Stabilized Dynorphin Derivatives for Modulating Antinociceptive Activity in Morphine Tolerant Rats: Effect of Different Routes of Administration

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ABSTRACT

Dynorphins, such as dynorphin A(1–13) (Dyn A(1–13)), have been shown to enhance analgesia in morphine-tolerant animals, despite their very short half-life after intravenous administration. The potential use of dynorphins in humans is therefore of interest. This laboratory has recently evaluated the metabolic fate of stabilized dynorphin derivatives. This study was conducted to evaluate whether such stabilized derivatives, ie, [N-Met-Tyr¹]-Dynorphin A(1–13) (N-MT Dyn A, stabilized at the N-terminal end) and [N-Met-Tyr¹]-Dynorphin A(1–13) amide (N-MT Dyn A amide, stabilized at the C- and N-terminal ends), would enhance the antinociceptive activity of morphine not only after intravenous administration but also after subcutaneous and pulmonary delivery. Intravenous administration of N-MT Dyn A (5 µmol/kg) and N-MT Dyn A amide (5 µmol/kg) to morphine-tolerant rats resulted in significantly higher tail-flick latencies than those observed for the saline group. These effects could be observed for up to 2.0 ± 0.1 hours after intravenous administration of N-MT Dyn A and for up to 3.4 ± 1.4 hours for N-MT Dyn A amide. The time-averaged effects of both peptides were similar. After pulmonary delivery of the same dose, derivatives remained active. The duration of the effects after pulmonary administration of the amide was 4.4 ± 2.5 hours while that of N-MT Dyn A was slightly shorter (2.8 ± 0.9 hours). No effect was observed after subcutaneous administration of N-MT Dyn A. These results suggest that pulmonary delivery of stabilized dynorphin derivatives represents a possible alternative to intravenous administration.

KEYWORDS: dynorphin derivatives, morphine, pulmonary delivery, tail-flick test, suppression of tolerance.

INTRODUCTION

Chronic use of opioids leads to behavioral and neurological changes including tolerance, physical dependence, abstinence syndrome, and self-administrative behavior in humans.

Dynorphin A(1–13) (Dyn A(1–13)), a metabolic fragment of Dyn A(1–17), was first isolated by Goldstein from porcine pituitary.¹

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In vivo studies have shown that Dyn A(1–13) itself does not have a significant analgesic effect,^{2–4} however the peptide facilitates analgesia in morphine-tolerant mice.^{5,6}

Based on previous studies,^{7–11} Dyn A(1–13) is considered to have possible significant clinical potential in modulating the tolerance development in patients under opioid therapy. However, the peptide has a markedly short half-life in human plasma¹² and is broken down mainly through amino- and carboxy-peptidases. One way to increase the stability of peptides is through chemical modification of the peptide. A variety of Dyn A analogs have been evaluated and studied for their stability.^{13–16} We evaluated N- and C-terminal modifications of dynorphin A(1–13).¹⁷ Among the peptides tested, [N-Met-Tyr¹]-Dynorphin A(1–13) amide (N-MT Dyn A amide) proved to be the most stable derivative, with a half-life of more than 2 hours (130 minutes) in vitro.¹⁷

Previous studies using Dyn A(1–13) showed that the activity of dynorphins was affected by their route of administration. Activity was observed after intravenous and intrathecal administration, but not intracerebroventricular or subcutaneous administration in morphine-dependent mice and rats.^{7,16,18} While these findings suggest a peripheral site of action for the tolerance-attenuating effects of Dyn A(1–13),^{7,16,19} it was of interest to evaluate whether the use of stabilized dynorphin derivatives would be suitable for other routes of administration, similar to investigations evaluating noninvasive routes of administration for insulin. An alternative route to intravenous administration of these compounds, if effective, may represent a more practical delivery approach.

Therefore, the aims of this study were to test whether compounds modified on the N- and C-termini of Dyn A(1–13), viz, [N-Met-Tyr¹]-Dynorphin A(1–13) and [N-Met-Tyr¹]-Dynorphin A(1–13) amide, are effective in modulating the antinociceptive effects in a morphine-tolerant rat model after intravenous administration, and to assess whether pulmonary and subcutaneous administration might be suitable routes of administration for these peptides.

