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# Regulation of the lipopolysaccharide signal transduction pathway by 17β-estradiol in macrophage cells

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**Abstract**

We have previously shown that 17β-estradiol ($E_2$) prevents the activation of brain macrophages, i.e. microglia cells, both in vitro and in vivo. Hormone exerts this inhibitory effect by inhibiting pro-inflammatory gene expression. In this study we further investigated on the molecular mechanism of $E_2$ action in the RAW 264.7 macrophage cell line. We show here that these cells express the α-isoform of the estrogen receptor (ERα) and not ERβ. Similarly to its activity in brain macrophages, $E_2$ is able to inhibit the activation program induced by lipopolysaccharide (LPS) in RAW 264.7 cells, as shown by the inhibitory effect of hormone on the morphological conversion and matrix metalloproteinase-9 (MMP-9) expression induced by the endotoxin. In addition, we demonstrate that hormone treatment is not associated with a reduction in the steady-state expression of Toll-like receptor-4 (TLR-4) and CD14, two components of the LPS receptor complex. Our results further confirm the anti-inflammatory role of ERα in macrophages and propose that the mechanism of hormone action on macrophage reactivity involves signaling molecules which are down-stream effectors of the LPS membrane receptors.


*Keywords:* Estrogen receptor-alpha; RAW cells; Microglia; MMP-9; TLR-4; CD14; Expression

## 1. Introduction

Macrophages are patrolling cells of the innate immunity, involved in the recognition of foreign pathogens and tissue injury, in the elimination of toxic molecules and in the reconstitution of tissue integrity. Macrophage cells orchestrate these diverse pathways by producing several different mediators, such as nitric oxide (NO), cytokines, including interleukin 1β (IL-1β) and tumor necrosis factor-α (TNFα), and matrix degrading enzymes, such as metalloprotease-9 (MMP-9). The production of these molecules, although beneficial for killing bacteria and further activating the immune system, has been hypothesized to damage the surrounding tissue when chronically or errouneously stimulated, especially in the central nervous system (CNS). The resident macrophages of the CNS are microglia cells. Upon activation by bacterial invasion or by chemical or mechanical injuries, microglial cells undergo a series of morphological and biochemical modifications that lead to the activation of immuno-inflammatory response. The activation of microglial cells has been proposed to play a pathogenic role in CNS disease, like Alzheimer's (AD), multiple sclerosis, AIDS-associated dementia and post-traumatic lesions and may contribute to trigger neurodegeneration [1,2].

One of the most potent inflammatory agent is lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. It is known that LPS has multiple and different effects on macrophages, as it regulates cellular functions, such as cell motility or morphology, and stimulates the synthesis of inflammatory mediators and cytokines. During the past few years, great progress has been made in understanding the mechanisms of LPS-induced host responses. Genetic analysis revealed that Toll-like receptor-4 (TLR-4) is a critical signal transducer for LPS after its binding to CD14, a membrane anchored protein [3,4]. Multiple biochemical and genetic studies support the concept that CD14 facilitates LPS action by binding and retaining LPS on the cell surface, but does not participate directly in signaling [5]. The main function of CD14 is to catalyze the transfer of LPS from the extracellular space to the membrane and then transfer it to the TLR-4 complex. TLRs are transmembrane proteins consisting of

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multiple copies of leucine-rich repeats in the extracellular domain and a conserved Toll/interleukin-1 receptor (TIR) homology domain in the cytoplasmic tail [6]. Similarly to other TIR-containing receptors, such as IL-1R and IL-18R, TLRs utilize down-stream effectors, including myeloid differentiation protein 88 (MyD88), IL-1R-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF6) to activate both nuclear factor (NF)-κB and mitogen-activated protein kinase signalling cascades [7–9]. Expression of TLR-4 and CD14 is restricted to a small number of cell types, including myeloid cells (monocytes, macrophages, dendritic cells and granulocytes); TLR-4 expression has also been described in endothelial cells and B-cells [10]. Transcriptional regulation of CD14 and TLR-4 is certainly an interesting focus of inquiry for the understanding of LPS signaling, since both their basal level of transcription and its regulation in myeloid cells may influence responses to LPS.

