Cryobiology 75 (2017) 100–105

Contents lists available at ScienceDirect

Cryobiology

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

Mild hypothermia pretreatment protects hepatocytes against ischemia reperfusion injury via down-regulating miR-122 and IGF-1R/AKT pathway

Qi Xiao [a], Qi Fa Ye [a, b, *], Wei Wang [a], Bi Qi Fu [c], Zhi Ping Xia [b], Zhong Zhong Liu [b], Xing Jian Zhang [b], Yan Feng Wang [b]

[a] Department of Transplant Surgery, The Third Xiangya Hospital, Central South University, Changsha, 410013, PR China
[b] Hubei Key Laboratory of Medical Technology on Transplantation, Zhongnan Hospital of Wuhan University, Institute of Hepatobiliary Diseases of Wuhan University, Transplant Center of Wuhan University, Wuhan, 430071, PR China
[c] Department of Rheumatology and Immunology, The First Affiliated Hospital of Nanchang University, Nanchang, 330006, PR China

ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form
5 November 2016
Accepted 13 January 2017
Available online 14 January 2017

Keywords:
Mild hypothermia pretreatment
microRNA-122
Ischemia reperfusion
IGF-1R
AKT

ABSTRACT

Background: Mild hypothermia has been well known as an effective way to reduce ischemia reperfusion injury (IRI), while the mechanisms are still unclear. More and more evidences have indicated that miRNAs should been involved in the regulation of IRI and expecially some miRNAs have shown temp-responsiveness for temperature variation. Therefore, the role of miR-122 in mild hypothermia pretreatment after IRI was investigated.
Methods: We established a LO2 cell anoxia-reoxygenation injury model to simulate liver IRI. Five groups of differently pretreated L02 cells were studied. ALT, AST and LDH as well as cell viability were measured. Flow cytometric analysis was used to evaluate the apoptosis. The expression of miR-122 was quantified by qRT-PCR. Insulin-like growth factor 1 receptor (IGF-1R), protein kinase B (p-AKT), AKT, forkhead box O3a (p-FOXO3a) and Caspase3 were examined using western blot analysis.
Results: We found that mild hypothermia pretreatment could reduce the hepatocellular injury and induce a significant down-regulation in miR-122 expression after IRI. However, those effects of protection were attenuated by overexpressed miR-122 blockade. We further demonstrated that down-regulation of miR-122 promoted IGF-1R translation and AKT activity, suppressed FOXO3a activity and Caspase3 expression after mild hypothermia pretreatment, which was abrogated by miR-122 mimic.
Conclusion: Our data clearly demonstrate that mild hypothermia pretreatment can down-regulate miR-122 to protect hepatocytes against IRI through activation IGF-1R/AKT signaling pathway and inhibit cells apoptosis.

1. Introduction

Hepatic ischemia reperfusion injury (IRI) is a complex disease associated with surgical procedures, which is a lackage of blood supply results in hypoxia and then followed by resumption of blood flow [12]. IRI causes a series of pathological responses which lead to cell and organ damage that in turn affect the post operative hepatic function and survival [17,26]. Since last decade the prevention and treatment of IRI have become more and more important for research. Mild hypothermia has received significantly attention as a means of protecting organs against IRI [15,20], and can improve survival in animal of hemorrhagic shock [16]. However, its protective mechanisms are not yet fully understood.

MiRNAs have emerged as a novel class of endogenous, small, noncoding oligonucleotides with imperfect complementarity predominantly to the 3′ untranslated region (UTR) of target mRNAs, which negatively regulate gene expression via mRNA degradation or cleavage contributing to down-regulation of protein levels [3]. MiRNAs play a significant role in cell physiological processes, including proliferation, differentiation and apoptosis [1,24]. More and more evidences have indicated that miRNAs are involved in the regulation of IRI [7,30]. Previous study [18] showed that miR-122 is

* Corresponding author. Department of Transplant Surgery, The Third Xiangya Hospital, Central South University, Changsha, 410013, PR China.
E-mail address: yqf_china@163.com (Q.F. Ye).

http://dx.doi.org/10.1016/j.cryobiol.2017.01.005
0011-2240/

exclusively and abundantly expressed in hepatocytes, constituting 70% of the total hepatic miRNAs. At the same time, most studies [6,19]have revealed that cytotoxic or viral induced hepatocellular necrosis is associated with an increase in the level of miR-122. Experimental study [2] first investigated that the level of circulating miR-122 could become a biomarker of acuter hepatic IR. Few targets of miR-122, including serum response factor, cylin G1, IGF-1R, a disintegrin and metalloprotease family-10 and -17, have been experimentally validated [4,10,27]. Study demonstrated that miR-122 suppresses IGF-1R expression and then attenuates IGF-1R/AKT singnaling, which represses cyclin D1 expression and cell proliferation [33].

Currently, the expression profiles of miR-122 in mild hypothermia pretreatment are not identified. In this context, we hypothesize that mild hypothermia pretreatment protect hepatocytes against IRI, in part, via down-regulation of miR-122. Using a vitro model, we investigated the effect of mild hypothermia pretreatment on the outcome of liver IRI and the expression of miR-122. With overexpression of miR-122, we further determined the role of miR-122 and its downstream target IGF-1R.

