

## Endomorphin-1 and -2 inhibit human vascular sympathetic norepinephrine release: lack of interaction with $\mu_3$ opiate receptor subtype<sup>1</sup>

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**KEY WORDS** opioid peptides; endomorphins; morphine; arteries; saphenous vein; vascular endothelium; nitric oxide; norepinephrine; monocytes; granulocytes

**AIM:** To determine if endomorphin-1 (End-1) and -2 (End-2) interact with  $\mu_3$  opiate receptor subtype and in this way cause vascular hypotension. **METHODS:** Amperometric nitric oxide (NO) determinations associated with opiate binding displacement analysis and preloaded [<sup>3</sup>H]norepinephrine KCl stimulated release in human vascular tissues from sympathetic nerve fibers *in vitro*. **RESULTS:** The endomorphins did not release NO from human monocytes, granulocytes, saphenous vein, and internal thoracic artery endothelium and did not displace opiate alkaloid binding to  $\mu_3$  receptor. However, they did inhibit KCl-stimulated [<sup>3</sup>H]norepinephrine release from vascular nerves. **CONCLUSION:** The data strongly suggested that End-1 and -2 caused hypotension by blocking sympathetic vascular sympathetic activity.

The newly discovered opioid peptides, endomorphin-1 (End-1, Tyr-Pro-Trp-Phe-NH<sub>2</sub>) and endomorphin-2 (End-2, Tyr-Pro-Phe-Phe-NH<sub>2</sub>), exhibit a high affinity for  $\mu$  opiate receptor<sup>[1]</sup>, produce short acting antinociception<sup>[2]</sup>, and exhibit vasodilatory actions in rat

and rabbit<sup>[3,4]</sup>. In this regard, we demonstrated the presence of an opioid peptide insensitive and opiate alkaloid selective receptor, designated  $\mu_3$ , on human monocytes, granulocytes, and human endothelial vascular tissues<sup>[5-9]</sup>, and also demonstrated the ability of opiate alkaloids, eg, morphine, to initiate vasodilation by  $\mu_3$  coupling to constitutive nitric oxide (NO) release, including from human vascular endothelium. Thus, we sought to determine if the novel opioid peptides End-1 and End-2 could stimulate NO release via  $\mu_3$  coupling.

### MATERIALS AND METHODS

Discarded human saphenous vein and internal thoracic artery were obtained from patients at University Hospital (State University of New York at Stony Brook); these vessels are normally used for coronary artery bypass grafting. The vessels were cut into 3-mm ring sections. The sections were cut and the flat tissue, endothelial side up, was used for NO determinations. Patient eligibility included admission for elective surgery only for atherosclerotic heart disease. Patients with chronic illnesses such as diabetes or cancer as well as acute processes (eg, infections, trauma) were not eligible. Vascular tissues used for the potassium-stimulated release of preloaded [<sup>3</sup>H]norepinephrine ([<sup>3</sup>H]NE), presumably from nerve terminals, were cut into uneven sections (saphenous vein 1 cm; internal thoracic artery 6 mm), reflecting the level of adrenergic nervous innervation (larger piece for the saphenous vein).

Human granulocytes and monocytes were obtained from the Long Island Blood Services (Melville NY). Cells were separated by the

<sup>1</sup> Project supported in part by NIMH COR 17138 (GBS, TVB), NIDA 09010 and the Research Foundation and Central Administration of the State University of New York (GBS), and NIH Fogarty INT 00045 (MS and GBS).

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Received 1998-02-24

Accepted 1998-06-19

standard Ficoll-Hypaque method<sup>(5,10)</sup>. The immunocytes, following their separations, were washed 3 times in RPMI medium (RPMI, HEPES 25 mol·L<sup>-1</sup>, Grand Island Biological Co, NY).

*Mytilus edulis* immunocytes were obtained<sup>(5,10)</sup>. All tissues were exposed to various pharmacological agents following a 10-min observation period.

Morphine was obtained from Winthrop Pharmaceuticals and naloxone was obtained from End laboratories. End-1 and -2 were obtained from Peptide International (Louisville KY).

