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Estradiol inhibits fMLP-induced neutrophil migration and superoxide production by upregulating MKP-2 and dephosphorylating ERK

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ABSTRACT

Estrogen has been reported to inhibit neutrophil infiltration related inflammation and suppress neutrophils migration in vitro, but the underlying mechanism is not fully understood. By using HL-60 differentiated neutrophil-like cells (dHL-60) and human neutrophils, we examined the effect of 17-β estradiol (E_2) on cell migration and superoxide production in response to chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) and explored the mechanisms involved. We found that fMLP significantly induced dHL-60 cell and neutrophil migration and superoxide production, which was inhibited by ERK inhibitor PD98059. E_2 significantly inhibited fMLP-induced dHL-60 cell and neutrophil migration and superoxide production at both physiological and pharmacological concentrations. Mechanistic studies showed that pretreatment of these cells with E_2 rapidly elevated the protein level of mitogen-activated protein kinase phosphatase 2 (MKP-2) and inhibited fMLP-induced ERK phosphorylation. Pretreatment of these cells with estrogen receptor (ER) antagonist ICI 182780 reversed the inhibition of fMP-induced cell migration and superoxide production, and the induction of MKP-2 expression and the suppression of fMP-induced ERK phosphorylation by E_2. However, pretreatment of cells with G-protein coupled ER antagonist G15 had no such effect. Collectively, these results demonstrate that fMLP stimulates neutrophil chemotaxis and superoxide production through activating ERK, and indicate that ER-mediated upregulation of MKP-2 may dephosphorylate ERK and contribute to the inhibitory effect of E_2 on neutrophil activation by fMLP. Our study reveals new mechanisms involved in the anti-inflammatory activity of estrogen.

1. Introduction

Neutrophils constitute the first line of host defenses against invading microorganisms. They migrate to the site of inflammation and infection along a concentration gradient of locally produced chemotactic molecules, including formyl-peptides derived from bacterial proteins or mitochondria of disrupted cells [1]. The peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) was one of the first identified leukocyte chemoattractant. Activated neutrophils phagocytose invading microorganism or necrotic cells, produce and release proteinases, reactive oxygen species (ROS), proinflammatory mediators, and form neutrophil extracellular traps, which are beneficial for destroy of pathogens and the repair of injured tissues [1–3]. However, inappropriate activation of neutrophils also contributes to tissue damage during autoimmune and inflammatory diseases [1,4].

Beside its classical functions in reproduction, estrogen is an important regulator of immune and inflammatory responses. Clinical and experimental observations showed that estrogens significantly influenced the incidence and/or the course of several autoimmune diseases, as well as bacterial and parasitic infection [5–8]. In vivo studies showed that estrogen protected organ and tissue function by inhibiting neutrophil infiltration mediated inflammation under pathological conditions, such as ischemia reperfusion injury [9,10], and trauma-hemorrhage [11,12]. In vitro studies showed that 17β-estradiol (E_2) reduced

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the chemotaxis [13,14] and superoxide production of neutrophils [15–18], but the underlying mechanisms are not fully understood.

Estrogens exert their actions through the activation of three known estrogen receptors (ERs), ERα, ERβ and a G-protein coupled ER (GPER or GPR30). The genomic action of estrogen is mediated by nuclear ERs; the nongenomic action of estrogen is mediated by membrane ERs and GPER [19,20]. Activation of ERα, ERβ and GPER by estrogen can induce MAP kinase ERK1 and ERK2 (ERKs) phosphorylation in various types of cells through nongenomic pathways [21–24]. Besides activation of ERKs, GPER is reported to mediate the inhibitory effect of estrogen on epidermal growth factor induced ERKs phosphorylation in human breast carcinoma cells [25]. Activation of ERKs participates in various functions of neutrophils, such as chemotaxis, adhesion, phagocytosis, granule secretion and respiratory burst [26–32]. It has been reported that fMLP induces ERKs phosphorylation in neutrophils through formyl peptide receptor 1, and fMLP stimulate neutrophil migration and superoxide production through activating ERKs [27,29]. Whether estrogen inhibits fMLP induced neutrophil migration through interfering ERK phosphorylation by fMLP and which type of ER mediated the effect of estradiol remains investigation.

In this study, we examined the effect of E_2 on fMLP-induced chemotaxis and superoxide production in HL-60 differentiated neutrophil-like cells (dHL-60) [33,34] and human neutrophils, and explored the underlying mechanisms.

