Functionally differentiating two neuronal nitric oxide synthase isoforms through antisense mapping: Evidence for opposing NO actions on morphine analgesia and tolerance

(MOR-1/opioid/ν receptor)

YURI A. KOLESNIKOV, YING-XIAN PAN, ANNA-MARIE BABEY, SUBASH JAIN, ROGER WILSON, AND GAVRIL W. PASTERNAK*

The Cotzias Laboratory of Neuro-Oncology and Departments of Neurology and Anesthesiology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

Communicated by Paul A. Marks, Memorial Sloan–Kettering Cancer Center, New York, NY, May 9, 1997 (received for review February 19, 1997)

ABSTRACT Several isoforms of neuronal nitric oxide synthase (nNOS) have been identified. Antisense approaches have been developed which can selectively down-regulate nNOS-1, which corresponds to the full-length nNOS originally cloned from the brain, and nNOS-2, a truncated form lacking two exons which is generated by alternative splicing, as demonstrated by decreases in mRNA levels. Antisense treatment also lowers nNOS enzymatic activity. Down-regulation of nNOS-1 prevents the development of morphine tolerance. Whereas morphine analgesia is lost in control and mismatch-treated mice given daily morphine injections for 5 days, mice treated with antisense probes targeting nNOS-1 show no decrease in their morphine sensitivity over the same time period. Conversely, an antisense probe selectively targeting nNOS-2 blocks morphine analgesia, shifting the morphine dose-response curve over 2-fold to the right. Both systems are active at the spinal and the supraspinal levels. An antisense targeting inducible NOS is inactive. Studies with N0-nitro-L-arginine, which does not distinguish among NOS isoforms, indicate that the facilitating nNOS-2 system predominates at the spinal level while the inhibitory nNOS-1 system is the major supraspinal nNOS system. Thus, antisense mapping distinguishes at the functional level two isoforms of nNOS with opposing actions on morphine actions. The ability to selectively down-regulate splice variants opens many areas in the study of nNOS and other proteins.

Nitric oxide synthase (NOS) has become increasingly important as our understanding of its diverse biological actions has expanded, especially within the nervous system (1–7). In addition to the documented role of NOS in pain perception (7–10), the NOS inhibitor N0-nitro-L-arginine (NOArg) also blocks the development of morphine tolerance (11, 12). Observation of decreases in mRNA levels. Antisense treatment lowers nNOS enzymatic activity.

MATERIALS AND METHODS

Morphine sulfate was a gift from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). Halothane was obtained from Halocarbon Laboratory (Hackensack, NJ). 3H-L-arginine was purchased from NEN/Dupont. NOArg was purchased from Sigma. Dowex AG50WX-8-H+ resin was purchased from Bio-Rad.

Male CD-1 mice (24–32 g; Charles River Breeding Laboratories) were housed in groups of five with food and water ad libitum. Animals were maintained on a 12-h light/dark cycle. Compounds were administered intracerebroventricularly (i.c.v.) under light halothane anesthesia as previously reported (43).

Response latencies were determined using the radiant heat tailflick assay (44), with baseline latencies between 2 and 3 sec. We used a maximum cutoff score of 10 sec to minimize tissue damage. Antinociception was defined quantally as a doubling or greater of baseline tailflick scores, as previously reported (39, 40, 42). For convenience, the term analgesia is used synonymously with antinociception. Comparisons of single doses were performed using the Fisher Exact Test.

All phosphodiester antisense oligodeoxynucleotides were based upon the mouse sequence (32). Mismatch oligodeoxynucleotides were designed by switching the sequence of two base pairs, keeping the remaining sequence the same. All were purified in our laboratory and dissolved in 0.9% saline before injection (2 μl) (39, 40, 42) as indicated in the figure legends. NOS activity was measured (14, 45). All experiments were performed in triplicate.