**Direct measurement of NO release** NO release from the vessel fragments or immunocytes (10<sup>10</sup> cells·L<sup>-1</sup>) was measured directly using an NO-specific amperometric probe (World Precision Instruments, Sarasota FL)<sup>(11)</sup>. Briefly, the respective vessel or cardiac tissue, ie, right atria fragments, was placed endothelial side up, immunocytes were pelleted by mild centrifugation (300 × *g* for 5 min) in a superfusion chamber in 2 mL of PBS. A micromanipulator (Zeiss-Eppendorff) attached to the stage of an inverted microscope (Nikon Diaphot) was employed to position the amperometric probe 15 mm above the cell surface. The system was calibrated daily using different concentrations of the nitrosothiol donor *S*-nitroso-*N*-acetyl-*DL*-penicillamine (SNAP; SAP was used as the control for SNAP; Sigma, St Louis MO) to generate a standard curve. Baseline levels of NO release were determined by evaluation of NO release in PBS. Cells were stimulated with the respective ligand, and the concentration of NO gas in solution was measured in real-time with the DUO-18 computer data acquisition system (World Precision Instruments). The amperometric probe was allowed to equilibrate for 12 h in PBS prior to being transferred to the chamber containing the respective tissues, and manipulation of the tissue was performed only with glass instruments. Each experiment was repeated four times. The mean values for NO release were obtained at 2 min intervals. Each experiment was simultaneously performed with a control (vehicle alone) to exclude experimental drift in NO release unrelated to the study drugs.

To evaluate NO release, the tissues were exposed to either morphine or End-1 or -2. It

should be emphasized that the morphine-stimulated release of NO in these tissues has been demonstrated to be inhibited by naloxone, a potent opioid receptor antagonist, and inhibited by the nitric-oxide synthase (NOS) inhibitors, *N*-nitro-*L*-arginine and *N*<sup>ω</sup>-nitro-*L*-arginine methyl ester (*L*-NAME)<sup>(11-13)</sup>. Furthermore, vessels in which the endothelium was gently scraped off did not respond to morphine. These studies also found opioid peptides to lack the ability to release NO. Studies in which 2 drugs were added, the first application preceded the second by 5 min unless indicated otherwise.

Data were evaluated by *t*-test. Data acquisition was by the computer-interfaced DUO-18 software (World Precision Instruments). The experimental values were then transferred to Sigma-Plot and -Stat (Jandel, San Rafael CA) for graphic representation and evaluation. Data gatherers were unaware of the experimental treatments.

**Binding analysis** Human endothelium for binding analysis was obtained by gently scraping the cells from the otherwise intact vessels. Separate cell membrane suspensions were prepared<sup>(5-7,10)</sup>. The identity and characteristics of the μ<sub>3</sub> receptor become quite apparent in a displacement analysis using a tritiated opiate alkaloid ligand<sup>(5-7,10)</sup> and thus represents an important method for μ<sub>3</sub> recognition. [<sup>3</sup>H] Dihydromorphine ([<sup>3</sup>H]DHM) binding was performed in Tris 50 mmol·L<sup>-1</sup>, pH 7.4 at 35 °C without enzyme inhibitors for 90 min<sup>(5)</sup>. The membranes were then washed twice with ice-cold Tris buffer, resuspended to half the original volume in Tris-HCl 50 mmol·L<sup>-1</sup> (pH 7.4) and stored at -70 °C for binding assays. For IC<sub>50</sub> determination (defined as the concentration of drug which elicits half-maximal inhibition of specific [<sup>3</sup>H]DHM binding), aliquots of human vascular endothelial and granulocyte and monocyte membrane suspensions were incubated with nonradioactive opioid compounds at 6 different concentrations at 22 °C for 10 min and then with [<sup>3</sup>H]DHM at 4 °C for 60 min<sup>(5,7)</sup>. The  $\bar{x} \pm s$  for 3 experiments was recorded for each compound tested. Each point represented the mean of the experiments made with 4 membrane preparations.

