

Reticuline Exposure to Invertebrate Ganglia Increases Endogenous Morphine Levels

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Submitted: September 2, 2004

Accepted: September 12, 2004

Key words: **nitric oxide; opiate precursors; opiate metabolites; nervous tissue; invertebrates; mussels**

Neuroendocrinol Lett 2004; 25(5):323-330 NEL250504A01 Copyright © Neuroendocrinology Letters www.nel.edu

Abstract

OBJECTIVES: Given the presence of morphine, its metabolites and precursors in mammalian and invertebrate tissues, it became important to determine if exposing tissues to an opiate alkaloid precursor, reticuline, would result in increasing endogenous morphine levels.

METHOD: Endogenous morphine levels were determined by high pressure liquid chromatography coupled to electrochemical detection and radioimmunoassay following incubation of *Mytilus edulis* pedal ganglia with reticuline. Nitric oxide (NO) release was determined in real-time via an amperometric probe. Mu opiate receptor affinity for opiate alkaloid precursors was determined by a receptor displacement assay.

RESULTS: Morphine is present in the pedal ganglia of *Mytilus edulis* (1.43 ± 0.41 ng/mg \pm SEM ganglionic wet weight). Ganglia incubated with 50 ng of reticuline, a morphine precursor in plants, for 1 hour exhibited a statistical increase in their endogenous morphine levels (6.7 ± 0.7 ng/mg tissue wet weight; $P < 0.01$). This phenomenon is concentration dependent. The increase in ganglionic morphine levels occurs gradually over the 60 min incubation period, beginning 10 minutes post reticuline addition. We show that reticuline (10^{-6} M) does not stimulate ganglionic NO release in a manner resembling that of morphine (10^{-6} M), which releases NO seconds after its exposure to the ganglia and lasts for 5 minutes. With reticuline, there is a 3 minute delay, which is followed by an extended release period. Furthermore, in binding displacement experiments both reticuline and salutaridine (another morphine precursor) exhibit no binding affinity for the pedal ganglia mu opiate receptor subtype. This finding is further substantiated using the positive control of human monocytes where the mu3 opiate receptor subtype has been cloned.

CONCLUSION: Taken together, we surmise that the morphine's precursors are being converted to morphine. The experiments strongly indicate that pedal ganglia can synthesize morphine from reticuline.

Abbreviations:

NO	- Nitric oxide;
HPLC	- High pressure liquid chromatography;
THP	- tetrahydropapoverine;
PBS	- phosphate buffered saline;
RIA	- radioimmunoassay

Introduction

There is a body of evidence indicating that opiate alkaloids such as morphine, morphine-3- and 6- glucuronide, as well as the morphine putative precursor molecules (thebaine, salutaridine, norcocolarine, reticuline, tetrahydropapoverine (THP) and codeine) exist in vertebrates [12,22,14,55]. In invertebrates, specifically *Mytilus edulis*, the presence of morphine, morphine-6-glucuronide, morphine-3-glucuronide, codeine, THP and reticuline also have been reported [40,17,41,53, 56]. Taken together, these data provide important evidence supporting the hypothesis that animals have the ability to synthesize opiate alkaloids.

Based on the above reports, it is important to determine if a morphine precursor found in animal tissues has the ability to augment endogenous morphine levels, indicating that morphine synthesis is occurring. In the present report, we demonstrate that exposing *Mytilus edulis* pedal ganglia to low levels of reticuline results in enhancing ganglionic morphine levels, strongly suggesting, as in plants, this molecule leads to morphine biosynthesis [6].

Material and Methods

Mytilus edulis collected from the local waters of Long Island Sound were maintained under laboratory conditions for at least 14 days prior to being used in experiments. Mussels were kept in artificial seawater (Instant Ocean, Aquarium Systems, Mentor, Ohio) at a salinity of 30 PSU and at a temperature of 18°C as previously described [47].

Biochemical Analysis

For reticuline exposure, 400 animals were placed and maintained in artificial seawater at 24 °C whereas control animals (100) were exposed to vehicle (PBS). For the biochemical analysis, groups of 20 animals had their pedal ganglia excised at different time periods after incubation with reticuline.