The lypophilic estrogen molecule is able to diffuse through the plasma membrane and to interact with high affinity with specific endogenous proteins, the estrogen receptors (ERs); once activated by hormone binding, ERs regulate gene transcription in the nucleus and modulate the activity of cytoplasmic kinase complexes linked to diverse signaling pathways [11]. A number of studies have shown that brain macrophage function can be modulated by estrogens. In primary cultures of rat microglia estrogen blocks the production of several inflammatory signals, such as MMP-9, prostaglandin-$E_2$ and inducible nitric oxide synthase [12], while 17β-estradiol ($E_2$) attenuates superoxide release, phagocytic activity and cytokine production in diverse macrophage cells [13–16]. More recently, we showed that estrogen treatment results in a reduced inflammatory response in the brain [17], which is mediated by ERα and occurs by a blockade of pro-inflammatory gene transcription. The onset and progression of brain diseases associated with the activation of the inflammatory response, such as the experimental autoimmune encephalitis, the animal model of multiple sclerosis or ischemia, were shown to be negatively regulated by $E_2$ through the selective activation of ERα [18,19].

In this study, we investigated the expression and regulation of the α-isoform of ER in macrophage cells and assayed estrogen activity on LPS signaling events and molecular effectors. A better understanding of the specific and potent role of ERα in innate immunity may help finding novel pharmacological interventions in brain pathologies associated with a local inflammatory reaction.

## 2. Materials and methods

### 2.1. Animals

The study was conducted according to the guidelines of the Institutional Animal Care Committee.

### 2.2. Materials

Unless otherwise specified, chemicals were purchased from Merck (Darmstadt, Germany), culture media and additives from LifeTechnologies-Invitrogen (Paisley, Scotland, UK).

### 2.3. Cell culture

BV-2 cells were kindly provided by E. Blasi (Perugia, Italy) and RAW cells were purchased from ATCC (Manassas, USA). Under a humidified 5% $CO_2$/95% air atmosphere and at 37 °C, BV-2 cells were grown in DMEM-F12 medium + 10% fetal bovine serum (FBS). Media were supplemented also with 2 g/l sodium carbonate, 0.11 g/l sodium pyruvate, 5 ml/l of a 10,000 IU streptomycin and penicillin mix. Cells were splitted twice a week and plated in 10 $cm^2$ Petri dishes (Corning, Acton, MA, USA) at a density of $5 \times 10^5$ cells for BV-2 cell line and $10 \times 10^6$ cells/ml for RAW 264.7 cell line. For the experiments, cells were plated on 6-well dishes ($2 \times 10^6$ cells/well) in 10% FBS–DMEM. After 24 h, medium was removed and replaced with 10% dextran-coated charcoal (DCC)-treated FBS–DMEM without phenol red. The next day cells were incubated in serum-free medium for 4 h in the absence or presence of 10 nM $E_2$ or 100 nM ICI 182,780, an estrogen receptor antagonist (from Astrazeneca, London, UK); 0.5 μg/ml of *E. coli* LPS (isotype 0.111:B4, from Sigma) were added for 6 h to analyze gene transcription or for 16 h to assess cell morphological changes.

### 2.4. Microglial cell culture

Microglia were isolated from cultures of newborn rat brains, as previously described [17]. Briefly, cerebral cortices were isolated from 2 day old Sprague–Dawley rats (Charles River, Milan, Italy), stripped of the meninges minced in 7.5 ml Hanks' solution (Sigma, Milan, Italy) containing 10 mM Hepes buffer, dissociated by trituration in the presence of 1 mg/ml DNase (Sigma, Milan, Italy). Mixed glial cells were grown in MEM medium containing 0.6% glucose and supplemented with 20% fetal bovine serum (Euroclone, Celbio, Milan, Italy), 1% non-essential amino acids, 5 ml/l of a 10,000 IU streptomycin and penicillin mix, at 37 °C under a humidified 5% $CO_2$/95% air atmosphere, in 75 $cm^2$ flasks (Corning, New York, USA) at a density of $3 \times 10^6$ cells/flask. Cells were cultured for 8–10 days and medium was replaced every 3 days. Two days before the experiment, cells were harvested by gentle shaking the flasks at 37 °C for 90 min, cell suspension was centrifuged for 5 min at 800 × g and cell pellet was resuspended in 10% FBS–MEM at a density of $2 \times 10^5$ cells/ml. One ml of cell suspension was plated on 6-well dishes and grown for 24 h. Purity of microglial cells at this stage was about 95%. Cells were incubated for additional 24 h in DMEM + 10% DCC–FCS. On the day of the experiment, serum-free

DMEM was added to the cells for 4 h in the absence or presence of $E_2$, as indicated in each figure legend, followed by a further incubation of 16 h with or without LPS.