MATERIALS AND METHODS

Drugs

Morphine sulfate (Baxter Healthcare Corporation, Deerfield, IL) was dissolved in sterile pyrogen-free isotonic saline at a concentration of 10 mg/kg. N-MT Dyn A and N-MT Dyn A

amide were purchased from GenoMechanix, LLC (Gainesville, FL) and dissolved in saline at a concentration of 5 $\mu\text{mol/mL}$. The peptide purity was greater than 95%.

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC), University of Florida, an Associate for the Assessment Accreditation of Laboratory Animal Care (AAALAC)–approved facility. Male Sprague-Dawley rats (230–270 g) were obtained from Harlan Laboratories (Indianapolis, IN). Prior to each experiment, the animals were housed for at least 24 hours in a temperature- and humidity-controlled environment and fed ad libitum.

Animal Experiments

Experiments were performed after subcutaneous, intravenous, and pulmonary administration of dynorphin. Tail-flick latencies were measured for every group on day 1 before and 30 minutes after subcutaneous morphine injection (3 mg/kg). Rats were then made tolerant to morphine by injecting 10 mg/kg morphine sulfate subcutaneously every 12 hours for 5 days. On day 6, 6 hours after the last morphine injection, another set of tail-flick latencies was measured before and after dynorphin or saline administration (subcutaneous, intravenous, and pulmonary). Four animals each were used for the subcutaneous administration of saline, subcutaneous administration of N-MT Dyn A, intravenous administration of N-MT Dyn A, pulmonary administration of saline, and pulmonary administration of N-MT Dyn A, while 8 animals each were employed for the intravenous administration of N-MT Dyn A amide, intravenous administration of saline, and pulmonary administration of N-MT Dyn A amide.

Drug Administration

Evaluation of the effects of study medication on tail-flick latencies were performed 6 hours after the last morphine injection on study day 6.

Subcutaneous Administration

On day 6 of the experiment, 5 $\mu\text{mol/kg}$ N-MT Dyn A or saline in a volume of 1 mL was administered subcutaneously to morphine-tolerant rats.

Catheter Implantation and Intravenous Injection

Animals were anesthetized with a combination of ketamine (30 mg/kg) and xylazine (6 mg/kg) in a dose of 0.015 mL for implantation of a jugular catheter. The catheter (silicone tubing, size 0.025-inch inner diameter and 0.047-inch outer diameter from Helix Medical, Inc, Carpinteria, CA) was

inserted to the level of the right atrium and the distal end was passed through a rat jacket (Lomir Biomedical Inc, Malone, NY) to prevent the animal from reaching and damaging the catheter. Animals recovered fully from anesthesia before dynorphins were administered. This was also indicated by the tail-flick latencies of the placebo group, which were not significantly different from the baseline latencies obtained in nonanesthetized rats. Six hours after the last morphine injection, the rats were administered N-MT Dyn A or N-MT Dyn A amide (5 $\mu\text{mol/kg}$ in a volume of 500 μL) or saline (500 μL) through the jugular catheter.

Pulmonary Administration

The rats were anesthetized using a rodent anesthetic machine. They were placed in the induction chamber and received a combination of oxygen (1 L/min) and 5% Forane (isoflurane). Once the rat was fully anesthetized, 1 inch of a special round-tipped cannula attached to a delivery device for pulmonary administration of drugs (Penn-Century, Philadelphia, PA) was introduced into the trachea through the mouth. The solution of N-MT Dyn A and N-MT Dyn A amide (5 $\mu\text{mol/kg}$ in a volume of 100 μL) or saline (100 μL) was placed in the chamber of the device and instilled into the lungs with insufflations of 1.5 mL of air. The test drugs were administered 6 hours after the last morphine injection.

Measurement of Antinociceptive Effects

A tail-flick antinociceptive assay was employed^{20,21} (Tail Flick Analgesia Meter, 0570–001L, Columbus Instruments Int Corp, Columbus, OH). Thirty minutes before each experiment, the rats were moved to the experimental room to acclimatize them to the surroundings. For the measurements of tail-flick latencies, the rats were held in dark Plexiglas boxes with the tail positioned on the tail-flick apparatus for radiant heat stimulation. The tail-flick response was elicited by applying the heat stimulus to the dorsal part of the tail. The heat stimulus was adjusted to yield baseline latencies of 2 to 3 seconds, with a 12-second cut-off time to prevent tail damage. The baseline was determined on day 1 after saline injection and 30 minutes after injection of 3 mg/kg morphine given subcutaneously by using the median of 3 tail-flick tests separated by ~20 seconds. The tail-flick latencies were also measured on day 5, 6 hours after the last morphine injection (after the induction of tolerance before and after dynorphin injection at 15, 30, 45, 60, 90, 120, 150 minutes). Each data point for a given animal represents the average of 3 individual measurements.

