Original article **P61**

Characterization of estrogen receptor mediating neutrophil function

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Abstract

Estrogen and its receptors play important roles in the prevention of cardiovascular disease in women. 17β-estradiol (E2) has a direct role in the modulation of the innate immune function. 17β-E2 attenuates the production of pro-inflammatory cytokines including IL-1, IL-6 and TNF-α. IL-8 production is also decreased by 17β-E2 in activated monocytes. Estrogen also has significant effects on neutrophil functions. Estrogen effects are mediated principally by two receptor subtypes, ERα and ERβ; both are expressed in endothelial, vascular smooth muscle cells, and immune cells. Both estrogen receptor subtypes are also expressed on human neutrophils, but their roles in mediating estrogen effects on neutrophil functions are remained to be elucidated. The purpose of this study was, therefore, to characterize the estrogen receptor subtypes that mediate estrogen effects on neutrophil functions. Neutrophils were isolated from post-menopausal women venous blood by Dextran and Percoll gradient centrifugation. All compounds were primarily investigated for their cytotoxic effects on neutrophils using XTT assay. Neutrophils were pre-incubated with 17β-E2, PPT (a selective ERα agonist), and DPN (a selective ERβ agonist), then activated by LPS or fMLP for the study of IL-8 production or adhesion molecule expression, respectively. The IL-8 production of leukocytes was assayed using ELISA. Neutrophil surface adhesion molecule expression was measured using flow cytometry. Neutrophil chemotaxis and superoxide anion generation (SAG) were also investigated. The results showed that 17β-E2 and PPT inhibited fMLP-induced CD62L-selectin shedding, while DPN did not. All drugs did not affect fMLP-induced MAC-1 expression. Both 17β-E2 and PPT attenuated IL-8 production in a dose-dependent manner. These results suggest the role of ERα on L-selectin shedding and IL-8 production in neutrophils. In addition, neutrophil chemotaxis-induced by rh IL-8 was inhibited by 17β-E2 and PPT, whereas SAG was inhibited by 17β-E2, PPT, and DPN. These results suggest that the inhibitory effect of 17β-E2 on neutrophil chemotaxis is mediated via ERα while both ERα and ERβ play roles in SAG.

Introduction

Inflammation plays an important role in the pathogenesis of various cardiovascular diseases. Estrogen plays important roles in the prevention of cardiovascular diseases in women. 17β-E2 has a direct role in the modulation of innate immune function and mediates profound effects on monocyte and macrophage immune function (1). It attenuates the production of pro-inflammatory cytokines including IL-6 and TNFα. Interleukin-8 (IL-8) plays a critical role in the recruitment of leukocytes to areas of vascular injury and its production is also decreased by 17β-E2 in activated monocytes. 17β-E2 also has significant effects on neutrophil functions (2,3). Estrogen effects are mediated principally by two receptor subtypes; the ERα and the ERβ, both are expressed in endothelial cells, vascular smooth muscle cells and immune cells. Human neutrophils are known to express both estrogen receptors subtypes. The role of estrogen receptor subtypes in mediating estrogen effects on neutrophil functions are remained to be elucidated. The purpose of this study was, therefore, to investigate the estrogen receptor subtypes involving estrogen effects on

neutrophil functions including, surface adhesion molecule expression, IL-8 production, rh IL-8 -induced neutrophil chemotaxis, and superoxide anion generation.

Materials and methods

Isolation of human neutrophils

Peripheral venous blood was drawn from healthy postmenopausal woman using heparin as an anticoagulant. Human neutrophils (PMN) were isolated by Percoll density gradient centrifugation. Briefly, venous blood was mixed with an equal volume of Percoll, and the mixture centrifuged at room temperature. After centrifugation, PMN were washed with PBS. Any contaminating red cells were removed by hypotoniclysis. The cells were >99% viable as determined by trypan blue exclusion and were resuspended as required.

Cytotoxic assay

Neutrophils were incubated with 17β-E2 or PPT or DPN in a 96-well plate for 4 h. XTT was added into the plate and then incubated for 3 h. The absorbance was measured spectrophotometrically at 450/650 nm.

In Vitro culture of neutrophils for IL-8 assay

Neutrophils were resuspended in Iscoves's IMDM containing pen/strep. They were incubated with 0.01-10 μM of 17β-E2 or PPT or DPN for 1 h, further activated with LPS 10 ng/ml for 7 h. Supernatant were collected for IL-8 ELISA assay as manufacturer.

