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The development of morphine antinociceptive tolerance in nitric oxide synthase-deficient mice

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Abstract

Elevations in nitric oxide (NO) have been implicated in the development of morphine antinociceptive tolerance. This study was conducted to establish the role of specific isoforms of NO synthase (NOS) in morphine tolerance development using genetically modified mice. *Methods*: Three groups of mice (endothelial NOS [eNOS]-deficient, neuronal NOS [nNOS]-deficient, and NOS-competent) were used in this experiment. On Day 1, the analgesic response (radiant heat tail-flick) to a challenge dose of morphine (4 mg/kg) was determined over 3 hr. Tolerance was induced on Days 1–5 by administering morphine subcutaneously (10 mg/kg) or L-arginine, a NO precursor, intraperitoneally (200 mg/kg), twice daily. Analgesic response to the challenge dose was determined again on Day 6. *Results*: Following sustained morphine administration, nNOS-deficient mice exhibited less tolerance development when compared to the control group, although measurable tolerance still occurred. Mice deficient in eNOS evidenced a degree of tolerance similar to that of control. Prolonged L-arginine administration produced significant functional tolerance to morphine in NOS-competent and eNOS-deficient mice. The loss of morphine responsivity after L-arginine administration was similar to that after morphine pretreatment. L-Arginine did not affect the antinociceptive response to morphine in mice deficient in nNOS, suggesting that the small degree of morphine-induced tolerance in this group occurs through an alternate pathway. *Conclusions*: These data demonstrate the pivotal role of the neuronal isoform of NOS in development of morphine antinociceptive tolerance. Furthermore, tolerance development appears to be predominantly a NO-mediated process, but likely is augmented by a secondary (non-NO) pathway.

Keywords: Morphine; Tolerance; Nitric oxide; NOS-deficient mice

1. Introduction

Morphine is an opiate analgesic commonly used for the treatment of severe and chronic pain. The long-term efficacy of morphine often is limited by the development of tolerance to the analgesic effect. Despite extensive research efforts in the area of opioid tolerance, the exact mechanisms by which this phenomenon occurs remain largely unknown. Recent evidence suggests that nitric acid (NO), an endogenous molecule implicated in the regulation of a number of physiologic and pathogenic processes, is involved in the development of morphine tolerance [1].

NO is formed intracellularly through the action of nitric acid synthase (NOS). NOS has been classified into three isoforms, including neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). The activity of the constitutive isoforms, including nNOS and eNOS, is controlled by intracellular calcium, which in turn is regulated by excitatory amino acids interacting with the *N*-methyl-D-aspartate (NMDA) receptor. The inducible isoform, on the other hand, is not regulated by calcium. L-Arginine, the only known substrate of all isoforms of NOS, is oxidized to form NO and L-citrulline [2,3].

Several studies have implicated the NMDA/NOS system in the development of morphine tolerance. Much of the *in vivo* research evaluating the interplay between morphine and NO has focused on the effects of NOS inhibitors,

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Abbreviations: AUEC, area under the effect vs. time curve; E_{max}, maximal percent maximum possible effect; eNOS, endothelial nitric oxide synthase; k_{off}, rate constant associated with the loss of effect; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; % MPE, percent maximum possible effect; t_d, duration of effect; t_{max}, time at which maximum possible effect occurred.

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NMDA receptor antagonists, or exogenous administration of L-arginine on the pharmacodynamics of morphine. For example, it has been demonstrated that coadministration of morphine with NMDA receptor antagonists or non-isoform-specific NOS inhibitors, both of which reduce NO concentrations, resulted in attenuated development of antinociceptive tolerance [4–13]. Furthermore, pretreatment with or coadministration of L-arginine, which increases NO concentrations, resulted in the development of functional tolerance to the analgesic effects of morphine [1,4,14].

While a link between morphine antinociceptive tolerance development and NO production clearly exists, information regarding the contribution of different NOS isoforms to this process is less definitive. Several studies have investigated NOS isoform-specific effects on morphine antinociceptive tolerance through use of isoform discriminating NOS inhibitors. Evidence suggests that 7-nitroindazole, a selective inhibitor of the neuronal isoform, and antisense inhibition of nNOS impairs the development of tolerance [6,15], while inhibitors (both chemical and antisense) of the inducible isoform have not been found to alter downregulation of morphine responsivity [16–18]. Presently, no studies have evaluated the effects of selective eNOS inhibition on the development of morphine antinociceptive tolerance.

