Acta Pharmacologica Sinica (2009) 30: 1522–1528
 1671-4083/09 $32.00
www.nature.com/aps

Original Article

Fenoterol, a β_2-adrenoceptor agonist, inhibits LPS-induced membrane-bound CD14, TLR4/CD14 complex, and inflammatory cytokines production through β-arrestin-2 in THP-1 cell line

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Aim: To investigate the molecular mechanism and signaling pathway by which fenoterol, a β_2-adrenergic receptor (β_2-AR) agonist, produces anti-inflammatory effects.

Methods: THP-1, a monocytic cell line, was used to explore the mechanism of β_2-AR stimulation in LPS-induced secretion of inflammatory cytokines and changes of toll-like receptors (TLRs). We labeled TLR4 and CD14 using monoclonal anti-TLR4 PE-conjugated and anti-CD14 FITC-conjugated antibodies in THP-1 cells stimulated by β_2-AR in the presence or absence of lipopolysaccharide (LPS) and small, interfering RNA (siRNA)-mediated knockdown of β-arrestin-2, and then analyzed their changes in distribution by flow cytometry, Western blotting and confocal analysis.

Results: LPS-induced membrane-bound CD14, TLR4/CD14 complex levels and elevation of inflammatory cytokines were all significantly reduced by pre-incubation of fenoterol ($P<0.05$). However, the total level of CD14 and TLR4 was not significantly changed. Interestingly, confocal microscopy revealed redistribution of CD14 and TLR4/CD14 complex under β_2-AR stimulation. Furthermore, siRNA-mediated knockdown of β-arrestin-2 eliminated the anti-inflammatory effects and redistribution of CD14 and TLR4/CD14 complex stimulated by β_2-AR.

Conclusion: β_2-AR agonist exerts its anti-inflammatory effects by down-regulating TLR signaling in THP-1 cells, potentially resulting from β-arrestin-2 mediated redistribution of CD14 and TLR14/CD14 complex.

Keywords: β_2-adrenoceptor; toll-like receptors; β-arrestin-2; fenoterol; confocal microscopy; lipopolysaccharide

Acta Pharmacologica Sinica (2009) 30: 1522–1528; doi: 10.1038/aps.2009.153

Introduction

β_2-adrenergic receptor (β_2-AR) agonist is administered in a variety of clinical situations[1–5] mostly for its bronchodilating effects. Furthermore, the regulation of β_2-AR agonist on the production of inflammatory cytokines has been recognized. For example, it was shown that, salbutamol and albuterol, agonists of β_2-AR, could inhibit tumor necrosis factor (TNF)-α production by human mononuclear cells[6, 7], in addition, salbutamol exerts immunosuppressive effects through down-regulation of co-stimulatory molecules, inter-cellular adhesion molecule 1 (ICAM-1), CD40 and CD14 on monocytes[7], endocytosis of the TLR4 complex was pertinent to anti-inflammatory effects[8]. Whether β_2-AR stimulation mediated anti-inflammatory effects in monocytes depending on the endocytosis or redistribution of TLRs is not clear. Therefore, it is necessary to make clear the exact target of β_2-AR stimulation during the process of anti-inflammation.

Upon agonist binding, β-arrestins1/2 is recruited to the plasma membrane and interacts directly with two structural components of clathrin-coated pits, clathrin and AP-2, which promote the endocytosis of β_2-AR into early endosomes via clathrin-coated vesicles[9–10]. Moreover, TLR4 was also endocytosed by a dynamin and clathrin dependent mechanism and colocalized with lipopolysaccharide (LPS) on early sorting endosomes[11]. Therefore, we hypothesized that β_2-AR stimulation mediated β-arrestins' translocation was associated with redistribution of TLRs.

Lipopolysaccharide (LPS)-induced inflammation in THP-1 cells is a model to study TLRs[12]. As a receptor of LPS, TLRs play an important role during LPS-induced inflammation[13].

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Received 2009-06-21 Accepted 2009-09-16

Recent studies have reported that β_2-adrenergic agonist exert its "anti-inflammatory" effects in monocytic cells through the IκB/NF-κB pathway[6]. On the other hand, IκB/NF-κB is downstream signaling of TLR, which plays a pivotal role in regulating inflammatory gene expression and LPS-induced inflammation[13]. The exact relationship between β_2-AR-mediated anti-inflammatory effects and TLR signaling pathway remained to be elucidated in monocytes.