2. Materials and methods

2.1. Cell culture and treatment

Normal human liver L02 cells were obtained from Kunming Institute of Zoology (Chinese Academy of Sciences, Kunming, China). L02 cells were cultured in DMEM medium supplemented with 10% fatal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in 21% O2 and 5% CO2. The media was changed once per day. Five groups of differently treated L02 cells were studied. (1) Normal control (N) group was incubated with medium only; (2) normothermic pretreatment + IR (NH) group; (3) mild hypothermic pretreatment + IR (MH) group; (4) miR-122 mimic + mild hypothermic pretreatment + IR (miR-122 mimic) group; (5) scramble + mild hypothermic pretreatment + IR (scramble) group.

The cells in the MH group, miR-122 mimic group and scramble group were cultured at 32 °C (mild hypothermic) before ischemic and hypoxic, mild hypothermia was maintained for 6 h. The other groups were cultured for 6 h under normothermic (37 °C).

MiR-122 mimic and the scramble control were obtained from RiboBio (Guangzhou, China). The miR-122 overexpression study was performed using miR-122 mimic (200 nM) and its scramble control (200 nM). Cells were cultured to 30–50% confluence, and transfected with miR-122 mimic and scramble control using lipofectamine 2000 in serum-free Opti-MEM medium according to the manufacturer's instruction. Cells were cultured in fresh medium containing 10% FBS after transfection.

In order to simulate the ischemic and hypoxic environment, L02 cells were exposed to hypoxic by a gaseous mixture of 95% N2 and 5% CO2 (oxygen was expelled by nitrogen) in a 37 °C humidified environment, and cultured in 100% low glucose medium (FBS free) for 12 h. After ischemia and hypoxic, the cells were returned reoxygenation under normoxia conditions with 21% O2 and 5% CO2 at 37 °C and cultured in fresh medium (90% DMEM high glucose medium supplemented with 10% FBS) for 4 h to achieve reperfusion. Then the cells were harvested for further analyses.

2.2. Cell viability assay

Cell viability was measured with cell counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan) according to the manufacture's protocol. L02 cells of different groups were seeded on 96-well plates at 2×10^5/well. After different treatments, 10 μl of CCK-8 solution was added to each well and cells were incubated for another 2 h at 37 °C in a humidified CO2 incubator. The optical absorbance at 450 nm for each sample was measured with a microplate reader. Five separate experiments were conducted and each was performed in triplicated.

2.3. Annexin V and phosphatidylinositol (PI) binding staining

The assay of Annexin V and PI binding staining was performed with an Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions. In short, differently treated cells were digested with 0.25% trypsin without EDTA, and then washed twice with ice-cold PBS, centrifuged at 1200 rpm for 5 min. Cells were resuspended in 500 μl of binding buffer at a concentration of 1×10^6 cells/ml, 5 μl Annexin V-FITC and 5 μl PI were added. Cells were gently mixed and incubated for 15 min at 37 °C in the dark. Transfer 400 μl of cell suspension to flow tubes. Stained cells were analyzed by BD FACSCalibur flow cytometer.

2.4. Measurement of alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) levels

ALT, AST and LDH Levels, the indicator of hepatocellular injury, were measured using an automatic biochemistry analyzer.

2.5. Real-time PCR assay of microRNAs

Trizol reagent was used to extract total RNA from cell sample according to the manufacturer's instructions, a UV spectrophotometer was used to measure the purity and concentration of RNA and the RNA integerity was verified by agarose gel electrophoresis. Taqman RNA reverse transcription kit was used for reverse transcription of miR-122. Quantitative real-time PCR was conducted using a Quantitect SYBR Green RT-PCR kit (TaKaRa Bio, Tokyo, Japan) and an Applied Biosystems 7500 system. Data were analyzed using the comparative Ct ($2^{-\Delta\Delta ct}$) that was normalized to the U6. hsa-miR-122 loop primer:5-GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGA CCAAACACC; hsa-miR-122 F primer: 5′ TGCGCTGGAGTGTGACAATGGT 3′, R primer:5′ CCAGTGCAGGGTCC-GAGGTATT 3’; U6 F: 5′ CGCTTCGGCAGCACATATAC 3′ R: 5′ AAA-TATGGAACGCTTCACGA 3’.

2.6. Western blot analysis

All protein samples were performed in lysates buffer containing protease inhibitors and western blotting was performed following the standard procedures. Protein lysates were electrophoresed on 10% SDS-PAGE gels, and transferred onto nitrocellulose membranes (Bio-Rad, USA). The membranes were incubated with primary antibodies against IGF-1R, p-AKT, AKT, p-FOXO3a, Caspase3. Blotting of β-actin was used as a loading control. All the antibodies were purchased from Abcam. Nitrocellulose membranes were then washed with TBST repeatedly and incubated with secondary antibodies. ECL was used to visualize the immunoreactive bands. Signal intensities of all protein bands were assessed by the Quantity One software.

3. Statistical analysis

All experiments were independently repeated at least 3 times. Quantitative data were expressed as Mean ± SD. Comparision between two groups were performed using student's *t*-test, while the differences among ≥3 groups were studied and analyzed by one-way analysis of variance (ANOVA) for post hoc multiple comparisons. All differences were considered statistically significant at the $p < 0.05$.