Immunomodulation by \(17\beta\)-Estradiol in bivalve hemocytes

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Effects of \(17\beta\)-Estradiol on molluscan immunocytes

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Abstract

In mammals, estrogens have both dose- and cell-type specific effects on immune cells and may act as pro-inflammatory and anti-inflammatory stimuli depending on the setting. Evidence has been provided that in the bivalve mollusc *Mytilus* the natural estrogen 17β-estradiol (E₂) can affect neuro-immune functions. In this work the immunomodulatory role of E₂ in *Mytilus* hemocytes, the cells responsible for the innate immune response, was investigated. E₂, in a narrow concentration range (5-25 nM), rapidly stimulated phagocytosis and oxyradical production *in vitro*; higher concentrations inhibited phagocytosis. E₂-induced oxidative burst was prevented by the nitric oxide synthase inhibitor L-NMMA and by SOD, indicating the involvement of both NO and O₂⁻; NO production was confirmed by nitrite accumulation. The effects of E₂ were prevented by the antiestrogen Tamoxifen and by specific kinase inhibitors, indicating a receptor-mediated mechanism and involvement of p38 MAPK and PKC. E₂ induced rapid and transient increases in the phosphorylation state of PKC, as well as of a CREB-like transcription factor, as indicated by WB with specific anti-phospho-antibodies. Localization of ERα- and ERβ-like proteins in hemocytes was investigated by immunofluorescence confocal microscopy. The effects of E₂ on the immune function were also investigated *in vivo*, at longer exposure times (6 and 24 hrs) in the hemocytes of E₂-injected mussels. E₂ significantly affected hemocyte lysosomal membrane stability, phagocytosis and extracellular release of hydrolytic enzymes: lower concentrations resulted in immunostimulation, whereas higher concentrations were inhibitory. Overall, the obtained data indicate that the physiological role of E₂ in immunomodulation is conserved from invertebrates to mammals.

*Key words: estrogen, innate immunity, bivalves, hemocytes, kinase-mediated cell signaling, Mytilus*
INTRODUCTION

In mammals, estrogens exert a broad spectrum of activities on a wide variety of cells and tissues, including the immune system. Estrogens have both dose- and cell-type specific effects on immune cells and may act as pro-inflammatory and anti-inflammatory stimuli depending on the setting (5, 22, 24, 43). 17β-estradiol (E2) can modulate the function of neutrophil granulocytes, monocytes and macrophages: these cell types have been reported to express both intracellular and membrane Estrogen Receptors (ERs) and their response to E2 can be mediated by both nuclear, classical or ‘genomic’ pathways, as well as by rapid, ‘non-genomic’ mechanisms of action (2, 3, 24, 45). These latter may be initiated at either membrane or cytosolic locations (29, 39, 49) and can result in both direct local effects (such as modification or ion fluxes) and regulation of gene transcription secondary to activation of cytosolic kinase cascades (5, 22, 24, 29, 43).

Among invertebrates, estrogens have been identified in bivalve molluscs (25, 34, 40, 43), where their role has been mainly investigated in the control of gametogenesis (20, 21, 30, 33, 34). However, evidence has been provided that estrogen can represent an important signaling molecule involved in roles other than reproduction also in these organisms. In neural tissues of the marine mussel Mytilus spp., 17β-estradiol has been shown to down-regulate ganglionic microglial cells after surgical insult, which normally stimulates their egress from the tissue (46), as well as after fMLP activation (48). The effects of E2 on microglial cells were mediated by rapid induction of nitric oxide (NO) release by nervous tissue and were antagonized by classical antiestrogens. These data indicated a receptor-mediated event in Mytilus pedal ganglia (46), where a fragment of the ERβ was identified, showing 100% sequence identity to the human receptor (48).

In Mytilus, E2 was also shown to affect the digestive cells and circulating hemocytes, in particular at the level of the lysosomal function (6, 32). In bivalves, hemocytes are responsible for cell-mediated immunity through phagocytosis and various cytotoxic reactions (such as lysosomal enzyme and antimicrobial peptide release and production of reactive oxygen intermediates) (13, 41). We have
previously shown that addition of E\textsubscript{2} (in the low nM range) to hemocyte monolayers induced a moderate increase in cytosolic \([\text{Ca}^{2+}]\), destabilisation of lysosomal membranes, morphological changes, hydrolytic enzyme release and stimulated the bactericidal activity towards \textit{E. coli} (12); all these effects were rapid, occurring from seconds to minutes from \(E_2\) addition and were prevented by the antiestrogen Tamoxifen. The effects of \(E_2\) on mussel hemocytes were mediated by components of tyrosine kinase-mediated cell signalling (12), in particular the stress-activated p38 MAPK (Mitogen Activated Protein Kinase), and STAT-like proteins (Signal Transducers and Activators of transcription), that play a key role in the activation of these cells (8-10).

In this work, in order to clarify the effects and mechanisms of action of \(E_2\) in the immune response of mussel hemocytes, the effects of the hormone on phagocytosis and oxyradical production were evaluated \textit{in vitro}; estrogen signalling was investigated by use of specific kinase inhibitors and by evaluating the phosphorylation state of PKC (protein kinase C) and of the transcription factors CREB (cAMP responsive element binding protein) by electrophoresis and Western blotting with specific anti-phospho-antibodies. Intracellular localization of ER-like proteins was evaluated by immunofluorescence confocal microscopy. Finally, the possible immunomodulatory role of \(E_2\) \textit{in vivo} was investigated in hemocytes collected from mussels injected with different concentrations of the hormone.
MATERIALS AND METHODS

Animals

Mussels (Mytilus galloprovincialis Lam.) 4-5 cm long, were obtained from SEA (Gabice Mare, PU) and kept for 1-3 days in static tanks containing artificial sea water (ASW) (1 l/mussel) at 16°C. Sea water was changed daily.

