Therapeutic manipulation of peroxynitrite attenuates the development of opiate-induced antinociceptive tolerance in mice

Carolina Muscoli,1,2 Salvatore Cuzzocrea,3,4 Michael M. Ndengele,5 Vincenzo Mollicone,1,6 Frank Porreca,7 Francesca Fabrizi,6 Emanuela Esposito,4 Emanuela Masini,6 George M. Matuschak,5 and Daniela Salvemini6

Faculty of Pharmacy, University of Catanzaro “Magna Graecia,” Roccella di Borgia, Catanzaro, Italy. 2Centro di Neurofarmacologia Sperimentale, IRCCS Mondino-Università Tor Vergata di Roma, Rome, Italy. 3Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, University of Messina, Messina, Italy. 4IRCCS Centro Neurolesi “Bonino-Pulejo,” Messina, Italy. 5Department of Internal Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, Saint Louis University School of Medicine, St. Louis, Missouri, USA. 6IRCCS San Raffaele Pisana, Rome, Italy. 7Department of Pharmacology, University of Arizona, Tucson, Arizona, USA. 8Department of Preclinical and Clinical Pharmacology, University of Florence, Florence, Italy.

Severe pain syndromes reduce quality of life in patients with inflammatory and neoplastic diseases, often because chronic opiate therapy results in reduced analgesic effectiveness, or tolerance, leading to escalating doses and distressing side effects. The mechanisms leading to tolerance are poorly understood. Our studies revealed that development of antinociceptive tolerance to repeated doses of morphine in mice was consistently associated with the appearance of several tyrosine-nitrated proteins in the dorsal horn of the spinal cord, including the mitochondrial isoform of superoxide (O2−) dismutase, the glutamate transporter GLT-1, and the enzyme glutamine synthase. Furthermore, antinociceptive tolerance was associated with increased formation of several proinflammatory cytokines, oxidative DNA damage, and activation of the nuclear factor poly(ADP-ribose) polymerase. Inhibition of NO synthesis or removal of O2− blocked these biochemical changes and inhibited the development of tolerance, pointing to peroxynitrite (ONOO−), the product of the interaction between O2− and NO, as a signaling mediator in this setting. Indeed, coadministration of morphine with the ONOO− decomposition catalyst, Fe(III) 5,10,15,20-tetrakis(N-methylpyridinium-4-yl)porphyrin, blocked protein nitration, attenuated the observed biochemical changes, and prevented the development of tolerance in a dose-dependent manner. Collectively, these data suggest a causal role for ONOO− in pathways culminating in antinociceptive tolerance to opiates. Peroxynitrite (ONOO−) decomposition catalysts may have therapeutic potential as adjuncts to opiates in relieving suffering from chronic pain.

Introduction

Chronic, severe pain is a significant health problem (1). One third of Americans suffer from some form of chronic pain, and in over 30% it is resistant to analgesic therapy (1). The economic impact of pain is equally large, at approximately $100 billion annually (1). Opiate/narcotic analgesics, typified by morphine sulfate, are the most effective treatments for acute and chronic severe pain, but their clinical utility is often hampered by the development of analgesic tolerance as well as by de novo painful hypersensitivity to innocuous and noxious stimuli, phenomena observed in both animal and human studies (2–4). With respect to morphine in particular, tolerance necessitates escalating doses to achieve equivalent pain relief (5), even as morphine-induced hypersensitivity subverts the therapeutic impact of such dose increases (2–4). This complex pathophysiological cycle contributes to decreased quality of life in the growing population of subjects with chronic pain because of oversedation, reduced physical activity, respiratory depression, constipation, potential for addiction, and other side effects (5). Accordingly, there is great interest in new approaches to maintain opiate efficacy during repetitive dosing for chronic pain, without engendering tolerance or unacceptable side effects.

The mechanisms by which prolonged opiate exposure induces tolerance and hypersensitivity remain unclear, although a role for neuronal apoptosis (6, 7) and neuroimmune activation, which include glial cell activation and release of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 at the level of the spinal cord, have all been demonstrated (8–10). ONOO−, the product of the interaction between superoxide (O2−) and NO, is a potent proinflammatory and proapoptotic reactive species (11–13) recently implicated in the development of thermal hyperalgesia (defined as augmented pain intensity in response to painful stimuli) associated with inflammation and in response to spinal activation of the NMDA receptor (NMDAR) (14, 15). Other biologically relevant features of ONOO− include posttranslational tyrosine nitration and consequent modification of protein function (16) as exemplified by mitochondrial manganese O2− dismutase (MnSOD), the enzyme that normally keeps concentrations of O2− and NO under tight control (17). ONOO−-mediated nitration of MnSOD inactivates the enzyme, leading to an increase in O2− levels (18–22), an event favoring increased ONOO− formation, which

Nonstandard abbreviations used: CuZnSOD, cytosolic SOD; FeTM-4-PyP6, Fe(III) 5,10,15,20-tetrakis(N-methylpyridinium-4-yl)porphyrin; GS, glutamine synthase; GT, glutamate transporter; l-NAME, N-nitro-l-arginine methyl ester; MnSOD, manganese O2− dismutase; MnTBAP, Mn(III) 5,10,15,20-tetrakis(4-carboxylato-phenyl)porphyrin; NMDAR, NMDA receptor; O2−, superoxide; ONOO−, peroxynitrite; PARP, poly(ADP-ribose) polymerase; SOD, O2− dismutase.

