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Superoxide signaling in pain is independent from nitric oxide signaling

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Abstract

Two reactive oxygen species (ROS), nitric oxide (NO[•]) and superoxide ([•]O₂[−]), contribute to persistent pain. Using three different animal models where ROS mediate pain, this study examined whether NO[•] and [•]O₂[−] converge to peroxynitrite (ONOO[−]) or whether each has an independent signaling pathway to produce hyperalgesia. The hyperalgesia following spinal nerve ligation was attenuated by removing superoxide by TEMPOL or inhibiting NO[•] production by L-NAME, but not by removing peroxynitrite with FeTMPyP. Nitric oxide-induced hyperalgesia was not affected by removing superoxide but was reduced by a guanyl cyclase inhibitor. Superoxide-induced hyperalgesia was not affected by inhibiting NO[•] production but was suppressed by a PKC inhibitor. The data suggest that nitric oxide and superoxide operate independently to generate pain.

Keywords

hyperalgesia; pain signaling pathway; peroxynitrite; reactive oxygen species; ROS

Introduction

Two types of reactive oxygen species (ROS), nitric oxide (NO[•]) and superoxide ([•]O₂[−]), are critically involved in persistent pain [1]. It has been shown that nitric oxide is produced in the spinal dorsal horn neurons in response to extensive nociceptive inputs. And then it diffuses out and increases neurotransmitter release from primary afferent terminals, thereby contributing to central sensitization and persistent pain [2,3]. On the other hand, recent studies show that reducing spinal superoxide levels decreases central sensitization and hyperalgesia [4,5], thus indicating that superoxide ([•]O₂[−]) also plays a critical role for persistent pain. [•]O₂[−] is known to activate various kinases, including protein kinase C (PKC) [6], which is critical for sensitization of spinal neurons and persistent pain [4,5,7]. In addition, it also seems possible that [•]O₂[−] and NO[•] can be generated simultaneously and react with each other to produce highly toxic peroxynitrite. Therefore, peroxynitrite has been proposed as a converged downstream molecule of [•]O₂[−] and NO[•] in persistent pain conditions [8]. However, the relationships among [•]O₂[−], NO[•], and peroxynitrite in pain signaling pathways are not clear. The present study is thus conducted to determine whether [•]O₂[−] and NO[•] induce pain through independent pain pathways or use peroxynitrite as a common mechanism.

Methods

Animals

Adult wild-type male C57 BL/6J mice (8–10 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). All animal procedures were performed in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch. All behavioral experiments were conducted blindly with respect to the drug administration.

Preparation of animal models

Neuropathic pain model—Peripheral neuropathy was produced by a unilateral L5 spinal nerve ligation (SNL) [9]. Briefly, under isoflurane anesthesia, the left L5 spinal nerve was isolated and tightly ligated with 7-0 silk thread. Mechanical sensitivity was assessed before and seven days after ligation.

NO[•]-induced hyperalgesia—A nitric oxide releasing compound, NOC12, was intrathecally injected in order to produce NO[•]-induced hyperalgesia [10]. An optimal intrathecal (i.t.) dose of NOC12 (3 µg in 5 µl saline) for each mouse was determined by a preliminary study, based on a previous rat study [10]. Mechanical sensitivity was assessed before and at various times after NOC12 injection.

•O₂⁻-induced hyperalgesia—We recently introduced a •O₂⁻-induced hyperalgesia model [11] using a mitochondrial electron transport complex inhibitor. Briefly, stock solution (1 mM) of antimycin A (complex III inhibitor; mitochondrial superoxide generator) was prepared in absolute ethanol and diluted in saline just before each experiment. Antimycin A (0.137 µg in 5 µl of 5 % ethanol-saline) was injected intrathecally in normal mice. Mechanical sensitivity was assessed before and from 9 hr after antimycin A injection.