Data Analysis

The antinociception data obtained by tail-flick test were expressed as a percent of maximum possible effect (%MPE),^{22,23} which is calculated:

Figure 1. Time course of changes in MPE % after intravenous administration of 5 µmol/kg [N-Met-Tyr¹]-Dynorphin A(1-13) ($n = 4$, ■), [N-Met-Tyr¹]-Dynorphin A(1-13) amide ($n = 8$, ◆), or saline ($n = 4$, ▲). Each value represents the mean; the vertical bars represent the $\pm$ SEM.

$$[(T_1 - T_0)/(T_2 - T_0)] \times 100 \quad (1)$$

where T_0 was the tail-flick latency time before morphine treatment on day 1, T_1 was the test latency time, and T_2 was the cut-off time, arbitrarily set at 12 seconds. The difference between the cut-off time (T_2) and the animal's baseline reaction time (T_0) was taken to be 100% effect. Reaction times that were measured on the study day before administration of the test substance were subtracted from all other tail-flick data. These estimates were used for the calculation of the cumulative effects. Area under the effect time curve ($AUC_{(0-t)}$) was calculated over the experimental time range from these %MPE vs time profiles using the trapezoidal rule. The time until the effect was reduced to zero was estimated in the following way. Assuming the rate of disappearance of the effect will be zero order for a drug with receptor-based mode of action,²⁴ the terminal slope of the effect time profile (S) was calculated. Based on this slope (S) and the effect at the last measurement point (E_x), the additional time to reach zero effect (t_z) was calculated as E_x/S for individual animals. The extrapolated area was then calculated as $E_x \times t_z/2$ and added to ($AUC_{(0-t)}$) to obtain AUC_{tot} . These extrapolated areas for both derivatized peptides and all routes of administration were less than 15% of the total area (average $11\% \pm 14\%$). Area under the first momentum curve ($AUMC_{(0-t)}$) was calculated from Effect $\times$ time vs time plots. Extrapolation from the last measurement point was calculated as $E_x \times t \times t_z/2$ and added to $AUMC_{(0-t)}$ to obtain $AUMC_{tot}$. Mean effect time (MET) was calculated according to the following equation:

$$MET = AUMC_{tot}/AUC_{tot} \quad (2)$$

Unpaired, 2-sided Student t test was used to analyze the statistical difference between the dynorphin-treated group and saline (control) group; P values less than .05 were accepted as significant.

RESULTS

Effects of Intravenous Administration of [N-Met-Tyr¹]-Dynorphin A(1-13) and [N-Met-Tyr¹]-Dynorphin A(1-13) Amide in Morphine-tolerant Rats

Treatment with morphine on day 1 shifted the latencies from 2.4 ± 0.4 seconds (baseline) to 6.4 ± 1.8 seconds (P value = 1×10^{-8}). Latencies observed on day 6 before dynorphin treatment were 2.9 ± 0.9 seconds (not significantly different from baseline when adjusted for multiple comparisons). This result indicated that tolerance was produced after 5 days of morphine treatment.

After intravenous administration of the dynorphins (5 µmol/kg), latencies increased by ~4- to 5-fold with maximum latencies (7-10 seconds) observed after ~30 minutes. Figure 1 shows the time course of the effect on tail-flick latencies (expressed as the %MPE) after intravenous administration of 5 µmol/kg of N-MT Dyn A, N-MT Dyn A amide, or saline.

For both compounds, maximum effects were observed 0.5 hour postadministration (Figure 1). The areas under the effect-time profiles (extrapolated or nonextrapolated) of both compounds exceeded that of the saline group (Table 1). There was, however, no significant difference between the AUC estimates of the 2 modified peptides ($P = .325$), although the maximum effects appeared more pronounced for N-MT Dyn A. The duration of the effects (Table 1) could be monitored for up to 3 hours for the amide derivative. The MET was significantly longer for amide (1.3 ± 0.3 hours) than for N-MT Dyn A (0.5 ± 0.3 hour). Similarly, the terminal slope determined for the amide appeared less steep than that for the N-MT Dyn A derivative (Table 1).

