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SPINAL NERVE LIGATION–INDUCED ACTIVATION OF NUCLEAR FACTOR KAPPAB IS FACILITATED BY PROSTAGLANDINS IN THE AFFECTED SPINAL CORD AND IS A CRITICAL STEP IN THE DEVELOPMENT OF MECHANICAL ALLODYNIA

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Abstract—This study investigated the effect of 5th and 6th lumbar nerve (L5/L6) spinal nerve ligation (SNL) on activated nuclear factor kappaB (NFkBa) in nuclear extracts from the lumbar dorsal horn of the rat, and its relationship to prostaglandin (PG)-dependent spinal hyperexcitability and allodynia 3 days later. Male Sprague–Dawley rats, fitted with intrathecal (i.t.) catheters, underwent SNL- or sham-surgery. Paw withdrawal threshold (PWT), electromyographic analysis of the biceps femoris flexor reflex, and immunoblotting of the spinal cord were used. Both allodynia (PWT ≤4 g) and exaggerated A- and C-fiber-mediated reflex responses (AFRR and CFRR), featuring decreased activation thresholds and evoked hyperexcitability, were evident only in nerve-ligated animals. This was preceded by an increase in NFkBa in the ipsilateral lumbar dorsal horn at 12 h which was still present 3 days after SNL. The amount of NFkBa in the ventral horns was unchanged compared with sham-controls. Blocking the activation of spinal NFκB, either directly with ammonium pyrrolidedithiocarbamate (PDTC; 100 μg i.t.) or indirectly with S(+)-ibuprofen (100 μg i.t.) administered immediately after SNL, prevented the SNL-induced expression of spinal cyclooxygenase-2 and the development of spinal hyperexcitability and allodynia 3 days later. R(−)-Ibuprofen and vehicle had no effect. These results demonstrate that NFκB is not only activated by SNL, but that spinal PG generated in the affected spinal cord from the onset of nerve injury facilitates this process. NFκB is a critical antecedent in the development of spinal PG-dependent hyperexcitability and allodynia in the SNL model.

Key words: NFκB, spinal prostaglandins, spinal nerve ligation, spinal hyperexcitability, allodynia, cyclooxygenase.

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Abbreviations: AFRR, A-fiber-mediated reflex responses; ANOVA, analysis of variance; CFRR, C-fiber-mediated reflex responses; COX, cyclooxygenase; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglia; iNOS, inducible nitric oxide synthase; i.t., intrathecal; L5, 5th lumbar nerve; L6, 6th lumbar nerve; NFκB, nuclear factor kappaB; NFκBa, activated nuclear factor kappaB; PDTC, ammonium pyrrolidedithiocarbamate; PG, prostaglandin; PGE_2, prostaglandin E2; PWT, paw withdrawal threshold; sGC, soluble guanylate cyclase; SNL, L5/L6 spinal nerve ligation.

Nuclear factor kappaB (NFκB) is constitutively expressed in glia and neurons (Lu et al., 2003; Lee et al., 2004; Yan et al., 2004; Tsatsanis et al., 2006) where it is stored in the cytoplasm as an inactive complex with the inhibitory factor, IκB (Egan and Toruner, 2006). Following cell activation, IκB is phosphorylated by protein kinases such as IκB kinase alpha, releasing NFκB from the complex. NFκB is then free to translocate into the nucleus where it is activated (NFκBa) and binds to specific response elements of the DNA initiating gene transcription.

Experimental nerve injuries such as partial ligation, complete transection or chronic constriction of the sciatic nerve, or spinal cord compression trigger the activation of NFκB (Sakaue et al., 2001; Pollock et al., 2005; Bethea et al., 1998). As a modulator of gene transcription (Lu et al., 2003; Lee et al., 2004; Yan et al., 2004; Tsatsanis et al., 2006), NFκBa plays a critical role in cell survival, apoptosis, and synaptic plasticity (Shishodia and Aggarwal, 2002; Piva et al., 2006; Bubici et al., 2006; Albensi and Mattson, 2000). Thus, it is well positioned to affect the functional outcomes induced by nerve injury. Consistent with this hypothesis are reports that blocking the transcriptional effects of NFκBa with ammonium pyrrolidedithiocarbamate (PDTC) or its decoy significantly attenuated the mechanical allodynia normally elicited by chronic constriction of the sciatic nerve (Ebersberger et al., 2006), spinal cord injury (La Rosa et al., 2004; Jiménez-Garza et al., 2005), or treatment with intrathecal (i.t.) dynorphin (Laughlin et al., 2000). Understanding the pharmacology of these acute signaling events provides a scientific basis for safe and effective interventions to mitigate the chronic and often debilitating outcomes of nerve injury.

