NOS Inhibitor Antagonism of PGE₂-Induced Mechanical Sensitization of Cutaneous C-Fiber Nociceptors in the Rat

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INTRODUCTION

Tissue injury and inflammation result in the release of prostaglandins and other inflammatory mediators, some of which cause pain and hyperalgesia. The peripheral administration of prostaglandin E₂ (PGE₂), the most thoroughly studied hyperalgesic inflammatory mediator, produces mechanical hyperalgesia in animals (Ferreira 1981; Ferreira et al. 1978; Taiwo and Levine 1988) and humans (Moncada et al. 1978), and sensitizes primary afferent nociceptors to mechanical stimuli. The reduction of mechanical threshold and increase in number of action potentials to sustained mechanical stimulation induced by intradermal application of PGE₂ was blocked by L-NMA, but not D-NMA. It is suggested that NO contributes to nociceptor sensitization induced by hyperalgesic prostaglandins.

MATERIALS AND METHODS

Animal preparation

Male Sprague-Dawley rats (280–380 g) from Bantin and Kingman (Fremont, CA) were anesthetized with pentobarbital sodium (initially 50 mg/kg ip, with additional doses given throughout the experiment to maintain areflexia) and their trachea were cannulated. Anesthetized rats were positioned supine, with the left hindlimb secured at the ankle and the skin on the medial aspect of the thigh incised and retracted to form a pool which was filled with warm mineral oil. The saphenous nerve was then exposed and dissected free for electrophysiological studies. At the end of experiments, rats were killed with an overdose of pentobarbital. Animal care and use conformed to NIH guidelines for care and use of laboratory animals.

Electrophysiological recording

Extracellular recordings were made from C-fiber afferents in the fascicle of the saphenous nerve in vivo. This nerve was dissected free from accompanying blood vessels at two sites 20–32 mm apart. At the distal site, bipolar silver-wire stimulating electrodes were placed under the nerve to allow electrical stimulation of the nerve, to determine conduction velocity. At the proximal site, a portion of the nerve was desheathed and fine filaments were dissected from the nerve with sharpened jeweler’s forceps, and placed on a silver-wire recording electrode. The conduction velocity of a fiber was determined by dividing the distance between the stimulating and recording electrodes by the latency from the stimulus to the first action potential. The nerve was crushed proximal to the recording site to block the conduction of action potentials to the spinal cord, which would otherwise evoke hindlimb reflexes. Fascicles were teased from the nerve, with the use of a voltage amplitude (window) discriminator, until a single C-fiber was activated at a given receptive field or when a single unit could be easily distinguished by the height of its action potential. There were between one and three C-fibers per fascicle. Fibers with conduction velocities <2 m/s were classified as C-fibers and used in experiments.
The action potential corresponding to the C-fiber whose receptive field was identified was determined by the latency delay technique, in which a mechanically induced orthodromic spike produced a delay in the electrically induced orthodromic spike (Handwerker et al. 1991; Iggo 1958).

**Mechanical stimuli**

The mechanically sensitive receptive field of each C-fiber was located and mapped using a 7.5 g von Frey hair (VFH). This intensity of VFH was previously demonstrated to be able to activate more than 95% of all mechanically sensitive C-fibers (Ahlgren et al. 1992). The location on the fiber’s receptive field that was most sensitive to mechanical stimulation was marked by a felt tip pen and was the target for all further mechanical stimulation. Mechanical threshold was determined by the use of stimuli of ascending and descending intensity with calibrated VFH (Ainsworth, London, UK) and defined as the lowest force that elicited two or more spikes within 1 s, in ≥6 of 10 trials. Sustained threshold stimulation was performed using a calibrated VFH. The VFH was placed by hand on the receptive field for 10 s. The C-fiber activity was recorded on video tape (Vetter; Rebersburg, PA). Triplicate trials of sustained stimulation were given at 1 min intervals. Acute and sustained (10 s) threshold intensity stimulation was performed before and 3 and 5 min after the intradermal injection of agents.

