17-β estradiol down regulates ganglionic microglial cells via nitric oxide release: Presence of an estrogen receptor β transcript

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Abstract

OBJECTIVES: In earlier studies we have demonstrated that 17-β-estradiol and an estrogen cell surface receptor can be found on various human cells where they are coupled to nitric oxide release. We also demonstrated the presence of estrogen signaling in *Mytilus edulis* ganglia. In the present report, we sought to determine a function for these ganglionic estrogen receptors, transcending a reproductive role for estrogen.

MATERIAL & METHODS: Ganglionic microglial egress from excised pedal ganglia was examined microscopically following pharmacological treatments designed to determine a role for 17-β-estradiol in microglial regulation via nitric oxide. Additionally, we examined the tissue by RT-PCR and sequence analysis for the estrogen receptor β gene.

RESULTS: In ganglia incubated with varying concentrations of 17-β-estradiol-BSA there is a significant drop in microglial egress at the 24 hour observation period (58.7 ± 7.4 vs. 17-β-estradiol-BSA exposed = 14.7 ± 1.5; P<0.01), which can be antagonized by tamoxifen and significantly diminished by L-NAME, a nitric oxide synthase inhibitor. By RT-PCR and sequence analysis *Mytilus edulis* pedal ganglia was found to express a 266 bp fragment of the estrogen receptor-β gene, which exhibits 100% sequence identity with the human counterpart.

CONCLUSION: These data suggest that 17-β-estradiol-BSA is working on estrogen cell surface receptors since 17-β-estradiol-BSA does not enter the cytoplasm and that these receptors are coupled to constitutive nitric oxide release. This study demonstrates that 17-β-estradiol can down regulate microglial fMLP induced activation and activation following ganglionic excision.
Introduction

Ongoing studies in our laboratory have demonstrated that intercellular signaling involving neuropeptides is quite conservative in that the messenger molecules and their respective receptors have been conserved during the course of evolution as noted by their presence in invertebrates. This is especially true for opioid peptides and opiate alkaloids [1,2,3,4]. Other studies indicate this is equally true for cytokines [5,6]. interleukin (IL)-1, Tumor Necrosis Factor (TNF) and IL-6-like materials have also been found in invertebrates, specifically in the marine mollusc *M. edulis* [7,8,9]. Here, these cytokine-like molecules were found to alter the behavior and conformation of immunocytes from *Mytilus* as well as those of man.

Given the strong and remarkable parallelisms in intercellular communication between man and molluscs, we sought to investigate the effects of another signal molecule, namely, estrogen, on invertebrate microglial behavior [10]. 17-β-estradiol conjugated to bovine serum albumin (E₂-BSA) appears to have the ability to diminish the phenomenon of microglial egress and activation from excised invertebrate ganglia maintain in culture [11,12]. Furthermore, 17-β-estradiol (E₂) also has the ability to down regulate spontaneously active microglia as well as those stimulated by fMLP. The results demonstrate for the first time that E₂ can down regulate invertebrate ganglion glia cells, demonstrating a neural role transcending reproduction. In addition, by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and sequence analysis, we have observed a significant base pair fragment of the estrogen receptor (ER)-β gene in *M. edulis* pedal ganglia total RNA, and sequencing of this fragment revealed a 100% sequence identity to human.

Material and Methods

*Mytilus edulis* were harvested from the shores of Long Island Sound. The animals were maintained for periods of up to three months in an internal marine aquarium system developed to eliminate bacteria [13].

For *in vitro* observations of glia, the excised ganglia of *M. edulis* were bathed for 1–24 hrs in a mixture of heated and filtered seawater and *M. edulis* hemolymph, also filtered and heated (50:50), containing antibiotics (50 mg streptomycin, 30 mg penicillin, 50 mg gentamicin in 100 ml, pH 7.5) and then transferred to a microwell culture dish. The pedal ganglia of *M. edulis* were immersed in the physiological saline to which the centrifuged cell-free hemolymph or physiological saline plus antibiotics were added 50% by volume. The hemolymph was inspected by light microscope to ascertain its cell-free status and then subjected to UV radiation for 1 h to further eliminate bacteria. This technique has been described elsewhere in detail [12,14].