Hemolymph collection, preparation of hemocyte monolayers and hemocyte treatments

Hemolymph was extracted from the posterior adductor muscle of 10-12 mussels for each experiment, using a sterile 1 ml syringe with a 18 G1/2” needle. With the needle removed, hemolymph was filtered through a sterile gauze and pooled in 50 ml Falcon tubes at 4°C. Hemolymph serum was obtained by centrifugation of whole hemolymph at 200 x g for 10 min and the supernatant was sterilised through a 0.22 µm pore size filter. Hemocyte monolayers were prepared as previously described (10). Briefly, aliquots of 0.5 ml of hemolymph, corresponding to about 1-2 x 10⁶ cells, were seeded onto glass cover slips (40 by 22 mm) placed in plastic culture dishes and incubated at 16°C for 30 min to allow for cell attachment. Non-adherent hemocytes were subsequently removed by gently washing the preparations with sterilised ASW. Hemocyte monolayers were added with 1.5 ml of hemolymph serum and kept before use at 16°C.

Hemocytes were incubated at 16°C with E₂ (from 10 mM stock solutions in ethanol suitably diluted in ASW) for different periods of time, as indicated in each experiment. Untreated and control vehicle hemocyte samples were run in parallel. In experiments with antiestrogens, Tamoxifen (final concentration 100 nM, from a 10 mM stock solution in ethanol) was added 10 min before E₂. In experiments with kinase inhibitors, before E₂ addition hemocyte monolayers were pre-treated for 20 min with 20 µM SB203580 (for p38 MAPK) or 2.5 µM GF109203X (for PKC) as previously described (10, 15). For each experiment, control hemocyte samples were run in parallel. Triplicate preparations were made for each sample. All incubations were carried out at 16°C.
Phagocytosis assay

Phagocytosis of neutral red-stained zymosan by hemocyte monolayers was used to assess the phagocytic ability of hemocytes according to (36) with slight modifications (4). Neutral red-stained zymosan in 0.05 M Tris-HCl buffer-(TBS), pH 7.8 (added with 2% NaCl to maintain osmotic conditions) was added to each monolayer at a concentration of about 1:50 hemocytes:zymosan diluted in ASW in the presence or absence of E₂, and allowed to incubate for 60 min. Monolayers were then washed three times with TBS, fixed with Baker’s formol calcium (4%v/v formaldehyde, 2% NaCl, 1% calcium acetate) for 30 min and mounted in Kaiser’s medium for microscopical examination with a Vanox (Olympus Italy 1.2.1, MI) optical microscope. For each slide, the percentage of phagocytic hemocytes was calculated from a minimum of 200 cells. The effect of E₂ on phagocytosis was also compared to that induced by human recombinant Tumor Necrosis Factor-α (TNFα, 200 nM) (4).

Oxidative burst

Extracellular oxyradical production by mussel hemocytes was measured by the reduction of cytochrome-c (36) with slight modifications. Haemolymph was extracted into an equal volume of TBS (0.05 M Tris-HCl buffer, pH 7.6, containing 2% NaCl). Aliquots (500 µl) of hemocyte suspension in triplicate were incubated with 500 µl of cytochrome-c solution (75 µM ferricytochrome-c in TBS), with or without E₂. Cytochrome-c in TBS was used as a blank. Samples were read at 550 nm at different times (from 0 to 60 min) and the results expressed as change in OD per mg protein. Detection of intracellular oxyradicals was also carried out using the nitroblue tetrazolium (NBT) assay (36) as described in (1). Aliquots (500 µl) of hemocyte suspension in triplicate were diluted 1:1 with TBS added with 2% NaCl and incubated for 60 min with an equal volume of a NBT solution (0.1% NBT) in the presence or absence of E₂ at room temperature in the dark. Samples were then centrifuged (180 x g, 5 min) and the pellet resuspended in 70% methanol for fixation. Samples were again centrifuged, the supernatant discarded and the pellet air dried. The
pellet was then resuspended in 1.1 ml of extraction medium (6 ml of 2 M KOH+7 ml DMSO) to dissolve the insoluble formazan formed, mixed by vortexing and centrifuged at 3500 x g for 20 min at 10°C). Supernatants were read at 620 nm and the results expressed as OD 620/mg protein. Experiments were carried out also in the presence of 1 mM L-NMMA (N\textsuperscript{G}-methyl-L-arginine), or 300 U/ml (SOD-superoxide dismutase) or after sample pre-incubation with Tamoxifen or kinase inhibitors at the concentrations indicated above. The effects of E\textsubscript{2} on extracellular and intracellular oxyradical production were compared to those induced by 10 µg/ml PMA (phorbol myristate acetate) in the same experimental conditions.

**Nitrite production**

NO production by mussel hemocytes was evaluated as described in (51) by the Griess reaction, that quantifies the nitrite content (NO\textsubscript{2}) of supernatants. Aliquots of hemocyte suspensions (1,5 ml) were incubated at 16°C with E\textsubscript{2} (25 nM) or vehicle (ethanol) for different periods of time (0-5 hrs) in the presence and absence of Tamoxifen or inhibitors (L-NMMA, SB203580, GF109203X). Every 30 min samples were immediately frozen and stored at -80°C until use. Prior to analysis, samples were thawed and centrifuged (12000 x g for 30 min at 4°C) and the supernatants analyzed for NO\textsubscript{2} content. Aliquots (200 µl) in triplicate were incubated for 10 min in the dark with 200 µl of 1% (w/v) sulphanilamide in 5% H\textsubscript{3}PO\textsubscript{4} and 200 µl of 0.1% (w/v) N-(1-naphtyl)-ethylenediamine dihydrochloride. Samples were read at 540 nm and the molar concentration of nitrite in the sample was calculated from standard curves generated using known concentrations of sodium nitrite. The effect of E\textsubscript{2} on nitrite accumulation was compared to that induced by PMA (50 µg/ml) in the same experimental conditions.