2. Materials and methods

2.1. Reagents

17β-Estradiol (E_2), fMLP, ferricytochrome C and superoxide dismutase (SOD) were purchased from Sigma-Aldrich (St. Louis, MO). PD98059 was obtained from Cell Signaling Technology (Danvers, MA). Thiazolyl blue tetrazolium bromide (MTT) was from Beyotime Biotechnology (Shanghai, China). ICI 182780 and G15 were from ApexBio (Houston, USA). RPMI 1640 medium was from Gibco (New York, USA). PD98059, E_2, ICI 182780 and G15 were dissolved in DMSO and diluted with different buffers in different experiments before use. In the following experiments, the control vehicle contained the same concentration of DMSO as in single drug treatment, or contained the final concentration of DMSO as in multiple-drug treatment.

2.2. Cell isolation, culture and treatment

Neutrophils were isolated from venous blood of healthy adult male donors by density gradient centrifugation using a Neutrophil Separation Kit (Tianjin Haoyang Commercial Co., Ltd., Tianjin, China). Briefly, 5 ml of heaprinized blood was laid carefully onto 5 ml of the separation solution in a tube, and centrifuged at 500 *g* for 25 min at room temperature. The second layer of cells (neutrophils) was collected, incubated with red blood cell lysis buffer at 37 °C for 5 min, and washed with PBS. Neutrophils were suspended in Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+} ($HBSS^-$) and kept at 4 °C before use. The viability of neutrophils was > 98%, as determined by Trypan blue exclusion test. The protocol was approved by the Ethics Committee of Ruihua Affiliated Hospital of Soochow University and blood samples were obtained with the donors' written informed consent.

HL-60 cells were cultured at density of 2×10^6/ml in RPMI 1640 medium containing L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 10% fetal bovine serum at 37 °C in a humidified incubator with 5% CO_2, and differentiated into neutrophils-like cells (dHL-60 cells) by incubation with 1.3% dimethyl sulfoxide (DMSO) for 4 days as previously described [35]. The expression of CD11b before and after differentiation was examined by Western blot.

Neutrophils or dHL-60 cells were washed with PBS or $HBSS^-$ twice, suspended in HBSS (with Ca^{2+} and Mg^{2+}) and incubated with indicated concentrations of E_2, PD98059 or vehicle for 15 min, then treated with or without 100 nM fMLP for different periods of time at 37 °C. The reaction was terminated by adding cold PBS or $HBSS^-$. The expression of MKP-2 and phosphorylation of ERK1/2 was examined by Western blot.

2.3. Cell viability assay

Cell viability was determined by MTT assay. dHL-60 cells seeded in a 96-well plate were stimulated with vehicle, different concentrations of E_2, fMLP, or the combination of E_2 and fMLP for 3 h. Human neutrophils seeded in a 96-well plate were stimulated with vehicle, G15 or ICI 182780 for 3 h. Ten microliter of MTT at 5 mg/ml was added to each well, and the cells were incubated for 4 h at 37 °C in a humidified incubator with 5% CO_2. One hundred microliter MTT solvent (0.1 g/ml sodium dodecyl sulfate, 10 mM HCl and 5% isobutanol) was added to each well to solubilize formazan crystals. After shaking for 12 h at 37 °C, the optical density (OD) was measured at 570 nm with a reference filter of 630 nm using a microplate reader.

2.4. Superoxide measurement

Superoxide production was measured by SOD-inhibitable cytochrome C reduction assay [36]. Briefly, dHL60 cells or human neutrophils (1×10^6/ml) were suspended in HBSS and supplemented with 0.5 mg/ml ferricytochrome C with or without 150 U/ml SOD. After incubation at 37 °C for 5 min, cells were added into 96-well microplate (200 μl/well) and treated with different concentrations of E_2 or 20 μM PD98059 for 15 min. Superoxide production was stimulated with 1 μM fMLP and measured by recording absorbance at 550 nm over a 21-min period with a microplate reader (Thermo Scientific instruments, Multiskan Go).

2.5. Chemotaxis assay

Chemotaxis of neutrophils or dHL-60 cells was measured by the transwell migration assay. Cells suspended in chemotaxis buffer (RPMI 1640 with 1% BSA, 25 mM Hepes, pH 7.4) at 1×10^6 cells/ml were treated with vehicle or different drugs (E_2, PD98059, ICI 182780, G15, E_2 + ICI 182780, E_2 + G15) for 15 min at 37 °C. Then 100 μl of cell suspension was placed in the upper compartment of a transwell chamber featuring uncoated polyester membrane with 3 μm pores (Costar, Corning, NY), and 600 μl of chemotaxis buffer containing 100 nM fMLP was added to the bottom chamber. After incubation for 60 min at 37 °C in a humidified incubator with 5% CO_2, the membrane was fixed and stained. The cells migrated through the membrane were counted in 5–10 fields under a microscope with a magnification of 400×. The results are expressed as the mean ± SD of migrated cells/field of triplicate samples. In addition, untreated neutrophils were placed in the upper compartment of a transwell to examine cell migration in response to E_2, G15 or ICI 182780 in the bottom chamber.