In the present study, we aimed to explore the underlying mechanism of the anti-inflammatory effects mediated by β_2-AR stimulation in THP-1 cells. We first investigated if LPS-induced cytokines could be suppressed by fenoterol via ELISA assay. To confirm fenoterol' anti-inflammatory effect, down-regulated LPS-induced membrane-bound TLR4/CD14 complex and CD14 level in THP-1 cells on stimulation of β_2-AR were verified by flow cytometry. Then, we discovered that the total level of CD14 and TLR4 was not significantly changed by Western blotting, but interestingly, redistribution of CD14 and TLR4/CD14 complex mediated by β_2-AR stimulation was found by confocal analysis. Lastly, anti-inflammatory effects and redistribution of CD14 and TLR4/CD14 complex mediated by β_2-AR stimulation were abolished by siRNA-mediated knockdown of β-arrestin-2, which might play an important role in crosstalk of β_2-AR and TLR[14].

Materials and methods

Cell culture

The human monocytic cell line THP-1 (obtained from the cell center of Peking Union Medical College) was cultured in RPMI-1640 medium (Sigma Chemical Co, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 g/mL streptomycin at 37 °C in 5% CO_2 in a humidified incubator. Cells were centrifuged and resuspended with fresh medium at 10^6/mL and incubated for another 24 h before use. The cells were washed and distributed into sterile microtiter plates at 10^6/mL in RPMI-1640 medium containing 2% FBS stimulated with 0.1 μg/mL of *Escherichia coli* 0111:B4 LPS (Sigma) for 24 h (unless indicated otherwise) at 37 °C in the presence or absence of β_2-AR agonists (fenoterol) and antagonists (ICI 118551) (both from Sigma).

Downregulation (siRNA) of the β-arrestin-2

Cells were split at least 24 h prior to transfection and transfected with siRNA designed against β-arrestin-2 or control siRNA using the Oligofectamine™ transfection reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the optimized procedure recommended by the producer as described elsewhere[15]. The siRNA sequence targeting β-arrestin-2 is 5′ AAGGACCGCAAAGUGUUUGUG 3′ (Shanghai GeneChem Co, Ltd, Shanghai,China). All assays were performed 72 h following transfection of siRNA. The inhibitory efficiency of siRNA probes was assessed by measuring knockdown of the β-arrestin-2 protein by Western blotting analysis.

ELISA assay

Concentrations of interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α) from cell supernatants were determined by use of an ELISA system (R&D Systems, Minneapolis, MN) according to the manufacturer. The detection limits of ELISA for IL-8 and TNF-α were 10 pg/mL.

Flow cytometry

The expression of CD14 and TLR4/CD14 complex in THP-1 cells was determined by flow cytometry. After LPS stimulation in the presence or absence of fenoterol, the cells (10^6/sample) were washed once with PBS, then incubated at 4 °C for 30 min with a combination of anti-CD14 FITC-conjugated (clone 61D3, 10 g/mL; eBioscience) and anti-TLR4 PE-conjugated antibodies (clone HTA125, 10 g/mL; eBioscience). After washing, cells were analyzed by use of a FACS Calibur (Becton Dickinson Biosciences, San José, CA, USA), and data were analyzed by use of the CELL QUEST Program (Becton Dickinson).

Western blotting and immunoprecipitation

After treatment, THP-1 cells were lysed in 10 mmol/L HEPES, pH 7.9, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethyl-sulfonylfuoride. Cell membrane proteins were prepared using the Plasma Membrane Protein Extraction Kit (Applygen Technologies Inc., Beijing, China). Cell membrane protein or cytoplasmic protein extracts, 60−90 μg were separated by 10% SDS-PAGE and electrotransferred onto anti-trocellulose membrane (Bio-Rad, Hercules, CA, USA). TLR4, CD14, and β-arrestin-2 were detected with use of mouse monoclonal anti-human TLR4, CD14, and β-arrestin-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Zhong Shan Jin Qiao Co, China), and enhanced chemiluminescence (Pierce Biotechnology). Band intensities were determined using computer program Image-J and were presented as the mean±SEM of the x-fold change over the respective control that was arbitrarily defined. For immunoprecipitation, 100 μg of membrane protein was incubated with 20 μL protein G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) pre-equilibrated in lysis buffer and 10 μL of polyclonal antibodies for 4 h at 4 °C. Samples were then centrifuged for 10 s, and the pellets were washed three times with 1 mL of lysis buffer. Bound proteins were eluted by the addition of 15 μL of SDS sample buffer and boiling for 5 min and then analyzed by SDS-PAGE and immunoblotting.

