Research report

Pharmacodynamics of morphine-induced neuronal nitric oxide production and antinociceptive tolerance development

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Abstract

Elevated nitric oxide (NO) production has been implicated in the development of morphine antinociceptive tolerance. This study was conducted to establish the temporal relationship between morphine-induced increases in neuronal NO and loss of pharmacologic activity. Five groups of rats equipped with microdialysis probes in the jugular vein and hippocampus received an intravenous infusion of saline or morphine (0.3, 1, 2, or 3 mg/kg/h) for 8 h. Morphine concentrations in the blood and hippocampal microdialysate were determined by LC/MS-MS; NO production was quantified with an amperometric sensor implanted in the contralateral hippocampus. Antinociceptive effect was monitored at selected time points during and following infusion by electrical stimulation vocalization. The data were fit with a pharmacokinetic/pharmacodynamic model to obtain parameters governing morphine disposition, stimulation of NO production, antinociception, and antinociceptive tolerance development. An additional three groups of rats were pretreated with L-arginine, the NO precursor (100, 300, or 500 mg/kg/h for 8 h), to elevate NO concentrations prior to morphine infusion. Morphine administration resulted in a dose-dependent increase in NO production; the time course of altered NO production coincided with the development of antinociceptive tolerance. L-arginine pretreatment initially enhanced morphine-induced analgesia early in the morphine infusion. However, this NO-associated increase in opioid response dissipated rapidly due to a dominant NO-induced loss of antinociception. Pharmacodynamic modeling suggested that this latter effect was consistent with a hyperalgesic response. These data define a strong, time-dependent relationship between morphine-induced stimulation of NO production and tolerance development, identify specific NO-induced alterations in nociceptive processing after morphine administration, and indicate that NO is a key mediator of antinociceptive tolerance development.

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Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Opioids: anatomy, physiology, and behavior

Keywords: Hyperalgesia; Morphine tolerance; Microdialysis; Nitric oxide; Pharmacokinetic/pharmacodynamic modeling

1. Introduction

Prolonged administration of opioids leads to the development of tolerance to a variety of biologic effects, including analgesia, as evidenced by increased dosage requirements to maintain pain control. Numerous homologous and heterologous cellular adaptations lead to altered opioid receptor processing in the presence of prolonged agonist exposure (for reviews, see Refs. [18,29,36,51]). In addition to molecular changes, opioid-induced modulation of endogenous substances, including cholecystokinin, dynorphin, agmatine, and nitric oxide (NO), has been observed in opioid-tolerant states [2,4,15,25,53]. One or more of these mediators could be involved in initiating or maintaining the complex cascade of time-dependent receptor and cellular changes that ultimately result in opioid tolerance.

Neuronal NO, while participating in maintenance of physiologic processes through its action as a chemical messenger, also has been identified as a key modulator of opioid tolerance development [4]. Neuronal NO is produced intracellularly through the activity of the neuronal...
isoform of NO synthase (NOS), which converts L-arginine to NO and L-citrulline. This enzyme is regulated by intracellular calcium and the N-methyl-D-aspartate (NMDA) receptor. NO also is produced by endothelial and inducible NOS isoforms, which are regulated and expressed differentially from the neuronal enzyme [44]. Due to the lability of NO and the path lengths involved, NO produced by the endothelial isoform of NOS does not act as a neurotransmitter.

Coadministration of opioids with chemical modulators of the NMDA/NOS system that reduce NO production, including NMDA receptor antagonists and NOS inhibitors, results in attenuated antinociceptive tolerance development [6,8,9,30,39,40,54]. Likewise, increases in NO production in response to exogenous L-arginine administration, in combination with and/or preceding opioid administration, result in an increased rate and extent of functional tolerance development [4,6,8]. The opioid tolerance-inducing effects of NO have been linked specifically to neuronal production of NO. Evidence for this relationship is, however, indirect and based primarily on the use of NOS isoform-selective chemical inhibitors [7,54]. More recent data obtained in mice genetically deficient in NOS isoforms also support a key role of neuronal NO in opioid tolerance [22]. Perturbations in NO production in the absence of opioid exposure do not alter nociceptive processing, suggesting that this endogenous molecule specifically modulates opioid actions to attenuate pharmacologic activity [22].

To date, the temporal relationships between opioid exposure, NO production, and antinociceptive tolerance development have not been explored. The present experiments were conducted with the prototypical opioid morphine to evaluate the kinetics of altered NO production in response to extended μ-opioid agonist administration, and to correlate the loss of pharmacologic effect with altered NO production through comprehensive pharmacokinetic/pharmacodynamic modeling.

