Sex Differences in Estrogen Receptor α and β Levels and Activation Status in LPS-Stimulated Human Macrophages

Sex differences in estrogen receptor α and β levels and activation status in LPS-stimulated human macrophages

Sex-dependent LPS modulation of estrogen receptors

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Abstract

Immune function, inflammation and atherosclerosis display sex differences and are influenced by 17β-estradiol through estrogen receptors subtypes ERα and ERβ. Male tissues express active ERs, but their possible involvement in inflammation in males has never been assessed. Macrophages express both ERα and ERβ and offer the opportunity to evaluate the role of ER levels and activation in inflammation. We assessed the ability of lipopolysaccharide (LPS) to modulate, in a sex-specific way, the expression and the activation status of ERα and ERβ in blood monocytes-derived macrophages (MDMs) from men and women. MDMs were incubated with 100 ng / ml LPS for 24 h and used to evaluate ERα, ERβ, P- ERα, p38 and P-p38 expression by Western Blotting.

In basal conditions, ERα and ERβ were significantly higher in female MDMs than in male MDMs. LPS up-regulated ERα and ERα phosphorylation in both sexes, with a significantly higher effect observed in male MDMs, and down-regulated ERβ level only in female MDMs. p38 and P-p38 proteins, indicative of ERβ activity, did not show sex differences both in basal conditions and after LPS treatment. Finally, ERα / ERβ and P-ERα / ERα ratios were significantly higher in male MDMs than in female ones.

Our data indicate, for the first time, that LPS affects ERα but not ERβ activation status. We identify a significant role of ERα in LPS-mediated inflammatory responses in MDMs, which represents an initial step in understanding the influence of sex in the relationship between LPS and ERα.

Keywords: estrogen receptors, lipopolysaccharide, inflammation, sex differences

Number of Figures: 3

Introduction

Macrophages play a significant role in immunity and immune responses. These cells reside in every tissue of the body and are activated in the initial steps of inflammation (Fujiwara and Kobayashi, 2005) and atherosclerosis (Moore et al., 2013; Moore and Tabas, 2011). A large body of clinical and preclinical evidence has demonstrated that 17β-estradiol (E2), the most active of estrogen hormones, plays a key role in both inflammation and atherosclerosis (Arnal et al., 2004; Lizcano and Guzman, 2014). In particular, the menstrual cycle and pregnancy influence the etiology and course of chronic inflammatory diseases and the increase in the incidence of disorders characterized by a strong inflammatory component (e.g., osteoporosis, atherosclerosis, diabetes, and arthritis) is typical in post-menopausal women, whereas pre-menopausal women are less prone than males to these diseases (Villa et al., 2015). Furthermore, E2 reduces the production of pro-inflammatory cytokines including interleukin-6, tumor necrosis factor-α (TNF-α) and macrophage inhibitory factor (Hsieh et al., 2007; Messingham et al., 2001; Suzuki et al., 2008).

All E2 anti-inflammatory effects are mediated by both estrogen receptors subtypes (ERα and ERβ), members of nuclear receptor family, which are widely distributed in several parts of male and female reproductive system as well as in various non-reproductive tissues such as brain, bone and the cardiovascular system (Ascenzi et al., 2006). ER-mediated cellular responses to E2 have been loosely grouped into two interconnected categories: nuclear and rapid extra-nuclear signals. It is now known that both ER subtypes share the same molecular action mechanisms which are strictly dependent on the intracellular localization of ERs (Ascenzi et al., 2006). In addition, it has been reported that ERs have a key role in inflammation, as the down-regulation of their expression has been reported in mouse microglia (Sierra et al., 2008), and the activation of ERα led to an anti-inflammatory effect in lipopolysaccharide (LPS) -induced brain inflammation (Vegeto et al., 2003). It is widely accepted that abnormalities in ER levels and signaling lead to different types of pathological conditions. For example, ERα expression increases at early stages of cancer where acts as a tumor promoter, whereas, ERβ levels are reduced during carcinogenesis and cancer progression acting as a tumor suppressor (Jia et al., 2015). At the present, the contribution of the balance of ERα and ERβ levels and activation status to E2 anti-inflammatory effects is completely unknown.

