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# *Modulation of Opioid Actions by Nitric Oxide Signaling*

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**Nitric oxide (NO) plays pivotal roles in controlling physiological functions, participates in pathophysiological intervention, and is involved in mechanisms underlying beneficial or untoward actions of therapeutic agents. Endogenous nitric oxide is formed by three isoforms of nitric oxide synthase: endothelial, neurogenic and inducible. The former two are constitutively present mainly in the endothelium and nervous system, respectively, and the latter one is induced by lipopolysaccharides or cytokines mainly in mitochondria and glial cells. Constitutively formed nitric oxide modulates the actions of morphine and related analgesics by either enhancing or reducing antinociception. Tolerance to and dependence on morphine or its withdrawal syndrome are likely prevented by nitric oxide synthase inhibition. Information concerning modulation of morphine actions by nitric oxide is undoubtedly useful in establishing new strategies for efficient antinociceptive treatment and for minimizing noxious and unintended reactions.**

THE labile molecule NO has been widely recognized to play pivotal roles in the regulation of physiologic functions; in contrast, it also participates in pathophysiological intervention. Nitric oxide (NO) synthesis was first found in vascular endothelial cells,[1–4] central and peripheral nerve cells and fibers,[5] and macrophages.[6] Investigations on the functional role of this inorganic molecule have been extended to other organs and tissues in the whole body. The NO/cyclic guanosine monophosphate (cyclic GMP) signaling pathway contributes to mechanisms underlying the action of therapeutic agents, the most promising through mechanisms of enhancing the action of endogenous NO is phosphodiesterase-5 inhibitors such as sildenafil[7] and its congeners.[8] Morphine and other opioid agonists exert analgesia through $\mu$-opioid receptors at spinal and multiple supraspinal sites. δ-Opioid receptor agonists also are potent analgesics in animals and humans. In animals, agonists for $\kappa$-receptors produce analgesia that is mediated at spinal sites. Some literature reports on opioid analgesics research have revealed that nitric oxide is involved in therapeutic actions but can also have untoward effects. Information concerning nitric oxide that undoubtedly plays important roles in modulating analgesic effects of opioids and their side effects would provide us with clues for establishing strategies for appropriate opioid therapy to enhance analgesic actions while minimizing tolerance, dependence, withdrawal syndrome, and other side effects.

Our previous review article[9] summarized the involvement of nitric oxide in the actions of a variety of anesthetic agents without discussing the opioid analgesics, although these are useful for anesthesia and inevitably important for intolerable pain. The present article describes the involvement of endogenous nitric oxide in the antinociceptive effects of morphine and other related analgesics and the tolerance, dependence, and withdrawal syndrome associated with the use of these drugs.

## Synthesis and Actions of NO

Nitric oxide is produced when L-arginine is transformed to L-citrulline through catalysis by nitric oxide synthase (NOS) in the presence of oxygen and cofactors. $Ca^{2+}$ is required for the activation of neuronal NOS (nNOS, NOS I) and endothelial NOS (NOS III) but not inducible NOS (iNOS, NOS II). nNOS, mostly a soluble enzyme, is constitutively expressed in the brain,[5] peripheral nerves, and kidneys. Endothelial NOS is also constitutively expressed mostly in particulate fractions of the endothelial cell.[10] iNOS is not constitutively expressed but is induced mainly in macrophages in response to bacterial lipopolysaccharide and cytokines. The synthesis of nitric oxide by these NOS isoforms is inhibited by L-arginine analogs, including $N^G$-monomethyl-L-arginine (L-NMMA),[11] $N^G$-nitro-L-arginine (L-NA),[12,13] L-NA methylester (L-NAME),[12] and asymmetric dimethylarginine.[14] 7-Nitroindazol (7-NI) is one of the most promising nNOS inhibitors introduced.[15] Aminoguanidine has a long history as a selective iNOS inhibitor.[16] Nitro compounds, including nitroglycerin, sodium nitroprusside, and S-nitroso-*N*-acetylpenicillamine, are capable of liberating nitric oxide and are called nitric oxide donors.

Endothelial nitric oxide causes vasodilatation, decreased vascular resistance, lowered blood pressure, inhibition of platelet aggregation and adhesion, inhibition

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of leukocyte adhesion and transmigration, and reduced vascular smooth muscle proliferation. Nitrovasodilators *via* release of nitric oxide activate soluble guanylyl cyclase and produce cyclic GMP from guanosine triphosphate in smooth muscle cells. Methylene blue, oxyhemoglobin, and 1H[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one[17] inhibit guanylyl cyclase activity. Accumulation of cyclic GMP causes activation of cyclic GMP-dependent protein kinase that is involved in the adenosine triphosphate (ATP)-sensitive $K^+$-channel opening to produce spinal or peripheral antinociception[18–20] and in $Na^+$,$K^+$-ATPase activation.[21] Cyclic GMP-dependent protein kinase phosphorylates the serotonin transporter at Thr-276 and increases its activity by modifying the substrate permeation.[22,23] Cyclic GMP is degraded by phosphodiesterase type 5 to 5′-GMP. Cyclic GMP is degraded by phosphodiesterase type-5 to 5′-GMP.

Nonadrenergic noncholinergic inhibitory responses to autonomic nerve stimulation are mainly mediated through nitric oxide synthesized by nNOS; Nitric oxide plays a crucial role as a neurotransmitter from the peripheral efferent nerves in cerebral blood vessels.[24] Afferent nitrergic nerves control some aspects of sensory information processing. There is evidence that nitric oxide is the neurotransmitter released from primary sensory nerves that mediates mesenteric vasodilatation.[25] Nitric oxide offers important roles in afferent signaling of pain through the dorsal horn of the spinal cord and in autonomic control through nitrergic innervation.[26] Many of the homeostatic actions of spinal afferents are brought about by release of transmitters (nitric oxide and calcitonin gene-related peptide) from their peripheral endings.[27]

Nitric oxide functions mainly as a neuromodulator in the brain. Nitric oxide signaling appears to be essential for neural plasticity; that is, long-term potentiation in the hippocampus and long-term depression in the cerebellum. Glutamate participates mainly in synaptic interactions; with the help of nitric oxide, the strength of excitatory input might be nonsynaptically signaled to the surrounding monoaminergic neurons in the brain. Nitric oxide formed by *N*-methyl-D-aspartate (NMDA)-receptor activation diffuses to adjacent nerve terminals to modulate neurotransmitter release.[28] Nitric oxide may act at several levels of the nervous system to develop hyperexcitability, resulting in hyperalgesia or allodynia.[29,30] On the other hand, nitric oxide-releasing molecules, such as nitroparacetamol (NOX-701) resulting from the combination of paracetamol and a nitrooxybutyrol moiety, has been shown to be effective in acute nociception and in neuropathic pain.[31,32]

Under pathologic conditions (*e.g.*, during inflammation), high levels of nitric oxide are produced after iNOS expression is induced, mainly in macrophages. Nitric oxide possesses protective/destructive duality inherent in every other major component of the immune response. This labile molecule exerts beneficial effects by acting as an antibacterial, antiparasitic, antiviral agent or as a tumoricidal agent; on the other hand, high levels of nitric oxide, if uncontrolled, elicits detrimental effects that are produced because nitric oxide reacts with concomitantly produced superoxide anions, thereby generating highly toxic compounds such as peroxynitrite and hydroxyl radicals.