**Lysosomal membrane stability**

Lysosomal membrane stability in mussel hemocytes was evaluated by the Neutral Red Retention time assay as previously described in *in vitro* (9, 12, 14, 15) and *in vivo* (11) experiments following
(31). Hemocyte monolayers on glass slides were incubated with 30 µl of a NR solution (final concentration 40 µg/ml from a stock solution of NR 20 mg/ml DMSO); after 15 min the excess dye was washed out, 30 µl of artificial sea water were added, and slides were sealed with a coverslip. In 
in vitro experiments, hemocyte monolayers were incubated with E₂ fro 30 min before addition of NR (12). Every 15 min, hemocytes were examined under an optical microscope and the percentage of cells showing loss of the dye from lysosomes in each field was evaluated. For each time point, 10 fields were randomly observed, each containing 8-10 cells. The endpoint of the assay was defined as the time at which 50% of the cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded). The stimulatory effect of E₂ on phagocytosis was compared to that induced by hemocyte incubation with E. coli (strain MG155) in the same experimental conditions as previously described (9).

*Immunolocalization of ER-like receptors*

Immunolocalization of ER-like receptors in hemocytes was evaluated by Confocal Laser Scanning Microscopy (CLSM). Hemocyte monolayers, seeded on polylysine coated glass coverslips, were fixed for 30 min at room temperature in 4% paraformaldehyde dissolved in 5 mM PBS (Na-phosphate buffer solution), pH 7.4, added with 2% NaCl. All subsequent steps were carried out at room temperature in the presence of PBS added with NaCl. Cells were washed 3 times with PBS, permeabilized for 10 min with 0.2% Triton X-100 in PBS, and treated for 30 min with 2% BSA (Bovine Serum Albumin) in PBS (PBS-BSA), to minimize possible unspecific binding of the antisera subsequently employed. Hemocytes were then incubated with human polyclonal anti-ERα (anti-rabbit) or anti-ERβ (anti-goat) antibodies (1:50 in PBS-BSA) for 60 min. Cells were washed three times in PBS-BSA and incubated for 60 min with donkey anti-rabbit IgG – rhodamine labelled (Santa Cruz), or rabbit anti-goat IgG-Cy3 labeled (Santa Cruz), respectively, as secondary antibodies (1:250 in PBS-BSA) following the manufacturer’s instructions. Cells were then washed three times in PBS and coverslips were mounted on glass slides using a glycerol-
DABCO (1,4-diazabicyclo[2.2.2]octane 5% in a solution made of 90% glycerol and 10% PBS 10x) anti-fading mixture.

Slides were mounted on the stage of a Leica inverted microscope which is part of a LEICA TCS SL confocal laser scanning microscopy (CLSM) system. Hemocytes were observed using a 63x immersion objective (numerical aperture = 1.32). Illumination was provided by an Argon ion laser filter. A TRITC wide filter ($\lambda_{exc} = 543$ nm) and a Cy3 filter ($\lambda_{exc} = 520$ nm) were used to visualize ER$\alpha$-like and ER$\beta$-like immunoreactivity. Neutral density filters and reduced laser powers were utilised to reduce photobleaching nearly to zero. To focus hemocytes, samples were viewed under reduced transmitted light illumination; then, in confocal fluorescence mode, 10 video frames, coming from 500 nm adjacent sections, were averaged. The confocal images (1024 x 1024 bits resolution) were viewed on a high resolution monitor and saved to disk.

**Electrophoresis and Western blotting**

Levels of phosphorylated PKC and CREB in whole cell extracts from hemocyte monolayers were determined using phosphospecific antibodies as previously described (9, 11, 14). Supernatants from each culture dish were discarded and each hemocyte monolayer was lysed with 1 ml of ice-cold lysis buffer [50 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 1% (w/v) SDS (sodium dodecyl sulfate), 1 $\mu$g ml$^{-1}$ pepstatine, 10 $\mu$g ml$^{-1}$ leupeptine, 2 mM sodium orthovanadate, 10 mM NaF, 5 mM EDTA, 5 mM NEM (N-ethylmaleimide), 40 $\mu$g ml$^{-1}$ PMSF (phenylmethylsulphonyl fluoride), 0.1% Nonidet-P40] and sonicated for 45 sec at 50 W. Samples were boiled for 4 min and then centrifuged for 10 min at 14,000 x g to remove insoluble debris. Supernatants were mixed 1:1 (v:v) with sample buffer (0.5 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 4% 2-mercaptoethanol, 0.05% Bromophenol blue) and samples (normalised for protein content before loading to 30 $\mu$g of protein) were resolved by 10% (for PKC) or by 12% for CREB SDS-polyacrylamide gel electrophoresis (27). Pre-stained molecular-mass markers were run on adjacent lanes. The gels were electroblotted and stained with Coomassie Blue (53). Blots were probed with human recombinant specific anti-
phospho-PKC(pan) (1:1000), anti-phospho-PKC(α/βII) (1:1000) or anti-phospho-CREB (1:1000), as primary antibodies, and horseradish-peroxidase-conjugated goat anti-rabbit IgG (1:3000) as secondary antibodies. Nitrocellulose membranes were stripped for 30 min at 50°C with stripping buffer (62.5 mM Tris-HCl, pH 6.7 containing 10 mM betamercaptoethanol and 2% SDS) and reprobed with anti-actin antibodies (1:1000) as loading controls (9,14) or with anti-CREB (1:1000) (11). Immune complexes were visualized using an enhanced chemiluminescence Western Blotting analysis system (Amersham- Pharmacia Corp.) following the manufacturer’s specifications. Western blots films were digitized (Chemidoc-Biorad) and band optical densities were quantified using a computerized imaging system (QuantityOne). Relative optical densities (arbitrary units) were normalized for the control band in each series.