Morphine Determination, Solid Phase Extraction

The extraction protocol, using internal or external morphine standards, was performed in a room where the animals were not maintained to avoid morphine contamination. Single use siliconized tubes were used to prevent the loss of morphine. *Mytilus edulis* pedal ganglia also were extensively washed (3 times) with PBS (0.01M NaCl 0.132 mM, NH₄HCO₃ 0.132 mM; pH 7.2) prior to extraction (3 times centrifugation at 1000 rpm, 1 min, then discard the PBS) to avoid exogenous morphine contamination. Tissues were dissolved in 1N HCl and sonicated using a Fisher scientific sonic

dismembrator 60 (Fisher Scientific, USA). The resulting homogenates were extracted with 5 ml chloroform/isopropanol 9:1. After 5 min at room temperature, homogenates were centrifuged at 3000 rpm for 15 min. The three phases were separated in the following order: 1) The lowest layer corresponding to the organic phase; 2) The intermediate phase corresponding to precipitated proteins; and 3) The top aqueous supernatant phase containing morphine. The supernatant was collected and dried with a Centrivap Console (Lab-conco, Kansas City, Missouri). The dried extract was then dissolved in 0.05% trifluoroacetic acid (TFA) water before solid phase extraction. Samples were loaded on a Waters Sep-Pak Plus C-18 cartridge previously activated with 100% acetonitrile and washed with 0.05% TFA-water. Morphine elution was performed with a 10% acetonitrile solution (water/acetonitrile/TFA, 89.5%:10%:0.05%, v/v/v). The eluted sample was dried with a Centrivap Console and dissolved in water prior to high pressure liquid chromatography (HPLC) analysis.

Radioimmuno-assay (RIA) determination

The morphine RIA determination is a solid phase, quantitative RIA, wherein ¹²⁵I-labeled morphine competes for a fixed time with morphine in the test sample for the antibody binding site. The commercial kit employed is from Diagnostic Products Corporation (USA). Because the antibody is immobilized on the wall of a polypropylene tube, simply decanting the liquid phase to terminate the competition and to isolate the antibody-bound fraction of radiolabeled morphine is sufficient. The material is then counted in a Wallac, 3", 1480 gamma counter (Perkin Elmer, USA). Comparison of the counts to a calibration curve yields a measure of the morphine present in the test sample, expressed as nanograms of morphine per milliliter. The calibrators contain, respectively, 0, 2.5, 10, 25, 75 and 250 nanograms of morphine per milliliter (ng/mL) in PBS. Reticuline and salutaridine do not cross-react with the antibody. The detection limit was 0.5 ng/ml.

HPLC and electrochemical detection of morphine in the sample

The HPLC analyses were performed with a Waters 626 pump (Waters, Milford, MA) and a C-18 Unijet microbore column (BAS). A flow splitter (BAS) was used to provide the low volumetric flow-rates required for the microbore column. The split ratio was 1:9. Operating the pump at 0.5 ml/min, yielded a microbore column flow-rate of 50 μ l/min. The injection volume was 5 μ l. Morphine detection was performed with an amperometric detector LC-4C (BAS, West Lafayette, Indiana). The microbore column was coupled directly to the detector cell to minimize the dead volume. The electrochemical detection system used a glassy carbon-working electrode (3 mm) and a 0.02 Hz filter (500mV; range 10 nA). The cell volume was reduced by a 16 μ m gasket. The chromatographic system was controlled by Waters Millennium³² Chromatography Manager V3.2

software and the chromatograms were integrated with Chromatograph software (Waters).

Morphine was quantified in the tissues by the method described by Zhu and Stefano [52]. This method was carried out in the following manner: The mobile phases were: Buffer A: 10 mM sodium chloride, 0.5 mM EDTA, 100 mM sodium Acetate, pH 5.0; Buffer B: 10 mM sodium chloride, 0.5 mM EDTA, 100 mM sodium Acetate, 50% acetonitrile, pH 5.0. The injection volume was 5 μ l. The running conditions were: from 0 min 0% buffer B; 10 min, 5% buffer B; at 25 min 50% buffer B; at 30 min 100% buffer B. Both buffers A and B were filtered through a Waters 0.22 μ m filter and the temperature of the whole system was maintained at 25 °C. Several HPLC purifications were performed between each sample to prevent residual morphine contamination remaining on the column. Furthermore, mantle tissue was run as a negative control, demonstrating a lack of contamination [55].