Fifth and 6th lumbar nerve (L5/L6) spinal nerve ligation (SNL) is a widely used nerve injury model inducing mechanical allodynia in experimental animals (Kim and Chung, 1992; Hefferan et al., 2003a,b). This model is characterized by early changes in and dependence on spinal prostaglandin (PG) signaling 7–10 days after SNL (Hefferan et al., 2003b; O'Rielly and Loomis, 2006, 2007). These changes include the up-regulation of cyclooxygenase-2 (COX-2) and EP_{1-3} receptors in the affected dorsal horn, a marked increase in pharmacodynamic sensitivity to prostaglandin E2 (PGE_2) in the lumbar cord (O'Rielly and Loomis, 2006, 2007), and the onset of brush-evoked (i.e. non-noxious) release of PGE_2 into spinal CSF (Hefferan et al., 2003b). Transcription of the COX-2 gene, which reaches maximum protein expression 3 days after SNL

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(O'Rielly and Loomis, 2006), is known to be modulated by NFκBa (Tsatsanis et al., 2006), and COX-2-derived spinal PG plays a critical role in the development of SNL-induced allodynia (O'Rielly and Loomis, 2006, 2007; Hefferan et al., 2003a,b). Thus, SNL represents a logical neuropathic pain model in which to determine if the activation of NFκB in the affected spinal cord is an immediate and necessary step in the development of allodynia.

To test this hypothesis, NFκBa was determined in nuclear extracts from the ipsilateral and contralateral lumbar dorsal horn 12 h, 1 day and 3 days after SNL- or sham-surgery. To assess the transcriptional effect of NFκBa, the expression of COX-2 protein was determined using Western analysis. PDTC, an inhibitor of NFκB, was used to probe the functional relationship between NFκBa in the affected spinal cord, the induction of spinal COX-2, and the development of mechanical allodynia and spinal hyperexcitability 3 days after SNL. The latter was investigated using the A- and C-fiber-mediated responses of the biceps femoris reflex (AFRR and CFRR, respectively), and paw withdrawal threshold (PWT) was used to confirm mechanical allodynia. The possibility that spinal PG, generated immediately after SNL, might facilitate the initial activation of NFκB and, in turn, its downstream transcriptional effect on COX-2 was also investigated.

EXPERIMENTAL PROCEDURES

Studies were approved by, and experiments conducted in accordance with the guidelines of the Institutional Animal Care Committee of Memorial University of Newfoundland (St. John's, NL, Canada) which requires that every attempt be made to minimize the number of animals used and their suffering.

Animals

Male Sprague–Dawley rats (130–150 g) were obtained from the Vivarium of Memorial University of Newfoundland (St. John's, NL, Canada) and housed in standard cages with woodchip bedding. Animals had free access to food and water. A 12-h light/dark cycle (lights on at 07:00 h) was used throughout.

I.t. catheterization

I.t. catheters (6.5 cm-length terminating near the lumbar enlargement) were implanted according to method of Yaksh and Rudy (1976) as modified by Hefferan et al. (2003a). Catheters were sterilized with 70% alcohol and filled with sterile saline. Under halothane anesthesia, the catheter was inserted through an incision in the atlanto-occipital membrane of the cisterna magna. The catheter was externalized behind the head and sealed with a piece of stainless steel wire. I.t. catheters (inner diameter, i.d. 240 μm, outer diameter, o.d. 290 μm) were constructed from triple lumen PE-5 tubing (Spectranetics, Colorado Springs, CO, USA) using the modified method of Marsala et al. (1995). Rats with normal motor, grooming and feeding behavior were housed separately and allowed to recover for 2 days before SNL- or sham-surgery.

