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Full Length Research Paper

17 -estradiol attenuates LPS-induced interleukin-8 production by human peripheral blood monocytes through estrogen receptor- activation

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Proper regulation of the immune response is essential for immune homeostasis. Several proinflammatory cytokines released from activated monocytes mediate inflammation, including interleukine-8 (IL -8) which recruits neutrophils to the site of inflammation. 17 -Estradiol (E2) has a direct role in the modulation of the innate immune function and mediates profound effects on immune function of the monocytes. The effects of 17 -E2 are mediated principally by two receptor subtypes, ER and ER ; both are expressed in monocytes. The aim of this study was, therefore, to characterize the estrogen receptor subtypes that mediate the estrogen effects on LPS-activated IL-8 production by human peripheral blood monocytes. 17 -E2 and PPT attenuated the production of IL- 8 by LPS-activated monocytes in a dose-dependent manner and these effects can be reversed by ICI182, 780. These results suggested a role of ER on the attenuating effect of 17 -E2 on IL-8 production by human peripheral blood monocytes.

Key words: Estrogen receptor , monocytes, interleukin 8.

INTRODUCTION

Inflammation contributes to the pathogenesis of various diseases. Circulating blood monocytes play an important role in the inflammation response, which is crucial for the modulation of innate immune function. Monocytes migrate to the site of inflammation, become activated and then release a broad variety of proinflammatory cytokines including interleukin-8 (IL-8). IL-8 has numerous roles including inflammation, cell recruitment, wound healing, angiogenesis, and metastasis. Among the diverse biological functions of IL-8 is the recruitment of neutrophils to the sites of inflammation where they aid in mediating pathogen clearance (Hersh et al., 1998). However, excessive neutrophil infiltration leads to tissue damage and contributes to severe inflammation.

17 -E2 has a direct role in the modulation of innate immune function and mediates profound effects on monocyte and macrophage immune function (Annechien et al., 2005). It attenuates the production of pro-inflammatory cytokines including IL-6 and TNF . Moreover, the production of the IL- 8 is also decreased by 17 -E2 in monocytes that have been challenged with LPS via NF-κB blockage (Pioli et al., 2007). Human monocytes are known to express both estrogen receptors subtypes; the ER and the ER , and estrogens have significant effects on its immune functions (Mor et al., 2003; Khan et al., 2005). Although 17 -E2 has been shown to directly affect monocyte function, relatively little is known about the role of the estrogen receptor subtypes in mediating estrogen effects in this immune cells. The purpose of this study was, to characterize the estrogen receptor subtypes that mediate the estrogen effects on LPS-activated IL-8 production by human peripheral blood monocytes

EXPERIMENTAL

Reagents

17 -estradiol and Enhanced Avian HS RT-PCR (HSRT100) were obtained from Sigma, U.S.A. 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-

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triyl)trisphenol(PPT;a selective ER agonist), 2,3-bis(4-Hydroxyphenyl)-propionitrile(DPN;a selective ER agonist), and 7a,17b-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol(ICI182,780; ER antagonist) were obtained from Tocris Bioscience. Iscove's modified Dulbecco's modified Dulbecco's medium (IMDM) was obtained from GIBCO, U.S.A. DouSet® ELISA human CXCL8/IL-8 was obtained from R&D Systems®, U.S.A. High Pure RNA Isolation Kit was obtained from Roche, Germany. SYBR® Safe DNA Gel Strain 10,000X conc. in DMSO was obtained from Invitrogen, U.S.A.

Isolation of human peripheral blood monocytes

All experiments were carried out in accordance with the Documentary Proof of Ethical Clearance Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. Peripheral venous blood was drawn from healthy postmenopausal donors that were not on hormone replacement therapy (HRT). Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll- hypaque centrifugation (Fuss et al., 2009). Briefly, venous blood was diluted with an equal volume of normal saline, and the mixture was laid over Ficoll-hypaque then centrifuged at room temperature. After centrifugation, PBMCs were washed with normal saline 3 times. The cells were >99% viable as determined by trypan blue exclusion and were resuspended in Iscove's DMEM at a concentration of 4×106 cells/ml, and then plated out in 48-well plates at a concentration of 2×106 cell per well. After incubation at 37°C in humidified 5% CO_2 for 1 h, non-adherent cells were removed by washing wells with Iscove's DMEM (Rossi et al., 1998).