Macrophages represent a good experimental model to evaluate the role played by ER levels and activation in inflammation in that these cells express both ERα and ERβ (Jensen and Pioli, 2008; Murphy et al., 2009). Furthermore, ERα levels are higher in monocytes derived macrophages (MDMs) from women taking oral contraceptives versus non-users, whereas ERβ is more expressed and is also more active in untreated women (Campesi et al., 2012). ERs play, also, a pivotal role in monocyte survival regulation (Vegeto et al., 2001) and in inflammation in different cells systems (Cuzzocrea et al., 2000). In particular, ERs activity has been shown to augment and dampen innate immune signaling pathways in macrophages (Cunningham and Gilkeson, 2011; Cutolo et al., 1995).

Several sex differences have been reported in immune function, inflammation and atherosclerosis (Berkley et al., 2006; Imahara et al., 2005; Lansky et al., 2012; Ngo et al., 2014; Oertelt-Prigione, 2012). As example, the LPS-induced TNF-α release (the most central inflammatory mediator rapidly secreted after interaction with pathogens) is different in men and women (Campesi et al., 2013). Moreover, normal human female alveolar macrophages are able to kill the ingested pneumococci better than men and authors suggest a role for E2 (Yang et al., 2014).

Altogether, these data sustain the ability of E2 to modulate ER levels in macrophages and the anti-inflammatory role of this hormone in females. However, no information is available on the possible involvement of ERs in inflammation in male, although male tissues express active ERs [(Ascenzi et al., 2006) and literature cited therein]. Therefore, we aimed to assess the ability of LPS, a bacterial cell-wall component of Gram-negative and a component of tobacco (Sebastian et al., 2006) to modulate, in a sex-specific way, the expression and the activity of ERα and ERβ in MDMs from young adult, and healthy men and women.

Materials and methods

Population

The study was approved by the local ethical committee of ASL1 of Sassari. Informed consent was obtained, and blood sampling was performed during a voluntary blood donation. Seven healthy adult men and 7 healthy adult women aged between 21-35 years were enrolled. All women were fertile and premenopausal, with regular menstrual cycles (28-30 days) without reported precocious and /or surgically-induced menopause, were free from contraceptives use for at least 3 months to ensure an adequate wash-out period and were all analysed

during the follicular phase of their menstrual cycles. All procedures were conducted in accordance with the Declaration of Helsinki.

Monocytes isolation, differentiation and treatment

Monocytes of individual subjects were isolated from 30 ml of blood withdrawn from healthy men and women according to Campesi et al. (2012). Purified monocytes were obtained by adhesion; non-adherent cells (mainly lymphocytes) were removed by gentle washes with phosphate buffer. MDMs were prepared from monocytes cultured for 8 days in a 5% CO_2 incubator at 37°C in RPMI 1640 medium containing 20% fetal bovine serum, 2 mM glutamine, 10 mM HEPES and antibiotics / antimycotics. This procedure allowed to obtain an homogeneous population of MDMs, which appeared as adherent cells with their typical elongated morphology: prominent nucleus with flatly outspread cytoplasm and multiple pseudopodia. After 8 day of culture, MDMs were incubated with 100 ng / ml LPS for 24 h and subsequently lysed for western blotting analysis.