2.6. Immunoblotting

Neutrophils, HL-60, or dHL-60 cells were centrifuged, lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. The supernatant was collected by centrifugation and the protein concentration was measured using the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Western blotting was performed following standard protocols. Primary antibodies against p38, JNK, phosphorylated ERK, p38 and JNK, as well as MKP-2 were from Cell Signaling Technology (Danvers, MA), anti-ERK antibody was obtained from Abcam (London, UK). HRP-conjugated secondary antibody was from Bioss (Beijing, China). The target proteins were detected using ECL luminescent liquid (Beyotime Biotechnology, Shanghai, China). Representative blots from at least three independent experiments are shown. The Western blot bands were quantified with ImageJ software.

The intensity of each target protein band is divided by the intensity of the internal loading control for that sample. The ratio of target protein to loading control of cells with treatment is then divided by the ratio of untreated control cells.

2.7. Statistical analysis

All results are expressed as mean ± SD. Statistical differences between two groups were analyzed with unpaired two-tailed Student's *t* test. Data sets that involved more than two groups were assessed using One Way ANOVA followed by Dunnett or Bonferroni post hoc test. All statistical analyses were performed using SPSS 19.0 software. A p value of less than 0.05 was considered significantly different.

3. Results

3.1. E_2 inhibited neutrophil chemotactic response to fMLP

We first examined the effect of E_2 on dHL-60 cell and human neutrophil migration in response to fMLP. HL-60 cells were differentiated into neutrophil-like cells (dHL-60) by DMSO. Western blot showed that the expression of CD11b, a marker of neutrophil, increased significantly after 4 days of DMSO treatment (Fig. 1A), indicating successfully differentiation of HL-60 cells into neutrophil-like cells. MTT assay showed that treatment of dHL-60 cells with E_2 at 1, 100 nM; with fMLP at 0.1, 1 μM, or with the combination of E_2 and fMLP for 3 h had no significant effect on cell viability (Fig. 1B). While fMLP significantly induced human neutrophil and dHL-60 cell migration (Fig. 1C, D), E_2 itself at 1 and 100 nM had no effect on neutrophil migration (Fig. 1C). Pretreatment with E_2 at 100 nM significantly inhibited the chemotactic response of dHL-60 cells to fMLP (Fig. 1D). The inhibitory effect of E_2 on fMLP-induced cell migration was observed in human neutrophils at 1 and 100 nM (Fig. 1E), indicating that E_2 suppresses neutrophil migration at both physiological and pharmacological concentrations.

3.2. E_2 reduced fMLP-induced superoxide production in dHL-60 cells and neutrophils

We then examined the effect of E_2 on fMLP-stimulated superoxide production in human neutrophils. One micromole fMLP significantly induced superoxide production in human neutrophils. E_2 itself at 1 and 100 nM showed weak inductive effect on superoxide production in neutrophils (Fig. 2A). E_2 at 1 and 100 nM both inhibited fMLP-induced superoxide production in human neutrophils (Fig. 2B). These data demonstrate that physiological and pharmacological concentrations of E_2 inhibit fMLP-induced superoxide production in neutrophils.

3.3. E_2 inhibits fMLP-induced ERK phosphorylation in dHL-60 cells and neutrophils

It has been reported that ERK activation plays important role in fMLP-induced neutrophil chemotaxis [27]. Consistently, our results showed that pretreatment of dHL-60 cells or human neutrophils with ERK inhibitor PD98059 significantly attenuated the migration of these cells in response to fMLP (Fig. 3A, B). Furthermore, we found that pretreatment of dHL-60 cells or human neutrophils with PD98059 significantly reduced superoxide production induced by fMLP (Fig. 3C, D), which indicate that ERK plays a critical role in fMLP-induced neutrophil superoxide production. To determine if E_2 inhibits fMLP-induced neutrophil migration and superoxide production through inhibiting ERK activation by fMLP, we examined the effect of E_2 on ERK phosphorylation by fMLP. We found that pretreatment of dHL-60 cells

