17 β -estradiol down-regulates lipopolysaccharide-induced MCP-1 production and cell migration in vascular smooth muscle cells

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Abstract

Atherosclerosis is an inflammatory disease where lipopolysaccharide (LPS) triggers the release of inflammatory cytokines that accelerate its initiation and progression. Estrogen has been proven to be vasoprotective against atherosclerosis; however, the anti-inflammatory function of estrogen in the vascular system remains obscure. In this study, we investigated the effect of estrogen on LPS-induced monocyte chemoattractant protein-1 (MCP-1; listed as CCL2 in the MGI database) production in vascular smooth muscle cells (VSMCs). LPS significantly enhances MCP-1 production and this is dependent on nuclear factor κ B (NF κ B) signaling, since the use of NF κ B inhibitor pyrrolidine dithiocarbamate or the silencing of NF κ B subunit p65 expression with specific siRNA largely impairs LPS-enhanced MCP-1 production. On the contrary, 17 β -estradiol (E₂) inhibits LPS-induced MCP-1 production in a time- and dose-dependent manner, which is related to the suppression of p65 translocation to nucleus. Furthermore, p38 MAPK is rapidly activated in response to LPS, while E₂ markedly inhibits p38 MAPK activation. Transfection with p38 MAPK siRNA or the use of p38 MAPK inhibitor SB203580 markedly attenuates LPS-stimulated p65 translocation to nucleus and MCP-1 production, suggesting that E₂ suppresses NF κ B signaling by the inactivation of p38 MAPK signaling. LPS promotes VSMCs migration and this is abrogated by MCP-1 antibody, implying that MCP-1 may play a major role as an autocrine factor in atherosclerosis. In addition, E₂ inhibits LPS-promoted cell migration by downregulation of MCP-1 production. Overall, our results demonstrate that E₂ exerts anti-inflammatory property antagonistic to LPS in VSMCs by reducing MCP-1 production, and this effect is related to the inhibition of p38 MAPK/NF κ B cascade.

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Introduction

Atherosclerosis is an inflammatory disease (Ross 1999). It is characterized by the formation of atherosclerotic plaques where cellular immune response takes place. Bacterial peptidoglycans, chlamydiae, or viruses have been found in the atherosclerotic lesions (Laman *et al.* 2002, Liu *et al.* 2006). Among these etiological factors, lipopolysaccharide (LPS), an outer-membrane component of Gram-negative bacteria, is the most important one responsible for vascular inflammation (Lehr *et al.* 2001, Kiechl *et al.* 2002). In fact, LPS even at low levels constitutes a strong risk factor for the development of atherosclerosis (Wiedermann *et al.* 1999), and it constitutes the trigger for events related to a possible link between periodontitis and atherosclerosis (Hettne *et al.* 2007). Similarly, in rat models, chronic exposure to LPS leads to atherosclerotic lesions in arteries (Smith *et al.* 2009). By binding its central signaling receptor Toll-like receptor 4, LPS activates a cluster of cells, including monocytes, macrophages, lymphocytes,

vascular endothelial cells (VECs), and vascular smooth muscle cells (VSMCs; Galkina & Ley 2009). These cells then produce a variety of inflammatory cytokines, which are responsible for the initiation and acceleration of atherosclerotic lesions (Liao 1996, Loppnow *et al.* 2008).

Monocyte chemoattractant protein-1 (MCP-1; listed as CCL2 in the MGI database), a member of the CC chemokine family, is one of the inflammatory cytokines that can be produced by VECs and VSMCs in response to LPS (Yang *et al.* 2005, Anand *et al.* 2009). The deleterious effects of this cytokine in the cardiovascular system have been demonstrated in MCP-1 or MCP-1 receptor CCR2 knockout mice, with those mice developing smaller atherosclerotic lesions (Boring *et al.* 1998, Gu *et al.* 1998). In contrast, mice overexpressing MCP-1 had larger lesions (Aiello *et al.* 1999). The mechanisms underlying the pro-atherogenic effects of MCP-1 are complex. One crucial mechanism is that MCP-1 recruits and enhances VSMC migration towards subendothelial area, thus accelerating the progression

of atherosclerosis (Spinetti *et al.* 2004, Ma *et al.* 2007). In this regard, it has been reported that LPS is able to stimulate VSMCs migration (Lin *et al.* 2007). These observations highlight the possibility that MCP-1 may act as an important mediator in LPS-induced cell migration.