Confocal analysis

A standard immunocytoplasmic staining protocol was used[16]. Briefly, after LPS stimulation in the presence or absence of fenoterol and siRNA-mediated knockdown of β-arrestin-2, THP-1 cells were cultured in a chamber slide (Zhong Shan Jin Qiao Co, China) for 20 min, then fixed with ice-cold acetone for 20 min and stained with PE-conjugated monoclonal antibodies for mouse anti-human TLR4 (HTA125) and FITC-conjugated monoclonal antibodies for mouse anti-human CD14 (61D3) for 24 h at room temperature, then washed with

PBS twice and stained with Hoechst-33342 (Sigma-Aldrich) for 15 min to visualize the nuclei, washed with PBS twice, then mounted with use of Antifadent Mountant Solutions (Zhong Shan Jin Qiao Co, China) and viewed under a confocal laser scanning microscope (LSM 510 META, Zeiss, Germany).

Statistical analysis

Experiments were repeated at least three times. Data are presented as mean±SEM. The statistical significance of the differences between the means of the groups was determined by one-way ANOVA followed by Bonferroni *post-hoc* test. *P* values of <0.05 were considered statistically significant.

Results

Fenoterol inhibits LPS-stimulated IL-8, TNF-α release from THP-1 cells

The concentration of IL-8 increased about 20-fold on stimulation with LPS (0.1 μg/mL) in THP-1 cells. The elevated concentration of IL-8 was significantly decreased by pre-incubation with up to 10^{-6} mol/L fenoterol. Furthermore, this effect was largely attenuated in the presence of 10^{-6} mol/L ICI118551, the antagonist of β_2-AR (Figure 1A). Similar results were found for TNF-α (Figure 1B).

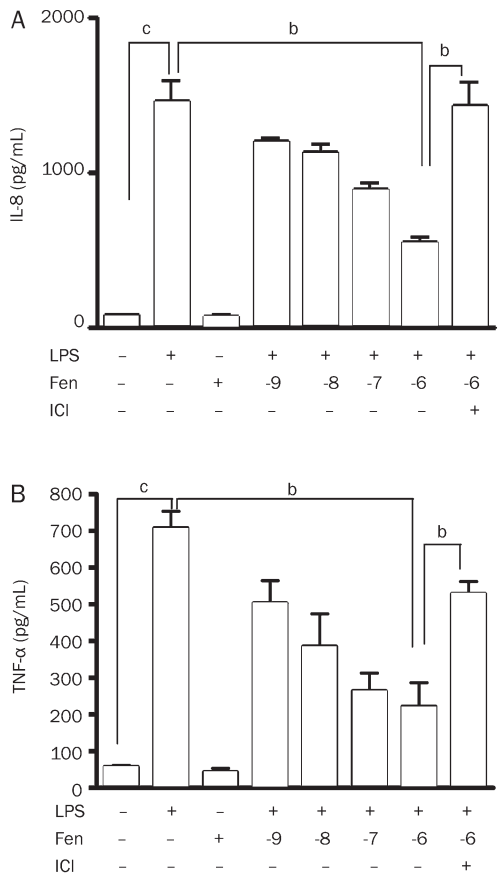

Figure 1. Concentration of LPS-stimulated IL-8 and TNF-α in cell supernatants determined by ELISA in the presence or absence of fenoterol (lg mol/L) and ICI118551 (10^{-6} mol/L). (A) Inhibitory effect of fenoterol on IL-8 production from THP-1 cells stimulated for 24 h with LPS (0.1 μg/mL). (B) Inhibitory effect of fenoterol on TNF-α production from THP-1 cells stimulated for 24 h with LPS (0.1 μg/mL). Data are presented as mean±SEM. $^bP<0.05$, $^cP<0.01$.

Fenoterol down-regulates membrane-bound TLR4/CD14 complex and CD14 in THP-1 cells

After LPS (0.1 μg/mL) stimulation for 24 h, the effect of fenoterol (10^{-6} mol/L) on change of the membrane-bound TLR4/CD14 complex and CD14 levels in THP-1 cells was examined by flow cytometry. Although LPS-induced TLR4 expression was not significantly changed with β_2-AR stimulation (data not shown), the membrane-bound TLR4/CD14 complex and CD14 levels in THP-1 cells were significantly decreased on incubation with fenoterol, pre-incubation with ICI118551 for 30 min abolished the effect of down-regulation of TLR4/CD14 complex and CD14 mediated by fenoterol (Figure 2A). Similar results were found by Western blotting (Figure 2B).

