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REVIEW ARTICLE

The American lobster, *Homarus americanus*, contains morphine that is coupled to nitric oxide release in its nervous and immune tissues: Evidence for neurotransmitter and hormonal signaling

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Abstract

OBJECTIVES: The study was designed to determine if morphine was present in lobster tissues. It was also important to determine, as in other animals, if its levels would change in response to stress. In this regard, it was also important to determine if lobster immune and neural tissues express the mu opiate receptor subtype, which was coupled to constitutive nitric oxide synthase derived nitric oxide release.
METHODS: *Homarus americanus* were used in these experiments. Morphine was purified in lobster tissues via high pressure liquid chromatography coupled to UV detection. It was quantified via radioimmunoassay (RIA) and was identified via quadruple time of flight – mass spectrometry. Animals were subject to 2 forms of trauma, namely pereiopod-ablation or lipopolysaccaride (LPS) –injection, and morphine levels determined in nerve cord or hemolymph. Real-time nitric oxide production was determined via an amperometric probe. RT-PCR was used to determine the presence of a μ opiate receptor transcript.
RESULTS: In *Homarus americanus* hemolymph and nerve cord morphine was found. RIA revealed morphine levels of 3.36 pg/mg +/– 0.48 SEM (N=8) in nerve cord and 717.88 pg/ml +/– 56.77 SEM (N=58) in hemolymph. In stressed (pereiopod-ablated or LPS-injected) animals, the endogenous morphine levels initially increased significantly by 24% for hemolymph and 48% for nerve cord. By day 5, the stressed and control values for endogenous morphine, in both tissues, was lower and non-distinguishable. In both hemocytes and neural cells, morphine, not met-enkephalin, stimulated constitutive nitric oxide release in a naloxone antagonizable manner, demonstrating a mu opiate receptor mediated phenomenon and suggesting the presence of the mu opiate receptor subtype, μ_3, since it is opiate alkaloid selective and opioid peptide insensitive. RT-PCR revealed the presence of a μ opiate receptor transcript in *Homarus* neural and immune tissues, which exhibits a 100% sequence identity with its human counterpart.
CONCLUSION: Taken together, after eliminating all sources of contamination, morphine is present in lobster tissues, potentially demonstrating hormonal and neurotransmitter functions that are involved in the animals' stress response.

INTRODUCTION

In numerous studies, morphine has been identified in neural and immune tissues of various mollusks by biochemical and immunocytochemical techniques [8,15,16,18,25], including animal parasites [7,10,11,27]. Morphine metabolites have also been identified in these reports. Recently, the morphine precursor, reticuline, was also found in mussels [30]. Furthermore, after exposing mussel ganglia to reticuline, the endogenous ganglionic morphine levels rose significantly, demonstrating that these tissues can synthesize this opiate alkaloid [31]. These reports also demonstrate that invertebrate endogenous morphine processes respond to various stressors [5,13], exhibiting increases in its level in various tissues, including nervous [15,21].

These studies prompted us to determine if morphine, and its associated signaling processes, were present in *Homarus americanus* and whether, as in mollusks, this system would respond to various stressors. In the present report, we demonstrate the presence of morphine, as well as its coupling to constitutive nitric oxide release via a mu opiate receptor subtype in lobster tissues. Moreover, endogenous morphine levels do change in response to different types of stressors.

MATERIALS AND METHODS

Adult *Homarus americanus,* at the intermolt stage, were obtained from commercial fish stores on Long Island, NY, and kept in marine aquaria described in detail elsewhere [24]. They were housed 6 per 200 gallon tank and maintained on a 10-h light / 14-h dark regime. Twenty four laboratory maintained lobsters (controls) were monitored throughout a stabilization period of 15 days. Hemolymph was obtained from the haemocoelomic space between the carapace and first body segment using 3 ml-syringes (21G). These samples were immediately half-diluted with modified Alsever solution (MAS), a Na/Citrate-based anticoagulant (27 mM Na-citrate, 336 mM NaCl, 115 mM Glucose, 9 mM EDTA) [14]. Hemolymph samples (0.5–2ml/animal) were then centrifuged at 700G and 4°C for 10 minutes in 15-ml high density polypropylene tubes (Falcon). Supernatants were separated from hemocyte pellets by transfer into new tubes. For stress experiments, 34 animals were subjected to different trauma (24 to pereiopod ablation and 10 to 1µg-injections of lipopolysaccharide (LPS) in order to simulate a bacterial assault). Hemolymph and lobster nerve cord samplings for morphine determinations (control and stressed groups) were performed at T_0 and at 1, 3, 5, 10 and 15 days thereafter.