Hormone replacement therapy (HRT) is believed to induce cardiovascular protection (Mendelsohn & Karas 1999), while clinical trials have challenged this assumption (Rossouw *et al.* 2002). Recently, the Women's Health Initiative subgroup analysis suggested that HRT is still effective to reduce cardiovascular risk in young postmenopausal women (Rossouw *et al.* 2007). The cardiovascular protective effects of estrogen are largely attributed to its direct actions on vasculature (Fu & Simoncini 2007). Currently, it is well recognized that estrogen functions as an immunomodulator in the cardiovascular system. It exerts anti-inflammatory effects on the vasculature through different mechanisms. On the other hand, estrogen also elicits pro-inflammatory changes under certain conditions (Chakrabarti *et al.* 2008). Estrogen has been shown to antagonize LPS-induced inflammatory responses in several organ systems, such as brain (Pozzi *et al.* 2006), lung (Speyer *et al.* 2005), and vasculature (Corbacho *et al.* 2007, Liu *et al.* 2009). Nevertheless, the intrinsic mechanisms of how estrogen acts as an anti-inflammatory agent against LPS still need to be elucidated. In the present study, we investigated the inhibitory effect of estrogen on LPS-stimulated VSMCs migration and the role of MCP-1 in this effect. In particular, the signaling events recruited by estrogen receptors (ERs) were characterized.

Materials and methods

Cell cultures and treatments

Cell culture

VSMCs were isolated from rat thoracic aorta (female Sprague-Dawley, 6–10 weeks old, provided by the Animal Center of Sun Yat-sen University; Certificate number: SCSK (Yue) 2008–2009/0034) by enzymatic dispersion and cultured over several passages. Cells were grown in a 5% CO₂ atmosphere at 37 °C in DMEM without phenol, and supplemented with penicillin and streptomycin, and 10% fetal bovine serum charcoal was stripped (steroid free and delipidated, FCS; Biowest, S181F-500, Nuaille, France). Experiments were performed with cells from passages 5 to 10. Before experiments, cells were kept in phenol red-free DMEM containing 1% FBS for 48 h. Cultures were pretreated with different concentrations of 17 β -estradiol (E₂; Sigma) for the indicated times and subsequently stimulated with *Escherichia coli* LPS

(Sigma) for another 24 h. Whenever an inhibitor was used, the compound was added 30 min before starting the treatments. SB203580, pyrrolidine dithiocarbamate (PDTc), pertussis toxin (PTX), and wortmannin (WM) were purchased from Sigma-Aldrich. 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), 2,3-bis-(4-hydroxyphenyl)-propionitrile (DPN), and ICI 182 780 were purchased from Tocris Cookson (Bristol, UK).

Immunoblottings

Cell lysates were separated by SDS-PAGE. Antibodies used were as follows: p38 MAPK and phospho-p38 MAPK (9210; Cell Signaling, Danvers, MA, USA), actin (sc-1615; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and nuclear factor κ B (NF κ B) p65 (sc-8008, Santa Cruz). Primary and secondary antibodies were incubated with the membranes by standard technique. Immunodetection was accomplished using enhanced chemiluminescence. Chemiluminescence was acquired with a quantitative digital imaging system (Quantity One, Bio-Rad) allowing to check for saturation. Overall emitted photons were quantified for each band, particularly for loading controls, which were homogeneously loaded.

ELISA for MCP-1 protein

MCP-1 concentrations in culture medium were measured using an ELISA kit (KRC1011, BioSource, Invitrogen Corporation) according to the manufacturer's instructions. In brief, cells in 24-well plates were serum deprived at 70% confluence. After treatments, media supernatants were collected and centrifuged at 9000 g (10 000 r.p.m.) for 1 min. The supernatants were stored at –80 °C until the assay. Experiments were performed in quadruplicate and verified on at least two occasions. Results were expressed as total supernatant MCP-1 per milligram cell protein (mean $\pm$ s.d.).

