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Increased production of mitochondrial superoxide in the spinal cord induces pain behaviors in mice: The effect of mitochondrial electron transport complex inhibitors

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Abstract

Scavengers of reactive oxygen species (ROS) have been shown to produce a strong antinociceptive effect on persistent pain, and mitochondria are suggested to be the main source of ROS in the spinal dorsal horn. To explore whether excessive generation of mitochondrial superoxide alone can induce pain, the effect of mitochondrial electron transport complex inhibitors on the development of mechanical hyperalgesia was examined in mice. Intrathecal injection of an electron transport complex inhibitor, antimycin A or rotenone, in normal mice resulted in a slowly developing but long-lasting and dose-dependent mechanical hyperalgesia. The levels of mechanical hyperalgesia after antimycin A, a complex III inhibitor, were higher than that with rotenone, a complex I inhibitor. A large increase of mitochondrial superoxide in the spinal dorsal horn and a strong antinociceptive effect of ROS scavengers, phenyl-*N-tert*-butylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were observed in antimycin A-treated mice. The study indicates that the enhanced production of spinal mitochondrial superoxide alone without nerve injury can produce mechanical hyperalgesia.

Keywords

Mitochondrial ETC; Antimycin A; Rotenone; Mechanical hyperalgesia; ROS

An emerging concept in pain research is that reactive oxygen species (ROS) play a critical role in persistent pain. Supporting evidence is that pain behaviors and hyper-responsiveness of spinal dorsal horn neurons are dramatically reduced by ROS scavengers [10,12,21] and elevated ROS levels are observed in the injured peripheral nerve and/or spinal cord in animal models of persistent pain [8,10,14,16,21].

The major types and sources of ROS that are critical in pain generation, however, are unknown. Elevated levels of mitochondrial superoxide are observed in the spinal dorsal horn neurons in both spinal nerve-ligated neuropathic rats [14] and mice with intradermal capsaicin-induced pain [16]. In addition, a superoxide dismutase (SOD) mimetic, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), is highly effective in reducing pain in several animal models of persistent pain [9,10,12,14,16]. These results suggest that superoxide generated from mitochondria in spinal dorsal horn neurons might be the main ROS source in persistent pain and their removal by an SOD mimetic relieves pain. Despite a possible connection between mitochondrial superoxide production and pain, there has been no direct evidence that the

increased mitochondrial superoxide production leads to the development of spinal ROS-mediated pain.

There are multiple sources of ROS in nervous tissue: superoxide generation from mitochondrial oxidative phosphorylation; activation of phospholipase A2 leading to an arachidonate cascade; activation of proteases leading to conversion of xanthine dehydrogenase to xanthine oxidase; and nitric oxide (NO) production by activation of NO synthase [2,5,7]. Among those, mitochondria have been recognized as the major source of intracellular ROS in neurons during excitotoxicity [7] and long-term potentiation (LTP) in hippocampal CA1 neurons [1,11]. In addition, the primary ROS type produced by mitochondria is superoxide released mainly from the mitochondrial electron transport complexes (ETCs) I and III [2,4,18].

To explore whether elevation of spinal superoxide alone will induce pain, the effect of ETC inhibitors on mechanical hyperalgesia was tested in mice. Either a complex III inhibitor, antimycin A, or a complex I inhibitor, rotenone, was injected intrathecally in normal mice and mechanical hypersensitivity of the hind paws was measured. To confirm superoxide involvement in hyperalgesia, the effect of two superoxide scavenging compounds on pain behaviors was examined and the levels of mitochondrial superoxide was examined in the spinal dorsal horn by using a mitochondrial superoxide sensitive fluorescent dye, MitoSox Red.

Adult wild-type male C57 BL/6J mice (8–10 weeks old) were purchased from Jackson Labs. (Bar Harbor, ME) and used for this study. All procedures with animals were performed in accordance to an animal protocol approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch. The experiments were conducted blindly, since the examiners of pain behaviors and tissue sections were blinded to the drug treatment.