## Effects of Morphine on NO Synthesis and Release

*Morphine on NOS*

Acute and chronic morphine treatment produced an increase in $Ca^{2+}$-dependent NOS in mouse brain; the acute effect of morphine was blocked by coadministration of naloxone.[33] In rat spinal cord, repeated administration of morphine increased the NOS mRNA level, the effect being accompanied by an increase in both the number of NOS-positive cells and the optical density of NOS-immunoreactivity, indicating that repeated morphine administration increases NOS biosynthesis.[34] At 24 h after treatment with morphine (25 mg in pellet), there was decreased NOS activity in all brain regions and the spinal cord of the mouse, and NOS activity increased at 48 and 72 h after the treatment in both the cerebellum and cortex; implantation of a naltrexone pellet in conjunction with a morphine pellet blocked the changes in NOS activity.[35] The authors suggested that the initial decrease in NOS activity is related to enhanced motor activity, whereas the increase in NOS activity is associated with tolerance-physical dependence development. Morphine increased the invertebrate immunocyte intracellular $Ca^{2+}$ level that was mediated by $\mu$3-opioid receptors and was associated with stimulating nitric oxide production.[36] The opioid stimulation of intracellular $Ca^{2+}$ levels appears to regulate constitutive NOS activity. Higher numbers of nNOS-positive cells were observed in the hippocampal dentate gyrus of wild-type mice repeatedly treated with either morphine or cocaine than in saline-treated wild-type mice; moreover, $\mu$-opioid receptor knockout mice showed higher morphine- or cocaine-induced nNOS expression levels in the dentate gyrus than saline-treated wild-type mice.[37] The knockout mice showed a higher morphine-induced nNOS expression level or a lower cocaine-induced nNOS expression level than morphine- or cocaine-treated wild-type mice. The authors suggested that morphine and cocaine sensitization is differentially regulated by the $\mu$-opioid receptors in the knockout mice *via* the nNOS systems in the dentate gyrus.

In contrast, Barjavel and Bhargava[38] demonstrated that NOS activity, as determined by the rate of conversion of [$^3$H]arginine into [$^3$H]citrulline, was inhibited by the $\kappa$-opioid receptor agonist U-50,488H but only at a high concentration (0.1 mM) and was not affected by selective $\mu$- and $\delta$-opioid receptor agonists in rat cerebral cortex, indicating that drugs acting at $\mu$-, $\delta$-, and $\kappa$-receptors have no direct action on central NOS activity *in vitro*. NOS activity was found to be unchanged in the brainstem and cerebellum of mice treated with morphine.[39]

Systemic administration of diacetylmorphine reduced the number of reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase-positive (nitric oxide-synthesizing) neurons in the rat brain raphe nuclei, and this effect was blocked by naloxone.[40] Acute and chronic administration of morphine suppressed NADPH diaphorase-positive neurons in rat brain cervical nuclei, and naloxone reversed the morphine actions.[41]

*Morphine on NO Release*

Acute exposure of human saphenous or internal thoracic artery endothelium or rat microvascular endothelial cells to morphine resulted in nitric oxide release *via* the $\mu$3-opiate receptor subtype.[42,43] Intravenous morphine increased norepinephrine, acetylcholine, and nitrite in spinal dorsal horn microdialysate in anesthetized sheep, and these effects were antagonized by intrathecal injection of the $\alpha_2$ adrenoceptor blocker idazoxan, atropine, or L-NMMA.[44] Spinally released nitric oxide appears to play a role in the analgesic effects of systemic opioids. Fimiani *et al.*[45] noted that nitric oxide release was mediated through the $\mu$3-opioid receptor, and morphine-stimulated nitric oxide release was higher in human surgical specimens of nonsmall-cell lung carcinoma than in those of the normal lung. Zhu *et al.*[46] provided evidence that morphine biosynthesis occurs in rat brain amygdala, and morphine releases nitric oxide in limbic tissues. Morphine and dopamine induced a transient surge of nitric oxide production in endometrial glandular epithelial cells, free of endothelial cells, isolated from human endometrial specimens.[47] How increased nitric oxide release contributes to regulation of endometrial cell functions is indeed an intriguing mechanism to investigate. The $\mu$-opioid receptor-specific antagonist $\beta$-funaltrexamine inhibited nitric oxide release from endomorphine 1–treated rodent and human immune cells.[48] There was evidence suggesting that by inhibiting nNOS and reducing nitric oxide levels, asymmetric dimethylarginine decreases $\mu$-opioid receptor constitutive activity in mice.[49]

On the other hand, Pu *et al.*[50] obtained findings that suggest the existence of a dual-control mechanism composed of the excitatory NMDA and the inhibitory $\mu$-opioid receptors in modulating cyclic GMP/nitric oxide release in the medial preoptic area of the rat brain.

## Modulation of Morphine Actions by NO Analgesia

*Supraspinal Site of Action*

The central analgesic effect of morphine as tested by the rat paw pressure and tail flick tests was inhibited by intracerebroventricular injection of methylene blue and potentiated by an inhibitor of cyclic GMP phosphodiesterase, but it was not blocked by the NOS inhibitors L-NMMA and *N*-iminoethyl-L-ornithine.[51,52] Therefore, activation of the cyclic GMP system, not *via* nitric oxide release, may be involved in the mechanism of the central analgesic effect of morphine. Antinociception induced by the muscarinic receptor agonist (+)-cis-dioxolane, but not $\beta$-endorphin, given supraspinally is likely mediated by the direct activation of an nitric oxide/cyclic GMP system, and the activation by the muscarinic agonist potentiates the antinociception induced by intracerebroventricular $\beta$-endorphin, but not that by $\mu$-, $\delta$-, or $\kappa$-opioid receptor agonists.[53] Intracerebroventricular L-NA diminished the morphine-induced analgesia, and L-arginine administered by the same route increased the analgesic effect of morphine, indicating that increased nitric oxide synthesis may potentiate morphine analgesia.[54] L-Arginine and the nitric oxide donor 3-morpholinosydnoimine intracerebroventricularly administered to mice produced antinociceptive effects that were blocked by naloxone and also by intracerebroventricular administration of a rabbit antiserum against rat dynorphin 1-13; the antinociceptive effect of L-arginine was antagonized by an inhibitor of nNOS (table 1).[55] The mechanisms for the antinociceptive action of L-arginine and 3-morpholinosydnoimine appear to be mediated by dynorphin and dependent on nitric oxide. Javanmardi *et al.* [56] noted that mesencephalic morphine antinociception was reduced when MK-801 and L-NAME were microinjected sequentially into the rostral ventromedial medulla in rats, and this reduction was not significantly different from the effects of MK-801 or L-NAME alone, implying that NMDA receptors and nitric oxide production in the rostral ventromedial medulla modulate the transmission of opioid pain-inhibitory signals from the periaqueductal gray.

On the other hand, L-NAME produced an opioid-independent antinociception in the mouse, probably by a direct effect within the brain.[57] In awake dogs, L-NA or morphine perfused through the fourth ventricle increased the nociceptive threshold, and combined morphine/L-NA perfusions produced a greater antinociceptive effect than seen when morphine was given alone, suggesting that endogenous nitric oxide, produced at supraspinal sites, acts as a nociceptive mediator.[58] Additional information in large mammals is required to extrapolate data to humans. In mice, chronic intraperitoneal administration of L-arginine decreased the antinociceptive response to subcutaneously administered morphine-6-$\beta$-D-glucuronide, a potent metabolite of morphine, whereas the response to intracerebroventricularly administered morphine-6-$\beta$-D-glucuronide was unaffected by L-arginine treatment; the decreased antinociceptive response to subcutaneous morphine-6-$\beta$-D-glucuronide was reversed by L-NA, suggesting that the decreased antinociceptive response of peripherally administered morphine-6-$\beta$-D-glucuronide by L-arginine may be related to a decrease in morphine-6-$\beta$-D-glucuronide entry into brain structures responsible for antinociceptive action.[59] Similar results were also obtained with morphine.[60,61] Bhargava *et al.*[62] obtained evidence suggesting that chronic intraperitoneal administration of