Cell immunofluorescence

VSMCs were grown on coverslips. After treatment, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X for 5 min. Blocking was performed with 3% BSA for 30 min. Cells were incubated with NF κ B p65 antibody (sc-8008, Santa Cruz) at 4 °C overnight. After washing, a FITC-conjugated secondary antibody (K00018968; Dako North America Inc., Dako, Carpinteria, CA, USA) was used for 1 h at room temperature. The nuclei were counterstained with 4'-6-diamidino-2-phenylindole (Sigma). Cells were mounted with fluorescence mounting medium (10027230, Dako) and covered with a coverslip. Samples were analyzed with Olympus fluorescence FV1000 confocal microscope.

Transfection experiments and luciferase reporter assay

On-TARGETplus SMARTpool siRNA reagents against rat p38 MAPK α (NM-031020) and control siRNA (D-001810-01-05) were purchased from Dharmacon (Thermo Fisher Scientific Inc., San Jose, CA, USA). p65 siRNA (antisense 5'-AAGAGCATCATGAAGAA-GAGTCCTGTCTC-3', sense 5'-AAACTCTTCTTCATGATGCTCCCTGTCTC-3') was designed by Riobio (Riobio Biotech Corporation, Guangzhou, China) according to a previous report (Lianxu *et al.* 2006). VSMCs were transfected with siRNA using Lipofectamine (Invitrogen) according to the protocol. Cells (40% confluent) were serum-starved for 1 h, followed by incubation with 100 nM target siRNA or control siRNA for 6 h in serum-free media. The serum-containing media were then added (10% serum final concentration) for 42 h before experiments, and/or functional assays were conducted. Target protein silencing was assessed through protein analysis up to 48 h after transfection.

NF κ B transient transfection assays have been reported previously (Wu *et al.* 2008). The cells were transfected using the SuperFect reagent (Qiagen) with a NF κ B luciferase reporter plasmid (Clontech) and a SV40 promoter-driven β -galactosidase expression plasmid to normalize the transfection efficiency. Twenty-four hours later, the culture medium was replaced, and cells were pretreated with E₂ for 12 h following by stimulation with LPS. After 24 h, the cells were lysed, and the luciferase activity was measured according to the manufacturer's instruction using a luminometer.

Cell migration assays and transwell experiments

Adherent cells (2×10^5) were scraped off from the bottom of a culture plate using a pipette tip to create a cell-free (wounded) area. The cell culture was washed with PBS to remove cell debris, and then 2 ml of DMEM containing steroid-deprived FBS and gelatin (1 mg/ml) were added. Cells were incubated with LPS alone or in combination with E₂ for 48 h, and migration was

Figure 1 LPS enhanced MCP-1 production in VSMCs. (a) VSMCs were exposed to increasing concentrations of LPS (0.01–10 µg/ml) for 24 h, and MCP-1 level was measured using ELISA. ** $P < 0.01$ versus control. (b) MCP-1 production was enhanced by treating with LPS (0.1 µg/ml) for the indicated times. ** $P < 0.01$ versus control. (c) VSMCs were pretreated with various concentrations of E₂ (10^{-9} – 10^{-6} mol/l) for 24 h, and subsequently stimulated with 0.1 µg/ml LPS for another 24 h. Then MCP-1 production was measured. ** $P < 0.01$ versus control; # $P < 0.05$ versus LPS group. ## $P < 0.01$ versus LPS group. (d) VSMCs were pretreated with E₂ (10^{-8} mol/l) for different times, and subsequently stimulated with 0.1 µg/ml LPS for another 24 h. MCP-1 production was measured. ** $P < 0.01$ versus control; # $P < 0.05$ versus LPS group. Each experiment was performed in quadruplicate and verified on at least two occasions. Results were expressed as total supernatant MCP-1 per milligram cell protein (mean $\pm$ s.d).

monitored. Fresh medium and treatment were replaced for every 12 h. Cells were digitally imaged, and migration distance was measured by using phase-contrast microscopy.