While the observations to date suggest a prominent role of the neuronal isoform in morphine tolerance development, the absolute selectivity of the inhibitors used in these investigations is in doubt, leading to uncertainty regarding the actual roles of different NOS isoforms. *In vitro*, 7-nitroindazole exhibits 10-fold selectivity for nNOS compared to the iNOS isoform [19]. Furthermore, administration of non-selective inhibitors of NOS increases blood pressure due to reduced eNOS activity; the fact that 7-nitroindazole does not produce hypertension [20] also suggests minimal inhibition of eNOS by 7-nitroindazole. However, in the absence of specific quantitation of isoform-specific NOS activity *in vivo* following 7-nitroindazole administration, the specific inhibition of nNOS is questionable.

In order to establish definitively the contributions of NOS isoforms to morphine tolerance development, morphine pharmacodynamics in animals genetically deficient in different NOS isoforms could be evaluated and compared. The objective of the experiments reported in this communication was to investigate the effects of genetic alterations in constitutive NOS isoforms on the development of morphine antinociceptive tolerance in mice.

2. Materials and methods

2.1. Materials

Morphine sulfate and L-arginine were purchased from Sigma Chemical Co. All other reagents used in this study were obtained from commercial sources and were of the highest purity available.

2.2. Animals

Male C57BL/6J (background strain), C57BL/6J-Nos3^{tm1Unc} (eNOS-deficient) [21], and C57BL/6J-Nos1^{tm1Plh} (nNOS-deficient) [22] were purchased from Jackson Laboratory at 4–6 weeks of age and were housed individually in a temperature-controlled facility (72 ± 2°F). Animals were allowed free access to food and water and were maintained on a 12-hr light cycle (7.00 a.m. to 7.00 p.m.). 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.

2.3. Assessment of morphine antinociception

The radiant heat tail-flick test was used to quantify antinociception. A small area of the tail was exposed to the lamp at 2 cm (first arm of the experiment; morphine tolerance induction) or 2.5 cm (second arm of the experiment; L-arginine tolerance induction) from the distal end. Lamp intensity was adjusted to produce a baseline latency of 2–4 s. A cutoff latency (10 s) was used to avoid tissue damage. Baseline latency was assessed in duplicate and recorded as an average. Antinociception was expressed as percent of maximum possible effect (% MPE):

$$\%\,\mathrm{MPE} = \left(\frac{\text{Test latency} - \text{Baseline latency}}{\text{Cutoff latency} - \text{Baseline latency}}\right) \times 100$$

2.4. Experimental design

Three groups (N = 6/group) of mice (eNOS-deficient, nNOS-deficient, and control) were used in this experiment. On Day 1, the analgesic response to a challenge dose of morphine (4 mg/kg) was determined at timed intervals over 3 hr. A complete response time course was constructed in order to generate a gradient of responses, ranging from 0 to 100% of maximum, in individual mice after a single administration of morphine. Tolerance was induced on Day 1 (after initial pharmacologic testing) through Day 5 by administering morphine (10 mg/kg subcutaneously) twice daily. Analgesic response to the challenge dose was redetermined on Day 6. Following a 90-day washout period, the second arm of the experiment was conducted to assess the functional tolerance to morphine induced by L-arginine (200 mg/kg intraperitoneally) twice daily for 5 days. The morphine challenge dose (pre- and post-L-arginine treatment), measurement of antinociceptive response, and timing of tail-flick tests were identical to the first arm of the study. However, a point on the tail slightly more proximal (2.5 cm from the distal end) was used for antinociceptive assessment to avoid potential changes in baseline response

from repeated exposure to the heat lamp during the first arm of the experiment.

2.5. Data analysis

Area under the effect vs. time curve (AUEC) following the subcutaneous morphine challenge dose (4 mg/kg) was calculated with the linear trapezoidal method. Maximal % MPE elicited by the morphine challenge dose (E_{max}) and time at which E_{max} occurred (t_{max}) were determined by inspection of the individual effect vs. time profiles. Duration of action (t_d) was defined as the duration of time over which the morphine challenge dose elicited >10% MPE. The rate constant associated with loss of effect after t_{max} (k_{off}) was calculated by linear regression of effect vs. time data.

