Biochimica et Biophysica Acta 1831 (2013) 1426–1434

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Lysophosphatidic acid promotes secretion of VEGF by increasing expression of 150-kD Oxygen-regulated protein (ORP150) in mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 28 November 2012
Received in revised form 18 April 2013
Accepted 13 May 2013
Available online 21 May 2013

Keywords:
Lysophosphatidic acid (LPA)
150-kD Oxygen-regulated Protein (ORP150)
Vascular endothelial growth factor (VEGF)
Mesenchymal stem cells (MSCs)

ABSTRACT

We previously reported that transplantation of lysophosphatidic acid (LPA) treated mesenchymal stem cells (MSCs) enhances capillary density in the myocardium and improves myocardial function in the ischemic heart. This effect may be mediated through the release of paracrine factors by MSC and potentially involves pro-angiogenic molecules such as vascular endothelial growth factor (VEGF). In this study, we examined the pharmacological and molecular regulation of VEGF secretion induced by LPA in rat MSCs. We showed that LPA stimulated VEGF secretion in MSCs but not in cardiomyocytes or cardiac fibroblasts. LPA-induced VEGF secretion occurred at the post-transcriptional levels and was mediated through the classical ER/Golgi-dependent protein secretory route. LPA also increased ORP150 protein expression. Inhibition of ORP150 upregulation by siRNA knockdown attenuated LPA-induced VEGF secretion. On the other hand, diazoxide, an activator of K_{ATP} channel, markedly inhibited LPA-induced ORP150 expression and VEGF secretion. Meanwhile, ATP concentration dependently increased VEGF secretion. In addition, L-Glutamate and NH_4Cl significantly reduced VEGF secretion. Furthermore, inhibition of two major subtypes of LPA receptors by Ki16425 and specific siRNA for LPA receptors prevented LPA-induced VEGF secretion and ORP150 expression. Lastly, inhibition of Gi protein that couples with LPA receptors by PTX and siRNA knockdown had no effect on LPA-induced VEGF secretion. Taken together, our findings demonstrate that LPA promotes VEGF secretion at the post-translation level by up-regulating ORP150 expression. Both LPA1 and LPA3 are involved in the LPA-induced VEGF secretion that is independent of Gi protein coupling but associated with the inactivation of K_{ATP} channels and inhibition of Na^+/K^+-ATPase activity.

1. Introduction

Mesenchymal stem cells (MSCs) have been considered as a potential source of cell therapy in ischemic heart disease and heart failure because of their great promise in improving heart function and neovascularization [1,2]. The therapeutic effects of MSCs result partly from the release of paracrine factors, including vascular endothelial growth factor (VEGF) that induce angiogenesis and protect the myocardium against ischemic injury [3–6]. In this regard, it has been reported that stem cells over-expressing VEGF have a favorable impact on the improvement of myocardial function in ischemic heart diseases [7–9]. Therefore, inducing VEGF production in MSCs may contribute to their therapeutic potential.

Lysophosphatidic acid (LPA, 1-acyl-2-lyso-sn-glycero-3-phosphate), as a simple endogenous bioactive phospholipid, is present in most tissues and biological fluids at nM to μM concentrations and is produced by the activated platelet [10]. LPA is a serum-derived growth factor that is involved in a wide range of biological processes including neurogenesis, angiogenesis, would healing, immunity and carcinogenesis [11–13]. Our previous studies indicate that LPA increases VEGF secretion from MSCs under hypoxia and serum deprivation (hypoxia/SD) conditions, which may contribute to the increased capillary density seen in the ischemic myocardium after transplantation of LPA-treated MSCs when compared to those without LPA treatment [14]. However, the precise mechanisms by which LPA regulates the expression or secretion of VEGF in MSCs have not been fully elucidated.