**Table 1. Evidence for NO as an Analgesic Mediator at Supraspinal, Spinal, and Peripheral Sites in Experimental Animals**

| Author, Year | Species | Method | Analgesic (Route) | Treatment (Route) | Effect | Analgesic Mechanism |
|---|---|---|---|---|---|---|
| Pataki and Telegdy, 1998[54] | Mouse | Tail-flick test | Morphine (icv) | L-arginine (icv) | Potentiation of analgesia | NO |
| Javanmardi *et al.*, 2005[56] | Rat | Tail-flick test | Morphine (PAG) | L-NAME (RVM) | Inhibition of analgesia | NO |
| | | | MK-801 (RVM) | | Inhibition of analgesia | NMDA receptor |
| Kolesnikov *et al.*, 1997[66] | Mouse | | Morphine (sc, ith) | AS to nNOS-2 (ith) | Block of analgesia | NO *via* nNOS-2 |
| | | | | AS to nNOS-1 (ith) | No effect | |
| Song *et al.*, 1998[67] | Rat | Paw withdrawal from heat source | Morphine (iv) | NOSI, NO scavenger or $\alpha_2$-AB (ith) | Inhibition of analgesia | NO, $\alpha_2$-Adrenoceptor |
| Hayashida *et al.*, 2003[68] | Rat | Formalin-test | BLF (ith) | L-NAME (ith) | Inhibition of analgesia | NO |
| | | | Morphine (ith) BLF + morphine* | $\mu$-Antagonist (ith) | Inhibition of analgesia | $\mu$-Opioid receptor |
| Chen *et al.*, 2003[69] | DM rat | Paw pressure test | DPDPE (ith) | L-NMMA (ith) or carboxy-PTIO (ith) | Inhibition of analgesia | NO |
| Durate *et al.*, 1990[76] | Rat | Paw pressure test | ACh (paw) | L-NMMA (ip) | Inhibition of analgesia | NO/cyclic GMP |
| | | | ACh and SNP (paw) | MY 5445 (paw) | Potentiation of analgesia | |
| Aquirre-Banuelos and Granados-Soto, 1999[80] | Rat | Formalin-test | Morphine (paw) | L-NAME (paw) | Inhibition of analgesia | NO |
| | | | Morphine + dipyron* (paw) | L-NMMA (paw) | Inhibition of analgesia | NO |
| Nozaki and Yamamoto, 1998[79] | Rat | Formalin-test | Morphine (paw) morphine + FK409* (paw) | carboxy-PTIO (paw) | Inhibition of analgesia | NO $\alpha_2$-Adrenoceptor |
| Mixcoatl-Zexuatl *et al.*, 2000[81] | Rat | Formalin-test | Morphine (paw) | L-NAME (paw) or ODQ (paw) | Inhibition of analgesia | NO/cyclic GMP |
| | | | | Sildenafil (paw) | Potentiation of analgesia | |
| Jain *et al.*, 2003[82] | Rat and mouse | Carrageenin (rat) Acetic acid–writhing (mouse) | Sildenafil (paw, ip) | L-NAME (paw, ip) | Inhibition of analgesia | NO |
| | | | Morphine + sildenafil* (paw) | MB (paw, ip) | Inhibition of analgesia | Cyclic GMP |
| Hayashida *et al.*, 2004[83] | Rat | Formalin-test | Morphine (paw) | BLF (paw) | Potentiation of analgesia | |
| | | | Morphine + BLF (paw) | L-NAME (paw) | Inhibition of analgesia | NO |
| Oritz *et al.*, 2005[84] | Rat | Formalin-test | Codeine (paw) | L-NAME, MB, GLC, 4-AP, or TEA (paw) | Inhibition of analgesia | NO/cyclic GMP/ $K^+$-channel |

* Potentation by combined use.

4-AP = 4-aminopyridine; $\alpha_2$-AB = $\alpha_2$-adrenoceptor blocker; ACh = acetylcholine; AS = antisense; BLF = bovine lactoferrin; carboxy PTIO = nitric oxide scavenger; DM = diabetes mellitus; DPDPE = [D-Pen2,D-Pen5]-enkephalin; FK409 = nitric oxide releaser; GLC = glibenclamide; GMP = guanosine monophosphate; icv = intracerebroventricular; ip = intraperitoneal; ith = intrathecal; iv = intravenous; L-NAME = $N^G$-nitro-L-arginine methylester; L-NMMA = $N^G$-monomethyl-L-arginine; MB = methylene blue; MY 5445 = cyclic GMP phosphodiesterase inhibitor; nNOS = neuronal nitric oxide synthase; NO = nitric oxide; NOSI = NOS inhibitor; ODQ = soluble guanylyl cyclase inhibitor; PAG = periaqueductal grey; RVM = rostral ventromedial medulla; sc = subcutaneous; SNP = sodium nitroprusside; TEA = tetraethylammonium.

L-arginine reduced the antinociceptive effect of morphine by increasing brain NOS activity and by decreasing the concentration of morphine in certain brain regions and the spinal cord in mice. Acute activation of the nitric oxide system by L-arginine administration attenuated morphine antinociception, possibly by inhibiting its uptake in central sites (midbrain and spinal cord) involved in antinociceptive actions.[63] Streptozotocin-induced diabetes in mice markedly decreased the antinociceptive effect of intracerebroventricularly administered morphine and increased the urinary nitrite concentration; administration of aminoguanidine improved the effect of morphine and attenuated the increase in urinary nitrite concentration, indicating that an increase in nitric oxide formation by iNOS may be responsible for the observed decrease in antinociceptive effect of morphine in diabetic mice.[64]

### *Spinal Site of Action*

Nitric oxide acts as a modulator of dorsal horn spinal cord nociceptive pathways. NOS immunoreactivity was

**Table 2. Evidence for NO as an Algesic Mediator at Supraspinal, Spinal, or Systemic Sites in Experimental Animals**

| Author, Year | Animal | Method | Analgesic (Route) | Treatment (Route) | Effect | Mechanism |
|---|---|---|---|---|---|---|
| Pelligrino *et al.*, 1996[58] | Dog | Hindpaw withdrawal threshold | Morphine (icv) | L-NA (icv) | Increased nociceptive threshold | NO as a nociceptive |
| Grover *et al.*, 2000[64] | DM mouse | Tail-flick test | Morphine (icv) | Aminoguanidine (ip) | Potentiation of analgesia | NO formed by iNOS as a nociceptive |
| Przewlocki *et al.*, 1993[71] | Rat | Tail-flick and paw pressure tests | Morphine (ith) | L-NAME (ith) or Hemoglobin (ith) | Potentiation of analgesia | NO as a nociceptive |
| | | | | SIN-1 | Inhibition of analgesia | |
| Yamaguchi and Naito, 1996[70] | Rat | Tail-flick on hot plate | Morphine (ith, ep, iv) L-NAME (ith, ep, iv) | L-NAME (ith, ep, iv) | Potentiation of analgesia | NO as a nociceptive |
| Machelska *et al.*, 1997[72] | Rat | Tail-flick and paw pressure tests | Morphine, $\mu$-agonist, or $\delta$-agonist (ith) | L-NAME or 7-NI (ith) | Potentiation of analgesia | NO as a nociceptive<br>$\mu$- and $\delta$-opioid receptor |
| Li and Clark, 2001[73] | Mouse | Hot plate test | Morphine (ith) | | Increase in cGMP production | |
| | | | Morphine (ith) | nNOS/HO-2 null | No effect on cGMP | |
| | | | Morphine (ith) | L-NAME (ith) | Inhibition of morphine-stimulated cGMP | NO as a nociceptive |
| | | | | Sn-P (ith) | | CO as a nociceptive |
| Brignola *et al.*, 1994[89] | Mouse | Hot plate, tail-flick, and acetic acid–writhing | Morphine (icv) | L-arginine (ip) | Inhibition of analgesia | L-arginine/NO as a nociceptive |
| | | | | L-NMMA (ip) | Block of L-arginine action | |
| | | | | L-NAME (ip) | | |

7-NI = 7-nitroindazole; CO = carbon monoxide; DM = diabetes mellitus; ep = epidural; HO-2 = heme oxygenase-2; icv = intracerebroventricular; iNOS = inducible nitric oxide synthase; ip = intraperitoneal; ith = Intrathecal; iv = intravenous; L-NA = $N^G$-nitro-L-arginine; L-NAME = L-NA methylester; L-NMMA = $N^G$-monomethyl-L-arginine; NO = nitric oxide; SIN-1 = nitric oxide donor; Sn-P = HO inhibitor.

present in both humans and rats with similar distribution, being present in primary sensory neurons of dorsal root ganglia and their afferent terminals in the dorsal horn of spinal cord.[65] nNOS-immunoreactive interneurons were found in the superficial layer of the dorsal horn and the intermediolateral cell column.

