Vascular Pharmacology 62 (2014) 150–161

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Activation of liver X receptor enhances the proliferation and migration of endothelial progenitor cells and promotes vascular repair through PI3K/Akt/eNOS signaling pathway activation

Jie Yu, Qiang Wang, Hang Wang, Wei Lu, Wei Li, Zhexue Qin, Lan Huang *

Institute of Cardiovascular Diseases of PLA, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China

ARTICLE INFO

Article history:
Received 15 February 2014
Received in revised form 21 April 2014
Accepted 26 May 2014
Available online 2 June 2014

Keywords:
Endothelial regeneration and repair
LXR
EPCs
Proliferation
Migration

ABSTRACT

Vascular endothelial injury is a major cause of many cardiovascular diseases. The proliferation and migration of endothelial progenitor cells (EPCs) play a pivotal role in endothelial regeneration and repair after vascular injury. Recently, liver X receptor (LXR) activation has been suggested as a potential target for novel therapeutic interventions in the treatment of cardiovascular disease. However, the effects of LXR activation on endothelial regeneration and repair, as well as EPC function, have not been investigated. In the present study, we demonstrate that LXRs, including LXRα and LXRβ, are expressed and functional in rat bone marrow-derived EPCs. Treatment with an LXR agonist, TO901317 (TO) or GW3965 (GW), significantly increased the proliferation and migration of EPCs, as well as Akt and eNOS phosphorylation in EPCs. Moreover, LXR agonist treatment enhanced the expression and secretion of vascular endothelial growth factor in EPCs. LXR agonists accelerated re-endothelialization in injured mouse carotid arteries in vivo. These data confirm that LXR activation may improve EPC function and endothelial regeneration and repair after vascular injury by activating the PI3K/Akt/eNOS pathway. We conclude that LXRs may be attractive targets for drug development in the treatment of cardiovascular diseases associated with vascular injury.

1. Introduction

Vascular endothelial injury triggers hypertension, atherosclerosis, and vascular disease in patients with risk of cardiovascular events. It is also a major cause of restenosis and thrombosis after angioplasty. Endothelial regeneration and repair can prevent thrombosis and suppress vascular smooth muscle cell (VSMC) proliferation after arterial injury. Therefore, accelerating the re-endothelialization of injured arteries and restoring endothelial function are useful strategies for preventing vascular injury-induced diseases [1].

Endothelial regeneration requires the proliferation and migration of neighboring endothelial cells (ECs) [2]. Endothelial progenitor cells (EPCs) are derived from the bone marrow (BM) and spleen, or they can be exogenously infused [3]. Accumulating evidence has demonstrated that the number of EPCs present in the systemic circulation is augmented in response to certain cytokines or tissue ischemia [4]. EPCs home to sites of endothelial injury [3,4], where they promote re-endothelialization directly by incorporating into the recovering endothelium [4,5] and indirectly by producing and releasing angiogenic growth factors [6]. Accordingly, treatment strategies that enhance the contribution of resident EPCs in the adult vasculature and increase migration, proliferation, and remodeling in response to angiogenic cues may facilitate recovery of the injured endothelium.

Liver X receptors (LXRs), including LXRα and LXRβ, are members of the nuclear receptor (NR) superfamily of ligand-activated transcription factors that heterodimerize with the retinoid X receptor (RXR) [7,8]. Like most other NRs that form heterodimers with RXR, LXRs reside within the nucleus and have been established as key modulators of lipid metabolism and inflammatory signaling [9]. Indeed, LXR activation recently has been suggested to be a potential target for novel

Abbreviations: VSMC, vascular smooth muscle cell; EC, endothelial cell; EPCs, endothelial progenitor cells; BM, bone marrow; LXRs, liver X receptors; RXR, retinoid X receptor; TO, TO901317; GW, GW3965; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; ECGS, endothelial cell growth supplement; PI3K, phosphatidylinositol 3′-kinase; LY, LY294002; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; MNCs, mononuclear cells; FBS, fetal bovine serum; ECGS, endothelial cell growth supplement; FACS, fluorescence-activated cell sorting; HPFs, high-power fields; RT-PCR, reverse transcriptase-PCR; eoEPCs, early-outgrowth EPCs; loEPCs, late-outgrowth EPCs; ABCA1, ATP-binding cassette transporter A1; ERs, estrogen receptors; PPAR-γ/δ, peroxisome proliferator-activated receptor-γ/δ.