Extraction

Extraction experiments using internal or external morphine standards were performed in different rooms to avoid contamination of the biological samples. Single-use siliconized tubes were used to prevent the loss of compounds. Each nerve cord sample was weighed and washed with phosphate buffered saline (Invitrogen, USA) prior to extraction (3 times in 1 ml), and then separately homogenized in 1N HCl (0.1g/ml) with a sonic dismembrator (Fisher Scientific, USA). HCl was added to hemolymph samples at 0.5N. Homogenates, from both nerve cord and hemolymph, were extracted with 5 ml of 9:1 (v/v) chloroform/isopropanol. After 5 min at room temperature, homogenates were centrifuged at 4000 rpm for 15 min. The three resulting phases were separated in the following order: (1) the lower layer, corresponding to the organic phase; (2) the intermediate phase, containing precipitated proteins; and (3) the top, aqueous phase, containing morphine. The resulting supernatants were collected and dried with a Centrivap Console (Labconco, Kansas City, Missouri). The dried extracts were then dissolved in 1 ml of 0.1% trifluoroacetic acid (TFA) water before solid phase extraction. Samples were loaded on a Sep-Pak Plus C-18 cartridge (Waters, Milford, MA) previously activated with 100% acetonitrile and washed with 0.1% TFA-water. Morphine elution was performed with a 30% acetonitrile solution (water/acetonitrile/ TFA, 69.9: 30: 0.1, v/v/v). The eluted samples were dried with a Centrivap Console and re-dissolved in 200µl of 0.1% TFA water prior to HPLC and subsequent RIA analysis. Additionally, the media, reagents and water were also examined for the presence of morphine.

HPLC and UV detection

Morphine in either tissue was analyzed by high pressure–liquid chromatography (HPLC). Analyses were performed with a Waters 626 pump (Waters, Milford, MA) equipped with a UV detector (Waters 2487, dual λ absorbance detector, West Lafayette, IN). Separation was achieved with a Waters Spherisorb 3µm ODS2 column. The injection volumes varied (50–100 µl) depending on tissue sample. The chromatographic system was controlled by Waters Millennium^{32} Chromatography Manager V3.2 software and the chromatograms were integrated with Chromatograph software (Waters) [9].

The mobile phase consisted of buffer A: 10 mM heptane sulphonic acid sodium salt, 30mM sodium phosphate monobasic (pH was adjusted to 3 with phosphoric acid) and buffer B: 50% buffer A, 50% acetonitrile. Running conditions were: t = 0, 5% buffer B, t = 10 min, 5% buffer B, t = 25 min, 100% buffer B, t = 30 min, 5% buffer B. Standard chromatograms were generated using 10 µg morphine in buffer A. Wave lengths 254nm and 210nm were used for morphine detection. Sensitivity was approximately 1 µg per injection volume.

HPLC fractions corresponding to morphine elution were analysed for either morphine identity verification, by quadrupole time of flight (Q-TOF) – mass spectrometry, or for morphine quantification, using radioimmuno-assay of appropriate HPLC peaks, which corresponded to authentic standards. Several HPLC purifications procedures (i.e., using buffer A

only), including the use of negative tissue, i.e., lobster tail muscle, were performed between each sample to prevent residual alkaloid contamination. HPLC runs were also run without tissue samples, yielding negative morphine peaks. Furthermore, the fraction of a blank chromatography run, corresponding to the elution of morphine was checked by Q-TOF mass spectrometry analysis, confirming that no alkaloids remained in the system. HPLC analyses of tail muscle tissue, which yielded negative data, were used as control. All reagents and tank water materials used in these experiments were also run to make sure this material did not arise from contamination [29]. Only new low-binding polypropylene tubes were used in all phases of the study.

Radioimmuno-assay (RIA) determination

The morphine RIA determination is a solid phase, quantitative RIA, wherein ^{125}I-labeled morphine competes for a fixed time with morphine in the test sample for the antibody binding site. 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 radiolabelled morphine is sufficient. RIA was performed in accordance with the protocol of the commercial kit (Coat-a-Count® Serum Morphine Assay) from Diagnostic Product Corporation (USA). Briefly, 25 µl of sample (i.e., from HPLC fractions corresponding to morphine's elution time) were pipetted into polypropylene tubes coated with morphine antibodies. One ml of radiolabelled morphine-I^{125} was then added and the solution incubated at room temperature for one hour. Tubes were decanted with equal thoroughness and reminiscent radioactivity was measured with 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 (ng/ml). The result is plotted in a semi-logarithmic system of coordinates subsequent to calibration using morphine standards containing 0, 2.5, 10, 25, 75 and 250 ng/ml, in human serum ($r^2 = 0.998$). The detection limit was 0.5 ng/ml. The extraction efficiency, using an internal standard was 86%.

Mass Spectrometry

Quadrupole time of flight – mass spectrometry (Q-TOF-MS) offers an effective way of demonstrating endogenous alkaloids in biological tissues [25,26,28]. A commercial Q-TOF mass spectrometer equipped with a Z-spray nano-electrospray ion source (Micromass, Manchester, UK) was used. The instrument was calibrated in MS/MS mode using 1 pmol/µl [Glu]–Fibrinopeptide B (Sigma) in a methanol/water/acetic acid (50:49/1, v/v/v) mixture. Argon (12 psi) was used as collision gas in both MS and MS/MS modes. The capillary- and cone voltages were 950 V and 30 V, respectively. One µl of acetonitrile/water/formic acid (50:49:1, v/v/v) solution containing the collected sample was loaded into a gold-coated capillary and sprayed at a flow rate of 30 nl/min. In a typical MS/MS experiment, protonated molecules were selected as precursor ions by the quadrupole mass filter (MS1) and induced to fragment by collision with argon, and resulting product ions were analyzed by the TOF mass analyzer (MS2). The collision energy was varied between 25 eV and 35 eV in order to obtain informative MS/MS spectra in which both precursor and major fragment ions were abundant. The instrument was operated in the positive ion mode.