Assessment of neutrophils L-selectin shedding and CD 11 b expression (4)

Neutrophils resuspended in PBS were incubated with 0.01-10 μM of 17β-E2 or PPT or DPN for 4 hrs, and then further activated with fMLP 10^{-7} M for 30 min then incubated with human CD26L and CD11b antibodies for 30 min. CD62L and CD11b expression were measured using flow cytometry.

Determination of Neutrophil Chemotaxis (5)

Neutrophil chemotaxis was measured in a 96 well chemotaxis chamber. The bottom wells of the chamber were filled with fMLP. The upper wells with the installed filter were filled with neutrophils which had been treated with the various concentrations of 17β-E2 or PPT or DPN for 10 min. The Chamber was incubated for 45 min at 37°C. The filter was then removed, washed, fixed and stained. Chemotaxis was quantified spectrophotometrically measuring absorbance at 550 nm

Determination of Superoxide Anion Generation (SAG) (6)

Neutrophil SAG was determined by spectrophotometric evaluation of the reduction of ferricytochrome C (Fe^{3+}) to ferrocytochrome C (Fe^{2+}) in the presence of cytochalasin B. Neutrophils were resuspended in PBS containing cytochrome C and cytochalasin B and were preincubated with various concentrations of 17β-E2 or PPT or DPN or PBS, for 10 minutes, then further incubated for 10 minutes with fMLP. After terminating the reaction, aliquots were dispensed into 96-well plate and the absorbance at 550 nm was measured.

Results

1. Cytotoxicity

Incubation of human neutrophils with 17β-E2, PPT, DPN (0.01-10 μM) for 4 h and 8 h caused no cytotoxic effect.

2. IL-8 production of LPS-activated neutrophils from post-menopausal woman

Both 17β-E2 and PPT at the concentrations of 0.01-1μM attenuated LPS-induced IL-8 production by human neutrophils in a dose-dependence manner (Fig.1). Dexamethasone, a

reference compound, exhibited strong inhibition of IL-8 production in LPS-activated neutrophils. However, DPN had no effects on LPS-activated IL-8 production in human neutrophils.

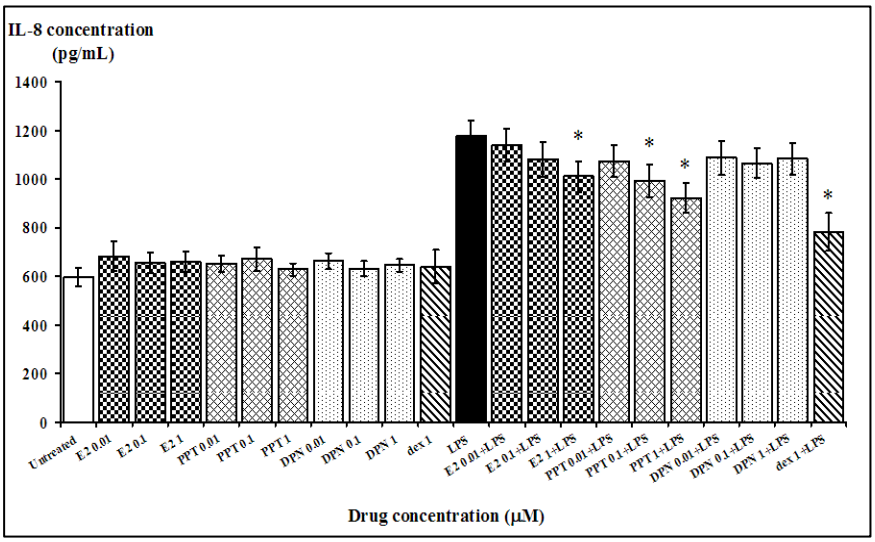


Figure 1. The effects of 17β-E2, PPT, DPN (0.01-1μM) on IL-8 levels in LPS-activated neutrophils from post-menopausal women. The values are expressed as means ± S.E.M. of six different donors. *p<0.05 indicates a significant difference from LPS-treated neutrophils.

3. Neutrophil adhesion molecule expression

17β-E2 and PPT at the concentrations of 0.1-1 μM inhibited fMLP-induced CD62L-selectin shedding in neutrophils, while PPT at the concentration of 10 μM tended to enhanced the shedding of CD62L-selectin. In contrast, DPN did not affect CD62L-selectin shedding in neutrophils (Fig.2A). All drugs did not inhibited fMLP-induced an increase in CD11b expression (Fig.2B).