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Glutamate-mediated neurotoxicity that often accompanies antinociceptive response when compared with responses observed in animals that received an equivalent volume of saline (naive group). On the other hand, a significant loss to the antinociceptive effect of the acute injection of morphine was observed in animals that received repeated administration of morphine over 4 days (morphine group; Mor). Coadministration of morphine over 4 days with (A) \(\alpha\)-NAME (1–10 mg/kg/d), (B) MnTBAP\(^{3+}\) (1–10 mg/kg/d), or (C) FeTM-4-PyP\(^{3+}\) (3–30 mg/kg/d) inhibited the development of tolerance in a dose-dependent manner. Results are expressed as mean ± SEM for 12 animals. \(*P < 0.001\) for vehicle versus naive; \(\dagger P < 0.001\) for morphine versus vehicle; \(\ddagger P < 0.001\) for morphine plus drug versus morphine alone.

Collectively we show in this study that formation of ONOO\(^-\) in the spinal cord plays a critical role in the development of morphine-induced antinociceptive tolerance through at least 3 biochemical pathways: (a) posttranslational nitration; (b) neuroimmune activation and release of proinflammatory cytokines; and (c) oxidative DNA damage and poly(ADP-ribose) polymerase (PARP) activation. Thus our studies provide a valid pharmacological basis for developing ONOO\(^-\) decomposition catalysts as potent adjuncts to opiates in the management of chronic pain, addressing an issue of major clinical and socioeconomic importance while laying the basis for interventions with strong therapeutic potential.

**Results**

The development of morphine-induced tolerance is associated with protein tyrosine nitration and this is inhibited by \(N^\alpha\)-nitro-L-arginine methyl ester and MnTBAP\(^{3+}\). When compared with animals receiving an equivalent injection of saline (naive group), acute injection of morphine (3 mg/kg) in animals that received saline over 4 days (vehicle group) produced a significant near-maximal antinociceptive response (percentage of maximum possible antinociceptive effect, ranging from 90% to 95%) (Figure 1). The antinociceptive effect of the acute dose of morphine was not associated with the appearance of tyrosine-nitrated proteins in the superficial layers of the dorsal horn as detected by immunohistochemistry (Figure 2). On the other hand, when compared with the antinociceptive response to acute morphine in the vehicle group, repeated administration of morphine over the same time course (morphine group) led to the development of antinociceptive tolerance as evidenced by a significant loss of its antinociceptive response (Figure 1). Baseline latencies in vehicle and morphine groups were statistically insignificant from each other and ranged between 6 and 8 seconds (\(n = 12\)). The development of tolerance was associated with the appearance of tyrosine-nitrated proteins in the superficial layers of the dorsal horn as detected by...
immunohistochemistry (Figure 2). No staining was observed in the ventral horn (Figure 2A). MnSOD but not cytosolic SOD (CuZnSOD; Figure 3, A and D), the GT GLT-1 (Figure 4A), and GS (Figure 4C) were proteins found to be nitrated in the dorsal horn as shown by immunoprecipitation. Representative gels obtained from 6 animals are shown in Figures 3 and 4; the composite (n = 6 animals) of the densitometry data resulting from these experiments is shown in Table 1. Posttranslational nitration of MnSOD (Figure 3A) led to functional enzymatic inactivation as evidenced by loss of its catalytic activity to dismutize $O_2^-$ as measured spectrophotometrically (n = 12) (Figure 3B). In contrast, the enzymatic activity of CuZnSOD was not affected (n = 12) (Figure 3E). Coadministration of morphine with the nonspecific NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME; ref. 33) or with the nonspecific O$_2^-$ scavenging agent MnTBAP$^{3-}$ [Mn(III) 5,10,15,20-tetrakis(4-carboxylatophenyl) porphyrin]; ref. 34, 35] inhibited in a dose-dependent manner (1–10 mg/kg/d, n = 12) the development of antinociceptive tolerance (Figure 1). When tested alone at the highest dose, neither L-NAME nor MnTBAP$^{3-}$ (both at 10 mg/kg/d) had antinociceptive effects. Thus on day 5 hot plate latencies following a s.c. injection of saline in vehicle-treated animals or in animals that received L-NAME or MnTBAP$^{3-}$ were statistically insignificant (n = 12) (Figure 3B). Inhibition of antinociceptive tolerance by L-NAME or MnTBAP$^{3-}$ was associated with a reduction in nitrotyrosine staining in the superficial layers of the dorsal horn (Figure 2B); attenuation of posttranslational nitration of MnSOD (Figure 3A), GLUT-1, and GS (n = 6) (Figure 3A and Figure 4, A and C); and protection of the enzymatic inactivation of MnSOD (n = 12) (Figure 3B). These results suggest that inhibition of tolerance by L-NAME or MnTBAP$^{3-}$ is secondary, at least in part, to inhibition of posttranslational nitration of MnSOD, GLUT-1, and GS.

