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Knockout of CNR1 prevents metabolic stress-induced cardiac injury through improving insulin resistance (IR) injury and endoplasmic reticulum (ER) stress by promoting AMPK-alpha activation

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ABSTRACT

Obesity and diabetes are associated with diabetic cardiomyopathy (DCM). However, the pathogenesis of DCM is not fully understood. Cannabinoid receptor gene (CNR1) has been a drug target for the treatment of obesity. Here, we reported that CNR1 expression was increased in high fat diet (HFD)-induced heart of mice. Following, the wild type ($CNR1^{+/+}$) and CNR1-knockout ($CNR1^{-/-}$) mice were employed and subjected to HFD treatments for 16 weeks to further investigate the effects of CNR1 on DCM. The results indicated that CNR1 knockout mice after HFD feeding exhibited a significant decrease of body weight and lipid accumulation in serum. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) analysis indicated that HFD-induced insulin resistance was attenuated by CNR1 deficiency. HFD-triggered cardiac dysfunction was also improved by CNR1 knockout using echocardiographic analysis. Further, CNR1 suppression increased expressions of genes promoting fatty acid oxidation, and mitochondrial biogenesis. Also, TUNEL staining showed that CNR1 inhibition markedly reduced apoptotic levels in heart tissue sections of HFD-fed mice. Importantly, HFD-induced insulin resistance was prevented by CNR1-knockout through decreasing p-$IRS1^{Ser}$ expressions, and increasing phosphorylated insulin receptor substrate 1 (p-$IRS1^{Tyr}$), phospho-AMP-activated protein kinase α (AMPKα) and phospho-acetyl-CoA carboxylase α (ACCα) expressions in heart tissue samples. In addition, CNR1 knockout impeded endoplasmic reticulum (ER) stress caused by HFD via down-regulating phospho-protein kinase-like ER kinase (PERK), phospho-eukaryotic initiation factor-2α (eIF2α), activating transcription factor 4 (ATF4) and ATF6 in heart tissue samples. Of note, we found that CNR1 knockout-improved insulin resistance, ER stress and lipid accumulation was diminished by AMPKα suppression using its inhibitor, Compound C. Therefore, the results demonstrated that therapeutic CNR1 inhibition could alleviate the progression of DCM.

1. Introduction

Diabetic cardiomyopathy (DCM) suggests a strong association with heart failure and diabetes and a major cause of morbidity and mortality in diabetic patients [1]. Obesity and type 2 diabetes (T2D) promote the risk for cardiomyopathy [2]. Insulin resistance plays an essential role in regulating obesity and T2D development. There are studies suggesting that insulin resistance is involved in HFD-induced DCM in murine animals [3,4]. Additionally, clinical studies have demonstrated that patients with cardiovascular diseases are always closely associated with insulin resistance [5]. Further, several factors, such as lipid accumulation and ER stress, are critical reasons that contribute to DCM [6]. On the one, lipotoxicity cardiomyopathy is a significant result of excessive lipids accumulation in heart, a major threat to human with DCM [7]. On the other, endoplasmic reticulum is an organelle where proteins form their appropriate structures. However, various proteins become unfolded or misfolded when exposed to stimuli, such as hyperglycemia, disturbance of calcium homeostasis and overexpression of abnormal proteins, activating ER stress eventually [8]. ER stress is a pathogenic molecular mechanism related to diabetes, and is often associated with obesity [9]. However, presently the pathogenesis of DCM associated with insulin resistance and ER stress is not fully understood.

Cannabinoid receptor gene (CNR1), encoding the type 1 cannabinoid receptor (CB1), is a superfamily of G protein-coupled

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receptors, which is recognized to stimulate multiple signaling pathways modulating cell survival/death and energy metabolism [10,11]. CNR1 plays an important role in hepatic insulin resistance [12]. CNR1 is identified as one of the genes with significantly increased levels in subcutaneous and omental adipose tissue in animal model with insulin resistance [13]. CNR1 modulates food intake in hypothalamus [14]. And thus, in obesity the CNR1 system is increased, both centrally and peripherally [15]. Given the effects of CNR1 on obesity and insulin resistance, we hypothesized that CNR1 might play a critical role in regulating DCM.

In the study, we aimed to investigate whether CNR1 was an essential factor related to the progression of DCM induced by HFD. We discovered that CNR1 was selectively increased in hearts of obese animals and in individuals with metabolic syndrome. Phenotypic analysis indicated that HFD-induced cardiac injury was attenuated in CNR1-knockout mice. Insulin resistance, lipid accumulation and ER stress were prevented in CNR1-deficient mice. Mechanistic studies indicated that CNR1 deletion-improved DCM induced by HFD was, at least partly, dependent on AMPKα activity.