Nitric oxide assay

Ten pedal ganglia (per determination) dissected from *M. edulis* were bathed in 1 mL sterile phosphate buffered saline (PBS). Experiments used morphine at a final concentration of 10^{-6} M, naloxone at 10^{-6} M, and 1 μ g of reticuline. For the opiate receptor antagonist experiments, ganglia were pretreated with naloxone for 10 min prior to reticuline addition. NO release was monitored with an NO-selective microprobe manufactured by World Precision Instruments (Sarasota, FL). The sensor was positioned approximately 100 μ m above the respective tissue surface. Calibration of the electrochemical sensor was performed by use of different concentrations of a nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine (SNAP), as previously described by [29]. The NO detection system was calibrated daily. The probe was allowed to equilibrate for 10 min in the incubation medium free of tissue before being transferred to vials containing the ganglia for another 5 min. Manipulation and handling of the ganglia was only performed with glass instruments. Data was acquired using the Apollo-4000 free radical analyzer (World Precision Instruments, Sarasota, FL). The experimental values were then transferred to Sigma-Plot and -Stat (Jandel, CA) for graphic representation and evaluation.

Binding experiments

Human monocytes served as a positive control since the μ 3 opiate receptor subtype, which is coupled to NO release, has been cloned from these cells [7]. The monocytes were obtained from the Long Island Blood Center (Melville, Long Island) and processed as previously described in detail [40,5,30]. An additional 100 excised pedal ganglia were washed and homogenized in 50 volumes of 0.32 M sucrose, pH 7.4, at 4 °C, by the use of a Brinkmann polytron (30 s, setting no. 5) as were the human monocytes. The crude homogenate is centrifuged at 900 x g for 10 min at 4 °C, and the supernatant is reserved on ice. The whitish crude pellet is resuspended by homogenization (15 s, setting

no. 5) in 30 volumes of 0.32 M sucrose/Tris-HCl buffer, pH 7.4, and centrifuged at 900 x g for 10 min. The extraction procedure is repeated one more time, and the combined supernatants were centrifuged at 900 x g for 10 min. The resulting supernatants (S1') are used immediately. Prior to the binding experiment, the S1' supernatant is centrifuged at 30,000 x g for 15 min. and the pellet (P2) is washed once by centrifugation in 50 volumes of the sucrose/Tris-HCl. The P2 pellet is then re-suspended with a Dounce hand-held homogenizer (10 strokes) in 100 volumes of buffer. Binding analysis is then performed on the cell membrane suspensions. Aliquots of membrane suspension (0.2 ml, 0.12 mg of membrane protein) are incubated in triplicate at 22 °C for 40 min with the appropriate radiolabeled ligand in the presence of dextrorphan (10 mM) or levorphanol (10 mM) in 10 mM Tris-HCl buffer, pH 7.4, containing 0.1% BSA and 150 mM KCl. Free ligand is separated from membrane-bound labeled ligand by filtration under reduced pressure through GF/B glass fiber filters (Whatman); filters were presoaked (45 min, 4 °C) in buffer containing 0.5% BSA. The filters are rapidly washed with 2.5 ml aliquots of the incubation buffer (4 °C), containing 2% polyethylene glycol 6000 (Baker). They are assayed by liquid scintillation spectrometry (Packard 460). Stereospecific binding is defined as binding in the presence of 10 mM dextrorphan minus binding in the presence of 10 mM levorphanol. Protein concentration is determined in membrane suspensions (prepared in the absence of BSA). For IC₅₀ determination (defined as the concentration of drug which elicits half-maximal inhibition of specific ³H-dihydromorphine binding (for μ 3), an aliquot of the respective tissue-membrane suspension is incubated with non-radioactive opioid compounds at 6 different concentrations for 10 min at 22 °C and then with ³H-dihydromorphine for 60 min at 4 °C as previously noted in detail [40]. The mean \pm S.E.M. for three experiments is recorded for each compound. All agents Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE) and naltrexone are from Sigma Chemical Co. (St.Louis, MO).