Fenoterol enhances redistribution of LPS-stimulated TLR4/CD14 complex and increases membrane-bound β-arrestin-2 expression in THP-1 cells

The total protein expression of CD14 and TLR4 in THP-1 cells was not significantly changed by treatment with fenoterol (10^{-6} mol/L) or LPS (0.1 μg/mL) (Figure 3Aa, 3Ad). However, confocal microscopy revealed that the membrane-bound TLR4/CD14 complex was reduced in level with pre-incubation of fenoterol (10^{-6} mol/L) (Figure 3Bb) in LPS-stimulated THP-1 cells (Figure 3Ba) and redistribution of TLR4/CD14 complex under stimulation with β_2-AR was abolished with pre-incubation of ICI118551 for 30 min (Figure 3Bc). Meanwhile, membrane-bound β-arrestin-2 was increased by treatment with fenoterol (10^{-6} mol/L) for 3 min (Figure 3C).

Silencing β-arrestin-2 abolished the anti-inflammatory effects and redistribution of LPS-induced TLR4/CD14 complex stimulated by β_2-AR

The siRNA used almost abrogated β-arrestin-2 expression in THP-1 cells (Figure 4A). To determine whether the β-arrestin-2 siRNA could affect anti-inflammatory effects and redistribution of LPS-stimulated TLR4/CD14 complex on stimulation with β_2-AR, afer transfection with siRNA designed against β-arrestin-2 or control siRNA, THP-1 cells were stimulated with LPS in the presence or absence of fenoterol as described before. As shown in (Figure 4B, 4C), anti-inflammatory effects and redistribution of CD14 and TLR4/CD14 complex mediated by β_2-AR stimulation were abolished by siRNA-mediated knockdown of β-arrestin-2, while not abolished by control siRNA (data not shown).

Discussion

LPS-induced inflammatory response was abolished in mice deficient in MyD88$^{-/-}$, an important downstream signaling molecule of TLRs, suggesting that TLRs play a central role in the pathogenic microorganism-mediated inflammatory response[13, 17]. On the other hand, we noticed that β_2-AR signaling exerted anti-inflammatory effect[6, 7]. Therefore, further

