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NADPH-oxidase 2 activation promotes opioid-induced antinociceptive tolerance in mice

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Abstract

The analgesic effectiveness of long-term opioid therapies is compromised by the development of antinociceptive tolerance linked to the overt production of peroxynitrite (ONOO⁻, PN), the product of the interaction between superoxide (O₂⁻, SO) and nitric oxide (NO), and to neuroinflammatory processes. We have recently reported that in addition to post-translational nitration and inactivation of mitochondrial MnSOD, activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase holoenzyme (NOX) in the spinal cord is a major source for the overt production of superoxide-derived PN during the development of morphine-induced antinociceptive tolerance. However, the NOX complex involved in these processes is not known. The objective of these studies is to identify a potential role for the NOX2 complex, an enzyme involved in inflammation. Mice lacking the catalytic subunit of NOX2 (*Nox2*^{-/-}) or its regulatory subunit, *p47^{phox}* (*p47^{phox}*^{-/-}), developed antinociceptive tolerance similar to wildtype (*wt*) mice after three days of continuous morphine. However, while *wt* mice continue to develop tolerance by day six, morphine analgesia was restored in both *Nox2*^{-/-} and *p47^{phox}*^{-/-} mice. Moreover, the loss of *Nox2* or *p47* did not affect acute morphine analgesia in naïve mice. In *wt* mice, antinociceptive tolerance was associated with increased activation of NOX, nitration of MnSOD, and proinflammatory cytokines production in spinal cord. These events were markedly attenuated in *Nox2*^{-/-} and *p47^{phox}*^{-/-} mice and instead, there was enhanced formation of antiinflammatory cytokine (IL4 and IL10) production. These results suggest that NOX2 activity provides a significant source of superoxide-derived PN to undertake post-translational modifications of mitochondrial MnSOD and to engage neuroinflammatory signaling in the spinal cord associated with opioid-induced antinociceptive tolerance. Thus, NOX2 may provide a potential target for adjuvant therapy to protect opioid analgesia.

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Keywords

NADPH-oxidase; antinociceptive tolerance; neuroinflammation; superoxide; morphine

Background

Opioid/narcotic analgesics, typified by morphine, are the most effective treatments for acute and chronic severe pain. However their clinical utility is often hampered by the development of analgesic tolerance, which requires escalating doses to achieve equivalent pain relief (Foley, 1995). This complex pathophysiological cycle represents a critical barrier to the quality of life of these patients due to the resulting oversedation, reduced physical activity, constipation, respiratory depression, high potential for addiction, and other side-effects (Foley, 1995). Adaptive modifications in cellular responsiveness have been proposed as contributing to tolerance (Taylor and Fleming, 2001). An alternative hypothesis with *in vivo* evidence in animals (Mao et al., 1995) and in humans (Arner et al., 1988) is that chronic opioid receptor stimulation triggers the activation of anti-opioid systems that reduce sensory thresholds, thereby resulting in hypersensitivity to tactile and noxious thermal stimulation (Simonnet and Rivat, 2003). As a corollary to this hypothesis, such opioid-induced hypersensitivity paradoxically diminishes the net analgesic effect of the opioid agonist (Ossipov et al., 2003, Simonnet and Rivat, 2003). Although a significant body of evidence has been gathered to suggest that tolerance and hypersensitivity share similar underlying cellular and molecular mechanisms, evidence also supports the existence of distinct molecular mechanisms (Ferrini et al., 2013). While progress has been made in modifying this drug class to improve their formulated delivery, pharmacokinetics, and potential for abuse; little progress has been made in preventing the development of antinociceptive tolerance and hyperalgesia.