It has been suggested that LPA may increase the expression of VEGF through transcriptional regulation in human ovarian cancer cells [15]. LPA promotes VEGF expression in cancer cells via induction of HIF-1α expression and its binding to the promoter of VEGF [16], or by the activation of c-Myc and Sp-1 transcription factors [17]. However, it is not known whether LPA regulates VEGF secretion at the transcriptional or post-transcriptional level. Additionally, chaperones in the endoplasmic reticulum (ER) play a critical role in the post-translational regulation of secretory proteins [18,19] and the 150-kDa Oxygen-regulated protein (ORP150), a stress-inducible ER chaperone, is

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http://dx.doi.org/10.1016/j.bbalip.2013.05.003

reported to contribute to VEGF transport in C6 glioma cell and macrophages [20,21]. Thus, in this study, we sought to identify regulatory mechanisms involved in LPA-induced secretion of VEGF in MSCs at the transcriptional or the post-translational level.

2. Materials and methods

2.1. Materials

Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were from Invitrogen (San Diego, CA, USA). Lysophosphatidic acid (oleoyl C: 18:1) was from Avanti Polar Lipids (Alabaster, AL, USA) and pertussis toxin (PTX) from Biomol Research Labs (Plymouth Meeting, PA, USA). Brefeldin A was from Beyotime Inst. (Haimen, Jiangsu, China). Diazoxide, 1-butanol and Ki16425 were from Sigma-Aldrich (St. Louis, MO, USA). A rabbit polyclonal anti-ORP150 antibody was from Abcam (Cambridge, UK) and an anti-GRP78 antibody from Biosynthesis Biotechnology Co. (Beijing, China). A mouse monoclonal anti-VEGF antibody and horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ORP150-siRNA, LPA1-siRNA, LPA3-siRNA, GNAI2-siRNA, GNAI3-siRNA, negative-siRNA and Lipofectamine 2000 transfection reagent were purchased from Invitrogen.

2.2. Cell culture and treatment

The MSCs were isolated from Sprague–Dawley rats (Vital River Laboratory Animal Inc., Beijing, China, http://www.vitalriver.com.cn) as previously described [22]. Bone marrow was collected from rat tibia and femur, plated in IMDM supplemented with 10% heat-inactivated fetal bovine serum and incubated at 37 °C in a humid atmosphere containing 5% CO_2. The medium was replaced after 24 h and non-adherent hematopoietic cells were removed. Adherent MSCs were further grown in the medium, replacing the latter every 2 days. Cells were used for experimentation at passage 3, and were switched to the IMDM containing 2% FBS for an additional 24 h before stimulation with LPA. Inhibitors or activators were pre-incubated for the indicated time with MSCs before the addition of LPA. Gene interference studies were performed using siRNA duplexes targeting ORP150 (ORP150-siRNA), LPA1 (LPA1-siRNA), LPA3 (LPA3-siRNA), Gi protein (GNAI2-siRNA, GNAI3-siRNA) and the scrambled siRNA controls (negative-siRNA). The siRNA were transfected into MSCs cultured in 6-well plates, using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. After 24 h incubation, the cells were treated with LPA at indicated concentrations and times.

2.3. ELISA for VEGF quantification

MSCs in 6-well plates were grown to 70% confluence in complete medium and then incubated for an additional 24 h in IMDM containing 2% FBS followed by LPA treatments (5, 25 and 50 μM) for 6 to 24 h. The levels of VEGF present in the culture supernatants were quantified using a rat VEGF ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 50 μl of supernatant from each well was added to the ELISA microplate coated with a monoclonal antibody against rat VEGF. The plate was incubated for 2 h at room temperature followed by an hour's incubation with a secondary polyclonal antibody conjugated to horseradish peroxidase. The substrate solution was added to the wells for 30 min and reactions were stopped by addition of a stop solution. The microplates were read at 450 nm on a Bio-Rad Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). All samples and standards were measured in duplicate. Values were normalized for cell number (pg $.10^5$ cells/ml) and expressed as mean ± SD. For comparative purposes, similar studies were carried out in parallel in rat cultured cardiomyocytes and cardiac fibroblasts to determine whether LPA induced a cell specific effect. The results were normalized to total protein production in each type of cells in order to compare among various types of cells.