Kolesnikov *et al.*[66] noted that an antisense probe selectively targeting nNOS-2 blocked morphine analgesia and suggested that the facilitating nNOS-2 system predominates at the spinal level over the supraspinal level. Intravenous administration of morphine produced antinociception in rats, and an $\alpha_2$-adrenoceptor antagonist, NOS inhibitors, and a nitric oxide scavenger that were intrathecally injected produced attenuation of morphine-induced antinociception.[67] It appears that a spinal $\alpha_2$-adrenergic mechanism is involved in antinociception from intravenously administered morphine and that spinal nitric oxide mediates antinociception produced by morphine. Spinally applied bovine milk-derived lactoferrin (BLF) produced $\mu$-opioid receptor-mediated analgesia that was reversed by coadministration of L-NAME in the rat formalin test, and it potentiated the analgesia induced by morphine, suggesting that BLF acts as an enhancer of the spinal $\mu$-opioidergic system *via* a nitric oxide-mediated mechanism.[68] Rats rendered diabetic with streptozotocin developed a mechanical hyperalgesia, and intrathecal [D-Pen2,D-Pen5]-enkephalin, a $\delta$-opioid receptor agonist, increased the withdrawal threshold in response to noxious pressure in diabetic rats to a greater extent than in normal rats; intrathecal L-NMMA or the nitric oxide scavenger carboxy PTIO diminished the analgesic action of the $\delta$-opioid receptor agonist in both normal and diabetic rats.[69] Spinal endogenous nitric oxide seems to contribute to the analgesic action of intrathecal [D-Pen2,D-Pen5]-enkephalin in both normal and diabetic neuropathic pain conditions. Table 1 summarizes the data indicating that morphine and nitric oxide show antinociceptive effects and that inhibitors of the nitric oxide/cyclic GMP pathway attenuate the analgesic effect of morphine or other opioids.

In contrast, there are findings indicating that NOS inhibition results in analgesic action and potentiates morphine-induced antinociception (table 2). Morphine or L-NAME given intrathecally, epidurally, or

intravenously produced antinociceptive effects as assessed by tail flick latency in response to thermal stimulation, and coadministration of small doses of L-NAME and morphine produced reductions of the median effective doses for morphine.[70] L-NAME given *via* three different routes appears to have a synergistic antinociceptive interaction with morphine in response to thermal stimulation. L-NAME and morphine coadministered intrathecally elicited a profound and long-lasting antinociception, which was abolished by intrathecal administration of the NO donor 3-morpholinosydnoimine.[71] Intrathecally injected 7-NI and L-NAME enhanced antinociception induced by morphine or by agonists of $\mu$- and $\delta$-opioid receptors in rat tail-flick, paw pressure, and formalin tests; however, coadministration of L-NAME and a $\kappa$-opioid receptor agonist produced antinociception in the paw pressure test only, showing that the inhibition of spinal NOS appears to potentiate the $\mu$- and $\delta$-mediated spinal antinociception and, to a lesser extent, $\kappa$-mediated spinal antinociception.[72] The use of the nNOS-selective inhibitor 7-NI excludes the possible involvement of cerebral vasoconstriction and systemic blood pressure increase in the enhancing effect of NOS inhibition on morphine actions. L-NAME lost the ability to potentiate the analgesic actions of intrathecally administered morphine in nNOS null-mutant mice, and the heme oxygenase inhibitor no longer potentiated morphine-induced analgesia in mice lacking a functional heme oxygenase gene.[73] In addition, the intrathecal injection of the cyclic GMP analog caused hyperalgesia in the hot plate assay; in spinal cord slices from either nNOS or heme oxygenase null-mutant mice, morphine did not stimulate cyclic GMP production. The authors suggested that spinal monoxide generation modifies the acute analgesic actions of morphine.

Behavioral responses, such as vocalization and agitation, to intrathecal injection of high-dose morphine in rats were not reversed by naloxone but were inhibited by pretreatment with NMDA-receptor antagonists and L-NAME; the intrathecal injection of morphine evoked increases in nitric oxide metabolites and glutamine in the extracellular fluid of dorsal spinal cord that were reduced by antagonists against L-NAME and NMDA receptors, suggesting that the excitatory action of high-dose intrathecal morphine may be mediated by an NMDA–nitric oxide cascade in the spinal cord.[74] These authors[75] also noted that injections of formalin into the plantar surface of the paw evoked a biphasic spinal release of nitrite/nitrate and a transient release of glutamate; these effects, together with flinching and licking/biting, were reduced by intrathecal, combined administration of L-NAME and morphine, suggesting that L-NAME may enhance morphine-induced antinociception through an increased inhibition of nitrite/nitrate and glutamate releases evoked by formalin injection at the spinal cord level.

*Peripheral Site of Action*

Acetylcholine and sodium nitroprusside, which releases nitric oxide nonenzymatically, caused antinociception in the rat paw made hyperalgesic with prostaglandin $E_2$, and these analgesic effects were enhanced by intraplantar injection of the inhibitor of cyclic GMP phosphodiesterase MY5445 and blocked by the guanylyl cyclase inhibitor methylene blue; the analgesia induced by acetylcholine, but not sodium nitroprusside, was blocked by L-NMMA, and L-arginine had no effect on prostaglandin-induced hyperalgesia but caused analgesia in paws inflamed with carrageenin.[76] Peripheral analgesia induced by acetylcholine and morphine was potentiated by MY5445 and blocked by L-NMMA, whereas central morphine analgesia was potentiated by MY5445 but not affected by L-NMMA, suggesting that nitric oxide causes peripheral analgesia *via* stimulation of the nitric oxide/guanylyl cyclase system and that the central analgesic effect of morphine is associated with activation of the cyclic GMP system that is not mediated by nitric oxide.[51] Involvement of the L-arginine/nitric oxide/cyclic GMP pathway in peripheral morphine analgesia was also reported.[77,78] FK409, a nitric oxide releaser, alone had no effect on the number of flinches induced by formalin injection in rats; however, when administered after intraplantar morphine, FK409 depressed the agitation behavior, and this inhibitory effect was reversed by naloxone and carboxy-PTIO.[79] In the rat formalin test, morphine (10 $\mu$g, ipsilateral intraplantar injection) produced antinociception. However, contralateral injection of morphine did not produce any antinociceptive effect, an indication that the local administration of morphine did not result in a systemic drug distribution.[80] Moreover, in the dipyrone-morphine combination study, the dose of morphine used (1.25 $\mu$g) was lower than the dose used for the study with morphine alone. Coadministration of sildenafil, an inhibitor of phosphodiesterase-5, enhanced the antinociceptive effect of morphine; and pretreatment of the paw with L-NAME, 1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, or naloxone blocked the effect of the combination of sildenafil and morphine, suggesting that sildenafil itself produces antinociception and increases that induced by morphine, probably through the inhibition of cyclic GMP degradation.[81] Sildenafil also exhibited an antinociceptive effect against the paw pressure test in rats and the writhing test in mice, and pretreatment with L-NAME, methylene blue, or naloxone (intraplantar injection) blocked the effect of the sildenafil-morphine combination.[82] Peripheral coadministration into the paw of morphine with a subeffective dose of BLF produced a potentiated antinociceptive effect compared to that of morphine alone in the rat formalin test, and this poten-

tiating effect was reversed by L-NAME and a $\mu$-opioid receptor antagonist.[83] Local peripheral injection of codeine produced an antinociception in the rat formalin test, and local pretreatment of paws with L-NAME, methylene blue, ATP-sensitive $K^+$ channel inhibitors (glybenclamide and tolbutamide), nonselective voltage-dependent $K^+$ channel inhibitors (4-aminopyridine and tetraethylammonium), or naloxone prevented codeine-induced antinociception.[84] Codeine appears to activate the opioid receptor- nitric oxide-cyclic GMP-$K^+$ channels pathway to produce its effect.

Stanojevic *et al.*[85] obtained data suggesting that the rat strain (Albino Oxford and Dark Agout)–dependent opposing effects of $\beta$-endorphin on paw inflammation are mediated through $\delta$- and $\kappa$-opioid receptors and probably involve changes in the production of reactive oxygen species.

*Actions of Systemically Administered Morphine*

On the basis of the phenyl benzoquinone-induced abdominal constriction test[86] in mice, morphine, mepyramine ($H_1$-receptor antagonist), and L-arginine produced antinociception; L-arginine increased the antinociceptive effect of morphine and mepyramine, and L-NAME decreased the antinociception induced by these agents in combination with L-arginine.[87] It appears that morphine and mepyramine produce peripheral antinociception through involvement of the L-arginine/nitric oxide cascade or other related pathways of nociceptive processes induced by nitric oxide. Brief and continuous footshock stress (3 min) induced a naloxone-insensitive antinociception that was not altered by either L-NAME, aminoguanidine, or L-arginine in mice; in contrast, prolonged and intermittent footshock (30 min) induced a naloxone-reversible antinociceptive effect that was blocked by L-NAME but not by aminoguanidine or L-arginine. In addition, morphine increased the antinociceptive effect of prolonged footshock, and this increase was inhibited by L-NAME but not by aminoguanidine.[88] Based on these observations, the authors concluded that nitric oxide of constitutive origin may be selectively involved in an opioid-mediated type of footshock stress antinociception in mice.