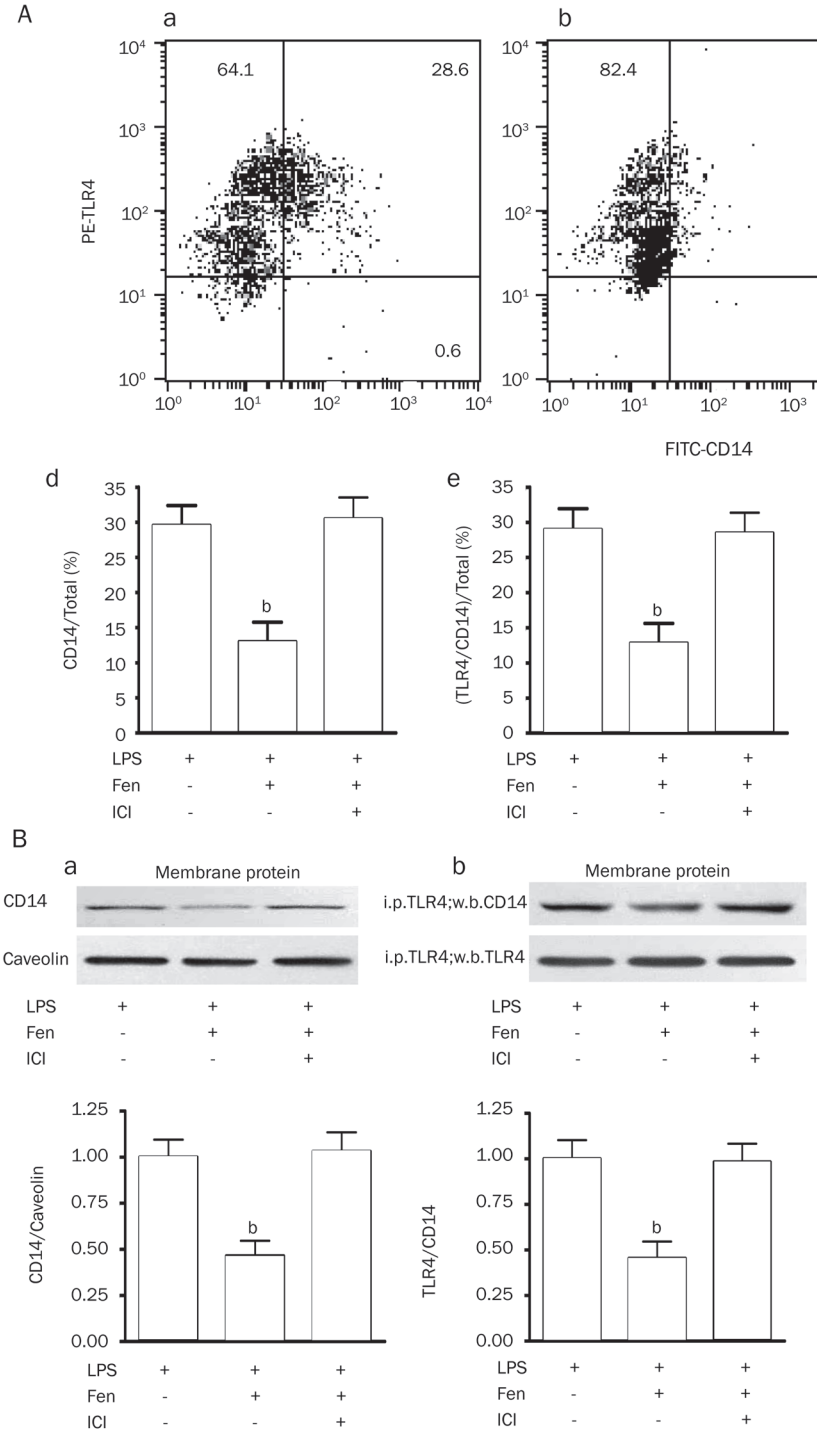

Figure 2. Expression of membrane-bound TLR4, CD14, and TLR4/CD14 complex in THP-1 cells by two-staining flow cytometry method (PE-TLR4 and FITC-CD14) and immunoprecipitation and immunoblotting. (A) (a) Expression of TLR4, CD14 and TLR4/CD14 complex in THP-1 cells stimulated by LPS (0.1 μg/mL) for 24 h (representative experiment). (b) Fenoterol (10^{-6} mol/L) for 24 h down-regulates LPS-stimulated membrane-bound CD14 and TLR4/CD14 complex in THP-1 cells (representative experiment). (c) Pre-incubation of ICI118551 for 30 min abolished fenoterol-induced down-regulation of membrane-bound CD14 and TLR4/CD14 complex (representative experiment). (d) Down-regulating effect of fenoterol (10^{-6} mol/L) for 24 h on LPS-stimulated membrane-bound CD14 in THP-1 cells. Data are presented as mean±SEM. $^{b}P<0.05$ *vs* LPS or LPS+Fen+ICI118551 group. (e) Down-regulating effect of 24 h fenoterol (10^{-6} moL/L) on LPS-stimulated membrane-bound TLR4/CD14 complex in THP-1 cells. Data are presented as mean±SEM. $^{b}P<0.05$ *vs* LPS or LPS+Fen+ICI118551 group. (B) (a) Down-regulating effect of fenoterol (10^{-6} moL/L) for 24 h on LPS-stimulated membrane-bound CD14 in THP-1 cells by Western blotting. Data are presented as mean±SEM. $^{b}P<0.05$ *vs* LPS or LPS+Fen+ICI118551 group. (b) Down-regulating effect of fenoterol (10^{-6} mol/L) for 24 h on LPS-stimulated membrane-bound TLR4/CD14 complex in THP-1 cells by immunoprecipitation and immunoblotting. Data are presented as mean±SEM. $^{b}P<0.05$ *vs* LPS or LPS+Fen+ICI118551 group.

study is needed to elucidate the relationship between β_2-AR-mediated anti-inflammatory effects and TLR signaling pathway.

To understand the mechanism of β_2-AR-mediated TLR regulation, TLR binding structure and its co-factors first need to be considered. All TLRs are type I transmembrane receptors, characteristic of a highly variable extracellular region, including a leucine-rich repeat domain involved in ligand binding and an intracellular tail containing a highly conserved region, the Toll/Interleukin-1 Receptor (TIR) homology domain, which mediates interaction between TLRs and downstream signaling molecules[13]. Activation of TLR4 is initiated as follows: the binding of the LPS binding protein (LBP)/LPS complex to membrane CD14 (mCD14), then binding and forming the TLR4/CD14 complex and activating TLR4, which activates signal transduction pathways and induces inflammatory