Western blotting

MDMs cells were cultured for 8 days and then were lysed (Cell Lysis Buffer, Cell Signalling Technology, Danvers, MA, USA). The protein concentration was quantified using the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). For the Western blot analysis, 30 µg of solubilized proteins were electrophoretically resolved by 4% – 15% SDS-PAGE (100 V, 2 h, 24°C) and then transferred to a PVDF membrane (250 mA, 65 min, 4°C) using a mini-PROTEAN tetracell system (Bio-Rad, Milano, Italy). The membranes were blocked in 5% (w/v) skim milk (Sigma-Aldrich, Milano, Italy) in 150 mM Tris buffer (Sigma-Aldrich, Milano, Italy) and 20 mM Tris-HCl, pH 7.2 (Sigma-Aldrich, Milano, Italy,) at 24°C for 1 h. Then membranes were incubated overnight at 4°C with the following antibodies: rabbit ERα and mouse ERβ (Santa Cruz Biotechnology, Segrate, Milano Italy) (1:100), rabbit P-ERα(Ser118), rabbit p38, and rabbit P-p38 (Cell Signalling Technology, Danvers, MA, USA) (1 : 1,000). After P-ERα(Ser118) and P-p38 determination, the nitrocellulose membranes were stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL, USA) for 10 min at room temperature and then probed with anti-ERα and anti-p38 antibodies, respectively. The level of phosphorylated / unphosphorylated protein has been assayed on the same electrophoresis, but the relative figures have been splitted to better evidence the

differences between male and female samples. The rabbit polyclonal antibody anti-β-tubulin (Cell Signalling Technology, Danvers, MA, USA) (1:1,000) was used as loading control to normalize ER levels; no difference in β-tubulin levels were reported between sexes as demonstrated by Western blot images After washing, the blots were incubated for 1 h with anti-rabbit IgG and anti-mouse IgG, horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Milano, Italy) (1:2,000). Antibody binding was detected using a chemiluminescence reaction (Cell Signalling Technology, Danvers, MA, USA) with the Bio-Rad Chemi Doc instrument (Berkeley, CA, USA). Band volume analysis was performed using the Image Lab 4.0 software (Bio-Rad Laboratories, Berkeley, CA, USA).

Statistical analysis

Data were reported as the mean ± SD. Statistical analysis was performed by Two Way Analysis of Variance (2-Way ANOVA) followed by the Pairwise Multiple Comparison Procedures to analyse the effect of sex and treatment with LPS, using Sigma-Stat 3.1 software (Systat Software, Erkrath, Germany). For the analysis of P-ERα, which was undetectable in basal conditions, an unpaired Student's t –tests was used to compare males and females after LPS exposure. The distribution of samples was assessed by the Kolmogorov-Smirnov and Shapiro tests. A $P \leq 0.05$ was considered statistically significant.

Results

Population

The 2 cohorts were well-matched for age and body mass index, and, as expected, men were taller and weight more than females.

LPS effect on ERα levels in male and female MDMs

ERα has a key role in the E2 anti-inflammatory effects (Sierra et al., 2008; Vegeto et al., 2003), this prompted us to first evaluate the effect of LPS on the levels and activation status of this ER subtype in male and female MDMs. The 2-Way ANOVA demonstrated that there is a statistically significant interaction between sex and treatment ($P < 0.001$). In particular, under basal conditions, the expression of ERα was significantly different in MDMs obtained from adult healthy men and adult healthy women (**Fig. 1**). Indeed, ERα expression was 2 times higher in MDMs obtained from women in comparison with MDMs obtained from men

(**Fig. 1A**). LPS exposure (100 ng / ml, 24 h) up-regulated ERα level by 26 times in male and 9 times in female MDMs (**Fig. 1A**).

It is well recognized that ERα activities (both nuclear and extra-nuclear) should be preceded by receptor phosphorylation at 118 serine residue (ERαS118) (Ali et al., 1993; La Rosa et al., 2012). In unstimulated cells, P-ERαS118 levels were undetectable in MDMs of both sexes, indicating that the receptor is inactive. However, LPS exposure (100 ng / ml, 24 h) up-regulated ERα phosphorylation in both sexes even if the effect was significantly higher in male MDMs than in female ones (**Fig. 1B**).

LPS effect on ERβ levels in male and female MDM cells.

The 2-Way ANOVA demonstrated that there is a statistically significant interaction between sex and treatment ($P < 0.001$). In basal conditions, ERβ expression appeared four times higher in female MDMs than in male cells sustaining the possible role played by this receptor subtype in E2 anti-inflammatory effects (**Fig. 2A**). In contrast to the effect on ERα, LPS exposure (100 ng / ml, 24 h) did not affect ERβ expression in male MDMs, whereas it significantly down-regulated ERβ level in female MDMs (**Fig. 2A**).