Fig.2A

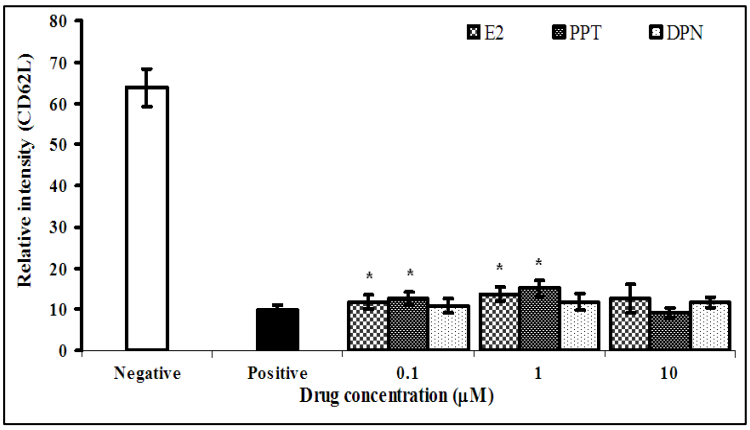
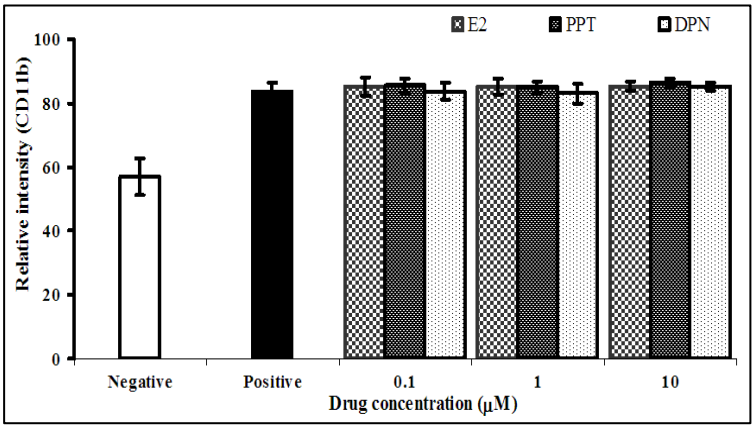


Figure 2. Effects of 17β-E2, PPT, DPN (0.1-10 μM) on fMLP-induced CD62L-selectin shedding of human neutrophils (Fig.2A) and $CD_{11}b$ (MAC-1) expression on surface of human neutrophils (Fig.2B). The values are expressed as means±S.E.M. of five different donors. *p<0.05 indicates a significant difference from positive control.

Fig.2B



4. Neutrophil Chemotaxis

Both 17β-E2 and PPT inhibited rh IL-8-induced neutrophil chemotaxis of which PPT exerted higher potency than 17β-E2, whereas, DPN had a very low potency (Fig 3). Indomethacin exhibited strong inhibition of neutrophil chemotaxis. It appears that the inhibitory effects of 17β-E2 on neutrophil chemotaxis-induced by rh IL-8 is mediated through the estrogen receptor alpha subtype (ERα).