For intrathecal injection of mitochondrial ETC inhibitors, a complex III inhibitor, antimycin A, and a complex I inhibitor, rotenone, were purchased from Sigma Co. (St. Louis, MO). The stock solutions of antimycin A and rotenone were prepared at 1 and 5 mM concentrations in absolute ethanol, respectively and stored at -70°C . Injection solutions were prepared fresh just before each experiment by diluting the stock solution with olive oil to appropriate concentrations. To examine the dose response of ETC inhibitors, four different doses of antimycin A (0, 10, 20 and 50 μM , 5 μl /ea, i.t.) and rotenone (0, 10, 100, and 500 μM , 5 μl /ea, i.t.) were tested. Since the amount of ethanol increased as the concentration of the complex inhibitors increased, control groups (0 μM of drug) received the vehicle (5 μl) containing the maximum ethanol concentration in olive oil, 5% and 10%, for antimycin A and rotenone, respectively. The drug was injected intrathecally in normal mice and foot withdrawal frequencies to von Frey stimuli (vF) were measured. Foot withdrawal frequencies were assessed before and at various times after intrathecal injection of antimycin A or rotenone. All intrathecal injections were performed at the L5–L6 intervertebral space via a modified direct lumbar puncture under isoflurane anesthesia as described previously [12].

Mechanical sensitivity of the hind paw was assessed by measuring foot withdrawal frequencies in response to vF stimuli (vF# 3.61, 0.52 g bending force, Stoelting Co., Wood Dale, IL). Behavioral testing was done following the procedures described previously [16]. In brief, each animal was placed in a plastic chamber on top of a mesh screen platform and a von Frey filament was applied perpendicular to the stimulation site for 2–3 s. An abrupt withdrawal of the foot with or without licking of the foot during stimulation or immediately after stimulus removal was considered a positive response. Based on our preliminary study ($n = 6$), the base of fourth toe and midplantar region of the paw consistently became more sensitive after intrathecal injection of antimycin A (50 μM). The above two sensitive areas on each hind paw were chosen as stimulation sites and both hind paws were tested. Thus, mechanical sensitivity was measured

from four areas in each animal. Ten stimuli were applied to each area with a vF filament at a 10–20 s interval. The data obtained from all four areas from each animal were summed to yield a total number of foot withdrawals. The percent of foot withdrawal frequency (FWF) was calculated by the formula: $FWF = (\text{total number of foot withdrawals}/40 \text{ stimuli}) \times 100$.

To test if hyperalgesia was due to superoxide elevation in the spinal cord, the effect of superoxide scavengers on the ETC-induced hyperalgesia was examined. Two different superoxide scavengers were tested: phenyl-*N-tert*-butylnitrone (PBN) and TEMPOL from Sigma Co. (St. Louis, MO). PBN or TEMPOL was dissolved in saline and injected intraperitoneally (i.p.) at a volume of 100 μ l/20 g body weight. Control mice received the same volume of saline. Mice received either PBN (100 mg/kg; $n = 7$), TEMPOL (300 mg/kg; $n = 6$) or saline ($n = 7$) intraperitoneally 9 h after i.t. antimycin (50 μ M). Behavioral tests were performed up to 4 h after a scavenger injection.

To visualize mitochondrial superoxide in the spinal dorsal horn, 10 μ l of MitoSox Red (33 μ M in a 1:1 mixture of dimethylsulfoxide and saline; Molecular Probes, Eugene, OR) was injected intrathecally in normal mice ($n = 12$). Fifteen hours after MitoSox Red injection, mice received either antimycin A (50 μ M, 5 μ l, i.t., $n = 6$) or vehicle (5% ethanol in olive oil, 5 μ l, i.t., $n = 6$). Nine hours after antimycin or vehicle injection, all mice were perfused through the left ventricle with saline followed by a fixative containing 4% paraformaldehyde. The L4–L5 spinal cord segments were removed, post-fixed overnight, and then equilibrated in 30% sucrose. The spinal cords were cross-sectioned by a cryostat at 30 μ m thickness and the sections were mounted on gelatin-coated slides and air dried. The sections were examined under an epifluorescent microscope (Olympus BX50) with a rhodamine filter. Images of the spinal dorsal horn, including the superficial (laminae I–II) and deep (laminae III–V) layers, were taken from 10 randomly selected sections from each animal with a Q-Imaging Retiga 2000R digital camera. The exposure time, brightness and contrast were held constant for all images. All images were taken with a 63 $\times$ oil immersion objective lens and stored as image files. From each sampled image, the numbers of MitoSox Red positive profiles were quantified by densitometric analysis [13,20]. In brief, images were converted into gray-scale picture with 8-bit codification (1280 $\times$ 1024 pixels), giving a signal intensity from 0 (darkest pixel) to 255 (brightest pixel). The number of pixels showing MitoSox Red-positive profiles was quantified by using the Image-Pro Plus (Media Cybernetics, Silver Spring, MD) image analysis system. The cut-off value of pixel intensity for the negative profiles was determined from 20 random samples. Then, the number of pixels with intensity greater than the cut-off value (112) was counted as MitoSox Red stained profiles after adjusting the background to 46 (dark gray) in all images. Data were expressed as the number of MitoSox Red-positive pixels over a field area of 1280 $\times$ 1024 pixels. Representative fluorescent images of MitoSox Red labeled samples were acquired by laser-scanning confocal microscopy (Olympus).