We previously identified the E2-dependent activation of the p38 component of the MAPK family as a conserved pathway in ERβ-based E2 signaling (Galluzzo et al., 2007). The ERβ-mediated activation of the p38/MAPK, which occurs in several E2 target cells (Acconcia et al., 2005; Galluzzo et al., 2007), is important both for the regulation of ER expression and for the transcriptional activity of this receptor subtype (Caiazza et al., 2007). LPS treatment significantly induced the levels of p38, without sex differences, although this compound did not affect the phosphorylation status of this kinase and thus its activation status (**Figs. 2B and 2C).** Consequently, no significant variation in the level of P-p38 / p38 ratio were reported (**Fig. 2D**), indicating that the level of activation of ERβ was not different between sexes after LPS exposure (**Figs. 2B, 2C, and 2D**).

LPS effect on ERα/ERβ ratio in male and female MDM cells.

The differences above reported between male and female ERs levels prompted us to calculate the ratio between receptor subtypes. **Figure 3A** shows that the ERα / ERβ ratio was significantly higher in male MDMs than in female ones. Intriguingly, the activation status of ERα (i.e P-ERα / ERα ratio) was significantly higher in male MDMs (**Fig. 3B**).

Discussion

A plethora of literature have demonstrated that ERα and ERβ exert opposite effects on E2-regulated cellular proliferation; these data have evidenced the strict correlation existing between the perturbation of ER subtype-specific expression and different clinical outcomes in cancer patients (Ascenzi et al., 2006; Kim and Kim, 2014; Malorni et al., 2008). Less information is available on the contribution of the balance of ERα and ERβ levels and their activation status to the well-known E2 anti-inflammatory effects. In addition, none of this information has never evaluated the possible involvement of ERs in inflammation in male, although male tissues express active ERs (Ascenzi et al., 2006). Here, we assessed the ability of LPS to modulate, in a sex-specific way, the expression of ERα and ERβ in MDMs from young adult, and healthy men and women. Moreover, when activated, both ERs trigger different and complex signal transduction pathways, thus, we selected ERα Ser118 phosphorylation and ERβ-dependent p38 activation as markers of receptor activation (Acconcia et al., 2005; Ali et al., 1993; Galluzzo et al., 2007; La Rosa et al., 2012). These two parameters represent a new tool in the study of ER biology being involved and, thus, allowing to investigate both nuclear (transcriptional) and extra-nuclear (rapid activation of signaling cascade) receptor activities.

Results reported here show that 24 h LPS exposure modifies the expression of the ERα and ERβ, the activity of the ERα in MDMs and these changes are sex-dependent. In particular, LPS significantly up-regulates the ERα level and this up-regulation is more evident in male than in female cells. On the other hand, the ERβ level is down-regulated only in female MDMs. The above data also indicate that LPS strongly perturbs the ERα / ERβ balance inducing a net increase of ERα level in both sexes. Considering that ERα can be activated in a ligand-dependent manner, via E2, or in a ligand-independent manner, via several signal transduction pathways including p44/42 MAPK (McGlynn et al., 2013) and PI3K/AKT (Ascenzi et al., 2006) it is likely that LPS activates ERα in a ligand independent way. Interestingly, human MDMs maintain sex differences after 8 days in culture. This result is in line with previous data that demonstrated that freshly isolated rat female- and male-derived vascular smooth muscle cells maintain several sex differences at least for the first eight passages in culture (Malorni et al., 2008; Pellegrini et al., 2014; Straface et al., 2009).