### Analysis of norepinephrine (NE) release

The effects of the endomorphins on NE release from the vascular sympathetic nerves was assessed. The vessels were incubated (2 pieces as indicated above) at 28 °C for 30 min in 1 mL of Krebs' containing 0.1 % ascorbic acid and [<sup>3</sup>H]NE (6 × 10<sup>5</sup> dpm; specific activity, 1.16 TBq · mol<sup>-1</sup>) (New England Nuclear) with constant shaking. After being incubated, the vessels were washed twice in 2 mL of saline (phosphate buffered saline). An average of 1430 and 1010 cpm were present in the internal thoracic artery, and saphenous vein segments, respectively, at the start of the superfusion. A four-channel peristaltic pump (Rainin) maintained the flow rate (0.2 mL · min<sup>-1</sup>) into an opening at the bottom of the chamber. Drugs and chemicals were added to the perfusion solution. The superfusate was collected from an outflow opening with one superfusate fraction (1 mL) collected every 5 min. Vessels were first superfused with saline containing KCl 50 mmol · L<sup>-1</sup> alone (control) or together with drug. Finally, the vessel segments were again perfused with saline for the remainder of the experiment. Radioactivity of the collected superfusate was determined by liquid scintillation counting<sup>[14,15]</sup>. The values represented the percentage of the total radioactivity in the tissue released during the 5-min period ending at the time indicated. Each value was the average of 2 separate experiments, and the variation was less than 1.3 % for all values. The <sup>3</sup>H-labeled substance released was then analyzed with TLC to insure that the material was NE. The results showed that 85 % of the released radioactivity was NE.

### RESULTS

Amperometric determination of NO demonstrated that End-1 did not stimulate NO release as did morphine in saphenous vein endothelial cells, even at higher concentrations (10 nmol · L<sup>-1</sup>) (Fig 1).

Exposure of various tissues (granulocytes, monocytes, saphenous vein, internal thoracic artery and invertebrate immunocytes) to either endomorphin did not result in NO release (Fig 2).

In examining the displacement analysis of the endomorphins for [<sup>3</sup>H]DHM binding to the  $\mu_{23}$  opiate receptor subtype on the various tissues,

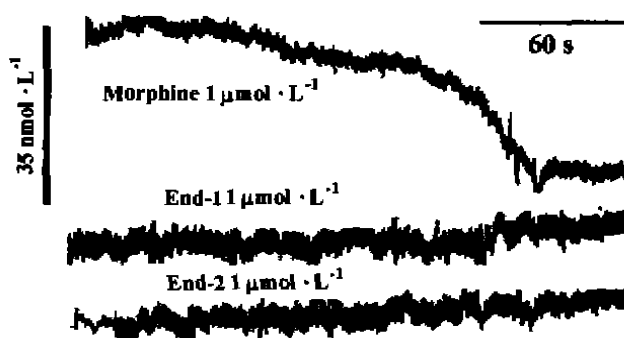


Fig 1. Representative nitric oxide determinations in real-time from saphenous vein segments stimulated by opiate and opiate ligands.

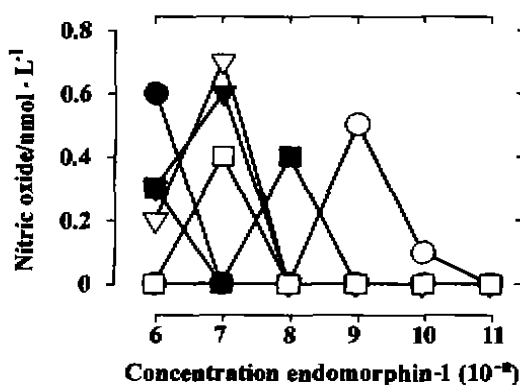


Fig 2. End-1 did not release NO from tissues previously shown to release NO when stimulated by morphine. Each experiment was replicated 3 times. Control (●), granulocytes (○), monocytes (▽), saphenous endothel (∇), Int Thor endothel (■), mytilus immunocytes (□).

the endomorphins exhibited a lack of affinity for this receptor compared to opiate alkaloids (Tab 1<sup>[5]</sup>), supporting the lack of NO production by these peptides. In this regard, the lack of affinity of kappa and delta-type ligands is as expected<sup>[5-9]</sup>.

Since the endomorphins did not release NO, we surmised that they might be initiating vasodilation by blocking peripheral adrenergic NE release<sup>[14,15]</sup>.

