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Low-concentration of perifosine surprisingly protects cardiomyocytes from oxygen glucose deprivation

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ABSTRACT

Here we found that low-concentration of perifosine, an Akt inhibitor, surprisingly protected cardiomyocytes from oxygen glucose deprivation (OGD)/re-oxygenation. In H9c2 cardiomyocytes, non-cytotoxic perifosine (0.1–0.5 μM) suppressed OGD/re-oxygenation-induced reactive oxygen species (ROS) production, p53 mitochondrial translocation and cyclophilin D complexation, as well as mitochondrial membrane potential (MMP) reduction. Molecularly, perifosine activated AMP-activated kinase (AMPK) signaling to increase intracellular NADPH (nicotinamide adenine dinucleotide phosphate) content in H9c2 cells. On the other hand, AMPK inhibition by AMPKα1 shRNA-knockdown in H9c2 cells significantly reduced perifosine-induced NADPH production, and alleviated perifosine-mediated antioxidant and cytoprotective activities against OGD/re-oxygenation. In primary murine cardiomyocytes, perifosine similarly activated AMPK signaling, and offered significant protection against OGD/re-oxygenation, which was largely attenuated with siRNA knockdown of AMPKα1. We demonstrate an unexpected function of perifosine (low-concentration) in protecting cardiomyocytes from OGD/re-oxygenation.

1. Introduction

Ischemic heart disease is a major health threat, causing large mortalities annually in China and worldwide [1,2]. Over the past decades, a number of interventional technologies for ischemic heart disease therapy have been developed, resulting in significant alleviation of disease progression, and dramatic improvement patients' life quality [3,4]. Meanwhile, conservative drug treatment is still a valuable and safe method for treatment of ischemic heart disease [3,4].

Oxygen glucose deprivation (OGD) has been applied in cultured cardiomyocytes to mimic ischemic heart damages. Our group [5] and others [6,7] have shown that serve and/or sustained OGD (>1 h) suppresses mitochondrial complex-I activity, thus inhibiting mitochondrial respiratory chain. When coupled with re-oxygenation, OGD will induce production of a large amount of superoxide and other reactive oxygen species (ROS), causing oxidative stress and cell programmed-necrosis (but not apoptosis) [5]. Our previous study has shown that salidroside protected H9c2 rat cardiomyocytes from OGD/re-oxygenation damages through activating of NF-E2-related factor 2 (Nrf2) signaling [5].

Perifosine, an Akt inhibitor, is the first orally bioactive alkylphospholipid [8,9]. It is currently being tested as a anti-cancer drug [8,9]. Preclinical studies have demonstrated that perifosine could exert profound cytotoxic effect against a number of human cancer cells [8,9]. Perifosine blocks Akt activation by disrupting Akt recruitment to the plasma membrane. Yet, recent studies have demonstrated other actions by perifosine. For example, perifosine could activate AMP-activated kinase (AMPK) signaling [10,11]. Further, perifosine is shown to inhibit lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α production in macrophages [12]. In the current study, we show that non-cytotoxic low-concentration of perifosine surprisingly protects cardiomyocytes from OGD/re-oxygenation, possibly via activating AMPK signaling.

2. Material and methods

2.1. Chemicals and reagents

Salidroside and perifosine were purchased from Sigma (St.

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Louis, MO). Antibodies against cyclophilin D (Cyp-D), voltage-dependent anion channel (VDAC), p53 and (β-) actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies utilized in the study were provided by cellular signaling Tech (Beverly, MA).

2.2. H9c2 cell culture

As described in our previous study [5], rat embryonic ventricular H9c2 cardiomyocytes were maintained in DMEM medium, supplemented with 10% FBS, penicillin/streptomycin, and 4 mM L-glutamine, in a CO_2 incubator at 37 °C.

2.3. Primary culture of murine cardiomyocytes

The protocol was described in literature [13–15] with minor modifications. Briefly, the ventricles of neonatal mice were minced in Hanks balance solution, and were incubated with Liberase (Roche Diagnostics, Shanghai, China) for 15 min at 37 °C. Myocardiocytes were then digested in 0.5 mg/mL collagenase I (Sigma, St. Louis, MO), and incubated at 37 °C for 30 min. The cell suspension was filtered through a 70 μm cell strainer, and centrifuged. Cells were then suspended in M-199 medium supplemented with 10% FBS and 5 mM D-glucose, and plated for 30 min to remove non-myocardiocytes. The myocardiocytes were plated at a density of 0.5×10^6 cells/mL in M-199 supplemented with 10% FBS. A confluent monolayer of spontaneously beating cells was formed and considered as primary murine cardiomyocytes. The animal procedures were approved by Institutional Review Board and IACUC of Nantong University.