### 2.5. Morphologic evaluation

This analysis allowed to evaluate the reactivity of RAW cells towards LPS and $E_2$. Cell morphology was assessed on fixed cells counterstained with cresyl violet. Cells with small cell bodies emanating several thin, branched processes were scored as resting, while round-shaped cells bearing a compact aspect were scored as activated cells.

### 2.6. RT-PCR

#### 2.6.1. RNA preparation

Cells were harvested by centrifugation, washed twice in PBS and resuspended in Bio/RNA-X Cell™ (Bio/Gene, Kimbolton Cambs, UK). RNA was isolated according to the manufacturer instructions.

#### 2.6.2. cDNA preparation

One microgram RNA was denatured at 70 °C with 10 pmol oligo-$dT_{(12–18)}$ (Perkin-Elmer, Milan, Italy) in 15 μl final volume. Primers–RNA mixes were cooled at room temperature for 15 min, dNTPs (Pharmacia, Milan, Italy) and MuMLV reverse transcriptase (RT) (Promega, Milan, Italy) were added at 200 μM and 1 U/μl final concentration, respectively, in a final volume of 25 μl. The RT reaction was performed at 37 °C for 1 h, then the enzyme was inactivated at 75 °C for 5 min. Control reactions without addition of the RT enzyme were performed for each sample.

#### 2.6.3. PCR

One microliter cDNA was incubated with 400 nM dNTPs, 200 nM each primer and 2 U of DynaZyme DNA polymerase (Finezyme OY, Espoo, Finland) in 25 μl final volume. The following primers (MWG Biotech, Ebersberg, Germany) were used: for ER-α, primer α-1a (5′-GTGCCGGATATGGGAAAGGATG-3′) and primer α-1b (5′-GAAGAGTTTGTG TGCCTCAAAT-3′), resulting in 296 bp-long products; for MMP-9, primers 350a (5′-GGCACCATCATAACATCA-3′) and 625b (5′-GCCCAGCGACCACAACTC-3′), resulting in 293 bp products; for TLR-4, m534a (5′-TTGAAGACAAGGC-ATGGCATGG-3′) and m1041b (5′-TCTCCCAAGATC-AACCGATG-3′), resulting in 507 bp-long products; for CD14, m668a (5′-GATCTGTCTGACAACCCTGAGT-3′) and m935b (5′-GTGCTCCAGCCCAGTGAAAGA-3′), resulting in a 267 bp-long amplification product. The PCR reactions were performed as follows: for ERα, 95 °C for 5 min followed by 40 cycles at 92 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min. For the other genes, 95 °C for 30 s, then 30 cycles at 94 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min. PCR reactions were performed on a Perkin-Elmer Thermal Cycler 480.

### 2.7. ERα immunofluorescence and confocal laser scan microscopy

RAW cells were grown in 24-well plate on glass coverslips for 2 days, then fixed for 10 min in 4% paraformaldehyde in 0.1 M PBS (pH 7.5) at room temperature. Cells were washed three times with PBS and incubated for 30 min at room temperature with Blocking solution (10% goat serum, 1% BSA, 0.5% Tween-20 in PBS). Cells were incubated with 400 μl/glass coverslip of a PBS solution containing 1:500 dilution of the anti-human ERα monoclonal antibody (1D5, from Zymed Lab, San Francisco, CA, USA) and 1% goat serum, o/n at 4 °C. Cells were washed three times in PBS and incubated with the secondary antibody Alexa-fluor 488 goat-anti mouse form Molecular Probes (Leiden, The Netherlands) for 60 min at RT. After 3× 5 min washes in PBS, coverslips were mounted with a mixture 50% PBS + 50% glycerol. ERα immunofluorescence was imaged with a Radiance 2100 confocal laser scanning microscope (Bio-rad, Milan, Italy) based on a Eclipse TE2000-S Microscope (Nikon, Milan, Italy) and operating in the simultaneous acquisition mode. Images were taken at the magnification of 180× and converted to black and white using standard computer program.

