

Nicotinamide adenine dinucleotide phosphate-diaphorase and c-fos expression in spinal cord neurons following noxious stimulation

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ABSTRACT

الأهداف: هذه الدراسة تهدف إلى التحقيق في أدنين نيكوتيناميد ديناكوتيد فوسفات-ديافوراز (NADPH-d) و (fos) في الجملة العصبية للحبل الشوكي بعد إجراء التنبيه الضار.

الطريقة: تم فحص الحبل الشوكي في الجرذ بعد ظهور جين (c-fos) المبكر مباشرة و نتريك أوكسيد المحتوى على نيوروناز بساعة واحدة بعد الحقن في الجانب الواحد بالفورمالين في ظهر الكف وذلك باستعمال تقنيات الكيمياء المناعية النسيجية لفوز الكيمياء النسيجية ل (NADPH-d). أجريت التجربة في عام 2004م و عام 2006م بمركز إيج الجامعي لبحوث الدماغ في مدينة أزمير بتركيا.

النتائج: تمت مراقبة زيادة نيوروناز (fos) ذو التنشيط المناعي في 10 من الجرذان الذكور نوع سبراجو- داولي و (NADPH-d) الموجب نيوروناز في موضع الحقن. حوالي 20% من نيوروناز (fos) ذو التنشيط المناعي كان ايجابي (NADPH-d) في حقن الفورمالين، بينما لم يتم ملاحظة وجود تصنيف مزدوج في موضع الجانب المقابل. بالإضافة إلى مراقبة العلاقة الوثيقة لسير العملية الإيجابي لدى (NADPH-d) مع (fos) ذو التنشيط المناعي.

خاتمة: تدعم نتائج هذه الدراسة فرضية أن عوامل انسداد أوكسيد النتريك قد تخدم كبديل محتمل في علاج فرط الحس بالألم عقب التعرض للإلتهاب أو إصابة في العصب المحيطي.

Objectives: To investigate nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and fos expression in spinal cord dorsal horn neurons following noxious peripheral stimulation.

Methods: Expression of the immediate-early gene c-fos and nitric oxide containing neurons one hour after unilateral formalin injection to the dorsal hind paw was investigated in rat lumbar spinal cord, using fos immunohistochemistry and NADPH-d histochemical techniques. The experiments were performed in 2004 and 2006 at Ege University Center for Brain Research in Izmir, Turkey.

Results: In 10 adult male Sprague-Dawley rats, an increase in fos-immunoreactive neurons was observed ipsilateral, and NADPH-d positive neurons equally ipsi- and contralateral to the formalin injection site. Approximately 20% of fos-immunoreactive neurons were NADPH-d positive ipsilateral to the formalin injection, whereas no double labeling was observed in the contralateral side. Also, a close relation of NADPH-d positive processes with fos-immunoreactive nuclei were also observed.

Conclusion: The results of this study support the hypothesis that nitric oxide synthase blocking agents may serve as a possible alternative in treatment of hyperalgesia following inflammation and peripheral nerve injury.

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Fos is a nuclear phosphoprotein product of the mammalian c-fos proto-oncogene and is rapidly and transiently expressed in response to noxious inputs in the brain and spinal cord.¹⁻⁵ Fos protein expression can be detected using the fos immunohistochemistry technique that is used as a marker of the activation of neurons by noxious stimulation.^{3,5} Recent immunohistochemical or in situ hybridization studies with fos have observed an increase in fos expression following mechanical, thermal, and chemical noxious stimulation in laminae I-II and V, VI, and area X neurons of the dorsal horn neurons.^{6,7} These laminae correspond to the terminal sites of primary afferent fibers and overlap with the distribution of neurons previously detected to be involved in nociception using tract-tracing and

electrophysiological methods.^{8,9} Nitric oxide (NO) is a novel neurotransmitter involved in nociceptive transmission.^{10,11} Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemical reaction shows nitric oxide synthase (NOS) activity.¹² Herdegen et al⁴ found an increase in the number of neurons labeled by NOS antibodies or stained for NADPH-d in rat dorsal horn following subcutaneous injection of formalin in the hind paw. All these previous data revealing an involvement of NO and c-fos in nociception propose a co-localization of NADPH-d and fos expression in dorsal horn neurons following noxious peripheral stimulation. The results of former studies show discrepancies, some observing co-localization of NADPH-d and fos,^{4,13} while others^{14,15} showed no co-localization, which led us to further investigate NADPH-d and fos expression in spinal cord dorsal horn neurons following noxious peripheral stimulation.

