Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

ENPP2 protects cardiomyocytes from erastin-induced ferroptosis

Yu-Ting Bai [a, c], Rong Chang [c], Hua Wang [b], Feng-Jun Xiao [b], Ri-Li Ge [a, d, *], Li-Sheng Wang [b, **]

[a] Research Center for High Altitude Medicine, Qinghai University, Xining, 810001, PR China
[b] Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing, 100850, PR China
[c] Qinghai Provincial People's Hospital, Xining, PR China
[d] Key Laboratory for Application of High Altitude Medicine in Qinghai Province, Qinghai, Xining, PR China

ARTICLE INFO

Article history:
Received 11 March 2018
Accepted 14 March 2018
Available online xxx

Keywords:
ENPP2
Cardiomyocytes
Ferroptosis
Erastin

ABSTRACT

Ferroptosis is an iron- and oxidative-dependent form of regulated cell death and may play important roles in maintaining myocardium homeostasis and pathology of cardiovascular diseases. Currently, the regulatory roles of lipid signals in regulating cardiomyocytes ferroptosis has not been explored. In this study, we show that ENPP2, as a lipid kinase involved in lipid metabolism, protects against erastin-induced ferroptosis in cardiomyocytes. The classical ferroptosis inducer erastin remarkably inhibits the growth which could be rescued by the small molecule Fer-1 in H9c2 cells. Adenovirus mediated ENPP2 overexpression modestly promotes migration and proliferation and significantly inhibits erastin-induced ferroptosis of H9c2 cells. ENPP2 overexpression leads to increase the LPA level in supernatant of H9c2 cells. H9c2 cells express the LPAR1, LPAR3, LPAR4 and LPAR5 receptors. The supernatant of ENPP2 transduced cardiomyocytes could protects the cells from erastin-induced ferroptosis of H9c2 cells. Furthermore, we observed that ENPP2 overexpression regulates ferroptosis-associated gene GPX4, ACSL4 and NRF2 expression and modulates MAPK and AKT signal in H9c2 cells. Collectively, these findings demonstrated that ENPP2/LPA protects cardiomyocytes from erastin-induced ferroptosis through modulating GPX4, ACSL4 and NRF2 expression and enhancing AKT survival signal.

1. Introduction

Various types of cell death such as apoptosis, ferroptosis and autophagic cell death play an important role in myocardial homeostasis and pathology [1,2]. Ferroptosis is a recently identified type of regulated cell death characterized by the iron-dependent accumulation of lipid ROS [3]. It is regulated by multiple signal pathways such as α6β4 integrin and NRF2-Keap1 axis [4,5]. Many regulators such as glutathione peroxidase 4 (GPX4), NRF2, p53, heme oxygenase-1 and acyl-CoA synthetase long-chain family member 4 (ACSL4) have been identified to be involved in regulation of cell ferroptosis [6–8]. Ferroptosis has been implicated in the pathology of a variety of diseases such as tumors, tissue or organ injury, stroke, ischemia-reperfusion injury and kidney degeneration [9,10]. However, its biological roles and regulation pathways in heart diseases remain poorly understood. New insight into observation of cardiomyocytes ferroptosis may provide new diagnostic and therapeutic approaches for cardiovascular diseases.

Bioactive lipids such as lysophosphatidic acid (LPA) and sphingosine-phosphate (S1P) play important roles in both physiological and pathophysiological processes including angiogenesis, inflammation, fibrosis and carcinogenesis [11–13]. LPA, a bioactive phospholipids with diverse functions, acts as an autocrine/paracrine messenger by activation of its G protein-coupled receptors [14]. Autotaxin (ATX), also termed ENPP2, is a secreted enzyme important for generating LPA [15]. Disturbances in normal ATX-LPA signaling is associated to a range of diseases including cardiovascular disease. Cardiomyocytes express LPA receptor subtypes including LPA1-LPA5. These receptors mediate LPA-induced hypertrophy of neonatal cardiac myocytes via activation of Gi and Rho [16]. LPA also mediated augmentation of cardiomyocytes lipoprotein lipase and involved in both acute and chronic ischemic cardiac damage [17,18]. However, the regulatory roles of ATX-LPA signaling

* Corresponding author. Research Center for High Altitude Medicine, Qinghai University, Xining, 810001, PR China.
** Corresponding author. Department of Experimental Hematology, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing, 100850, PR China.
E-mail addresses: geriligao@hotmail.com (R.-L. Ge), wangls@bmi.ac.cn (L.-S. Wang).

https://doi.org/10.1016/j.bbrc.2018.03.113
0006-291X/

in regulating of cardiomyocytes characteristics have less been explored. In this study, we investigated the regulatory roles of ATX-LPA signaling in erastin-induced ferroptosis of cardiomyocytes and elucidate its mechanisms.

2. Materials and methods

2.1. Cell culture

The rat embryonic cardiomyoblast H9c2 cells were purchased from the ATCC. The H9c2 cells were maintained in DMEM medium (Gibico, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone, Logan, UT) and propagated at 37 °C under 5% CO2.

2.2. Cell viability assays

H9c2 cells were cultured in 96-well plates at 5000 cells/well and treated with erastin (Tocris, Minneapolis, MN, USA) or Ferrostatin-1 (Fer-1) (Sigma-Aldrich, St Louis, MO, USA) at different concentrations for 24 h. Then CCK-8 (Dojindo Molecular Technologies, Kumamoto, Japan) were added and re-incubated at 37 °C under 5% CO2 for 3 h. The absorbance at a wavelength of 450 nm was measured by a microplate reader. The percentage of cell viability was calculated according to the following formula: Percent of cell viability = OD treatment group/OD control group × 100%.