Data are presented as mean $\pm$ S.E.M. (standard error of the mean). Behavioral data were analyzed by one- or two-way repeated-measurement analysis of variance (ANOVA), followed by post hoc testing using the Holm–Sidak method, and quantitative data of MitoSox Red imaging between two groups were tested by using unpaired Student's *t*-tests. *P*-values ≤ 0.05 were considered statistically significant.

The time courses of mechanical hyperalgesia following mitochondrial ETC inhibitor treatment in normal mice are shown in Fig. 1A and B. All mice developed mechanical hyperalgesia, as shown by an increase in FWF, following intrathecal administration of a complex III inhibitor, antimycin A. The development of hyperalgesia was slow and gradual, and the first significant change was detected at least 3–5 h after antimycin treatment. Once developed, the hyperalgesia lasted for more than 24 h and then returned to the pretreatment control level by 48 h. The peak responses were observed approximately 12 h after antimycin A treatment (Fig. 1A) and reached

54.0±10.4% foot withdrawal responses with the highest dose of 50 µM. For the dose response study, three different doses of antimycin A (10, 20 and 50 µM) produced graded levels of hyperalgesia while vehicle treatment did not result in hyperalgesia.

The levels of mechanical hyperalgesia that developed after intrathecal injection of the complex I inhibitor, rotenone, were significantly lower even with the highest dose of 500 µM. Furthermore, hyperalgesia developed more slowly and persisted longer than with antimycin A. Thus the first significant hyperalgesia was detected 5–8 h after rotenone treatment and persisted up to 48 h. The peak response values were also low, reaching up to a 29.2±2.9% increase compared to the pretreatment values 8–12 h after treatment. The results showed that inhibition of spinal mitochondrial ETC led to a slow and long-lasting mechanical hyperalgesia in normal mice, and the complex III inhibitor, antimycin A, produced a severer hyperalgesia than did the complex I inhibitor, rotenone. The vehicle-treated group did not show any significant change in foot withdrawal frequencies compared to the pretreatment value.

To examine whether the antimycin A-induced hyperalgesia is due to superoxide elevation, we tested the effect of two superoxide scavenging compounds: PBN and TEMPOL. When the hyperalgesia was fully established 9 h after 50 µM antimycin A treatment (i.t., 5 µl), either PBN (100 mg/kg) or TEMPOL (300 mg/kg) was injected systemically (i.p.) and mechanical hypersensitivity was measured 0.5, 1, 2, and 4 h after the scavenger treatment. A broad-spectrum ROS scavenger, PBN, greatly reduced the hyperalgesia to 17.5±6.1% and 17.9±2.5% in foot withdrawal frequencies 0.5 and 1 h after PBN treatment, respectively, compared to 49.6±8.5% and 58.9±5.8% with saline treatment. The SOD mimetic, TEMPOL, also had potent antinociceptive effect 0.5 h after TEMPOL, but its effect was shorter lasting than that of PBN (Fig. 1C).

To verify the increased levels of superoxide during the hyperalgesia after i.t. antimycin A, we visualized spinal superoxide by using a mitochondrial superoxide specific fluorescent dye, MitoSox Red. The amount of oxidized MitoSox Red, which showed a red fluorescence, was quantified in the spinal dorsal horn of antimycin A-treated mice and compared to that of the vehicle-treated group. The spinal dorsal horn of antimycin A-treated mice showed a greater number of red fluorescent granules with higher red density (Fig. 1E) compared to the vehicle-treated mice (Fig. 1D). When quantified, the total MitoSox Red stained area (averaged total number of pixels) of the antimycin A group showed 3.8- and 4.1-fold increases in the superficial (laminae I–II) and deep dorsal horn (III–V), respectively, compared to that of the vehicle group (Fig. 1F). There was no significant difference in the MitoSox Red stained area between the superficial and the deep layers of the dorsal horn. Thus, the results indicate that there is a significant increase of superoxide levels in the mitochondria of the spinal dorsal horn after antimycin A treatment.