Behavioral testing

Mechanical sensitivity was assessed by measuring foot withdrawal frequencies in response to von Frey stimuli (von Frey number 3.61, 0.41 gram force, Stoelting Co., Wood Dale, IL) [11]. The animal was placed in a plastic chamber on top of a mesh screen platform. The von Frey filament was applied perpendicularly to the base of the fourth toe for neuropathic mice (1 area; ipsilateral paw to surgery) and the base of the fourth toe and midplantar region (four areas; both paws) for antimycin- or NOC12-induced hyperalgesia. Ten stimuli were applied to each area at a 10–20 s interval. An abrupt withdrawal with or without licking of the foot, during stimulation or immediately after stimulus removal, was considered a positive response. Foot withdrawal frequency was quantified as = (total number of positive foot withdrawals/total stimuli) x 100. The mechanical sensitivity was assessed before (0) and 20, 40, 60, 90 and 120 min after i.t. injection of test compounds.

Drugs and i.t. Administration

The tested drugs and their intrathecal doses were: a ROS scavenger, phenyl N-tert-butyl nitrone (PBN; 100 µg), a superoxide scavenger, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL; 100 µg), a NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 100 µg), a peroxynitrite decomposition catalyst, 5, 10, 15, 20-tetrakis (N-methyl-4-pyridyl) porphyrinato iron III (FeTMPyP; 1 µg), a PKC inhibitor, GF109203X (1.6 pg), and a guanyl cyclase inhibitor, methylene blue (MB; 5 µg; from Harleco, Gibbstown, NJ). All chemicals except MB were purchased from Sigma Chemical (St Louis, MO). All drugs were dissolved in 0.9 % saline (except GF109203X) and administered intrathecally in a volume of 5 µl. GF109203X was stored in 100 %

dimethyl sulfoxide (DMSO) at a concentration of 4.5×10^5 pg/ μ l and diluted with saline to the proper concentration immediately before use. The above intrathecal doses, except FeTMPyP, are based on previous reports [8,12]. For intrathecal FeTMPyP, the maximum tolerable dose 1 μ g, that showed no abnormal behaviors such as paralysis, itching and tail biting, was determined from our preliminary study and used for the present study. All mice were injected by a modified direct transcutaneous intrathecal method using a disposable 30-gauge needle [13]. Vehicle groups received the same volume of saline or vehicle (0.00007 % DMSO in saline; for the vehicle control group of GF109203X experiment).

Nitrotyrosine immunohistochemistry

Seven days after SNL surgery, the L4-L5 spinal cord segments were removed, post-fixed for 4 hr, cryoprotected in 30% sucrose, cryosectioned at 10 μ m in thickness and mounted on gelatin coated slides. The sections were incubated with anti-nitrotyrosine (a peroxynitrite marker; 1:100; Sigma) and anti-NeuN (a neuronal marker; 1:2000; Chemicon), followed by secondary antibodies conjugated with fluorescent dyes, Alexa Fluor 568 (red) and 488 (green), respectively. A positive control for nitrotyrosine immunostaining was prepared by adding 110 mM peroxynitrite (Upstate) to cord sections of normal mice. Spinal dorsal horn was examined and photographed with a 40 $\times$ objective on a laser-scanning confocal microscope (Olympus).

Data analysis

Data are presented as mean $\pm$ SEM (standard error of the mean) and analyzed by one- or two-way repeated-measurement analysis of variance (ANOVA), followed by post hoc testing using the Holm-Sidak method. P values ≤ 0.05 were considered statistically significant.

Results

Both superoxide and nitric oxide are involved in generation of neuropathic pain

In the neuropathic mice, intrathecal injection of either a superoxide scavenger, TEMPOL, or a non-selective NO $\cdot$ synthase inhibitor, L-NAME, greatly reduced mechanical hyperalgesia. This significant antihyperalgesic effect peaked at 20 min and lasted up to 40 min after injection, compared to the vehicle group. A peroxynitrite decomposition catalyst, FeTMPyP, however, did not reduce mechanical hyperalgesia (Fig. 1A & 1B). The nitrotyrosine immunostaining, an indicator of the presence of peroxynitrite, was not detected in the spinal cord of neuropathic mice (Fig. 1D). The results indicate that NO $\cdot$ and $\cdot$ O $_2^-$, but not peroxynitrite, are critical contributors for neuropathic pain.