Tolerance development may affect AUEC, E_{max}, or t_d. In the present experiment, changes in AUEC were used as the primary indicator of tolerance, as AUEC is reflective of overall (i.e. integrated) pharmacologic response. The degree-of-tolerance development was expressed as a percentage loss of responsivity (% tolerance) and was calculated from the pre- and post-treatment AUEC estimates as:

$$\%\,\text{Tolerance} = \left(1 - \frac{\text{AUEC}_{\text{Day 6}}}{\text{AUEC}_{\text{Day 1}}}\right) \times 100$$

While somewhat limited in scope, E_{max} and t_d can provide additional information regarding the influence of tolerance on morphine pharmacodynamics, and therefore were included in subsequent statistical comparisons. ANOVA and Student's t test were used, where appropriate, to determine the statistical significance of differences between experimental groups. In situations in which the basic assumptions underlying these parametric tests were violated, the analogous nonparametric test was employed. In all cases, the criterion for statistical significance was $P < 0.05$.

3. Results

No differences in response to the initial (Day 1) morphine challenge dose, expressed as the AUEC, were observed between mouse strains prior to treatment with morphine (Table 1) (ANOVA, $P = 0.296$). The Day 1 morphine response was consistently higher (by ~25%) in all three mouse strains during the second arm of the experiment. However, no difference was observed between strains for the Day 1 response (AUEC) in this second arm ($P = 0.598$). Furthermore, comparison between groups (within each arm of the study) for other Day 1 pharmacodynamic parameters including E_{max}, t_{max}, and k_{off} (Tables 1 and 2) revealed no differences (E_{max}: $P = 0.791$ and $P = 0.280$; t_{max}: $P = 0.293$ and $P = 0.189$; k_{off}: $P = 0.760$ and $P = 0.648$, for arms 1 and 2, respectively).

Table 1
Pharmacodynamics of morphine-associated antinociception following a subcutaneous morphine challenge dose (4 mg/kg) before (Day 1) and after (Day 6) morphine pretreatment

Experimental group	Day 1	Day 6	P value
	Pharmacodynamic parameter		
	$AUEC_{(0–3\ hr)}$ (% MPE × hr)		
nNOS-deficient	8400 ± 900	5900 ± 1700	0.016
eNOS-deficient	8000 ± 2000	900 ± 600	<0.001
NOS-competent	9000 ± 3000	1800 ± 1200	0.003
	E_{max} (% MPE)		
nNOS-deficient	93 ± 11	71 ± 18	0.013
eNOS-deficient	87 ± 12	21 ± 8	<0.001
NOS-competent	90 ± 17	32 ± 13	<0.001
	t_{max} (hr)		
nNOS-deficient	0.8 ± 0.3	0.46 ± 0.10	0.084
eNOS-deficient	0.6 ± 0.2	0.38 ± 0.14	0.141
NOS-competent	0.8 ± 0.3	0.6 ± 0.4	0.456
	t_d (hr)		
nNOS-deficient	2.8 ± 0.4	1.8 ± 0.3	<0.001
eNOS-deficient	2.3 ± 0.6	0.8 ± 0.4	0.005
NOS-competent	2.5 ± 0.6	1.2 ± 0.5	0.014
	k_{off} (% MPE/hr)		
nNOS-deficient	0.61 ± 0.09	0.50 ± 0.09	0.054
eNOS-deficient	0.55 ± 0.10	N.D.	N.D.
NOS-competent	0.6 ± 0.2	N.D.	N.D.

Data are presented as mean ± SD. P values reflect paired comparisons within each mouse strain. N.D.: insufficient data >10% MPE.

The average effect vs. time profiles on Day 1 vs. Day 6 for the mice treated with chronic morphine (first arm of the study) are shown in Fig. 1. Antinociceptive tolerance based on AUEC (Table 1) developed to a statistically significant extent in all three mouse strains (Table 1) after 5 days of morphine treatment. However, the magnitude of tolerance differed between the different strains. Tolerance to morphine was marginal (~30% loss of response) in the nNOS-deficient mice (Fig. 2). In contrast, the eNOS-deficient and NOS-competent mice evidenced profound tolerance in response to the 5-day course of morphine treatment (~90 and 80% loss of response to the challenge dose, respectively). Similarly, tolerance decreased the maximal effect and reduced the duration of action in all three mouse strains, although the absolute changes in the eNOS-deficient and NOS-competent mice were substantially larger than those in the nNOS-deficient animals. The time of peak effect did not differ between Days 1 and 6 in any of the experimental groups (Table 1), suggesting that overall morphine disposition was not affected. Likewise, k_{off} did not change with 5 days of morphine treatment in the nNOS-deficient group (Table 1); k_{off} could not be calculated due to the lack of sufficient data above 10% effect (i.e. no measurable duration of action in tolerant animals).