For the determination of microglial cell numbers and conformational changes, the ganglia or cells of *M. edulis* were placed in a ring of Vaseline on a slide and protected by a cover slip. The substances to be tested (e.g., E₂-BSA) were added to the incubation medium, followed by final adjustments. Following a 24 h incubation period, the ganglionic explants of *M. edulis*, with or without E₂-BSA, tamoxifen, L-NAME and in combination, were examined for the numbers of cells having moved into the incubation medium. The microglia were also examined for cell shape changes as noted extensively elsewhere [12,15,16] by image analysis at a magnification of 100x [12]. In other experiments these cells were exposed to fMLP (10⁻⁹ M) for 30 min then washed 3 times with the incubation medium. These cells were later exposed to E₂-BSA for periods of up to 4 hrs.

The determination of the degree of activation in glial cells (amoeboid vs. round conformation) that egressed 24 hours earlier from excised ganglia was based on measurements of cellular area and perimeter by use of the Compix image analysis software [17]. A 400μm viewing diameter was selected for determining the number of cells having moved into the culture dish and for frame grabbing and conformational determinations.

Ligands

Ganglia were stimulated with various concentrations of E₂-BSA (10⁻¹¹ to 10⁻⁶ M of 17-β-E₂) (Sigma, St. Louis, MO), which has been shown to activate human and invertebrate estrogen cell surface receptors that are coupled to constitutive nitric oxide release [10,18,19,20]. They were also exposed to either tamoxifen (10⁻⁷M) (Sigma, St. Louis, MO), estrogen receptor antagonist (n = 4), or L-NAME, a nitric oxide synthase inhibitor (n = 4) at concentrations previously demonstrated to be efficacious in this tissue [10,18,19,20]. Tamoxifen was added to the milieu 5 min before E₂-BSA.

Statistical analysis was by way of the *student’s t-test*. Each curve was compared to a control performed at the same time using cells obtained from the same animal in order to minimize variations. Each evaluation measured or counted from 71 to 109 cells. Experiments were replicated three times and the resulting mean was graphed ± SEM.

Isolation of Total RNA

*M. edulis* pedal ganglia were excised, washed in PBS, and total RNA was isolated with the RNeasy Protect Mini Kit (Qiagen, Stanford, CA). The ganglia were resuspended in 600 µl buffer RLT and homogenized using a hand-held homogenizer. One volume (600 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-Polymerase Chain Reaction
First strand cDNA synthesis was performed using random hexamers (GIBCO, BRL, Gaithesburg, MD). 2 µg of total RNA isolated from pedal ganglia was denatured at 95°C and reverse transcribed at 40°C for 1 hr using Superscript II Rnase H-RT (GIBCO BRL, Gaithesburg MD). 10µl of the RT product was added to the PCR mix containing specific primers for the ER-β gene and Taq DNA polymerase (GIBCO, BRL, MD). PCR reactions were denatured at 95°C for 5 minutes followed by 40 cycles at 95°C for 1 min, 57°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) and visualized by ethidium bromide staining. The only ER-β-specific primers used in the PCR reactions that yielded a specific PCR product amplified a 266 bp fragment at map position 1477–1499 (Primer–5’gctcatctttgctccagatcttg 3’), and at map position 1742–1721 (primer – 5’caatcacccaaaccaaagcatc3’).

Sequencing of the cloned PCR Products
PCR products obtained from the PCR reaction for the ER-β were electrophoresed on a 2% agarose gel. The bands corresponding to the expected size fragment were excised, purified with the Qiagen Qiaprep kit. The DNA fragment was cloned into the TOPO TA cloning vector (Invitrogen), and then used to transform chemically competent E. coli cells. Purified plasmid DNA containing the 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
E2 effect on microglial egression
In the course of microglial egress from excised pedal ganglia we demonstrate that the process does not occur at a steady rate during the initial 24 hour observation period (Figure 1). There is an initial lag lasting approximately 8 hours, followed by a period of substantial microglia egress up to 24 hours (Figures 1 and 2), which appears to be the peak egress time [12].