Nitric oxide (NO) determination

For NO determination, hemocyte pellets were resuspended in sterile saline solution –SSS-(15.0 mM $CaCl_2$, 7.0 mM KCl, 12.5 mM $MgCl_2$. $6H_2O$, 7.4 mM $NaHCO_3$) and about 10^6 cells were placed in different wells of a 96-well plate (Falcon) in ~200 µl media (25% lobster plasma and 75% PBS). Hemocyte samples were allowed to adhere for 2 hrs and the media was removed and replaced with 200 µl of PBS prior to NO determination. Nerve cord sections (80–100 mg) were placed in 1.5ml low-binding pre-siliconized microcentrifuge tubes containing 1ml PBS.

NO release from either hemocytes or nerve cords was directly measured using an NO-specific amperometric probe (30 µm, 0.5 mm, World Precision Instruments, Sarasota, FL). In certain instances, the tissues were allowed to recover for 2 hours in incubation medium and used again for the NO determination. A micromanipulator (World Precision Instruments, Sarasota, Fl), which was attached to the stage of an inverted microscope (Nikon Diaphot, Melville, NY), was used to position the amperometric probe 15 µm above the tissue. The system was calibrated daily by nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine (SNAP), resulting in the liberation of a known quantity of NO (World Precision Instruments). The amperometric probe was allowed to equilibrate for at least 10 min prior to being transferred to the well containing the tissue. Morphine-stimulated NO release was evaluated at final concentrations of 10^{-6} to 10^{-9}M. The receptor antagonist, naloxone, was added at 10^{-6} M 5 min prior to morphine addition. The addition of the opioid peptide Met-enkephalin was also evaluated at 10^{-7} M and 10^{-9} M. In addition, the animal's own extracted morphine was also added. Each experiment was repeated three times along with a control, which was just exposed to vehicle (i.e., PBS).

Molecular Biology

Isolation of Total RNA

Lobster tissue (i.e., ventral nerve cord, hemocyte pellets, etc) was placed in 1.5 ml tubes and immediately processed. Total RNA was isolated from about 200 mg of nerve cord and from about 10^7 hemocyte cells with the RNeasy Protect Mini Kit (Qiagen, Stanford, CA). RNA was analyzed on a 1% agarose gel and concentrations were determined spectrophotometrically.

Reverse Transcription-Coupled Polymerase Chain Reaction (RT-PCR)

One μg of total RNA isolated from lobster tissue was denatured at 95°C and first-strand cDNA synthesis was performed using random primers (Invitrogen, Carlsbad, CA). Reverse transcription was done at 40°C for 1 hr using Superscript II Rnase H-RT (Invitrogen, Carlsbad, CA). Ten microliters of the RT product was added to the PCR mix containing specific primers for the μ opioid receptor gene (Table 1) and Taq DNA polymerase (Invitrogen, Carlsbad, CA). The PCR reaction was denatured at 95°C for 5 minutes followed by 40 cycles at 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and then an extension step cycle at 72°C for 10 min. PCR products were analyzed on a 2% agarose gel (SIGMA, St. Louis, MO) stained with ethidium bromide.

Sequencing of the Cloned Products

The bands corresponding to the expected size fragment (i.e., 441bp) were excised, purified with the Qiaquick gel extraction kit (Qiagen, CA), ligated into the pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA), and then transformed TOP10 chemically competent cells (Invitrogen, CA). Transformed cells were grown overnight in a 37°C incubator shaker, and plasmid DNA was purified using the Qiaprep miniprep kit (Qiagen, Stanford, CA) and then sequenced (Seqwright, Houston, TX).

Real Time PCR

Primers and probes specific for the μ_3-opiate receptor gene (Table 2), and the internal control gene, β-actin, were designed by the software Primer