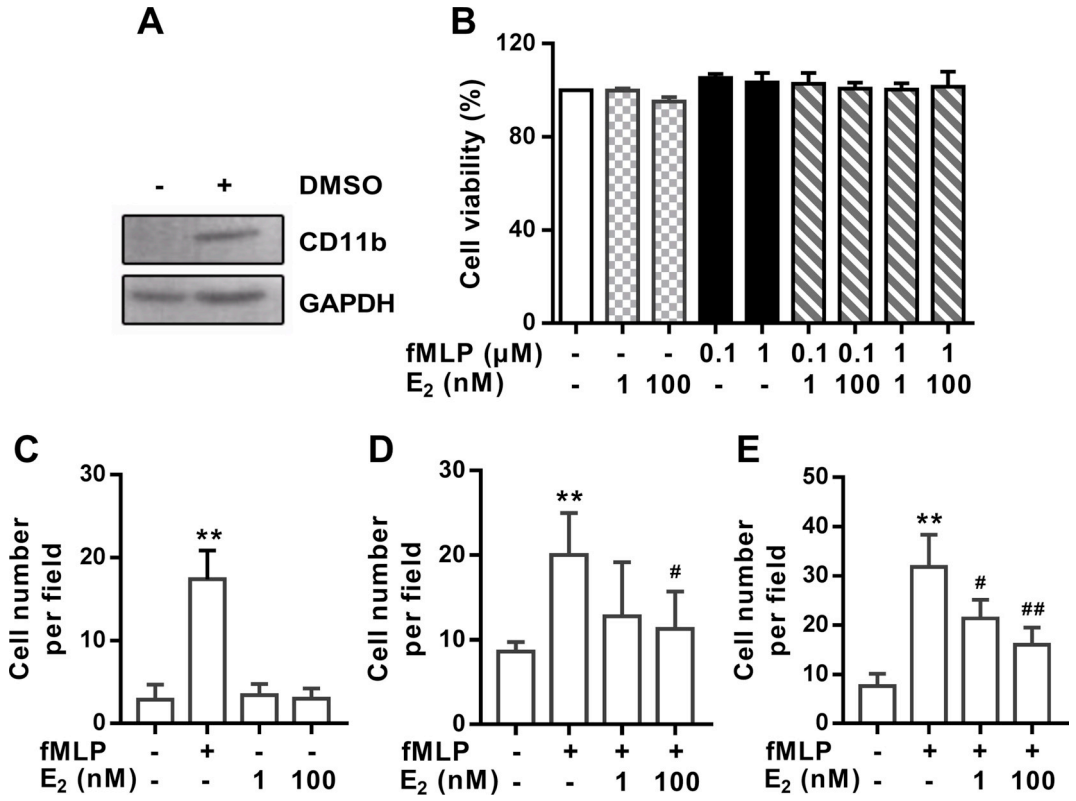

Fig. 1. Estradiol inhibits neutrophils chemotactic response to fMLP. A. HL-60 cells were treated with 1.3% DMSO for 4 days and examined for CD11b expression by Western blot. B. dHL-60 cells were treated with different concentrations of estradiol (E_2), fMLP or the combination of E_2 and fMLP for 3 h. Cell viability was measured by MTT assay. C. Human neutrophils were examined for cell migration in response to 0.1 μM fMLP and different concentrations of E_2. D-E. dHL-60 cells (D) or human neutrophils (E) were incubated with or without different concentrations of E_2 for 15 min, and examined for cell migration in response to 0.1 μM fMLP. Data are mean ± SD, $n = 5$. $^{**}p < 0.01$, compared with untreated cells. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with cells in response to fMLP.

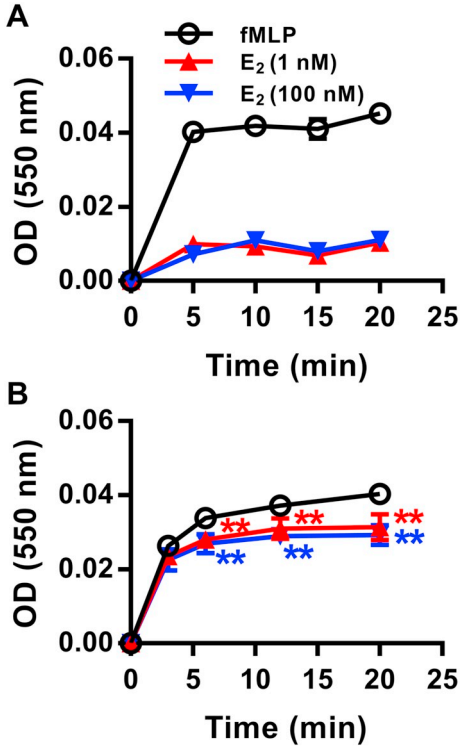

Fig. 2. Estradiol suppresses fMLP-induced superoxide production in neutrophils. A. Human neutrophils were stimulated with 1 μM fMLP or different concentrations of estradiol (E_2) and measured superoxide production at different periods of time after stimulation. B. Human neutrophils pretreated with or without different concentrations of E_2 for 15 min were stimulated with 1 μM fMLP and examined for superoxide production after stimulation. Data are mean ± SD. n = 5. **p < 0.01, compared with cells in response to fMLP alone.

