

Morphine Down Regulates Human Vascular Tissue Estrogen Receptor Expression Determined by Real-Time RT-PCR

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Abstract

Human vascular endothelial cells express the estrogen receptor- β (ER- β), which can be modulated by the opiate alkaloid morphine.

OBJECTIVES: To determine if morphine is capable of down regulating the ER- β receptor in a similar fashion as the μ opiate receptor since they are both coupled to constitutive nitric oxide synthase derived nitric oxide release.

METHODS AND RESULTS: Endothelial cells obtain from human vascular tissues (saphenous vein, atria and primary saphenous vein cells) were treated with 1 μ M morphine plus or minus the μ opiate receptor antagonist naloxone or CTOP (10 μ M) for 24 h at 37°C. Total RNA was isolated from treated and untreated primary endothelial cells, and specific primers and a probe were used to determine the ER- β gene expression by real-time RT-PCR. Cells treated with morphine exhibited a down-regulation of ER- β , whereas naloxone and CTOP were able to partially block the morphine effect. In addition, the 266 bp fragment generated by RT-PCR using the same primers as in the real-time PCR was sequenced and revealed a 100% sequence identity as the authentic ER- β gene sequence.

CONCLUSIONS: These results indicate that ER- β is expressed in human vascular endothelial cells, and morphine appears to regulate this receptor in a similar fashion as the μ opiate receptor.

Introduction

Recently, we have demonstrated the expression of the estrogen receptor (ER) subtype β on human endothelial and that it is coupled to constitutive endothelial nitric oxide synthase (eNOS) derived nitric oxide (NO) release [1]. This finding also has physiological significance due to the fact that in rats it can selectively affect median eminence neurosecretory processes [2].

Furthermore, over the last six years we have also demonstrated that human endothelial cells express on a pharmacological, biochemical and molecular level the opiate selective and opioid peptide insensitive opiate alkaloid receptor subtype designated μ_3 , see [3–7]. Additionally, we have demonstrated that morphine and the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) causes a down regulation of the mu receptor gene expression in human vascular endothelium and mononuclear cells [5,8]. These reports also documented that morphine stimulates eNOS derived NO release, which may modulate median eminence neurosecretion [9], demonstrating a common process, i.e., endothelial NO release, mediated by estrogen and morphine.

Given this NO convergence by both signal molecules we sought to determine if there is a relationship between them. In this regard, we demonstrate that morphine can down regulate the expression of the ER- β receptors found on human endothelial cells.

Materials and Methods

Material

Saphenous vein (SV) segments were obtained from patients undergoing elective coronary artery bypass grafting (CABG) for atherosclerotic coronary artery disease. Saphenous vein segments were obtained from patients (four post-menopausal women, mean age 74.5 ± 10.3 years and eight men mean age 57.6 ± 13.3 years). This material is regarded as waste and the institutional review board approved the project. Patients with chronic illnesses, such as diabetes or cancer as well as acute processes (e.g., known infections, trauma), were excluded. In all patients undergoing CABG, the analgesic management included induction with fentanyl (fentanyl citrate adjusted for pH) or sufentanil (N-[4(methoxymethyl)-1-[2-(2-thienyl)ethyl]-4-piperidinyl]-N-phenylpropanamide 2-hydroxy-1,2,3-propanetricarboxylate) up to 15 $\mu\text{g}/\text{kg}$. Maintenance was achieved with the same agents. Parts of SV that were not used in the CABG procedure were stored in an electrolyte solution at 4°C (500 cc plasmalyte with 5000 U heparin), and these specimens were immediately transported on ice to the laboratory for processing. Immediately upon arrival, each blood vessel was washed in phosphate buffered saline (PBS). SV specimens were prepared as previously described [10].

Primary human saphenous vein endothelial cells were obtained from Clonetics (Walkersville, MD). The cells were seeded in a 75 cm^2 flask at 1×10^7 cells in

EGM2 media (Clonetics) as recommended and incubated at 37°C with 5% CO_2 .