Both endomorphins inhibit the KCl-stimulated [<sup>3</sup>H]NE release, in a concentration-dependent manner, from [<sup>3</sup>H]NE preloaded saphenous vein and internal thoracic artery segments (Fig 3).

Furthermore, naloxone 100 nmol · L<sup>-1</sup> administered 5 min prior to the endomorphins

Tab 1. Displacement of [ $^3\text{H}$ ] dihydromorphine ( $1 \text{ nmol} \cdot \text{L}^{-1}$ ) by opioid ligands in various human tissue membrane suspensions.  $n=3$  experiments,  $\bar{x} \pm s$ .

| Ligand             | Vein           | Artery         | Granulocytes   | Monocytes      |
|--------------------|----------------|----------------|----------------|----------------|
| <b>Agonists</b>    |                |                |                |                |
| $\delta$ -Agonist  |                |                |                |                |
| DPDPE              | > 1 000        | > 1 000        | > 1 000        | > 1 000        |
| $\mu$ -Agonist     |                |                |                |                |
| End-1              | > 1 000        | > 1 000        | > 1 000        | > 1 000        |
| End-2              | > 1 000        | > 1 000        | > 1 000        | > 1 000        |
| DAMGO              | > 1 000        | > 1 000        | > 1 000        | > 1 000        |
| DHM                | $19.0 \pm 2.1$ | $21.0 \pm 3.0$ | $18.4 \pm 2.9$ | $20.6 \pm 3.8$ |
| Morphine           | $21.0 \pm 2.2$ | $22.0 \pm 3.2$ | $23.1 \pm 3.7$ | $21.4 \pm 3.3$ |
| $\kappa$ -Agonist  |                |                |                |                |
| Dynorphin 1-17     | > 1 000        | > 1 000        | > 1 000        | > 1 000        |
| <b>Antagonists</b> |                |                |                |                |
| Naltrexone         | $34.0 \pm 5.1$ | $37.0 \pm 3.8$ | $43.7 \pm 6.8$ | $47.6 \pm 9.3$ |
| Naloxone           | $49.0 \pm 7.7$ | $49.0 \pm 6.1$ |                |                |

DPDPE = ( $D$ -Phe<sup>2</sup>,  $D$ -Phe<sup>5</sup>)-enkephalin;

DAMGO = [Tyr- $D$ -Ala<sup>2</sup>, Gly- $N$ -Me-Phe<sup>4</sup>, Gly(ol)<sup>5</sup>-enkephalin].

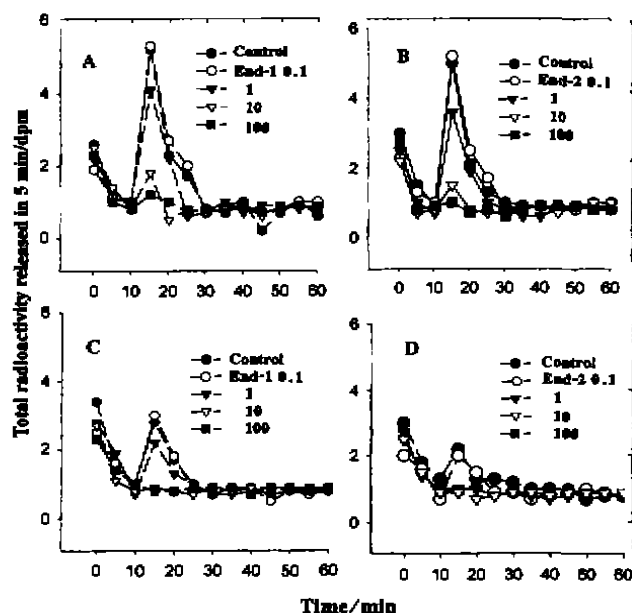


Fig 3. End-1 and -2 ( $\text{nmol} \cdot \text{L}^{-1}$ ) concentration-dependent inhibition of [ $^3\text{H}$ ]NE release from human vascular tissues. A and B: segments of the internal thoracic artery exposed to End-1 and -2, respectively. C and D: saphenous veins exposed to the same peptides as indicated. Each experiment was performed in triplicate.