Several neurobiological mechanisms for opioid-induced hyperalgesia and antinociceptive tolerance have been proposed. For example, increasing evidence implicates a role for neuroinflammatory processes (Johnston et al., 2004, Christie, 2008) and glial-derived enhancement of glutamatergic signaling (Trujillo and Akil, 1991, Mao et al., 2002). Proinflammatory cytokines (e.g., TNF α and IL1 β) enhance the neuronal sensitivity to both noxious and non-noxious stimuli by increasing glutamatergic signaling (Beattie et al., 2002, Gustafson-Vickers et al., 2008, Kawasaki et al., 2008, Gu et al., 2011), blocking inhibitory GABA signaling (Gustafson-Vickers et al., 2008, Kawasaki et al., 2008) or indirectly stimulating the release of pronociceptive mediators, such as prostaglandins (Narita et al., 2008). These neuroinflammatory mechanisms are further complemented by enhanced glutamatergic signaling due in part through reductions in glutamate transporter (i.e., GLT-1, GLAST, and EAAC1) activity and expression, which results in sustained elevated concentrations of glutamate within the synapse (Mao et al., 2002, Yang et al., 2008). We have reported that in the spinal cord, peroxynitrite (PN) formation, the diffusion-limited product of superoxide and nitric oxide (Beckman et al., 1990), is central to these processes (Dang and Christie, 2012). Systemic delivery of PN decomposition catalysts (PNDCs) in mice and rats not only blocks morphine-induced antinociceptive tolerance (Muscoli et al., 2007, Batinic-Haberle et al., 2009), but does so by attenuating the activation of redox-

sensitive transcription factors (NF κ B) and MAPK kinases (ERK, p38 kinase) and the increased formation of various proinflammatory cytokines (Muscoli et al., 2007). PNDCs also block post-translational nitration and inactivation of glutamate transporters, GLAST and GLT-1, and glutamine synthetase (Salvemini and Neumann, 2009). Moreover, PNDCs block the post-translational nitration and inactivation of mitochondrial manganese superoxide dismutase (MnSOD) found in the spinal cord during the development of morphine tolerance (Muscoli et al., 2007). MnSOD is a key mitochondrial protein regulating superoxide levels leaking from oxidative phosphorylation pathways and its inactivation provides a “feed-forward” mechanism resulting in the accumulation of superoxide and PN.

In addition to mitochondria and inactivated MnSOD, NOX is a significant source of superoxide in the development of central sensitization associated with inflammatory hyperalgesia (Ibi et al., 2008) and recently has been shown to contribute to morphine tolerance (Doyle et al., 2010). The NOX family consists of five membrane-bound flavoenzymes (Nox1–5) that produce superoxide and two enzymes (Duox1 and 2) that mainly produce superoxide-derived hydrogen peroxide (see for review (Bedard and Krause, 2007)). The activities of the Nox1–3 isoforms are inducible upon phosphorylation and membrane translocation of cytosolic regulatory complexes (Bedard and Krause, 2007). For example, Nox2 is typically quiescent in resting cells, but when activated, the cytosolic complex of p47 $phox$, p40 $phox$, p67 $phox$, and rac1/2 translocates to the membrane Nox2/p22 to form the active NOX2 holoenzyme (Bedard and Krause, 2007). The activity of Nox4 is constitutive (Bedard and Krause, 2007) and Nox5 appears to be Ca²⁺-regulated (Bánfi et al., 2001) without the need for cytosolic regulatory complexes (Bedard and Krause, 2007). Both antinociceptive tolerance and hyperalgesia are attenuated by intrathecal administrations of inhibitors that prevent the association of the regulatory complex with the Nox1, 2, or 3 catalytic core, such as apocynin, or prevent the electron transfer to FAD, such as diphenyleneiodonium (DPI) (Doyle et al., 2010). However, these inhibitors are not isoform-specific (O'Donnell et al., 1993, Heumüller et al., 2008) and therefore, little is known about which Nox isoforms are involved. Moreover, each inhibitor has off-target properties that include innate antioxidant (apocynin) (Heumüller et al., 2008) and NOS-inhibiting (DPI) (Stuehr et al., 1991) activities that could contribute to their effects in tolerance. Despite these limitations, there seems to be a role of NOX in antinociceptive tolerance. In mice lacking Nox1, acute morphine analgesia was potentiated and the development of tolerance from chronic morphine was suppressed (Ibi et al., 2008). However, the loss of Nox1 does not provide the full attenuation afforded by apocynin, DPI, or PNDCs. Therefore, contributions of other Nox isoforms are possible. We hypothesize that the NOX2 holoenzyme may be a major contributor of superoxide. NOX2 has long been associated with inflammatory processes (see for review (Sorce and Krause, 2009, Sareila et al., 2011)) and is found in both neurons and microglia (Guemez-Gamboa et al., 2011). Moreover, NOX2 is upregulated in microglia in spinal nerve transection models and its deletion attenuates the development of neuropathic pain and associated neuroimmune activation and oxidative stress in the spinal cord (Kim et al., 2010). Using mice deficient in the Nox2 or its regulatory protein, p47 $phox$ (see for review (Groemping and Rittinger, 2005)), we investigated the possibility that NOX2 holoenzyme contributes to the production of proinflammatory cytokines and peroxynitrite in the spinal cord that underlie opioid-induced tolerance.