* Corresponding author. Tel./fax: +86 2368755601.
E-mail address: simpls@tmmu.edu.cn (L. Huang).

http://dx.doi.org/10.1016/j.vph.2014.05.010
1537-1891/

therapeutic interventions in human cardiovascular disease [10,11]. Two LXR agonists, TO901317 (TO) and GW3965 (GW), have been shown to prevent the development of atherosclerosis in murine models through efficient reverse cholesterol transport and net inhibition of plaque inflammation [12]. In addition, these compounds have been shown to affect the function of ECs and VSMCs and to promote angiogenesis [13,14].

Drugs and cytokines known to improve EC function and promote angiogenesis can be used to encourage re-endothelialization after vascular injury [15]. Moreover, TO and GW have been found to alleviate EPC adhesion and migration in vitro and in a mouse model [16,17]. Based on these findings, we speculate that LXR activation might promote endothelial regeneration and repair after vascular injury via regulation of EPC function.

2. Materials and methods

2.1. Reagents

CoStar cell culture plates were obtained from Fisher Scientific (Waltham, MA). Culture medium and serum were obtained from Gibco (Grand Island, NY). Recombinant rat vascular endothelial growth factor (VEGF) (564-RV-010), basic fibroblast growth factor (bFGF) (2099-FB-025), epidermal growth factor (EGF) (2028-EG-200), and the mouse VEGF ELISA Quantikine kit (MMV00) were obtained from R&D Systems (Minneapolis, MN). Endothelial cell growth supplement (ECGS), fibronectin and FITC-UEA-I were purchased from Sigma (St. Louis, MO). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated LDL (DiI-ac-LDL) was acquired from Molecular Probes (Eugene, OR). FITC-conjugated anti-rat CD133 (bs-0395R), CD34 (bs-0646R), CD31 (bs-0468R), and VEGFR-2 (ER-1542) antibodies, and the corresponding isotype control IgGs were obtained from Bios (Beijing, China). The LXR agonists, TO and GW, the phosphatidylinositol 3′-kinase (PI3K) inhibitor LY294002 (LY), and the nitric oxide synthase (NOS) inhibitor (L-NAME) were all purchased from Sigma. The Akt inhibitor (Akt inhibitor II) was purchased from Calbiochem-Novabiochem Ltd. (Nottingham, UK).

Anti-rat LXRα (sc-1202) and LXRβ (sc-1001) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-rat Akt (21054) and phospho-Akt (Ser473; 11054) antibodies were acquired from Signalway Antibody (Pearland, TX). Anti-rat eNOS (9572) and phospho-eNOS (Ser1177; 9571) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-rat VEGF antibodies (ab46154) were from Abcam (Cambridge, MA). Anti-rat GAPDH antibody (AB-P-R 001) was obtained from Hangzhou Goodhere Biotechnology Co., Ltd. (Hangzhou, Zhejiang, China).

2.2. Cell culture and characterization

Of note, a specific problem arises when cells are expanded and cultured ex vivo; culture conditions, including supplements, such as FCS and cytokines, and plastic, can rapidly change the phenotype of the cells [37]. In this study, EPCs were isolated and characterized following our established protocol, with some modifications [18,19]. Briefly, mononuclear cells (MNCs) were isolated from the BM of Sprague–Dawley rats (male, 150–180 g; Chongqing, China) by density gradient centrifugation (Lymphoprep 1.083, Tianjin, China). MNCs were seeded on fibronectin-coated cell culture plates (1×10^6 cells/cm^2) and cultured using M199 supplemented with 20% fetal bovine serum (FBS), VEGF (50 ng/mL), bFGF (5 ng/mL), EGF (10 ng/mL), ECGS (75 μg/mL), heparin (100 μg/mL), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 5% CO_2 incubator at 37 °C. Four days after culture, nonadherent cells were removed by washing with phosphate-buffered saline (PBS). The medium was changed every 3 days. EPCs cultured for 7 to 10 days were used for the experiments.