In vivo $E_2$ exposure experiments

Mussels were injected with different concentrations of $E_2$: 50 µl of $E_2$ solutions 0.1, 0.5 and 2 µM, containing, respectively 5, 25 and 100 pmoles of $E_2$ (from a 10 mM stock solution in ethanol diluted in ASW) were injected into the posterior adductor muscle of groups of 10-12 mussels using a sterile 0.1 ml syringe as previously described (11). This range of concentrations was chosen taking into account that the average volume of hemolymph that can be withdrawn from mussels of this size (4-5 cm) is 0.6-1 ml. Experiments were repeated three times. For each experiment, a parallel set of control mussels were injected with 50 µl of a solution of ASW containing an equal amount of ethanol (≤0.005%). Mussels were then placed in plastic tanks containing ASW at 16 °C (0.5 l/mussel). At different times after injection (6 and 24 hrs), hemolymph was withdrawn using a sterile syringe and hemocytes were utilised for determination of lysosomal membrane stability, phagocytosis, and hydrolytic enzyme release. Lysosomal enzyme release was evaluated by measuring lysozyme activity in the extracellular medium (12) following (18) and data were expressed as lysozyme equivalents/mg protein/ml using Hen egg-white (HEW) lysozyme as a standard.
Data analysis

The results are the mean of at least 3 experiments in triplicate ± SD. Data from densitometric analyses of Western blots are the mean ± SD of 3 independent experiments. Statistical analysis was performed by using the Mann-Whitney U test with significance at P ≤ 0.05.

Chemicals

All reagents were of analytical grade. E$_2$ was from Sigma (St. Louis, MO). Rabbit polyclonal anti-phospho PKC (pan) (ser660), anti-phospho PKCα/βII (Thr638/641), anti-phospho-CREB (Ser133), and anti-CREB were obtained from New England Biolabs Inc. (Beverly, USA). Anti ERα (H-184) and anti-ERβ (N-19) antibodies were from Santa Cruz Biotechnology (CA). Anti-β-actin antibodies (rabbit polyclonal) were from Sigma. Prestained LMW and HMW molecular mass markers were from Biorad (CA). All other reagents were purchased from Sigma (St. Louis, MO).
RESULTS

Effects of E₂ on hemocyte functional parameters

The effects of E₂ on hemocyte phagocytosis are reported in Fig. 1. A significant stimulation was observed at 5 and 25 nM E₂ (+26 and +37% respectively; P≤0.05), whereas higher concentrations of E₂ (50 nM) inhibited the phagocytic process (-32%; P≤0.05) (Fig. 1A). E₂-stimulated phagocytosis was prevented by cell pre-treatment with the antiestrogen Tamoxifen and with the specific p38 MAPK inhibitor SB302580; a similar effect was observed with the PKC inhibitor GF109203X (Fig. 1B). At the concentration tested, neither inhibitor showed cytotoxic effects in mussel hemocytes or affected lysosomal membrane stability (10, 15) and phagocytosis (4) in control cells.

The effects of E₂ on oxyradical production by mussel hemocytes are reported in Fig. 2. As shown in Fig. 2A, E₂ (25 nM), induced a rapid and time-dependent increase in extracellular oxyradical production, evaluated spectrophotometrically as cytochrome c reduction, reaching a maximal 2-fold increase with respect to controls at 60 min (P≤0.05); the effect of E₂ was prevented by hemocyte pre-treatment with Tamoxifen. A significant increase was also observed with lower E₂ concentrations (5 nM) (+50%; P≤0.05). As shown in Fig. 2B, addition of the O₂⁻ scavenger SOD, that significantly decreased the basal level of oxyradical production in control hemocytes, did not affect E₂-induced cyt c reduction; on the other hand, the NOS inhibitor L-NMMA prevented the effect of E₂. Similar results were obtained with both SB302580 and GF109203X (Fig. 2B).

E₂ (25 nM) induced a two-fold increase in intracellular oxyradical production, evaluated by the NBT reduction assay, with respect to controls (P≤0.05) (Fig. 2C). The effect was abolished by both SOD and L-NMMA. Moreover, E₂-induced NBT reduction was prevented by Tamoxifen, as well as by SB302580 and GF109203X.

NO production in hemocyte supernatants was evaluated by the Griess reaction, that measures the concentration of the stable nitrite product. In control hemocytes, little nitrite production was observed over a period of 0-6 hrs; addition of E₂ (25 nM) induced a significant increase in the
concentration of nitrite from 30 min to 4 hrs (not shown); although a large variability in the time course of E2-induced NO production was observed among different hemocyte pools, in all experiments maximal nitrite concentration was observed at 4 hrs and the results are reported in Fig. 2D. The effect was prevented by cell pre-treatment with L-NMMA, as well as with Tamoxifen and with p38 MAPK and PKC inhibitors. Higher concentrations of E2 (50 nM) did not affect oxyradical production (data not shown).

