Published in final edited form as:
Neurosci Lett. 2010 October 11; 483(2): 85–89. doi:10.1016/j.neulet.2010.07.013.

Spinal NADPH Oxidase is a Source of Superoxide in the Development of Morphine-Induced Hyperalgesia and Antinociceptive Tolerance

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Abstract

The role of superoxide and its active byproduct peroxynitrite as mediators of nociceptive signaling is emerging. We have recently reported that nitration and inactivation of spinal mitochondrial superoxide dismutase (MnSOD) provides a critical source of these reactive oxygen and nitrogen species during central sensitization associated with the development of morphine-induced hyperalgesia and antinociceptive tolerance. In this study, we demonstrate that activation of spinal NADPH oxidase is another critical source for superoxide generation. Indeed, the development of morphine-induced hyperalgesia and antinociceptive tolerance was associated with increased activation of NADPH oxidase and superoxide release. Co-administration of morphine with systemic delivery of two structurally unrelated NADPH oxidase inhibitors namely apocynin or diphenyleneiodonium (DPI), blocked NADPH oxidase activation and the development of hyperalgesia and antinociceptive tolerance at doses devoid of behavioral side effects. These results suggest that activation of spinal NADPH oxidase contributes to the development of morphine-induced hyperalgesia and antinociceptive tolerance. The role of spinal NADPH oxidase was confirmed by showing that intrathecal delivery of apocynin blocked these events. Our results are the first to implicate the contribution of NADPH oxidase as an enzymatic source of superoxide and thus peroxynitrite in the development of central sensitization associated with morphine-induced hyperalgesia and antinociceptive tolerance. These results continue to support the critical role of these reactive oxygen and nitrogen species in pain while advancing our knowledge of their biomolecular sources.

Keywords

superoxide; peroxynitrite; morphine antinociceptive tolerance; NADPH oxidase

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Superoxide ($O_2\cdot^-$) and its downstream signaling mediator, peroxynitrite ($ONOO^-$, PN), have emerged as powerful pronociceptive reactive oxygen and nitrogen species [37–38]. Indeed, their involvement in central sensitization has been reported during the development of thermal hyperalgesia associated with acute and chronic inflammation [7–8,19,30,48,52–53], in response to spinal activation of the N-methyl-D-aspartate receptor (NMDAR) [27], in the development of orofacial pain [53], and in the development of opiate-induced hyperalgesia and antinociceptive tolerance [4,25,31]. Furthermore, and as an extension to the above findings, a role for nitroxidative stress (herein defined as stress induced in the presence of $O_2^{\bullet-}$, PN and related species) was supported using a variety of non-selective agents [5,26,37] such as phenyl N-tert-butylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL). Indeed, PBN or TEMPOL showed efficacy in inflammatory [19] and neurogenic pain [13,21,39–40], visceral pain [51], neuropathic pain [13,33,42,47], and chemotherapy-induced pain [20]. These findings led us to put forth the hypothesis that targeting $O_2\cdot^-$ and PN should lead to development of novel analgesics for the management of pain [37–38]. Clearly unraveling the enzymatic sources in the production of $O_2\cdot^-$ and PN and understanding the signaling pathways engaged by these species in nociceptive processing is of paramount importance [37–38]. Inactivation of manganese superoxide dismutase (MnSOD), the enzyme that normally keeps [$O_2\cdot^-$] under tight control, [24] is a central source for $O_2\cdot^-$-derived PN in several diseases driven by overt production of PN [22]. Such enzymatic inactivation results from nitration of Tyr-34 by PN in a manganese-catalyzed process [23]. In a series of studies, our group revealed that spinal nitration and inactivation of MnSOD provides a critical “feed-forward” mechanism that allows for the accumulation of $O_2\cdot^-$ and PN during the development and maintenance of central sensitization [4,25,27,31,52], findings confirmed and subsequently extended by others [39–40]. Another important $O_2\cdot^-$-generating enzyme system is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [29] recently implicated in the development of central sensitization associated with inflammatory hyperalgesia [17]. This $O_2\cdot^-$-generating enzyme, is dormant in resting cells and produces $O_2\cdot^-$ only upon activation. The principal regulation of NADPH oxidase is post-translational and depends on the assembly of several membrane-bound and cytosolic components to form an active enzyme complex [3]. In resting cells, the enzyme consists of two membrane-bound components, gp91*phox* and p22*phox*, and several cytosolic components, including p47*phox*, p40*phox*, p67*phox*, and rac1/2 [3]. Gp91*phox* is a flavocytochrome and the catalytic core of the enzyme. Upon activation, the cytosolic components translocate to the membrane and associate with membrane components to form an assembled, activated, and $O_2\cdot^-$-producing enzyme complex [3]. Although this enzyme is best characterized in immune cells and leukocytes for its involvement in $O_2\cdot^-$production, it is now known that various protein components of NADPH oxidase are expressed in neurons, astrocytes, and microglia [1,14,49]. Importantly, $O_2\cdot^-$ auto-augments its formation by up-regulating the expression of the Rac1 and gp91*phox* subunits of the holoenzyme creating a self-perpetuating cascade [28,35]. *Therefore, we hypothesize that post-translational nitration and inactivation of MnSOD and activation of NADPH oxidase represent two pathways that operate in synchrony to maintain central sensitization.* To this end and in order to extend our previous studies and address our hypothesis, we investigated in this study whether the NADPH oxidase contributes to central sensitization associated with the development of morphine-induced hyperalgesia and antinociceptive tolerance by providing an additional source of $O_2\cdot^-$.