2. Materials and methods

2.1. Patients and samples

Heart tissue samples of diabetic heart failure were obtained from patients with history of diabetes mellitus and metabolic syndrome (MS) for more than 5 years exhibiting clinical symptoms associated with heart failure were included and patients with history of myocardial infarction, ischemic heart disease, or coronary artery diseases were excluded from the study. The control group included patients without history of diabetes mellitus, or cardiovascular diseases. Written informed consent was obtained from all subjects or from the families of the heart donors. The study was approved by the First Affiliated Hospital of Xinxiang Medical College (Henan, China).

2.2. Animals and treatments

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Institute of Model Animal of the First Affiliated Hospital of Xinxiang Medical College. The animals received humane care following the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Male wild type ($CNR1^{+/+}$) C57BL/6 background mice, ob/ob, db/db, and lean mice were purchased from the Laboratory Animal Center, Medical College of Zhengzhou University (Zhengzhou, China). Ob/ob, db/db, and lean mice were fed a chow. CNR1-knockout mice ($CNR1^{-/-}$) mice were designed and purchased form Cyagen Biosciences Inc (USA). 8-week-old male mice on a C57BL/6 background (22–24 g) were kept in a standard environment with a 12-h light-dark cycle. A mouse model of DCM was established by feeding the mice a HFD (D12492, Research Diets, USA) for 16 weeks. Mice that were fed a normal chow (D12450B, Research Diets) served as controls. Mice were divided into 4 groups (n = 8/group), including $CNR1^{+/+}$/Chow, $CNR1^{-/-}$/Chow, $CNR1^{+/+}$/HFD, and $CNR1^{+/+}$/HFD. After feeding for 16 weeks, blood pressure (BP) including diastolic and systolic BPs was determined by tail-cuff manometry (Visitech Systems, Cary, NC) as previously described [16]. Then, all mice were sacrificed for further research.

2.3. Cell isolation and treatment

Adult mouse cardiomyocytes were isolated as described [17]. The isolated myocardiocytes were then cultured in high-glucose DMEM medium (Gibco, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS (Invitrogen, USA) and maintained at 37 °C and 5% CO_2. Palmitate (200 μM, Sigma Aldrich, USA) was added to cells for 24 h. A769662 was purchased from LC Laboratories (Woburn, USA). Compound C was purchased from EMD Millipore (Billerica, USA).

2.4. Cardiac function analysis

Cardiac function was calculated in anesthetized mice (ketamine 80 mg/kg and xylazine 12 mg/kg, i.p.) with the two-dimensional guided M-mode echocardiography (Philips SONOS 5500) equipped with a 15–6 MHz linear transducer (Phillips Medical Systems, USA). Left ventricular (LV) anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M-mode [18]. Fractional shortening, ejection fraction (EF), left ventricular end-systolic (LVIDs), and left ventricular end-diastolic (LVIDd) were calculated as previously described [19].

2.5. Biochemical measurements

The concentration of triglyceride (TG), total cholesterol (TC) and free fatty acid (FFA) was measured by using commercial kits (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China) following the manufacturer's protocol. Serum insulin levels were measured using insulin ELISA kit (Crystal Chem, USA) following the manufacturer's protocol.

2.6. Western blot analysis

Cardiomyocytes or heart tissue samples were homogenized in lysis buffer (KeyGen Biotech, Nanjing, China) containing protease and phosphatase inhibitors. Protein concentration was measured using BCA protein assay (Pierce, UAS). Proteins were separated using 10% SDS-PAGE and transferred to PVDF membrane (BioRad, USA). Following incubation with primary and secondary HRP-conjugated antibody (Supplementary Table 1), bands were detected using ECL reagents (Pierce). The blots were detected on Kodak film developer (Fujifilm, Japan) and normalized to the loading control GAPDH.

2.7. Real time-quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from heart tissues or cells using Trizol regent (KeyGen Biotech) following the manufacturer's instructions. Quantitative real-time RT-PCR was performed using 7300 real-time PCR system (Applied Biosystems, USA). Primer sequence used in our study was synthesized by Generay Biotech (Shanghai, China) and listed in Supplementary Table 2. The mRNA signal was normalized to GAPDH. The mean value of each experiment, performed in triplicate, was used to determine the relative mRNA level.

2.8. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

To evaluate GTT, mice were intraperitoneally injected with glucose (1.5 g/kg) after an overnight fasting (8 h), and venous blood was collected after injection at 0, 30, 60, 90 and 120 min from the tail of each mouse. The glucose was measured using a glucose meter (G832631, Roche, USA). To assess ITT, a single dose of Novolin R regular insulin (Novo Nordisk A/S, Denmark) (0.5 units/kg) was intraperitoneally administered to mice after fasting for 8 h, and the blood glucose level was measured using glucose meter as described above.