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Original article

FAT10 attenuates hypoxia-induced cardiomyocyte apoptosis by stabilizing caveolin-3

Qiongqiong Zhou[a,1], Xiaogang Peng[b,1], Xiao Liu[a], Leifeng Chen[c], Qinmei Xiong[a], Yang Shen[b], Jinyan Xie[b], Zhenyan Xu[a,b], Lin Huang[a], Jinzhu Hu[a], Rong Wan[b], Kui Hong[a,b,*]

[a] Department of Cardiovascular Medicine, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, China
[b] Jiangxi Key Laboratory of Molecular Medicine, Nanchang, Jiangxi 330006, China
[c] Department of General Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, China

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ABSTRACT

FAT10, a member of the ubiquitin-like-modifier family of proteins, plays a cardioprotective role in response to hypoxic/ischemic injury. Caveolin-3 (Cav-3), a muscle-specific caveolin family member, is involved in cardiomyocyte apoptosis. However, the link between FAT10 and Cav-3 in ischemic cardiomyocytes is unclear. In the present study, we found that both FAT10 and Cav-3 were upregulated in ischemic myocardial tissues and in hypoxic cardiomyocytes. Furthermore, our results demonstrated that FAT10 inhibits hypoxia-induced cardiomyocyte apoptosis by increasing Cav-3 expression. Importantly, following myocardial infarction, knockout of FAT10 aggravated cardiac dysfunction and increased cardiomyocyte apoptosis by reducing Cav-3 expression. Additionally, Cav-3 was degraded by the ubiquitin–proteasome system (UPS) in cardiomyocytes. Mechanistically, we found that FAT10 stabilizes Cav-3 expression by inhibiting ubiquitination-mediated degradation in cardiomyocytes. Together, these findings revealed a novel role of FAT10 in protection against ischemia-induced injury via stabilization of Cav-3, providing evidence that the FAT10/Cav-3 axis may be a potential therapeutic target for patients with ischemic heart conditions.

1. Introduction

The loss of cardiomyocytes in the ischemic zone reduces cardiac contractility and impedes angiogenesis and repair [1]. In addition to cardiomyocyte necrosis, cardiomyocyte apoptosis also causes loss of cardiomyocytes during the ischemia process [2]. Although knowledge regarding the signaling pathways in cardiomyocyte apoptosis has rapidly expanded, therapeutic targeting of this cellular process is currently unavailable [3]. However, given the complexity of ischemia progression, it seems that many important apoptosis-regulating genes have not been clearly defined.

Caveolins (Cavs) are the fundamental components of caveolae, which compartmentalize and modulate signal transduction in many cell types [4]. The caveolin family consists of three members: Cav-1, Cav-2, and Cav-3 [5]. Increasing evidence confirms that the role of Cav-3 in cardiovascular diseases have attracted extensive attention [6,7]. Under stress adaptation, Cav-3 is essential for protective effects against various injuries in the heart [8,9]. Recent studies have demonstrated that Cav-3 plays a very important role in cardiomyocyte apoptosis. For instance, Cav-3 overexpression substantially attenuates ischemic-induced cardiac injury [8]. Additionally, upregulation of Cav-3 expression significantly reduces TNF-α-induced cardiomyocyte apoptosis [9]. A previous study demonstrated that Cav-3 expression is upregulated through activation of both the p38 and PI3-kinase/AKT pathways in myoblasts [10], but the regulatory mechanisms of Cav-3 expression in cardiomyocytes, especially in the ischemic myocardium, are still unclear. Another previous study found that Cav-1 is subject to ubiquitin-dependent degradation [11]. However, whether Cav-3 is degraded by the ubiquitin-proteasome system (UPS) remains unclear.

The UPS is largely responsible for the degradation of proteins, thereby regulating regulation of cardiac dysfunction in the ischemic heart [12]. In recent years, several ubiquitin-like proteins have been identified, including small ubiquitin-like modifiers (SUMOs), interferon-stimulated gene 15 (ISG15), and human leukocyte antigen (HLA)-F adjacent transcript 10 (FAT10) [13,14]. FAT10, which contains 165 amino acids arranged in two in-tandem ubiquitin-like domains, is

* Corresponding author at: Department of Cardiovascular Medicine, Jiangxi Key Laboratory of Molecular Medicine, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, China.
E-mail address: hongkui88@163.com (K. Hong).
[1] Z. Q. and P. X. contributed equally to this work.

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strongly upregulated by pro-inflammatory cytokines in a variety of cells [15,16]. Our recent study also provided evidence that FAT10 expression is strongly induced in the ischemic myocardium and that its increased expression may be caused by inflammatory cytokines, such as TNF-α and IFN-γ [17]. Similar to ubiquitin, FAT10 is a well-known signal for proteasomal degradation, but increasing evidence has shown that the functions of FAT10 extend beyond protein degradation [18–21]. Our previous studies also demonstrated for the first time that FAT10 stabilizes specific substrates by antagonizing their ubiquitination [19–21]. Interestingly, FAT10 is involved in various cellular processes, such as cell cycle regulation, immune response and cell apoptosis [22,23]. We also found that FAT10 protects cardiomyocyte against apoptosis in response to hypoxic/ischemic injury [17]. However, the mechanism by which FAT10 suppresses cardiomyocyte apoptosis remains unclear.