Results

Morphine was identified in the ganglionic extraction by reverse phase HPLC using a gradient of acetonitrile following liquid and solid extraction, and was compared to an authentic standard (Figure 1). The material exhibited the same retention time as authentic morphine, confirming earlier studies that also identified this material via mass spectrometry (Figure 1; [52]). The electrochemical detection sensitivity of morphine is 80 picograms. The concentration of morphine was 1.43 ± 0.41 ng/mg \pm SEM ganglionic wet weight as determined by the Chromatogram Manager 3.2 commercial software (Millennium³², Waters, Milford, MA) extrapolated from the peak-area calculated for the external standard. Ganglia incubated with 50 ng of reticuline for 1 hour exhibited a statistical increase in their endogenous morphine levels (6.7 ± 0.7 ng/mg tissue wet weight; $P < 0.01$) (Figure 1).

The electrochemical results are compatible with the RIA quantification (Figure 2), which yields a control ganglionic level of morphine at 1.33 ± 0.61 ng/mg tissue wet weight \pm SEM. Incubation with various concentrations of reticuline increases ganglionic morphine levels after one hour in a concentration dependent manner (Figure 2). Exposure of excised ganglia to 100 ng of reticuline yields about 14.53 ± 4.6 ng/

mg morphine (Figure 2; $P < 0.001$). The increase in ganglionic morphine levels occurs gradually over the 60 min incubation period, beginning 10 minutes post reticuline addition (Figure 3). From these studies, we estimate that approximately 24% of the reticuline gets converted to morphine. Blank runs between morphine HPLC determinations did not show a morphine residue with RIA, nor did any signs of its presence occur with mantle tissue. Incubation of 50 ng of reticuline