The inhibitory effects of L-NAME or MnTBAP$^{3-}$ were not attributable to acute antinociceptive interactions between L-NAME or MnTBAP$^{3-}$ and acute morphine doses, since the response to acute morphine at 3 different doses (0.3–3 mg/kg, n = 10) in animals treated with the highest dose of L-NAME or MnTBAP$^{3-}$ (10 mg/kg/d, n = 10) or their vehicle over 4 days was statistically insignificant (Figure 5).

The development of morphine-induced tolerance is associated with increased cytokine formation, oxidative DNA damage, and PARP activation and is inhibited by L-NAME and MnTBAP$^{3-}$. On day 5 compared with the naive group, acute injection of morphine (3 mg/kg, n = 12) in vehicle-treated mice did not increase dorsal horn tissue levels of TNF-α, IL-1β, or IL-6 as measured by ELISA using commercially available kits (Figure 6); did not increase levels of 8-OHdG, a marker of oxidative DNA damage (Figure 7A); and did not increase the activity of PARP measured as described previously (36) (Figure 7B). On the other hand, acute injection of morphine in mice in the morphine group led to a significant increase in TNF-α, IL-1β, and IL-6 (n = 12) (Figure 6); increased the levels of 8-OHdG (n = 12); and increased PARP activity (n = 12) (Figure 7) in dorsal horn tissues. This increase was attenuated by coadministration of morphine over 4 days with L-NAME or MnTBAP$^{3-}$ (both at 10 mg/kg/d, n = 12) (Figures 6 and 7).

ONOO$^-\cdot$ formed by NO and $O_2^-$ is a key mediator in the biochemical events leading to antinociceptive tolerance. Because NO is known to react with $O_2^-$ at a diffusion-limited rate to form ONOO$^-\cdot$ (12), results obtained with L-NAME and MnTBAP$^{3-}$ indirectly suggest that ONOO$^-\cdot$ from these reactive species is the common denominator in the molecular and biochemical pathways leading to antinociceptive tolerance. The important role of ONOO$^-\cdot$ in tolerance was confirmed by the use of a well-known and -characterized ONOO$^-\cdot$ decomposition catalyst,
Prolonged use of opiates results in antinociceptive tolerance, such that higher doses are required to achieve equivalent analgesia (5). By contrast, an alternative hypothesis is that stimulation of opioid receptors over time triggers activation of antiopioid systems that in turn reduce sensory thresholds, thereby resulting in hypersensitivity (42). As a corollary to this hypothesis, such opioid-induced hypersensitivity paradoxically diminishes the net analgesic effect of the opioid agonist (40, 43, 44). Adaptative modifications in cellular responsiveness and particularly desensitization and downregulation of opioid receptors are at the origin of this phenomenon (42). By contrast, an alternative hypothesis is that stimulation of opioid receptors over time triggers activation of antiopioid systems that in turn reduce sensory thresholds, thereby resulting in hypersensitivity (42). As a corollary to this hypothesis, such opioid-induced hypersensitivity paradoxically diminishes the net analgesic effect of the opioid agonist (40, 43, 44). Adaptative modifications in cellular responsiveness and particularly desensitization and downregulation of opioid receptors are at the origin of this phenomenon (42). By contrast, an alternative hypothesis is that stimulation of opioid receptors over time triggers activation of antiopioid systems that in turn reduce sensory thresholds, thereby resulting in hypersensitivity (42). As a corollary to this hypothesis, such opioid-induced hypersensitivity paradoxically diminishes the net analgesic effect of the opioid agonist (40, 43, 44). Adaptative modifications in cellular responsiveness and particularly desensitization and downregulation of opioid receptors are at the origin of this phenomenon (42). By contrast, an alternative hypothesis is that stimulation of opioid receptors over time triggers activation of antiopioid systems that in turn reduce sensory thresholds, thereby resulting in hypersensitivity (42). As a corollary to this hypothesis, such opioid-induced hypersensitivity paradoxically diminishes the net analgesic effect of the opioid agonist (40, 43, 44). Adaptative modifications in cellular responsiveness and particularly desensitization and downregulation of opioid receptors are at the origin of this phenomenon (42). By contrast, an alternative hypothesis is that stimulation of opioid receptors over time triggers activation of antiopioid systems that in turn reduce sensory thresholds, thereby resulting in hypersensitivity (42). As a corollary to this hypothesis, such opioid-induced hypersensitivity paradoxically diminishes the net analgesic effect of the opioid agonist.
Support for this alternative hypothesis has been evidenced in vivo in animals (2, 45, 46) and humans (3, 47, 48). Thus it is thought that analgesic tolerance arises when pain facilitatory systems become sensitized or hyperactive after repeated opioid use. Our focus herein has been to address the involvement of ONOO− in the maladaptive process of altered nociceptive signaling within the dorsal horn of the spinal cord, as it relates to the clinical problem of morphine-induced tolerance.