Transwell experiments were performed as previously described ([Jiang *et al.* 2010]). In brief, cells were seeded into the upper chamber of the transwell chamber (Corning Life Sciences, Lowell, MA, USA). After incubation with LPS alone or in combination with E₂, cells that had invaded to the lower surface of the membranes were fixed with methanol for 10 min and

stained with hematoxylin. The cells on the lower side of the membrane were counted and averaged in six high-power fields (200×) with a light microscope.

Statistical analysis

All values are expressed as mean ± s.d. Statistical differences between mean values were determined by ANOVA, followed by the Fisher's protected least significance difference test. All differences were considered significant at $P < 0.05$.

Results

E₂ inhibited LPS-induced MCP-1 production in VSMCs

VSMCs were exposed to increasing concentration of LPS (0.01–10 µg/ml) for 24 h, and the MCP-1 production was measured by using ELISA. Consistent with data from others (Yang *et al.* 2005), LPS induced MCP-1 production in a concentration-dependent manner, with maximal effect at 10 µg/ml (Fig. 1a). In order to avoid cytotoxic effect, we used a relatively low dose of 0.1 µg/ml LPS as the optimal concentration to assess the time course. As shown in Fig. 1b, MCP-1 production was elevated within 6 h after LPS (0.1 µg/ml) treatment, and was maximal at 24 h.

To investigate the anti-inflammatory role of E₂, VSMCs were pretreated with various concentrations of E₂ (10⁻⁹–10⁻⁶ M) for 24 h, followed by the stimulation with 0.1 µg/ml LPS for another 24 h. Pretreatment with different concentrations of E₂ all caused a significant decrease in LPS-induced MCP-1 production (Fig. 1c). In addition, LPS-induced MCP-1 production was markedly inhibited by pretreatment with 10⁻⁸ M E₂ for 12 h, and this inhibitory effect reached a plateau between 24 and 48 h (Fig. 1d).

E₂ reduced MCP-1 production via the inactivation of NFκB signaling

To characterize the intracellular events implicated in LPS action, we utilized several signaling cascade inhibitors relevant to TLR4 signalings. The blockade of p38 MAPK activity with SB203580 at 20 µM, the concentration widely used in other similar studies (Fatima *et al.* 2001, Gorlach *et al.* 2001), largely reduced MCP-1 production (Fig. 2a). Likewise, the NFκB pathway inhibitor PDTC also led to a marked reduction of MCP-1 production (Fig. 2a). However, no additive

effect was found when both inhibitors were used together (Fig. 2a). On the contrary, neither the G protein inhibitor pertussis toxin (PTX, 100 ng/ml), and of PI3K inhibitor wortmannin (WM-30 nM/l). MCP-1 production was measured. These results suggested that p38 MAPK and NFκB pathways may be involved in LPS-stimulated MCP-1 production.

To confirm the role of NFκB pathway in LPS-induced MCP-1 production, we silenced NFκB subunit p65 expression by using specific siRNA (Fig. 2b). As a result, the silencing of p65 markedly impaired LPS-induced MCP-1 production (Fig. 2c). Indeed, challenge with LPS enhanced nuclear NFκB subunit p65 expression as early as 30 min, and the LPS increase reached the maximum at 1 h (Fig. 2d). This was further confirmed by confocal microscopy showing that while cells are in a quiescent state, p65 protein was mainly located in cytoplasm and only 8% of total cells showed a strongly positive nuclear stain, whereas under LPS stimulation, p65 protein translocated from cytoplasm into nucleus, with 85% of cells positively stained (Fig. 2e and f).

Moreover, to determine whether LPS increases transcriptional activation of NFκB-dependent gene expression, we employed an NFκB promoter luciferase construct. LPS induced approximately a fourfold increase in NFκB promoter-driven luciferase activity (Fig. 2h). On the contrary, the p38 MAPK inhibitor SB203580 efficiently prevented p65 nuclear translocation and luciferase activity (Fig. 2e–h), implying that p38 MAPK is indispensable for NFκB activation.

Pretreatment with 10⁻⁸ M E₂ for 24 h reversed the LPS-evoked p65 expression and reduced the nuclear translocation of p65 in VSMCs (Fig. 2e–g). It also reduced LPS-stimulated luciferase activity (Fig. 2h). Together, these results indicate that E₂ reduces MCP-1 production through the inactivation of NFκB signaling.