We have previously shown that E2–induced destabilisation of lysosomal membranes in mussel hemocytes was mediated by p38 MAPK activation (12). As shown in Fig. 3, the effect of E2 on lysosomal membrane stability was prevented by cell-pre-treatment with the PKC inhibitor GF109203X.

**Immunofluorescence microscopy of ER-like receptors**

Intracellular localisation of ER-like receptors in hemocyte monolayers was investigated by immunofluorescence labelling and confocal laser scanning microscopy (CLSM), utilising both anti-ERα and anti-ERβ antibodies and the results are shown in Fig. 4. Prominent ERα immunoreactivity was observed in the nuclei; cytoplasmic staining was also observed, except for large intracellular vacuoles (Fig. 4A). ERβ immunoreactivity was extranuclear; a strong signal was observed in the perinuclear region, and punctate cytoplasmic staining indicated association with small intracellular organelles (Fig. 4B).

**Effect on PKC phosphorylation**

The effect of E2 on hemocyte PKC phosphorylation was first evaluated by Western blotting with anti-phospho-PKC(pan) antibodies as previously described (9, 14) and the results are shown in Fig. 5. The antibody, that detects α, βI, βII, δ, ε and η isoforms of mammalian PKC only when phosphorylated at a C-terminal residue homologous to ser660 of human PKC βII, recognizes two phosphorylated protein bands of approximately 70 and 75 kDa, respectively, in mussel hemocytes
like in protein extracts from mammalian cells (9). As shown in Fig. 5A, E$_2$ (25 nM) induced a rapid and transient increase in phosphorylation of the 75 kDa protein band; densitometric band analysis (Inset) revealed that the effect was maximal at 5 min (about 5-fold with respect to controls; P≤0.05).

On the other hand, phosphorylation of the 70 kDa protein band was decreased. The 75 kD PKC isoform was previously shown to correspond to PKC$\alpha$ and $\beta$ isoforms, as evaluated with a specific anti-phospho-PKC$\alpha/\beta$I antibody, directed towards phosphorylated Thr638/641 residues (14). As shown in Fig. 5B, E$_2$ induced an increase in phosphorylation of the PKC$\alpha/\beta$I protein band (Inset: up to 3-fold with respect to controls at 5 min; P≤0.05). An anti-actin blot is shown as loading control (Fig. 5C). As previously demonstrated (9), no significant changes in PKC phosphorylation were observed in control hemocytes (not shown).

**Effect on CREB phosphorylation**

The effect of E$_2$ on the phosphorylation state of CREB was evaluated as previously described (11) using specific antibodies directed towards the phosphorylated Ser133 of CREB, and the results are reported in Fig. 6. E$_2$ induced a rapid increase in the phosphorylation state of CREB with respect to controls (Fig. 6A), whereas the level of total, unphosphorylated CREB did not change (Fig. 6B). Densitometric band analysis (Inset) revealed that the effect was maximal at 5 min (4-fold with respect to controls; P≤0.05).

**In vivo effects of E$_2$ on hemocyte function**

The possible *in vivo* effects of exogenous E$_2$ on hemocyte function were investigated in mussels injected with different amounts of E$_2$. Hemocyte parameters were evaluated in cells collected from mussels injected with 5, 25 and 100 pmoles of the hormone after 6 and 24 hrs, and the results were compared to those obtained in hemocytes from vehicle-injected mussels (Fig. 7). E$_2$ treatment induced a significant and concentration-dependent decrease in lysosomal membrane stability at both times of exposure (Fig. 7A). A particularly large lysosomal membrane destabilisation (≥50%) was
observed with 25 and 100 pmoles E₂ (nominal concentration in the hemolymph). With regards to the phagocytic activity, a small but significant increase was observed only after 6 hrs with the lowest E₂ concentration (+16% with respect to controls, P≤0.05) (Fig. 7B). On the other hand, higher concentrations inhibited phagocytosis; with 100 pmoles the effect was observed after only 6 hrs (-23% with respect to controls, P≤0.05) and with both 25 and 100 pmoles after 24 hrs (-17 and -37%, respectively; P≤0.05).

E₂ also stimulated extracellular enzyme release, evaluated as lysozyme activity in the extracellular medium (Fig. 7C). The lowest concentration tested induced a large increase in lysozyme release at 24 hrs post-injection (+231% with respect to controls, P≤0.05); higher concentrations induced smaller but significant increases at both 6 and 24 hrs (+70 and +31% at 25 pmoles E₂ and +35 and +23% at 100 pmoles, respectively; P≤0.05).
DISCUSSION

The results demonstrate that E$_2$ in vitro can rapidly affect both phagocytosis and oxyradical production in *Mytilus* hemocytes; the effects were prevented by the antiestrogen Tamoxifen. The results confirm and expand previous observations indicating that E$_2$, in the same experimental conditions, induced Tamoxifen-sensitive lysosomal membrane destabilization, morphological changes, degranulation of hydrolytic enzymes, and stimulation of bactericidal activity (12).