Figure 1. The effect of E2-BSA on microglial exodus over 24 hours and its antagonism by concomitant tamoxifen (Tam;10^{-7} M) or L-NAME (10^{-4} M) exposure. The experiments were replicated 4–6 times. The mean value of these trials is represented along with the standard error of the mean. Statistical significance (P<0.01) was determined by a one-tailed students t-test comparing E2-BSA migration numbers to that of the controls. Tamoxifen (Tam;10^{-7} M) or L-NAME (10^{-4} M) exposure compared to E2-BSA at 8 to 24 hours are significant at the P<0.01 level.

Figure 2. Microglial exodus over a 24 hour period and its antagonism by varying concentrations of E2-BSA. The experiments were replicated 4–6 times. The mean value of these trials is represented along with the standard deviation. Statistical significance (P<0.01) was determined by a one-tailed students t-test comparing migration numbers at 24 hours to 10^{-8} to 10^{-6} M E2-BSA exposed values.
In ganglia incubated with varying concentrations of E2-BSA there is a significant drop in their egress at the 24 hours observation period (58.7 ± 7.4 vs. E2-BSA exposed = 14.7 ± 1.5; P<0.01), which can be antagonized by tamoxifen and significantly diminished by L-NAME exposure (Figure 1). Tamoxifen or L-NAME, given alone, caused no noticeable change in any of the experiments (data not shown). These data suggest that E2-BSA is working on estrogen cell surface receptors since E2-BSA does not enter the cytoplasm [10] and this receptor is coupled to constitutive nitric oxide release.

Of the cells that respond to ganglionic surgical excision by leaving the tissue, if left alone for another 24 hours, about 13.1% remain active (amoeboid-form factors of 0.6 or less) while the rest appear round (form factors of more than 0.8) and inactive (Figure 3). E2-BSA exposure to these activated cells in culture, caused the number of amoeboid cells to decrease to 6.1% (P<0.01) whereas cells incubated alone or with E2-BSA and tamoxifen or E2-BSA and L-NAME exhibited control levels of microglial activation of 12% and 17%, respectively (Figure 3). At the 4 hour observational period the percent of active cells increase, approaching control values. Furthermore, these rapid responses also support a cell surface location for the estrogen receptor. In other studies we demonstrate that M. edulis microglia express a functional cNOS-derived NO release system coupled to other types of receptors as well [21] and that E2-BSA stimulates cNOS derived NO in real-time in M. edulis ganglia within two minutes of exposure to the tissue [10].

It also was of interest to determine if E2-BSA was capable of blocking the microglial activation induced by fMLP (Figure 4). After an initial exposure of these cells to fMLP (10^-8 M) and their subsequent washing, and then E2-BSA incubation, there was a significant diminishing of the number of activated microglia compared to cells exposed to fMLP alone (P<0.01) and cells exposed to fMLP; E2-BSA and tamoxifen and cells exposed to fMLP; E2-BSA and L-NAME (Figure 4). Thus, E2-BSA diminishes the excitation caused by fMLP, a well established immunocyte excitatory agent.

**RT-PCR and Sequence Analysis**

In previous studies we have demonstrated expression of ER-β in human endothelial cells via RT-PCR [18]. Furthermore, this report also demonstrated that ER-β was coupled to ecNOS-stimulated NO release in endothelial cells. We now demonstrate by RT-PCR (Figure 5) and sequence analysis that M. edulis ER-β exhibits 100% sequence identity with its human counterpart determined by the NCBI BLAST nucleotide computer search program (Figure 6).
Discussion

The present report demonstrates the following:

1) *M. edulis* pedal ganglia maintained in culture after surgical removal, over the course of 24 hours, emit microglia;
2) E2-BSA significantly reduces the microglial egress;
3) Concomitant treatment of the ganglia with E2-BSA and an estrogen receptor antagonist, tamoxifen, or the nitric oxide synthase inhibitor L-NAME effectively restores the glial egress to the level found in control preparations;
4) Spontaneously active invertebrate microglia can be down regulated by E2-BSA; and
5) fMLP activated microglia can also be down regulated by E2-BSA. Taken together these results suggest that endogenous estrogen may play a role in down regulating microglial activation and/or maintaining them in a down regulated state ready for activation, i.e., disinhibition.