To confirm the EPC phenotype after 7 days of culture, cells were incubated with 2.4 μg/mL DiI-ac-LDL for 1 h. Cells were fixed in 4% paraformaldehyde, counterstained with FITC-labeled UEA I (10 μg/mL), and washed with PBS. Samples were viewed under an inverted fluorescence microscope and a laser scanning confocal microscope (Leica TCS SP5, Germany). Cells that were double-positive for DiI-acLDL and FITC-UEA-I were identified as differentiated EPCs, as reported previously [14]. To confirm the phenotype further, fluorescence-activated cell sorting (FACS) analysis was performed after 7 days in culture. Most previous studies suggest that EPCs are characterized by the expression of three markers, CD133, CD34, and the vascular endothelial growth factor receptor-2 (VEGFR-2) and that cells with these characteristics are localized predominantly in the bone marrow [20,21]. Therefore, cells in the present study were fixed with 4% paraformaldehyde and labeled with FITC-conjugated anti-rat CD133, CD34, CD31, and VEGFR-2 antibodies. Matched isotype antibodies served as controls.

2.3. Cell proliferation assay

Cell proliferation was evaluated using a colorimetric MTS assay kit (Promega Corporation, Madison, WI), as described previously [22]. EPCs were detached after 7 days in culture, and 1.5×10^4 cells were reseeded into individual wells of a 96-well plate. After 24 h of culture in fully supplemented growth medium, the medium was changed to M199 (supplemented with 0.5% bovine serum albumin [BSA], and lacking FBS and ECGS) for 12 h. Cells were incubated in 20% FBS-supplemented with M199 and the LXR agonist TO (0, 0.5, 2, and 5 μM) or GW (0, 0.5, 2, and 5 μM) for the indicated times. After 48 h in culture, proliferation was determined using the MTS dye. The optical density (OD) at 490 nm was measured using a plate reader. Cell viability was measured by the trypan blue exclusion assay, according to the manufacturer's instructions. LY (10 μM), Akt inhibitor II (15 μM), and L-NAME (100 μM) were added 30 min before the addition of LXR agonists, where indicated.

Simultaneously, we detected the effect of TO (2 μM) or GW (5 μM) treatment on EPC proliferation by performing cell counts, as previously described [23]. Briefly, cells were left untreated or incubated with an LXR agonist, TO (2 μM) or GW (5 μM). After 7 days in culture, attached cells were stained to detect uptake of DiI-acLDL and UEA-1 expression, as described above. Dual-positive cells were deemed EPCs and quantified by examining fifteen random microscopic fields (×200) [23].

2.4. Cell migration assay

The cell migration assay was performed using a transwell chamber (8-μm pore size), as previously described [24]. The lower surface of the membrane was precoated with gelatin (0.1% w/v, Sigma). The EPCs used for the experiments were serum-starved in M199 (supplemented with 0.5% BSA, and lacking FBS and ECGS) for 8 h, pretreated with LY (10 μM)/L-NAME (100 μM) as indicated, and treated with various concentrations of TO or GW for 24 h, as indicated. Cells (5×10^4) were seeded onto the upper chamber and incubated at 37 °C. LXR agonists and LY (10 μM)/L-NAME (100 μM) were added to the upper and lower chambers, as indicated. Chemotaxis was achieved by adding 20% serum to the lower chamber. After 16 h, the chambers were fixed with 4% paraformaldehyde and stained with DAPI for 10 min. Cells on the upper surface were removed with a cotton swab. Cells that had migrated to the lower surface were counted using a fluorescence microscope. In all cases, five randomly selected high-power fields (HPFs) were counted per membrane. The migration activity is reported as the mean number of migrated cells.