## 3. Results

### 3.1. Estrogen receptor-alpha expression in macrophage-like cells

We have previously shown that ERα is expressed in primary cells of rat microglia [12], as well as in human circulating monocyte-derived macrophages and monoblastoid cells [20]. With the intention to better characterize the mechanism of action of estrogen in inflammatory cells, in the present study we assayed two cell lines of macrophagic origin, namely RAW 264.7 and BV-2 cells, for the presence of estrogen receptors. By means of RT-PCR we first assessed the expression of the ERα RNA and compared receptor expression levels with those obtained from primary microglia cells from newborn rats. As shown in Fig. 1A, RAW cells express ERα RNA at a similar level compared to microglia cells. On the other hand, the RT-PCR assay in BV-2 cells did not show any detectable band for ERα, suggesting that this receptor is either not present or it is expressed at undetectable levels by our RT-PCR assay (data not shown). The reason why BV-2 cells do not express ERα is unknown, although it could be ascribed to the transformed phenotype of these cells. Our results are consistent with a previous report showing ERα expression in RAW cell line [19].

### 3.2. ERα subcellular localization in RAW cells

Confocal microscopy allows to finely detect the localization of proteins inside the cell. We used this technique

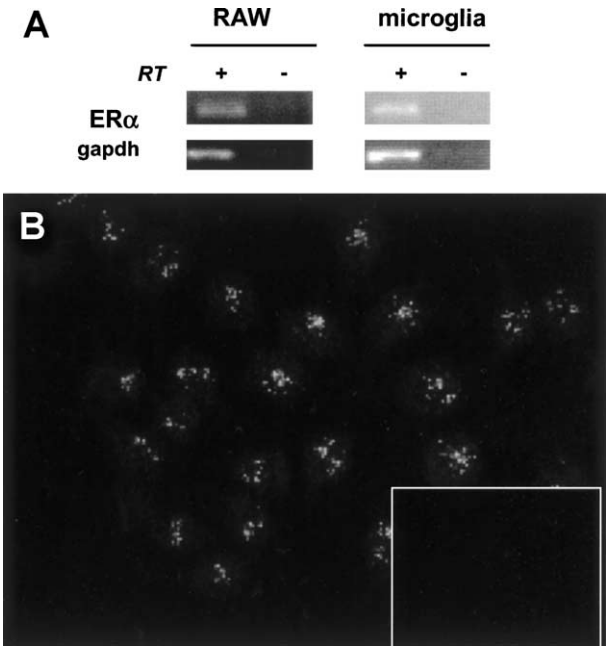


Fig. 1. ERα RNA expression in macrophage cells. (A) RAW cells and primary cultures of rat microglia were analyzed by RT-PCR for the presence of the mRNA encoding ERα. Cells were cultured in standard growth conditions; RNA was extracted and retrotranscribed using the reverse transcriptase enzyme (RT+); to exclude the presence of genomic DNA contaminations, reactions were also performed omitting the RT (RT−). (B) Confocal image of ERα protein detected in RAW cells by immunocytochemistry performed in the presence of $10^{-9}$ M 17β-estradiol added for 30 min, by using ERα specific 1D5 primary antibody. (Inset, B) Specificity of receptor immunoreactivity was confirmed by omitting the primary antibody. Image colors were converted to black and white using standard computer programs.

to evaluate ERα protein distribution in RAW cells. An antibody specifically directed against ERα reveals the expression of this receptor in RAW 264.7 cells (see Fig. 1B), which is homogeneously detected in the cell nucleus of about 99% cells. Specificity of receptor immunoreactivity was confirmed by omitting the primary antibody (inset in Fig. 1B). These results show that ERα protein is expressed in RAW cells, where it accumulates in the nuclear compartment.

### 3.3. Estrogen and RAW cell morphological activation

We then characterized the effect of $E_2$ on RAW cells reactivity induced by LPS. As shown in Fig. 2, LPS addition results in a modification of cell morphology, giving rise to cells roundly shaped and full of vacuoles. Interestingly, $E_2$ administration before the endotoxin strongly inhibits this morphological conversion, resulting in cells bearing branched processes and thin cell bodies, the typical appearance of a resting cell phenotype. Therefore, similarly to the effect of hormone in microglia cells, induction of a reactive phenotype is prevented by $E_2$ also in RAW cells.