Cell viability assay

Monocytes were incubated with 17 -E2 or PPT or DPN for 36 h. XTT was added into the plate and then incubated for 3 h. The absorbance was measured spectrophotometrically at 450/650 nm (Roehm et al., 1991).

In vitro culture of leukocytes for IL-8 assay

Monocytes were resuspended in Iscoves's IMDM containing 10% autologous serum supplemented with penicillin/streptomycin. They were incubated with 10.8 - 10.4 M of 17 -E2 or PPT or DPN for 24 h, and then treated or not with 10 ng/ml LPS for an additional 12 h. For experiments in which ER binding was inhibited, monocytes were preincubated with 10-6 M of the ER antagonist ICI182, 780 for 2 h before treatment with 17 -E2 or PPT or DPN and 10 ng/ml LPS. At the conclusion of these incubations, total RNA was extracted from these cells and supernatants were collected for IL-8 ELISA assay as manufacturer.

Enzyme-Linked Immunoabsorbent Assay (ELISA)

The level of IL-8 in supernatant was quantified using the human CXCL8 Quantikine ELISA kit (R&D Systems). Samples collected from cells treated with LPS were diluted 1/25 before analysis. The absorbance was measured spectrophotometrically at 450/570 nm.

RNA extraction and RT-PCR

Total RNA was isolated from monocytes using High Pure RNA Isolation Kit. The cDNA was synthesized from 1 µg of total RNA using the HSRT 100 kit (Eastlund and Mueller, 2001). From each sample, 1 µl of 500 µ M each dNTP and 1 µl of 2.5 µM. Random nonamers were added and then incubated at 70°C for 10 min. After that, 2 µ l of 10X buffer for AMV-RT, 1 µl of 1 U/µl RNase inhibitor and 1 µl of 1 U/µl Enhanced avian RT were added and then incubated at 45°C for 50 min. After cDNA generation was completed, the samples were placed on ice for subsequent PCR amplification.

The PCR analyses were carried out in a volume of 50 µl containing 1 µl of 200 µM each dNTP, 5 µl of Template DNA (cDNA) from RT reaction, 1 µl of JumpStart AccuTaq LA DNA polymerase mix and 1 µl of PCR primers. The sense primer for human IL-8 is 5'-ATTTCTGCAGCTCTGTGTGAA-3' and the antisense primer is 5'-AACTTCTCCCGACTCTTAAGT-3'. The sense primer for human GAPDH (as the internal standard) is 5'-TCTTCTTTTCGCGCAG-3' and the antisense primer is 5'-GGGGGCAGAGATGATGACC-3'. The amplification conditions for IL-8 were the following steps: initial activation at 94°C for 10 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The amplification conditions for GAPDH were the following steps: initial activation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 68°C for 1 min. The PCR product size of IL-8 and GAPDH are 255 and 413 bp, respectively. The PCR products were separated on 2% agarose gels and visualized using SYBR® Safe DNA Gel staining.

RESULTS

Cytotoxicity

Incubation of human monocytes with 10-8 -10- 6 M of 17 -E2, PPT, DPN, ICI182, 780, dexamethasone caused no cytotoxic effect, while PPT exhibited cytotoxic effect at the concentration of 10-4 M with 48% (Data not shown).

Kinetic study of IL-8 production from LPS-activated monocytes

Monocytes were incubated with LPS 10 ng/ml for 1, 2, 4, 8, 10, 12, and 14 h. After incubation, supernatants were collected for interleukin-8 ELISA assay. The IL-8 level was measured spectrophotometrically at a wavelength of 450 nm with a reference of 570 nm and each reaction mixture was done in triplicate and the values were averaged. When peripheral blood monocytes were activated with LPS 10 ng/ml, less amount of IL-8 was excreted during 1-4 h. The secretion of IL-8 was rapidly increased after 6 h and the maximum concentration of IL-8 in the culture medium of monocytes was observed after 12 h (Figure 1). Therefore, this incubation period of time was selected to use for the measurement of the effects of 17 -E2, PPT and DPN on IL-8 production by LPS-activated monocytes.