Results of our studies using indirect pharmacological approaches to inhibit the formation of ONOO− (l-NAME and MnTBAP3+) and a direct pharmacological approach to catalytically decompose ONOO− (FeTM-4-PyP5+) reveal that ONOO− is a key signaling molecule in morphine-induced antinociceptive tolerance, contributing to its development via at least 3 biochemical pathways as summarized in Figure 8. A biologically relevant feature of the involvement of ONOO− and related species in pathophysiological conditions is posttranslational tyrosine nitration and consequent modification of protein function (16). Protein nitration is increasingly recognized as an important occurrence during cell signaling and regulation of protein activity (49). Several proteins are now known to be nitrated by ONOO−, with subsequent loss or gain of function associated with this modification (16). We have recently reported that nitration and enzymatic inactivation of mitochondrial MnSOD plays a key role in the development of hyperalgesia occurring during the development of inflammation (15) and in response to NMDA receptor activation (14). Here we show that nitration and subsequent enzymatic inactivation of mitochondria of morphine-induced antinociceptive tolerance contributes to the development of morphine-induced antinociceptive tolerance in the maladaptive process of altered nociceptive signaling within the dorsal horn of the spinal cord, as it relates to the clinical problem of morphine-induced tolerance.

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morphine-induced antinociceptive tolerance may involve altered nociceptive signaling at several levels of the neuroaxis; characterization of events in other central nervous system tissues is an exciting avenue for future studies.

Chronic administration of morphine promotes neuroimmune activation as evidenced by activation of spinal cord glial cells, production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, and spinal sensitization (8–10). Thus inhibitors of glial cell metabolism and/or anticytokine approaches block morphine-induced antinociceptive tolerance and hyperalgesia (8–10). The possible mechanisms for chronic morphine-induced glial cell activation are not known with certainty. μ-Opiate receptors are present on microglia and astrocytes (57), but acute administration of morphine does not activate these cells (10). On the other hand, morphine primes glial cells for enhanced production of proinflammatory cytokines (58). Our results suggest that ONOO⁻ is a signaling molecule involved in the increased formation of TNF-α, IL-1β, and IL-6. As reported by our group and others, a mechanism by which ONOO⁻ leads to the generation of such proinflammatory cytokines is through activation of redox-sensitive transcription factors such as NF-κB and AP-1 as well as activation of MAPK kinases such as p38 kinase (59–61). These results suggest that attenuation of morphine tolerance by ONOO⁻ decomposition catalysts such as FeTM-4-PyP³⁺ may be secondary to the suppression of repeated morphine-induced spinal neuroimmune activation promoted by ONOO⁻.

It has become increasingly recognized that under severe oxidative and nitrosative stress situations, excessive DNA damage causes overactivation of the nuclear enzyme PARP, a critical intracellular mechanism of neurotoxicity and cell death (11, 62, 63). ONOO⁻ is one of the reactive species that has been considered to be a major oxidant responsible for DNA strand breakage, which then activates the enzyme (11). PARP activation induces excitotoxic transsynaptic morphological changes in superficial dorsal horn “dark neurons” in morphine-induced antinociceptive tolerance and hyperalgesia as well as in neuropathic pain (64, 65). As shown in Figure 7, repeated administration of morphine led to oxidative DNA damage as evidenced by an increase in the levels of 8-OHdG in the spinal cord and increased PARP activity, and these events were blocked by FeTM-4-PyP³⁺ (or L-NAME and MnTBAP³⁻). Our results support the work by Mayer and colleagues as they confirm activation of PARP (64) and extend these earlier observations by establishing a role of ONOO⁻ in PARP activation during antinociceptive tolerance to morphine.

The mechanisms leading to ONOO⁻ formation are not known although several possibilities exist, including μ and NMDA receptor activation as well as activation of glial cells and cytokine formation, as these are associated with the synthesis and production of its precursors, namely NO and ONOO⁻ (13, 66–71).