This study demonstrated that intrathecal administration of a mitochondrial ETC inhibitor produces mechanical hyperalgesia in normal mice. Thus, the data suggest that elevated spinal ROS levels by increased production of mitochondrial superoxide leads to central sensitization and consequently pain without peripheral nerve injury or tissue inflammation.

The involvement of reactive oxygen species (ROS) in pain has been shown in a number of studies mainly by demonstrating the antinociceptive effect of antioxidants [10,12,21] and/or increased levels of ROS or their by-products in specific tissues in animal models of persistent pain [6,14,16]. While it has become clear that ROS are important in pain, many questions still remain unanswered: e.g., what specific types of ROS are critical; what are the sources of ROS; where are the main action sites; by what mechanism ROS does induce pain. The types and sources of ROS in the central nervous system are multiple: superoxide generation from mitochondrial oxidative phosphorylation; activation of phospholipase A2 leading to an

arachidonate cascade; activation of proteases leading to conversion of xanthine dehydrogenase to xanthine oxidase; and NO production by activation of NO synthase [2,5,7]. The present study, however, provides evidence that an increase of mitochondrial superoxide generation in the spinal cord alone is sufficient to produce pain in the foot, suggesting that central sensitization might have been established in the spinal cord by elevated levels of superoxide. Furthermore, the data further suggest that mitochondria may be a critical source of ROS in persistent pain conditions. However, considering that superoxide easily transmutates to other types of ROS in *in vivo* system [2], the possibility of involvement of other types ROS besides superoxide in central sensitization cannot be ruled out.

Then the question is how the pain induced by mitochondrial ETC inhibitor relates to the persistent pain resulted from nerve injury or tissue inflammation. It is well known that persistent pain is triggered by excessive and/or repetitive nociceptive input that leads to the establishment of central sensitization. The excessive nociceptive input is also shown to drive the increased production of mitochondrial superoxide by activating glutamate receptors, particularly NMDA receptors [17,23]. Thus, we propose that an intense nociceptive input drives an increased production of mitochondrial superoxide either by enhanced mitochondrial oxidative phosphorylation or decreased efficacy of mitochondria due to excessive Ca^{2+} accumulation [2,17]. This leads to elevated spinal ROS levels, which in turn sensitizes spinal dorsal horn neurons and thus results in pain. A strong antinociceptive effect and a significant reduction of spinal dorsal horn neuron responses by antioxidants [12], and increased mitochondrial superoxide levels in the dorsal horn neurons [14] in animal models of persistent pain further support the hypothesis that the elevated spinal ROS is a critical factor for inducing central sensitization and thus pain. The fact that pain can be produced by simply generating more superoxide from mitochondria suggests that ROS may be a critical intermediate between intense nociceptive input and central sensitization.

Mitochondrial superoxide generation by ETC inhibitors has been extensively studied in cells of *in vitro* culture system. Although it has been shown that 1–10 μM of antimycin A or rotenone seems to be sufficient to produce superoxides in cultured cells [4,19], the amounts of ETC inhibitors required for superoxide generation in an *in vivo* system without tissue damage have not been identified. In our preliminary study, we tested the effect of intrathecal injection of 5 μl of antimycin A at concentrations of 10–100 μM or of rotenone at 10–500 μM . Antimycin A 100 μM produced severely impaired gait and paralysis of hind legs in all treated mice. With 50 μM antimycin A, 3 out of 42 mice showed mild impaired gait for 2 h and then recovered, thus data from these three mice were not included in the presented data. Thus 0, 10, 20 and 50 μM of antimycin A were used for this study. After rotenone, even when using with 500 μM , the maximum attainable solubility in 5 μl of vehicle (10% ethanol in olive oil), no abnormal behaviors were displayed. Thus 10, 100, and 500 μM of rotenone were tested in the experiment. Because all mice treated with ETC inhibitors recover to normal sensitivity levels by 48–60 h after treatment, we speculate that the temporary hyperalgesic effect of ETC inhibitors is due to elevated levels of ROS but not from permanent damage to cells. This point was also confirmed by systemic administration of the superoxide scavengers, PBN and TEMPOL, which produce transient antinociception in antimycin A-induced hyperalgesic mice. Thus this is the first time that an artificial elevation of spinal ROS by increased endogenous mitochondrial superoxide production has been demonstrated to induce hyperalgesia in mice. The study further indicates that complex III may contribute significantly more to superoxide generation than complex I, because levels of hyperalgesia are significantly higher with antimycin A compared to rotenone.