2. Methods

2.1. Chemicals

Morphine sulfate was purchased from Spectrum Chemical (New Brunswick, NJ). [3H]morphine was obtained from American Radiolabeled Chemical (St. Louis, MO). All other reagents and solvents used in this study were obtained from commercial sources and were of the highest purity available.

2.2. NO sensor and calibration

Neuronal NO production was quantified with an electrochemical sensor (amino 7003F-EH; diameter 0.6 mm, 3 mm sensing element; Harvard Apparatus, Holliston, MA) as described previously [20]. The electrical signal was captured with an inNO-T® recorder and inNO® software (Harvard Apparatus). The NO sensors were calibrated with nitrite solutions, which were converted chemically to NO with acidic potassium iodide. Sensors were polarized in aqueous solution for a minimum of 12 h prior to calibration or implantation into an animal.

2.3. Animal preparation

Adult male Sprague–Dawley rats (320–370 g) were purchased from Charles River Laboratories (Raleigh, NC) and were housed in a temperature-controlled facility (72±2 °F). Animals were allowed free access to food and water and were maintained on a 12-h light cycle (7 am–7 pm). Animals were acclimated for a minimum of 1 week prior to experimentation. All procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, and were conducted in accordance with accepted standards for laboratory animal care.

To investigate morphine-induced NO production and tolerance development, rats were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg). A silicone rubber cannula (Helix Medical, Carpinteria, CA) and a vascular microdialysis probe (10 mm; Bioanalytical Systems, West Lafayette, IN) were implanted in the right femoral and jugular veins, respectively. Both vascular implants were exteriorized at the dorsal neck region. An intracerebral guide cannula (Bioanalytical Systems) was placed in the left hippocampal structure, and an amperometric NO sensor was placed in the right hippocampal structure, using a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and the following coordinates: bregma −5.6; lateral ±5; and DV-3 (guide cannula) or DV-7 (amperometric sensor). Dental cement was used to keep placements stationary. Two copper wires (22 gauge) were inserted intradurally at 1 and 3 cm from the tail root for assessment of antinociception. An 18-gauge needle with the hub removed was inserted through the dermal layer. The copper wire was inserted through the barrel of the needle, which then was removed from the dermis. The wire ends were wrapped around the tail and secured with electrical tape. Instrumented rats were maintained in a Raturm® apparatus (Bioanalytical Systems) that allowed free locomotion without disrupting implanted probes, cannulae, or wires. The NO sensor was connected to the inNO-T® recorder to maintain an electrical charge across the sensor during postsurgical recovery.

To investigate the effect of increased NO production in response to L-arginine infusion on morphine-associated antinociception, rats were anesthetized as described above. For this experiment, surgical preparation consisted of implanting only the femoral vein cannula and copper wires for electrical stimulation vocalization. These animals also were maintained during the recovery period in a Raturm® apparatus.

Venous cannulae were filled with heparinized saline (20 U/ml) to maintain patency during the recovery period. All
animals were allowed a minimum of 36 h to recover prior to experimentation.

2.4. Experimental protocol

2.4.1. Morphine-induced NO production and tolerance development

On the day of experimentation, the insert was removed from the guide cannula in the left hippocampus and replaced with a brain microdialysis probe (BAS BR-4). The dimensions of the microdialysis probe and the NO sensor were identical to ensure similar experimental conditions on both sides of the brain. The brain and vascular microdialysis probes were perfused throughout the entire experiment at a rate of 2.5 μl/min with artificial cerebrospinal fluid (aCSF) and phosphate-buffered saline (PBS), respectively. The microdialysis probes were perfused for 1 h prior to initiating morphine infusion.

Five treatment groups were examined (n=3–5/group). Following a 40-min baseline assessment of NO production, morphine (administered as morphine sulfate diluted in normal saline) was infused (1.2 ml/h) at a rate of 0.3, 1, 2, or 3 mg/kg/h into the femoral vein for 8 h starting at 9 am. Control rats received a saline infusion at an equivalent flow rate. Microdialysate samples (50 μl) were collected every 20 min from the brain and blood microdialysis probes. Hippocampal NO was quantified continuously throughout the experiment. Following termination of the morphine infusion, microdialysate samples were collected and NO concentrations were monitored for an additional 2 h. Antinociceptive effect was determined at timed intervals throughout the experimental period.