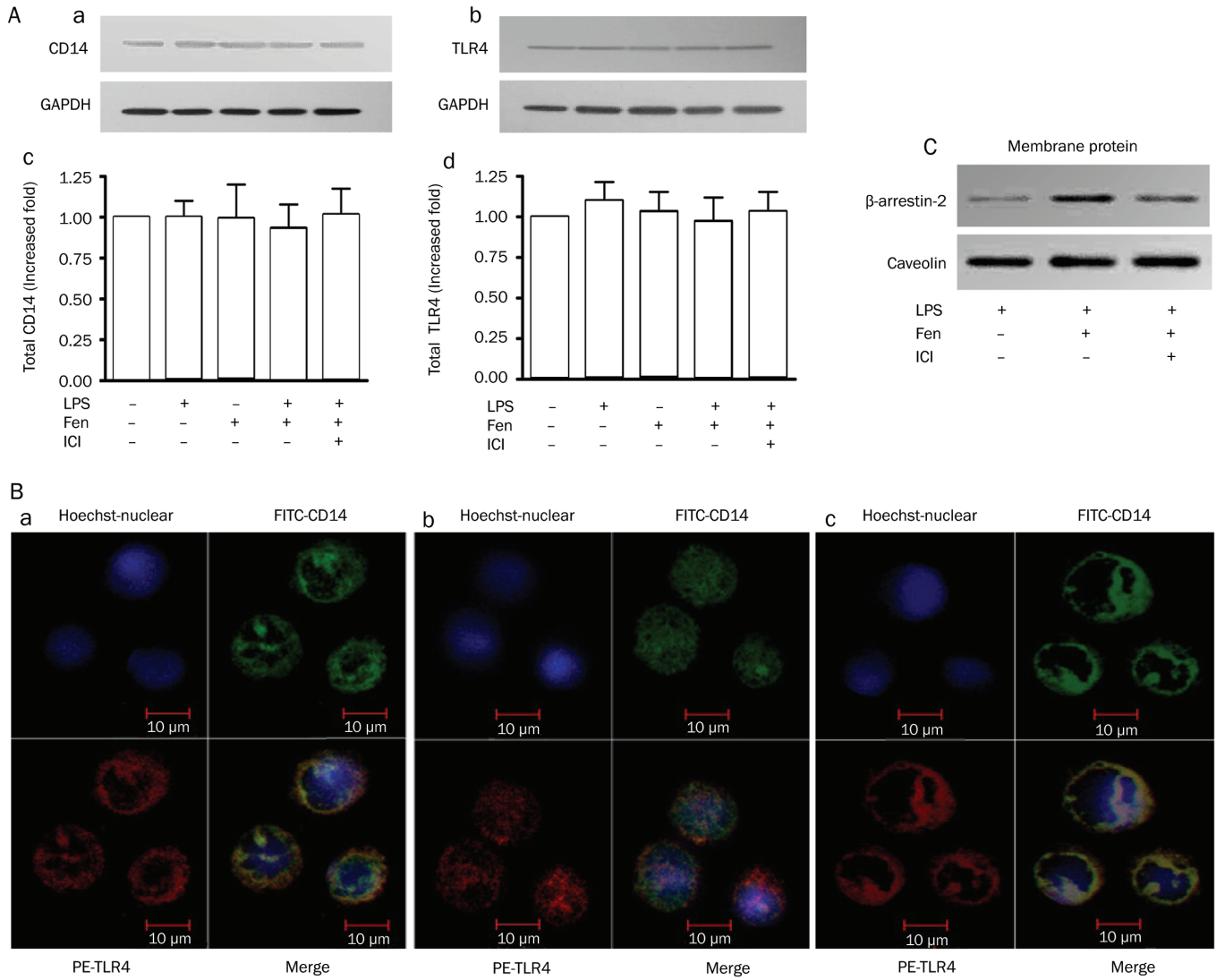

Figure 3. Expression of CD14, TLR4, and membrane-bound β-arrestin-2 in the presence or absence of fenoterol by Western blotting. Distribution of LPS-stimulated TLR4/CD14 complex on stimulation with β_2-AR examined on confocal analysis. (A) (a,c) Representative Western blotting and analysis of CD14 and GAPDH protein expression; (A) (b,d) Representative Western blotting and analysis of TLR4 and GAPDH expression. GAPDH was used as an internal loading control. (B) (a) Confocal analysis of LPS-stimulated TLR4/CD14 complex in THP-1 cells; (b) Confocal analysis of redistribution of LPS-stimulated TLR4/CD14 complex from THP-1 cells under stimulation with β_2-AR. (c) Redistribution of LPS-stimulated TLR4/CD14 complex under stimulation of β_2-AR with pre-incubation of ICI118551 in THP-1 cells. (C) Expression of membrane-bound β-arrestin-2 in the presence or absence of fenoterol for 3 min, LPS and ICI118551 by Western blotting analysis (representative experiment).

