Journal of Molecular and Cellular Cardiology 75 (2014) 111–121

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

β-Blocker carvedilol protects cardiomyocytes against oxidative stress-induced apoptosis by up-regulating miR-133 expression

Chaoqian Xu [a,b,1], Yingying Hu [a,1], Liangyu Hou [a], Jin Ju [a], Xiaoguang Li [a], Ning Du [a], Xiaoxiang Guan [a], Zhenhong Liu [a], Tianze Zhang [c], Wei Qin [a], Nannan Shen [a], Muhammad U. Bilal [a], Yanjie Lu [a,b], Yong Zhang [a,b,*], Hongli Shan [a,b,*]

[a] Department of Pharmacology (State-Province Key Laboratories of Biomedicine — Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), Harbin Medical University, Harbin, Heilongjiang, China
[b] Institute of Cardiovascular Research, Harbin Medical University, Harbin, Heilongjiang, China
[c] Department of Cardiothoracic Surgery, The Second Affiliated Hospital of Harbin Medical University, China

ARTICLE INFO

Article history:
Received 10 May 2014
Received in revised form 26 June 2014
Accepted 15 July 2014
Available online 25 July 2014

Keywords:
Oxidative stress
Apoptosis
Carvedilol
miR-133
Caspase-3
Caspase-9

ABSTRACT

Oxidative stress is a causal factor and key promoter of a variety of cardiovascular diseases associated with apoptotic cell death by causing deregulation of related genes. Though carvedilol, a β-adrenergic blocker, has been shown to produce cytoprotective effects against cardiomyocyte apoptosis, the mechanisms are not fully understood. The present study was designed to investigate whether the beneficial effects of carvedilol are related to microRNAs which have emerged as critical players in cardiovascular pathophysiology via post-transcriptional regulation of protein-coding genes. In vivo, we demonstrated that carvedilol ameliorated impaired cardiac function of infarct rats and restored miR-133 expression. In vitro, carvedilol protected cardiomyocytes from H_2O_2 induced apoptosis detected by TUNEL staining and MTT assays, and increased miR-133 expression in cardiomyocytes. Overexpression of miR-133, a recognized anti-apoptotic miRNA, produced similar effects to carvedilol: reduction of reactive oxygen species (ROS) and malondialdehyde (MDA) content and increment of superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) level, so as to protect cardiomyocytes from apoptosis by downregulating caspase-9 and caspase-3 expression in the presence of H_2O_2. Transfection with AMO-133 (antisense inhibitor oligodeoxyribonucleotides) alone abolished the beneficial effects of carvedilol. Caspase-9-specific inhibitor z-LEHD-fmk, caspase-3-specific inhibitor z-DEVD-fmk, caspase-9 siRNA and caspase-3 siRNA were used to establish caspase-3 as a downstream target of miR-133. In conclusion, our data indicated that carvedilol protected cardiomyocytes by increasing miR-133 expression and suppressing caspase-9 and subsequent apoptotic pathways.

1. Introduction

Cardiovascular disease is the main cause of death and birth defects all over the world [1]. Oxidative stress is one of the common responses to cardiac injuries in a variety of cardiovascular diseases [2]. Over-production of oxidative stress causes damage to a variety of cellular macromolecules, including lipids, DNA and proteins [3]. Increased ROS level contributes critically to the advance of atherosclerosis, hypertension, heart failure and acute myocardial infarction (MI) [4]. Oxidative stress is a major inducer of cardiomyocyte apoptosis [5]. However, the mechanisms underlying oxidative stress-induced apoptosis in cardiomyocytes are poorly understood.

It has been well recognized that microRNAs (miRNAs, miRs) play a central role in regulating some key protein-coding genes related to cardiovascular disease [6–10]. Among the known miRNAs, miR-133 is found to be specifically expressed in adult cardiac and skeletal muscles [11,12]. Recent functional studies indicate that miR-133 produces an anti-apoptotic effect, protecting cardiomyocytes against apoptotic cell death under stress [13].

β-Adrenergic blockers have been widely used for the treatment of cardiovascular diseases, such as hypertension, hyperlipidemia, and coronary heart disease, especially for preventing sudden cardiac death in patients suffering acute or chronic MI [14–16]. Carvedilol, a nonselective β-adrenoceptor antagonist, is a multiple-action drug that has potent antioxidant properties [17]. Several experimental models of ischemia–

Abbreviations: ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; SOD, superoxide dismutase; MDA, malondialdehyde; GPx, glutathione peroxidase; AO/EB, acridine orange/ethidium bromide; MI, myocardial infarction; AMO, antisense inhibitor oligodeoxyribonucleotides.

* Corresponding authors at: Department of Pharmacology (State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), Harbin Medical University, Heilongjiang 150081, China. Tel./fax: +86 451 86671354.
E-mail addresses: hmuzhangyong@hotmail.com (Y. Zhang), shanhongli@ems.hrbmu.edu.cn (H. Shan).
[1] Contributed equally to this work.

http://dx.doi.org/10.1016/j.yjmcc.2014.07.009
0022-2828/

reperfusion (IR) have already underlined the cardioprotective effects of carvedilol [18]. Although studies have shown that carvedilol also inhibits cardiomyocyte apoptosis [19], the exact mechanism is still unknown. In this study, we investigated whether carvedilol protects cardiomyocyte from apoptosis by influencing miRNA under pathological conditions relevant to human cardiac disease.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethic Committees of the Harbin Medical University. Experimental procedures were approved by the Animal Care and Use Committee of Harbin Medical University, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Rat model of myocardial infarction (MI) and drug administration

Healthy male Wistar rats (200–250 g) were used and kept under standard animal room conditions at room temperature (23 ± 1 °C), with constant humidity of 55 ± 5%. Rats were randomly divided into three groups: sham, MI and MI + Car groups. Left anterior descending coronary artery ligation was performed to induce myocardial infarction (MI) as described previously [20]. In the MI + Car group, rats were intragastrically administered with carvedilol (10 mg/kg/day) for two weeks prior to subsequent measurements.