Effects of Subcutaneous Administration of [N-Met-Tyr¹]-Dyn A(1-13)

Figure 2 shows the time course of the effect on tail-flick latencies after subcutaneous administration of N-MT Dyn A or saline. No difference between the control and treatment groups was observed.

Effects of Pulmonary Administration of [N-Met-Tyr¹]-Dynorphin A(1-13) and [N-Met-Tyr¹]-Dynorphin A(1-13) Amide in Morphine-tolerant Rats

Figure 3 shows the time-effect curves after pulmonary administration of 5 µmol/kg of N-MT Dyn A, N-MT Dyn A amide, or saline. AUC estimates obtained for N-MT Dyn A

Table 1. Pharmacodynamic Properties

Peptide	Average AUC (MPE %*hr)	Terminal Slope (%*hr ⁻¹)	Time of Effect (hr)	Mean Effect Time (hr)
Intravenous				
Saline Control	-2 ± 25			
N-MT-Dyn	73 ± 34 (73 ± 35) (<i>P</i> = .325)	-51 ± 10 (<i>P</i> = .07)	2.0 ± 0.1 (<i>P</i> = .08)	0.5 ± 0.3 (<i>P</i> = .002)
N-MT-Dyn amide	85 ± 43 (94 ± 42) (<i>P</i> = .0972)	-30 ± 25 (<i>P</i> = .02)	3.3 ± 1.3 (<i>P</i> = .238)	1.3 ± 0.3 (<i>P</i> = .491)
Pulmonary				
Saline Control	-4 ± 12			
N-MT-Dyn	49 ± 18 (56 ± 14) (<i>P</i> = .420)	-33 ± 20 (<i>P</i> = .005)	2.8 ± 0.9 (<i>P</i> = .17)	0.6 ± 0.1 (<i>P</i> = .02)
N-MT-Dyn amide	54 ± 47 (74 ± 78)	-9 ± 7	4.4 ± 2.5	1.1 ± 0.4

Area under the curve (AUC) estimates were calculated over the observation period. AUC estimates in parenthesis represents interpolated values to infinity.

The *P* values in italic compare data of N-MT-Dyn with N-MT-Dyn amide groups for a given route of administration. The *P* values given in bold compare iv and pulmonary data for a given peptide.

Figure 2. Time course of changes in MPE % after subcutaneous administration of 5 μmol/kg [N-Met-Tyr¹]-Dynorphin A(1-13) (*n* = 4, ■) or saline (*n* = 4, ▲). Each value represents the mean; the vertical bars represents the ± SEM.

Figure 3. Time course of changes in MPE % after pulmonary administration of 5 μmol/kg [N-Met-Tyr¹]-Dynorphin A(1-13) (*n* = 4, ▲), [N-Met-Tyr¹]-Dynorphin A(1-13) amide (*n* = 8, ▼), or saline (*n* = 4, ■). Each value represents the mean; the vertical bars represents the ± SEM.

and N-MT Dyn A amide did not differ significantly, with both estimates representing about two thirds of those observed after intravenous administration of the same dose (Table 1). Antinociceptive effects of the amide derivative could be monitored for up to 4 hours. METs for N-MT Dyn A and N-MT Dyn A amide were 0.6 and 1.1 hours, respectively. Terminal slopes determined for N-MT Dyn A amide were less steep than for the N-MT Dyn A derivative and those determined from the corresponding intravenous data.

DISCUSSION

Different methods for inducing experimental morphine tolerance, such as subcutaneous administration of morphine 2 or 3

times a day for 3 to 8 days²² or implanting morphine pellets⁹ can be found in current literature. We found that subcutaneous injection of morphine sulfate twice a day was both easy to perform and well tolerated by the rats. Before starting the repeated injections of morphine sulfate, a single, analgesic dose (3 mg/kg) was used to test the analgesic effect of morphine on rats. The tail-flick latencies increased 2- or 3-fold as compared with saline administration. After chronic morphine administration the tail-flick latencies were statistically not different from the baseline indicating that tolerance was observed.