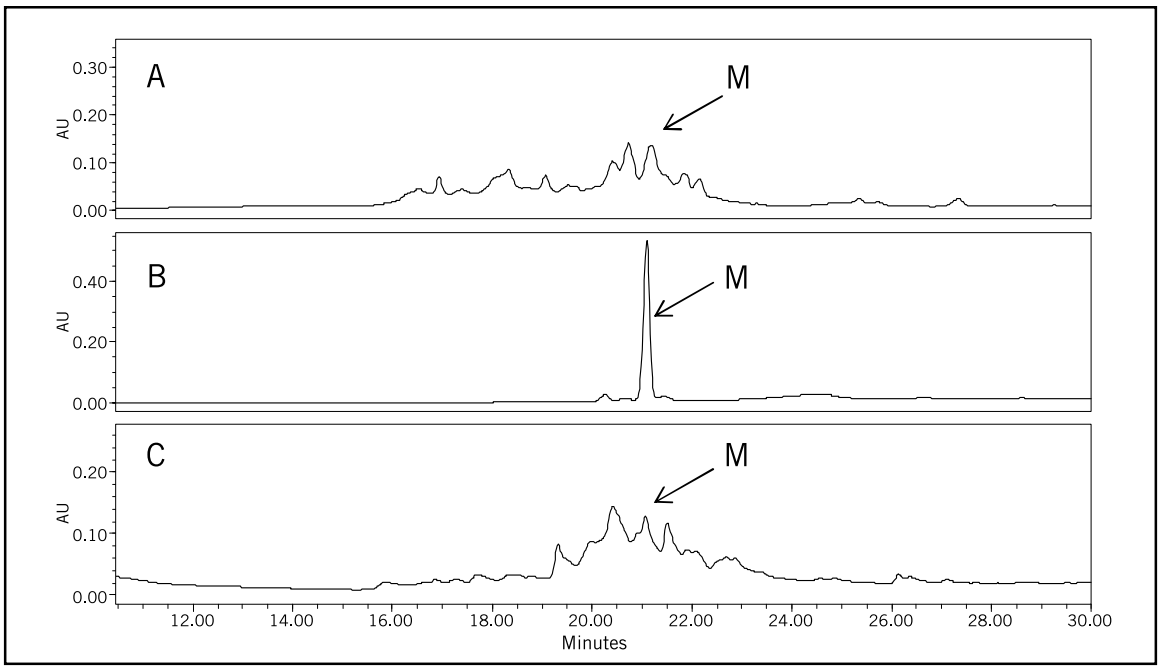

Figure 1. Comparison of HPLC chromatograms exhibiting a morphine (M) retention time of 21 min from *Homarus americanus* nerve cord (A), (B) morphine standard (10μg) and (C) hemolymph.

Table 1. Primers used for conventional PCR

Forward primer	5′-GGTACTGGGAAAACCTGCTGAAGATCTGTG-3′
Reverse primer	5′-GGTCTCTAGTGTTCTGACGAATTCGAGTGG-3′

Table 2. Primers and Taqman Probe for MOR sequence used in Real Time-Polymerase chain reactions.

Primer / probe	Sequence
Forward primer	5′-ATGCCAGTGCTCATCATTAC-3′
Reverse primer	5′-GATCCTTCGAAGATTCCTGTCCT-3′
Taqman Probe*	5′-CGCCTCAAGAGTGTCCGCATGCT- 3′

*The Taqman probe was constructed with the 5′-reporter dye, 6-carboxyfluorescein (FAM), and a 3′-quencher dye, 6-carboxy-tetramethyl-rhodoamine (TAMRA).

Table 3. Nitric Oxide Production by Lobster Hemocytes (~10^6 cells per tube) N=3

Treatment	NO nM (± SEM)
Control	0.6 ± 0.1
Morphine 10^{-10} M	0.7 ± 0.1
" " 10^{-9} M	1.7 ± 0.6
" " 10^{-8} M	5.7 ±1.2
" " 10^{-7} M	19.8 ± 2.8**
" " 10^{-6} M	29.5 ± 3.8**
Lobster Morphine*	10.6 ± 2.2**
Plus Naloxone (10^{-6} M)	1.7 ± 0.3
Plus L-NAME (10^{-4} M)	0.2 ± 0.0
Met-Enkephalin (10^{-6} M)	0.8 ± 0.1

*Approximately 30 nM morphine;
**One Way ANOVA (control vs. treatment) reveals statistical significance at the $P < 0.01$ level.

