of nitric oxide

syntheses by isothioureas: cardiovascular and antinociceptive effects. Pharmacol. Biochem. Behav. 55 (2), 179–184.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32, 77–88.

Herdegen, T., Kovary, K., Leah, J., Bravo, R., 1991. Specific temporal and spatial distribution of Jun, Fos, Krox-24 proteins in spinal neurons following noxious transsynaptic stimulation. J. Comp. Neurol. 313, 178–191.

Herdegen, T., Rüdiger, S., Mayer, B., Bravo, R., Zimmermann, M., 1994. Expression of nitric oxide synthase and colocalisation with Jun, Fos and Krox transcription factors in spinal cord neurons following noxious stimulation of the rat hindpaw. Mol. Brain Res. 22, 245–258.

Hou, W.Y., Shyu, B.C., Chen, T.M., Shieh, J.Y., Sun, W.Z., 1997a. Protein synthesis inhibitor cyclohexamide dose-dependently decreases formalin-induced c-Fos protein and behavioral hyperalgesia in rats. Neurosci. Lett. 227, 99–102.

Hou, W.Y., Shyu, B.C., Chen, T.M., Lee, J.W., Shieh, J.Y., Sun, W.Z., 1997b. Intrathecally administered c-*fos* antisense oligodeoxynucleotide decrease4 formalin-induced nociceptive behavior in adult rats. Eur. J. Pharmacol. 329 (1), 17–26.

Hunt, P.S., Pin, A., Evan, G., 1987. Induction of c-*fos*-like protein in spinal cord neurons following sensory stimulation. Nature 383 (13), 632–634.

Kaltschmidt, C., Kaltschmidt, B., Neumann, H., Wekerle, H., Baeuerle, P.A., 1994. Constitutive NF-κB activity in neurons. Mol. Cell. Biol., 3981–3992.

Kleinert, H., Wallerath, T., Fritz, G., Ihrig-Biedert, I., Rodriguez-Pascual, F., Geller, D.A., Forstermann, U., 1998. Cytokine induction of NO synthase II in human DLD-1 cells: roles of the JAK-STAT, AP-1 and NF-kappa B-signaling pathways. Br. J. Pharmacol. 125 (1), 193–201.

Lantéri-Minet, M., De Pommery, J., Herdegen, T., Weil-Fugazza, J., Bravo, R., Menétrey, D., 1993. Differential time course and spatial expression of Fos, Jun, and krox-24 proteins in spinal cord of rats undergoing subacute or chronic somatic inflammation. J. Comp. Neurol. 333, 223–235.

Marks-Konczalik, J., Chu, S.C., Moss, J., 1998. Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor kappa B-binding sites. J. Biol. Chem. 273 (35), 22201–22208.

Meller, S.T., Gebhart, G.F., 1993. Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain 52, 127–136.

Menétrey, D., Gannon, A., Levine, J.D., Basbaum, A.I., 1989. Expression of c-*fos* protein in internerons and projection neurons of the rat spinal cord in response to noxious somatic, articular, and visceral stimulation. J. Comp. Neurol. 285, 177–195.

Norris, P.J., Faull, R.L.M., Emson, P.C., 1996. Neuronal nitric oxide synthase (nNOS) mRNA expression and NADPH-diaphorase staining in the frontal cortex, visual cortex and hippocampus of control and Alzheimer's disease brains. Mol. Brain Res. 41, 36–49.

Shibuki, K., 1993. Cerebellar long-term depression enabled by nitric oxide, a diffusible intercellular messenger. Ann. N.Y. Acad. Sci. 707, 521–523.

Sun, W.Z., Shyu, B.C., Shieh, J.Y., 1996. Nitrous oxide or halothane, or both, fail to suppress c-*fos* expression in rat spinal cord dorsal horn neurons after subcutaneous formalin. Br. J. Anaesth. 76, 99–105.

Swett, J.E., McMahon, S.B., Wall, P.D., 1985. Long ascending projection to the midbrain from cells of lamina I and nucleus of the dorsolateral funiculus of the rat spinal cord. J. Comp. Neurol. 238, 401–416.

Todoroki, S., Goto, S., Urata, Y., Komatsu, K., Sumikawa, K., Ogura, T., Matsuda, I., Kondo, T., 1998. High concentration of L-arginine suppresses nitric oxide synthase activity and produces reactive oxygen species in NB9 human neuroblastoma cells. Mol. Med. 4 (8), 515–524.

Togashi, H., Sasaki, M., Frohman, E., Taira, E., Ratan, R.R., Dawson, T.M., Dawson, V.L., 1997. Neuronal (type I) nitric oxide synthase regulates nuclear factor kappa B activity and immunologic (type II) nitric oxide synthase expression. Proc. Natl. Acad. Sci. U.S.A. 94 (6), 2676–2680.

Traub, R.J., Herdegen, T., Gebhart, G.F., 1993. Differential expression of c-*fos* and c-*jun* in two regions of rat spinal cord following noxious colorectal distention. Neurosci. Lett. 160, 121–125.

Yashpal, K., Mason, P., Mckenna, J.E., Sharma, S.K., Henry, J.L., Coderre, T.J., 1998. Comparison of the effects of treatment with intrathecal lidocaine given before and after formalin on both nociception and Fos expression in the spinal cord dorsal horn. Anesthesiology 88, 157–164.