2.3. Adenovirus production and transduction of H9c2 cells

Adenovirus vector carrying the ENPP2 gene (Ad.ENPP2) was purchased from ViGene Biosciences(Shandong, China). The H9c2 cells were infected with Ad.ENPP2 and control vector (Ad.Null) at 20 multiplicity of infection (MOI) for 48 h, then these cells were applied for further assays including proliferation, migration and ferroptosis.

2.4. RNA extraction and real-time quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted from H9c2 cells by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized using RevertAidTM First Strand cDNA Synthesis Kit (Thermo Scientific, Wilmington, DE) according to the manufacturer's instructions. The LPAR1-5 primers were designed and synthesized in Tsingke (Beijing, China) and the primers were as follows: LPAR1, sense 5′-tcttctgggccattttcaa-3′ and antisense 5′-gccgttggggttctcgtt-3′; LPAR2, sense 5′-caatctgccgcttgactgga-3′ and antisense 5′-taaccagcaggttggtcagcaata-3′; LPAR3, sense 5′-tcttaggagccttcgtggtgt-3′ and antisense 5′-gctgatgctgtcctccaggta-3′; LPAR4, sense 5′-agtgcgagttgcccgtttac-3′ and antisense 5′-ttagttcaaaccaaactctgacacc-3′; LPAR5, sense 5′-tgccaattcttcagccaaca-3′ and antisense 5′-ggaagacccagagagccaga-3′; β-actin, sense 5′-ggagattactgccctggctccta-3′ and antisense 5′-gactcatcgtactcctgcttgctg. qPCR was performed for amplifying target genes by using the SYBR® Premix Ex Taq kit (TaKaRa, Dalian, China).

The primers of ENPP2 and GAPDH were purchased from Applied Biosystems (ENPP2 ID: Hs00905125_m1; GAPDH ID: Hs02758991_g1) and were carried out according to protocol of the TaqMan® Gene Expression Assays (Applied Biosystems, FosterCity, CA, USA).

2.5. Western blotting

Total proteins were extracted from H9c2 cells using RIPA buffer containing 1% Phenylmethane sulfonylfluoride (PMSF). The concentrations of protein were determined by a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Equivalent amounts of proteins were loaded on 10% SDS polyacrylamide gels for electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline for 2 h and then labeled with primary antibodies (anti-GAPDH [Cell Signaling], anti-ENPP2 [abcam], anti-GPX4 [abcam], anti-ACSL4 [abcam], anti-NRF2 [abcam], anti-AKT [Cell Signaling], anti-P-AKT [C31E5E, Cell Signaling], anti-p44/42 MAPK [Erk1/2, Cell Signaling], anti-P-p44/42 MAPK [T202/Y204, Cell Signaling]). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibodies (ZhongShan Golden Bridge Biotechnology, Beijing, China) and visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, USA).

2.6. Wound healing assays

Scratched a line across the monolayer with a sterile pipette tip after transfection cells were confluent on 6-well plates. Cell debris were washed and obtained the images of the wound. Cells migrating into the scratched area were acquired, counted and imaged after cells were incubated for 6 h and 12 h.

2.7. Cell migration

Cell migration was also performed using transwell chambers (8 μM membrane pores; Cambridge, MA, USA). After transfection, cells were resuspended with serum-free medium at 2×10^4/well in the top well. DMEM with 15% FBS was added to the lower chamber. After incubation for 10 h, cells that migrated to the lower of the filter membranes were fixed and stained with crystal violet. Then the number of cells was counted under microscope.

2.8. Measurement of reactive oxygen species production

$1 \times 10^{\cdot 5}$ cells/well were seeded in 6-well plates. Cells were treated with DMSO (control), erastin (5 μM) and Ferrostatin-1 (1 μM) for 6 h. Cells were harvested by trypsinization and collected by centrifuge, then washed with Hanks Balanced Salt Solution (HBSS, Gibico) and resuspended in HBSS containing C11-BODIPY581/591 (1 μM, ThermoFisher Scientific) incubated for 10 min at 37 °C. ROS were assayed using fluorescence activated cell sorting (FACS Calibur) and analyzed using Cell Quest software (BD Biosciences, San Jose, CA).

2.9. ELISA analysis

Supernatants of H9c2 cells with or without ENPP2 overexpression were subjected to ELISA analysis for their content of LPA. LPA ELISA kits purchased from Guduo Biotechnology (Shanghai, China) were used according to the manufacturer's instructions.

2.10. Statistical analyses

All experiments were repeated at least three times. The results are presented as mean ± SD. ANOVA and Student's t-tests were performed using Graphpad Prism software (Graphpad Software, La Jolla, CA, USA). A probability level of 0.05 ($P < 0.05$) was considered to be statistically significant.

3. Results

3.1. Erastin induces ferroptosis of H9c2 cardiomyocytes

Erastin is a classic ferroptosis activator and triggers cell ferroptosis in a variety of cell types. To establish the ferroptosis model of

Please cite this article in press as: Y.-T. Bai, et al., ENPP2 protects cardiomyocytes from erastin-induced ferroptosis, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.03.113