A comparison of the average effect vs. time profiles on Day 1 vs. Day 6 for mice treated with chronic L-arginine

Table 2
Pharmacodynamics of morphine-associated antinociception following a subcutaneous morphine challenge dose (4 mg/kg) before (Day 1) and after (Day 6) L-arginine pretreatment

Experimental group	Day 1	Day 6	*P* value
	Pharmacodynamic parameter		
	$AUEC_{(0–3\ hr)}$ (% MPE × hr)		
nNOS-deficient	10900 ± 1600	10600 ± 1700	0.624
eNOS-deficient	10100 ± 1100	710 ± 1600	<0.001
NOS-competent	10700 ± 1900	2000 ± 200	<0.001
	E_{max} (% MPE)		
nNOS-deficient	100 ± 0.16	96 ± 6	0.176
eNOS-deficient	96 ± 8	61 ± 32	0.035
NOS-competent	100 ± 0	78 ± 23	0.062
	t_{max} (hr)		
nNOS-deficient	1.0 ± 0.3	0.7 ± 0.3	0.175
eNOS-deficient	0.7 ± 0.3	0.33 ± 0.13	0.062
NOS-competent	0.7 ± 0.4	0.33 ± 0.13	0.050
	t_d (hr)		
nNOS-deficient	3.0 ± 0.0	2.2 ± 0.4	0.063
eNOS-deficient	2.5 ± 0.6	1.0 ± 0.5	0.003
NOS-competent	2.5 ± 0.6	1.0 ± 0.5	<0.001
	k_{off} (% MPE/hr)		
nNOS-deficient	0.7 ± 0.1	0.67 ± 0.20	0.300
eNOS-deficient	0.63 ± 0.16	0.45 ± 0.16	0.026
NOS-competent	0.72 ± 0.16	0.52 ± 0.12	0.040

Data are presented as mean ± SD. *P* values reflect paired comparisons within each mouse strain.

(arm 2 of the study) is provided in Fig. 3. Following prolonged L-arginine administration, AUEC analysis revealed functional tolerance to morphine (Table 2) was induced in eNOS-deficient and NOS-competent mice (~90 and 75% loss of response, respectively). The nNOS-deficient mice, on the other hand, did not exhibit a reduced morphine effect on Day 6 when compared to Day 1. E_{max} was decreased in both the eNOS-deficient and NOS-competent mice, but not in nNOS-deficient animals, although the difference in the NOS-competent mice only approached statistical significance. Duration of action was decreased, and k_{off} increased, in eNOS-deficient and NOS-competent mice, but not nNOS-deficient mice, after L-arginine treatment. The time to maximum effect tended to be lower after 5 days of L-arginine administration in all three mouse strains. While these differences approached statistical significance, they represented relatively subtle changes in the effect vs. time profile.

4. Discussion

The differential tolerance development observed between the three strains of mice (nNOS-deficient, eNOS-deficient, and NOS-competent) following prolonged morphine administration (Figs. 1 and 2) strongly implicates nNOS as the key isoform governing NO-mediated morphine tolerance development. This observation is consistent with the hypothesis of a local NO effect in the central nervous system being an important, but likely

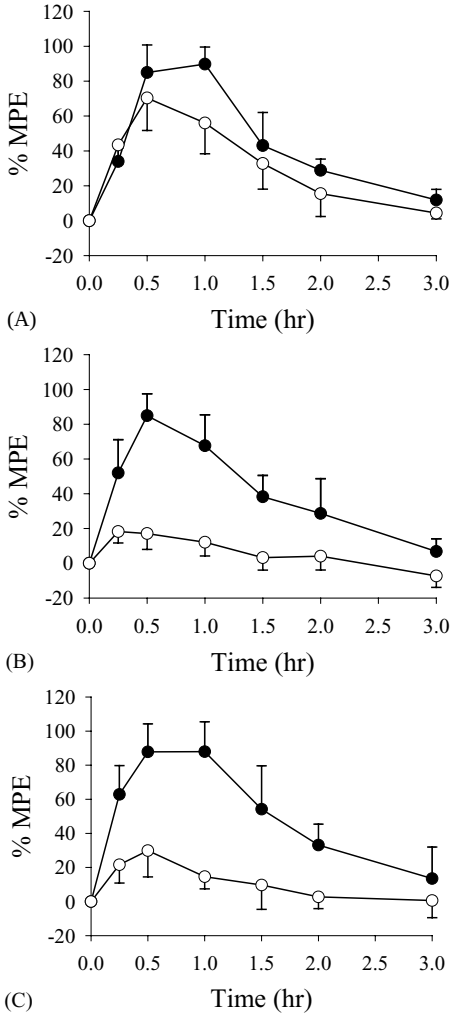

Fig. 1. Time course of morphine-associated antinociception (4 mg/kg challenge dose) prior to (●) or following 5 days of (○) morphine treatment (10 mg/kg twice daily) in (A) nNOS-deficient, (B) eNOS-deficient, and (C) NOS-competent mice. Data are presented as mean ± SD (N = 6/group).