Figure 2 E₂ inhibited LPS-induced MCP-1 production through suppression of NFκB pathway. (a) VSMCs were exposed to 0.1 µg/ml LPS for 24 h in the absence or presence of p38 MAPK inhibitor SB203580 (SB-2 × 10⁻⁵ mol/l), of NFκB inhibitor PDTC (2 × 10⁻⁵ mol/l), of G protein inhibitor pertussis toxin (PTX, 100 ng/ml), and of PI3K inhibitor wortmannin (WM-30 nM/l). MCP-1 production was measured. ***P* < 0.01 versus control; **P* < 0.05 versus LPS group. (b) VSMCs were transfected with scrambled siRNA or p65-targeted siRNAs for 48 h. After that, the level of p65 protein expression was detected by western blot. Actin was used as a loading control. (c) VSMCs were transfected with scrambled siRNA or p65-targeted siRNAs for 48 h. Then, cells were exposed to 0.1 µg/ml LPS for 24 h, and MCP-1 production was measured. ***P* < 0.01 versus corresponding control; ##*P* < 0.01 versus corresponding control. (d) VSMCs were exposed to 0.1 µg/ml LPS for different times as indicated. Cell nucleus was extracted, and p65 protein expression was detected by western blot. Nuclear marker histone H3 was used as a loading control. p65 densitometry values were adjusted to histone H2 intensity, and then normalized to expression from the control sample. ****P* < 0.001 versus control; ***P* < 0.01 versus control. (e) VSMCs were exposed to 0.1 µg/ml LPS for 1 h with or without pretreatment with E₂ (10⁻⁸ mol/l) or SB203580 (SB-2 × 10⁻⁵ mol/l) for 24 h. Subcellular localization of the p65 NFκB subunit was assayed with cell immunofluorescence technique. VSMCs were stained with an antibody versus p65 (FITC; green staining), and nuclei were counterstained with DAPI (blue staining). (f) Cells were counted in six different fields, and the percentage of cells with p65 nuclear staining was expressed as means ± s.d. ***P* < 0.01 versus control; ****P* < 0.01 versus LPS. (g) VSMCs were treated as indicated. Cell nucleus was extracted, and p65 protein expression was detected by western blot. (h) VSMCs were transfected with NFκB promoter luciferase construct, and then treated for 12 h with E₂ (10⁻⁸ mol/l), followed by stimulation with 0.1 µg/ml LPS for 24 h. After that, the NFκB reporter was measured and normalized to β-galactosidase activity. Values shown represent the mean ± s.d. of triplicate experiments. ***P* < 0.01 versus control; ##*P* < 0.01 versus LPS.

E₂ suppressed NFκB cascade via the dephosphorylation of p38 MAPK pathway

We further checked the role of p38 MAPK in LPS-recruited signalings. By using specific siRNA for p38 MAPK, we silenced its expression in VSMCs (Fig. 3a). As a consequence, the silencing of p38 MAPK significantly reduced MCP-1 production induced by LPS (Fig. 3b). In addition, LPS resulted in a rapid increase of p38 MAPK phosphorylation (Fig. 3c). These data indicate that p38 MAPK is activated by LPS and is responsible for MCP-1 production.

To clarify the relevance between p38 MAPK and the NFκB cascade, we silenced their expression by using specific siRNA. LPS-induced p65 expression in the nucleus was prevented in p38 MAPK siRNA-transfected VSMCs compared with control (Fig. 3d), while the silencing of p65 expression did not alter LPS effect on p38 MAPK phosphorylation (Fig. 3e). These findings imply that p38 MAPK functions as the upstream of NFκB cascade.

Pretreatment with 10^{-8} M E₂ for 12, 24, and 48 h all decreased LPS-stimulated p38 MAPK phosphorylation (Fig. 3f). This inhibitory effect was mimicked by the ERα (ESR1) agonist PPT, but not by the ERβ (ESR2) agonist DPN (Fig. 3g). Moreover, the inhibitory effect of E₂ on p38 MAPK phosphorylation was prevented by ER antagonist ICI 182 780 (Fig. 3g).