Interleukin-8 production of LPS-activated monocytes

After being activated with LPS 10 ng/ml, monocytes maximally produced IL-8 and the detected level from the cultured medium was up to 30,000 pg/ml, whereas those

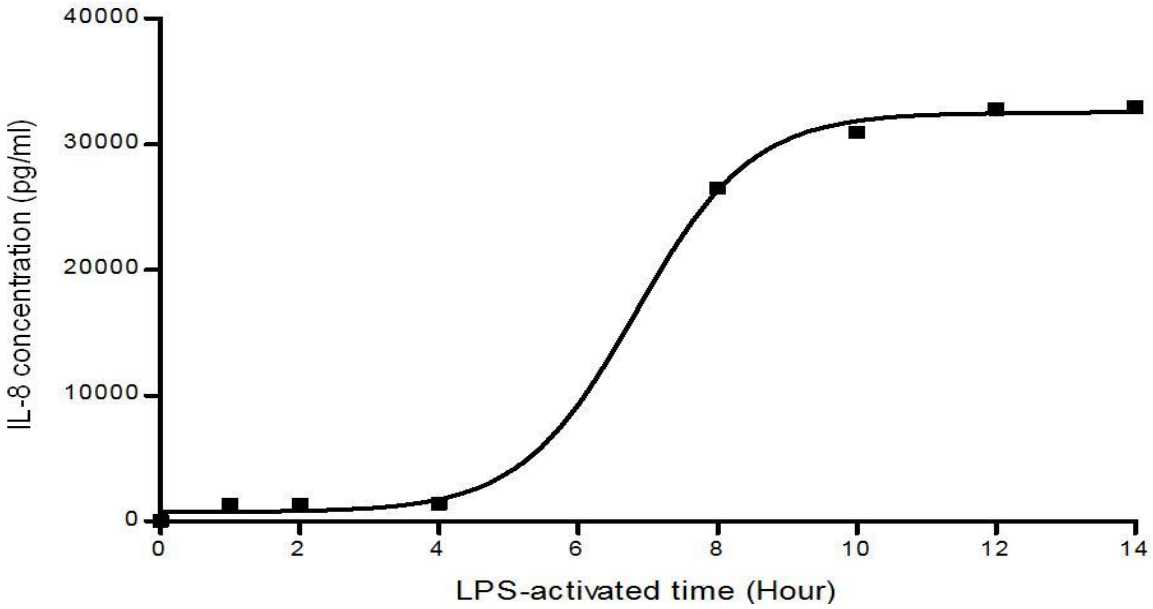

Figure 1. Kinetics study of IL-8 production from LPS-activated monocytes from post-menopausal women. Monocytes were activated by 10 ng/ml LPS for 1, 2, 4, 8, 10, 12, and 14 h.