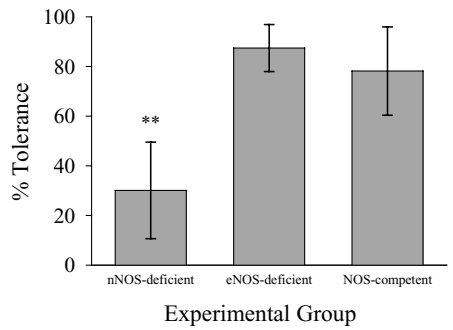

Fig. 2. Percent tolerance development, defined as percent loss of response to a 4 mg/kg challenge dose of morphine, in nNOS-deficient, eNOS-deficient, and NOS-competent mice after 5 days of morphine treatment (10 mg/kg twice daily). Data are presented as mean ± SD. $^{**}P < 0.001$ compared to NOS-competent.

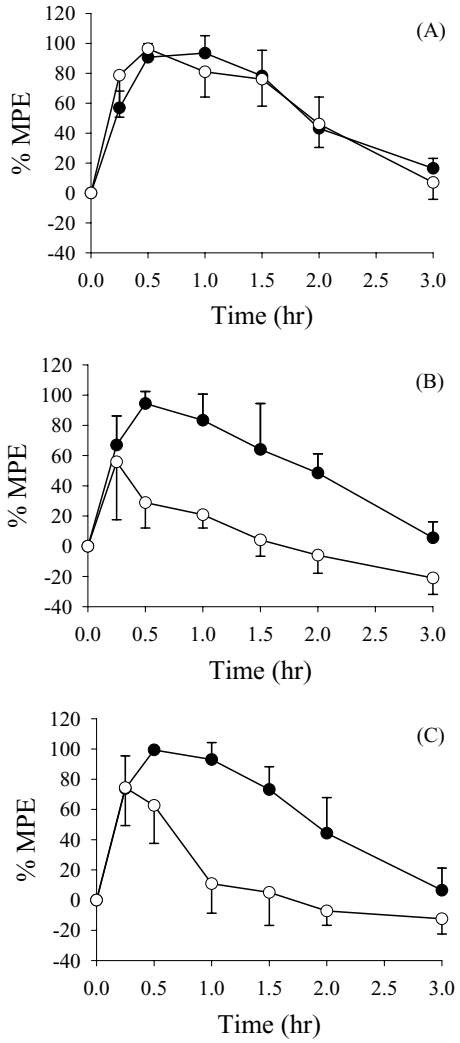

Fig. 3. Time course of morphine-associated antinociception (4 mg/kg challenge dose) prior to (●) or following 5 days of (○) L-arginine treatment (200 mg/kg twice daily) in (A) nNOS-deficient, (B) eNOS-deficient, and (C) NOS-competent mice.

not the singular, driving force for opioid tolerance development. Previously reported observations support this interpretation. The predominantly expressed splice variant nNOS-1 was identified as being responsible for antinociceptive tolerance development through use of antisense inhibition of expression; a second (less abundantly expressed) splice variant appeared to reduce single-dose analgesic efficacy [18,23]. The nNOS-deficient mice used in the present study have a targeted mutation that reduces the majority of nNOS, including nNOS-1 [22]. In contrast to the significant role of nNOS, the eNOS isoform does not appear to contribute to attenuation morphine-associated antinociception, as tolerance developed in these animals to an extent similar to that in NOS-competent mice.