E$_2$ significantly stimulated the phagocytic process in a narrow concentration range (5-25 nM), whereas higher concentrations (50 nM) were inhibitory. Along with increased phagocytosis, stimulation of the oxidative burst was observed, as indicated by measurement of extracellular and intracellular oxyradical production utilising standard methods for bivalve hemocytes (54). The extent of stimulation was similar to that observed following challenge of *Mytilus* hemocytes with PMA (1, this work) or laminarin (1). Addition of SOD prevented only E$_2$-stimulated intracellular oxyradical production, this indicating an increase in superoxide (O$_2^-$) generation associated with increased phagocytic activity. Moreover, both extra- and intracellular oxyradical production were prevented by addition of the NOS (nitric oxide synthase) inhibitor L-LMMA, that prevents NO synthesis in molluscan hemocytes (44). A similar effect was induced by L-NAME (data not shown). NO production was confirmed by the significant increase in nitrite concentration, with a maximum after 4 hrs of E$_2$ addition. In the same experimental conditions, a similar, although smaller effect was induced by PMA (50 µg/ml). The effect was prevented by Tamoxifen and L-NMMA, suggesting that in *Mytilus* hemocytes E$_2$ induces NO synthesis through a receptor-mediated event, as previously demonstrated in ganglionic tissue (46). Since in bivalve molluscs phagocytosis and oxyradical production through the action of NADPH oxidase and NOS represent important non-specific defense mechanisms (1, 23, 35, 44, 50), the results demonstrate that E$_2$ can act as an immunostimulant on mussel hemocytes. In invertebrates, NO can be produced both rapidly and after a latent period through constitutive (cNOS) and inducible (iNOS) NO synthase isoforms, with cNOS modulating basal NO actions that are further enhanced by iNOS-derived NO (44).
considering that the nitrite determination usually misses constitutive cNOS-derived NO release and the observed time course of NO production observed in response to E₂, our data support the hypothesis that rapid E₂ signaling subsequently leads to increased NO production probably through disinhibition of a iNOS form in the hemocyte. (44).

In vertebrate phagocytes, the oxidative burst is mediated by several intracellular pathways, including MAPKs, Ca²⁺-dependent and Ca²⁺-independent PKC isoforms, leading to NADPH oxidase activation (52). Both E₂-stimulated phagocytosis and oxyradical production (including NO) were prevented by cell pre-treatment with SB302580, confirming that, as previously demonstrated, the stress-activated p38 MAPK represents a target for the action of E₂ in these cells (12). In particular, both 5 and 25 nM E₂ induced a rapid (from 5 min) and transient increase in the level of phosphorylated p38 (12). The p38 MAPK inhibitor prevented the effect of E₂ on lysosomal membrane stability (12), a sensitive parameter of in vitro exposure to E₂ in Mytilus cells (6, 32). In mussel hemocytes, the effects of E₂ on all the functional parameters tested, including lysosomal membrane stability, were prevented by GF109203X and in fact PKC, and in particular a PKCα/βII-like isoform (14), were rapidly and transiently phosphorylated in response to E₂. In the same conditions, E₂ induced a small but significant increase in cytosolic [Ca²⁺], indicating that [Ca²⁺]-mediated cell signaling may participate in activation of PKC-like classical isoforms (12). PKC is involved in rapid estrogen signalling through both ER-dependent and ER-independent mechanisms (19, 50). Since activation of p38 MAPK and PKC has been shown to play a key role in the response of Mytilus hemocytes to bacterial challenge (9, 10), overall the results demonstrate that estrogen signaling through both p38 MAPK and PKC is present in invertebrate cells and participates in immunostimulation.

E₂ also induced a transient increase in the phosphorylation of a CREB-like protein previously identified in mussel hemocytes (11). CREB (cAMP Responsive Element Binding Protein), a transcription factor whose activation is mediated by serine/threonine kinases, is involved in the cross-talk between estrogen, estrogen receptors and cytosolic kinase cascades, including MAPKs
CREB-like proteins have been identified in the nervous system of gastropod molluscs, where they play a role in neuronal plasticity and learning (42). In mussel hemocytes, in vivo exposure to the xenoestrogen Bisphenol A significantly decreased phosphorylation of CREB at Ser133 (11); on the other hand, large CREB phosphorylation is rapidly induced by bacterial challenge with *E. coli* (Canesi, unpublished results). The results here reported demonstrate that E₂ signaling in mussel hemocytes involves CREB-like proteins like in mammalian cells.

Overall, the results further support the hypothesis that rapid activation of kinase pathways represents a significant mode of action by the natural estrogen 17β-estradiol in *Mytilus* hemocytes in vitro. In *Mytilus* ganglia, the effects of the membrane-impermeant E₂-BSA conjugate were similar to those of free E₂, suggesting an E₂-mediated signaling pathway at the cell surface coupled to NO production (46). In hemocytes, E₂-BSA, like E₂, induced lysosomal destabilisation and MAPK and STAT activation; however, part of the E₂-BSA was rapidly internalised by the cells and therefore seemed unsuitable to demonstrate the extracellular action of the hormone (12). E₂-BSA stimulated the oxidative burst but it induced a slight decrease (10-15%), rather than an increase in phagocytosis of zymosan particles (data not shown).

The results obtained with the antiestrogen Tamoxifen indicate a possible role for ER-like receptors in mediating the rapid effects of E₂. We previously demonstrated the presence of immunoreactive ERα- and ERβ-like proteins of approximate MW of 70 and 49 kDa, respectively, by electrophoresis and WB of soluble hemocyte protein extracts utilising antibodies directed against a specific region in the amino-terminal domain of the receptor forms ERα and ERβ, respectively, which is the highly divergent domain between the two mammalian forms (12). The results of confocal microscopy utilising the same antibodies confirm the presence of ER-like proteins in control hemocytes. ERα immunoreactivity showed a prominent nuclear localization, and a diffuse signal in the cytoplasm; on the other hand, ERβ-like proteins were observed at extranuclear sites, with most cells showing a strong signal in the perinuclear region. A distinct immunostaining was also observed in cytoplasmic organelles: large vacuoles showed a characteristic absence of immunoreactivity to ERα antibodies,
whereas punctate ERβ immunostaining indicated association with smaller vacuoles. In *Mytilus* spp., granular hemocytes, the dominant cell type in the hemolymph, are characterised by a low nucleus/cytoplasm ratio, high phagocytic activity and capacity for oxyradical production (54). The distinct association of the two ER-like proteins with intracellular granules may reflect the heterogeneity of these organelles in terms of enzyme composition (lysosomal hydrolytic enzymes and oxidases) (16, 37) and the possible role of lysosomal compartments in ER degradation (39). E2-induced localisation of ER in perinuclear lysosomes was first demonstrated by Szego (49) and by other Authors (39, and references quoted therein), supporting a role for lysosomes in nucleocytoplasmic communication in the response to estradiol. Moreover, recent studies indicated that in different cell types perinuclear localization of ERβ isoforms was due to association of the ER with the mitochondria (7, and references quoted therein). Our results, although preliminary, suggest that in mussel hemocytes ER localization in lysosomal compartments is highly likely; however, experiments are in progress in order to identify the nature of ER-positive vacuoles utilising lysosomal and mitochondrial markers in control and E2-treated hemocytes.