gene expression[13]. Thus, we speculated that a change in the membrane-bound TLR4/CD14 complex level might affect the activation of TLR4. Furthermore, in the present study, we identified that reduced inflammatory response mediated by β_2-AR stimulation was related to the change of membrane-bound TLR4/CD14 complex (Figure 2) but not total protein expression of TLR4 in monocytes (Figure 3A). Interestingly, despite no significant change in total protein expression of TLR4 with β_2-AR stimulation, confocal microscopy revealed redistribution of the TLR4/CD14 complex (Figure 3B). A previous study showed that human corneal epithelial cells express TLR2 and TLR4 intracellularly but not at the cell surface and fails to respond to LPS even on artificial translocation of LPS[18]. Thus, membrane-bound TLRs play a central role in LPS-induced inflammatory response, and β_2-AR mediated reduction of membrane-bound TLRs was responsible for the reduced inflammatory response in monocytes.

Whether the β_2-AR-mediated anti-inflammatory effect depends on the inhibition of the receptor level or downstream signaling of TLRs is still in debate. There have been some reports that the anti-inflammatory effect of β-receptor activation was associated with a change in content of IκB/NF-κB, extracellular signal-regulated kinase 1/2 (ERK1/2) or p38[6, 19], whether these changes were the direct effect of

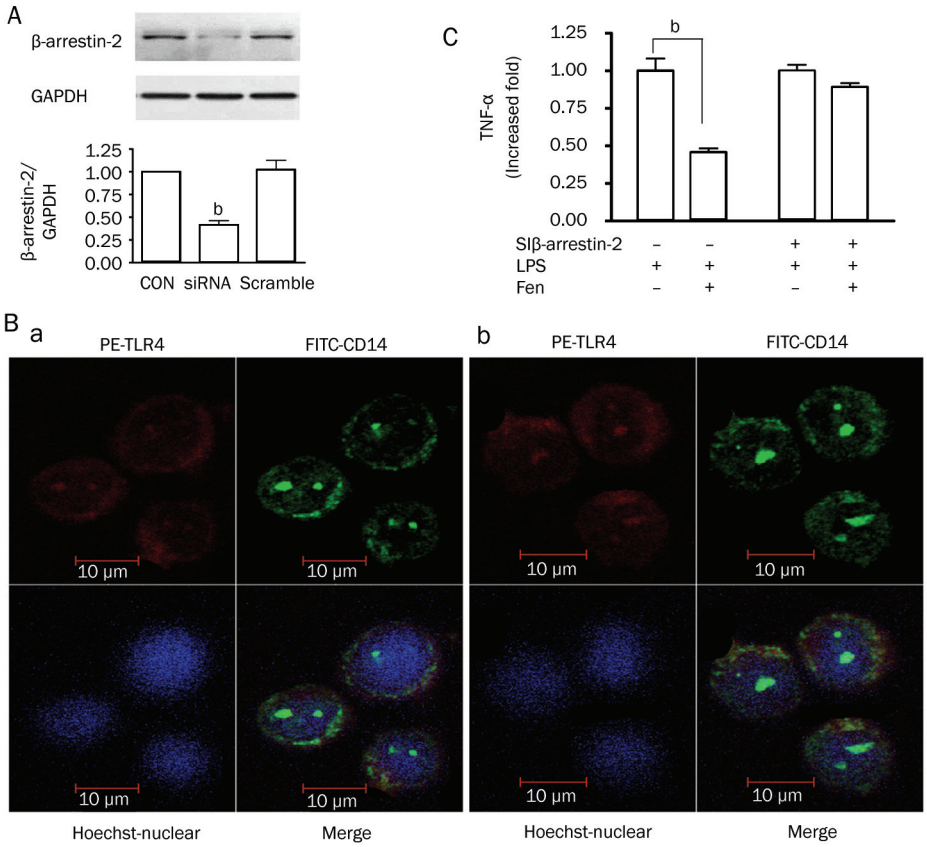

Figure 4. Effects of β-arrestin-2' down-regulation on the anti-inflammation and redistribution of LPS-stimulated TLR4/CD14 complex stimulated by β_2-AR. (A) Effect of β-arrestin-2 small interfering RNA (siRNA) for 72 h on the expression of the β-arrestin-2 protein. Data are presented as mean±SEM. $^bP<0.05$ *vs* control or scramble. GAPDH was used as an internal loading control. (B) After transfection with siRNA designed against β-arrestin-2 for 72 h, confocal analysis of LPS-stimulated TLR4/CD14 complex in THP-1 cells (a); Confocal analysis of redistribution of LPS-stimulated TLR4/CD14 complex in THP-1 cells under stimulation with β_2-AR (b). (C) After transfection with siRNA designed aga inst β-arrestin-2 for 72 h, THP-1 cells were stimulated with LPS for 24 h in the presence or absence of fenoterol, anti-inflammatory effects stimulated by β_2-AR was abolished when β-arrestin-2 was knocked down. $^bP<0.05$ *vs* LPS 0.1 μg/mL.