E₂ inhibited LPS-provoked VSMCs migration via the downregulation of MCP-1 production

Finally, we explored the roles of LPS and E₂ in VSMCs migration by using wound assay and by the modified Boyden chamber assay. Exposure of VSMCs to LPS significantly enhanced VSMCs migration distance and migrating cell numbers, which was significantly inhibited by pretreatment with E₂ (Fig. 4a–d). Meanwhile, MCP-1 antibody largely reduced LPS-enhanced cell migration distance and numbers of migrating cells into

the membrane (Fig. 4a–d). MCP-1 antibody or E₂ use alone did not display a significant effect on cell migration (Fig. 4a–d).

Discussion

Estrogen deters atherosclerosis in healthy women, but the exact mechanism is still a matter of investigation. Previously, we and others have demonstrated that estrogen induces vasodilatation, and regulates vascular cell growth and migration as well as protects cardiomyocytes from injury (Simoncini *et al.* 2006, Fu *et al.* 2007, Liu *et al.* 2007, Kublickiene *et al.* 2008). The present study reveals that estrogen exerts anti-inflammatory activity in VSMCs by reducing LPS-induced MCP-1 production, thus inhibiting cell migration. These salutary effects may converge into a protective action of estrogen on the vessels from atherosclerotic lesions.

Atherosclerosis is an inflammatory disease, and estrogen may function as an anti-inflammatory factor (Chakrabarti *et al.* 2008). Indeed, menopause and ovariectomy generate a low grade of systemic inflammation, which can be reversed by the administration of estrogen (Abu-Taha *et al.* 2009). This may be related to the regulatory effects of estrogen on the production of inflammatory cytokines (Georgiadou & Sbarouni 2009). For instance, an *in vivo* study showed that transdermal estrogen therapy reduces circulating levels of several cytokines, including MCP-1 (Yasui *et al.* 2009). In parallel, studies *in vitro* demonstrate that estrogen reduces MCP-1 production in several cells, such as peripheral blood mononuclear cells and immature dendritic cells (Bengtsson *et al.* 2004, Yuan *et al.* 2008). The present study provides the first evidence that estrogen directly acts on VSMCs by decreasing LPS-stimulated MCP-1 production, which adds new insight into estrogen's anti-inflammatory functions in cardiovascular system.

Figure 3 E₂ suppressed NFκB cascade via the dephosphorylation of p38 MAPK pathway. (a) VSMCs were transfected with scrambled siRNA or p38 MAPK-targeted siRNAs for 48 h. After that, the level of p38 MAPK protein expression was detected by western blot. (b) VSMCs were transfected with scrambled siRNA or p38 MAPK-targeted siRNAs for 48 h. Then, cells were exposed to 0.1 μg/ml LPS for 24 h, and MCP-1 production was measured. ***P* < 0.01 versus corresponding control; ##*P* < 0.01 versus corresponding control. (c) VSMCs were exposed to 0.1 μg/ml LPS for different times, and phosphorylated p38 MAPK (p-p38 MAPK) protein expression was detected by western blot. p-p38 MAPK densitometry values were adjusted to total p38 MAPK intensity, and then normalized to expression from the control sample. ***P* < 0.01 versus control. (d) VSMCs were transfected with scrambled siRNA or p38 MAPK-targeted siRNAs for 48 h. Then, cells were exposed to 0.1 μg/ml LPS for 1 h, and nuclear content of p65 expression was detected. p65 densitometry values were adjusted to histone H3 intensity, and then normalized to expression from the control sample. ****P* < 0.001 versus control; ##*P* < 0.01 versus LPS treatment without transfection. (e) VSMCs were transfected with scrambled siRNA or p65-targeted siRNAs for 48 h. Then, cells were exposed to 0.1 μg/ml LPS for 15 min, and p-p38 MAPK expression was detected. p-p38 MAPK densitometry values were adjusted to actin intensity, and then normalized to expression from the control sample. ***P* < 0.01 versus control. (f) VSMCs were pretreated with E₂ (10^{-8} mol/l) for different times, and subsequently stimulated with 0.1 μg/ml LPS for 15 min. Cells were lysed, and p-p38 MAPK expression was detected. p-p38 MAPK densitometry values were adjusted to total p38 MAPK intensity, and then normalized to expression from the control sample. ***P* < 0.01 versus control; ##*P* < 0.01 versus LPS. (g) VSMCs were pretreated with E₂ (10^{-8} mol/l) alone or in combination with ICI 182 780 (10^{-6} mol/l), PPT (10^{-8} mol/l), and DPN (10^{-8} mol/l) for 24 h, and subsequently stimulated with 0.1 μg/ml LPS for 15 min. Cells were lysed, and p-p38 MAPK expression was detected. ***P* < 0.01 versus control; ##*P* < 0.01 versus LPS.