However, there is evidence suggesting that nitric oxide counteracts the analgesic actions of morphine. L-Arginine administered orally or intraperitoneally, but not intracerebroventricularly, reduced the antinociceptive effect of morphine assessed in mice by using the hot plate, tail flick, and acetic acid-induced writhing tests; L-NMMA and L-NAME reversed the effects of L-arginine.[89] Although L-NAME alone did not show any antinociceptive activity, it potentiated morphine-induced analgesia in mice; L-NAME dramatically augmented the analgesic effect of morphine in the late dark period at 19 h after the lights were turned on.[90] Morphine, L-NAME, or both increased the nociceptive threshold for a criterion response to thermal stimuli for rats, and exposure to a magnetic field abolished the analgesic effects of morphine or L-NAME when applied separately but not when injected together relative to rats that had received these drugs and had been exposed to the sham field.[91] Homayoun *et al.* [92] demonstrated that acute administration of cyclocsporin A, known to decrease nitric oxide production in nervous tissues, or L-NAME enhanced the antinociception induced by administration of morphine, whereas chronic pretreatment with cyclosporine A or L-NAME did not affect morphine-induced antinociception. L-NAME, but not the iNOS inhibitor L-canavanine, enhanced the pain threshold and potentiated morphine-induced analgesia in mice.[93] L-NAME changed the non-analgesic effect of codeine to highly significant analgesia, and naloxone abolished the L-NAME-codeine–induced analgesia, indicating that the nitric oxide modulatory effect on the opioid analgesic codeine may be exerted, at least in part, through opioid receptors.[94] Experimental diabetes in mice decreased the antinociceptive effect of morphine, and green tea extract reversed the morphine analgesia; coadministration of green tea extract and L-NAME further increased the antinociceptive effect of morphine, the stimulating effect being attenuated by administration of L-arginine. In addition, diabetes increased plasma nitrite levels that were decreased by green tea extract.[95] Increased nitric oxide formation may be responsible for the decreased antinociceptive effect of morphine in diabetic mice, and green tea extract appears to restore the antinociceptive effect of morphine by inhibition of nitric oxide production.

The analgesic effect of morphine injected intraperitoneally exhibited biologic time-dependent differences in the thermally induced algesia in mice, whereas exogenously administered peroxynitrite exhibited either an algesic or analgesic effect, depending on the circadian time of its injection; concomitant administration of peroxynitrite and morphine reduced morphine-induced analgesia.[96]

*Human Studies*

Randomized studies on patients with cancer pain who reported pain despite taking 80–90 mg of oral morphine daily indicated that the daily consumption of oral morphine on day 30 was greater in the group that received an additional 20 mg of oral morphine and the group that received 500 mg of oral dipyrone at 6-h intervals than in the group that received a 5-mg nitroglycerin (nitric oxide donor) patch daily and the group that received 0.5 mg/kg oral ketamine at 12-h intervals, suggesting that low-dose ketamine and transdermal nitroglycerin are effective coadjuvant analgesics.[97] In conjunction with their opioid tolerance-sparing function, joint delivery of ketamine or nitric oxide donors with opiates may be of benefit in cancer pain management. In another randomized, double-blind study on cancer pain patients[98] who complained of pain despite taking 80 to 90 mg of oral

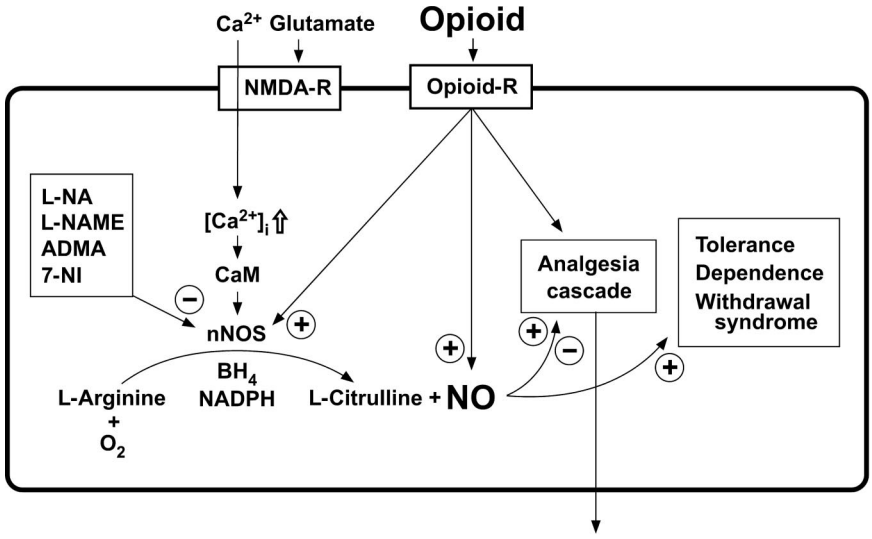


**Fig. 1. Possible mechanism underlying interactions between μ-opioid receptor agonists and nitric oxide (NO) generated *via* NMDA-receptor stimulation in antinociceptive processes. Effects of NO on tolerance to and dependence on opioids and their withdrawal syndrome are also presented. NMDA-R, *N*-methyl-D-aspartate-receptor; opioid-R, opioid receptor; $[Ca^{2+}]i$, intracellular concentrations of $Ca^{2+}$; CaM, calmodulin; $BH_4$, tetrahydrobiopterin; NADPH, reduced nicotinamide adenine dinucleotide phosphate; +, stimulation; –, inhibition; 7-NI, 7-nitroindazol; ADMA, asymmetrical dimethyl arginine; L-NA, $N^G$-nitro-L-arginine; L-NAME, L-NA methylester; nNOS, neuronal nitric oxide synthase.**

morphine daily and were allowed to freely manipulate their daily morphine consumption at the time the test drug (placebo patch or 5 mg/24-h nitroglycerin patch) was administered, these authors found that daily consumption of oral morphine was smaller in the nitroglycerin group compared with the placebo group, when evaluated on the fourteenth day after initiation of the study; patients from the placebo group in general complained of somnolence, compared with the nitroglycerin group. Delivery of nitric oxide donors together with opioids may be of significant benefit in cancer pain management. This idea was further evaluated by Iohom *et al.*[99] who performed studies on patients undergoing breast surgery with axillary clearance who were randomly allocated into one of two groups: group S received a standard intraoperative and postoperative analgesic regimen (morphine, diclofenac, dextropropoxyphene + acetaminophen), and group N received a continuous paravertebral block (for 48 h) and acetaminophen and parecoxib. They found that 80% of the patients in group S and no patient in group N developed chronic postsurgical pain, and the plasma concentrations of nitrite/nitrate were greater in group N compared with group S 48 h postoperatively. Whether increased nitric oxide production is involved in the analgesic efficacy could not be determined.

Figure 1 summarizes the scheme of NMDA-receptor stimulation that mediates nNOS activation *via* an increase in intracellular $Ca^{2+}$ concentrations and thereby the release of nitric oxide that modifies the opioidergic analgesic cascade. The nNOS activity and nitric oxide release are enhanced mainly by μ-opioid receptor stimulation. Up-to-date reports in the literature on studies employing experimental animals have led to the conclusion that nitric oxide appears to participate in potentiation of analgesia induced by morphine administered to peripheral sites (paw) *via* μ-opioid receptors; the nitric oxide/cyclic GMP pathway may be involved (table 1). However, some studies with intrathecally administered morphine and NOS inhibitors have led to conclusions that endogenous nitric oxide, possibly formed through nNOS, plays significant roles as an antinociceptive agent and/or an enhancer of morphine antinociception (table 1), but other researchers report that NOS inhibitors enhance the antinociceptive effect of morphine or other opioids (table 2). Differences in animal species, NOS inhibitors, and nitric oxide scavengers employed in the various experiments do not appear to account for the opposite findings. A difference is only seen in diabetic mice, in which nitric oxide derived from iNOS acts as a nociceptive when morphine is administered intraventricularly.[54] Nitric oxide formed in large amounts by iNOS may contribute to lowering the pain threshold. Sousa and Prado[100] noted that low doses of the nitric oxide donor 3-morpholinosydnoimine reduced and higher doses enhanced or had no effect on the mechanical allodynia evoked by chronic ligature of rat sciatic nerve, suggesting that nitric oxide produces a dual effect in a model of neuropathic pain. However, it might be too speculative to postulate that excessive concentrations of nitric oxide produced through iNOS or nNOS *via* activation of NMDA receptors during nociceptive stimuli (tail-flick, hot plate tests mainly used in studies appearing in table 2) participate in promoting nociception. At present, there is only limited information available to support the beneficial use of nitric oxide as an analgesic in patients. However, data on patients are quite important because one frequently faces the problem of species variations (primate *vs.* subprimate mammals) in the actions and mechanisms of action of endogenous molecules or therapeutic compounds. More data on healthy individuals and patients are required to introduce a qualified strategy of opioidergic analgesic treatment.