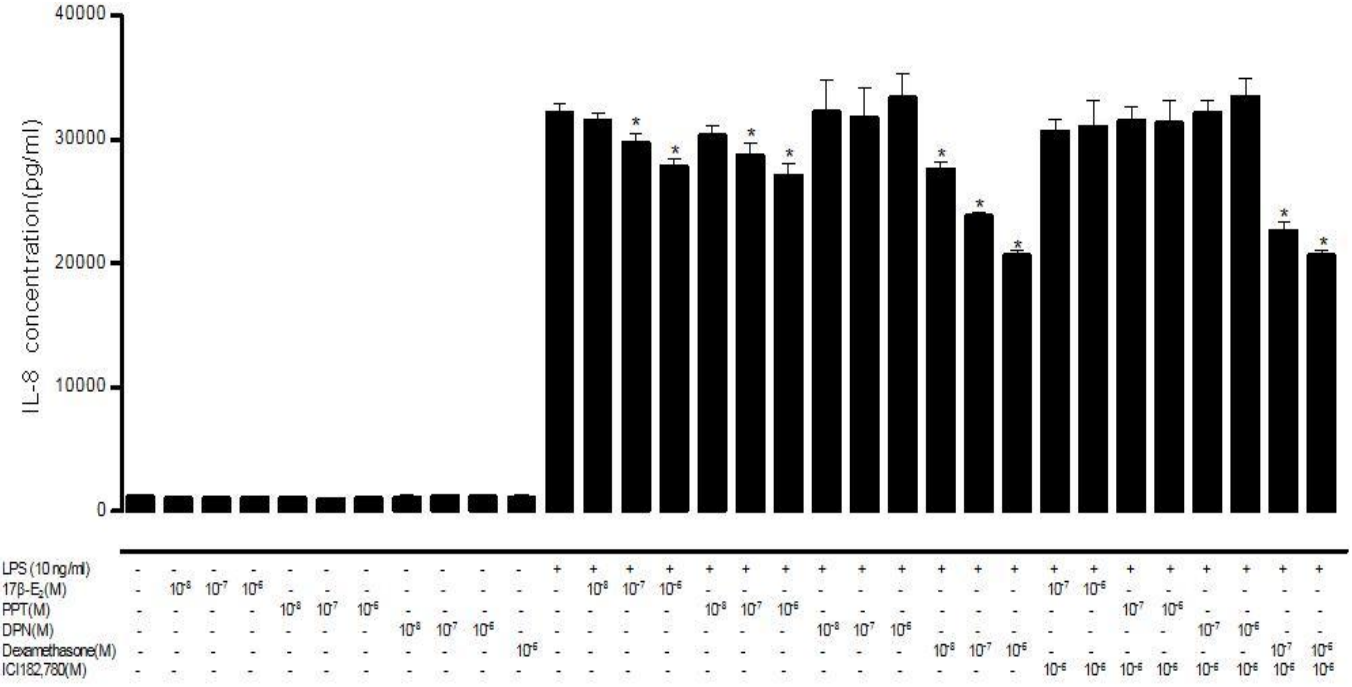

Figure 2. Effects of 17β-E2, PPT, and DPN (10-8 – 10-6 M) on IL-8 levels in LPS-activated monocytes from post-menopausal women. The values are expressed as means ± S.E.M. of seven different donors. *p<0.05 indicates a significant difference from LPS-treated monocytes.

from untreated cells and the cells that were only treated with 17 - E2 or PPT or DPN or dexamethasone were below the level of 1000 pg/ml, indicating that the drugs by themselves did not induce IL-8 production (Figure 2). Also, ICI182, 780 alone did not affect IL-8 production (data not shown). Both 17 -E2 and PPT at the