All experiments were performed in accordance with the International Association for the Study of Pain and the National Institutes of Health guidelines on laboratory animals welfare and the recommendations by Saint Louis University Institutional Animal Care and Use Committee. For all experiments animals were placed in a plastic restrainer for antinociception test and these were habituated to handling and testing equipment at least 20–

30 minutes before experiments. All experiments were conducted with the experimenters blinded to treatment conditions. Male CD-1 mice (24–30g; Charles River Laboratory) were housed 4–5 per cage, maintained under identical conditions of temperature (21 ± 1°C) and humidity (65% ± 5%) with a 12-hour light/12-hour dark cycle, and allowed food *ad libitum*. The tail flick test, which measures withdrawal latencies of the tail from a noxious radiant heat source, was used to measure thermal nociceptive sensitivity [11]. The intensity of the heat stimulus was adjusted so that the mouse flicked its tail after 2–4 s. A cut-off time of 15 s was imposed to prevent tissue damage. Mice received a subcutaneous (*sc*, 0.2 ml) injection of morphine (20 mg/kg) or its vehicle sterile saline twice daily at 0800–0900 h and 1600–1700 h for 4 days. Apocynin (4-hydroxy-3-methoxy-acetophenone; 25, 50 and 100 mg/kg/day), DPI (diphenyleneiodonium, 0.25, 0.5 and 1 mg/kg/day) or their vehicle were given by intraperitoneal (*i.p*) injections (0.2 ml) twice a day and 15 minutes before each morphine injection over the 4 days for the drugs to be distributed more constantly. On day 5, and approximately 16 h after the last morphine injection, each mouse was tested twice on the tail flick and the latency (s) reaction times averaged to obtain a baseline. Mice were then challenged with acute subcutaneous (*s.c*) injection of morphine (10 mg/kg) to study the expression of tolerance. Data obtained were then converted to percentage maximal possible antinociceptive effect (%MPE) as equal to: (response latency−baseline latency)/(cut off latency−baseline latency) × 100. At least six mice per group were used. After the behavioral tests, animals were sacrificed with CO_2 and rapidly decapitated to harvest spinal cord tissues from the lumbar enlargement segment of the spinal cord (L4–L6).