Spinal NO $\cdot$ induced foot hyperalgesia is independent from $\cdot$ O $_2^-$ action

Since intrathecal FeTMPyP failed to reverse mechanical hyperalgesia in neuropathic mice, we speculated that $\cdot$ O $_2^-$ and NO $\cdot$ might act independently for pain generation without converging to peroxynitrite. First, we tested whether NO $\cdot$ could generate pain without any interaction with endogenous $\cdot$ O $_2^-$ (Fig. 2A). Intrathecal injection of NOC12, a NO $\cdot$ -releasing compound, induced hyperalgesia on the hind paw in normal mice. The NO $\cdot$ -induced hyperalgesia was inhibited by i.t. co-administration of a general ROS scavenger, phenyl N-tert-butyl nitrone (PBN), or a guanyl cyclase inhibitor, methylene blue (MB), but not by a superoxide scavenger, TEMPOL (Fig. 2B). The data suggest that spinal NO $\cdot$ may induce hyperalgesia via the NO $\cdot$ -cGMP pathway without involvement of superoxide.

Spinal $\text{O}_2^{\bullet -}$ induced foot hyperalgesia is independent from NO $^{\bullet}$ action

In the next experiment, we tested whether endogenous NO $^{\bullet}$ was involved in the spinal $\text{O}_2^{\bullet -}$ induced foot pain model (Fig. 2C). Intrathecal injection of antimycin A, an $\text{O}_2^{\bullet -}$ generator, produced hyperalgesia on the hind foot. This $\text{O}_2^{\bullet -}$ -induced hyperalgesia was inhibited by a superoxide scavenger, TEMPOL, and by a protein kinase C (PKC) inhibitor, GF 109203X, but not by a NO $^{\bullet}$ synthase inhibitor, L-NAME (Fig. 2D). The data indicate that spinal $\text{O}_2^{\bullet -}$ can contribute to pain via the $\text{O}_2^{\bullet -}$ -PKC pathway, without any interaction with endogenous nitric oxide.

Discussion

Highly toxic peroxynitrite, the reaction product of NO $^{\bullet}$ and $\text{O}_2^{\bullet -}$, has been proposed as the culprit for persistent pain [8,14]. This is based on indirect evidence that: 1) theoretically, NO $^{\bullet}$ and $\text{O}_2^{\bullet -}$ rapidly combine to yield peroxynitrite (ONOO $^-$), six times faster than the removal of $\text{O}_2^{\bullet -}$ by superoxide dismutase (SOD) [15]; and 2) inhibition of NO $^{\bullet}$ or $\text{O}_2^{\bullet -}$ with L-NAME or TEMPOL, respectively, produces analgesia in rodent neuropathic pain models [8,14]. Consistent with the previous reports [1,8], TEMPOL and L-NAME produced potent analgesic effects in neuropathic mice in this study, thus confirming that both superoxide and nitric oxide critically contribute to neuropathic pain. The lack of antihyperalgesic effect of FeTMPyP and nitrotyrosine immunostaining in the neuropathic mice, however, suggests that NO $^{\bullet}$ and $\text{O}_2^{\bullet -}$ may operate via two independent pathways, without converging to peroxynitrite formation. This point is further confirmed by showing that NO $^{\bullet}$ - and $\text{O}_2^{\bullet -}$ -induced hyperalgesia is mediated through cGMP and PKC signaling pathways, respectively, without the presence of the other ROS in the system. In addition to having independent signaling pathways, NO $^{\bullet}$ and $\text{O}_2^{\bullet -}$ seem to function at different sites. NO $^{\bullet}$ is well known as a retrograde messenger because it is produced in the postsynaptic neuron but diffuse back to presynaptic terminals and potentiate glutamate release through modulation of cGMP [16,17]. In contrast, $\text{O}_2^{\bullet -}$ seems to be produced in and sensitizes the spinal dorsal horn neurons, thus mainly functioning via postsynaptic mechanisms. The fact that TEMPOL suppresses only the central sensitization component without affecting the peripheral component in capsaicin-induced hyperalgesia and the fact that most superoxide-producing cells are spinal dorsal horn neurons [4,5,7,13] support postsynaptic action of $\text{O}_2^{\bullet -}$. Thus it may be reasonable to see that superoxide and nitric oxide function independently at post- and pre- synaptic sites, respectively, while both are contributing to the same persistent pain.