Methods. The experiments were performed on male Sprague-Dawley rats (n=10, 250-350 g). Fifty μ l of 5% formalin was injected subcutaneously into the dorsal surface of the left hind paw. Following a one-hour induction period, rats were anesthetized with Pentothal sodium (40 mg/kg, intraperitoneally) and perfused with 4% formaldehyde. Lumbar spinal cord segments (L3-L5) were cut on a cryostat at 25 μ m and free-floating sections were first processed with fos immunohistochemistry (primary antibody rabbit c-fos 1:2000, secondary antibody biotinylated anti-rabbit IgG 1:200, Oncogene Sci. Inc., Cambridge, USA). The reaction was visualized using hydrogen peroxidase and diaminobenzidine (DAB) as chromogen. Sections were then processed for NADPH-d histochemical reaction (0.1 mg/ml nitroblue tetrazolium and 0.5 mg/ml NADPH tetrasodium salt, Sigma, USA) for 30-60 minutes. Sections were examined under light microscope. The number of NADPH-d-positive and fos-immunoreactive neurons in laminae I-II, III-IV, V-VI, VII-IX, and X ipsilateral, and contralateral to the

injected hind paw were counted in every fifth section through the L3-L5 segments. Since sections were at least 200 μ m apart, no correction was made for double counting. All experiments were performed in 2004 and 2006 at Ege University Center for Brain Research in Izmir, Turkey. The guidelines on ethical standards for investigations of experimental pain in animals were followed.¹⁶ The Ethical Committee of the Ege University, Faculty of Medicine, Izmir, Turkey approved all protocols.

Results. Fos immunoreactivity was observed as dark brown round to ovoid nuclei, extending rostro-caudally from spinal segments L3-L5. The average number of fos-immunoreactive neurons, one hour after formalin injection, was greatly increased (120.5 \pm 4 neurons/section) in the segments of the ipsilateral spinal cord, when compared to the contralateral site (7.9 \pm 0.7 neurons/section). Neurons were predominantly located in laminae I-II and III-IV, ipsilateral to the formalin injection. The NADPH-d staining was observed as a dark blue color in bodies and dendritic processes. Formalin injection resulted in bilateral NADPH-d staining, 74 \pm 3.5 neurons/section in the ipsilateral and 59 \pm 4.1 neurons/section in the contralateral side. The NADPH-d positive neurons were mainly located in laminae I-II, III-IV, and X. In this study, we observed numerous fos/NADPH-d double-labeled neurons (8.1 \pm 0.4 neurons/section) ipsilateral to the formalin injection, whereas no double labeling was observed in the contralateral side (Table 1). A total of 21.1% of fos-immunoreactive neurons were NADPH-d positive (Table 1, Figures 1a-d). Another observation was that many fos-immunoreactive nuclei were directly apposed or in close proximity to NADPH-d positive cell bodies or dendritic processes (Figure 1c).

Discussion. Previous studies have shown a close relationship between NO and fos expression in the CNS. For example, nitroglycerin, which forms NO in

Table 1 - Number (mean \pm SD) of c-fos, nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and c-fos/NADPH-d positive neurons per section in the ipsilateral and contralateral lumbar spinal cord one hour after formalin injection.

Variable	Side	Total	Laminae I-II	Laminae III-IV	Laminae V-VI	Laminae VII-IX	Lamina X
Fos	ipsilateral	120.5 \pm 4	33.4 \pm 6.9	44.1 \pm 3.4	11.5 \pm 1.3	16.4 \pm 2.6	15.1 \pm 2.9
	contralateral	7.9 \pm 0.7	2.7 \pm 0.4	1.8 \pm 0.6	1.1 \pm 0.3	1 \pm 0.4	1.3 \pm 0.3
NADPH-d	ipsilateral	74 \pm 3.5	28.1 \pm 5.6	17.4 \pm 3.4	9.4 \pm 3.5	10.2 \pm 0.6	8.9 \pm 1.3
	contralateral	59 \pm 4.1	21.9 \pm 4.7	13.9 \pm 2.8	7.9 \pm 1.6	8.9 \pm 2.1	6.4 \pm 2.4
Fos/NADPH-d	ipsilateral	28 \pm 1.9	9.5 \pm 2.1	5.1 \pm 3.1	3.9 \pm 2.3	6.4 \pm 3.1	3.1 \pm 1.6
	contralateral	-	-	-	-	-	-