In summary, our results have established a key role for ONOO⁻ in the development of morphine-induced tolerance, providing what is, to our knowledge, a novel mechanistic rationale for development

### Table 1

Posttranslational nitration of MnSOD, GLT-1, and GS during the development of morphine antinociceptive tolerance

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Nitrated protein</th>
<th>Total lysate</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MnSOD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>267.3 ± 45.6</td>
<td>1,801.4 ± 3.5</td>
<td>1,752.3 ± 34.1</td>
</tr>
<tr>
<td>Vehicle</td>
<td>397.6 ± 32.2</td>
<td>1,889.3 ± 88.6</td>
<td>1,765.3 ± 73.2</td>
</tr>
<tr>
<td>Morphine</td>
<td>1,653 ± 143.4³⁺</td>
<td>1,963 ± 75.4</td>
<td>1,789.3 ± 75.4</td>
</tr>
<tr>
<td>Morphine + L-NAME (10 mg/kg)</td>
<td>378.4 ± 28.6³⁻</td>
<td>1,973.7 ± 67.2</td>
<td>1,834.3 ± 73.1</td>
</tr>
<tr>
<td>Morphine + MnTBAP³⁻ (10 mg/kg)</td>
<td>403.1 ± 78.3³⁻</td>
<td>1,982.1 ± 56.2</td>
<td>1,867 ± 53.7</td>
</tr>
<tr>
<td>Morphine + FeTM-4-PyP³⁺ (30 mg/kg)</td>
<td>396.2 ± 95.4³⁻</td>
<td>1,945.8 ± 45.9</td>
<td>1,854 ± 62.7</td>
</tr>
<tr>
<td><strong>CuZnSOD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>609.2 ± 45.7</td>
<td>1,934.5 ± 56.2</td>
<td>1,201 ± 34.3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>678.3 ± 36.3</td>
<td>1,989.3 ± 35.7</td>
<td>1,267.8 ± 23.6</td>
</tr>
<tr>
<td>Morphine</td>
<td>1,620.1 ± 34.7³⁺</td>
<td>1,894.5 ± 45.1</td>
<td>1,204.5 ± 78.2</td>
</tr>
<tr>
<td>Morphine + L-NAME (10 mg/kg)</td>
<td>698.6 ± 89.2³⁻</td>
<td>1,843.1 ± 67.2</td>
<td>1,215.4 ± 54.3</td>
</tr>
<tr>
<td>Morphine + MnTBAP³⁻ (10 mg/kg)</td>
<td>745.2 ± 67.1³⁻</td>
<td>1,904.2 ± 55.7</td>
<td>1,254.8 ± 87.1</td>
</tr>
<tr>
<td>Morphine + FeTM-4-PyP³⁺ (30 mg/kg)</td>
<td>703.37 ± 95.4³⁻</td>
<td>1,891.4 ± 68.3</td>
<td>1,237.9 ± 68.3</td>
</tr>
<tr>
<td><strong>GLT-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>207.1 ± 14.7</td>
<td>1,654.2 ± 42.1</td>
<td>1,519.2 ± 45.1</td>
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<tr>
<td>Vehicle</td>
<td>224.6 ± 23.2</td>
<td>1,623.1 ± 32.4</td>
<td>1,567.7 ± 44.9</td>
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<tr>
<td>Morphine</td>
<td>921.4 ± 15.7³⁺</td>
<td>1,612.8 ± 21.3</td>
<td>1,534.6 ± 77.8</td>
</tr>
<tr>
<td>Morphine + L-NAME (10 mg/kg)</td>
<td>198.7 ± 35.2³⁻</td>
<td>1,615.4 ± 56.3</td>
<td>1,590.3 ± 64.5</td>
</tr>
<tr>
<td>Morphine + MnTBAP³⁻ (10 mg/kg)</td>
<td>212.4 ± 20.4³⁻</td>
<td>1,599.2 ± 18.5</td>
<td>1,546.5 ± 89.4</td>
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<tr>
<td>Morphine + FeTM-4-PyP³⁺ (30 mg/kg)</td>
<td>195.7 ± 24.1³⁻</td>
<td>1,634 ± 58.7</td>
<td>1,562.9 ± 73.5</td>
</tr>
<tr>
<td><strong>GS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>0</td>
<td>1,667.1 ± 16.4</td>
<td>1,654 ± 34.2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>1,698.4 ± 45.7</td>
<td>1,678.9 ± 23.6</td>
</tr>
<tr>
<td>Morphine</td>
<td>0</td>
<td>1,672.3 ± 37.4</td>
<td>1,705.3 ± 37.9</td>
</tr>
<tr>
<td>Morphine + L-NAME (10 mg/kg)</td>
<td>0</td>
<td>1,669.1 ± 56.7</td>
<td>1,653.2 ± 25.9</td>
</tr>
<tr>
<td>Morphine + MnTBAP³⁻ (10 mg/kg)</td>
<td>0</td>
<td>1,661.9 ± 29.3</td>
<td>1,675 ± 43.7</td>
</tr>
<tr>
<td>Morphine + FeTM-4-PyP³⁺ (30 mg/kg)</td>
<td>0</td>
<td>1,692 ± 45.4</td>
<td>1,710.2 ± 56.2</td>
</tr>
</tbody>
</table>