Methods

Materials

Wildtype (C57BL/6J) and genetic knockout (*Nox2*^{-/-} or *p47^{phox}*^{-/-}) mice were obtained from Charles River (Milan Italy) or Jackson Laboratories (Bar Harbor, ME, USA). CD1 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The minipumps for morphine administration were from Alza (Mountain View, CA, USA). eTM-4-PyP⁵⁺ was purchased from Caymen Chemical Company (Ann Arbor, MI, USA). Mouse anti-3NT antibodies conjugated to agarose and rabbit anti-MnSOD antibodies were from Millipore (Billerica, MA, USA). Peroxidase-conjugated bovine anti-mouse IgG was obtained from Jackson ImmunoResearch (West Grove, PA, USA). Cytokine ELISA kits were purchased from R & D Systems (Minneapolis, MN, USA). The bicinchoninic acid assay was from Pierce (Rockford, IL, USA). Western blot materials were from Bio-Rad (Hercules, CA, USA). Morphine, cytochrome c kit and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental animals

Male *Nox2*^{-/-} or *p47^{phox}*^{-/-} mice (24–30g), their strain-and age-matched wild-type (*wt*) cohorts (C57BL/6J), or CD-1 mice were housed 4–5 per cage, and maintained under identical conditions of temperature ($21 \pm 1^\circ\text{C}$) and humidity ($65\% \pm 5\%$) with a 12-hour light/12-hour dark cycle, and allowed food *ad libitum*. All experiments were conducted with the experimenters blinded to treatment conditions. The knockout animals are healthy and do not exhibit abnormal acute pain responses. All experiments were performed in accordance with the International Association for the Study of Pain and the National Institutes of Health guidelines on laboratory animal welfare, and regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986) and the USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA). All animal experiments and care were approved by the Saint Louis University Institutional Animal Care and Use Committee, the Institutional Animal Care and Use Committee (Council directive # 87-848, October 19, 1987, Minist re de l'Agriculture et de la For t, Service V t rinaire de la Sant et de la Protection Animale, permission # 92-256 to SC) and the University of Messina Review Board.

Osmotic pump implantation

Mice, were lightly anesthetized with isoflurane and were subcutaneously (s.c.) implanted (in the interscapular region) with primed osmotic minipumps (Alzet 1007D), to deliver saline at 0.5 $\mu\text{l/h}$ or morphine at 60 $\mu\text{g } \mu\text{l}^{-1} \text{ h}^{-1}$ over 6 days as described (King et al., 2007, Vera-Portocarrero et al., 2007). Minipumps were filled according to manufacturer's specifications. The use of the osmotic pump ensures a continuous subcutaneously delivery of morphine avoiding intermittent periods of withdrawal. Mice were tested for analgesia at 2h following minipump implant to verify that they are analgesic - approximately 100% analgesia was achieved. This helps verify that the pumps are working well, which is typically not a problem.

Behavioral tests

Acute thermal nociception was measured using the tail flick test that measures withdrawal latencies of the tail from a noxious radiant heat source with baseline latencies of 2–3 sec and a cut-off time of 10 sec to prevent tissue injury (D'Amour, 1941). Morphine-induced antinociceptive tolerance was indicated by a significant ($P < 0.05$) reduction in tail flick latency 30 min after a challenge with an acute dose of morphine sulfate (6 mg/kg, given *i.p.*). Data obtained were converted to percentage maximal possible antinociceptive effect (%MPE) as follows: (response latency–baseline latency)/(cut off latency–baseline latency) $\times$ 100. The ED₅₀ values for acute morphine were calculated from %MPE (0–100%) using a normalized non-linear regression analysis with a standard slope (Hill-slope = 1) in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA). On day 6 and after the behavioral tests, spinal cord tissues from the lumbar enlargement segment of the spinal cord (L5–L6) were removed and tissues processed for immunohistochemical, Western blot and biochemical analysis.