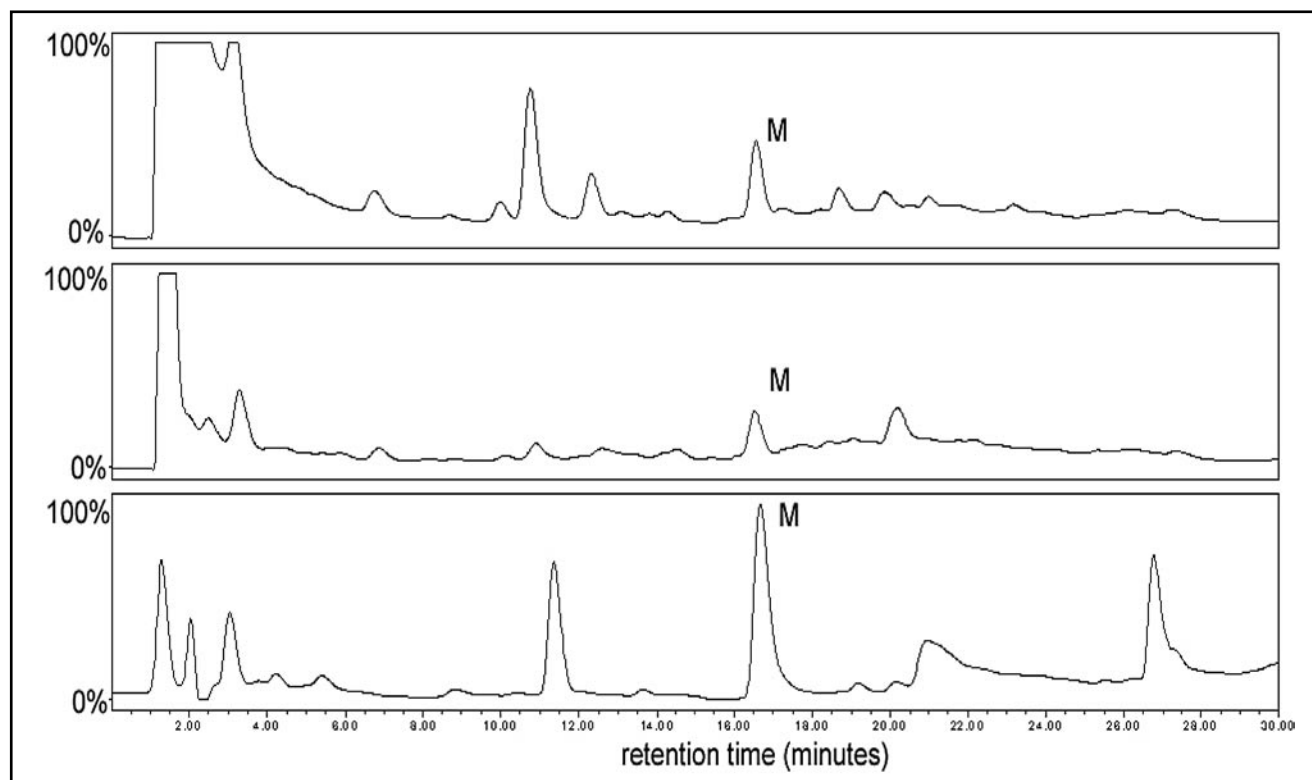


Figure 1. HPLC chromatogram of ganglia extraction. Top. Ganglia incubated with 50 ng of reticuline for 1 hour exhibit a level of 5 ng/mg morphine tissue wet weight. Middle. Control ganglia. Bottom. External morphine standard 15 ng.

Table I. Reticuline and salutaridine stimulated ganglionic nitric oxide release. Ligand stimulated NO levels appear to be similar. Peak NO release times differ, suggesting reticuline and salutaridine do not directly stimulate ganglionic NO release. Furthermore, morphine stimulated NO release occurs following a 16.1 ± 5.6 sec latency whereas reticuline's and salutaridine's latency are 2.9 ± 0.8 and 3.1 ± 1 min, respectively. Each experiment was repeated 4 times and the mean \pm SEM shown in the table. Reticuline's and salutaridine's NO peak time and latency before NO production rose at 10 nM were statistically different ($P < 0.01$) from those values recorded for morphine and dihydromorphine.

LIGAND	NO Peak Level (nM)	NO Peak Time (min)
Control	0.9 ± 0.1	None
Morphine (10^{-7} M)	24.3 ± 3.1	0.8 ± 0.2
Reticuline (10^{-7} M)	17.6 ± 3.8	9.2 ± 1.5
Salutaridine (10^{-7} M)	18.5 ± 3.3	8.9 ± 1.3
Dihydromorphine (10^{-7} M)	23.2 ± 3.7	0.9 ± 0.3

Table II. Displacement of ^3H -dihydromorphine (DHM; $\text{nmol}\cdot\text{L}^{-1}$) by opioid ligands in various tissue membrane suspensions. One hundred per cent binding is defined as bound ^3H -DHM in the presence of $10 \mu\text{M}$ dextrorphan minus bound ^3H -DHM in the presence of $10 \mu\text{M}$ levorphanol. IC_{50} is defined as the concentration of drug which elicits half-maximal inhibition of specific binding. The mean \pm SD. for three experiments is given. DPDPE = (D-Pen², D-Pen⁵)-enkephalin.

LIGAND	Pedal Ganglia	Monocytes
δ-agonist		
DPDPE	>1000	>1000
μ-agonist		
Reticuline	>1000	>1000
Salutaridine	>1000	>1000
Dihydromorphine	22 ± 2.3	19.1 ± 3.3
Antagonists		
Naltrexone	31 ± 7.1	34.6 ± 8.2

Figure 2. Reticuline incubation of *Mytilus edulis* ganglia. Ganglia incubated with 1, 10, 50, 100 ng of reticuline for 60 min. Morphine concentrations were obtained by RIA. One Way ANOVA analysis shows the morphine level in ganglion incubated with reticuline were significantly higher than control at 50 and 100 ng of reticuline. One ganglion weighs about 1.7 mg.