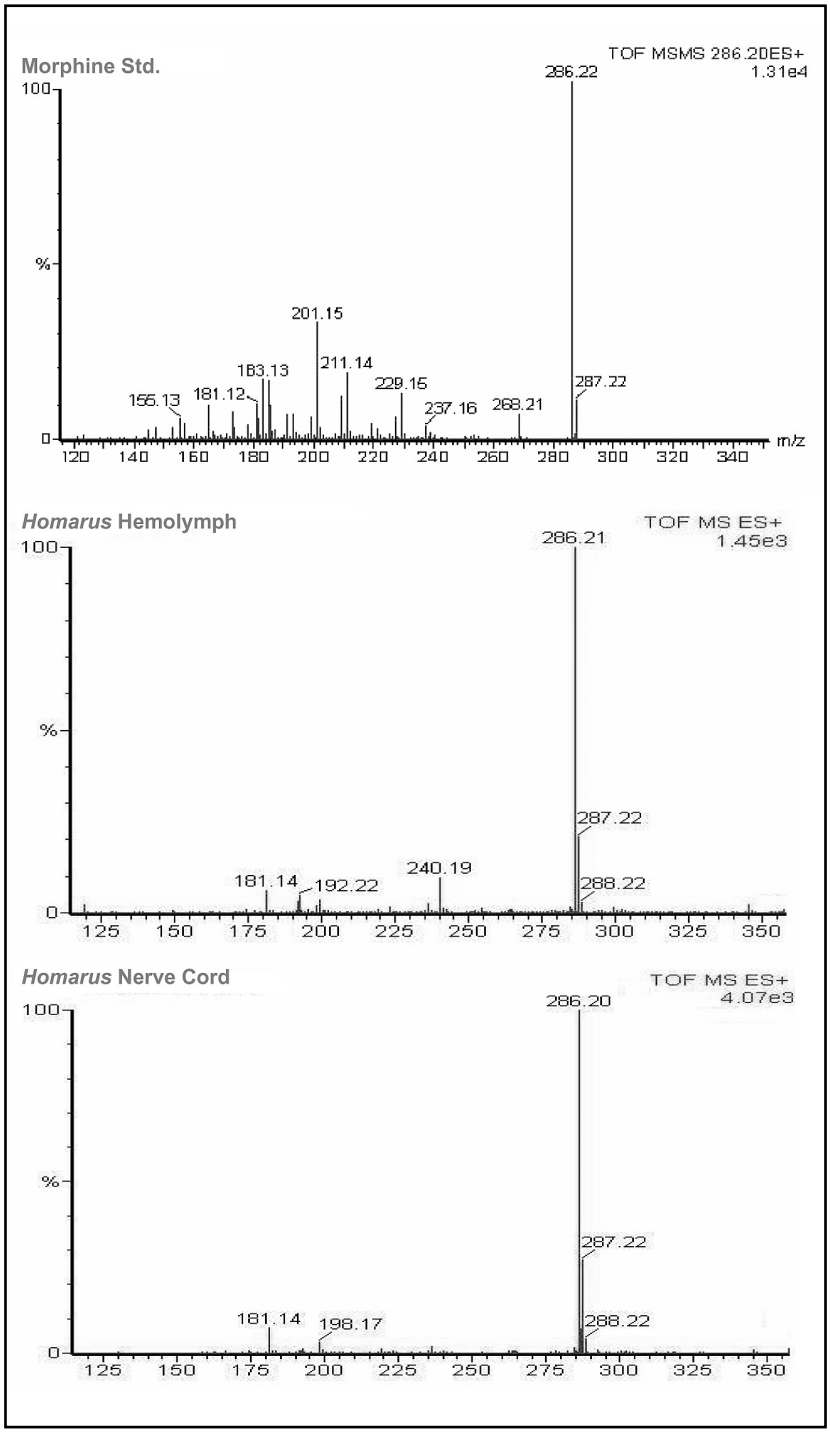

Figure 2. Q-TOF MS analysis.
Positive mass identification of HPLC fractions corresponding to morphine's elution time from nerve cord (bottom) and hemolymph (middle) with authentic morphine (top). An ion at *m/z* 286.2 was consistently detected in lobster tissue samples.

Express (Applied Biosystems). The 2X universal master mix (Applied Biosystems) containing the PCR buffer, $MgCl_2$, dNTP's, and the thermal stable AmpliTaq Gold DNA polymerase was used in the PCR reactions. In addition, 200 µM reverse and forward primers, 100 µM Taqman probe, 10 µl of RT product generated from lobster tissues (nerve cord or hemocytes) and Rnase/DNase-free water was added to the master mix to a final volume of 50 µl. The PCR reaction mixture was transferred to a MicroAmp optical 96-well reaction plate and incubated at 95 °C for 10 min to activate the Amplitaq Gold DNA polymerase and then run for 40 cycles at 95 °C for 30s and 60 °C for 1 min on the Applied Biosystems GeneAmp 7500 sequence Detection System. The PCR result was analyzed with the GeneAmp 7500 SDS software (Applied Biosystems).

RESULTS

In specific HPLC fractions from lobster hemolymph and nerve cord, a compound with an identical retention time to an authentic morphine standard was consistently detected at 21 minutes (Figure 1). We further analyzed tail muscle, media and marine water for the presence of morphine (data not shown). It was found not to be present within the sensitivity of the assay, as determined by HPLC coupled to UV detection and RIA. Additionally, Q-TOF-MS analysis demonstrated unambiguous identification of the HPLC isolated material as morphine in both hemolymph and neural tissues (Figure 2). In the MS spectra, an ion at *m/z* 286.2, corresponding to protonated morphine, was consistently observed (Figure 2).

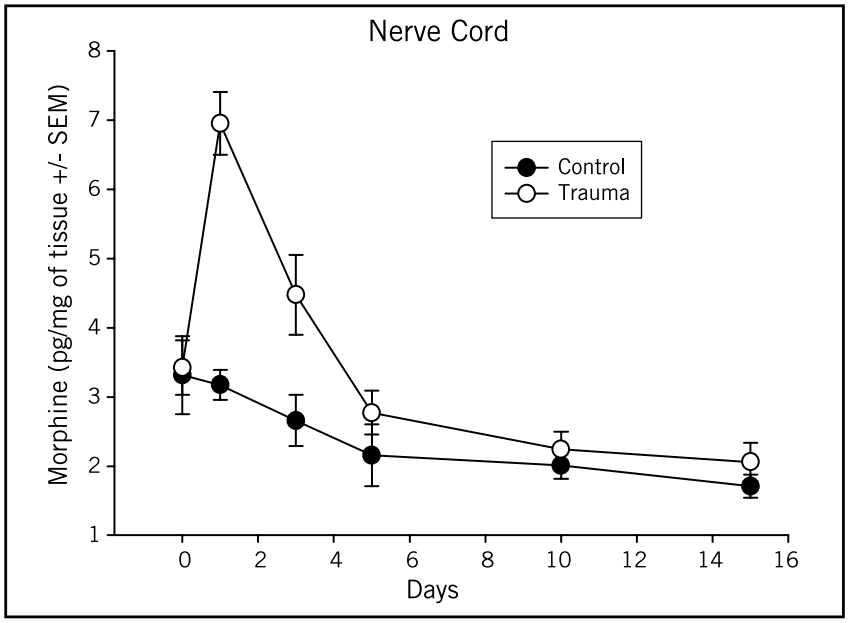

Figure 3. Morphine levels (before and after trauma) in lobster nerve cord quantified by RIA. Trauma = pereiopod ablation at day 0, One Way ANOVA (control vs. trauma): Day 1: P = <0.001; Day 3: P = 0.038, Control: N=24; Trauma: N=24