The possibility that E2 may affect the hemocyte function also *in vivo*, at longer exposure times, was investigated in mussels injected with different concentrations of E2. In hemocytes from control and E2-injected mussels functional parameters that have been shown to be affected by E2 *in vitro*, i.e. lysosomal membrane stability, extracellular lysozyme release and phagocytosis (12, this work), were evaluated. The results show that in mussels injected with E2 a concentration-dependent decrease in lysosomal membrane stability was observed with respect to controls at both 6 and 24 hrs post-injection. These results demonstrate that also *in vivo* the lysosomal function represents a sensitive target for the action of the hormone in mussel cells as previously shown *in vitro* (6, 12, 32). The lowest concentration tested (5 pmoles) induced a large increase in extracellular release of lysosomal hydrolytic enzymes, comparable to that induced by *E. coli* (38). The smaller effect observed at higher concentrations may be due to unspecific degranulation associated to cell damage, as indicated by the large decrease in lysosomal membrane stability observed in these conditions.
Similarly, the lowest concentration of $E_2$ stimulated phagocytosis only at 6 hrs post-injection, whereas at longer exposure times, and with higher concentrations, a decrease in phagocytosis was observed.

The effects and mechanisms of action of $E_2$ at longer incubation times \textit{in vivo} may be more complex than those involved in the rapid effects on cell signalling observed \textit{in vitro}: they may be mediated by both membrane and intracellular signaling pathways, involving or not ER-like receptors, or reflect the integrations of actions at different receptor pools like in mammalian cells (29). In addition to the direct effect on the hemocytes, exposure to $E_2$ can also affect the activity of steroid metabolising enzymes in other mussel tissues (gonad and digestive gland) that metabolise exogenous $E_2$ to an esterified form (25), thereby affecting the concentration of free $E_2$ in the hemolymph; $E_2$ exposure may also affect the levels of endogenous modulators that have been shown to affect the immune function in bivalves (4, 26, 47).

Steroid concentrations reported in the literature in bivalve tissues widely differed (from pg to ng) depending on the species, tissue, sexual maturation stage and on the technique utilised (21). The only available data on estrogen content in the hemolymph are those reported by the group of George Stefano in \textit{Mytilus edulis} (46, 55). Although the concentration of free $E_2$ was not evaluated in the present study at different times post-injection, preliminary data, obtained utilising a commercial competitive chemiluminescent enzyme immunoassay kit (Immulite 2000 Estradiol), indicate that a only a small fraction of the injected $E_2$ was retained in hemolymph serum (Canesi L., unpublished observations). Further research utilising $E_2$ dissolved in sea water and measurements of $E_2$ isoforms in the hemolymph with appropriate analytical techniques are needed to clarify the effects of exogenous $E_2$ on mussel immune function \textit{in vivo}.

In mammalian immunocytes, estrogens induce both dose- and cell-type specific responses, thus resulting in pro-inflammatory or anti-inflammatory effects (2, 5, 22, 24, 43, 45). Overall, the results indicate that in \textit{Mytilus} hemolymph changes in the concentrations of $E_2$ in a narrow range may elicit immunostimulation through activation of the signaling pathways involved in the immune response.
Higher concentrations can downregulate the hemocyte function probably through impairment of the mechanism involved in controlled membrane fusion events during the phagocytic process leading to lysosomal membrane destabilisation (this work, 6), lysosomal enlargement and increased protein degradation (6). In bivalve immunocytes, the lysosomal vacuolar system, that plays a key role in different aspects of the immune function, seems to represent a significant target for the action of E2. Synthetic estrogens, such as 17α-ethynylestradiol, diethylstilbestrol and mestranol showed similar effects, although at much higher concentrations (15; Canesi L., unpublished data). In mussels, the effects of the natural estrogen 17β-estradiol on the immune function may be of relevance in physiological conditions. Overall, our data demonstrate that in bivalve molluscs 17β-estradiol can modulate the immune function both in vitro and in vivo and support the hypothesis that the role of estrogens in non reproductive functions is conserved in evolution.
REFERENCES


44. **Stefano GB, and Ottaviani E.** The biochemical substrate of nitric oxide signaling is present in primitive non-cognitive organisms. *Brain Res* 924: 82-89. 2002.


Figure Legends

Fig. 1 - Effects of 17β-estradiol (E$_2$) on phagocytosis of NR-conjugated zymosan particles. A) Effects of different concentrations of E$_2$ (5-50 nM); B) Effects of E$_2$ (25 nM) on hemocytes pre-incubated with the antiestrogen Tamoxifen (100 nM) (TAM/E$_2$), or the specific kinase inhibitors SB203580 (20 µM) (SB/E$_2$) and GF109203X (2.5 µM) (GF/E$_2$) (see methods). C= control. The effect of TNF-alpha (200 nM) is also shown as positive control. Data are the mean±SD of four experiments in triplicate. * = P≤0.05.