Repeated administration of morphine led to nitration of MnSOD, GLT-1, and GS but not of CuZnSOD as evidenced by immunoprecipitation. Total levels of these proteins did not change with the various treatments as measured by Western blot analysis. All results are expressed as densitometry units (mean ± SEM) for 6 gel results obtained from different animals. ^P < 0.001 for morphine alone versus vehicle; *P < 0.01 for morphine plus drug versus morphine alone.
of ONOO$^-$-targeted approaches to alleviate the burden of suffering from chronic severe pain by restoring the sensitivity and therapeutic efficacy of opiates. The broader implications of our findings on the importance of protein tyrosine nitration in this setting further suggest that a comprehensive approach to understanding the functional relevance of posttranslational tyrosine nitration and modification may identify additional non-opiate pharmacological strategies for multimodality therapy of chronic pain. Considering the appreciable molecular, biochemical, and pharmacological similarities between opiate-mediated hypersensitivity, and hypersensitivity associated with chronic neuropathic pain from diabetes mellitus and other sensory neuropathies (2, 8, 72), ONOO$^-$ may be a viable therapeutic target in both conditions.

Methods

Induction of morphine-induced antinociceptive tolerance in mice

Male CD-1 mice (24–30 g; Charles River Laboratory) were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Saint Louis University Health Science Center and in accordance with the NIH Guidelines on Laboratory Animal Welfare and the University of Catanzaro “Magna Graecia” in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with European Economic Community regulations. The IACUC of Saint Louis University Health Science Center and the University of Catanzaro “Magna Graecia” approved all studies. Mice were housed 5–7 per cage and maintained under identical conditions of temperature (21 ± 1°C) and humidity (60% ± 5%) with a 12-hour light/12-hour dark cycle and allowed food ad libitum. Nociceptive thresholds were determined by measuring latencies (in seconds) of mice placed in a transparent glass cylinder on a hot plate (Ugo Basile) maintained at 52°C. Determination of antinociception was assessed between 7:00 am and 10:00 am. All injections were given i.p. or s.c. in a 0.1-ml volume at approximately 7 am and 4 pm. Drugs or saline were given before each dose on morphine. Responses indicative of nociception included intermittent lifting and/or licking of the hindpaws or escape behavior. Hot plate latencies were taken in mice from all groups on day 5 before (baseline latency) and 40 minutes after an acute dose of morphine (0.3–3 mg/kg) or its vehicle (saline) (response latency). Baseline values from all groups as measured on day 5 before injection of the acute dose of morphine or saline were statistically insignificant and ranged between 6 and 8 seconds. Results are expressed as percentage of maximum possible antinociceptive effect, which was calculated as follows: (response latency − baseline latency) / (cut-off latency − baseline latency) × 100. A cut-off latency of 20 seconds was employed to prevent tissue damage. Six to twelve mice per group were used.

Figure 5

On day 5 acute injection of different doses of morphine (0.3–3 mg/kg) in animals that received saline over 4 days produced a significant dose-dependent antinociceptive response when compared with responses obtained in naive group. The antinociceptive response to morphine was not altered in animals that were treated over 4 days with L-NNAME (10 mg/kg/d), MnTBAP$^{3–}$ (10 mg/kg/d), or FeTM-4-PyP$^{5+}$ (30 mg/kg/d), indicating lack of acute interaction between morphine and L-NNAME, MnTBAP$^{3–}$, or FeTM-4-PyP$^{5+}$. Results are expressed as mean ± SEM for 10 animals. *P < 0.001 for the vehicle group when compared to values obtained in the absence of morphine.

Figure 6

Acute injection of morphine (3 mg/kg) on day 5 in animals that received saline over 4 days (vehicle group) did not increase dorsal horn tissue levels of TNF-α (A), IL-1β (B), or IL-6 (C) when compared with animals that received an equivalent volume of its vehicle (naive group). On the other hand, acute administration of morphine in animals that received repeated administration of morphine (morphine group) led to a significant increase in TNF-α, IL-1β, and IL-6 in dorsal horn tissues. This increase was attenuated by coadministration of morphine over 4 days with L-NNAME (10 mg/kg/d), MnTBAP$^{3–}$ (10 mg/kg/d), or FeTM-4-PyP$^{5+}$ (30 mg/kg/d). Results are expressed as mean ± SEM for 12 animals. *P < 0.001 for morphine alone versus vehicle; †P < 0.001 for morphine plus drug versus morphine alone.
and all experiments were conducted with the experimenters blinded to treatment conditions. Unless specified, all drugs were purchased from Sigma-Aldrich. Charges on MnTBAP\(^+\) and FeTM-4-PyP\(^{3+}\) were omitted for clarity on all Figures. The following experimental groups were used.