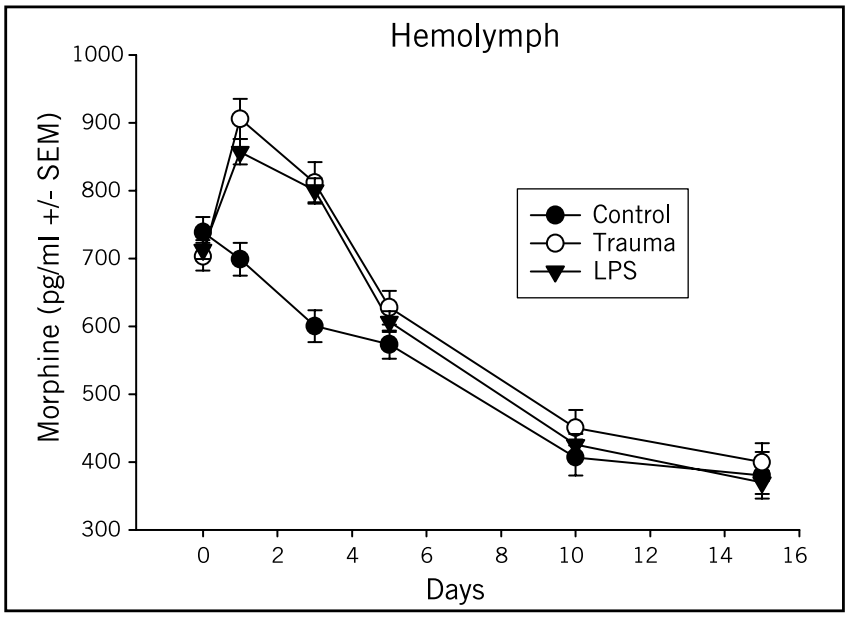

Figure 4. Morphine levels (before and after trauma) in lobster hemolymph quantified by RIA. Trauma = pereiopod removal at day 0; LPS = 1µg injections at day 0, One Way ANOVA: Day 1: Control vs. Trauma: P = <0.001; Control vs. LPS: P = 0.001, Day 3: Control vs. Trauma: P = <0.001; Control vs. LPS: P = <0.001, Control: N=24; Trauma: N=24; LPS: N=10

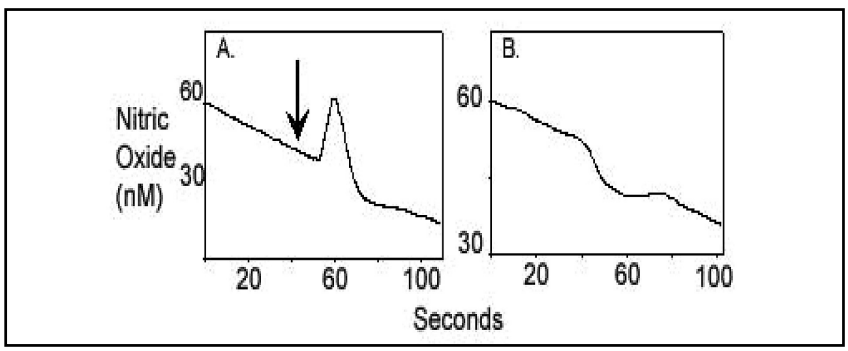

Figure 5. Morphine-induced nitric oxide production by lobster hemocytes (10^6 cells per sample) measured amperometrically in real-time. **A.** Arrow represents the time at which morphine (10^{-6} M) was added to the incubation wells containing lobster hemocytes, resulting in a 30 nM release. **B.** Hemocytes were exposed to naloxone (10^{-6} M) for 5 min. and then exposed to morphine (10^{-6} M) at the 40 sec time interval shown, resulting in a blockade of NO release, demonstrating an opiate receptor mediated process.

Figure 6.

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Lobster:   1    ggtactgggaaaacctgctgaagatctgtgttttcatcttcgccttcattatgccagtg 59
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human µ3:  403  ggtactgggaaaacctgctgaagatctgtgttttcatcttcgccttcattatgccagtg 461

Lobster:   60   ctcatcattaccgtgtgctatggactgatgatcttgcgcctcaagagtgtccgcatgctc 119
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human µ3:  462  ctcatcattaccgtgtgctatggactgatgatcttgcgcctcaagagtgtccgcatgctc 521

Lobster:   120  tctggctccaaagaaaaggacaggaatcttcgaaggatcaccaggatggtgctggtggtg 179
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human µ3:  522  tctggctccaaagaaaaggacaggaatcttcgaaggatcaccaggatggtgctggtggtg 581

Lobster:   180  gtggctgtgttcatcgtctgctggactcccattcacatttacgtcatcattaaagccttg 239
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human µ3:  582  gtggctgtgttcatcgtctgctggactcccattcacatttacgtcatcattaaagccttg 641

Lobster:   240  gttacaatcccagaaactacgttccagactgtttcttggcacttctgcattgctctaggt 299
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human µ3:  642  gttacaatcccagaaactacgttccagactgtttcttggcacttctgcattgctctaggt 701