*Tolerance*

The analgesic response to subcutaneous morphine given daily was abolished within 5 days in mice, and coadministration of L-NA with morphine prevented the

development of tolerance for at least 11 days.[101] Together with the finding that the NMDA-receptor antagonist MK-801 prevented the development of tolerance to morphine, the authors suggested that morphine tolerance involves the activation of NMDA receptors followed by the subsequent release of nitric oxide. Preventive effects of competitive and noncompetitive NMDA antagonists and L-NA against the development of morphine tolerance were also reported.[102] Administration of L-NA or L-NAME along with morphine prevented the development of tolerance to morphine and also attenuated some signs of morphine dependence in mice.[103] Downregulation by antisense treatment of nNOS-1 prevented the development of morphine tolerance in mice.[66] Neurons in rat locus coeruleus expressed opioid receptors, and these neurons exhibited tolerance to chronic administration of opioids; the average median effective dose (obtained from curves for morphine dose *vs.* single cell extracellular activity response) for morphine of the locus coeruleus cells from rats who received L-NA injections and morphine pellets was similar to that in cells from control rats, and the median effective dose of cells from morphine pelleted rats who received saline injections was substantially higher.[104] Nitric oxide inhibition appears to attenuate the development of tolerance to morphine in locus coeruleus neurons. Cyclosporin A that decreases nitric oxide production in nervous tissues, L-NAME, and a combination of the two at *per se* noneffective doses inhibited the induction and expression of tolerance to morphine-induced antinociception, and aminoguanidine did not alter morphine tolerance, suggesting the involvement of decreasing nitric oxide production through constitutive NOS, but not iNOS, in the modulation of morphine tolerance.[105]

After implantation of morphine pellets in mice, the analgesic response was abolished on the third day, coadministration of L-NA with the pellets markedly retarded the development of tolerance, and L-NA slowly reversed preexisting tolerance; L-NA did not prevent tolerance to analgesia mediated by a $\kappa$1 or $\kappa$3 agonist.[106] Multiple injections of [D-Pen2,D-Pen5]enkephalin ($\delta$1-opioid receptor agonist), deltorphin II ($\delta$2-opioid receptor agonist), or morphine resulted in the development of tolerance to their analgesic action in mice; concurrent administration of L-NA or L-NMMA had no effect on the development of [D-Pen2,D-Pen5]enkephalin or deltorphin tolerance but inhibited the development of morphine tolerance.[107,108] Development of tolerance to the antinociceptive activity of morphine and the $\kappa$-opioid receptor agonist U-50,488H was inhibited by 7-NI, which did not modify the development of tolerance to the activity of [D-Pen2,D-Pen5]enkephalin in mice.[109] It appears that inhibition of nNOS activity inhibits tolerance to the antinociceptive activity of $\mu$- and $\kappa$- opioid receptor agonists but not $\delta$-opioid receptor agonists. Development of acute antinociceptive tolerance to intracerebroventricular morphine in mice was blocked by L-NAME, L-NMMA, 7-NI, and 3-bromo-7-NI and also by a guanylyl cyclase inhibitor.[110] Nitric oxide formed by nNOS acting through the cyclic GMP pathway appears to mediate the development of acute antinociceptive tolerance. Subchronic administration of 7-NI attenuated the development of morphine tolerance to the cellular and analgesic effects of $\mu$-opioid receptor agonists in rats.[111] Herraez-Baranda *et al.*[112] provided evidence indicating that $\kappa$-opioid receptors and nNOS in the same intracellular network of the rat periaqueductal gray appears to control the development of morphine tolerance and dependence. Blockade of nitric oxide overproduction, the consequence of NMDA-receptor activation by aminoguanidine *via* inhibition of iNOS, attenuated the development of morphine tolerance and dependence.[113]

In mice implanted subcutaneously with morphine pellets, NOS activity measured from the rate of conversion of [$^3$H]arginine to [$^3$H]citrulline in the cerebral cortex and cerebellum increased; morphine-implanted mice had higher Vmax values, but the Km values did not differ from those of control mice.[114] Chronic treatment with morphine seems to increase NOS activity in the brain without modifying its substrate affinity. L-Arginine accelerated tolerance when it was coadministered with morphine and decreased morphine's potency in mice when given alone.[39] In superfused rat hippocampal slices, the amplitude of population spikes recorded by a glass microelectrode was increased by morphine; after continuous morphine superfusion, this effect was deteriorated (*i.e.*, tolerance developed), which also increased the nitrite level in the superfusate; cosuperfusion of L-arginine with morphine further increased the nitrite level and facilitated the development of morphine tolerance.[115] Morphine administration increased the nitric oxide production in hippocampal microdialysate in rats; the time course of altered nitric oxide production coincided with the development of antinociceptive tolerance.[116] After sustained morphine administration, nNOS-deficient mice exhibited less tolerance development compared to the control group, although measurable tolerance still occurred; mice deficient in endothelial NOS showed a degree of tolerance similar to that of the control animals.[117] In addition, tolerance development appears to be predominantly a nitric oxide-mediated process, but it is likely augmented by a secondary (non–nitric oxide) pathway. Heinzen *et al.* [118] provided evidence for important nitric oxide-induced alterations in $\mu$-opioid receptor functionality that directly lead to the development of opioid antinociceptive tolerance. Asymmetric dimethylarginine, a major circulating form of methylarginines in humans and animals, competes with L-arginine for the active site of NOS isoforms.[119] Kielstein *et al.*[120] noted that increased nitric oxide production in mice resulted in an enhanced development of tolerance

to morphine; nitric oxide increased constitutive $\mu$-receptor activity; 7-NI attenuated morphine withdrawal in opioid-dependent rats. It was hypothesized that by inhibiting nNOS and reducing nitric oxide levels, asymmetric dimethylarginine may decrease $\mu$-opioid receptor constitutive activity, resulting in alteration of the analgesic dose–response curve of morphine. According to Muscoli *et al.*,[121] morphine-induced antinociceptive tolerance in mice was associated with increased formation of proinflammatory cytokines and oxidative DNA, and inhibition of nitric oxide synthesis or removal of superoxide blocked these biochemical changes and inhibited the development of tolerance; coadministration of morphine with a peroxynitrite decomposition catalyst attenuated protein nitration and the observed biochemical changes and prevented the development of tolerance. Peroxynitrite decomposition catalysts may have therapeutic potential as adjuncts to opioids in relieving suffering from chronic pain.

Chronic administration of morphine resulted in the development of tolerance to the analgesic action of morphine, and L-NMMA attenuated the tolerance in rats at a higher dose than in mice.[122] Induction phase L-arginine slowed the development of opioid tolerance and physical dependence, while L-NAME and L-NMMA led to a higher degree of tolerance but had no effect on the development of physical dependence; expression-phase NOS inhibition attenuated morphine tolerance and reduced the incidence of withdrawal signs.[123] A 5-day treatment with increasing doses of morphine in rats induced antinociceptive tolerance, which was attenuated by L-NAME, whereas tolerance to the effect of morphine on thyrotropin (decrease) and prolactin (increase) levels was not modified by L-NAME.[124] Both nitric oxide-dependent and nitric oxide-independent mechanisms may be involved in the development of tolerance to the various effects of morphine. Additional experimentation is needed to determine the mechanism underlying the nitric oxide action on morphine tolerance.