A blood sample was obtained at the end of the infusion and centrifuged, and the plasma was collected to calculate the in vivo recovery of morphine by the vascular microdialysis probe. Fractional recovery of morphine by the probe was estimated by dividing the morphine microdialysate concentration by the plasma morphology concentration (collected at the midpoint of microdialysis collection interval). Radiolabeled morphine (0.1 μM) was perfused (2.5 μl/min) for a total of 40 min through the brain microdialysis probe to determine fractional loss across the probe membrane immediately following the experiment [12]. Recovery-corrected morphine concentrations were determined by dividing the morphine concentration from the microdialysate sample by the fractional recovery of morphine by the specific probe.

Following experimentation, the animals were sacrificed, brain tissue was removed, and placement of the microdialysis probe and NO sensor was assessed visually to ensure proper hippocampal location.

2.4.2. Effect of L-arginine-associated increases in NO on the antinociceptive effects of morphine

Three groups of rats (n=3/group) received L-arginine at a rate of 100, 300, or 500 mg/kg/h (pH 7–8) by infusion into the femoral vein (1.2 ml/h) for 8 h starting at 9 am. These infusion rates were shown previously to result in incremental increases in neuronal NO [23]. Antinociceptive effect was assessed at 0, 4, and 8 h following initiation of the L-arginine infusion. At 9 am the following day (16 h post-L-arginine infusion cessation), the rats received a 3-mg/kg/h morphine infusion for 4 h. Antinociceptive effect was determined at timed intervals during the morphine infusion.

2.5. Analysis of morphine

Plasma samples were prepared by liquid–liquid extraction as described previously [47]. Concentrations of morphine in extracted plasma, or in blood or hippocampal microdialysate samples (20 μl), were determined by LC-MS-MS following a threefold dilution with 50% methanol. Nalorphine (internal standard; 10 μl, 400 ng/ml in DMSO) was added to each sample, which was mixed by vortex and injected (10 μl; Agilent 1100 wellplate autosampler) onto a Phenomenex 2.0×30-mm 4 μM Synergi Max-RP column (Phenomenex, Torrance, CA) maintained at room temperature. Chromatographic separation was achieved with a mobile phase gradient of 10 mM ammonium acetate (pH 6.8; 95–5% linear gradient for 0–2 min, 5% for 2–2.5 min, and 95% for 2.5–3 min) and a flow rate gradient (0.75–1.5 ml/min linear gradient for 0–2 min, 1.5 ml/min for 2–3 min) (Agilent 1100 series binary pump). Morphine and nalorphine were quantitated using multiple reaction monitoring (286.1→201.1 and 312.2→201.2, respectively) (Turbo Ionspray of a PE-Sciex API-4000 triple quadrupole mass spectrometer; AB Sciex Instruments). The entire column effluent was diverted from the mass spectrometer from 0 to 0.5 min and from 2.5 to 3 min. The system was returned to initial conditions at 3 min and allowed to reequilibrate for 1 min. Standard curves were prepared in relevant microdialysis perfusion fluid (PBS or aCSF) or in morphine-free plasma. The lower limit of quantitation was 0.5 ng/ml; intraday and interday coefficients of variation were <15%.

2.6. Assessment of morphine antinociception

Antinociception was determined at timed intervals during each experimental treatment by electrical stimulation vocalization [17]. A GRASS s48 stimulator (Grass Telefactor, West Warwick, RI) was used to deliver voltage to the intradermal copper wires in the rat tail. The voltage (125 pulse/s, 1.6 ms pulse width) was increased by 1-V increments from 0.5 V to a maximum of 11.5 V (to prevent tissue damage) until a vocalization response was audible. Response was recorded in volts. Baseline response was taken as the average of two trials obtained at 15-min intervals. Antinociception was expressed as a percentage of the maximum possible effect (%MPE):

\[ \%MPE = \left( \frac{\text{Test response} - \text{Baseline response}}{\text{Cutoff voltage} - \text{Baseline response}} \right) \times 100 \]
2.7. Data analysis

Data collected by the NO sensor were smoothed using time averaging over 10-s intervals, followed by eigendecomposition to remove electrical interferences [20]. Further time averaging over 20-min intervals was performed in order to reduce the number of data points for subsequent pharmacokinetic/pharmacodynamic modeling. Baseline NO was expressed as the averaged smoothed output signal over the 40 min prior to initiation of morphine infusion. All NO data are expressed as percent change from the saline treatment group (% saline) to correct for circadian changes in NO production [3,21]. Autosignal Software (Clecom Software, Edgbaston, Birmingham, UK) was used to perform the smoothing and deionising algorithm. Area under the NO concentration–time curve (AUC) was calculated using the linear trapezoidal method. Peak effect (E_{max}) and time to peak effect (t_{max}) were determined by visual inspection of the effect versus time data for each animal. The extent of tolerance development was estimated by dividing the effect at the end of the morphine infusion (the point of maximum tolerance development in the confines of the present experiment) by the peak effect (E_{max}), subtracting the value from 1, and multiplying by 100. Analysis of variance (ANOVA) was used to determine the statistical significance of differences between groups. Bonferroni corrections were applied when multiple comparisons were made to control. SigmaStat (SPSS, Chicago IL) was used for statistical analyses. In all cases, the criterion for statistical significance was p<0.05.