Lobster:   300  tacacaaacagctgcctcaacccagtcctttatgcatttctggatgaaaacttcaaacga 359
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human µ3:  702  tacacaaacagctgcctcaacccagtcctttatgcatttctggatgaaaacttcaaacga 761

Lobster:   360  tgcttcagagagttctgtatcccaacctcttccaacattgagcaacaaaactccactcga 419
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human µ3:  762  tgcttcagagagttctgtatcccaacctcttccaacattgagcaacaaaactccactcga 821

Lobster:   420  attcgtcagaacactagagacc 441
           |||||||||||||||||||||||
Human µ3:  822  attcgtcagaacactagagacc 843
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Figure 6. Sequence of the mu conserved region obtained by conventional PCR from *Homarus* nerve cord showing 100% homology with the conserved region (441bp) of human µ3

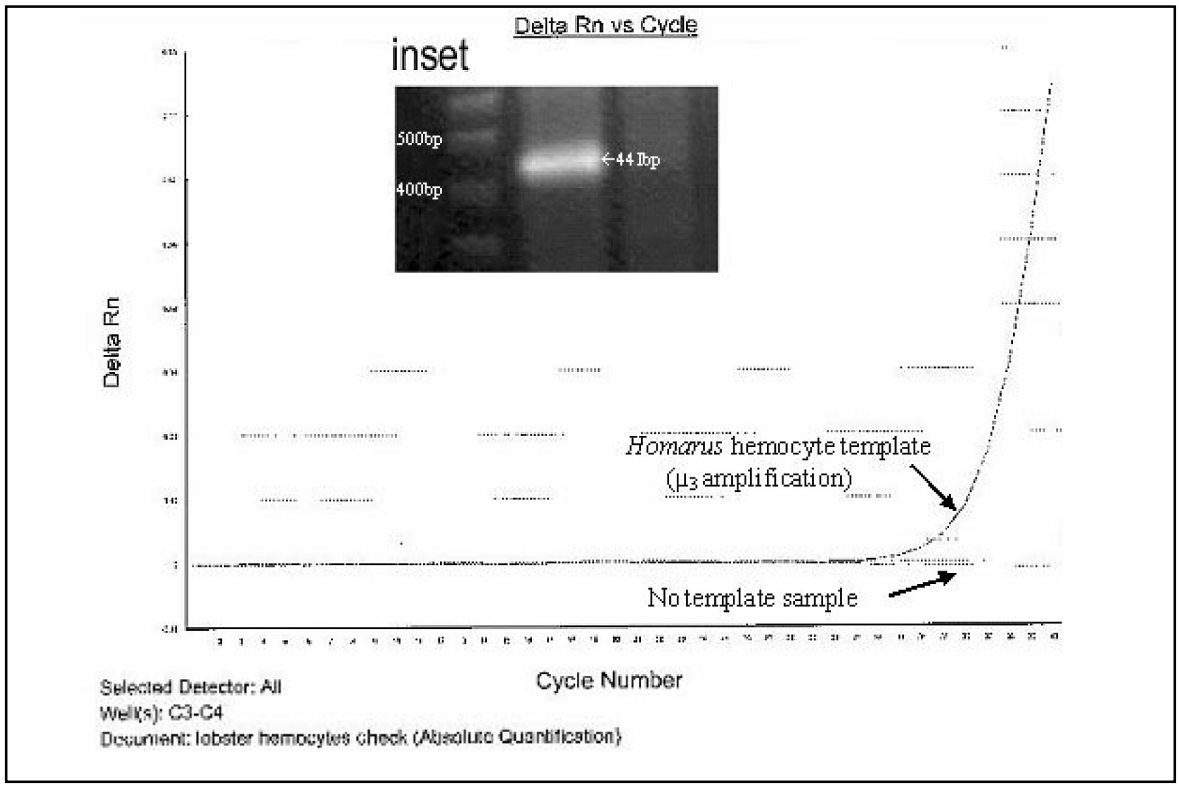

Figure 7. Real Time-PCR showing amplification of µ3 receptor in lobster hemocyte template. Inset: Agarose gel from conventional PCR product showing the mu conserved region (441bp) expressed in lobster nerve cord

Quantification of samples via RIA, following HPLC isolation, revealed morphine levels of 3.36 pg/mg +/– 0.48 SEM (N=8) in nerve cord and 717.88 pg/ml +/– 56.77 SEM (N=58) in hemolymph at day 0. After 15 days, the endogenous morphine levels decreased to almost half the original values in animals left undisturbed (see Figures 3 & 4). In stressed (pereiopod-ablated or LPS-injected) animals, quantification of HPLC isolated material via RIA revealed that morphine levels significantly increased 1 to 3 days after trauma (Figures 3 & 4) by an average of 24% for hemolymph and 48% for nerve cord. In hemolymph, following pereiopod ablation, significant increases in morphine levels were observed on day 1 ($P<0.001$) and day 3 ($P<0.001$). Control values were 698.72 pg/ml +/– 23.96; SEM for day 1 and 600.11 pg/ml +/– 23.59 SEM for day 3 versus 905.55 pg/ml +/– 29.61; SEM (day 1) and 811.28 pg/ml +/– 30.44; SEM (day 3). In nerve cord, statistically significant increases in endogenous morphine levels were also observed in day 1 ($P<0.001$) and day 3 ($P <0.038$) following pereiopod ablation. Control values were 3.17 pg/mg +/– 0.22; SEM and 2.66 pg/mg +/– 0.37; SEM on days 1 and 3, respectively, whereas stress values were 6.95 pg/mg +/– 0.45; SEM (day 1) and 4.48 pg/mg +/– 0.58 SEM (day3). Increases in endogenous morphine levels in hemolymph were also significant on day 1 ($P<0.001$) and day 3 ($P<0.001$), following LPS (1 μg) injection, exhibiting values of 857.16 pg/ml +/– 18.62; SEM and 800.26 pg/ml +/– 17.78; SEM, respectively. By day 5, the stressed and control values for endogenous morphine in either nerve cord or hemolymph, were both lower and non-distinguishable from controls (Figures 3 & 4). Statistics were obtained by One Way ANOVA with multiple comparisons versus control group (Holm-Sidak method).