Preliminary evidence for the presence of estrogen signaling in invertebrates has been noted in molluscs, specifically in the octopus [22,23,24]. These reports strongly suggest that an estrogen-like material and an unidentified ER are involved with the reproductive functions in the octopus. Furthermore, we have demonstrated following HPLC purification of E2 from *M. edulis* ganglionic tissue, the presence of E2 by RIA and nano-electrospray quadrupole time of flight tandem mass spectrometry (ES-Q-TOF-MS) analysis [10]. We further found that when either exogenous or endogenous (purified HPLC fraction) E2 was added to pedal ganglia there was an immediate concentration-dependent NO release measured amperometrically [10]. E2-BSA also stimulated NO release, suggesting mediation by a membrane surface receptor [10]. Tamoxifen, an estrogen receptor antagonist, inhibited the action of both E2 and E2-BSA, further supporting the presence of an estrogen receptor. In addition, by Western blot analysis with anti-ER-β antibodies we observed a 55 kDa protein in both the membrane and cytosolic fractions in pedal ganglia as well as in human leukocytes, which have been previously shown to express ER-β [10]. These results directly support the observations made in the present report that estrogen acutely stimulates NO release within pedal ganglia, i.e., microglia via an estrogen cell surface receptor. These data also suggest that this estrogen signaling pathway appeared
much earlier along the evolutionary timetable than previously thought. In this context, in human tissues (vascular endothelial cells, neutrophils and monocytes), various ERs were found on the cell surface that were coupled to constitutive nitric oxide synthase (cNOS)-derived nitric oxide (NO) release [18,19,20,25].

The assignment of microglial status to the cells that have egressed from excised invertebrate and vertebrate ganglia has been noted in another report [12]. Briefly, the evidence supporting the microglial status is two-fold: (a) In response to surgical trauma, a subset of reactive glial cells exhibit a number of features characteristic of the animals’ immunocytes; (b) These structural and functional commonalities parallel those between microglia and macrophages observed in vertebrates under comparable experimental conditions [26,27]. The critical aspects of this concept can be summarized as follows:

1. In mammals and invertebrates comparable mobile immune cells are known to enter various tissues, including nervous tissues [10,28].
2. In the process of transformation to resident glia, these immunocytes appear to undergo similar conformational changes and some biochemical dedifferentiation [29].
3. During microglial egress from lesioned neural tissue, in excised ganglia maintained in incubation medium, they regain some of their inherent characteristics, including an amoeboid conformation, adhesiveness, and motility [10].
4. An important criterion for the immunoregulatory potential of these mobilized microglial cells and their counterpart in vertebrates is their phagocytotic activity. This capacity is made use of in the uptake and removal of degenerating neural structures, demonstrated in mammals as well as invertebrates.
5. In both groups of animals, the egress of reactive microglial cells from lesioned nervous tissue and their conformational changes are counteracted by exogenous morphine, a process corresponding to the inhibiting effect of this drug in immune cells [10,12,14]. Thus, it would appear that the small non-neuronal cells found mostly juxtaposed to nerve cell bodies are indeed microglia.

The data presented in this report demonstrates that estrogen has the ability to modulate microglial function, down regulating the cells or maintaining them in a state of readiness since estrogen can be detected in hemolymph constitutively as well as in the ganglia along with an estrogen receptor [10]. Furthermore, we surmise that estrogen may exert a protective role as well, since it limits microglial activation, preventing “noise-like” influences from inappropriately activating these cells. In this regard, we further surmise that inappropriate activation of these immune active cells may liberate substances, i.e., IL-1 (present in M. edulis neural tissues [5]), which may cause damage to nerve cells.

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REFERENCES