2.8. Pharmacokinetic/pharmacodynamic modeling

Group-averaged morphine concentrations in blood and brain microdialysate, neuronal NO production, and antinociceptive effect data for the four morphine treatment groups were fit simultaneously with the model shown schematically in Fig. 1. This model was based on the assumption of linear disposition of morphine (distribution into the brain and systemic elimination). The effect of morphine on NO production was treated as an indirect response [24] in which morphine stimulated the rate of NO production; the relationship between stimulation of NO production and brain morphine concentration was modeled as a sigmoidal relationship. A hypothetical effect compartment was incorporated to account for an evident delay in both NO production and the NO-associated decrease in antinociceptive effect; concentrations in this hypothetical compartment (NOBR*) are determined by equivalent rate constants into (k_{ie}) and out of (k_{eo}) the effect compartment [49]. The concentrations of morphine in the blood (MOR_{BL}) and brain (MOR_{BR}), as well as NO concentration in the brain (NO_{BR}), were modeled versus time with the following differential equations:

\[
\frac{dMOR_{BL}}{dt} = k_0 V_{BL} - MOR_{BL} \left( \frac{k_{10} - k_{12}}{C_0} \right) + k_{21}MOR_{BR} \left( V_{BR} / V_{BL} \right)
\]

\[
\frac{dMOR_{BR}}{dt} = k_{12}MOR_{BL} \left( V_{BL} / V_{BR} \right) - k_{21}MOR_{BR}
\]

\[
\frac{dNO_{BR}}{dt} = k_{on} \left( 1 + \frac{E_{max} \cdot MOR_{BR}^2}{EC_{50a} + MOR_{BR}^2} \right) - k_{off}NO_{BR}
\]

\[
\frac{dNO_{BR}^*}{dt} = k_{eo}NO_{BR} - k_{le}NO_{BR}^*
\]

where \( k_0 \) is the zero-order infusion of morphine into the rat (via the femoral vein); \( k_{10} \) is the first-order rate constant

Fig. 1. Scheme depicting the model fit to the morphine disposition data, neuronal NO concentrations, and antinociceptive effect. Parameter designations are defined in the text.
governing elimination of morphine from the blood; $k_{12}$ and $k_{21}$ are the first-order rate constants mediating movement of morphine between the blood and the brain; $V_{BL}$ and $V_{BR}$ are the apparent volumes of distribution of morphine in the blood and the brain, respectively; $k_{on}$ is the apparent zero-order rate constant mediating neuronal NO production; $k_{off}$ is the first-order rate constant governing degradation of NO; $E_{max,s}$ is the maximum possible percent stimulation of neuronal NO production ($k_{on}$) by $\text{MOR BR}_E$; $EC_{50,s}$ is the $\text{MOR BR}_E$ that elicits a 50% reduction in $k_{on}$; and $\gamma_S$ represents the shape factor governing the relationship between percent stimulation of $k_{on}$ and $\text{MOR BR}_E$. 

The loss of antinociceptive effect due to $\text{NO BR}_E$ concentrations was modeled as NO acting as an inverse agonist with the following integrated equation:

$$E = \frac{E_{max,E} \text{MOR BR}^\gamma}{EC_{50,E} + \text{MOR BR}} - \frac{I_{max,E} (\text{NO BR}^*)^{\gamma_I}}{IC_{50,E}^{\gamma_I} + (\text{NO BR}^*)^{\gamma_I}}$$

where $E_{max,E}$ is the maximum possible antinociceptive effect produced by $\text{MOR BR}_E$; $EC_{50,E}$ is the $\text{MOR BR}_E$ that elicits a 50% maximum response; $\gamma_E$ is the shape factor governing the relationship between antinociceptive effect and $\text{MOR BR}_E$; $I_{max,E}$ is the maximum possible inverse effect produced by $\text{NO BR}_E^*$; $IC_{50,E}$ is the $\text{NO BR}_E^*$ that produces an effect equal to 50% of the maximum possible inverse effect; and $\gamma_I$ is the shape factor dictating the relationship between $\text{NO BR}_E$ and the inverse antinociceptive effect.