Changes in mRNA levels of the nNOS isoforms were determined using reverse transcription–PCR (RT-PCR). Total RNA was extracted from mouse periaqueductal gray (PAG) region using an RNAeasy Mini kit (Qiagen, Chatsworth, CA). The first-strand cDNAs were reverse-transcribed from the total RNA with random hexamers and used in the following PCRs with [α-32P]dCTP. The amount of RNA added was determined by PCR using a set of mouse β-2-microglobulin primers (CLONTECH), yielding an amplified 373-bp fragment. The expression of NOS-1 mRNA was measured by PCR using a sense primer A (5′-CGCAGCTATCCGCTATGCC-3′) and a antisense primer B (5′-CGCCGCTCATCGCCTATGGC-3′).

Abbreviations: NOS, nitric oxide synthase; NOArg, N0-nitro-L-arginine; nNOS, neuronal NOS; RT-PCR, reverse transcription–PCR; PAG, periaqueductal gray; i.c.v., intracerebroventricular; i.t., intrathecal.

*To whom reprint requests should be addressed: Department of Neurology, Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. e-mail: pasterng@mskmail.mskcc.org.
3°, nt 1468–1487 of the mouse nNOS; GenBank accession no. D14552) and an antisense primer B (5′-CAATCCACACCCAGTCCGCG-3′, corresponding to nt 2057–2076), yielding a 517-bp fragment. The PCR was carried out for 25 cycles, each cycle consisting of a 30-sec melting step at 94°C, a 30-sec annealing step at 63°C, and a 40-sec extension step at 72°C. To assess the expression of nOS-2 mRNA, the double-stranded cDNAs were amplified by the first PCR using the above first-strand cDNAs as template only with the sense primer A for 15 cycles, each cycle consisting of a 1-min melting step at 94°C, a 1-min annealing step at 63°C, and a 1-min extension step at 72°C. The PCR products were then digested with the DraII, which cut twice in the predicted NOS-1 fragments, but not in the NOS-2 fragments. The digested PCR products were used as templates in the second PCR with the sense primer A and the antisense primer B for 35 cycles using the same program as for NOS-1. The amplified fragment size for NOS-2 is 203 bp. All the PCR products were separated in 1.5% thin agarose gel and then exposed to x-ray film. The corresponding bands observed on the film were cut out and counted.

RESULTS
NOS Antisense Actions on mRNA and Enzyme Activity. In view of the limited selectivity of NOArg, we have employed an antisense approach based upon paradigms previously worked out in our laboratory (39–42, 46). We focused upon the full-length nNOS (nNOS-1) and the isoform lacking two exons (nNOS-2), which correspond to exons 9 and 10 in the human homolog (32, 33) (Fig. 1a). The levels of nNOS-2 in the brain are quite low, with an abundance less than 10% of that of nNOS-1 and with highest values in the cerebellum and the spinal cord (32). Using RT-PCR, we also confirmed the presence of nNOS-2 in all brain regions examined and its low abundance relative to nNOS-1. Spinal cord levels of NOS-2 were about 60% of those in the cerebellum, the region with the highest levels (data not shown). Levels in the PAG were only about 40% of those in the cerebellum, while the cortex had the lowest levels (28%). To explore the roles of these isoforms, we designed antisense probes selective for nNOS-1 (antisense B, D and E) or NOS-2 (antisense C) or probes targeting both (antisense A and F).

First, we examined the efficacy and specificity of the down-regulation of mRNA levels in the periaqueductal gray (Fig. 1 b and c), a region with high nNOS levels (48) and which is important in opioid analgesia (49). Antisense F, which targets both nNOS isoforms, reduced the levels of NOS-1 and NOS-2 mRNA by 48% and 64%, respectively, using an RT-PCR approach to enhance sensitivity. Antisense C, which selectively targets NOS-2, lowered the levels of NOS-2 mRNA by 76% without noticeably affecting the levels of NOS-1. Conversely, antisense D reduced NOS-1 mRNA levels by 67% without interfering with NOS-2 mRNA levels. Thus, antisense approaches can selectively down-regulate individual splice variants.

Antisense treatment with a common probe reduced nNOS enzymatic activity. Using the conversion of [3H]arginine to [3H]citrulline to examine NOS activity (14), we observed that antisense F lowered the levels of NOS activity by almost 35% (P < 0.05; Fig. 2), a reduction comparable to that seen against the mRNA levels and similar to antisense results in other systems (37, 39, 46). The inactivity of the mismatch control confirms the selectivity of the effect.