with PD98059 significantly decreased ERK phosphorylation induced by fMLP (Fig. 3E). While E_2 had no significant effect on ERK phosphorylation (Fig. 3F), it inhibited fMLP-induced ERK phosphorylation in a dose dependent manner (Fig. 3G). The inhibitory effect of E_2 on ERK phosphorylation induced by fMLP was also observed in human neutrophils (Fig. 3H). In addition to ERK, MAPKs p38 and JNK are also involved in the migration of neutrophils and other types of cells [37,38]. We found that pretreatment of neutrophils with E_2 had no significant on p38 and JNK phosphorylation in response to fMLP (Fig. 3I). All together, these results demonstrate that E_2 inhibits neutrophil migration and superoxide production in response to fMLP through inhibiting ERK activation by fMLP.

3.4. E_2 upregulated MKP-2 expression in dHL-60 cells and neutrophils

To further explore the mechanisms involved in the inhibition of ERK phosphorylation by E_2 in fMLP-stimulated neutrophils, we examined the effect of E_2 on the expression of MKP-2, a phosphatase which could dephosphorylate ERK. We found that E_2 rapidly induced MKP-2 expression in dHL-60 cells. E_2 upregulated MKP-2 expression in dHL-60 cells in dose- and time-dependent manners (Fig. 4A). E_2 also enhanced MKP-2 expression in human neutrophils (Fig. 4B). These results indicate that E_2 may reduce fMLP-induced ERK phosphorylation by increasing the expression of MKP-2.

3.5. E_2 inhibits fMLP-induced neutrophil activation, ERK phosphorylation and upregulates MKP-2 expression through estrogen receptor

The physiological and pharmacological effects of E_2 are mediated by classic ERs and GPER (GPR30). By using ER antagonist ICI 182780 and GPER antagonist G15, we examined the involvement of these receptors in the inhibitory effect of E_2 on the activation of neutrophils by fMLP. As shown in Fig. 5A and B, pretreatment of human neutrophils with 1 μM ICI 182780 significantly attenuated the inhibitory effect of E_2 on fMLP-induced cell migration and superoxide production, indicating that E_2 inhibits neutrophil migration and superoxide production in response to fMLP through ER. Furthermore, we found that pretreatment of neutrophils with 1 μM ICI 182780 inhibited E_2-induced MKP-2 expression (Fig. 5C), and the inhibition of E_2 on fMLP induced ERK phosphorylation was reversed by ICI 182780 (Fig. 5D). However, pretreatment of neutrophils with 1 μM G15 had neither effect on the inhibitory effect of E_2 on fMLP-induced cell migration, superoxide production and ERK phosphorylation, nor the inductive effect of E_2 on MKP-2 expression (Fig. 5A-D). ICI 182780 and G15 at 1 μM both had no effect on neutrophil viability (Fig. 5E), neutrophil migration (Fig. 5F), MKP-2 expression (Fig. 5H), ERK phosphorylation (Fig. 5I), and fMLP-stimulated superoxide production (Fig. 5G)/ERK phosphorylation (Fig. 5J). These results indicate that E_2 inhibits fMLP induced neutrophils migration and superoxide production through upregulating MKP-2 which in turn dephosphorylats ERK.

4. Discussion

Estrogen has been reported to exert an anti-inflammatory effect by inhibiting neutrophil chemotaxis [13,14] and superoxide production [15–18]. In the present study, we found that pretreatment of dHL-60 cells and human neutrophils with E_2 at physiological and pharmacological concentrations both significantly inhibited fMP-induced cell migration and superoxide production. As fMLP has been reported to induce neutrophil chemotaxis and superoxide production through activating ERKs [26,27,29,39], we examined the effect of E_2 on ERK phosphorylation by fMLP in dHL-60 cells and human neutrophils. Although estrogen has been reported to induce ERKs phosphorylation through ERα, ERβ and GPER in different types of cells [21–24], we found that E_2 had no effect on ERKs phosphorylation in neutrophils (Figs. 3F, 5I). These data indicate that E_2 may affect ERK activity in a cell-dependent manner. We found that E_2 at physiological and pharmacological concentrations could significantly suppress fMLP-induced ERK phosphorylation, indicating that E_2 inhibits neutrophil activation in response to fMLP through inhibiting ERK activation.