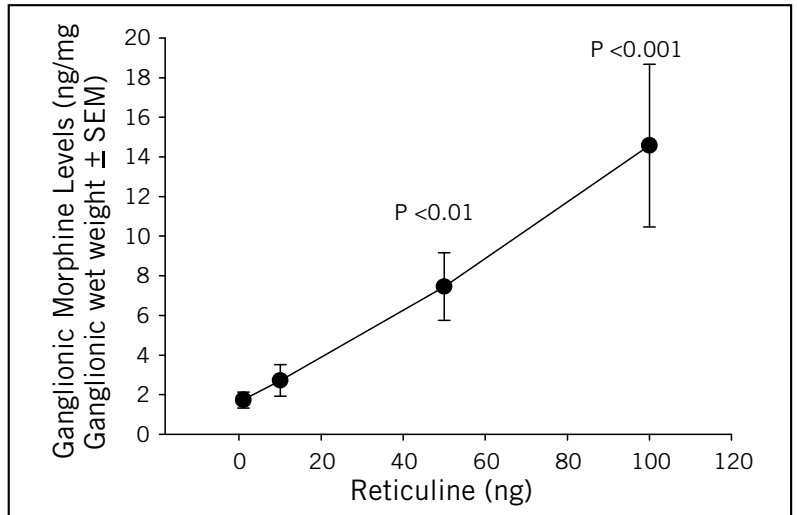


Figure 3. Reticuline (50 ng) incubation of *Mytilus edulis* ganglia over 60 min. The results of morphine concentration were obtained by RIA. One Way ANOVA analysis shows the morphine level in ganglia incubated with reticuline were significantly higher than control at 60 min.

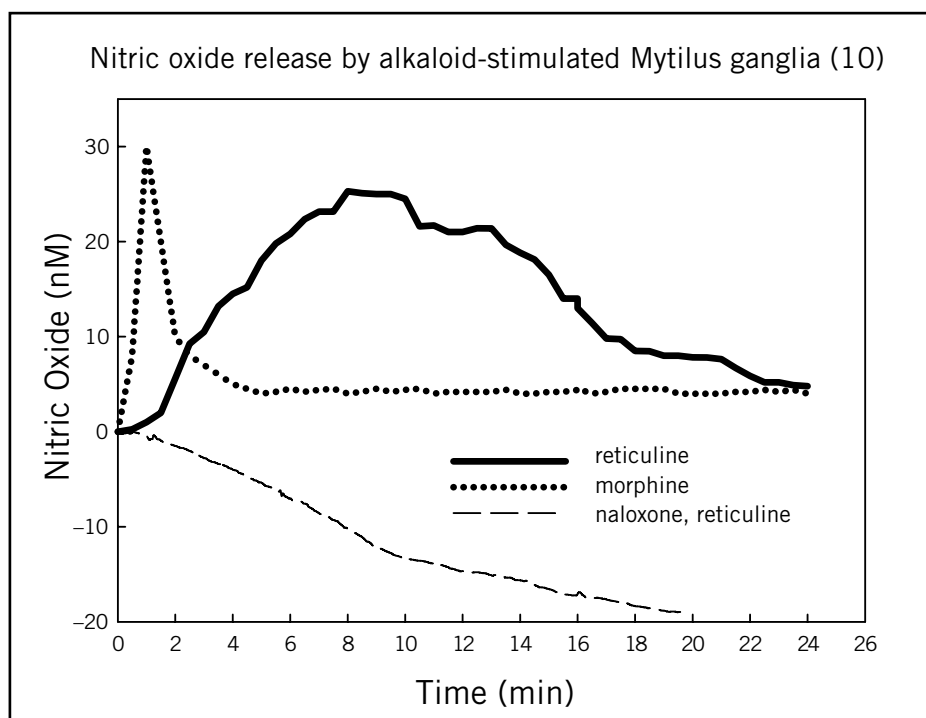
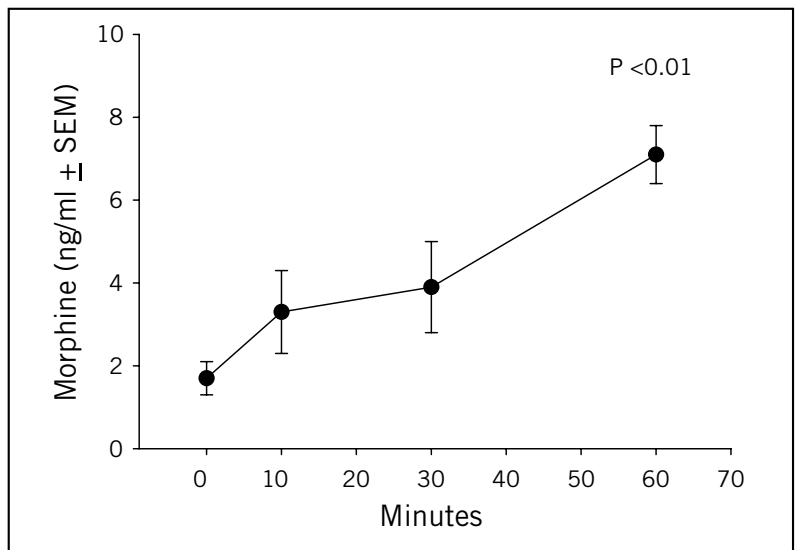