Neuropathy

Neuropathy was induced using the method of Kim and Chung (1992) as previously described (Hefferan et al., 2003a). Briefly, rats were anesthetized with halothane, the left L4 and L5 spinal nerves were isolated and separated, and the L5 and L6 spinal nerves were tightly ligated with 6–0 silk thread. In sham-controls, the L5 and L6 spinal nerves were isolated but not ligated. Except for NFκBa, which was determined 12 and 24 h after surgery, all animals were allowed to recover for 3 days before experimentation.

PWT

Mechanical allodynia was quantified by determining the PWT using von Frey filaments (Chaplan et al., 1994). Briefly, rats were placed in a plastic cage with a wire-mesh bottom allowing access to the plantar surface of the left hind paw. Following a 20-min acclimatization period, a control threshold was determined. Allodynia was defined as a PWT of $\leq$4 g (Chaplan et al., 1994; Hefferan et al., 2003a,b).

Electrophysiological recordings

Animal preparation. Rats were anesthetized with halothane and cannulae were placed in the trachea, left carotid artery and right external jugular vein. Halothane anesthesia was then replaced by sodium thiobutabarbitone (1 mg$\bullet$kg^{-1} i.v.; Inactin, Sigma-Aldrich, Oakville, ON, Canada). Adequate depth of anesthesia was assessed by testing for hind limb withdrawal and corneal reflexes which had to be absent. Blood pressure was continuously monitored via a left carotid artery catheter and the mean arterial pressure maintained between 100 and 130 mm Hg by additional anesthetic as required. Systolic blood pressure did not fall below 100 mm Hg throughout the experiment. In the event that the blood pressure dropped and consistently remained below 100 mm Hg, the experiment was stopped and the animal killed. Core temperature was maintained close to 37 °C using a homeothermic blanket system. The animal preparation was allowed to stabilize for at least 30 min prior to data collection.

Stimulation and recording protocol. Spinal flexor reflexes were evoked by s.c. electrical stimulation applied to the first toe of the hind paw. Needle location was based on the innervation pattern of the sural nerve (Wiesenfeld-Hallin, 1988) and square-wave pulses (0.2, 0.6, 1.0 Hz) of 1 ms duration were used. Stimulation at each frequency was repeated three times to ensure stability and extracellular electromyographic responses were recorded from the biceps femoris muscle using a pair of tungsten needle electrodes. Intervals of 3–5 min were introduced between successive stimulus trains to prevent a conditioning effect by the preceding stimulus. A low intensity supra-threshold stimulus refers to a voltage twice the AFRR activation threshold (5 pulses at 0.2 Hz). A high intensity supra-threshold stimulus refers to a voltage twice the CFRR activation threshold (20 pulses at 1.0 Hz).

Selection criteria for A- and C-fiber components. The AFRR and CFRR were distinguished on the basis of activation threshold and response latency. Electrical stimulation sufficient to activate the CFRR resulted in two distinct components; an early and late phase separated by a quiescent period of variable duration. The AFRR and CFRR were classified as those appearing $<$100 ms after the stimulus artifact, and $>$100 ms up to a maximum of 600 ms, respectively. These criteria were confirmed by examining the effect of i.t. morphine (100 μg) and naloxone (100 μg) on the AFRR and CFRR of the biceps femoris reflex in naive animals (O'Rielly and Loomis, 2007). Assuming a motor neuron conduction distance of 11 cm, a conduction velocity of 60m $\cdot$ s^{-1} (Chamberlain and Lewis, 1989), a synaptic delay of 0.5 ms at the neuromuscular junction, a minimum central synaptic delay of 0.5 ms, and an afferent conduction distance of 12 cm, the fastest afferent fibers mediating the late component of the reflex response had conduction velocities of approximately 1.2 m $\cdot$ s^{-1} (Lynn and Carpenter, 1982). The AFRR and CFRR intervals used in the present experiments are in agreement with previous reports on the rat biceps femoris reflex (Lynn and Carpenter, 1982; Herrero and Cervero, 1996a,b). No attempt was made to separate the