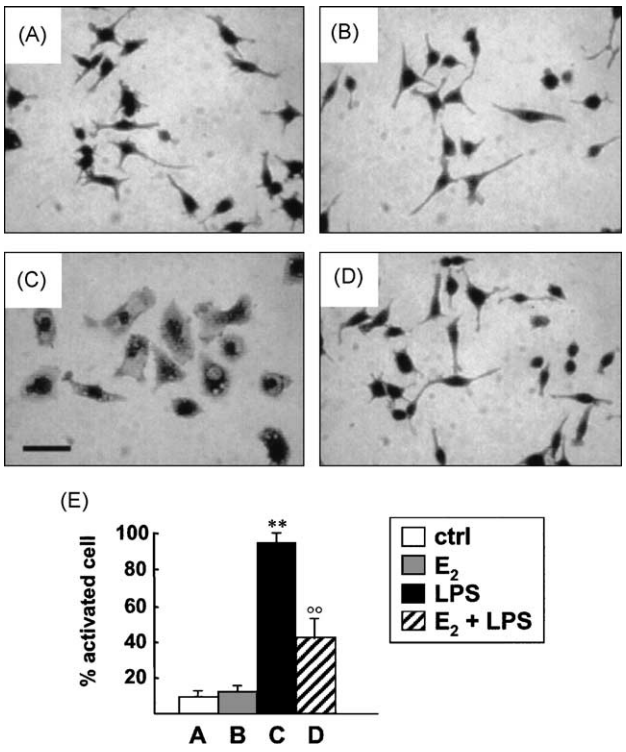


Fig. 2. Estrogen and RAW cells activation. (A–D) Immunocitochemistry assay for the evaluation of RAW cell morphology. Cells were grown in the absence (A) or presence of $10^{-9}$ M 17β-estradiol ($E_2$; B and D) for 4 h and then treated with 0.5 μg/ml LPS for 16 h (B and D). Cells were fixed in 4% paraformaldehyde and counterstained with cresyl violet. Photographs were taken with a digital photocamera and converted to black and white using conventional computer programs. Scale bar, 10 μm. (E) percentage of activated vs. total cells. Bars are from a single representative experiment performed in triplicate and repeated at least three times. Values represent the mean ± S.D. of at least 50 cells counted in each experimental point. $^{**}P < 0.01$ vs. control; $^{oo}P < 0.01$ vs. LPS.

### 3.4. Estrogen activity on LPS-induced MMP-9 expression in RAW cells

It is known that estrogen exerts its anti-inflammatory activity by modulating transcription of pro-inflammatory genes. We have previously shown that one of these genes is the matrix metalloproteinase-9, a proteolytic enzyme involved in tissue destruction and monocyte invasion. We therefore assayed MMP-9 gene expression induced by LPS in RAW cells in the absence or presence of $E_2$. As shown in Fig. 3, nanomolar concentrations of $E_2$ added 4 h before LPS result in 80% decrease of MMP-9 RNA levels induced by LPS. The use of a receptor antagonist in biological assays helps identifying the involvement of receptor molecules in response to selected ligands. We therefore utilized the ER antagonist ICI 182,780 to block ER activity and assayed hormone activity on MMP-9 expression. As shown in Fig. 3, addition of the ICI compound prevents the inhibitory activity of $E_2$ on MMP-9 induction. These results suggest that the endogenous ERα mediates the inhibitory activity of estrogen on LPS signaling.

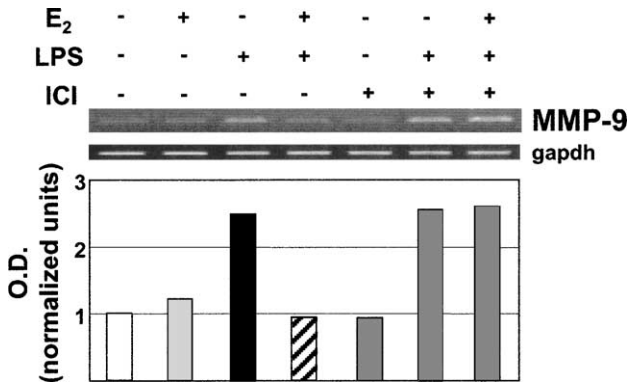


Fig. 3. Induction of MMP-9 expression is regulated by estrogen in RAW cells. Cells were treated for 4 h with $10^{-9}$ M 17β-estradiol ($E_2$) or with $10^{-7}$ M ICI 182,780 (ICI) and then with 0.5 μg/ml LPS for 6 h; total RNA was extracted and RT-PCR assay performed to amplify MMP-9 and gapdh RNAs. PCR amplification products were separated by electrophoresis on agarose gel and visualized by ethidium bromide staining. Bands were quantified by densitometry; the optical density (OD) of MMP-9 amplification products was normalized by the OD of gapdh bands. Graph is from a single experiment, representative of at least other three assays.