NADPH-oxidase activity

Spinal cord tissues (L4–L6) were homogenized in HEPES buffer (10 mM, pH 7.5, containing 250 mM sucrose, 1 mM EGTA, 25 mM potassium chloride, 10 μ g/ml soybean trypsin inhibitor, 2 μ g/ml aprotinin and 10 μ g/ml leupeptin) and centrifuged at $1000 \times g$. The NADPH cytochrome *c* reductase activity in these supernatants was measured using Cytochrome *c* Reductase (NADPH) Assay Kit following the manufacturer's instructions (Doyle et al., 2011).

MnSOD nitration assay

The formation of peroxynitrite and its nitrative capacity were assessed by the formation of 3-nitrotyrosine (NT) in MnSOD. Cytosolic fractions were obtained as described previously (Wang et al., 2004, Muscoli et al., 2010) and stored immediately at -80°C . The total NT protein fraction was immunoprecipitated using an agarose conjugated anti-3NT antibody and Western blot analyses of MnSOD protein levels in the immunoprecipitated fractions were performed as described (Wang et al., 2004, Muscoli et al., 2007). The levels of β -actin were measured to verify equal protein input for IP using a murine monoclonal anti- β -actin antibody (1:2000) and visualized with peroxidase-conjugated bovine anti-mouse IgG secondary antibody (1:5000) and enhanced chemiluminescence. The relative expression of each protein band was measured from scanned films using GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and Molecular Analyst (IBM) and normalized to β -actin bands.

Cytokine assays

Portions of spinal cord tissues were homogenized as previously described in PBS containing 2 mmol/L of phenyl-methyl sulfonyl fluoride (PMSF,) and tissue level of TNF α , IL1 β , IL4, and IL10 production in spinal cord (L4–L6) was assessed. The assay was carried out by using a colorimetric, commercial ELISA kits according to manufacturer's protocol. Cytokine levels were normalized to the total protein in the lysates as determined by bicinchoninic acid assay.

Statistical Analyses

Data are expressed as mean $\pm$ SD for an $n = 6$ mice per group. Time course behavioral data were analyzed by two-tailed, two-way repeated measures ANOVA with Bonferroni *post hoc* tests. Changes in NOX activity, protein nitration, and cytokine production between isogenic morphine- and vehicle-treated cohorts were analyzed by unpaired Student's *t*-test. Changes in these parameters amongst *wt*, *Nox2*^{-/-}, and *p47^{phox}*^{-/-} in response to morphine were analyzed by a two-tailed, one-way ANOVA with Dunnett's *post hoc* tests. Significance was accepted at $P < 0.05$.

Results

NOX2 is central to the development of morphine antinociceptive tolerance

Mice lacking the *Nox2* or *p47^{phox}* gene are viable, fertile, and have no outward adverse effects (Rivera et al., 2010). Defective NOX2 activity can decrease neuronal survival in the hippocampus (Dickinson et al., 2011), which may affect pain behavior. However, there were no observable differences in the baseline tail-flick latencies between the phenotype, indicating comparable nociceptive processing in all animals. When compared to its vehicle or baseline at day 0 (D0), the chronic administration of morphine (s.c., 60 μ g/ μ l/h, Fig. 1A,B) in *wt* mice produced a time-dependent decrease in the analgesic response to an acute morphine dose (6 mg/kg) on D3 and D6 indicating the development of antinociceptive tolerance. In both *Nox2*^{-/-} (Fig. 1A) and *p47^{phox}*^{-/-} (Fig. 1B) mice antinociceptive tolerance also developed by D3 with chronic administration of morphine, but this was absent by D6 (Fig. 1A,B).

The NOX2-independent early phase of morphine antinociceptive tolerance is attenuated by FeTM-4-PyP⁵⁺

We have reported that FeTM-4-PyP⁵⁺, a well-characterized dual superoxide dismutase and PNDC (Misko et al., 1998), attenuated antinociceptive tolerance when measured on D6 (Muscoli et al., 2007). However, tolerance developed in both *Nox2*^{-/-} (Fig. 1A) and *p47^{phox}*^{-/-} (Fig. 1B) mice by day 3; suggesting that early development of tolerance may not be dependent upon superoxide-derived PN. To test this, CD-1 mice were injected with the FeTM-4-PyP⁵⁺ (30 mg/kg/day, i.p.) for 5 days during continuous morphine administration. On days 0, 1, 3, and 6, mice received a s.c. dose of acute morphine (6 mg/kg) to assess antinociceptive responses via tail-flick assay. When compared to vehicle, tolerance was observed in CD-1 mice chronically treated with morphine from D3–D6 (Fig. 2). However, tolerance did not develop in mice co-treated with FeTM-4-PyP⁵⁺ (Fig. 2); implicating a role for NOX2-independent superoxide-derived PN formation during the early phases of developing morphine tolerance.