Model differential and integrated equations were fit to the mean data by nonlinear least squares regression using WinNonlin Software (Pharsight, Palo Alto, CA). Assessment of the goodness of fit of the model to the observed data was based on coefficients of variation and distribution of residual error. Akaike’s information criterion was used to compare the appropriateness of different model structures.

3. Results

Morphine concentrations in the blood and brain are presented in Fig. 2. Fractional recoveries of morphine by the vascular and brain microdialysis probes were 29±10% and 13±10%, respectively. Changes in morphine concentrations were dose-proportional in both the blood and the brain, suggesting that systemic elimination and transfer across the blood–brain barrier were not saturated at the doses employed in this experiment. Brain microdialysate concentrations produced by the lowest morphine infusion rate (0.3 mg/kg/h) approached the lower limit of detection of the analytical method; samples with concentrations below the limit of detection were treated as “missing” for the purpose of generating the group-averaged concentration–time profile.

Neuronal NO concentrations following morphine infusion were elevated above those in saline-treated animals (Table 1; ANOVA, $p=0.003$). Percent increase in NO versus time is shown for a representative animal for each treatment group in Fig. 3. The morphine doses employed allowed characterization of the complete concentration–response relationship for NO production as a function of brain morphine concentrations (sigmoidal relationship; Fig. 4). The lowest dose of morphine administered (0.3 mg/kg/h) represents a no-effect dose with respect to changes in NO. Incremental increases in NO were observed with the two intermediate morphine doses. No additional increase in NO production was observed between the 2- and 3-mg/kg/h morphine infusion groups, suggesting that maximal morphine-associated stimulation of NO production was attained.

During the first hour of morphine administration, antinociceptive effect was dose-proportional (Fig. 5A and B). Intravenous infusion of morphine at rates of 0.3, 1, 2, and 3 mg/kg/h resulted in peak response (mean±S.D.) of 9±4%, 26±4%, 52±6%, and 67±8% MPE, respectively. Despite maintenance of constant morphine brain concentrations at each dose rate, a time-dependent loss of response was observed in all treatment groups, consistent with the development of antinociceptive tolerance. The decrease in pharmacologic response coincided closely with morphine-induced increases in neuronal NO; both processes occurred between 2 and 4 h after initiation of the morphine infusion.
The time at which maximal analgesic effect was observed increased with increasing morphine dose, suggesting that one or both of the time-dependent processes (onset of effect and/or tolerance development) were dose-dependent. \( t_{\text{max}} \) for the treatment groups were 1.2 ± 0.8, 1.6 ± 0.5, 1.9 ± 1, and 2.8 ± 0.9 h (mean ± S.D.), corresponding to the 0.3-, 1-, 2-, and 3-mg/kg/h treatment groups, respectively. A dose-dependent relationship in extent of tolerance development also was observed. Animals treated with the 3-mg/kg/h morphine infusion had a 42 ± 10% reduction in effect over the 8-h experiment. Extent of tolerance development was approximately equivalent for the 1- and 2-mg/kg/h treatment groups (61 ± 14% and 57 ± 10% loss of effect over the course of the infusion, correspondingly). Antinociceptive effect for the 0.3-mg/kg/h treatment groups was evident shortly after initiation of the morphine infusion and became undetectable as time preceded suggesting complete (100%) tolerance development.

The pharmacokinetic/pharmacodynamic model depicted in Fig. 1 was fit simultaneously to the morphine concentration, NO production, and antinociceptive effect versus time data using a stepwise nonlinear least squares regression strategy. In the first stage of the analysis, blood and brain microdialysate concentrations of morphine, pooled across all animals, were fit with the pharmacokinetic portion of the model governing morphine disposition in blood and brain.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \text{AUC}_{\text{NO}} ) (% baseline) ± S.D.</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>-80 ± 50</td>
<td>-</td>
</tr>
<tr>
<td>0.3 MOR</td>
<td>-80 ± 50</td>
<td>1</td>
</tr>
<tr>
<td>1 MOR</td>
<td>-20 ± 20</td>
<td>0.129</td>
</tr>
<tr>
<td>2 MOR</td>
<td>12 ± 40</td>
<td>0.009</td>
</tr>
<tr>
<td>3 MOR</td>
<td>4.3 ± 20</td>
<td>0.011</td>
</tr>
</tbody>
</table>

\( p \) values represent comparisons to saline control.

\(^1\) Statistically significant elevation from saline treatment.