NOS Antisense and Morphine Analgesia. First, we examined the time course of antisense A effects following intrathecal (i.t.) administration. As anticipated, intrathecal antisense A administration blocked morphine analgesia in a time-dependent manner (Fig. 3). The mismatch probe was inactive, confirming the selectivity of this response. We then examined the relative importance of the two NOS isoforms at the spinal level using antisense C and D, which selectively target nNOS-2 and nNOS-1, respectively (Fig. 4a). Antisense C effectively blocked morphine analgesia (P < 0.001) while antisense D was inactive, inferring that nNOS-2 is important in mediating morphine analgesia and nNOS-1 is not. As before, the mismatch probes were inactive.
To further define the role of nNOS-2 in spinal morphine analgesia, we performed full morphine dose-response curves after administering antisense C spinally. The antisense treatment significantly shifted the dose-response curve over 2-fold, raising the ED50 from 4.3 mg/kg (2.9, 6.1) to 9.2 mg/kg (6.3, 13.3) in antisense-treated mice.

Supraspinal treatments revealed similar results. Antisense C again blocked morphine analgesia (Fig. 4b), confirming a supraspinal role for nNOS-2 in morphine analgesia as well. However, all the probes targeting nNOS-1 given supraspinally were inactive against morphine analgesia, including those targeting both nNOS isoforms.

**NOS and Morphine Tolerance.** NOArg given systemically prevents morphine tolerance in a daily injection paradigm (11, 12). NOArg at a dose (1 μg, i.c.v.) that did not enhance morphine analgesia blocked the tolerance seen with repeated morphine administration (P < 0.001) (Fig. 5a). Antisense A at two different doses had a similar effect (Fig. 5b). Tolerance developed in the mismatch control group as rapidly as the control group, confirming the specificity of the response. An antisense oligodeoxynucleotide targeting inducible NOS was inactive.

We then examined a series of different antisense and mismatch oligodeoxynucleotides given supraspinally against morphine tolerance (Fig. 6a). Like the control group, tolerance to the fixed morphine dose developed in all the mismatch groups by day 5. In contrast, all the probes targeting nNOS-1 blocked the development of tolerance.

The marked reduction in morphine analgesia following intrathecal NOArg and antisense C administration makes it impractical to explore them in this paradigm. Although intrathecal antisense D administration, which selectively targets nNOS-1, had no effect on morphine analgesia, it prevented morphine tolerance (Fig. 6b). Again, the corresponding mismatch oligodeoxynucleotide was not active.

**NOArg and Morphine Analgesia.** Given systemically, NOArg does not affect morphine analgesia despite its ability to block and reverse morphine tolerance (11, 12). However, a different picture emerged when NOArg was given centrally. Supraspinal NOArg significantly enhanced systemic morphine analgesia while spinal NOArg markedly reduced systemic morphine analgesia (Fig. 7a) in a dose-dependent manner (Fig. 7b). NOArg also reduced spinal morphine analgesia and potentiated supraspinal analgesia, but only when both agents were given at the same site (Fig. 7a). The lack of NOArg activity when it was given into a different site than morphine confirmed that their interactions were localized to the region. These opposite NOArg actions indicated a predominance of the nNOS-1 system supraspinally and the nNOS-2 system spinally.

**DISCUSSION**

NO has a number of important actions in the central nervous system, including the modulation of morphine actions. Although our initial investigations found that NO plays a major role in the production of morphine tolerance, our current studies reveal a far greater complexity, with two nNOS isoforms responsible for opposing actions of NO on morphine analgesia.

As observed in other studies, antisense treatment downregulates nNOS mRNA levels by 50–75%, a level similar to...
that seen with antisense studies on opioid receptors (46). This reduction in mRNA levels is accompanied by a smaller drop in enzymatic activity. Several factors may explain this difference. First, the decrease in mRNA levels would be expected to precede lower protein levels, partially explaining the lower drop in enzymatic activity. More important, the enzymatic assay does not have the selectivity of the antisense approach and a portion of the activity seen in these assays is likely to be due to isoforms other than nNOS.