MKP-2 (DUSP4) is a member of MAPK phosphatase family which negatively regulates MAPK signaling through dephosphorylation of ERKs, and/or c-Jun N-terminal kinase (JNK), p38 [40]. MKP-2 is preferentially inactivates ERK and JNK over p38 [40,41]. Despite our knowledge of the biochemical and structural basis for the catalytic mechanism of the MKP-2 and its function [41], we know much less about its regulation. MKP-2 can be upregulated at transcriptional level by HoxA10 and p53 [42,43]. GnRH induces MKP-2 gene transcription in pituitary gonadotropes via activation of PKC-ERK-Egr and Ca^{2+}-ERK pathways [44–46]. Human chorionic gonadotropin and cAMP up-regulates MKP-2 in Leydig cells through PKA and ERKs mediated MKP-2 transcription and a posttranslational modification that increases MKP-2 half-life [47]. ERK upregulates MKP-2 at posttranslational level by phosphorylation of MKP-2 that inhibits its degradation by proteasomes [48–50]. In our study, we found that E_2 rapidly (5 min) elevated MKP-2 protein level in dHL-60 cells and human neutrophils (Fig. 4), and pretreatment of these cells with E_2 significantly suppressed fMLP-induced ERK phosphorylation. These results indicate that E_2 inhibit fMLP-induced ERKs phosphorylation through upregulating MKP-2. As E_2 had no effect on ERKs phosphorylation in neutrophils, we propose that E_2 may increase MKP-2 level at posttranslational level through signaling molecules other than ERK. This possibility remains further investigation.

dHL-60 cells and human neutrophils express ERα, ERβ and GPER [51,52]. By using ER antagonist ICI 182780 and GPER antagonist G15, we demonstrated that E_2 attenuated fMLP-induced dHL-60 cell and