β-receptor stimulation or resulted from down-regulation of TLRs is still unknown. A recent study revealed that β_2-AR agonist exerts its anti-inflammatory effect through inhibiting the expression of membrane-bound CD14, a co-factor of TLRs, on monocytes [7]. The regulation of TLRs might be a potential target of the β_2-AR agonist. Our results further demonstrated that the reduced level of membrane-bound TLRs was responsible for the anti-inflammatory effect of β_2-AR agonist (Figure 2). As well, the decreased activation of NF-κB signaling was attributed to the down-regulation of membrane-bound TLRs. Whether the signaling of TLRs is a specific pathway for the β_2-AR-mediated anti-inflammatory effect still needs to be elucidated.

Upon agonist binding, β-arrestins1/2 is recruited to the plasma membrane and mediates desensitization and internalization of G-protein-coupled receptor (GPCR)[20]. However, β-arrestins have been considered as novel non-G protein-dependent signaling molecules and play functional roles in the regulation of a variety of signaling pathways and in the mediation of cross-talk between receptors[21–23]. For example, β-arrestin-2-dependent stabilization of cytosolic IκBα and inhibition of NF-κB activation following LPS stimulation are essential for rapid and sufficient production of NO in response to microbial attack[14]. Moreover, there is accumulating evidence that β-arrestin-2, which is expressed abundantly in the spleen, is functionally involved in some important immune responses, such as regulation of lymphocyte chemotaxis and homing[24, 25]. In the present study we used RNA interference against β-arrestin-2 to test its role in anti-inflammatory effects stimulated by β_2-AR. The specificity and efficiency of siRNA against β-arrestin-2 was demonstrated by Western blotting (Figure 4A). The translocation of β-arrestins1/2 to the plasma membrane was reported to interact directly with two structural components of clathrin-coated pits, clathrin and AP-2, promoting the endocytosis of β_2-AR into early endosomes via clathrin-coated vesicles[9, 10]. Moreover, TLR4 was also endocytosed by a dynamin and clathrin dependent mechanism and colocalized with LPS into early/sorting endosomes[11]. Therefore, we hypothesized that β-arrestins' translocation to the cell surface was associated with redistribution of TLRs on stimulation of β_2-AR. Meanwhile, fenoterol increased membrane-bound β-arrestin-2 expression, suggesting that β-arrestin-2 translocated to the cell surface on stimulation of β_2-AR in THP-1 cells (Figure 3C). Our study indicated that depletion of β-arrestin-2 abolished redistribution of CD14 and TLR4/CD14 complex mediated by β_2-AR activation (Figure 4B), suggesting that β-arrestin-2' translocation and β-arrestin-2/clathrin-dependent redistribution of TLRs was required for anti-inflammatory effects stimulated by β_2-AR. Further study needs to clarify β-arrestin-2/clathrin mediated redistribution of TLRs on stimulation of β_2-AR.

Taken together, we provided evidence that β_2-AR agonist exerts anti-inflammatory effects by down-regulating membrane-bound TLRs through β-arrestin-2. Down-regulation

of β-arrestin-2 significantly attenuates the anti-inflammatory effects mediated by fenoterol, suggesting that β-arrestin-2 is beneficial to protecting organism against invading pathogens. This finding has implications not only with regard to our understanding of molecular mechanism for the β_2-AR agonists' anti-inflammatory effects but also for the development of therapeutic agents targeting these pathways, which may be helpful for treatment of acute and chronic inflammatory diseases.

Acknowledgements

Project was supported by the National Key Basic Research Program (NKBRP) of People's Republic of China (No 2006CB503806) and the National Natural Science Foundation of China (No 30770939 and 30821001).

The authors would like to thank Dr Laura HERATY for language revision, and Dr Pei ZHANG for performing the confocal laser scanning microscope.

Author contribution

Bei HE, Ming XU, You-yi ZHANG designed research; Wei WANG performed research; Wei WANG contributed new analytical tools and reagents; Wei WANG, Bei HE, Ming XU wrote the paper.

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