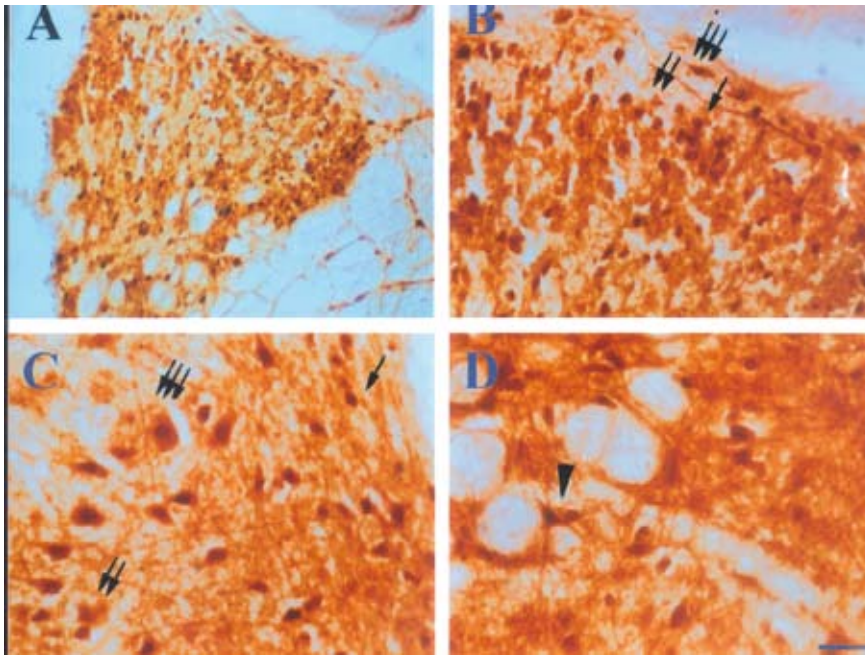


Figure 1 - Photomicrographs showing the distribution of fos-immunoreactive, nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) positive and double-labeled neurons (A-C) in the lumbar spinal cord one hour after formalin injection. D) Arrowhead points to the apposition of fos-immunoreactive nuclei to NADPH-d positive cell body and cytoplasmic process. ↓ - Fos-immunoreactive nucleus, ↓↓ - NADPH-d-positive neuron, ↓↓↓ - double-labeled neuron. Scale bar = 160 μm for A, 80 μm for B and 40 μm for C and D.

vitro, induces fos expression in various brain nuclei.¹⁷ The NO mediates fos expression induced by peripheral mechanical noxious stimulation in the spinal cord.¹⁸ Nitric oxide has also been shown to play an important role in the development of nociception and spinal fos expression through central sensitization mediated by chronic constriction of the sciatic nerve, a neuropathic pain model.¹⁹ In a study with intrathecal administration of N(G)-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, and unilateral sectioning of the spinal cord, Gao and Qiao²⁰ suggested that endogenous NO not only facilitates the perception of nociceptive inputs at the spinal level, but also enhances the descending inhibition upon spinal nociception. Lin et al²¹ suggested that spinal NO release might be one of the nociception mechanisms of the spinal cord. It has also been shown that soluble guanylate cyclase, a NO receptor, might be involved in the central mechanism of formalin-induced inflammatory hyperalgesia in the spinal cord.²² Co-localization of NO and fos in the rat spinal cord following noxious peripheral stimulation has been subject to former studies, yet the results of these studies show discrepancies. The limitation of these studies, as ours, is that spinal fos expression can also be induced by different mechanisms other than nociception, such as the stress response caused by the pain or the motor activity provoked by the stimulation.⁵ Lee et al¹⁴ and Traub et al¹⁵ did not find NO and fos

co-localization in the lumbar spinal cord following noxious stimulation of the hind paw, while Nazli et al²³ observed very rare co-localization. On the contrary, Herdegen et al⁴ found co-localization in the lumbar spinal cord following noxious stimulation of the hind paw and Leong et al¹³ in the rat spinal trigeminal nucleus following formalin injection into the lateral face of the rat. In this study, we observed that approximately 20% of fos-immunoreactive neurons were NADPH-d positive, which is in accordance with the studies of Herdegen et al⁴ and Leong et al.¹³ We also observed intensely stained NADPH-d positive perikarya and cytoplasmic processes lying next or apposed to Fos-immunoreactive nuclei.

The co-localization and close relationship of fos and NADPH-d can be explained as follows: noxious stimulation activates NMDA receptors that leads to increased production of NO.¹⁰ The NO diffuses from neuronal cell bodies or dendritic or axonal processes of these cells to adjacent neurons and activates guanyl cyclase that ultimately induces fos expression via the cGMP pathway. Most of the neurons in the spinal cord found to be fos-immunoreactive cells are interneurons.² These neurons may be excitatory or inhibitory, and in terms of neurotransmitters GABAergic, glycinergic, cholinergic, or glutamatergic.²⁴ The NO may affect the central sensitization of nociceptive neurons through activation of fos, and thus modulate the activity of neurons that release other peptides or amino acids.²⁵

However, not all NADPH-positive neurons in the spinal cord induce fos expression. While fos expression indicates activation of neurons in response to nociceptive stimulation, its absence in the NO containing neurons does not indicate that these neurons are not activated, as Dragunow and Faull²⁶ have shown that not all neurons express the gene when activated.

Both non-selective NOS inhibitor, L-NAME, and the selective NOS inhibitor 7-nitroindazole, reduce formalin-evoked fos expression in the dorsal horn of the rat spinal cord^{25,27,28} and licking behavior.²⁸ Thus, administrating agents that block NO synthesis may be one way to prevent the development of hyperalgesia after inflammation and perhaps even after peripheral nerve injury.²⁸

In conclusion, the results of this study show there are NADPH-d and fos co-localization, and a close relation of NADPH-d positive processes with fos-immunoreactive nuclei in spinal cord neurons following noxious peripheral stimulation. This anatomical relationship supports the hypothesis that NOS blocking agents may be used as an alternative in treatment of hyperalgesia following inflammation and peripheral nerve injury.

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