Peroxynitrite decomposition catalysts rapidly convert peroxynitrite to relatively harmless nitrate thus reducing the formation of cytotoxic hydroxyl radicals and protein nitration [15]. A peroxynitrite decomposition catalyst, FeTMPyP exhibits good specificity for peroxynitrite and does not affect NO $^{\bullet}$ and $\text{O}_2^{\bullet -}$ action. Moreover, it catalyzes peroxynitrite most rapidly among the iron-based catalysts [18] and almost exclusively to harmless nitrate [19]. In spite of rapid, specific OONO scavenging properties of FeTMPyP, a single injection of FeTMPyP did not affect mechanical hyperalgesia in neuropathic mice. In our preliminary study (n=5), a high dose of FeTMPyP (10 μg in 5 μl , i.t.) produced paralysis (n=2) and moderate doses (2 or 5 μg in 5 μl , i.t.) did not affect neuropathic pain while producing a transient paralysis in some mice (n=3). Contrary to our results, FeTMPyP has been shown to inhibit hyperalgesia or partially restore defective autonomic function in diabetic mice [20,21]. Since the FeTMPyP effect in the diabetic neuropathy has been observed after repeated, long-term systemic treatments (over 2 weeks) with a high dose, it is likely that peroxynitrite involvement in persistent pain is indirect because it induces slow cumulative cell damage [15].

In conclusion, our study suggests that NO $^{\bullet}$ and $\text{O}_2^{\bullet -}$ contribute to persistent pain via two separate and independent pathways.

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Fig. 1.

Effect of pharmacological interventions and nitrotyrosine immunostaining in neuropathic mice. (A) Schematic representation of the experimental design. (B) Effect of a superoxide scavenger, TEMPOL, a NOS inhibitor, L-NAME and a peroxynitrite decomposition catalyst, FeTMPy, on mechanical hyperalgesia in neuropathic mice. All drugs were administered intrathecally 7 days after spinal nerve ligation (SNL). D: day. * $P < 0.05$ compared with vehicle (saline). (C, D, E) Spinal dorsal horn with double immunostaining for nitrotyrosine (red) and NeuN (green) in normal (C) and neuropathic (D) mice and peroxynitrite-positive control (E). Nitrotyrosine immunostaining (red; a peroxynitrite marker) was not detected in neurons (green) of the normal and neuropathic dorsal horn. Bar = 50 μm .

Fig. 2.

Effect of pharmacological interventions on NO•- or •O₂⁻-induced hyperalgesia in mice. (A) Schematic representation of the experimental design for NO•-induced hyperalgesia. (B) Effect of a ROS scavenger, PBN, a guanyl cyclase (GC) inhibitor, MB, and a superoxide scavenger, TEMPOL, on NO•-induced hyperalgesia by i.t. NOC12, a NO• donor. Each drug (5 µl) was co-administered intrathecally with NOC12. cGMP: cyclic guanosine monophosphate. (C) Schematic representation of the experimental design for •O₂⁻-induced hyperalgesia. (D) Effect of a NOS inhibitor, L-NAME, a superoxide scavenger, TEMPOL, and a protein kinase C (PKC) inhibitor, GF109203X on •O₂⁻-induced hyperalgesia by i.t. antimycin A. Each drug (5 µl) was administered intrathecally 9 hours after antimycin injection. H: hour. *P<0.05 compared to vehicle [either saline (B) or 0.00007 % DMSO saline (D)].