Treatment

Primary human SV endothelial cells were stimulated with 1 μM of morphine. When specific receptor antagonists naloxone (10 μM) or CTOP (10 μM) were employed they were administered 10 min prior to the agonist. Tissues were exposed to the drugs for a total of 24 h. In the case of pure agonist exposure, 10 min prior to its administration PBS was added in the same volume. Controls consisted of tissues not exposed to drugs. All drugs were purchased from Sigma Chemical CO. (St. Louis, MO).

Isolation of Total RNA

Following incubation, the various human endothelial cells were pelleted by centrifugation and total RNA was isolated with the RNeasy Protect Mini Kit (Qiagen, Stanford, CA). Pelleted cells were resuspended in buffer RLT and homogenized by passing lysate 5 times through a 20-gauge needle fitted to a syringe. One volume (350 μL) of 70% ethanol was mixed with the homogenized lysate by pipetting. The samples were applied to an RNeasy mini spin column sitting in a 2-ml collection tube, and centrifuged for 15 sec at 10,000 rpm. For washing, 700 μL of buffer RW1 was added to the column, and then centrifuged as previously. The column is then transferred into a new 2-ml collection tube, and washed with 500 μL of buffer RPE. After centrifugation, the column was washed again with 500 μL of buffer RPE and centrifuged for 2 min at maximum speed to dry the RNeasy membrane. The column was transferred into a 1.5 ml collection tube and RNA was eluted with 50 μL of RNase-free water for 1 min at 10,000 rpm. RNA was analyzed on a 1% agarose gel and purity determined spectrophotometrically.

Reverse Transcription

First strand cDNA synthesis was performed using random hexamers (GIBCO, BRL, Gaithersburg, MD). 3 μg of total RNA isolated from human saphenous endothelial cells were denatured at 95°C and reverse transcribed at 40°C for 1 hr using Superscript II RNase H-RT (GIBCO BRL, Gaithersburg MD). Five microliters of the RT product was used for the real-time PCR reaction.

Taq-Man Probe Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Primers and probe specific for the estrogen- β receptor gene (Table 1) were obtained from Applied Biosystems. The 2X universal master mix (Applied Biosystems) containing the polymerase chain reaction (PCR) buffer, MgCl_2 , dNTP's, and the thermal stable AmpliTaq Gold DNA polymerase was used in the PCR reactions. In addition, 200 μM of reverse and forward primers, 100 μM Taqman probe, 5 μL of RT product and

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caatcacccaaacccaaagcatcggtcacggcggttcagcaagtgcagccagcttccggctgc 60
|||||
caatcacccaaacccaaagcatcggtcacggcggttcagcaagtgcagccagcttccggctgc 1683
Sbjct: 1742

Query: 61
tgtcagcatcctgggtcgctgtgaccagagggtacatactggaattgagcaggatcatgg 120
|||||
tgtcagcatcctgggtcgctgtgaccagagggtacatactggaattgagcaggatcatgg 1623
Sbjct: 1682

Query: 121
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|||||
ccttgacacagagatattccttgggtgagtttaactctcgaaaccttgaagttagttg 1563
Sbjct: 1622

Query: 181
ccaggagcatgtcaaagatttcagaattccttctacgcatttcccctcatccctgtcca 240
|||||
ccaggagcatgtcaaagatttcagaattccttctacgcatttcccctcatccctgtcca 1503
Sbjct: 1522

Query: 241          gaacaagatctggagcaaagatgagc 266
|||||
Sbjct: 1502          gaacaagatctggagcaaagatgagc 1477

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Figure 1. Sequence analysis of the estrogen receptor (ER-beta) 266 bp fragment from human saphenous endothelial cells. Comparison of the endothelial cell ER-beta gene sequence (Query) with the authentic ER-beta receptor (Sbjct) using the NCBI Blast nucleotide computer search program. 100% sequence identity is observed.

Table 1. Primers and Taqman Probe for Estrogen receptor β sequence used in Real Time-Polymerase chain reactions.