In the ROS scavenger effect study, a systemic dose of 100 mg/kg PBN or 300 mg/kg TEMPOL was used in this study. This is based on previous studies [10,12,16] and also on our preliminary study. A systemic 100 mg/kg PBN was very effective to relieve mechanical allodynia, a sign

of central sensitization, in neuropathic rats [10], suppress the dorsal horn neuron excitability in capsaicin-treated rats [12], and reduces the secondary hyperalgesia in capsaicin-treated mice [16]. Thus, evidence indicates that systemic PBN is highly effective in removing ROS from the spinal cord. To further confirm the spinal action of a ROS scavenger in antimycin-induced hyperalgesia, we performed an extra experiment by injecting PBN (200 µg in 5 µl) intrathecally in antimycin A-treated mice. Behavioral test showed that i.t. PBN greatly reduced FWF from $65.0 \pm 2.9\%$ to $22.5 \pm 12.5\%$ ($n = 6$) 1 h after PBN treatment. Thus, intrathecal PBN effect is similar to that of systemic PBN. In addition to the report that systemically injected PBN can be detected in the brain tissue as early as 15 min after injection [3], these data support that systemic ROS scavengers act through the spinal cord effectively. Furthermore, neither 100 mg/kg PBN nor 300 mg/kg TEMPOL influence mechanical sensitivity of foot in normal rodent, while the same dose significantly reduces the capsaicin-induced secondary hyperalgesia [16]. Our preliminary study also confirmed that 100 mg/kg PBN or 300 mg/kg TEMPOL did not change normal nociception in mice. Thus, we used a systemic injection method, rather than i.t. administration, of ROS scavengers in order to avoid complications of multiple i.t. injections.

While it is clear that elevated spinal ROS is correlated with induction of central sensitization, it is not known how elevated ROS induces central sensitization. Many studies indicate that ROS are involved in inducing cell proliferation and differentiation signaling pathways, besides their well-known role in cell death [15,22]. We speculate that elevation of ROS in the spinal cord modifies redox sensitive signaling enzymes and gates cellular mechanisms toward central sensitization. This proposed hypothesis needs to be examined and verified in future studies.

In conclusion, this study provides evidence that an artificial increase of superoxide production from mitochondria of lumbar spinal cord neurons by mitochondrial electron transport complex inhibitors leads to pain in the foot in normal mice. Thus, the data indicate that elevation of spinal ROS can be a sufficient factor for induction of central sensitization and that mitochondria may be the major ROS source.

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Fig. 1.

Increased mechanical hyperalgesia and mitochondrial superoxide following intrathecal mitochondrial complex inhibitor. Behavioral study (upper panels): development of mechanical hyperalgesia following intrathecal mitochondrial complex inhibitors in normal mice and antinociceptive effect of superoxide scavengers. (A) Intrathecal complex III inhibitor antimycin A-induced hyperalgesia. (B) Intrathecal complex I inhibitor rotenone-induced hyperalgesia. (C) Antinociceptive effects of superoxide scavenging compounds, PBN (100 mg/kg, i.p.) and TEMPOL (300 mg/kg, i.p.), on intrathecal complex III inhibitor antimycin A (50 μ M)-induced hyperalgesia. Histochemical assay of mitochondrial superoxide (lower panels): increase of mitochondrial superoxide in spinal dorsal horn following intrathecal complex III inhibitor antimycin A. (D and E) Representative confocal images of mitochondrial MitoSox Red fluorescence in spinal dorsal horn (deep layer) 9 h after intrathecal antimycin A (50 μ M) (E) or vehicle (D). (F) Quantitative data showing significant increases in MitoSox Red-stained area in antimycin A-treated group ($n = 6$) compared to vehicle group (5% ethanol in olive oil; $n = 6$). Bar represents 20 μ m. * $P < 0.05$ compared with vehicle or saline group. The data are expressed as mean $\pm$ S.E.M.