The antisense oligodeoxynucleotide common to both isoforms lowers the mRNA levels of both to a similar extent. We anticipated that Antisense D would selectively lower nNOS-1 mRNA levels because the targeted sequence is unique to that splice variant. Targeting nNOS-2 was less certain because all the exons comprising nNOS-2 also are present in nNOS-1. To overcome this problem we targeted the only unique aspect of the nNOS-2 mRNA, the splice site. By limiting the number of bases on either side of the splice site, we are able to down-regulate nNOS-2 without appreciably affecting nNOS-1. This ability to selectively down-regulate individual splice variants in vivo provides a method to independently define their functional activities, as illustrated with our current studies. Equally important, this approach can readily be extended to many other proteins, particularly those within the central nervous system.

mRNA levels because the targeted sequence is unique to that splice variant. Targeting nNOS-2 was less certain because all the exons comprising nNOS-2 also are present in nNOS-1. To overcome this problem we targeted the only unique aspect of the nNOS-2 mRNA, the splice site. By limiting the number of bases on either side of the splice site, we are able to down-regulate nNOS-2 without appreciably affecting nNOS-1. This ability to selectively down-regulate individual splice variants in vivo provides a method to independently define their functional activities, as illustrated with our current studies. Equally important, this approach can readily be extended to many other proteins, particularly those within the central nervous system.

The antisense results exposed an unanticipated pharmacological complexity for NO/opioid interactions. nNOS-2 is important in modulating both spinal and supraspinal morphine analgesia. Selectively targeting nNOS-2 with antisense C lowers morphine’s analgesic potency at both levels of the neuraxis. Although there is a dramatic loss of activity at single doses, this reflects only a 2- to 3-fold increase in ED50 values determined...
from dose–response studies. Thus, nNOS-2 appears to play a modulatory role rather than being an integral component of the analgesic circuit. Presumably, morphine would retain its analgesic activity with a complete loss of the nNOS-2 system, although its potency would be greatly lowered.

In contrast, morphine tolerance is modulated by nNOS-1 both supraspinally and spinaly. Five different antisense probes targeting nNOS-1 given supraspinally all blocked morphine tolerance, as does the nNOS-1 selective antisense given intrathecally. Several of these probes down-regulate both nNOS-1 and nNOS-2 without affecting analgesia. Presumably the loss of the facilitating nNOS-2 system is compensated by the concomitant down-regulation of the inhibitory nNOS-1 system, yielding little net change in the response to morphine either acutely or with repeated administration, manifested as a loss of tolerance. Thus, these studies associate the two nNOS isoforms with opposing effects on morphine analgesia both spinaly and supraspinally. nNOS-1 diminishes the analgesic actions of morphine while nNOS-2 enhances them.

The lack of effect of systemic NOArg on morphine analgesia (11, 12) probably reflects the simultaneous blockade of both facilitating and suppressive NO systems. NOArg has little selectivity among NOS isoforms and would be expected to block them all. If both facilitating and suppressive NO systems are simultaneously blocked, the net effect may be quite minimal. However, this lack of effect is no longer seen when NOArg is given either spinaly or supraspinally. At the spinal level, NOArg inhibits morphine analgesia, suggesting the predominance of the morphine-facilitating nNOS-2 system. This corresponds well with the relatively high levels of nNOS-2 reported in the spinal cord (32). Supraspinal NOArg enhances morphine analgesia, implying that inhibitory nNOS-1 systems play the major role at this site.

In conclusion, the current study separates the functional roles of two alternatively spliced isoforms of nNOS. This approach is suitable for exploring other functional roles of nNOS in the central nervous system. The ability to design active probes spanning splice sites as well as probes targeting individual exons provides further validation of the antisense mapping approach and may prove useful for exploring the functional roles of a wide variety of proteins.

We thank Drs. Jerome Posner and Rao Rapaka for their assistance in these studies. This work was supported in part through the National Institute on Drug Abuse by grant DA07242 and a Research Scientist Award (DA00220) to G.W.P. and a Research Scientist Development Award (DA00296) to Y.-X.P.