NOX2 is the major source of NOX activity in the late phase of antinociceptive tolerance development

When compared to vehicle (Veh), the total NOX activity in the spinal cord increased by D6 in *wt* mice chronically treated with morphine (Mor, Fig. 3). This morphine-induced NOX activity was completely lost in *Nox2*^{-/-} and *p47^{phox}*^{-/-} mice on D6 (Fig. 3).

The loss of NOX2 does not alter of the antinociceptive responses to acute morphine

When given an acute dose of morphine, naive *wt* mice exhibited a dose-dependent increase in thermal antinociception that peaked at 40 min post-morphine (Fig. 4; ED₅₀ at peak = 8.0 μmol/kg; 95%CI: 5.4–11.8 μmol/kg). Equivalent time- and dose-dependent responses were found in both the *Nox2*^{-/-} (Fig. 4; ED₅₀ at peak = 7.5 μmol/kg; 95%CI: 5.1–11.2 μmol/kg) and *p47^{phox}*^{-/-} (Fig. 4; ED₅₀ at peak = 8.6 μmol/kg; 95%CI: 5.9–12.7 μmol/kg) mice.

Morphine-induced NOX2 activity is a significant source of superoxide for the formation of peroxynitrite

Peroxyntirite is difficult to directly measure due to its highly reactive nature (Radi et al., 2002); however, nitrotyrosine (NT) formation from the reaction of PN with tyrosine residues on proteins serves as a good marker for PN formation (Radi et al., 2002). We have shown that the formation of NT occurs within the spinal cord during opioid-induced antinociceptive tolerance and hyperalgesia and is blocked by a peroxyntirite-decomposition catalyst; thus, confirming the presence of PN (Muscoli et al., 2007, Ndengele et al., 2009, Muscoli et al., 2010). To determine whether NOX2 is a source of superoxide in the formation peroxyntirite, we measured the level of tyrosine nitration in MnSOD in the spinal cord. Consistent with our previous studies (Muscoli et al., 2007, Ndengele et al., 2009, Muscoli et al., 2010), we found that nitration of MnSOD is increased with the chronic administration of morphine in *wt* mice compared to Veh *wt* mice (Fig. 5A,B). However, when compared to Mor *wt* mice, the level of nitrated MnSOD was largely attenuated in Mor *Nox2*^{-/-} and *p47^{phox}*^{-/-} mice despite a modest but significant increase in nitrated MnSOD over Veh KO mice (Fig. 5A,B).

NOX2 promotes proinflammatory cytokine production in the spinal cord

When compared to its vehicle, chronic administration of morphine in *wt* mice was associated with increased production of the proinflammatory cytokines, TNFα (Fig. 6A) and IL1β (Fig. 6B) in the spinal cord on D6. This increase was greatly attenuated in both *Nox2*^{-/-} and *p47^{phox}*^{-/-} mice. However, when compared to Veh, *p47^{phox}*^{-/-} mice, morphine (Mor) still induced a modest, but significant, increase in TNFα (Fig. 6A). In contrast to proinflammatory cytokines, the expression of antiinflammatory cytokines, IL4 (Fig. 6C) and IL10 (Fig. 6D) were modest, but significantly increased in Mor *wt* mice. In Mor *Nox2*^{-/-} and *p47^{phox}*^{-/-} mice both IL4 (Fig. 6C) and IL10 (Fig. 6D) expression were greatly enhanced. There were no significant differences in the levels of both proinflammatory and antiinflammatory cytokines between KO mice and *wt* when given vehicle (Veh).