Figure 4. Real-Time ganglionic nitric oxide release following reticuline (10^{-7} M), morphine (10^{-6} M) and naloxone (10^{-6} M) prior to reticuline exposure.

with mantle tissues did not produce detectable morphine (data not shown).

Previously, we demonstrated that pedal ganglia, which contain mu opiate receptors, respond to morphine exposure by releasing constitutive nitric oxide synthase derived NO in a naloxone and L-NAME sensitive manner [45,8]. In an attempt to substantiate the identity of newly synthesized morphine further, ganglia were examined for their ability to release NO in response to reticuline exposure (Figure 4; Table 1). We show that reticuline (10^{-7} M) does not stimulate ganglionic NO release in a manner resembling that of morphine (10^{-6} M), which releases NO seconds after its application to the ganglia and lasts for 5 minutes (Figure 4; Table 1). Instead, with reticuline, there is a 3 minute delay, which is followed by an extended release period occurring over 17 minutes (Figure 4). We surmise that this reticuline-stimulated release occurs because it is being converted to morphine, which is actually responsible for the release, as indicated by the time course of the morphine increase, following reticuline exposure (Figure 3).

Table 2 demonstrates that both reticuline and salutaridine, another putative morphine precursor [55], do not exhibit binding affinity for the pedal ganglia mu opiate receptor subtype, i.e., mu3 [8]. This finding is further substantiated using the positive control of human monocytes where the mu3 opiate receptor subtype has been cloned [7]. This result strongly suggests that the pre-treatment of the ganglia with naloxone (10^{-6} M) blocking the reticuline (10^{-7} M) stimulated release of NO (Figure 4) occurs by way of this precursor being converted to morphine since reticuline does not have an affinity for the mu opiate receptor (Table 2).

Discussion

The present study demonstrates the following:

- 1) Morphine is present in *Mytilus* pedal ganglia [40,43,53], confirming earlier reports, including those using mass spectrometry;
- 2) Exposing pedal ganglia to a putative morphine precursor, reticuline [46,55], results in significant increases in ganglionic morphine levels in a concentration and time dependent manner;
- 3) Reticuline stimulates ganglionic NO production, following a latency period, in a manner consistent with it being converted to morphine; and
- 4) Reticuline does not exhibit an affinity for the mu3 opiate receptor, again suggesting that NO release occurs because of its conversion into morphine.

Taken together, it is apparent that animals have the ability to synthesize morphine from reticuline.