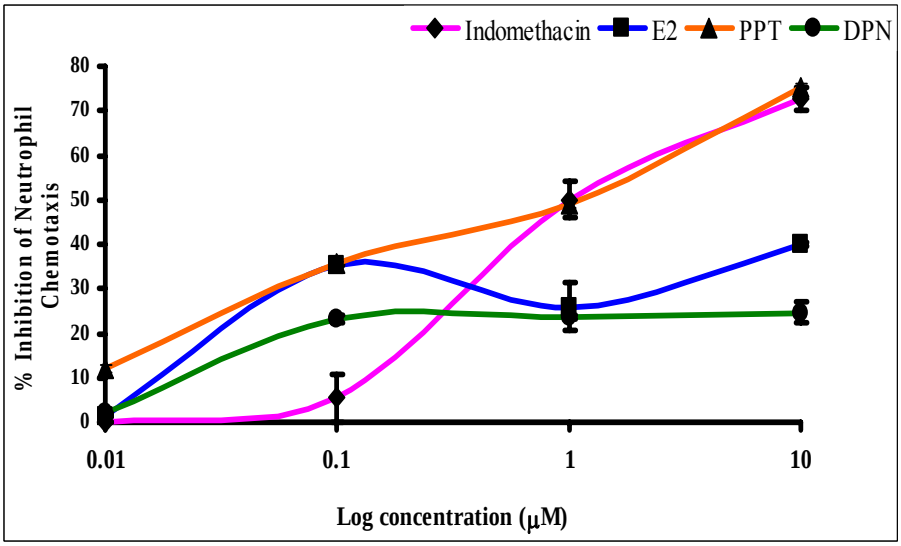


Figure 3. Log concentration-effect curves for inhibition of rh IL-8 (100 nM)-induced human neutrophil chemotaxis of 17β-E2, PPT and DPN. Indomethacin was used as a reference compound. The values are expressed as means±S.E.M. from five independent experiments.

5. Superoxide anion generation (SAG)

All drugs inhibited fMLP-induced human neutrophil SAG of which PPT exerted the greatest inhibitory effect (Fig.4). It demonstrates that the inhibitory effects of 17β-E2 on fMLP-induced human neutrophil SAG are mediated through both estrogen receptor alpha and beta subtypes (ERα and ERβ).

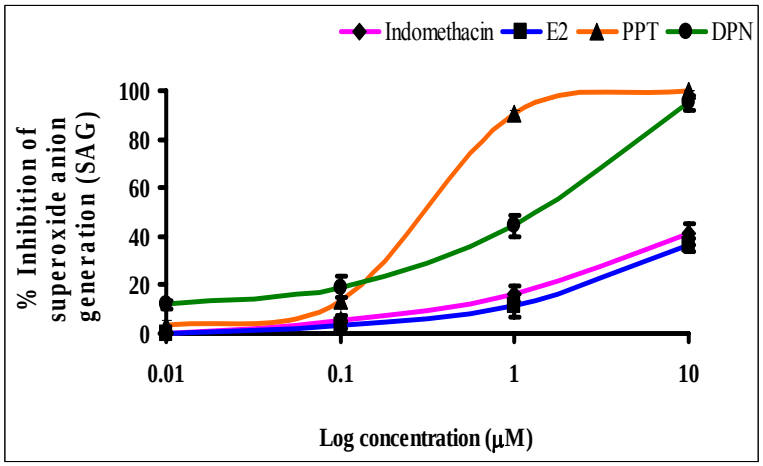


Figure 4. Log concentration-effect curves for inhibition of fMLP (100 nM)-induced human neutrophil SAG of 17β-E2, PPT and DPN. Indomethacin was used as a reference compound. The values are expressed as means±S.E.M. from five independent experiments.

Discussion

IL-8 is a chemotactic for neutrophils to sites of inflammation. It also induces shedding of L-selectin, expression of CD18 on PMNs, up-regulation of LFA-1 and transendothelial migration of neutrophils. The IL-8 production of neutrophils induced by LPS was inhibited in a dose-dependent manner by 17β-E2 and PPT, whereas DPN had no effects. The results reveal that the inhibitory effect of 17β-E2 on LPS-induced IL-8 production by human neutrophils is mediated via ERα. L-selectin plays a crucial role in leukocyte rolling and adhesion on endothelial cell surface. It has been reported that estrogen attenuates recruitment and adhesion of leukocytes to the endothelium offering a possible mechanism by which estrogens exert an anti-inflammatory effect (**7**). The results from this study showed that 17β-E2 and PPT (0.1-1μM) inhibited fMLP-induced CD62 L-selectin shedding in neutrophils, while DPN did not, suggesting that 17β-E2 and PPT mediated their inhibitory effects on CD62L-selectin shedding via ERα. While both 17β-E2 and PPT strongly inhibited rh IL-8-induced neutrophil chemotaxis, DPN possessed weak inhibitory effect. It seems that the inhibitory effects of 17β-E2 on neutrophil chemotaxis-induced by rh IL-8 is mediated via ERα. Both PPT and DPN inhibited fMLP-induced human neutrophil SAG stronger than 17β-E2 and indomethacin. The results showed that both ERα and ERβ involved in the inhibitory effects of 17β-E2 on fMLP-induced human neutrophil SAG. Taken together, these findings demonstrate that 17β-E2 exerts its inhibitory effects on LPS-induced IL-8 production by human neutrophils, CD62 L -selectin shedding, and rh IL-8-induced neutrophil chemotaxis via ERα, whereas its inhibitory effects on fMLP-induced neutrophil SAG is mediated via both estrogen receptor subtypes (ERα and ERβ).

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