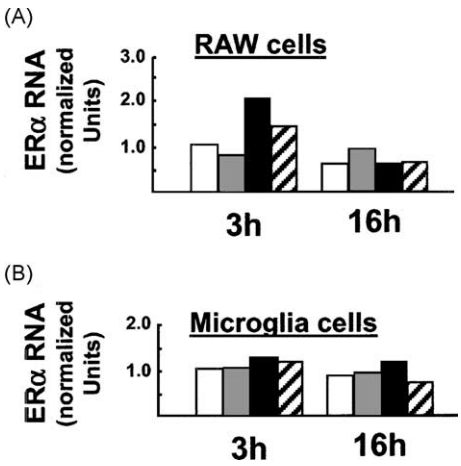


Fig. 4. ERα expression in the absence or presence of LPS and estrogen. (A) RAW cells or (B) primary cultures of microglia were either left untreated (open bars), treated with 10 nM 17β-estradiol ($E_2$; light gray bars) for 4 h, then for additional 6 or 16 h, as specified in each panel, with 0.5 μg/ml LPS (black bars) alone or with $E_2$ before LPS (dashed bars). Total RNA was extracted and RT-PCR assay performed to amplify ERα and gapdh RNAs. Amplification products were separated by electrophoresis on agarose gel and visualized by ethidium bromide staining. Bands were quantified using a densitometer; the optical density (OD) of ERα amplification products was normalized by the OD of gapdh bands. Graphs are from a single experiment, representative of at least other three assays.

Altogether, these results further confirm the anti-inflammatory activity of estrogen-activated ERα in macrophage cells and drive the exploitation of RAW 264.7 cells as a model system to recapitulate the mechanism of action of ERα in inflammation.

### *3.5. LPS activity on ERα expression in macrophage cells*

In order to exclude the possibility that the observed activity of $E_2$ could be ascribed to a modification in hormone receptor content, we evaluated the RNA levels for ERα after LPS and hormone treatments. As shown in Fig. 4, receptor mRNA levels remain similar after short (3 h) or long exposures (16 h) to hormone, LPS or the two molecules added sequentially. A 3 h treatment with LPS resulted in a two-fold increase in the ERα RNA level, which was counteracted by prior exposure to $E_2$; we believe that this slight increase does not lead to a significant increase in receptor content, since immunodetection assays for ERα did not show any modification in receptor levels (data not shown); in addition this effect is not observed in microglia, where hormone acts similarly with RAW 264.7 cells in terms of dosage and timing (data not shown). In summary, we conclude that LPS and hormone do not modify receptor expression in RAW 264.7 and microglial cells.

### *3.6. Estrogen activity on LPS signaling molecules in RAW 264.7 and microglial cells*

The Toll-like receptor-4 (TLR-4) and CD14 are key components in the signal transduction pathway for LPS [3]. Expression of these membrane-associated proteins has been shown to be under control of several extracellular signals, including LPS. We therefore asked whether the induction of TLR-4 and CD14 could be hindered by the hormone. We first addressed this issue in RAW cells and observed that $E_2$ alone did not modify the steady-state levels of TLR-4 and CD14 mRNAs (see Fig. 5). Addition of LPS to the medium resulted in an increase in both mRNAs, which was inhibited by 80% after $E_2$ pre-treatment. Therefore, hormone administration does not modify the steady-state levels of the LPS recognition molecules, seemingly leaving unaltered the early LPS-induced signaling processes in RAW cells. On the contrary, $E_2$ is able to limit the induction of TLR-4 and CD14 expression mediated by LPS. This latter mechanism may result in an inhibitory activity of $E_2$ on late events activated by LPS in this cellular system; however, it does not influence the early effects of LPS on MMP-9 transcription described above.

We then tested whether the negative effect of $E_2$ on TLR-4 and CD14 mRNA induction observed in RAW cells could be extended also to primary microglial cells. Similarly with RAW 264.7 cells, $E_2$ alone did not modify the steady-state levels of LPS receptors in microglia (Fig. 6). LPS induced an increase in the levels of TLR-4 and CD14 mRNAs, demonstrating that this bacterial endotoxin is able to control the expression of these proteins also in microglia cells. However, addition of $E_2$ did not prevent LPS action as observed in RAW 264.7 cells, suggesting that, in microglia, $E_2$ does not modify the LPS-induced expression of TLR-4 and CD14.