2.4. Flow cytometric analysis of cell apoptosis

The Annexin V-FITC/PI Apoptosis Detection Kit was used to evaluate apoptosis of cells. After being rinsed with ice-cold PBS, the cells were resuspended in 200 ml of a binding buffer. Ten microliter Annexin V stock solutions was added to the cells followed by a 30-min incubation at 4 °C. The cells were then further incubated with 5 μl of propidium iodide (PI) and immediately analyzed using a FACScan. Ten thousand events were acquired on a FACSC-LSR (Becton-Dickinson, San Jose, CA) and analyzed with the CellQuest (Becton-Dickinson) software.

2.5. Cell proliferation assay

Mesenchymal stem cells in IMEM with 2% FBS were seeded in 96-well plates at a density of 5×10^3 cells per well and cultured for 48 h. The cells were then treated with different concentrations of LPA (25 and 50 μM) for 24 h. Cell proliferation was measured with a BrdU Cell Proliferation Assay kit (Calbiochem, San Diego, CA, USA). Briefly, 20 μl of BrdU (10 μM) was added to each well and cultures were incubated at 37 °C for 4 h before treatment with LPA for 20 h. The cells were fixed with 1% paraformaldehyde for 20 min at room temperature and then exposed to the mouse anti-BrdU antibody for 1 h at room temperature. A secondary goat anti-mouse antibody was subsequently added for 30 min at room temperature and the plates washed before adding the substrate. Absorbance readings were measured at 450 nm with a Bio-Rad Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). Cell proliferation was determined from a standard curve correlating the absorbances to known cell densities determined in parallel.

2.6. Quantitative real time PCR

Total RNA was isolated from MSCs with Trizol reagent (Invitrogen) and then reverse-transcribed by M-MLV reverse transcriptase and oligo (dT) 18 primer. Quantitative real-time PCR (qPCR) was performed using SYBR Green I (Perkin Elmer Life Sciences, Boston, MA) in an ABI Prism 7300 thermocycler (Applied Biosystems, Foster City, CA, USA). All gene specific extron primers used in the present study were as follows: VEGF: 5′-TTC TAC AAT GAG CTG CGT GTG GC-3′ and 5′-CTC ATA GCT CTT CTC CAG GGA GGA-3′; LPA1: 5′-TCT TCT GGG CCA TTT TCA A-3′ and 5′-GCC GTT GGG GTT CTC GTT-3′; LPA3: 5′-TCT TAG GAG CCT TCG TGG TGT-3′ and 5′-GCT GAT GCT GTC CTC CAG GTA-3′; GNAI2: 5′-AAG ACC TGT CGG GCG TCA TC-3′ and 5′-GCG CTC CAG GTC ATT CAG GTA-3′; GNAI3: 5′-TGA AGA CTA CAG GCA TTG TGG AGA C-3′ and 5′-GTT CGG ATC TTT GGC CAC CTA-3′. The thermal profile for PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To obtain a relative quantitation, the results were normalized to those obtained for the corresponding GAPDH mRNAs. Each sample was run in duplicate.

2.7. Measurement of Na^+/K^+-ATPase activity

The Na^+/K^+-ATPase activity was determined as described [23]. Briefly, MSCs were homogenized and total protein quantified by the Bradford protein assay. The homogenate (400–500 μg of total proteins) was then mixed with 150 μl of a solution containing 80 mM histidine, 20 mM KCl, 6 mM $MgCl_2$, 2 mM EGTA, alamethicin (2 μg/ml), 30 μM digitonin, and 200 mM NaCl at pH 7.4. 10 μl of 300 mM ATP was added to the mixtures for 30 min at 37 °C. The ATP hydrolysis reaction was stopped by the addition of 75 μl of 50% trichloroacetic acid to each well. After centrifugation at 3000 rpm for 15 min, 50 μl of the sample supernatants was added to 100 μl of ammonium molybdate reagent and phosphate content was determined at 640 nm using a spectrophotometer. The Na^+/K^+-ATPase activity was calculated, and expressed as