**Drug injection**

Injection of saline, PGE2 (100 ng), and L-NMA (1 μg) plus PGE2 (100 ng) or D-NMA (1 μg) plus PGE2 (100 ng) was performed 1 mm away from the center of the fiber’s mechanical receptive field. All injections were in a volume of 2.5 μL. PGE2 and L-NMA or D-NMA were separated in the injection syringe by a tiny air bubble to prevent mixing before injection, and injected in the order of L-NMA or D-NMA then PGE2. PGE2 (Sigma; St. Louis, MO) was dissolved in ethanol and diluted to a final concentration with saline; and the final ethanol concentration was <1%. L-NMA and D-NMA (both Sigma; St. Louis, MO) were dissolved in saline to a concentration of 1%, as their stock solutions.

**Analysis of data**

Data are expressed as means ± SE. Statistical analyses were done using analysis of variance (ANOVA), t-test, χ2 test and Wilcoxon Signed Rank test, as appropriate. Differences were considered significant at the P < 0.05 level.

**R E S U L T S**

**Effect of NOS inhibitor on PGE2-induced reduction of mechanical threshold**

Before injection of saline, the range and average of the baseline mechanical threshold was 0.22–12.6 g and 1.93 ± 0.33 g (n = 38), respectively; before injection of PGE2 the range and average of the baseline mechanical threshold of saline and PGE2 treated group was 0.22–12.6 g and 1.89 ± 0.33 g (n = 38), respectively. The range and average of the baseline mechanical threshold of L-NMA group was 1.0–4.75 g and 2.02 ± 0.46 g (n = 38). The range and average of the baseline mechanical threshold of D-NMA group was 1.66–4.75 and 2.82 ± 0.60 g (n = 8). There were no statistically significant differences between baseline mechanical thresholds for these groups of C-fibers (all P > 0.05). Intradermal injection of PGE2 (100 ng) alone decreased the mechanical threshold in 18 of 38 (47.4%) C-fibers whereas intradermal injection of saline decreased the mechanical threshold in only 5 of 38 (13.2%) C-fibers (P < 0.005, Fig. 1A). Following intradermal injection of L-NMA (1 μg) plus PGE2 (100 ng), the mechanical threshold decreased in only 1 of 8 (12.5%) C-fibers and was unchanged in 7 of 8 (87.5%) C-fibers (P > 0.05, Fig. 1A). However, following application of D-NMA (1 μg) plus PGE2 (100 ng), the mechanical threshold decreased in 4 of 8 (50%) C-fibers and was unchanged in 4 of 8 (50%) C-fibers (P < 0.05, Fig. 1A).

**Effect of NOS inhibitor on PGE2-induced change in response to sustained threshold mechanical stimulation**

The average number of action potentials elicited by sustained threshold mechanical stimulation before saline and PGE2 was 17.0 ± 3.5 and 15.1 ± 3.6 (both n = 15, imp/10 s), respectively. After intradermal injection of PGE2 (100 ng), the number of action potentials was increased in 12 of 15 (80.0%) C-fibers whereas the number of action potentials was increased in only 5 of 15 (33.3%) C-fibers after saline. The increase in

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FIG. 1. Effects of nitric oxide synthase (NOS) inhibitor Nω-methyl-L-arginine (L-NMA) and its inactive stereoisomer Nω-methyl-D-arginine (D-NMA) on prostaglandin E2 (PGE2)-induced sensitization of C-fiber nociceptors to mechanical stimuli. A: effect of L-NMA and D-NMA on PGE2-induced reduction of von Frey hair mechanical threshold (saline and PGE2, both n = 38; L-NMA + PGE2 and D-NMA + PGE2, both n = 8; *P < 0.05, **P < 0.005, χ2 test and Wilcoxon Signed Rank test). B: effect of L-NMA and D-NMA on PGE2-induced change in response to sustained threshold mechanical stimulation [saline and PGE2, both n = 15; L-NMA + PGE2 and D-NMA + PGE2, both n = 8; *P < 0.05, **P < 0.005, analysis of variance (ANOVA) t-test]. Data are expressed as means ± SE.
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number of action potentials after PGE$_2$ was statistically significant ($P < 0.005$, Fig. 1B). Before intradermal injection of L-NMA (1 µg) plus PGE$_2$ (100 ng) the average number of action potentials was $18.4 \pm 5.4$ (imp/10 s, $n = 8$) and it increased in only 2 of 8 (25%) C-fibers. However, before injection of d-NMA (1 µg) plus PGE$_2$ (100 ng) the average number of action potentials was $14.5 \pm 2.1$ (imp/10 s, $n = 8$) and it increased in 5 of 8 (62.5%) C-fibers. The increase in number of action potentials produced by PGE$_2$ in the presence of d-NMA but not L-NMA was statistically significant ($P < 0.05$ and $P > 0.05$, respectively; Fig. 1B).