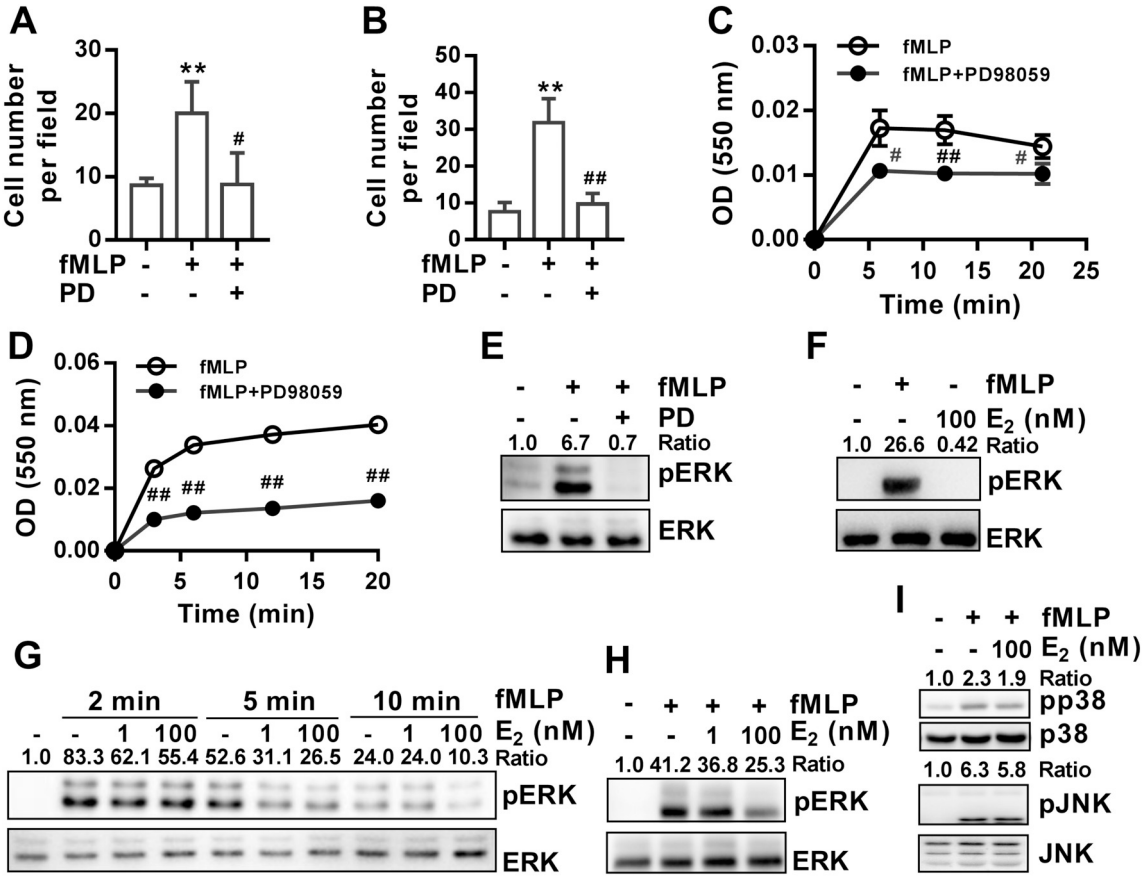

Fig. 3. Estradiol inhibits fMLP-induced dHL-60 cell/neutrophil migration and superoxide production through suppressing ERK phosphorylation in response to fMLP. A-E and G-I. dHL-60 cells (A, C, E,G) or human neutrophils (B, D, H,I) pretreated with different concentrations of estradiol (E_2) or 20 μM PD98059 (PD) for 15 min were examined for cell migration in response to 0.1 μM fMLP (A, B) and superoxide production (C, D) in response to 1 μM fMLP, or stimulated with 0.1 μM fMLP for 5 min (E, H,I) or different periods of time (G), and detected the phosphorylation of ERK, p38 or JNK by Western blot. F. Human neutrophils were stimulated with 0.1 μM fMLP or 100 nM E_2 for 5 min and examined for ERK phosphorylation. Data are mean ± SD, n = 5. **p < 0.01, compared with untreated cells. #p < 0.05, ##p < 0.01, compared with cells in response to fMLP. The images are representative results of 3 independent experiments.

human neutrophils migration and superoxide production through ER but not GPER (Fig. 5A,B). In support of our results, Ito et al. [13] reported that selective estrogen receptor modulators clomiphene and tamoxifen inhibited the chemotaxis of human neutrophils in response to fMLP. Abrahams et al. [17] reported that E_2 inhibited PMA-induced O^{2-} production in neutrophils via ER. Furthermore, our results showed that the upregulation of MKP-2 expression and inhibition of fMLP-induced ERKs phosphorylation by E_2 are also mediated by ER. These results support that E_2 suppresses fMLP-induced neutrophils migration and superoxide production via ER-mediated upregulation of MKP-2 expression and inhibition of ERKs activation.

In summary, we demonstrated that E_2 inhibited fMLP-induced neutrophil migration and superoxide production at physiological and pharmacological concentrations, and provided evidence that E_2 suppressed neutrophil activation by fMLP through ER mediated upregulation of MKP-2 and inhibition of ERKs phosphorylation. Our study reveals new mechanisms involved in the anti-inflammatory activity of estrogen.

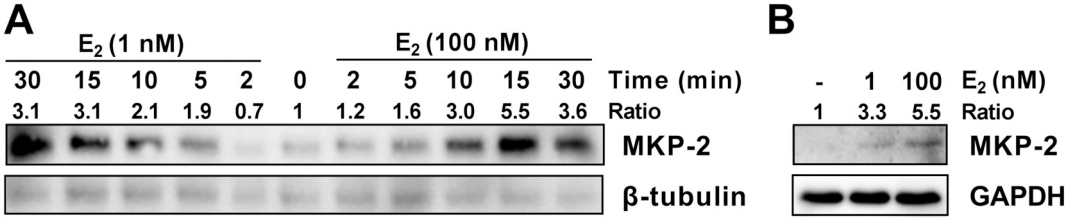

Fig. 4. Estradiol upregulates MKP-2 expression in dHL-60 cells and neutrophils. dHL-60 cells (A) or human neutrophils (B) were treated with different concentrations of estradiol (E_2) for different periods of time (A) or 15 min (B), and examined for MKP-2 expression by Western blot. The images are representative results of 3 independent experiments.