On the other hand, combined administration of BLF with morphine retarded the development of tolerance to morphine in mice; this effect of BLF was partially blocked by L-NAME or methylene blue and completely blocked by 7-NI, suggesting that BLF blocks the development of tolerance to morphine, possibly *via* the selective activation of nNOS.[125]

*Spinal Tolerance*

Intrathecal coadministration of L-NAME with spinal morphine produced only a small attenuation of tolerance in rats, showing little effect of nitric oxide on morphine tolerance at spinal sites.[126] There was evidence indicating that spinal cyclooxygenase activity, and to a lesser extent NOS activity, may contribute to the development and expression of opioid tolerance.[127] In a rat spinal model, coadministration of MK-801 inhibited the development of morphine tolerance; the binding affinity of [$^3$H]MK-801 was higher in lumbar spinal cords of morphine-tolerant rats than control rats, and constitutive expression of nNOS protein was also higher in the morphine-tolerant group, this upregulation being prevented by MK-801.[128] The authors suggested that morphine tolerance affects NMDA-receptor binding activity and increases nNOS expression in rat spinal cord. Pretreatment with midazolam inhibited the development of acute and chronic morphine tolerance in mice, and this was reversed by intrathecal injection of L-arginine; in chronic morphine-tolerant rats, midazolam decreased formalin-induced expression of Fos and Fos/NADPH diaphorase double-labeled neurons in the contralateral spinal cord and NADPH diaphorase-positive neurons in the bilateral spinal cord.[129] It appears that decreases in both the activity and expression of NOS (nNOS and iNOS) contribute to the inhibitory effect of midazolam on the development of morphine tolerance. Intrathecal 7-NI attenuated not only the development of morphine antinociceptive tolerance, but also the activation of p38 mitogen-activated protein kinase in the spinal microglia induced by chronic intrathecal administration of morphine.[130] Attenuation of morphine tolerance by nNOS inhibition appears to be associated with reducing p38 mitogen-activated protein kinase activation in the spinal microglia. On the basis of studies measuring the spinal gene expression of heme oxygenase, NOS, soluble guanylyl cyclase, and cyclic GMP-dependent protein kinase, Liang and Clark[131] noted that the carbon monoxide/nitric oxide-cyclic GMP signaling pathway was upregulated after chronic morphine exposure in mice. Xu *et al.*[132] provided evidence suggesting that activations of metabotropic glutamate receptor subtype-5 and NMDA receptors occur after the appearance of antinociceptive tolerance to morphine in rats and that the activations of these receptors appear to play a role in the development of tolerance and expression of spinal NOS through increased concentration of [$Ca^{2+}$]i and activation of protein kinase C.

In summary, morphine tolerance involves the activation of NMDA receptors followed by subsequent release of nitric oxide formed by nNOS but not by iNOS (fig. 1); the nitric oxide/carbon monoxide-cyclic GMP pathway may participate in the induced tolerance. Morphine tolerance also appears to be partly mediated by nitric oxide-independent mechanisms.

*Dependence*

Opioid tolerance and dependence are distinct phenomena, developing independently of each other.[133,134] Administration of L-NA reduced dependence to morphine in mice with implanted morphine pellets and reversed previously established dependence.[106] Chronic administration of morphine resulted in the development of physical dependence as evidenced by the appearance of a variety of symptoms including a stereotyped jump-

ing response following naltrexone injection; concurrent treatment with L-NMMA inhibited the naltrexone-induced jumping response, but not other responses.[122] L-NAME attenuated the expression phase of morphine dependence, but it did not modify the induction phase of morphine dependence and tolerance in mice.[93] Acute and chronic administration of agmatine that inhibits NOS activity[135] prevented morphine dependence/withdrawal in wild-type mice; in contrast, agmatine reduced only peripheral signs, not the central signs, of morphine physical dependence in nNOS knockout mice, indicating that the action of agmatine in reducing the central signs requires functional nNOS.[136] In addition to inhibition of NOS activity, the effects of agmatine are mediated through imidazoline receptors, $\alpha_2$-adrenoceptors, and blockade of NMDA receptors that may involve peripheral signs of morphine dependence.

On the other hand, Pataki and Telegdy[54] reported that neither L-NA nor L-arginine affected the signs of morphine dependence, as assessed by naloxone-precipitated withdrawal in mice. In morphine-dependent rats, 7-NI did not affect naloxone's discriminative stimulus effects, but it decreased naloxone-induced weight loss and abolished expression of withdrawal signs, such as diarrhea, scream on touch, tremor, and "wet dog" like shaking, suggesting different mechanisms for subjective and somatic components of opioid withdrawal.[137] In morphine-dependent rats, pretreatment with L-NA potentiated the cataleptic response to morphine and blocked morphine-induced hyperthermia.[138] In the induction phase of morphine dependence, the $\alpha_2$-adrenoceptor agonist clonidine intensified and yohimbine attenuated the degree of dependence; L-NAME did not affect the development of dependence, but it potentiated the effect of clonidine.[139] The $\alpha_2$-adrenergic pathway seems to functionally link with the nitric oxide pathway in the modulation of opioid dependence.

In heroin abusers, the levels of lipoperoxides in plasma and erythrocytes and the plasma level of nitric oxide increased with prolonged abuse and with increased daily quantity, whereas the plasma levels of vitamins C and E and $\beta$-carotene and the erythrocyte levels of superoxide dismutase, catalase, and glutathione peroxidase decreased.[140] The balance between oxidation and antioxidation in the heroin abusers appears to be seriously impaired.

In summary, NOS inhibitors prevent morphine dependence; central signs of morphine dependence may be associated with nitric oxide derived from nNOS (fig. 1). Some investigators report that NOS inhibitors do not affect or even potentiate the signs of morphine dependence.

*Withdrawal Syndrome*

L-NA and L-NAME intraperitoneally administered 1 h before naloxone reduced "wet dog" shakes and weight loss that were evoked in morphine-dependent rats given naloxone[141] or decreased naloxone-precipitated withdrawal jumping and diarrhea in morphine-dependent mice,[142] suggesting the involvement of nitric oxide in morphine withdrawal syndrome. NOS inhibition may contribute to treatment of the opioid withdrawal syndrome. Similar results were also obtained in studies using L-NAME and isosorbide dinitrate, a nitric oxide donor, injected shortly before naloxone in morphine-dependent rats.[143] Thorat *et al.*[144] obtained data suggesting that NOS inhibitors may be more beneficial than NMDA-receptor antagonists in managing the symptoms of morphine abstinence syndrome. L-NA and 7-NI attenuated naloxone-precipitated withdrawal signs, such as rearing, jumping, ptosis, rhinorrhoea, and irritability on touch, in morphine-dependent rats.[145] Mainly central, but not endothelial, nitric oxide may be involved in the expression of some opioid withdrawal symptoms. L-NA, L-NAME, 7-NI, and $N^5$-(1-iminoethyl)-L-ornithine, a potent inhibitor of endothelial NOS, administered shortly before morphine withdrawal produced decreases in weight loss, diarrhea, wet dog shakes, and grooming; 7-NI also reduced mastication, salivation, and genital effects, and clonidine produced similar effects to 7-NI, indicating that the nNOS inhibitor attenuates more signs of opioid withdrawal than inhibitors of other types of NOS without causing hypertension.[146] Insofar as hypertension is a component of opioid withdrawal in humans, the ability of 7-NI to attenuate morphine withdrawal in rats without eliciting a vasopressor response suggests that 7-NI may have human therapeutic potential.[147] These authors[148] also obtained data suggesting that constitutive NOS isoforms, but not iNOS, have a primary role in nitric oxide-mediated processes that modulate the opioid withdrawal syndrome in the rat. There was evidence suggesting that nNOS and phospholipase $A_2$, but not iNOS, play an important role in the expression of morphine-induced withdrawal syndrome in mice, possibly by increasing free radicals.[149] When administered intracerebroventricularly, both L-NA and L-NMMA inhibited natrexone-induced stereotyped jumping behavior in morphine-dependent mice.[150] Brain nitric oxide likely plays an important role in the expression of behavioral signs of morphine withdrawal syndrome. In morphine-dependent mice, subcutaneous L-NAME reduced the number of escape jumps and other motor symptoms of abstinence, together with a decrease in NOS activity in the cerebellum, indicating a hyperactivity of the L-arginine/nitric oxide pathway in opiate withdrawal.[151] Pretreatment with subcutaneous lamotrigine, a new antiepileptic compound, reversed the withdrawal-induced increase in cerebellar $Ca^{2+}$-dependent NOS activity and reduced the number of escape jumps and other motor symptoms of abstinence; MK-801 also showed similar effects on cerebellar NOS activity and motor symptoms.[152] Treatment of morphine-dependent rats with L-NAME enhanced osmotically-stimulated oxytocin secre-

tion during naloxone-precipitated withdrawal, and sodium nitroprusside inhibited oxytocin neurons during naloxone-precipitated morphine withdrawal.[153]