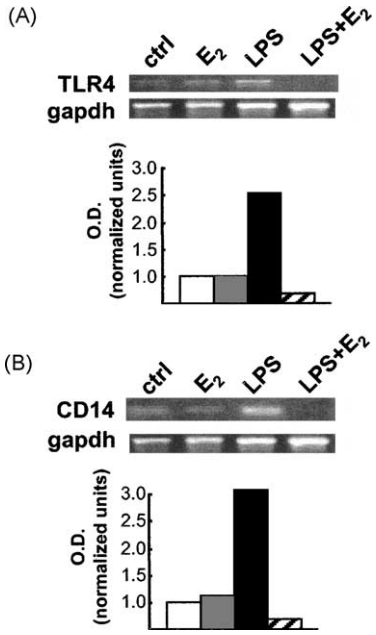


Fig. 5. LPS signal transduction pathway in RAW cells. Cells were either left untreated (open bars), treated with 10 nM 17β-estradiol ($E_2$; light gray bars) for 4 h, then for additional 6 h with 0.5 μg/ml LPS (black bars) alone or with $E_2$ before LPS (dashed bars). Total RNA was extracted and RT-PCR assay performed to amplify TLR-4 (A), CD14 (B) and gapdh RNAs. Amplification products were separated by electrophoresis on agarose gel and visualized by ethidium bromide staining. Bands were quantified by densitometry; the optical density (OD) of TLR-4 or CD14 amplification products was normalized by the OD of gapdh bands. Graphs are from a single experiment, representative of at least other three assays.

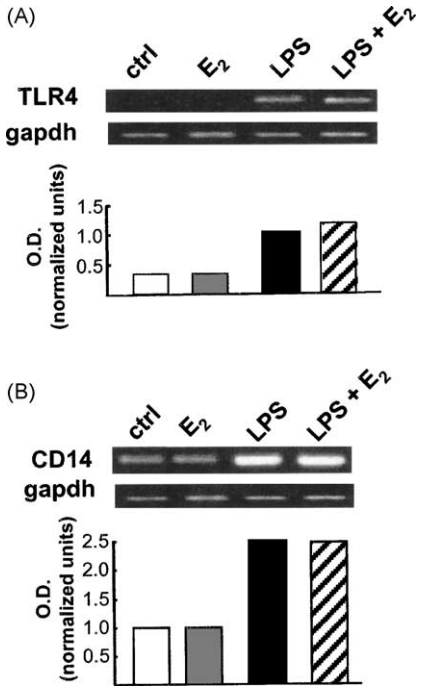


Fig. 6. LPS signaling in primary cultures of microglia from newborn rats. Cells were either left untreated (open bars), treated with 10 nM 17β-estradiol ($E_2$; light gray bars) for 4 h, then for additional 6 h with 0.5 μg/ml LPS (black bars) alone or with $E_2$ before LPS (dashed bars). Total RNA was extracted and RT-PCR assay performed to amplify TLR-4 (A), CD14 (B) and gapdh RNAs, as described in Fig. 5.

Altogether these results show that hormone does not alter the capability of macrophage cells to respond to LPS. We hypothesize that hormone inhibitory activity on LPS signaling is due to a modification in the activity of down-stream effectors of the LPS signaling molecules.