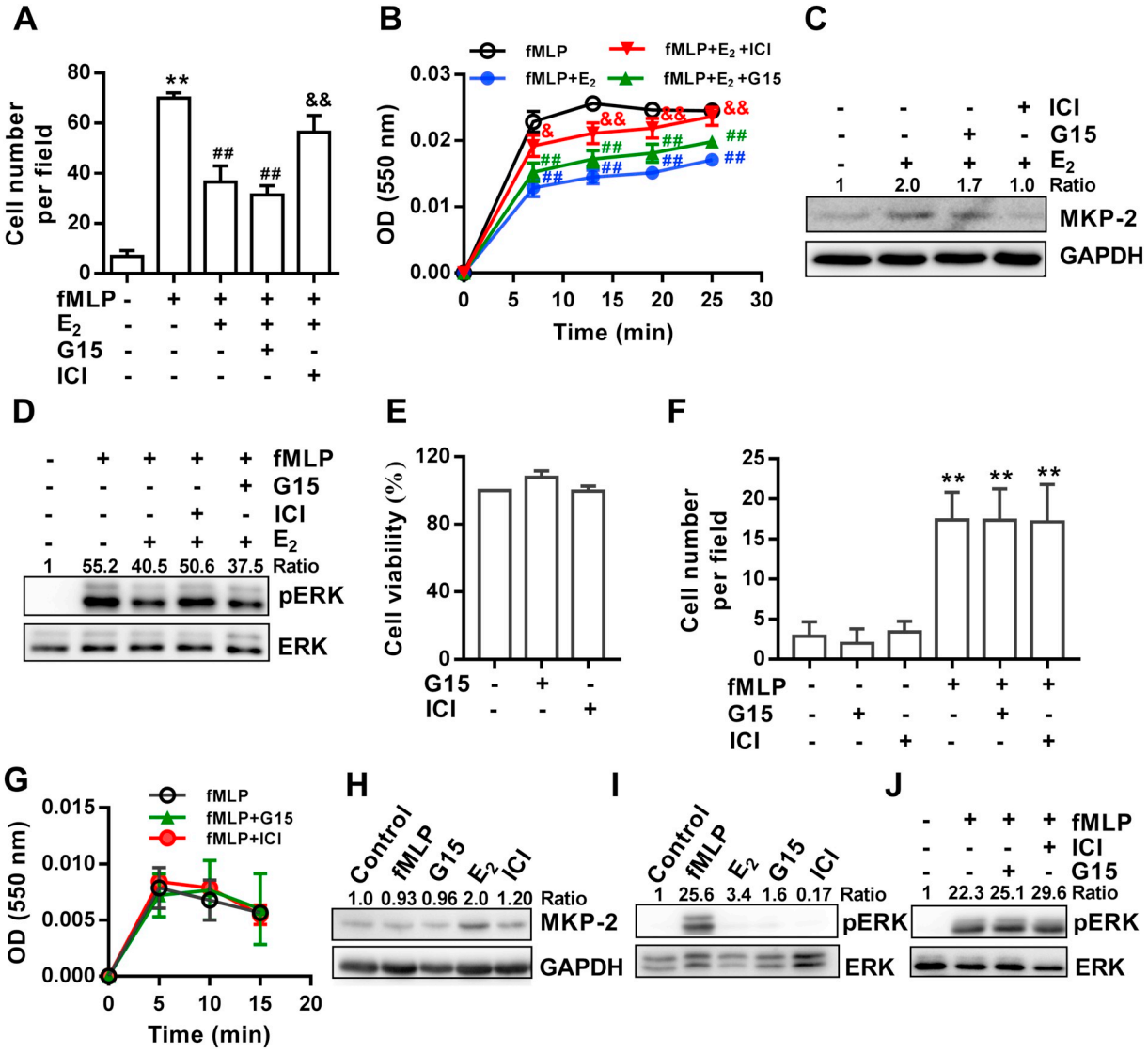

Fig. 5. Estradiol inhibits fMLP-induced cell migration, superoxide production, ERK phosphorylation, and upregulates MKP-2 expression in neutrophils through estrogen receptor. A-D. Human neutrophils pretreated with or without 1 μM G15 or ICI182780 for 30 min were incubated with estradiol (E_2) for 15 min, then examined for cell migration (A), superoxide production (B) and ERK phosphorylation (D) in response to fMLP, or detected MKP-2 expression (C). *E*-J. Human neutrophils treated with 1 μM G15 or ICI182780 were examined for cell viability (E), cell migration (F), MKP-2 expression (H), and ERK phosphorylation (I), fMLP-stimulated superoxide production (G)/ERK phosphorylation. fMLP concentrations: 0.1 μM to induce cell migration and ERK phosphorylation, 1 μM to induce superoxide production. Data are mean ± SD. n = 5. $^{**}p < 0.01$, compared with untreated cells; $^{\#\#}p < 0.01$, compared with cells treated with fMLP alone; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, compared with cells treated with E_2 and fMLP. The images are representative results of 3 independent experiments.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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