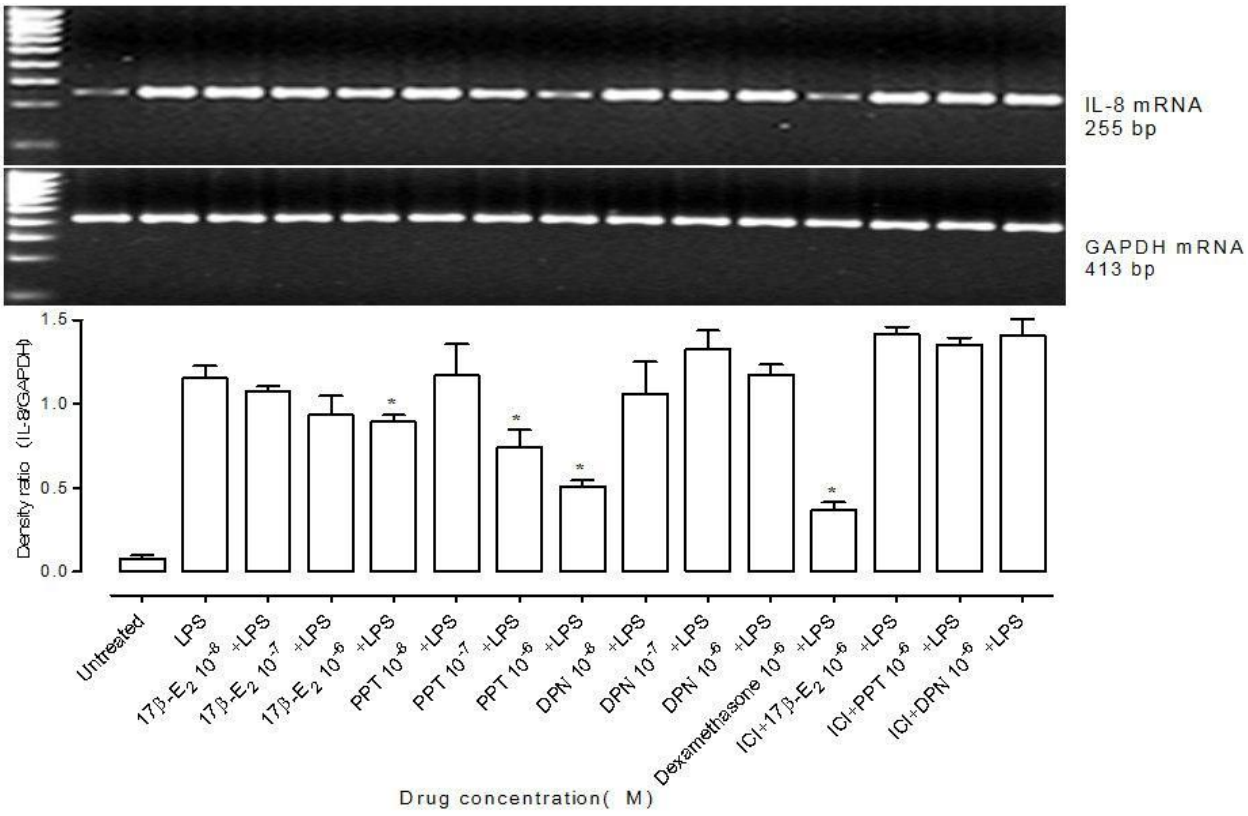

Figure 3. Effects of 17β-E2, PPT, and DPN (10-8- 10-6 M) on IL-8 mRNA expression in LPS-activated monocytes. The expression of the housekeeping gene, GAPDH, was performed for each sample as an internal control. *p<0.05 indicates a significant difference from LPS-treated monocytes.

concentration of 10-7–10-6 M significantly attenuated the LPS- induced IL-8 production by human monocytes in a dose dependent manner with IC50 of 17 - E2 and PPT were 6.0±1.1 and 5.2±0.4 M, respectively. Dexamethasone, a reference compound, exhibited strong inhibition of IL-8 production in LPS-treated monocytes. However, DPN did not attenuate LPS-activated IL-8 production by human monocytes. In addition, the inhibitory effect of 17 - E2 and PPT were reversed by the estrogen receptor antagonist ICI182, 780 while the effect of dexamethasone was not attenuated.

Interleukin- 8 mRNA expression of LPS-activated monocytes

RT-PCR analyses were performed to determine whether the inhibitory effects of 17β-E2 on the production of IL-8 in LPS-activated monocytes were related to the modulation of IL-8 mRNA levels. The IL-8 mRNA expression was markedly increased in response to LPS treatment, while its expression in untreated monocytes was low. As shown in Figure 3, 17β-E2 and PPT significantly inhibited the IL-8 Mrna expression of activated monocytes in a dose-dependent manner. In contrast, DPN at all concentration used did not affect IL-8 mRNA expression in LPS-activated monocytes. The expressions of housekeeping gene, GAPDH, were not affected by all concentration of drugs. The results of these drugs on IL-8 mRNA expression were consistent with the profile of the inhibitory effects of 17β-E2 and PPT on IL-8 production by activated monocytes.

DISCUSSION

The study demonstrated that the constitutive production of IL-8 from monocytes was minimal, and was not affected by all drugs pre-treatment. In contrast, activation of monocytes by LPS caused a significant increase in IL-8 production, and this IL-8 production was significantly attenuated by both 17 -E2 and PPT (a selective ER agonist) at the concentration of 10-7–10-6 M. This inhibition was dose dependent and was reversed by ICI182, 780, a pure ER antagonist. Dexamethasone, a reference compound as anti-inflammatory drug, exhibited strong inhibition of the IL-8 production in LPS-activated monocytes, and its inhibition could not be reversed by

ICI182, 780.

Dexamethasone is a steroid compound, exerts inhibitory effect on IL-8 production via the different receptor from that of 17 -E2. However, DPN (a selective ER agonist) did not cause any change in the IL-8 level. The finding of the reduction in IL- 8 levels of activated monocytes as seen in 17 -E2 and PPT treatment were consistent with the reduction in IL-8 gene expression. These results suggested that effect of 17 -E2 which LPS-induced IL-8 production by human monocytes was attenuated, is mediated through the estrogen receptor alpha (ER) activation. Our study agreed with Pioli et al. who demonstrated that 17 -E2 attenuates LPS-induced expression of chemokine CXCL8 in human peripheral blood monocytes via NF-κB blockage (Pioli et al., 2007). However, the level of IL-8 reduction with 17 - E2 was less than that demonstrated by Pioli et al. These may due to the IL-8 production from LPS-activated monocytes which was very high in this study as shown in the IL-8 kinetic assay. The mild reduction in IL- 8 levels of 17 -E2 and PPT may not possess any acute anti-inflammatory effect in the clinical level; however it may be useful in the long term exposing with these agents. Estrogens can mediate their effect through either classical genomic signaling or through non-genomic effects. Therefore, further investigation will clarify their signaling pathway and reveal the relevance of estrogen receptor in inflammatory diseases.

Conclusion

The present data suggested that the effect of 17 -E2 in which LPS-induced IL- 8 production by human peripheral blood monocytes was attenuated, is mediated through the activation of estrogen receptor alpha (ER). This study may lead to an enhanced understanding of estrogen receptor in the regulation of immune response.

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