2.4. OGD/re-oxygenation model

As previously described [5], H9c2 cells or primary murine cardiomyocytes were placed in an anaerobic chamber [5]. The medium was replaced with pre-warmed (37 °C) glucose-free balanced salt solution [5] at 37 °C for 1–4 h to produce oxygen and glucose deprivation (OGD) and then re-oxygenated [5,15]. Experimental parameters were assayed at 0.5–24 h following re-oxygenation.

2.5. Cell viability assay (MTT assay)

Cell survival was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) assay through the recommended protocol. The MTT cell viability OD value was utilized as a quantitative indicator of cell survival [5].

2.6. Flow cytometry assay of cell necrosis

Following treatment, H9c2 cells were detached, washed with PBS, and incubated in 500 μL binding buffer, and 5 μL of propidium iodide (PI) (Invitrogen) for 15 min in the dark [5]. Cells were then detected through fluorescence-activated cell sorting (FACS) with a Becton–Dickinson FACScan (Immunocytochemistry Systems, San Jose, CA). PI positive cells were gated as necrotic cells, and its percentage was utilized as H9c2 cell death percentage [5].

2.7. Detection of death in primary murine cardiomyocytes

Following treatment, primary murine cardiomyocyte death percentage was determined by counting cardiomyocytes through a hemocytometer after addition of trypan blue. Death percentage (%) = the number of trypan blue stained cardiomyocytes/the number of total cardiomyocytes (×100%).

2.8. Western blots

Western blots were performed as described previously [5,16]. Prior to blotting, a SDS-PAGE gel was stained with Coomassie blue (Sigma) to provide visual confirmation that equivalent loading. The intensity of each band in the blot was quantified through the ImageJ software (NIH) after normalization to corresponding loading controls.

2.9. Mitochondrial immunoprecipitation (Mito-IP)

Following treatment, the mitochondria of $1–2 \times 10^7$ H9c2 cells were isolated through the "mitochondria Isolation Kit" [5]. The mitochondria were then lysed [5]. Immunoprecipitation (IP) was performed using anti-Cyp-D antibody ([5,6]), and immune complexes were captured with protein G-Sepharose. Proteins were resolved by SDS-PAGE, p53-Cyp-D association was detected by the Western blots.

2.10. Real-time PCR

Total RNA of H9c2 cells was extracted through TRIzol reagents (Invitrogen, Shanghai, China), and reverse transcription was performed using TOYOBO ReverTra Ace-a RT-PCR kit (TOYOBO, Japan), and was described in detail in our previous study [5]. The primer sets for rat heme oxygenase-1 (HO-1) and quinone oxidoreductase 1 (NQO-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described [5].

2.11. ROS detection

ROS production was determined by carboxy-H2DCFDA staining method (see our previous study [5]). In brief, after treatment, cells were incubated with 5 μM carboxy-H2-DCFDA (Invitrogen). 5×10^5 cells/sample were resuspended in PBS, and were sent to flow cytometry analysis (Epics Altra, BECKMAN, CA). The percentage of fluorescence-positive cells, indicating ROS content, was recorded on a microplate spectrofluometer (molecular Devices) [5]. JC-10 OD was utilized as a quantitative measurement of MMP reduction [5].

2.12. Detection of mitochondrial membrane potential (MMP)

MMP was measured through JC-10 dye (Invitrogen) as described [5]. In brief, H9c2 cells with applied treatment/s were stained with 5.0 μg/mL of JC-10 for 5 min. Cells were then washed, and tested immediately on a microplate reader with an excitation filter of 485 nm.

2.13. NADPH assay

The intracellular NADPH (nicotinamide adenine dinucleotide phosphate) content was measured with the help from Dr. Dong's group [17,18]. In brief, following treatment, 1×10^6 H9c2 cells/primary murine cardiomyocytes per sample were lysed in 200 μL of extraction buffer (20 mM nicotinamide, 20 mM $NaHCO_3$, 100 mM Na_2CO_3) [18]. For NADPH extraction, the lysates were maintained at 60 °C for 30 min. Next, 160 μL of NADP-cycling buffer (100 mM Tris–HCl pH 8.0, 0.5 mM thiazolyl blue, 2 mM phenazine ethosulfate, 5 mM EDTA) containing 1.3 U of glucose-6-phosphate dehydrogenase (G6PD, Sigma) was added to the 96-well plate containing 20 μL of the cell extract. After a 1-min incubation in the dark at 30 °C, 20 μL of 10 mM glucose 6-phosphate (G6P, Sigma) was added to the mixture, and the change in absorbance at 570 nm was measured every 30 s for 4 min at 30 °C with a microplate