In the second stage of the analysis, the pharmacokinetic parameters governing morphine disposition obtained in the first stage were held constant, and the NO production data were fit with the pharmacodynamic submodel incorporating indirect morphine-induced stimulation of NO synthesis. In the final stage of analysis, all parameters recovered in steps (1) and (2) were held constant, and the antinociceptive effect data were fit with the antinociceptive effect submodel. Overall, the integrated model described the observed data.
well ([Figs. 2, 3, and 5]). The associated parameter estimates are compiled in Table 2. The model was consistent with first-order disposition of morphine in the blood and brain, morphine-induced enhancement of neuronal NO production, a direct relationship between brain morphine concentrations and antinociceptive effect, and a delayed NO-mediated hyperalgesic (reverse agonist) response. Interestingly, the model results suggested that the delay between NO production and the nociceptive effect is a function of the morphine dose administered (i.e., dose-dependent value of \( k_{oc} \); Table 2).

Following pretreatment of animals with L-arginine, tolerance development was accentuated (Fig. 6). This increase in functional tolerance was not due to a change in the baseline response to painful stimuli in the absence of morphine (data not shown). As expected, L-arginine pretreatment resulted in an enhanced extent of tolerance development (% tolerance: 65±5%, 81±10%, and 130±20% for L-arginine infusions of 100, 300, 500 mg/kg/h, respectively) when compared to control rats (% tolerance at 4 h: 27±20%). Morphine-induced antinociception following the highest L-arginine pretreatment produced an extent of tolerance greater than 100%. Interestingly, this hyperalgesia was reversed upon cessation of the morphine infusion in a time course consistent with the half-life of morphine elimination from the system. These findings are consistent with a morphine-induced hyperalgesic response. While the antinociceptive effect at 4 h was reduced significantly following pretreatment with L-arginine, the initial response to morphine was higher than expected. In rats treated with L-arginine at the 500-mg/kg/h dose, the antinociceptive response at 0.5 h was increased above that achieved in controls rats at 0.5 h (\( E_{0.5}^{l} \): 88±20% and 37±20%; L-arginine: 500 mg/kg/h and control rats, respectively; \( p=0.035 \)). The effect values at 0.5 h for the L-arginine 100- and 300-mg/kg/h-treated animals also were higher that control rats (\( E_{0.5}^{l} \): 58±20 and 51±8, in corresponding order) (Fig. 6), although these differences did not reach statistical significance.

### 4. Discussion

Opioid analgesics commonly are used in the treatment of chronic pain, but the loss of antinociceptive effect with prolonged administration often limits the utility of these agents. Tolerance to a number of biologic effects, including gastrointestinal transit time, respiratory depression, and analgesia, can develop and dissipate at different rates. The onset of antinociceptive tolerance occurs most rapidly [19,28]. Dose escalation to achieve pain control therefore may be limited by intolerable side effects. Extensive research has identified multiple mechanisms believed to govern the development of tolerance to this class of medications (for review, see Refs. [18,29,36,51]). However, the time-dependent evaluation of these processes in vivo and complete evaluation of these effects on antinociception in the intact organism still require elucidation. Purported upregulation of NO following morphine administration likely contributes to the complex cascade of events involved in downregulation of responsivity to opioids. Comprehensive characterization of morphine-induced upregulation of NO production and the resultant effects on morphine analgesia is needed to decipher the role of this chemical messenger in mediating antinociceptive tolerance.

The present data demonstrated clearly a time-dependent attenuation of effect that closely paralleled that of NO production. The mechanisms governing a potential NO-driven loss of effect with time could include alterations in the opioid receptors, modulation of receptor-mediated