Systemic and intracerebroventricular pretreatment of rats with L-NAME in a dose sufficient to inhibit brain NOS activity blocked the naloxone-precipitated locus coeruleus withdrawal response as measured with an *in vivo* voltammetric approach, and it reduced the naloxone-induced increase in the catechol oxidation current signal.[154] nitric oxide appears to play an intermediary role in the locus coeruleus neuronal hyperactivity associated with both acute and chronic morphine withdrawal. Jhamandas *et al.*[155] provided evidence by way of NADPH-diaphorase histochemistry for the activation of select populations of nitric oxide-synthesizing neurons in the paraventricular and supraoptic nuclei, and to a lesser extent in the brainstem nucleus tractus solitarius, during the opioid withdrawal syndrome. The soluble guanylyl cyclase may play an intermediary role in the genesis of locus coeruleus neuronal hyperactivity and behavioral signs of morphine withdrawal.[156] In anesthetized rats chronically treated with morphine, intraperitoneal L-NAME attenuated some signs of opioid withdrawal and also reduced the withdrawal-induced hyperactivity of locus coeruleus neurons; intraperitoneal 7-NI caused a complete blockade of the withdrawal-induced hyperactivity, and application of 7-NI to the vicinity of the locus coeruleus also caused a partial blockade, leading to the conclusion that opioid withdrawal may to be mediated by nitric oxide acting as an intermediate messenger in the locus coeruleus.[157] In morphine-treated freely moving rats, acute pretreatment with 7-NI or L-NA-p-nitroaniline (nNOS inhibitors) before naltrexone challenge attenuated the behavioral expression of morphine withdrawal and reduced the withdrawal-induced increase in 3,4-dihydroxyphenylacetic acid in the rat locus coeruleus, suggesting a role for nitric oxide in the expression of morphine withdrawal syndrome that may be mediated, at least in part, by locus coeruleus noradrenergic neurons.[158] Other noradrenergic nuclei may also be involved in the action of NOS inhibition.

Mice first made dependent to morphine that were then withdrawn by removal of pellets followed by a sublethal dose of lipopolysaccharide exhibited 100% lethality, and these animals had elevated serum tumor necrosis factor-$\alpha$ and nitric oxide levels and depressed interleukin-12 levels compared to controls; anti–tumor necrosis factor-$\alpha$ antibody given at the same time as the lipopolysaccharide challenge afforded protection to morphine-withdrawn mice.[159] Morphine withdrawal may sensitize the animals to lipopolysaccharide lethality *via* increased production of tumor necrosis factor-$\alpha$.

*Spinal Sites of Action*

Intrathecal administration of NMDA receptor antagonists, MK-801 and AP-7, reduced the expression of naloxone-precipitated cardiovascular and behavioral symptoms; L-NAME produced L-arginine-reversible inhibition of the cardiovascular component of withdrawal, but it had no effect on the expression of behavioral signs; in contrast, L-NA and L-NMMA inhibited only the expression of the behavioral signs.[160] Both spinal NMDA receptors and a nitric oxide–generating system are suggested to play a role in the expression of both the cardiovascular and behavioral components of naloxone-precipitated withdrawal. However, the fact that different structural analogs of L-arginine have different profiles of activity is puzzling. Naloxone-precipitated morphine withdrawal increased the expression of Fos protein, NADPH-diaphorase-positive neurons, and Fos/NADPH diaphorase double-labeled neurons in all the laminae of the rat spinal cord; intrathecal injection of nNOS antisense oligonucleotides inhibited the increase in Fos expression and $NMDA_{1A}$-receptor mRNA expression during morphine withdrawal and decreased the scores of morphine withdrawal symptoms.[161] The authors concluded that nitric oxide seems to mediate the increase of Fos protein and $NMDA_{1A}$-receptor mRNA expression in the spinal cord during morphine withdrawal. On the basis of immunohistochemical studies, morphine induced c-Fos expression in the striatum, cerebral cortex, and midline/intralaminar nuclei of the thalamus in rats; expression in the striatum, but not the thalamus or cortex, was blocked by 7-NI, and there was no colocalization of c-Fos and nNOS in any brain region, suggesting a role for nNOS in the neural circuits activated by morphine.[162] Cao *et al.*[163] provided evidence supporting the idea that cross talk between nitric oxide and the extracellular signal regulated kinase 1 and 2 signaling pathway mediates morphine withdrawal and withdrawal-induced spinal neuronal sensitization in morphine-dependent rats. Intrathecal injection of muscarinic $M_2$-receptor antisense oligonucleotides decreased the scores of morphine withdrawal symptoms; the expression of nNOS-positive neurons in the locus coeruleus increased in morphine-dependent rats and increased to a greater extent during morphine withdrawal, and intrathecal injection of $M_2$ antisense oligonucleotide inhibited the increase in nNOS expression.[164] $M_2$ muscarinic receptors of the spinal cord appear to mediate the increase of nNOS expression in the locus coeruleus during morphine withdrawal.

As presented so far, naloxone-precipitated withdrawal responses are attenuated by NOS inhibitors, possibly *via* the activation of guanylyl cyclase; nNOS-selective inhibitors are more effective than inhibitors of other types of NOS isoforms (fig. 1) and do not raise systemic blood pressure; these effects may be therapeutically beneficial for the prevention of withdrawal syndrome.

## Summary and Conclusion

The current article includes a summary of the interactions between morphine/other opioids and nitric oxide

in antinociception, dependence, tolerance, and withdrawal syndrome. Some investigators have reported that NOS inhibitors attenuate the antinociceptive effects of morphine; in contrast, others have shown that NOS inhibition augments morphine-induced analgesia in experimental animals. Limited studies on humans suggest the involvement of nitric oxide in morphine-induced antinociception. Dependence on morphine is either enhanced or inhibited by endogenous nitric oxide; however, tolerance to morphine and morphine withdrawal syndrome are potentiated by nitric oxide, as so far reported. Although the reason for the controversial results on morphine-nitric oxide interactions in experimental animals remains to be determined, information included in the present review should contribute to the construction of advanced strategies for therapy with morphine, with the goals of effectively eliminating nociception and minimizing side effects. However, further, consolidated studies on human materials, healthy individuals, and patients are eagerly awaited.

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## ANESTHESIOLOGY REFLECTIONS

### Bronze of M.T. "Pepper" Jenkins, M.D.

**Anesthesiologist-in-Chief at Dallas' University of Texas Southwest Medical Center (UTSWMC) from 1948–1981, Marion Thomas "Pepper" Jenkins (1917–1994) served as UTSWMC's first McDermott Chair after his professorship was funded by a grateful founding family of Texas Instruments, Inc. A codesigner of teaching laryngoscopes, Dr. Jenkins received worldwide attention after he pronounced the death of President John F. Kennedy at Parkland Memorial Hospital on November 22, 1963. After serving as American Society of Anesthesiologists (ASA) president in 1972, Dr. Jenkins received Distinguished Service Awards from the ASA in 1978 and from the American Medical Association a decade later. A trustee and vice-president of the Wood Library-Museum of Anesthesiology (WLM), Pepper delivered the WLM's 1993 Lewis H. Wright Memorial Lecture, titled "Epochs in Intravenous Fluid Therapy: From the Goose Quill and Pig Bladder to Balanced Salt Solutions." Afterwards he quipped, "Yes, Pepper loves salt." Pictured here is a bronze bust, "M.T. Pepper Jenkins, M.D.," completed by his friend and colleague, surgeon-sculptor Ben Wilson, M.D., just 1 yr after Dr. Jenkins' passing. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)**

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