( $100 \text{ nmol} \cdot \text{L}^{-1}$ ) blocked the endomorphin inhibition of [ $^3\text{H}$ ] NE release. Naloxone restored the End-1-inhibited [ $^3\text{H}$ ] NE release (Fig 1) from vein and artery segments to 8.5 %

and 8.2 %, respectively ( $P < 0.01$ ) and End-2 to 8.4 % and 8.6 %, respectively.

The endomorphins inhibited the KCl-stimulated release of [ $^3\text{H}$ ]NE in *Mytilus edulis* pedal ganglia preloaded with [ $^3\text{H}$ ]NE, a major invertebrate catecholamine<sup>[14]</sup> (Tab 2). This process was concentration dependent and inhibitable by naloxone (Tab 2).

Tab 2. Endomorphins inhibit potassium-stimulated [ $^3\text{H}$ ]nor-epinephrine release from *mytilus pedal* Ganglia. Data were replicated in triplicate,  $\bar{x} \pm s$ .  $^{\circ}P < 0.01$  vs controls.

| Treatment/ $\text{nmol} \cdot \text{L}^{-1}$             | % Total radioactivity released (SEM) |
|--|--------------------------------------|
| Control  | $8.7 \pm 1.3$                        |
| End-1 1  | $8.4 \pm 1.1$                        |
| End-1 10   | $4.5 \pm 0.7$                        |
| End-1 100  | $1.1 \pm 0.4^{\circ}$                |
| Control  | $8.9 \pm 1.0$                        |
| End-2 1  | $8.2 \pm 0.9$                        |
| End-2 10   | $3.9 \pm 0.9$                        |
| End-2 100  | $0.8 \pm 0.3^{\circ}$                |
| End-1 100 + L-NAME 0.1 $\text{mmol} \cdot \text{L}^{-1}$ | $1.1 \pm 0.5^{\circ}$                |
| End-2 100 + L-NAME 0.1 $\text{mmol} \cdot \text{L}^{-1}$ | $1.0 \pm 0.4^{\circ}$                |

## DISCUSSION

In the past we have demonstrated that opiate alkaloids, not opioid peptides, exhibited a strong and selective affinity for the  $\mu_3$  opiate receptor on human monocytes, granulocytes, and vascular endothelial cells<sup>[5-9]</sup>. The  $\mu_3$  receptor was coupled to NO release in these tissues<sup>[6,7,9]</sup>. This coupling resulted in vasodilation and a loss of immunocyte adherence to the vascular endothelial cells in fragments of the saphenous vein or internal thoracic artery<sup>[6,9,11]</sup>. Zadina *et al*<sup>[1]</sup> have demonstrated the presence of novel and naturally occurring opioid peptides, End-1 and -2, that exhibited potent  $\mu$ -associated activities. One of the activities was that of initiating hypotension in rat and rabbit<sup>[3,4]</sup>. Given this, and based on the results of our studies documenting the presence of the  $\mu_3$  opiate receptor on vascular tissue initiating vasodilation via NO, we examined the interactions of these novel peptides with the alkaloid selective receptor. In this regard, the data strongly demonstrate that End-1 and -2 exhibited no affinity for the  $\mu_3$  receptor. Additionally, the

peptides did not release endothelial NO, demonstrating an alternate method for their vasodilatory action.

In this regard, we demonstrate that these opioid peptides were capable of inhibiting the sympathetic release of NE in vascular and neural tissues, accounting for their hypotensive action<sup>(3,4)</sup>. This opioid-mediated process appeared to be quite common in both invertebrates and vertebrates<sup>(13)</sup>. We therefore conclude, as did Champion *et al*<sup>(3,4)</sup> that the hypotension induced by the endomorphins appears to be mediated by central nervous system processes or neural elements within the vasculature or both.

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内吗啡肽-1和-2抑制人血管交感神经去甲肾上腺素释放: 缺乏与 $\mu_3$ 阿片受体亚型的交互作用<sup>1</sup>

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关键词 阿片样肽; 内吗啡肽类; 吗啡; 动脉; 隐静脉; 血管内皮; 一氧化氮; 去甲肾上腺素; 单核细胞; 粒细胞

交感神经