Discussion

In this study, we demonstrate that NOX2 activity is a major source of superoxide for the production of peroxyntirite and promotes proinflammatory cytokine expression during the development of morphine-induced tolerance. By genetically knocking out NOX2 or its regulatory subunit, *p47^{phox}*, NADPH oxidase activity induced by chronic morphine was absent on D6 corresponding with the complete attenuation of morphine tolerance by D6, which are consistent with data obtained with pharmacological inhibitors of NOX activity (Doyle et al., 2009). However, even with the loss of NOX2 activity, morphine tolerance still developed from D0-D3 in similar fashion to that found in wildtype mice. Moreover, this

early NOX2-independent phase is also driven by superoxide-derived PN since a small, but significant, level of MnSOD nitration was observed in the absence of NOX2 (Fig. 4) on D6 and FeTM-4-PyP, when given daily systemically, attenuated morphine tolerance on D3. This suggests that other sources of superoxide are active and modulate early development of tolerance until there is a shift in the underlying mechanisms between D3 and D6 that center on the activation of NOX2 to maintain the drive towards tolerance.

The previous study linking NOX1 to the development of tolerance showed that NOX1 exerted continuous effects throughout the course of repeated morphine treatment beginning as early as 24 h after the first injection and was attributable to the downregulation of $G\alpha_{i2}$ in neurons, which in turn disrupted μ -opioid signal transduction (Ibi et al., 2008). The loss of NOX1 in genetic knockout not only partially attenuated tolerance, but also potentiated the analgesic effect of acute morphine in na ve mice. We did not observe this same increase in morphine analgesic effects in na ve NOX2 knockout mice, further suggesting that NOX2 activity and its effects result from a trigger later in the development of tolerance. However, morphine tolerance is associated with increased NMDAR-mediated glutamatergic signaling (Trujillo and Akil, 1991). NMDAR signaling can induce NOX2 activity in neurons to facilitate synaptic plasticity (Mao and Mayer, 2001). Thus, NOX2 may respond to and contribute to enhanced NMDAR signaling. Moreover, this enhanced NOX2 activity may in turn supplement NOX1 activity in disrupting μ -opioid signaling.

Another potential NOX2-dependent mechanism underlying tolerance is glial activation and the induction of proinflammatory processes. In neuropathic models of pain, the NOX2 activity in spinal cord microglia has been found to be responsible for increased superoxide production, proinflammatory cytokine production, and the development of allodynia and thermal hyperalgesia (Kim et al., 2010). In mice lacking NOX2-derived superoxide, we also find the attenuation of morphine antinociceptive tolerance corresponded with reduced levels of proinflammatory TNF α and IL1 β levels in the spinal cord, thus implicating NOX2 in the neuroinflammatory pathway. Growing evidence indicates that chronic morphine either through direct activation of Toll-like receptors (TLRs) (Hutchinson et al., 2009, Wang et al., 2012b) or indirectly through increased receptor-mediated signaling that include glutamatergic (Liu et al., 2011), purinergic (Horvath and DeLeo, 2009, Horvath et al., 2010), and/or ceramide/sphingosine 1-phosphate signaling (Ndengele et al., 2009, Muscoli et al., 2010) leads to glial cell hyperactivation and their increased production of proinflammatory cytokines. Blocking these pathways pharmacologically or using antiinflammatory strategies, such as anti-TNF α (i.e., Entanercept) (Hutchinson et al., 2008), direct recombinant IL10 (Lin et al., 2010) or indirect IL10 gene therapy (Johnston et al., 2004), protect morphine antinociceptive properties. Moreover, evidence suggests that subpicomolar morphine and micromolar naloxone, a μ -opioid antagonist, demonstrate antiinflammatory properties in TLR4-activated microglial cells independent of the opioid receptor through direct interactions with NOX2 lead to reductions in NOX2 activation (Qian et al., 2007, Wang et al., 2012a). This opioid-independent mechanism with morphine treatment is likely overshadowed by opioid-dependent events that lead to tolerance, however, it may contribute to the potentiation of morphine analgesia by naloxone (Qian et al., 2007, Wang et al., 2012a). Several of the reported triggers of glial activation in response to morphine are also known to induce NOX2 activity. For example, TLR signaling can activate NOX2 (Bae et al.,

2009) and activation of ionotropic and metabotropic glutamate receptors with exogenous glutamate increases Nox2 expression and superoxide production in microglia (Brennan et al., 2009, Girouard et al., 2009, Guemez-Gamboa et al., 2011). Likewise, the release of ATP in response to mitochondrial stress (Krysko et al., 2011) or during increased neurotransmitter release (Jo and Schlichter, 1999) can activate NOX2 through purinergic receptors, specifically P2X₇ (Moore and MacKenzie, 2009), which are found on microglia (Parvathenani et al., 2003). Thus, it is possible that between the NOX1-dependent loss of opioid receptor function, enhanced NMDAR activation, and the likely increased metabolic demand on mitochondria promotes the release of proinflammatory mediators that cause activation of NOX2-dependent inflammatory pathways in hyperactive glia.