Primer / probe	Sequence
Forward primer	5'-GCTCATCTTTGCTCCAGATCTTG-3'
Reverse primer	5'-CAATCACCCAAACCAAGCATC-3'
Taqman Probe*	5'-TGCTCCTGGCAACTACTTCAAGGTTTCGA- 3'

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

Rnase/DNase-free water was added to the master mix to a final volume of 50 μ L. 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 5700 sequence Detection System. Real-time (RT)-PCR for the β -actin reference gene (Applied Biosystems) was also performed using 1 μ L of the RT product. The PCR result was analyzed with the GeneAmp 5700 SDS software (Applied Biosystems). In order to determine the relative copy number of the target gene transcript and the reference gene, control cDNA generated from untreated endothelial cell total RNA was used to produce the standard curves (Figure 1).

Sequencing of the cloned PCR Products

PCR products obtained from the PCR reaction for ER- β were electrophoresed on a 2% agarose gel. The bands corresponding to the expected size (266 bp) fragment were excised, purified with the Qiagen Qiaprep kit. The DNA fragment was cloned into the TOPO TA cloning vector (Invitrogen), and then was used to transform chemically competent E. coli cells. Transformed cells were grown at 37°C overnight, and then

the plasmid was purified using the Qiagen plasmid mini prep kit.

Purified plasmid DNA containing the ER- β PCR fragment was sequenced with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems-Perkin-Elmer) and the ABI Prisms 310 Genetic Analyzer and ABI 377 DNA sequencer.

Results

Specific ER- β primers and a Taq-Man probe, designed to amplify a fragment of the coding region of the ER- β gene, were employed (Table 1). Sequence analysis of this transcript obtained from human vascular endothelial cells (primary, atrial, and SV performed separately) demonstrated 100% sequence identity to the authentic ER- β gene (Figure 1). By Real-time RT-PCR, we generated the amplification plot for the treated samples, primary human SV endothelial cells, and a standard curve plot for the ER- β gene (Figure 2). Analysis of the effect of morphine on ER- β gene expression by RT-PCR demonstrates that primary human saphenous vein endothelial cells treated with 1 μ M morphine for 24 h significantly diminished ER- β expression (Figure 3). Prior treatment of the primary human SV endothelial cells with 10 μ M of naloxone, a general opiate receptor antagonist, partially blocked the effect of morphine whereas CTOP, a specific μ opiate receptor antagonist, also only partially blocked the effect (Figure 3). Trypan blue staining demonstrated a cell survival rate of 91 % to 94 % after treatment.

Discussion

The present study demonstrates the following: 1) ER- β expression occurs in human vascular endothelial cells obtained from SV, atria and primary human saphenous vein endothelial cells; 2) Exposure of primary human SV endothelial cells to morphine for 24 hours down regulates ER- β expression in these cells; 3) Opiate receptor blockade by various antagonists partially

