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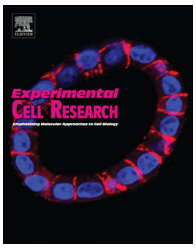

Research Article

Roles of mitochondrial fragmentation and reactive oxygen species in mitochondrial dysfunction and myocardial insulin resistance

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ABSTRACT

Purpose: Evidence suggests an association between aberrant mitochondrial dynamics and cardiac diseases. Because myocardial metabolic deficiency caused by insulin resistance plays a crucial role in heart disease, we investigated the role of dynamin-related protein-1 (DRP1; a mitochondrial fission protein) in the pathogenesis of myocardial insulin resistance.

Methods and Results: DRP1-expressing H9c2 myocytes, which had fragmented mitochondria with mitochondrial membrane potential ($\Delta\Psi_m$) depolarization, exhibited attenuated insulin signaling and 2-deoxy-D-glucose (2-DG) uptake, indicating insulin resistance. Treatment of the DRP1-expressing myocytes with Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (TMPyP) significantly improved insulin resistance and mitochondrial dysfunction. When myocytes were exposed to hydrogen peroxide (H_2O_2), they increased DRP1 expression and mitochondrial fragmentation, resulting in $\Delta\Psi_m$ depolarization and insulin resistance. When DRP1 was suppressed by siRNA, H_2O_2-induced mitochondrial dysfunction and insulin resistance were restored. Our results suggest that a mutual enhancement between DRP1 and reactive oxygen species could induce mitochondrial dysfunction and myocardial insulin resistance. In palmitate-induced insulin-resistant myocytes, neither DRP1-suppression nor TMPyP restored the $\Delta\Psi_m$ depolarization and impaired 2-DG uptake, however they improved insulin signaling.

Conclusions: A mutual enhancement between DRP1 and ROS could promote mitochondrial dysfunction and inhibition of insulin signal transduction. However, other mechanisms, including lipid metabolite-induced mitochondrial dysfunction, may be involved in palmitate-induced insulin resistance.

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Introduction

Myocardial energy deficiency is closely associated with both the initiation and progression of various heart diseases [1–4]. In physiological conditions, the myocardium utilizes energy from substrates (fatty acids and carbohydrates) according with the metabolic demand and environment [5]. Several studies have suggested that myocardial insulin resistance, where the myocardium is disturbed to use glucose as an energy substrate and depends on fatty acids (FAs), plays a crucial pathophysiological role in heart disease [6,7]. Because mitochondria mainly support the cardiac energy demand by producing more than 90% of intracellular ATP [5], previous studies have focused on mitochondrial dysfunction as a crucial cause of metabolic disorders and/or insulin resistance-associated heart diseases [8–10].

Mitochondria are dynamic organelles that continuously change their morphology through fusion and fission events in response to intracellular circumstances [11]. Mitochondrial fusion events are mediated by GTPase dynamin-family proteins, including mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy-1 (OPA1). The most relevant mitochondrial fission proteins are dynamin-related protein (DRP1) and fission 1 homologue protein (Fis1) [12,13]. Because mitochondrial morphology determines their function [11], the misregulation of mitochondrial dynamics may induce metabolic disorders through mitochondrial dysfunction [12,14]. Altered mitochondrial shape was observed in the muscle from type 2 diabetes, and the aberrant mitochondrial dynamics have been known to contribute to the development of insulin resistance [12,14]. Previous studies have also indicated the robust correlation between the alteration in mitochondrial morphology and cardiac diseases [8,9,13,15–17]. Mitochondrial fragmentation (fission) was apparent in failing myocardium caused by myocardial infarction, in which both the decrease in fusion proteins and increase in fission proteins were observed [16]. Furthermore, recent investigations have suggested that mutations in mitochondrial fusion genes led to cardiomyopathy [18,19].