Morphine's synthesis, including enzymes and precursors, has been demonstrated in the plant *Papaver somniferum* [23,24,26,25]. Morphine, codeine and thebaine have been identified in mammalian tissues [12,15,21,20,18], suggesting the ability for mammals to synthesize the morphine skeleton [50]. This evidence suggests that mammals synthesize morphine in a manner similar to that of the poppy plant, see

[55,34]. Supporting this view are the findings demonstrating morphine in various invertebrates, including parasites and free-living invertebrates identified by HPLC-coupled to electrochemical detection and Q-TOF-Mass Spectrometry [17,54,16,27]. We have also found reticuline, tetrahydropapoverine and codeine in invertebrate tissues along with the morphine metabolites, morphine-6-glucuronide and -3-glucuronide, providing additional evidence for this ability in evolutionarily diverse animals, as well as suggesting that this pathway has been conserved during evolution in both plants and animals [40,39,41,17,53,54]. Furthermore, the newly cloned mu3 opiate receptor subtype, found in human and invertebrate tissues, only responds to opiate alkaloids as opposed to opioid peptides, providing the means for endogenous opiate alkaloid signaling [40], adding additional support for endogenous morphine signaling. Invertebrate tissues express mu opiate receptors that are also coupled to NO release [8]. The lack of mu affinity for reticuline and salutaridine suggests that these compounds are indeed being converted to morphine since they cannot release NO directly.

The argument for a *de novo* biosynthetic pathway in animals can be supported with studies demonstrating the ability of animal enzymes to synthesize, through the same precursors, morphine in an identical stereo- and regio-specific manner to that of the poppy plant, see [55]. In particular, reticuline's conversion to morphine in mammalian and invertebrate tissues, as suggested by the present data, can occur in pigs, rats, sheep, and cows where the conversion of reticuline to salutaridine, the next step in the pathway, can be mediated by cytochrome P450 [1,2]. This enzyme catalyzes the phenol oxidative coupling reaction in the same NADPH and O₂ dependent manner as that of the plant. This enzyme is stereo-, regio-, and substrate specific, which may further demonstrate the ability of animals to synthesize morphine endogenously.

Thebaine, following salutaridine in the morphine biosynthetic pathway, also was found in mammalian brain [20]. This is the first morphian alkaloid formed en route to the formation of morphine. Rat microsomes demonstrated the ability to transform thebaine to oripavine through a cytochrome P450 2D1 enzyme [38]. Furthermore, morphine dehydrogenase was discovered in mammals [51]. This enzyme is NADPH-dependent and catalyzes the oxidation of morphine to morphinone as well as the reverse reaction [51] that produces morphine, adding to the evidence of the capability of animal enzymes to catalyze the necessary reactions to conclude the pathway.

With regard to reticuline, it is found in a variety of plants [56,10,33,35,49]. The fruit and bark of Annonaceae family of plants is used as an analgesic, diuretic, and cough suppressant [9], properties shared with morphine. *Graviola*, which also contains reticuline, is also used as an anti-hypertensive, anti-arthritic, and anti-diarrhea remedy [9], further supporting our hypothesis that this compound may yield morphine in

animal tissues since it shares pharmacological actions associated with morphine.

At the cellular level, reticuline reduces cytosolic calcium concentrations, inhibiting uterine contractions [32]. Kimura et al. [19] have isolated tetrahydroisoquinoline alkaloids from *Magnolia* and have shown that reticuline decreases contractions in guinea pig papillary muscle. Martin et al. [31] also have demonstrated the antispasmodic activity of isoquinoline alkaloids. These researchers speculated that the antispasmodic effects were due to an inhibition of inter- and/or extracellular calcium. In our laboratory, we have demonstrated that NO via morphine or estrogen stimulation can inhibit muscle contraction [44] and subsequently influence calcium mobility, as well as actin degranulation, accounting for the lack of muscle contraction [44]. Hence, via reticuline synthesis to morphine, these actions can also be attributable to morphine.

Moreover, other researchers have found that reticuline has antimicrobial, antiviral and molluscicidal properties [28,36,35,13]. This can also be explained by the synthesis of morphine via reticuline since morphine via the μ_3 opiate receptor subtype is coupled to NO release in brain, immune, vascular and gut tissues [42,30,37,11,48]. Thus, these actions may also be attributable to NO [3,4], following morphine synthesis.

Acknowledgements

This work was supported, in part, by the following grants: NIMH 47392 and DA 09010. The authors wish to express their gratitude to Dr. Meinhard H. Zenk and Dr. Trevor Robinson for providing reticuline.

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To cite this article: *Neuroendocrinol Lett* 2004; **25**(5):323–330