When compared to mice that received a subcutaneous injection of saline (Veh-Sal, n=6) over 5 days, injection of morphine over the same time frame (Veh-Mor, n=6) led to the development of antinociceptive tolerance (Fig. 1). This was indicated by a significant ($P<0.001$) reduction in tail flick latency over time (0–120 min) after an acute dose of morphine challenge (10 mg/kg) in rats receiving repeated morphine administration over 5 days compared to mice receiving repeated saline injection over the same interval (Fig. 1). The role of the NADPH oxidase was assessed by the use of apocynin or DPI, well-characterized inhibitors of the NADPH-oxidase [9,41,43–44]. Apocynin prevents serine phosphorylation of p47*phox* and blocks its association with gp91phox, thus blunting NADPH oxidase activation [41,44], whereas DPI forms adducts with FAD interrupting oxygen reduction through gp91*phox* [32]. These inhibitors exert beneficial effects in several animal models of nitroxidative stress including rheumatoid arthritis, diabetes, atherosclerosis, neurodegeneration, stroke and ischemia-reperfusion injuries [2,6,9–10,16,18,34,36,43,46]. We now show, for the first time, that activation of spinal NADPH oxidase plays a critical role in the development of morphine-induced antinociceptive tolerance. Indeed and as can be seen in Fig. 2, the development of morphine-induced antinociceptive tolerance was associated with increased activation of NADPH-oxidase ($P<0.001$) and superoxide formation in spinal cord tissues as measured by increased spectrophotometric absorbtion at 550nm by reduced cytochrome c using a commercially available kit (CY0100; Sigma, St. Louis, MO). Co-administration of morphine with daily (5 days) injections of apocynin (100 mg/kg/day, n=4) or DPI (1 mg/kg/day, n=4) blocked spinal NADPH oxidase activation ($P<0.01$) (Fig. 2) and blocked in a dose-dependent manner (25–100 mg/kg/day, n=6 for apocynin and 0.25–1 mg/kg/day, n=6 for DPI) the development of antinociceptive tolerance (Fig. 1) at doses devoid of motor function impairment (as tested on the Rotarod; n=4, not shown). Baseline values for tail flick latency from all groups on day 5 before injection of acute morphine, were statistically insignificant from each other and ranged between 2–3 sec. Also, inhibiting $O_2\cdot^-$ production with an acute injection of apocynin did not reverse established tolerance (n=4, not shown); thus confirming results obtained with other $O_2\cdot^-$-targeted approaches, which established that $O_2\cdot^-$ and PN contribute to events in the development, but not expression, of tolerance [4,25].

These results indicate that spinal activation of NADPH oxidase is a critical source of $O_2\cdot$ in the development of morphine-induced antinociceptive tolerance.

The role for spinal NADPH oxidase was confirmed in subsequent studies using intrathecal (*i.th*) delivery of apocynin. To this end, rats, under light isoflurane anesthesia, were subcutaneously implanted (in the interscapular region) with osmotic pumps (Alzet 2001; Alza, Mountain View CA), to deliver saline at 0.5 μl/h or morphine at 7.5 μg μl^{-1} h^{-1} for 7 days over 7 days as previously described [50]. Drugs or isovolumetric vehicle (10 μl followed by a 10 μl flush with sterile physiological saline) were given once a day for 6 days by *i.t.h* injections in rats with chronically implanted *i.t.h* catheter as described previously [45]. The method of Hargreaves and colleagues was used to assess changes in baseline nociceptive responses to a thermal nociceptive stimulus [15] with baseline latencies of 18–20 sec and a maximal cut-off time of 20 sec to prevent tissue damage. The tail flick test was used to measure thermal nociceptive sensitivity with baseline latencies of 4–5 sec and a cutoff time of 10 sec [11]. When compared to rats that received a chronic subcutaneous (*s.c*) infusion of saline (Veh-Sal, n=6) over 7 days, morphine infusions over the same time frame (Veh-Mor, n=6) led to **1)** the development of thermal hyperalgesia [15] as evidenced by a significant ($P<0.001$) reduction in paw-withdrawal latency on day 6 compared to paw-withdrawal latency from before implantation of the osmotic minipump (baseline) (Fig. 3A) and **2)** the development of antinociceptive tolerance (Fig. 3B). The latter was indicated by a significant ($P<0.001$) reduction in tail flick latency 30 min after challenge with an acute dose of morphine (6 mg/kg) given intraperitoneally (*i.p*) on day 6 in rats receiving chronic morphine infusion over 7 days compared to rats receiving an infusion of saline over the same interval (Fig. 3A). Co-administration of morphine with daily (6 days) *i.th* injections of apocynin (Apo-Mor; 0.1–0.6 nmol/day, n=6) blocked the development of hyperalgesia and antinociceptive tolerance (Fig. 3A, B) thus establishing the critical role of superoxide derived from spinal activation of the NADPH oxidase. When given alone daily and over 6 days to rats that received saline infusion (Veh-Sal), apocynin (0.6 nmol/day) had no effect (n=3, not shown).