Figure 4 E₂ inhibited LPS-provoked VSMCs migration via the downregulation of MCP-1 production. (a) Adherent VSMCs were scrapped to create a cell-free (wounded) area. Then, cells were incubated with 0.1 µg/ml LPS alone or in combination with E₂ (10⁻⁸ mol/l) or with antibody against MCP-1 (Ab versus MCP-1 – 1:100 dilution) for 48 h, and migration was monitored. Cells were digitally imaged, and migration distance was measured by using phase-contrast microscopy. Representative images of cell migration are shown. (b) Cell migration distances were measured, and values are presented as % of control. ***P* < 0.01 versus control; ##*P* < 0.01 versus LPS group. (c) After incubation with 0.1 µg/ml LPS alone or in combination with E₂ (10⁻⁸ mol/l) or with antibody against MCP-1 (Ab versus MCP-1 – 1:100 dilution) for 48 h, cells that had invaded to the lower surface of the membranes were fixed with methanol for 10 min, and then stained with hematoxylin. Representative images of transwell experiments are shown. (d) The cells on the lower side of the membrane were counted and averaged in six high-power fields (200×) with a light microscope. ***P* < 0.01 versus control; ##*P* < 0.01 versus LPS group.

The migration of VSMCs from arterial media to intima is an essential step in the pathogenesis of atherosclerosis. It has been reported that LPS promotes VSMCs migration, but the mechanism remains unclear (Lin *et al.* 2007). Our data show that LPS promotes VSMCs migration, which is markedly inhibited by MCP-1 antibody, indicating that MCP-1 is an important mediator for LPS action. On the other side, E₂ inhibits LPS-induced VSMCs migration, and this is possibly

related to the decreased MCP-1 production. MCP-1 belongs to the member of the CC chemokine family. It is expressed by various non-immune cells, including VECs and VSMCs (Charo & Taubman 2004). Elevated levels of MCP-1 are observed in atherosclerotic plaques, and mice overexpressing MCP-1 develops larger atherosclerotic lesions (Aiello *et al.* 1999). Currently, the role of MCP-1 in atherosclerosis is predominantly explained by its ability to recruit macrophages and monocytes to

the vessel wall (Harrington 2000). Apart from that, here we demonstrate that MCP-1 directly drives VSMCs migration. In parallel, MCP-1 can also be produced by VECs, and recruits VSMCs migration towards endothelium (Ma *et al.* 2007). Therefore, MCP-1 may function as a potent stimulator for VSMCs migration in both autocrine and paracrine manner. Notwithstanding, the detailed molecular basis for MCP-1's effect on cell migration is still lacking. In this regard, our previous work has demonstrated that actin cytoskeleton remodeling is an essential step for cell migration (Fu *et al.* 2008), and it could be modulated by MCP-1 (Lee *et al.* 2009). These observations hint at the possibility that MCP-1 may induce actin cytoskeleton rearrangement in VSMCs. This, however, is beyond the scope of the present work and will be investigated in future studies.

Our data show that during challenge with LPS, NFκB subunit p65 translocates into the nucleus. NFκB is linked to the development of numerous inflammatory disorders, including atherosclerosis. It is a heterodimeric protein composed of five different proteins (p50, p52, p65/RelA, RelB, and c-Rel). These dimers exist in the cytoplasm in inactive forms that bound to the inhibitory protein IκB (Hayden & Ghosh 2004). Various activating agents can mediate the dissociation of IκB from NFκB and thus translocation of the p65 subunit into the nucleus, where it activates transcription of target genes (Hayden & Ghosh 2004). Correspondingly, analysis of the promoter region of the human *MCP-1* gene demonstrates the binding site for NFκB (Rovin *et al.* 1995), and NFκB activation has been shown to upregulate MCP-1 production in VSMCs (Wang *et al.* 2000, Esteban *et al.* 2005). In line with these, we show that LPS enhances NFκB subunit p65 translocation into nucleus, and promotes NFκB promoter-driven luciferase activity. Moreover, the silencing of p65 markedly impairs MCP-1 production. These findings indicate that NFκB pathway is responsible for MCP-1 production induced by LPS.