**Naive group.** In this group, mice were injected twice a day with an i.p. injection of saline (vehicle used to deliver the drugs to the other groups over 4 days) and a s.c. injection of saline (vehicle used to deliver morphine to the other groups over 4 days). On day 5 mice received an i.p. injection of saline followed 15 minutes later by a s.c. injection of saline.

**Naive plus drug groups.** In these groups, mice were injected twice a day for 4 days with an i.p. injection of the highest dose of \( l \)-NAME (10 mg/kg/d), MnTBAP\(^+\) (10 mg/kg/d), or FeTM-4-PyP\(^{3+}\) (30 mg/kg/d) and a s.c. injection of saline. On day 5 mice received an i.p. injection of \( l \)-NAME (5 mg/kg). MnTBAP\(^+\) (5 mg/kg), or FeTM-4-PyP\(^{3+}\) (15 mg/kg), followed 15 minutes later by s.c. doses of acute morphine giving between 10% and 95% maximum antinociceptive responses within 40 minutes of administration (0.3 and 1 mg/kg, s.c.).

**Vehicle plus drug groups.** In these groups, mice were injected twice a day for 4 days with an i.p. injection of the highest dose of \( l \)-NAME (10 mg/kg/d), MnTBAP\(^+\) (10 mg/kg/d), or FeTM-4-PyP\(^{3+}\) (30 mg/kg/d) and a s.c. injection of saline. On day 5 mice received an i.p. injection of \( l \)-NAME (5 mg/kg), MnTBAP\(^+\) (5 mg/kg), or FeTM-4-PyP\(^{3+}\) (15 mg/kg), followed 15 minutes later by s.c. doses of acute morphine (3 mg/kg).

**Vehicle group.** In this group, mice were injected twice a day for 4 days with an i.p. injection of saline and a s.c. injection of morphine (20 mg/kg/d). On day 5 mice received an i.p. injection of saline followed 15 minutes later by a s.c. dose of acute morphine (3 mg/kg).

**Morphine plus drug groups.** In this group, mice were injected twice a day for 4 days with an i.p. injection of varying doses of \( l \)-NAME (1, 3, and 10 mg/kg/d), MnTBAP\(^+\) (1, 3, and 10 mg/kg/d), or FeTM-4-PyP\(^{3+}\) (3, 10, and 30 mg/kg/d), followed by s.c. injection of morphine (20 mg/kg/d). On day 5 mice received an i.p. dose of \( l \)-NAME (5 mg/kg), MnTBAP\(^+\) (5 mg/kg), or FeTM-4-PyP\(^{3+}\) (15 mg/kg), followed 15 minutes later by the s.c. dose of acute morphine (3 mg/kg).

**Morphine group.** In this group, mice were injected twice a day for 4 days with an i.p. injection of saline and a s.c. injection of morphine (20 mg/kg/d). On day 5 mice received an i.p. injection of saline followed 15 minutes later by a s.c. dose of acute morphine (3 mg/kg).

**Vehicle plus drug groups.** In these groups, mice were injected twice a day for 4 days with an i.p. injection of \( l \)-NAME (1, 3, and 10 mg/kg/d), MnTBAP\(^+\) (1, 3, and 10 mg/kg/d), or FeTM-4-PyP\(^{3+}\) (3, 10, and 30 mg/kg/d), followed by s.c. injection of saline (vehicle used to deliver morphine to the other groups over 4 days). On day 5 mice received an i.p. injection of saline followed 15 minutes later by a s.c. injection of saline.

**Vehicle group.** In this group, mice were injected twice a day for 4 days with an i.p. injection of saline and a s.c. injection of saline. On day 5 mice received an i.p. injection of saline followed 15 minutes later by a s.c. injection of saline.

**Figure 7** Acute injection of morphine (3 mg/kg) on day 5 in animals that received saline over 4 days (vehicle group) did not increase dorsal horn tissue levels of 8-OHdG and did not activate PARP when compared with animals that received an equivalent volume of its vehicle (naive group). On the other hand, acute administration of morphine on day 5 after repeated administration of morphine (morphine group) led to significant increase in dorsal horn tissue levels of 8-OHdG and substantially activated PARP (B). These biochemical changes were significantly attenuated by coadministration of morphine over 4 days with \( l \)-NAME (10 mg/kg/d), MnTBAP\(^+\) (10 mg/kg/d), or FeTM-4-PyP\(^{3+}\) (30 mg/kg/d). When compared with the naive groups, the acute dose of morphine did not increase levels of these cytokines. Results are expressed as mean ± SEM for 12 animals. \(^*\) \( P < 0.001 \) for morphine alone versus vehicle; \( ^{1\prime}P < 0.001 \) for morphine plus drug versus morphine alone.