Despite the identification of an apparent correlation between the misregulation of mitochondrial dynamics and myocardial metabolic deficiency, the precise mechanisms of how the misregulation of mitochondrial dynamics contributes to the pathogenesis of metabolic deficiency are still unclear. Moreover, it is still unknown whether these aberrant mitochondrial dynamics would induce myocardial insulin resistance. In this study, we focused on DRP1 (a mitochondrial fission protein) to investigate the mechanisms by which the misregulation of mitochondrial dynamics induces myocardial insulin resistance and studied the relationship between DRP1 and reactive oxygen species (ROS) in the pathogenesis of myocardial insulin resistance. Furthermore, because we had previously established an *ex vivo* myocardial insulin resistance model by exposing myocytes to palmitate, which mimics elevated serum FA through chronic adrenergic stimulation under heart failure [20], we also investigated the impact of DRP1 suppression using this *ex vivo* myocardial insulin resistance model.

Materials and methods

Cell culture, differentiation, and treatment

H9c2 rat cardiac myoblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in a growth medium, comprising Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin, in humidified air (5% CO_2) at 37 °C. The differentiated H9c2 myocytes were produced as previously reported [20]. In brief, the H9c2 myoblasts were cultured with the differentiated medium, which was composed of DMEM, 1% FBS, 2 mM glutamine, 1 mM pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin, for 5–7 days to induce differentiation. Some differentiated myocytes were treated with hydrogen peroxide (H_2O_2; 100 μM) for 1 h or palmitate (saturated fatty acid; 0.2 mM) for 24 h to induce myocardial insulin resistance. The palmitate solution was prepared by conjugating palmitate with fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) as previously described [20]. In brief, sufficient palmitate was dissolved in preheated 0.1 N NaOH and diluted 1:10 in pre-warmed (40–50 °C) DMEM, containing 12% (w/v) BSA, to yield a concentration of 2.0 mM (stock palmitate solution). The stock palmitate solution was filter-sterilized and subsequently diluted to 1:10 with the cell growth media (final concentration, 0.2 mM) for use in the following experiments. Mn(III)tetrakis (1-methyl-4-pyridyl)porphyrin pentachloride (TMPyP; 200 μM; Sigma-Aldrich, St. Louis, MO, USA) was applied to myocytes as a ROS scavenger 24 h before the experiment.

DNA constructs, reagents, and transfection into differentiated H9c2 myocytes

The double strand cDNA of rat DRP1 (*Rattus norvegics* dynamin 1-like, mRNA; BC085843.1) was synthesized with the insertion of restriction endonucleases (*Bam*HI for 5' and *Hind*III for 3'; Takara Bio INC., Otsu, JAPAN) and amplified using a clone vector (pBApo-CMV neo; Takara Bio INC., Otsu, JAPAN). The mock transfected myocytes, which were treated with transfection regents without cDNA, were compared with the DRP1-expressing myocytes. To evaluate the mitochondrial morphology, some myocytes were transfected with mitochondrial YFP (Clontec Laboratories, Inc., Mountain View, CA, USA). Transfection of cDNA into the differentiated H9c2 myocytes was performed employing Lipofectamine LTX reagent (Life Technologies Corporation, Carlsbad, CA, USA), according to the manufacturer's instructions, and the experiments were conducted 72 h after transfection according to the study protocol.

The siRNA target sequences of the rat DRP1 gene were designed using the TAKARA siRNA Design Support System (http://www.takara-bio.com). Among the six target sequences identified by this free online software, two target sequences were selected as follows: (1) 5'-GAACCGACAACAGGCAACUTT-3' and 3'-TTCUUGGCUGUUGUCCGUUGA-5' and (2) 5'-GUAUUGUAACACUAUUGAATT-3' and 3'-TTCAUAACAUUGUGAUAACUU-5'. The scrambled control siRNA was designed to target the following sequence: 5'-CCGUCCGCAUUAGUAGCGGTT-3' and 3'-TTGGCAGGCGUAAUCAUCGCC-5'. The siRNA were transfected with the differentiated H9c2 myocytes, employing Lipofectamine RNAiMAX reagent (Life Technologies Corporation), according to the manufacturer's instructions. The siRNA-expressing myocytes were analyzed 72 h after transfection.

Western blot analysis

Western blot analysis was performed as previously described [20]. In brief, whole cell proteins were obtained according to the