In summary, results derived from our studies have defined for the first time the importance of spinal NADPH oxidase as a source of $O_2\cdot^-$ and thus of its downstream signaling mediator PN in the development of antinociceptive tolerance. Since both neurons and glial cells possess functional NADPH oxidase [1,14,49], we expect both cell populations to contribute to the production of superoxide derived from the activation of this enzyme; we are currently testing this in our laboratories. Whereas in this paper we focused on the role of spinal NADPH oxidase, we are not excluding the likely possibility that this $O_2\cdot^-$-generating enzyme may, in addition, contribute to the development of morphine-induced hyperalgesia and antinociceptive tolerance at supraspinal sites. In support, we have recently reported that $O_2\cdot^-$ and PN contribute to the development of morphine-induced hyperalgesia and antinociceptive tolerance by acting both at spinal [4,25,31] and supraspinal sites [12].

Collectively these results continue to support our general hypothesis that targeting $O_2\cdot^-$ and PN is an evidence-based approach to develop novel therapeutics for managing pain of several etiologies [37–38].

Research Highlights

- Spinal NADPH-oxidase superoxide increases with morphine antinociceptive tolerance.
- Inhibition of NADPH-oxidase activity blocks morphine antinociceptive tolerance.

- Targeting spinal NADPH-oxidase may be a therapeutic pain management strategy.

Acknowledgments

Supported by R01 DA024074 and 1RC1AR058231 (DS). The authors declare no conflicts of interest. We would like to thank Dr Bill Neumann (Southern Illinois University Edwardsville) for helpful discussions and editing of this manuscript.

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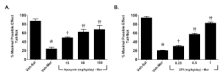

Fig. 1. The development of morphine antinociceptive tolerance is attenuated by inhibitors of the NADPH oxidase

On day 5, acute injection of morphine (10 mg/kg) in animals that received saline over 4 days (vehicle group, Veh-Sal) produced a significant antinociceptive response. On the other hand, a significant loss to the antinociceptive effect of the acute injection of morphine was observed in animals that received morphine over the same time-period indicative of antinociceptive tolerance (Veh-Mor). Co-administration of morphine over 4 days with **(A)** apocynin (25–100 mg/kg/day) or **(B)** DPI (0.25–1 mg/kg/day) inhibited the development of antinociceptive tolerance in a dose-dependent manner. Results are expressed as percent maximal possible antinociceptive effect (%MPE) with mean ± SEM for 6 animals. Data were analyzed by ANOVA with Dunnett's *post hoc* test where *P<0.001 for morphine alone vs. vehicle and † P<0.05 or †† P<0.001 for morphine plus apocynin or DPI vs. morphine alone.