In many reports, we have demonstrated that morphine, not opioid peptides, stimulates constitutive nitric oxide synthase (cNOS)-derived NO production in various invertebrate tissues [20]. In hemocytes (Figure 5, Table 3) and neural cord, morphine, not met-enkephalin, stimulated an almost immediate constitutive nitric oxide synthase derived NO release in a naloxone antagonizable manner. L-NAME, nitric oxide synthase inhibitor, also attenuated morphine's stimulatory action, demonstrating that morphine did stimulate NO release (Table 3). This demonstrates a mu opiate receptor mediated phenomenon and suggests the presence of the mu opiate receptor subtype, μ_3, since it is opiate alkaloid selective and opioid peptide insensitive [17].

Given the pharmacological data that a mu opiate receptor was present in lobster tissues, we sought to demonstrate its presence via molecular techniques. RT-PCR revealed the presence of a μ opiate receptor transcript in *Homarus* neural and immune tissues (Figures 6 & 7). In nerve cord, the opiate receptor transcript exhibited a 100% sequence identity (Figure 6) with its human counterpart. In hemocytes, the amount of cDNA obtained by conventional PCR was not sufficient for sequencing. However, real-time PCR, using specific proprietary primers for the μ_3 (opioid peptide-insensitive and opiate alkaloid-selective) receptor subtype [3], showed amplification of this receptor in *Homarus* hemocyte templates (Figure 7).

DISCUSSION

In the present study, we demonstrate that morphine is endogenous to the lobster's neural and immune tissues, which also expresses a mu opiate receptor transcript that exhibits high sequence identity to its human counterpart. Additionally, lobster morphine levels increase in response to acute stress, demonstrating its involvement in a stress response process. We also demonstrated that morphine cannot be found in tail muscle and that it is not present in the marine water, media and chemicals used in the present study, thereby removing the issue of contamination. Additionally, morphine signaling was coupled to cNOS-derived NO release in both tissues, a phenomenon that can be blocked by naloxone and attenuated by L-NAME, suggesting that this is an opiate signaling process coupled to nitric oxide release. Taken together, it appears that morphinergic signaling is present in lobster immune and neural tissues and that this chemical signaling molecule may functionally serve as a hormone and neurotransmitter.

To demonstrate a physiological role for lobster morphine, we determined if it was involved in the lobster's response to stress. Both LPS exposure, representing a bacterial type of stress, and pereiopod ablation, representing tissue trauma, revealed an initial significant increase in morphine levels in both neural and immune tissues. This increase was followed by morphine levels returning to control levels during the following days. Morphine's involvement in the stress response, as well as following a similar pattern of expression during and post stress, has been documented in invertebrates, mammals and human tissues [1,4–6,12,21–23]. The present data strongly suggests that this opiate alkaloid is being synthesized within the animal's neural tissue and being released into the hemolymph. Examination of lobster sea water over the course of 20 days reveals no morphine present, demonstrating that this material is produced by lobster tissues. The study also demonstrates that morphine may be used as a bioindicator of acute stress since its synthesis responded to different types of stress.

In addition, the presence of a mu opiate receptor, exhibiting high sequence identity with its mammalian counterpart, has also been demonstrated for *Mytilus edulis*, a marine bivalve [4]. In the present report, lobster mu opiate receptor transcripts exhibit 100% sequence identity with human material and can be found on neural and immune tissues where it is coupled to NO release. The association of this mu3-like receptor (since it is opiate alkaloid selective and opioid peptide insensitive) with immune and neural tissues also occurs in other invertebrates and in mam-

mals and the corresponding human tissues, suggesting that this phenomenon has a long evolutionary history and is important since it has been conserved [3,17,20,22,23].

Given the tissue associations and the coupling of morphine to NO release, especially lobster hemocytes, we surmise that there is a protective role for endogenous morphine [19,20,22,23]. In preliminary data, morphine level increases to trauma appear not to occur immediately but 6 hours post treatment, demonstrating a latency period. As in other animals, including man, this lack of immediate increase is associated with events allowing a proinflammatory response to take place [2,22]. The need for signaling cascades associated with restoring homeostasis is just as important as signaling cascades involved with meeting various forms of stress immediately. Following trauma, endogenous morphine restores homeostasis via NO release, ensuring that the body's response to stress is not exaggerated and a state of calm is restored [19,22].

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