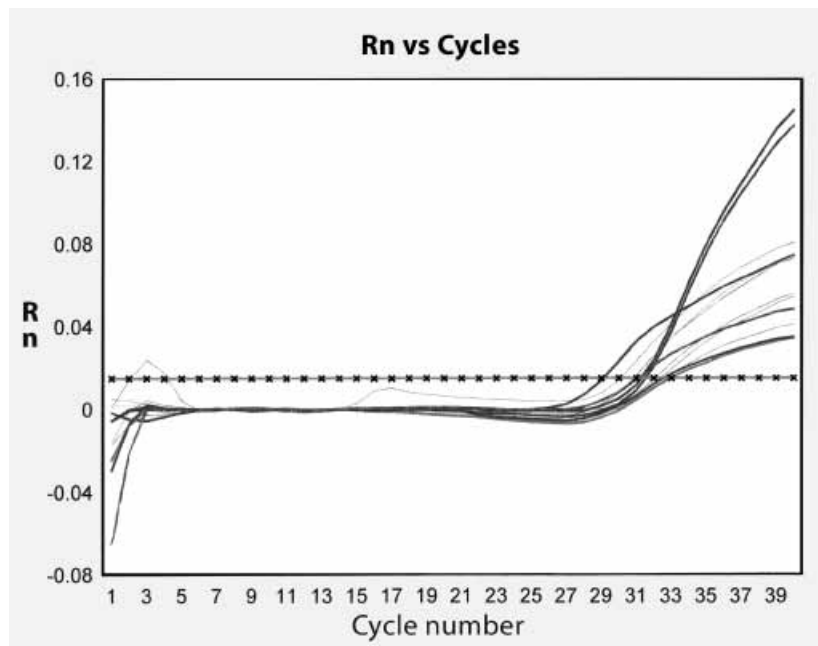


Figure 2. Real-time RT- PCR of the ER-beta standard curve using untreated SV endothelial cell total RNA. Analysis of real-time RT-PCR was performed using the ABI GeneAmp 5700 Sequence Detection System. The cDNA standards were serially diluted and used as templates ranging from .740 ng to 148 ng. The ratio of the quencher dye emission (dRn) is plotted against the cycle number (A and B). The input DNA templates were plotted against the Ct values (the fractional cycle number at which dRn crosses a fixed threshold baseline). The samples were all within the standard range.

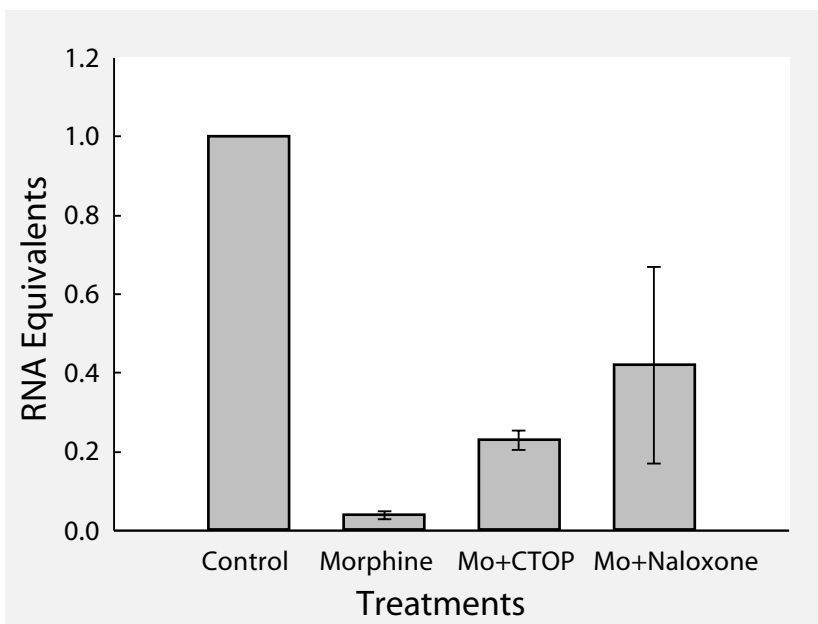


Figure 3. Effect of morphine on ER-beta expression in human saphenous vein endothelial cells. ER-beta gene expression on endothelial cells was determined by real-time RT-PCR. Cells treated with 1uM of morphine alone, or with 10 uM naloxone or CTOP for 24 h. The data shown represents three different experiments. MO = morphine

inhibits morphine stimulated inhibition of ER- β expression; and 4) Since the opiate antagonists didn't completely block the effect of morphine on the endothelial cells, it appears that morphine may be acting on these cells through a novel mechanism, which partially involves a μ opiate receptor mediated process. Taken together we surmise that these vascular signaling molecules can modulate each other's expression, suggesting that these processes have physiological relevance.

In previous reports from our group we have demonstrated that morphine liberates cNOS derived NO from vascular endothelial cells [3,10-14]. This naloxone antagonizable action of morphine is mediated by a μ opioid receptor found on this tissue see [7], which appears to be a μ splice variant [5]. This μ receptor subtype can also be up regulated by proinflammatory cytokines and down regulated by morphine[5]. In addition, this receptor expression was not found on human

smooth muscle cells or vein adventia [5]. Interestingly, we have demonstrated the presence of morphine and morphine 6 glucuronide in human vascular tissues, suggesting the existence of "local" opiate vascular signaling given the expression of a μ opiate receptor [15,16]. Furthermore, these same cells contain a cell surface estrogen receptor subtype, namely β , that is also coupled to cNOS derived NO release [1], suggesting an interaction between these signaling processes since cNOS derived NO represents a common end point.