**Figure 8** Illustration summarizing the key findings of this study depicting the role(s) of ONOO\(^-\) in the development of morphine-induced antinociceptive tolerance. Formation of ONOO\(^-\) in the spinal cord during repeated administration of morphine plays a critical role in the development of morphine-induced antinociceptive tolerance through at least 3 biochemical pathways: posttranslational nitration, neuroimmune activation, and release of proinflammatory cytokines, and oxidative DNA damage and PARP activation. Inhibition of its formation by removal of NO and \( O_2^- \) or by catalytically decomposing it by ONOO\(^-\) decomposition catalysts such as FeTM-4-PyP\(^{3+}\) blocked these pathways, leading to inhibition of antinociceptive tolerance.
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On day 5 and after the behavioral tests, spinal cord tissues from the lumbar enlargement segment of the spinal cord (L4–L6) and dorsal horn tissues were removed and tissues processed for immunohistochemical, Western blot, and biochemical analysis.

**Immunohistochemical detection of nitrated proteins**

Animals were anesthetized with pentobarbital and transcardially perfused with normal saline followed by 4% paraformaldehyde solution. Dorsal half of the spinal cord lumbar region enlargement (L4–L6) was processed for immunostaining as previously described (15). Monoclonal anti-nitrotyrosine antibody (1:100 in 10% normal horse serum; Calbiochem) was used as primary antibody and the samples processed for immunolabeling visualization by incubation with correspondent secondary antibody, A/B complex, and diamino-benzidine according to the manufacturer’s instructions (Vector ABC Elite Kit; Vector Laboratories). For immunoreaction specificity, some sections were incubated with either primary antibody or secondary antibody alone.

**Immunoprecipitation and Western blot analysis.** Dorsal half of the spinal cord lumbar region enlargement (L4–L6) were obtained as described previously (14, 15, 73). The resulting lysates samples were stored immediately at −80°C and immunoprecipitation of tyrosine nitrated protein followed by Western blot analysis performed as previously described (14, 15). For immunoprecipitation of nitrated proteins, a well-characterized affinity-purified anti-nitrotyrosine monoclonal antibody conjugated to agarose beads from Upstate Biotechnology was used according to the manufacturer’s instructions. To determine whether MnSOD, CuZnSOD, GLUT-1, and GS were nitrated, Western blot analysis of i.p. protein complex and total lysates were performed using antibodies specific to these proteins. Briefly, the immunoprecipitated proteins were resolved in 12% SDS-PAGE mini and proteins transferred to nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature in 1% BSA/0.1% thimerosal in 50 mM Tris-HCl (pH 7.4)/150 mM NaCl/0.01% Tween-20 (TBS/T), followed by incubation with rabbit polyclonal antibodies for MnSOD and CuZnSOD (1:2000; Upstate Biotechnology), GLUT-1 (1:1000; Alpha Diagnostic International), and monoclonal antibody for GS (1:4000; Transduction Laboratories). Membranes were then washed with TBS/T and incubated secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature. After washes, proteins were visualized by enhanced chemiluminescence (ECL or Femto kit; Amernaite Biosciences or Pierce Biotechnology, respectively). Rat brain lysate from Upstate Biotechnology that contained most of the proteins expressed by nervous tissue was used as positive control and for equal loading of membranes, and total lysates blots were stripped and reprobed with a monoclonal antibody specific for β-actin at a dilution of 1:2000. Quantification of protein bands of interest was determined by densitometry using ImageQuant 5.2 software (Molecular Dynamics).

**Measurement of MnSOD and CuZnSOD activities**

Dorsal half of the spinal cord lumbar region enlargement (L4–L6) were homogenized with 10 mM PBS (pH 7.4) in a Polytron homogenizer and then sonicated on ice for 1 minute (3 times, 20 seconds each time). The sonicated samples were subsequently centrifuged at 1,100 g for 10 minutes and SOD activity was measured in the supernatants. In brief, a competitive inhibition assay was performed that used xanthine-xanthine oxidase–generated O2·− to reduce nitroblue tetrazolium (NBT) to blue tetrazolium salt. The reaction was performed in sodium carbonate buffer (50 mM, pH 10.1) containing EDTA (0.1 mM), nitroblue tetrazolium (25 μM), and xanthine and xanthine oxidase (0.1 mM and 2 nM, respectively; Boehringer). The rate of NBT reduction was monitored spectrophotometrically (PerkinElmer Lambda 5 Spectrophotometer) at 560 nm. The amount of protein required to inhibit the rate of NTB reduction by 50% was defined as 1 unit of enzyme activity. CuZnSOD activity was inhibited by performing the assay in the presence of 2 mM NaN3 after preincubation for 30 minutes. Enzymatic activity was expressed in units per milligram of protein (15).

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Address correspondence to: Daniela Salvemini, Department of Internal Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, Saint Louis University School of Medicine, St. Louis 63104, USA. Phone: (314) 577-8856; Fax: (314) 577-8859; E-mail: salvem@dlsu.edu.

Carolina Muscoli and Salvatore Cuzzocrea contributed equally to this work.