## 4. Discussion

As direct targets of pharmaceuticals, estrogen receptors represent a promising biological system for the discovery of potent, selective and specific drugs to control the evolution of several disorders [21]. Macrophage cell lines expressing endogenous estrogen receptors represent valuable tools to study hormone activity on inflammatory cells and to discern the mechanism of action of hormone and hormone-related drugs. We here showed that RAW 264.7 cells synthesize ERα to similar levels as compared with rat microglia cells and this receptor content is not modified by hormone or LPS treatments. In addition, we observed that the blockade of LPS-mediated cell activation by $E_2$ is similar to that observed in microglia, since both the morphological activation and the induction of MMP-9 transcription could be blocked by hormone addition. Treatment with a receptor antagonist reverted the anti-inflammatory effect of estrogen, suggesting that the endogenous receptor is the mediator of estrogen action and that pharmacological tools are able to influence inflammation. Therefore, RAW 264.7 cells represent a faithful cellular system to study the mechanism of action of estrogen in inflammation. In addition, this cellular system can be exploited for a pharmacological interest in the identification of estrogen drugs that mimic the effect of the endogenous hormone on the immune and nonimmune regulatory functions of macrophages in different tissues. This is in line with the recent evidence showing that novel physiological targets exist outside the hypothalamic–pituitary–ovary axis, the system where the action of sex hormones was believed to be restricted. The anti-inflammatory role of estrogen might have an important role in preserving the brain from the chronic activation of microglia cells. Reactive microglia has been observed in neurodegenerative diseases and it has been hypothesized to contribute to neuronal damage [2,22,23]. The negative role played by inflammation on neuron loss is substantiated by recent findings, showing that non-steroidal anti-inflammatory drugs are efficacious in reducing the incidence and progression of AD [24]. Since a large number of studies points to a potential beneficial role of estrogens in different brain pathologies, it is possible that the inhibitory activity of estrogen on the inflammatory response associated with several brain diseases might be involved in the beneficial role played by this hormone in tissue preservation [25].

In this report, we investigated on the activity of estrogen on the upstream effectors of the LPS signal transduction pathway in macrophage cells. Although regulation of LPS receptor expression is matter of intense studies, and estrogen is known to play a key role in the control of the immune system, hormone activity on LPS receptor expression has been barely explored. LPS, the principal component of Gram-negative bacteria, triggers innate immune responses in macrophages through the interaction with CD14, a glycosyl-phosphatidylinositol-anchored membrane protein [26], and TLR-4, the transducing subunit of the LPS receptor complex [3,27]. In our study, we demonstrate a basal expression of CD14 and TLR-4 both in RAW cells and microglia, as already expected by the presence of stimulated changes in these cells and by previous reports showing that CD14 and TLR-4 expression is localized to brain parenchymal microglia [28–30]. These steady-state levels were not altered by estrogen, showing that sensitivity towards LPS and the early events activated by the endotoxin are not modified by pre-treatment with hormone. We observed that both TLR-4 and CD14 RNAs are increased by LPS in RAW and microglia cells. This up-regulation of LPS receptors expression by LPS itself, already reported by other authors, has been ascribed to a compensatory mechanism, at least in microglia [31]. In fact, it has been recently described that LPS is internalized and transported to the Golgi apparatus, concomitantly with a reduction in TLR-4 surface protein [32,33]; therefore, up-regulation of transcript expression may represent a compensatory mechanism to partially counteract ligand-induced TLR-4 down-regulation. Our observation on the activity of LPS on CD14 RNA levels in macrophages is in agreement with previous reports [30], while the effect of LPS on TLR-4 expression is different from observations already published [34,35]. The reason of this discrepancy is not known; however, it is possible that cells grown in culture acquire a distinct responsiveness to LPS that results in a different regulatory mechanism of the signaling molecules involved in the innate immunity program. In addition, regulation of TLR-4 expression has already been the object of conflicting results. In fact, it has been repeatedly found that LPS up-regulates TLR-4 steady-state transcripts in human monocytes and neutrophiles [7,36]; on the other hand, LPS decreased TLR-4 levels in mouse peritoneal macrophages and in constitutively expressing parenchimal and non-parenchymal regions of the brain [30]. The main goal of our study was to assess, however, the effect of estrogen on CD14 and TLR-4 induction. We observed that hormone affected LPS-induced increase in CD14 and TLR-4 expression only in RAW cells, suggesting that, in this cellular system, the inhibitory activity of estrogen on late phases of the LPS response could possibly be also ascribed to a down-regulation of LPS receptors. This conclusion needs further confirmation and a better characterization of the receptor protein levels. Most importantly, our results point to the major conclusion that the prominent effect of hormone on LPS activity in macrophages involves inhibition of signaling molecules down-stream of the LPS membrane receptors. Extensive data in the literature described intracellular targets for estrogen action, including distinct families of transcription factors and kinases [11,37,38]. Interactions between the ER and effectors of membrane receptors-coupled signals might also exist in inflammatory cells. Future clarification of the molecular mechanism underlying estrogen action on LPS signaling cascade will shed more light on the biological relevance of the role played by estrogen and estrogenic drugs in inflammation, and will possibly provide hints for the exploitation of the beneficial effects of estrogen in several human inflammatory diseases.

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