In a recent review concerning NO signaling we summarize various reports and conclude that cNOS derived NO release is modulated by autoregulation: that is, once stimulated it's further stimulation is diminished by NO itself [17]. Given the present data, we surmise that the diminished NO produced after the primary event occurs [17] is due to other cNOS coupled

receptors being down regulated, as is the case in the present report for estrogen following morphine exposure. We speculate that morphine down regulates ER- β expression to limit cNOS derived NO release, further suggesting that cNOS stimulation has many origins, i.e., estrogen, designed for different functions see [18,19]. This process, at the least, limits the local NO levels, further diminishing detrimental side effects that may be linked to NO see [19]. Indeed, this further suggests that under abnormal circumstances, e.g., uncoupling of these receptors to cNOS regulation, detrimental NO actions may emerge.

In the literature there is a small body of work that demonstrates that estrogen influences μ opioid receptor expression as well [20]. The binding of [3H]-diprenorphine in human neuroblastoma cells transfected with an estrogen receptor cDNA (SK-ER3 cell line) and then exposed to 17- β -estradiol revealed that the number of opioid receptors is reduced at 6 days [21]. In another study, laser-scanning microscopy demonstrated that estrogen treatment induces the translocation of μ -opiate receptor immunoreactivity from the membrane to an internal location in steroid-sensitive cell groups of the limbic system and hypothalamus [22].

On the contrary, others have found an increase in μ opioid receptor expression. Estrogen treatment alters μ -opiate receptor mRNA levels in different areas of the forebrain of ovariectomized female [23]. They report an increase in μ -opiate receptor mRNA levels in the ventromedial nucleus of the hypothalamus and arcuate nucleus after 48 h of 10 mg of 17- β -estradiol-3-benzoate treatment when compared to ovariectomized females. No effects of estrogen were observed on μ -opiate receptor mRNA levels in the posterior medial nucleus of the amygdala, hippocampus, caudate-putamen or the medial habenula [23]. Exposure of ovariectomized rats to estradiol for 48 h increased the density of [3H]naloxone binding in both the ventral and dorsal preammillary nuclei but not in other regions of the hypothalamus or amygdala [24]. These contradictions may be due to the lack of opiate alkaloid use [3,7,13,25]. Additionally, the various binding ligands employed can also react with other opioid receptor subtypes, i.e., δ .

Given the interaction demonstrated in the current study, we offer the following speculation. At first glance, it may appear that we have a redundant im-

munovascular down regulating process. However, we believe that each signaling system performs this common function, i.e., cNOS derived NO release, under different circumstances. Morphine, given its long latency before increases in its levels are detected, arises after trauma/inflammation to down regulate these processes in neural and immune tissues [3,26,27]. We surmise that estrogen, since testosterone or progesterone do not exert this NO generating action, provides an extra-degree of immunocyte and vascular down regulation in females. This is most probably due to both the immune and vascular trauma associated with cyclic reproduction activities, i.e., endometrial buildup, when a high degree of vascular and immune activities are occurring. Given the high degree of proliferative growth capacity during estrogen peak levels in this cycle, NO may function to enhance down regulation of the immune system to allow for these changes. In this regard, it is not difficult to understand the reports documenting various cancers with blocking estrogen actions and conversely reports documenting its anti-cancer protective actions see [28].

Since morphine, presumably via NO, down regulates ER receptor expression; we surmise that this phenomenon is involved with the tissues ability to down regulate its excitation to a level that does not compromise the tissues ability to respond to additional excitation see [19]. If one signal molecule down regulates the tissue's excitation potential the other signal molecule would down regulate it further. However, this is not the case. Since the other receptor is down regulated as well, morphine via NO down regulates its own expression [8]. This allows the tissue to recover or become excited if called upon. Furthermore, it would allow for a stronger excitatory response since an additive effect on down regulating influences would not be felt, regardless of whether or not they are expressed. Thus, we surmise this ability of morphine to down regulate ER expression is physiologically important and as such has biomedical implications.

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