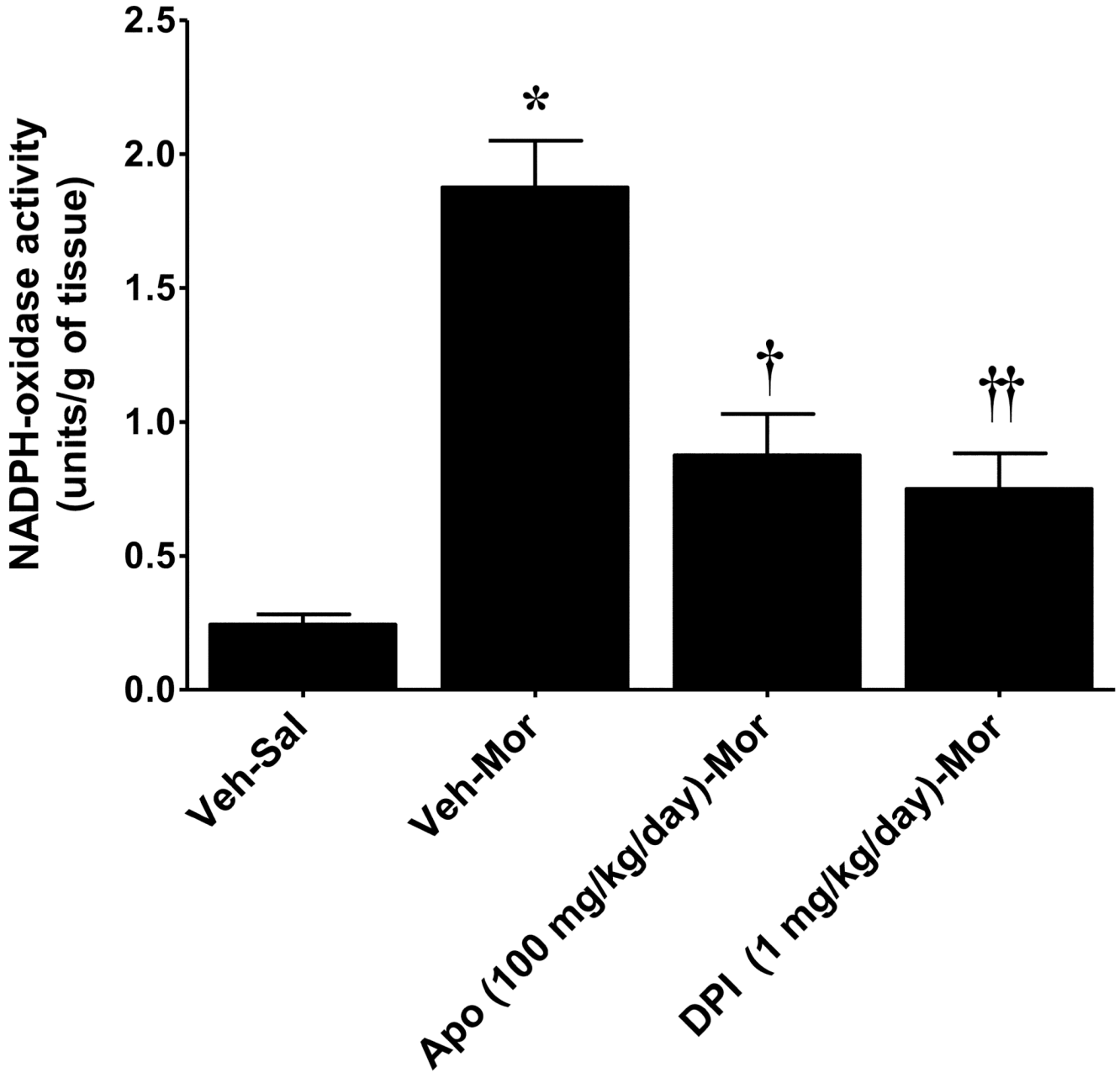

Fig. 2. Chronic morphine administration activate NADPH-oxidase superoxide production

When compared to animals receiving vehicle over 4 days, repeated administration of morphine over the same time frame led to **(A)** activation of the NADPH oxidase in spinal cord tissues from the lumbar enlargement (L4–L6). Co-administration of morphine over 4 days with apocynin (100 mg/kg/day) or DPI (1 mg/kg/day) attenuated the activation of NADPH oxidase. Results are expressed as NADPH-oxidase activity (units/g of tissue) with mean ± SEM for 4 animals. Data were analyzed by ANOVA with Dunnett's *post hoc* test where *P<0.001 for morphine alone vs. vehicle and † P<0.01 for morphine plus apocynin or DPI vs. morphine alone.

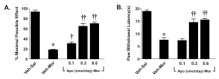

Fig. 3. Intrathecal delivery of apocynin attenuates the development of morphine-induced hyperalgesia and antinociceptive tolerance

When compared to rats that received a chronic *s.c* infusion of saline (Veh-Sal, n=6) over 7 days, infusion of morphine over the same time frame (Veh-Mor, n=6) led to the development of **(A)** thermal hyperalgesia as evidenced by a significant reduction in paw-withdrawal latency(s) on day 6 when compared to paw-withdrawal latency from before implantation of the osmotic minipump (baseline) and **(B)** antinociceptive tolerance. The development of morphine induced thermal hyperalgesia **(A)** and antinociceptive tolerance **(B)** were attenuated in a dose-dependent fashion by intrathecal delivery of apocynin (0.1–0.6 nmol/day). Results are expressed as mean ± SEM for n = 6 animals. Data were analyzed by ANOVA with Dunnett's *post hoc* test where *P<0.001 for morphine alone vs. vehicle and † P<0.05 or †† P<0.001 for morphine plus apocynin vs. morphine alone.