Fig. 2 - Effect of E$_2$ on oxyradical and nitrite production by mussel hemocytes.
A,B: Extracellular oxyradical production, evaluated as cyt c reduction as described in Methods. The effect of PMA (10 µg/ml, 60 min) is shown as a positive control.
A) Time course of cyt c reduction in control hemocytes and in hemocytes added with different concentrations of E$_2$ (E) and effect of Tamoxifen (Tam/E);
B) Effects of cell pre-treatment with different inhibitors on the cyt c reduction in control and E$_2$-treated hemocytes (25 nM) at 60 min. Control (C ); E= E$_2$; SB=SB203580, GF=GF109203X. SOD (300 mU/ml) and L-NMMA (1 mM) were utilised as described in Methods. C: Intracellular oxyradical production, evaluated as NBT reduction as described in Methods, in hemocytes incubated with E$_2$ (25 nM, 60 min) in the absence and in the presence of different inhibitors.
D: NO production, evaluated as nitrite concentration by the Griess reaction as described in Methods, in control hemocytes and hemocytes incubated with E$_2$ (25 nM, 4 hrs). The effect of E$_2$ on NO production was also evaluated in the presence of different inhibitors. NO production induced by PMA (50 µg/ml; 4 hrs) is shown as a positive control.

Data are the mean±SD of three experiments in triplicate. E$_2$ or PMA vs C : * = P≤0.05; inhibitors/E$_2$ vs E$_2$ alone: ° = P≤0.05.
Fig. 3 – Effect of hemocyte pretreatment with the PKC inhibitor GF109203X (20 min, 2.5 µM) on the lysosomal membrane destabilisation induced by 25 nM E$_2$. C = control. Lysosomal membrane destabilisation induced by hemocyte incubation with E. coli is shown as a positive control. Data are the mean±SD of four experiments in triplicate. * = P≤0.05.

Fig. 4 – Confocal laser scanning microscopic images of ER$\alpha$ (A) and ER$\beta$ (B) immunoreactivity in mussel hemocytes. Note that ER$\alpha$-like immunoreactivity is present not only in the nucleus, but also in the cytoplasm, except for large intracellular vacuoles. ER$\beta$-like immunoreactivity is mainly localized in the perinuclear region and in the cytoplasm, where it appears to be associated with small vacuoles. Scale bar = 10 µM.

Fig. 5 – Effect of incubation of mussel hemocytes with E$_2$ (25 nM) for different periods of time (5-60 min) on PKC phosphorylation. Protein extracts from control and E$_2$-treated hemocytes were subjected to 10% SDS-PAGE followed by Western blotting using polyclonal phosphospecific antibodies to anti-phospho-PKC(pan) (A) and anti-phospho-PKC$\alpha$/$\beta$II antibodies (B). An anti-actin blots is shown as a loading control (C). Bands were detected using enhanced chemioluminescence reagents (see Methods). Results are representative of three independent experiments. C = control. Insert: densitometric analysis of blots from three independent experiments (mean±SD). * = P≤0.05. Relative increases in band optical densities (arbitrary units) were normalised for the control band in each series.

Fig. 6 – Effect of incubation of mussel hemocytes with E$_2$ (25 nM) for different periods of time (5-60 min) on CREB phosphorylation. Protein extracts from control and E$_2$-treated hemocytes were subjected to 12% SDS-PAGE followed by Western blotting using polyclonal phosphospecific antibodies to CREB (p-CREB) (A), stripped and reprobed with antibodies directed towards the
unphosphorylated form (CREB) (B). Bands were detected using enhanced chemiluminescence reagents (see Methods). Results are representative of three independent experiments. C = control. 

Inset: densitometric analysis of blots from three independent experiments (mean±SD). * = P≤0.05. Relative increases in band optical densities (arbitrary units) were normalised for the control band in each series.

Fig. 7 – Effects of in vivo exposure to different concentrations of E₂ on hemocyte functional parameters: A) Lysosomal membrane stability, B) phagocytosis, C) hydrolytic enzyme release. Mussels were injected with different concentrations of E₂ (5, 25, 100 pmoles) or vehicle and hemocytes collected after 6 and 24 hrs post-injection were analysed as described in Methods. Data are the mean±SD of three experiments in triplicate. * = P≤0.05.
Fig. 1A

Phagocytosis (%) vs. E2 [nM]

Fig. 1B

Phagocytosis (%) by Treatment

- C
- E2
- Tam/E2
- SB/E2
- GF/E2
- TNF-alpha

* indicates statistical significance.
Fig. 3

NR Retention Time (min)

Treatment

C E2 GF/E2 E. coli

*
Fig. 5

E₂

C 5' 15' 30' 60'

p-PKCpan

p-PKC α/βII

Actin

A

B

C

Fold changes vs Controls

Time (min)

p-PKC(pan) 75 kD

p-PKC(pan) 70 kD

* *

Fold changes vs controls

Time (min)

p-PKC α/βII

* *
Fig 6

E₂

A  p-CREB

B  CREBtot

**  *  *  *

Fold changes vs controls

Time (min)
Fig. 7A

NRR Time (min)

- ○ 6 hrs
- ● 24 hrs

E₂ (nM)

Fig. 7B

Phagocytosis (%)

- ○ 6 hrs
- ● 24 hrs

E₂ (nM)

Fig. 7C

Lysozyme activity (%)

- ○ 6 hrs
- ● 24 hrs

E₂ (nM)