Pretreatment with E₂ reverses p65 nuclear translocation and NFκB promoter-driven luciferase activity, implying that E₂ may inhibit MCP-1 expression through the inactivation of NFκB signaling. In support of our findings, it has been shown that p65 nuclear translocation can be prevented by E₂ in VECs, macrophages, and breast cancer cells (Hsu *et al.* 2000, Ghisletti *et al.* 2005, Tiruppathi *et al.* 2008). Currently, little is known about the NFκB transport machinery. In this aspect, it has been reported that dynein-dependent transport may be a conserved mechanism in NFκB activation (Shrum *et al.* 2009). Intriguingly, ER is shown to interact with dynein light chain and regulate its functions (den Hollander & Kumar 2006). Thus, future studies will be needed to address the role of dynein in estrogen's inhibitory effect on NFκB signaling.

p38 MAPK is one of the three major MAPK signaling pathways that participate in inflammatory response (Kyriakis & Avruch 2001, Kaminska 2005). Our study demonstrates that p38 MAPK is rapidly phosphorylated in response to LPS. Silencing of p38 MAPK markedly inhibits LPS-induced p65 translocation to nucleus, leading to decreased MCP-1 production. These results imply that p38 MAPK functions as the critical upstream signal for NFκB. Likewise, it has been demonstrated that NFκB activity is dependent on p38 MAPK activation in other types of cells (Baeza-Raja & Munoz-Canoves 2004, Ryan *et al.* 2007). The exact mechanism of p38 MAPK-induced NFκB activation in VSMCs is poorly understood, and it may be relevant to the effects of p38 MAPK on IκB degradation or p65 transactivation (Baeza-Raja & Munoz-Canoves 2004). p38 MAPK has been verified as the primary determinant of CCL2 transcription, the gene that encodes MCP-1 protein (Lu *et al.* 2009). Therefore, we do not exclude the possibility that p38 MAPK itself translocates into nucleus and regulates the *MCP-1* gene, resulting in enhanced MCP-1 production in response to inflammatory stimuli.

We show that E₂ inhibits LPS-induced p38 MAPK activation in a dose- and time-dependent manner, suggesting that E₂ exerts anti-inflammatory activity by suppression of this pathway. The biological actions of E₂ in the inflammatory response require the participation of specific receptor, namely ERα or ERβ. By using the ER-selective ligands PPT and DPN, we demonstrate that ERα is necessary for p38 MAPK inhibition in VSMCs. These data are consistent with the previous reports showing that ERα is indispensable to estrogen's immune protective effects (Liu *et al.* 2003, Polanczyk *et al.* 2003, Vegeto *et al.* 2003). In contrast, several studies have reported that ERβ mediates estrogen's actions in inflammation (Baker *et al.* 2004, Xing *et al.* 2007). The cause of this discrepancy is still not well defined, and maybe related to the different cell types and the stimuli used in experimental settings.

Taken together, our findings demonstrate that E₂ inhibits LPS-stimulated VSMCs migration by reducing MCP-1 production. This effect is related to the inhibition of the p38 MAPK/NFκB signaling cascade, and ERα appears to be the dominant ER subtype in these events. The characterization of the novel mechanism of E₂ action may help to develop new pharmacological tools for the prevention of inflammatory response in cardiovascular system.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions statement

P Jiang and J Xu designed and carried out the experiments, analyzed the data, drafted, and revised the manuscript; S Zheng, J Huang, and Q Xiang carried out the experiments; X Fu reviewed and revised the manuscript; T Wang designed the experiments, analyzed the data, drafted, and revised the manuscript. All authors read and approved the final manuscript.

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