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**Table 2**
P pharmacokinetic/pharmacodynamic parameter estimates and corresponding coefficients of variation obtained from simultaneous modeling of morphine disposition, NO production, and antinociceptive effects. \( k_{oc} \) \((k_{oc}=k_{1e})\) estimates, which varied between morphine infusion rates, are defined in the table by \( k_{oc} \) low dose (0.3 and 1 mg/kg/h); \( k_{oc} \) intermediate dose (2 mg/kg/h); and \( k_{oc} \) high dose (3 mg/kg/h).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{BH} ) (l/kg)</td>
<td>2.5</td>
<td>25.9</td>
</tr>
<tr>
<td>( V_{HR} ) (l/kg)</td>
<td>26</td>
<td>22.6</td>
</tr>
<tr>
<td>( k_{10} ) (h(^{-1}))</td>
<td>2.17</td>
<td>26.3</td>
</tr>
<tr>
<td>( k_{12} ) (h(^{-1}))</td>
<td>2.15</td>
<td>45.2</td>
</tr>
<tr>
<td>( k_{21} ) (h(^{-1}))</td>
<td>1.77</td>
<td>10.8</td>
</tr>
<tr>
<td>( E_{max}^{l} ) (%stim)</td>
<td>0.19</td>
<td>36.8</td>
</tr>
<tr>
<td>( E_{50,a} ) (ng/ml)</td>
<td>15.2</td>
<td>36.7</td>
</tr>
<tr>
<td>( \gamma_s )</td>
<td>3.1</td>
<td>115</td>
</tr>
<tr>
<td>( k_{on} )</td>
<td>258</td>
<td>126</td>
</tr>
<tr>
<td>( k_{off} )</td>
<td>2.58</td>
<td>127</td>
</tr>
<tr>
<td>( E_{max,E} ) (%MPE)</td>
<td>100</td>
<td>21.9</td>
</tr>
<tr>
<td>( E_{50,E} ) (ng/ml)</td>
<td>26.8</td>
<td>29.8</td>
</tr>
<tr>
<td>( \gamma_E )</td>
<td>1.04</td>
<td>14.8</td>
</tr>
<tr>
<td>( I_{max,E} ) (%MPE)</td>
<td>2726</td>
<td>133</td>
</tr>
<tr>
<td>( IC_{50,E} ) (%SAL)</td>
<td>264</td>
<td>157</td>
</tr>
<tr>
<td>( \gamma_l )</td>
<td>5.2</td>
<td>52.4</td>
</tr>
<tr>
<td>( k_{oc} ) low-dose morphine (h(^{-1}))</td>
<td>1.56</td>
<td>19.3</td>
</tr>
<tr>
<td>( k_{oc} ) intermediate-dose morphine (h(^{-1}))</td>
<td>1.01</td>
<td>15.8</td>
</tr>
<tr>
<td>( k_{oc} ) high-dose morphine (h(^{-1}))</td>
<td>0.61</td>
<td>21.4</td>
</tr>
</tbody>
</table>

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second-messenger events, changes in morphine disposition resulting in decreased concentrations at the site of action, or the upregulation of an opposing system that counteracts opioid-associated analgesia. Mathematical representation of these potential mechanisms would differ. Consequently, a series of empirical models was developed and compared, with each model incorporating different potential mechanisms. Based on these analyses, a mechanism by which NO induced a hyperalgesic state provided the best overall description of the observed data. However, additional information regarding the specific effects of NO per se, as opposed to effects associated with increased NO production in response to morphine administration, was needed to ensure a correctly specified pharmacokinetic/pharmacodynamic model.

In order to characterize the effect of increased neuronal NO production on morphine-associated antinociception, an experiment was conducted in which incremental increases in hippocampal NO were achieved with intravenous infusion of L-arginine. L-arginine infusion regimens were selected based on previous work from this laboratory that established a dose–response relationship between brain NO and L-arginine [21]. The L-arginine infusion regimens (100, 300, and 500 mg/kg/h) employed in these studies resulted in respective increases in NO of approximately 12%, 36%, and 48% relative to saline-treated controls, thereby stimulating NO production across the dynamic range of morphine-associated increases in NO.

Consistent with previous literature, L-arginine pretreatment resulted in a dose-dependent reduction in antinociceptive effect (Fig. 7) [4–6], with the highest dose of L-arginine (500 mg/kg/h) resulting in a hyperalgesic response following 4 h of continuous morphine infusion. It should be noted that based on the dynamic range of morphine-induced NO production, detection of the hyperalgesic response would be unlikely following intravenous morphine administration alone. Fig. 7 shows the relationship between antinociceptive effect following a 4-h morphine infusion and steady-state NO concentrations (obtained through infusion of L-arginine at various rates). Based on the observed maximal concentrations of NO attained following morphine administration (~20% elevation), a reduction in analgesia due to upregulation of a NO-induced hyperalgesic response may occur, but a true inverse response would not be obvious. Despite the inability to detect hyperalgesic responses with morphine alone, the present results describing morphine-associated pain following L-arginine pretreatment, in combination with the associated pharmacodynamic modeling, suggest that NO facilitates the loss of analgesic response with time through induction of a hyperalgesic response pathway.