Previous studies conducted in morphine-tolerant rats showed a short but distinct effect after intravenous-administered dynorphin A(1-13) (20 to 40 minutes).⁷ The lack of any

antinociceptive activity when given alone at doses up to 50 mg/kg (30 μ mol/kg, 6 times higher than the doses used in this study) suggested that after this form of administration, underivatized Dyn A(1–13) is modulating the activity of morphine and does not independently exhibit antinociceptive activity.⁴ Assuming an identical mode of action for our dynorphin derivatives, one might speculate the lack of any antinociceptive activity of the dynorphin derivatives by themselves, especially as modulation at the tyrosine 1 position should prevent interaction with the opioid receptor system. However, we did not test the antinociceptive effects of these derivatives in the absence of morphine in nontolerant animals and a definite conclusion as to whether the derivatives modulate the morphine tolerance or show antinociceptive effects by themselves cannot be reached with the present data. What can be stated is that the derivatives induce antinociceptive responses in morphine-tolerant animals during morphine administration.

Because intravenously administered Dyn A(1–13) has a short half-life of less than 1 minute,²⁵ we were interested in evaluating stabilized dynorphin derivatives. In a previous study,¹⁷ the metabolic stability of N- and C-terminal modifications of Dyn A (1–13) derivatives in human plasma was investigated and it was reported that one of the derivatives, N-MT Dyn A amide, showed a half-life in human plasma that was more than 100 times longer than that of Dyn A(1–13). The same study revealed on a qualitative level that these modifications remained pharmacologically active. The present study assessed, on a more quantitative level, the pharmacological effects over time and compared it with a similar derivative that lacked protection of the C-terminus. The main purpose of this study was, however, to investigate whether other routes of administration, especially the pulmonary route, might represent a viable alternative to intravenous administration.

After intravenous administration, the N-MT Dyn A derivative (only partially protected because of the high activity of carboxypeptidases at the C-terminal end of Dyn A(1–13)) showed a duration of activity of ~2 hours, which was somewhat prolonged when compared with literature values for Dyn A(1–13) (20 to 40 minutes). The *in vivo* activity of the N-MT Dyn A amide (half-life of more than 100 minutes in human plasma) should provide much better protection than N-MT Dyn A. Indeed, N-MT Dyn A amide had a longer effect (up to 3.3 hours) *in vivo* with a mean effect time of 1.3 vs 0.5 hours (Figure 1, Table 1), than the nonamide derivative (Table 1). Based on the estimates of the mean effect times and the duration of the effect (Table 1), N-MT Dyn A amide seems to provide somewhat longer protection. However, these differences were modest. This observation might be related to several factors. First, stability tests *in vitro* might not reflect the pharmacokinetic properties *in vivo*. Second, generated metabolites might be active and therefore are responsible for the observed pharmacological effect. Further pharmacokinetic studies need to be performed for a more complete interpretation of these observations.

One drawback of intravenous administration is that repeated intravenous injections reduce the compliance of the patients. A variety of alternative administration routes have been proposed for proteins and peptides.²⁶ Previous studies with Dyn A(1–13) have shown that subcutaneous administration does not lead to pharmacological activity,¹⁶ suggesting that when administered subcutaneously only a small percentage of the peptide enters the bloodstream.¹⁶ These findings were supported by our experiments with the N-terminus-stabilized dynorphin derivative. However, at least theoretically, the low pharmacological activity observed after subcutaneous administration during the 1 hour of monitoring could be due to a slow absorption of the peptide. Thus, an effect might have been observed at later time points. However, such a slow onset of action would not be desirable from a pharmacological point of view.

Pulmonary delivery has been suggested for protein and peptide drugs,^{26,27} such as insulin, calcitonin, or growth hormones. In our experiments, both N-MT Dyn A and N-MT Dyn A amide were effective in enhancing antinociceptive activity in morphine-dependent rats after pulmonary administration (Figure 3). Overall the effect observed after pulmonary delivery was about two thirds of that observed after intravenous administration (although these differences were not statistically significant), indicating a high systemic availability after pulmonary delivery. The effect of N-MT Dyn A amide was observable for up to 4 hours (Table 1, with a slightly longer MRT than that of N-MT Dyn A) with no statistical difference seen between intravenous and pulmonary delivery.

Based on the comparison of duration of action of N-MT Dyn A and N-MT Dyn A amide, we conclude that both derivatives effectively modulate antinociceptive activity in morphine-tolerant rats after intravenous and pulmonary administration. Our results show that these derivatives produce measurable pharmacological activities for approximately 2 hours.

Our study supports, in general, the importance of chemically modified dynorphin derivatives, although further attempts should look at even more stable derivative, eg, by protecting metabolism through endopeptidases and to further evaluate hepatic metabolism. In addition, the study indicated that pulmonary delivery might represent a promising approach to deliver these drugs.

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