In the present study, using a combination of in vitro and in vivo approaches, we investigated the role of FAT10 in ischemic rat hearts and hypoxic cardiomyocytes. Our results showed that FAT10 inhibited hypoxia-induced cardiomyocyte apoptosis by increasing Cav-3 expression. Importantly, we found that FAT10 deletion markedly exacerbated cardiac dysfunction and increased cardiomyocytes apoptosis in myocardial infarction (MI) hearts by reducing Cav-3 expression. Further investigation revealed that Cav-3 was degraded by the UPS and that FAT10 antagonized Cav-3 ubiquitination-mediated degradation to stabilize its expression in cardiomyocytes. Taken together, our findings demonstrate that FAT10 attenuates hypoxia-induced cardiomyocyte apoptosis by stabilizing Cav-3.

2. Materials and methods

2.1. Ischemic animal model and surgical procedure

Newborn (6–10 g, 2 day) and adult male Sprague-Dawley rats (250–280 g, 10 week) were used in this study. All experiments and procedures were approved by the Animal Ethics Committee of Nanchang University and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Myocardial infarction (MI) was induced in male, 10-week-old Sprague Dawley (SD) rats via ligation of the left anterior descending artery (LAD). Regional ischemia of the heart was confirmed by ECG, which showed ST elevation. Control rats underwent sham operations without ligation of the LAD. Seven days after surgery, the rats were euthanized and their hearts were quickly excised for further evaluation.

2.2. Cardiomyocyte culture and hypoxia injury in neonatal rat heats

Neonatal rat cardiomyocytes were isolated from the ventricular myocardium of 2-day-old Sprague Dawley rats by enzymatic digestion according to a published protocol [24,25]. Then, cultured cardiomyocytes were sequentially subjected to hypoxic injury (CO_2/N_2 /O_2 at a 94:5:1 ratio) for different time (0 to 8 h).

In addition, the experimental design for assessing cultured cells in response to hypoxia was as follows. In the control group (Control), cardiomyocytes were subjected to hypoxia for 8 h. For the control shRNA lentivirus particles (sh-NC) or control vector lentivirus particles (Lv-vector) groups, cardiomyocytes were incubated in DMEM after 24 h of exposure to sh-NC or Lv-vector and then subjected to hypoxia for 8 h. In the Cav-3 shRNA lentivirus particles (sh-Cav-3) or FAT10 shRNA lentivirus particles (sh-FAT10) groups, cardiomyocytes were pre-treated with sh-Cav-3 or sh-FAT10 for 24 h in DMEM and then subjected to hypoxia for 8 h. The FAT10 lentivirus particles (Lv-FAT10) group included cardiomyocytes that were pre-treated with Lv-FAT10 for 24 h in DMEM and subsequently subjected to hypoxia for 8 h. The sh-NC + Lv-vector group was composed of cardiomyocytes pre-treated with sh-NC and Lv-vector (1:1) for 24 h in DMEM and subsequently subjected to hypoxia for 8 h. In the Lv-FAT10 + sh-Cav-3 group, cardiomyocytes were pre-treated with Lv-FAT10 and sh-Cav-3 (1:1) for 24 h in DMEM and then subjected to hypoxia for 8 h. In the sh-FAT10 + Lv-Cav-3 group, cardiomyocytes were pre-treated with sh-FAT10 and Cav-3 (1:1) for 24 h in DMEM and then subjected to hypoxia for 8 h. All in vitro experiments in cardiomyocytes were performed 3 times.

2.3. Generation of FAT10 knockout rat model

FAT10-knockout rats (FAT10-KO) were generated using the CRISPR-Cas9 technique in a Sprague Dawley (SD) background. In brief, the FAT10 gene contains two exons. The knockout allele lacks the 911 base pairs of exon 2, leading to a truncated protein of FAT10. Knockout of FAT10 was confirmed by Western blot and gene sequencing.

2.4. Histological analysis

Histological and immunohistochemical staining were performed as previously described [17,26]. In brief, heart sections were prepared at 4-μm thickness through a routine procedure. The sections were subsequently stained with hematoxylin and eosin (HE) and Masson's trichrome staining to determine the extent of fibrosis [26]. For immunohistochemical staining (IHC), the sections were incubated with primary antibodies against Cav-3 (Abcam, 1:200) and FAT10 (Santa Cruz, 1:200) [17].

2.5. Triphenyltetrazolium chloride (TTC) staining

Myocardial infarct size was determined by TTC staining as previously described [27]. Briefly, at the end of 7 days post-MI, the rat hearts were quickly excised and then sliced into 1.0-mm-thick sections. Next, the sections were incubated with 1% TTC at 37 °C for 15 min and then scanned. For infarct size measurement, TTC-stained areas and TTC-negative staining areas (infarcted myocardium) were measured. Myocardial infarct size was expressed as a percentage of the total LV area.

2.6. Analysis of cardiac function by echocardiography

The cardiac phenotypes of the rats were characterized using echocardiography before and after LAD ligation. The animals were anesthetized with 1% isoflurane and examined via non-invasive echocardiography (echocardiograph Vivid 7 ultrasound; GE).

2.7. Cardiomyocyte apoptosis by TUNEL staining and flow cytometry

Cell apoptosis was determined by TUNEL (terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labeling) assay using an in situ cell death detection kit (Roche, Indianapolis, IN, USA), or by flow cytometry after mixing cells with fluorescein isothiocyanate (APC)-labeled annexin-V and propidium iodide.

2.8. Cell lines and cell culture

H9C2 and HEK-293 cells were obtained from ATCC (Rockville, USA). The cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 mg/mL of penicillin and 50 mg/mL of streptomycin (Invitrogen, USA). All cell lines were maintained in an incubator in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.9. Plasmid and lentiviral constructs

GST-Cav-3, deletion forms of Cav-3 (1–54 aa, 55–106 aa and 107–151 aa) and Cav-3 mutant forms were purchased from Genechem