The present observation of an NO-induced hyperalgesic response to morphine administration supports an emerging hypothesis regarding one mechanism governing opioid tolerance development. This hypothesis postulates that reduced antinociceptive effect with time may be the result, in part or in full, of upregulation of hyperalgesic pathway, rather than through the traditional desensitization mechanisms [46,53]. Several investigations in experimental animals and human subjects have documented that opioids can induce a hyperalgesic state characterized by abnormal pain following intrathecal administration [1,14,35,38,53]. Hyperalgesic responses also have been observed in rodents receiving subcutaneous fentanyl bolus doses as evidenced by a reduced baseline pain threshold [11,27]. While less common than with spinal administration of opioids, there also have been case reports of opioid-induced hyperalgesic responses following oral or intravenous administration of morphine in humans [50].

The mechanisms surrounding a potential for opioid-induced pain have not been elucidated completely. However, it is known that excitatory amino acids interacting with the NMDA receptor, and the associated neurochemical pathways (including upregulation of NO), are involved in the well-characterized hyperalgesia observed following tissue injury and inflammation [10,16]. Due to the observation that antagonists of the NMDA receptor and inhibitors of NOS inhibit the hyperalgesic states associated with tissue injury and opioid tolerance development, it has been suggested that these neurochemical pathways may overlap, and that the excitatory states resulting from neuronal plastic changes associated with abnormal pain sensations following tissue damage also may be involved in opioid tolerance development [13,26,31,33,39,43,52]. Multiple investigations also have identified commonalities in the mechanisms surrounding tissue injury-induced and opioid tolerance-induced nociception including the upregulation of protein kinase C activity [32,34,41,45] and in the neuronal degenerative changes observed with both processes [37,42]. While additional work is needed to establish the molecular detail surrounding this potential mechanism of opioid tolerance, the experiments reported herein provide additional support for this hypothesis.

As described in the Results section, pharmacokinetic/pharmacodynamic modeling suggested that increasing doses
of morphine resulted in an increase in the delay between stimulated neuronal NO production and attenuation of antinociceptive effect, as evidenced by the trend of increasing $t_{\text{max}}$ values with increasing morphine dose. This observation can be accounted for by the presence of two time-dependent, NO-mediated processes. L-arginine pretreatment (500 mg/kg/h for 8 h) resulted initially (during the first 30 min of morphine infusion on day 2) in an enhanced magnitude of morphine-associated antinociception. This enhanced opioid response was reduced rapidly by the more dominant NO-induced hyperalgesic response (Fig. 6). A similar biphasic effect of NO on opioid antinociception has been reported previously. Pretreatment of mice with L-arginine resulted in a synergistic effect with single-dose morphine; in contrast, prolonged morphine exposure in the presence of L-arginine was associated with accelerated tolerance development [48]. Without specific data evaluating these two time-dependent phenomena independently, pharmacokinetic/pharmacodynamic modeling was unable to recover accurate estimates of parameters associated with these two processes. Nevertheless, it was possible to reproduce the observed changes by altering $k_{\text{on}}$ across treatment groups. In the presence of competing effects of NO on analgesia, the delay function then becomes an apparent delay that is actually a hybrid of two opposing time-dependent processes. The pharmacodynamic modeling indicated the existence of increasing $k_{\text{on}}$ values only for the two highest morphine treatment groups, suggesting that the NO-modulated process resulting in enhanced analgesia likely occurs to a pharmacologically relevant extent only when stimulation of NO production exceeds ~10%.

Interestingly, additional work in this laboratory has demonstrated that in vivo administration of L-arginine at doses of 100, 300, and 500 mg/kg/h results in a dose-dependent increase in agonist stimulation of $\mu$-opioid receptor G-protein activation (ex vivo GTP$\gamma$S binding analyses). However, L-arginine pretreatment followed by prolonged infusion of morphine resulted in a time-dependent attenuation of G-protein activation [23]. These observations support a dual role of NO in mediating morphine-associated analgesia, and also suggest that NO-mediated changes, in part, occur at the level of the receptor. However, additional work is required to establish specific molecular changes associated with NO-mediated receptor alterations, and to determine if these effects are linked to possible neuronal plasticity changes observed in hyperalgesic processes.

In conclusion, the results reported herein demonstrate that morphine administration leads to increases in neuronal NO production, and that stimulated NO production results ultimately in tolerance development through the induction of an opioid-induced hyperalgesic state. An interesting, but unexpected, observation was that NO also may have a competing mechanism that acutely enhances analgesic effects of opioids. Further investigation is needed to establish the molecular mechanisms governing these observations. While this work constitutes only a starting point for extensive investigations into the complex involvement of NO in receptor processing, establishing a time course between morphine-induced NO production and the resultant loss of antinociceptive effect is an essential first step in elucidating the substantial role of NO in mediating opioid tolerance.

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References