More importantly, once established, NOX2 activity maintains the development of tolerance in part through maintaining a shift in inflammatory/antiinflammatory balance toward an inflammatory state by providing positive feedback loops to maintain NOX2 activity and enhanced glial and neuronal activity. Superoxide can up-regulate Rac1 and NOX2 subunits expression to auto-augment its own formation (Puntambekar et al., 2005, Muzaffar et al., 2006). The release of TNF α can serve as an additional trigger of NOX2 activity through TNF α receptor-mediated signaling (Anilkumar et al., 2008). The loss of MnSOD activity raises the probability of peroxynitrite-mediated nitration of proteins of the mitochondrial respiratory chain and maintenance of the mitochondrial membrane potential (Castro et al., 2011). Inactivation of these proteins would compromise mitochondrial integrity and in turn trigger the release of danger associated molecular patterns (DAMPs) that can activate HMGB1 (Krysko et al., 2011), NLRP3 inflammasomes (Zhou et al., 2011), or TLRs (Krysko et al., 2011) and drive inflammation. Moreover, increased production of superoxide, and thus peroxynitrite, can enhance the nitration and inactivation glutamate uptake proteins, which in turn, compromises the termination of glutamatergic signaling providing for positive feedback on NMDA receptor signaling (Trotti et al., 1996, Mao et al., 2002, Muscoli et al., 2007, Yang et al., 2008, Muscoli et al., 2010) and further NOX2 activation.

The evidence of NOX2 activity, together with NOX1, in the spinal cord during repeated morphine dosing supports a central role of NOX-mediated nitroxidative stress and neuroinflammation in the development of morphine antinociceptive tolerance. Little is known about the contributions by other isoforms, such as NOX4, which has been linked to neuropathic pain (Kallenborn-Gerhardt et al., 2012). More importantly, there is a growing body of evidence for a central role of NOX activity in pain of various etiologies (Kim et al., 2010, Doyle et al., 2012, Kallenborn-Gerhardt et al., 2012). These studies confirm and underscore the critical contributions of nitroxidative stress in pain (see (Salvemini et al., 2011) for review). As an extension, understanding the mechanisms by which the NOX isoforms and nitroxidative stress responses are activated and upon which mechanisms they act will not only assist in designing effective adjunct therapies to maintain morphine efficacy, but could have broader impact on treating pain in general.

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Highlights

- NOX2 holoenzyme is critical for the development of antinociceptive tolerance
- NOX2 is a major source of superoxide-derived PN during late stages of tolerance.
- NOX2 activity drives proinflammatory cytokine production.
- The loss of NOX2 activity attenuates tolerance and reduces nitroxidative stress.
- The loss of NOX2 activity favors anti-inflammatory cytokine production.

Figure 1. NADPH-oxidase is central to the development of morphine antinociceptive tolerance
When compared to vehicle (□) in *wt* mice, repeated i.p. injections of morphine (20 mg/kg; ■; A,B) induced a time-dependent decrease in %MPE of an acute dose of morphine (10 mg/kg, i.p.). In *Nox2*^{-/-} (▲; A) and *p47*^{phox}^{-/-} (▼; B) mice, morphine induced a similar time-dependent decrease in %MPE by D3, but this was resolved by D6. The loss of *Nox2* (△; A) or *p47*^{phox} (▽;B) had no effect on behavior in vehicle-treated animals when compared to *wt* mice. Data are mean ± SD for n=6 mice and analyzed by two-way repeated measures ANOVA with Bonferroni comparisons. **P*<0.01 and ***P*<0.001 morphine vs. *t*₀; †*P*<0.001 *Nox2*^{-/-} or *p47*^{phox}^{-/-} vs. *wt* mice.