2.3. Echocardiography

Twenty-four hours after left anterior descending coronary artery ligation, a noninvasive transthoracic echocardiography method was used to evaluate the function of the left ventricle. After anesthetized with sodium pentobarbital (40 mg/kg, i.p.), rats were fixed on their backs with their fur shaved and skin cleaned. The parasternal long axis view was selected by using a high-frequency linear-array transducer. The parameters of heart structure and function were checked in the two-dimensional ultrasound-guided M-curve.

2.4. Cell culture

Cardiomyocytes were obtained from 1- to 3-day-old neonatal Wistar rats as described previously [21–23]. Newborn rats were cleaned with 75% ethanol, and then sacrificed by decapitation with heads dropped immediately into liquid nitrogen. Hearts were isolated and minced in serum-free DMEM, and then digested in 0.25% trypsin solution. Dispersed cells were suspended in DMEM containing 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin and centrifuged. Pooled cells were plated into culture flasks (non-coated) and 0.1 mM bromodeoxyuridine was added into the medium to deplete non-myocytes. Cardiomyocytes were incubated under a condition of 5% CO_2 at 37 °C for 48 h, and were subsequently treated with various concentrations of H_2O_2 (25, 50 and 100 μM) or carvedilol (0.1, 1 and 10 μM) for 24 h. Caspase-9-specific inhibitor z-LEHD-fmk (20 μM) and caspase-3-specific inhibitor z-DEVD-fmk (20 μM) were purchased from Merck, Germany.

2.5. Transfection of miRNA or siRNA

MiR-133 (5′-UUGGUCCCCUUCAACCAGCUGU-3′) and anti-miR-133 antisense oligonucleotides (AMO-miR-133) (5′-ACAGCUGGUUGAAG GGGACCAA-3′) were synthesized by Guangzhou Ribo Bio Co., Ltd. AMO-133 contained 2′-*O*-methyl modifications. Caspase-9 siRNA (5′-AUAUCUGCAUGUCUCUCGAUCUUCC-3′) and caspase-3 siRNA (5′-UACCAUUGCGAGCUGACAUUCCAGU-3′) were synthesized by Invitrogen, USA. Cardiomyocytes (1×10^5 per well) were starved in serum-free medium for 24 h before transfection with X-treme GENE siRNA transfection reagent (catalog #04476093001; Roche) according to the manufacturer's instructions.

2.6. Cell viability assay

Cells (2×10^4 cells/well) were seeded in a 96-well culture plate. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions. The absorbance was measured at 490 nm.

2.7. TUNEL assay

DNA fragmentation of individual cells was detected in situ by TUNEL with the In Situ Cell Death Detection Kit (TUNEL fluorescence FITC kit, Roche, Indianapolis, IN, USA) [24]. Cardiomyocytes grown on coverslips were washed with PBS containing (in mM) NaCl 137, KCl 2.7, Na_2HPO_4 4.3, KH_2PO_4 1.4, pH 7.4, and fixed in 4% paraformaldehyde solution for 1 h at 4 °C. The cells were permeabilized in a solution containing 0.1% Triton X-100 for 2 min, followed by incubation in freshly prepared TUNEL reaction mixture for 1 h at 37 °C in the dark. The coverslips were then washed with PBS. Next, the coverslips were mounted on slides with anti-fading solution and TUNEL staining was analyzed with a fluorescence microscopy (Eclipse 80i; Nikon Co., Tokyo, Japan).

2.8. Determination of oxidative stress

Detection of reactive oxygen species (ROS) accumulated in cardiomyocytes was carried out with the oxidation sensitive probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) [24]. Cells cultured on glass coverslips in 6-well plates were incubated with DCFH-DA (10 μM) for 30 min at 37 °C. After they were washed, the cells were fixed in 3% paraformaldehyde in PBS for 30 min. The images were taken by confocal microscopy.

Superoxide dismutase (SOD) activity, malondialdehyde (MDA) content and glutathione peroxidase (GPx) levels were tested with Biochemical Analysis Kits (Jiancheng Biotechnology Co., Nanjing, China) according to the respective manufacturers' protocols.

2.9. AO/EB staining

Acridine orange/ethidium bromide (AO/EB) double staining was used to detect the apoptotic cells as described previously [24]. Cardiomyocytes were harvested with 10 μl of prepared AO/EB working solution (100 μg/ml AO and 100 μg/ml EB in PBS) and then examined under a fluorescence microscope (Eclipse 80i; Nikon Co., Tokyo, Japan).

2.10. RNA isolation and real-time quantitative RT-PCR (qRT-PCR)

Total RNA from cultured neonatal cardiomyocytes after different treatments or heart tissue was extracted using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's protocols. The levels of miR-133 and caspase-9 mRNA were determined using SYBR Green I incorporation method on ABI 7500 fast Real Time PCR system (Applied Biosystems, USA), with U6 and GAPDH as internal controls for miR-133 and caspase-9, respectively.

2.11. Protein isolation and western blot analysis

Protein samples were extracted from cells with the procedures essentially the same as described in detail elsewhere [25]. For western blot analysis, 100 μg protein samples were fractionated by SDS–PAGE (12% SDS–polyacrylamide gel). The primary antibodies against total caspase-3 and total caspase-9 (Cell Signaling Technology, Danvers,