**DISCUSSION**

Accumulating evidence suggests that peripheral administration of PGE$_2$ sensitizes nociceptors (Ahlgren et al. 1997; Handwerker 1975; Kumazawa et al. 1993; Martin et al. 1987; Mizumura et al. 1993; Schaible and Schmidt 1988) by direct action on the nociceptor (England et al. 1996; Gold et al. 1996), and that this effect is mediated by the cAMP second messenger system (Ferreira and Nakamura 1979; Taiwo and Levine 1991; Taiwo et al. 1989). However, recent studies have shown that intracutaneous application of another signaling molecule, NO, also evokes pain in humans (Holthusen and Arndt 1994, 1995). In behavioral studies local administration of NOS inhibitors blocked hyperosmolar solution-induced (Kindgen-Milles and Arndt 1996), bradykinin-induced pain (Carrado et al. 1992; Kindgen-Milles and Arndt 1996), formalin-induced mechanical hyperalgesia and acetic acid-induced abdominal writhing (Moore et al. 1993), neuropathy-induced thermal hyperalgesia (Thomas et al. 1996), PGE$_2$-induced mechanical hyperalgesia (Aley et al. 1998), and carrageenan-induced thermal hyperalgesia (Lawand et al. 1997). Our results demonstrate that the NOS inhibitor, L-NMA, blocks PGE$_2$-induced reduction of C-fiber mechanical threshold and increase in number of action potentials evoked by sustained threshold stimulation. This result is consistent with the recent finding that NOS inhibitors block the allodynia induced by intrathecal administration of PGE$_2$ (Minami et al. 1995), which suggests that the hyperalgesia induced by PGE$_2$ acting at both central and peripheral terminals of nociceptors is mediated by NO.

Some studies have shown a peripheral antinociceptive effect of NO (Duarte et al. 1992; Kamei et al. 1994; Kabawata et al. 1992; Lorenzetti and Ferreira 1996), which may be the result of high doses of L-arginine (10 µg and 2 mg, respectively) because in the study that evaluated low and high doses, injection of the high dose ($\geq 10$ µg L-arginine) attenuated nociception whereas injection of the low dose (0.1–1 µg) enhanced nociception (Kabawata et al. 1992). In addition, administration of a NOS inhibitor is fundamentally different from administration of a NO precursor; for example, L-arginine may be metabolized to kyotorphin, an antinociceptive endogenous neuropeptide (Ueda et al. 1987).

Although NO often produces several of its effects by activation of guanylyl cyclase (Brennan and Breidt 1997; Moncada and Higgs 1993), we recently have shown that the contribution of NO to PGE$_2$-induced hyperalgesia is not mediated by a guanylyl cyclase-dependent modulation of the cAMP second messenger system (Aley et al. 1998). Because PGE$_2$ is a direct-acting hyperalgesic agent and NO is present in small-diameter dorsal root ganglion neurons (Qian et al. 1996; Zhang et al. 1993) we suggest that the NOS mediating PGE$_2$-induced sensitization of C-fibers is in the peripheral terminal of these neurons. Nevertheless, we cannot exclude other potential sources of NO. For example, prostaglandin-induced aqueous flare (Hiraki et al. 1996) and hyperosmolar solution-and bradykinin-induced pain (Kindgen-Milles and Arndt 1996) may be mediated by NO released from vascular endothelium.

In conclusion, the data for the present study supports the suggestion that PGE$_2$-induced decrease in mechanical threshold and increase in response to sustained threshold mechanical stimulation in C-fiber nociceptors is dependent on the NO second messenger system.

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**REFERENCES**