It has been reported that LPS binds to the Toll like receptor 4 (TLR4) (Takeda and Akira, 2005) which, in turn, enhances ERα expression whereas neither E2, nor ERα, impacted TLR4 expression, at least, in mesangial cells (Svenson et al., 2014). In contrast, Smith et al. (2011) demonstrated that LPS decreases the levels of ERα mRNA in microglia without any effect on ERβ mRNA, and Holm et al. (2010) reported that 4 days of LPS stimulation reduced both ERα and ERβ mRNA levels in endothelial cells. Finally, Toniolo et al. (2015) found that the association of LPS with interferon gamma decreases ERα levels. Although it is possible that LPS effects differ in dependence of animal species or tissue, data reported in literature and present data suggest that LPS effects could rely on the modulation of ER synthesis and degradation. Moreover, the evidence that the number of TLR4 is higher in blood monocytes isolated from boys than in girls (Bannister et al., 2013) well supports the higher LPS effect in male than in female MDMs.

The most important result of this research is the LPS ability to increase ERα phosphorylation without any effect on ERβ activation status. The ability of TLR ligands, including LPS, to increase ERα phosphorylation has been already reported in mesangial cells where it is associated to the increase of inflammatory response (Svenson et al., 2014). Moreover, LPS induces higher expression of TLR4 and greater release of TNF-α in male neutrophils than in female ones (Aomatsu et al., 2013), which is paralleled by a stronger activation of mitogen-activated protein kinases and phosphatidylinositol 3-kinase (Aomatsu et al., 2013). Together these data sustain the pivotal role played by ligand-independent ERα activation in LPS-induced inflammatory response and suggest that males are prone to the inflammation cascade as females.

Conclusions

Our findings sustain the key role played by ERα in LPS-induced inflammatory response and indicate that males are more sensitive to the LPS effects than females. Considering the role of macrophages in atherosclerosis the higher response of male MDMs respect to female ones can impact on a variety of key molecular mechanisms linked with important processes such as immune response and cardiovascular diseases (Moore et al., 2013; Moore and Tabas, 2011).

As a whole, the results reported here identify a significant impact / interaction of ERα in LPS-mediated inflammatory responses in MDMs representing an initial step in understanding the influence of sex in the relationship between LPS, its receptor and ERα.

Abbreviations

E2: 17β-estradiol; ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; IL-6: interleukin 6; LPS: lipopolysaccharide; MDMs: monocytes-derived macrophages; P-ERα: phosphorylated estrogen receptor alpha; P-p38: phosphorylated p38; TLR4: Toll like receptor 4; TNF-α : tumor necrosis factor-α

Competing interests

The authors declare that they have no competing interests.

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Figures legends

Fig. 1. Representative Western blot and densitometric analysis of ERα **(A)** and P-ERα **(B)** expression in male (black bar) and female (white bar) MDMs in basal conditions and after 24h exposure to 100 ng / ml LPS. The values are expressed as the means ± SD of at least 4 independent experiments for each sex, normalised on β -tubulin levels. The level of phosphorylated / unphosphorylated protein has been assayed on the same electrophoresis, but the relative figures have been splitted to better evidence the differences between male and female samples. Connectors represent statistical difference between basal condition and LPS stimulation; * and ** indicate a $P < 0.05$ and a $P < 0.001$ between male and female MDMs, respectively.

Fig. 2. Representative Western blot and densitometric analysis of ERβ **(A)**, p38 **(B)**, P-p38 **(C)** expression and P-p38 / p38 ratio **(D)** in male (black bar) and female (white bar) MDMs in basal conditions and after 24h exposure to 100 ng / ml LPS. The values are expressed as the means ± SD of at least 4 independent experiments for each sex, normalised on β -tubulin levels. The level of phosphorylated / unphosphorylated proteins has been assayed on the same electrophoresis, but the relative figures have been splitted to better evidence the differences between male and female samples. Connectors represent statistical difference between basal condition and LPS stimulation; * indicates a $P < 0.05$ between male and female MDMs.

Fig. 3. ERα / ERβ **(A)** and P-ERα / ERα **(B)** ratios calculated in in male (black bar) and female (white bar) MDMs in basal conditions and after 24h exposure to 100 ng / ml LPS. The values are expressed as the means ± SD of at least 4 independent experiments for each sex. Connectors represent statistical difference between basal condition and LPS stimulation; * and ** indicate a $P < 0.05$ and a $P < 0.001$ between male and female MDMs, respectively.