Figure 2. The NOX2-independent early phase of morphine antinociceptive tolerance development is attenuated by the dual SODm/PNDC, FeTM-4-PyP⁵⁺

When compared to its vehicle (□), tolerance was observed in CD-1 mice chronically treated with morphine (■) from D3-D6. Conversely, tolerance did not develop in mice co-treated with FeTM-4-PyP⁵⁺ (30 mg/kg/day, ▲). Data are mean ± SD for n=6 mice and analyzed by two-way repeated measures ANOVA with Bonferroni comparisons. * $P < 0.001$ morphine vs. vehicle; † $P < 0.001$ Morphine + FeTM-4-PyP⁵⁺ vs. Morphine.

Figure 3. Morphine activates NOX2

When compared to vehicle (Veh), chronic morphine administration (Mor) induced NOX activity in spinal cords of *wt* mice. This morphine-induced NOX activity was lost in *Nox2*^{-/-} or *p47^{phox}*^{-/-} mice. The loss of Nox2 or *p47^{phox}* had no effect on NOX2 activity in vehicle-treated animals when compared to *wt* mice. Data are mean $\pm$ SD for *n*=6 mice and analyzed by unpaired Student's *t*-test (morphine vs. vehicle) or one-way ANOVA with Dunnett's comparisons (*Nox2*^{-/-} or *p47^{phox}*^{-/-} vs. *wt* mice). **P*<0.001 morphine vs. vehicle; †*P*<0.001 *Nox2*^{-/-} or *p47^{phox}*^{-/-} vs. *wt* mice.

Figure 4. The loss of NADPH-oxidase activity does not alter acute morphine antinociception
 At peak analgesic effect ($t=40$ min), *wt* (○), *Nox2*^{-/-} (□), and *p47^{phox}*^{-/-} (▽) mice exhibited similar dose-dependent responses to morphine (i.p.; 1–10 mg/kg). Data are mean ± SD for $n=4$ mice and analyzed using normalized non-linear regression with a standard slope (Hill-slope = 1).

Figure 5. Morphine-induced NOX2 activity is a significant source of superoxide for the formation of peroxynitrite

When compared to Veh, morphine (Mor) significantly increases nitration of MnSOD, indicated by NT formation, in *wt*, *Nox2^{-/-}*, and *p47^{phox}^{-/-}* mice. However, when compared to Mor *wt* mice, MnSOD nitration was greatly attenuated in morphine-treated *Nox2^{-/-}*, and *p47^{phox}^{-/-}* mice. The loss of Nox2 or p47^{phox} had no effect MnSOD nitration in Veh animals when compare to Mor *wt* mice. Data are mean ± SD for n=6 mice and analyzed by unpaired Student's t-test (morphine vs. vehicle) or one-way ANOVA with Dunnett's comparisons (*Nox2^{-/-}* or *p47^{phox}^{-/-}* vs. *wt* mice). **P*<0.05, ***P*<0.01, and ****P*<0.001 morphine vs. vehicle; †*P*<0.001 *Nox2^{-/-}* or *p47^{phox}^{-/-}* vs. *wt* mice.

Figure 6. Morphine-induced NOX2 activity promotes proinflammatory cytokine production in the spinal cord

When compared to Veh, repeated morphine injections (Mor) significantly increases proinflammatory TNF α (A) in *wt* and *p47^{phox-/-}* mice and IL1 β (B) in *wt* mice. However, when compared to Mor *wt* mice, both TNF α (A) and IL1 β (B) were greatly attenuated in Mor *Nox2^{-/-}*, and *p47^{phox-/-}* mice. In contradistinction to proinflammatory cytokines, morphine induced modest increases in antiinflammatory IL4 (C) and IL10 (D) in *wt* mice when compared to Veh. However, when compared isogenic Veh or Mor *wt* mice, morphine induced greater increases in IL4 (C) and IL10 (D) in *Nox2^{-/-}*, and *p47^{phox-/-}* mice. The loss of Nox2 or p47^{phox} had no effect on cytokine expression in Veh animals when compare to Veh *wt* mice. Data are mean $\pm$ SD for n=6 mice and analyzed by unpaired Student's t-test (morphine vs. vehicle) or one-way ANOVA with Dunnett's comparisons (*Nox2^{-/-}* or *p47^{phox-/-}* vs. *wt* mice). * P <0.05, ** P <0.01, and *** P <0.001 morphine vs. vehicle; † P <0.001 *Nox2^{-/-}* or *p47^{phox-/-}* vs. *wt* mice.