Despite the fact that neuronal NO production presumably was absent, a small but statistically significant degree of morphine tolerance, based on AUEC analysis, was apparent in the nNOS-deficient group. This observation can be explained by either the presence of a non-NO-mediated tolerance mechanism, operating in parallel with the NO-driven event, or to the existence of residual brain NO production despite targeted disruption of nNOS gene. Previous evidence from other laboratories suggests that nNOS-deficient mice produce small amounts (<5% relative to NOS-competent animals) of NO in the brain despite the absence of the gene encoding the nNOS protein [22]. It is unclear at this time whether this residual activity is due to the presence of splice variants of the gene still remaining intact and functioning in these mice, or if the phenomenon is due to upregulation of other isoforms that compensate for the lack of nNOS in the central nervous system [22,24]. In order to elucidate the mechanism underlying the modest morphine-associated tolerance development in the nNOS-deficient mice, a second arm of the present study was conducted in which functional tolerance to morphine was induced by repeated L-arginine administration. Pretreatment with this requisite NO precursor attenuates the antinociceptive response to morphine [1,4,14]; if the residual tolerance to morphine observed in nNOS-deficient animals was related to NO production *via* a different isoform of the enzyme, some loss of response to morphine would be expected after L-arginine pretreatment. This arm of the experiment revealed a complete lack of functional tolerance in nNOS-deficient mice after L-arginine administration. In contrast, significant functional tolerance developed in both the eNOS-deficient and NOS-competent mice. Furthermore, the magnitude of functional tolerance was similar to that observed after a 5-day course of morphine administration. The inability of L-arginine to induce functional tolerance to morphine in nNOS-deficient mice clearly indicates that one or more NO-independent mechanisms likely contribute to downregulation of morphine-associated antinociception during the development of tolerance.

While multiple experiments have demonstrated the involvement of NO in morphine tolerance, little information exists regarding the mechanisms by which NO decreases morphine responsivity. The data presented herein not only represent important information regarding the specific NOS isoform responsible for morphine-associated tolerance, but also provide additional mechanistic insight. Previous evidence suggests that non-specific impairment of NO production leads to reduced nociceptive processing, which may be interpreted as an antinociceptive response. For example, administration of 7-nitroindazole produced a measurable analgesic response in experimental animals [20]. Likewise, a hypertensive state has been linked to reduced nociceptive processing [25]. This latter observation is intriguing in light of the fact that eNOS has a specific role in blood pressure regulation [21]. Given this apparently non-specific link between NO and nociception, in addition to the more specific relationship between neuronal NO and morphine pharmacology, the proximal mechanism underlying NO-related tolerance is unclear.

The attenuated response to morphine in functionally tolerant animals could be due to increased responsiveness to painful stimuli (i.e. increased nociceptive processing with increased NO production). In the present experiment, the three mouse strains consistently exhibited similar responses to the pretreatment morphine challenge dose, as evidenced by a lack of difference in AUEC, E_{max}, or t_d between mouse strains. In addition, this study provided an estimate of the slope of the effect vs. time relationship (k_{off}) which represents a hybrid of the elimination of the drug from the systemic circulation and the slope of the concentration–effect relationship. Specifically, for a drug that exhibits monoexponential elimination, k_{off} is the product of the slope of the concentration–effect curve and the first-order elimination rate constant [26]. Assuming that alterations in NO do not change the pharmacokinetics of morphine, the lack of difference in k_{off} between mouse strains on Day 1 suggests that absence of NO does not alter the morphine concentration–effect relationship. Taken together, these observations suggest that reduced NO formation in the nNOS-deficient and eNOS-deficient animals did not alter nociception in a non-specific manner. Furthermore, no changes in baseline response were observed on Days 1 and 6 for any of the experimental groups in the L-arginine treatment group (data not shown), suggesting that NO does alter nociceptive processing not non-specifically (i.e. the change occurs only in the presence of exogenous agonist).

Because increased brain NO appears to decrease the response to morphine as opposed to increasing the response to the nociceptive stimulus, alterations in NO production could affect either opioid disposition at the site of action (pharmacokinetic tolerance) or opioid receptor pharmacology (pharmacodynamic tolerance). The observation that k_{off} was reduced between Days 1 and 6 in the eNOS-deficient and nNOS-deficient groups could be the result of either a change in the slope of the concentration–effect relationship or a change in the elimination of the drug. The results of this experiment suggest that increased NO production likely affects one of these two factors. Further experimentation is required, however, to identify the precise mechanism by which NO induces functional tolerance to morphine.

Opioid tolerance is a complex phenomenon that involves one or more of several purported mechanisms, including opioid receptor downregulation, alterations in binding of the peptide to the receptor, modulation of the G-protein-coupled receptor activation, alterations of downstream receptor processes, and possible changes in drug disposition to the receptor site [27,28]. While the intricacies of this process have yet to be elucidated, the data presented herein suggest a major role of neuronal NO in modulating loss of antinociceptive effect during prolonged morphine administration, and provide insight into the manner by which NO alters morphine antinociceptive response.

Acknowledgments

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