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Mitochondrial binding of α-enolase stabilizes mitochondrial membrane: Its role in doxorubicin-induced cardiomyocyte apoptosis

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ABSTRACT

α-Enolase is a metabolic enzyme in the catabolic glycolytic pathway. In eukaryotic cells, the subcellular compartmentalization of α-enolase as well as its multifaceted functions has been identified. Here, we report that α-enolase is a regulator of cardiac mitochondria; it partially located in the mitochondria of rat cardiomyocytes. Doxorubicin treatment displaced α-enolase from mitochondria, accompanied by activation of mitochondrial cell death pathway. Furthermore, in isolated mitochondria, recombinant α-enolase significantly alleviated Ca^{2+}-induced loss of membrane potential, swelling of matrix and permeabilization of membrane. In contrast, mitochondria from α-enolase knockdown H9c2 myoblasts underwent more severe membrane depolarization and swelling after Ca^{2+} stimulation. In addition, α-enolase was further identified to interact with voltage dependent anion channel 1 in the outer membrane of mitochondria, which was weakened by doxorubicin. Collectively, the present study indicates that mitochondria-located α-enolase has a beneficial role in stabilizing mitochondrial membrane. In cardiomyocytes, the displacement of α-enolase from mitochondria by doxorubicin may involve in activation of the intrinsic cell death pathway.

Introduction

Anthracyclines (e.g., doxorubicin – Dox[1]) are the most effective chemotherapy drugs available in the management of hematological and solid tumors including leukemia and breast cancer. However, the clinical application of these drugs is limited by the risk of serious cardiotoxicity, resulting in a permanent loss of cardiomyocytes [1]. Numerous studies suggested that Dox-induced reactive oxygen species (ROS) generation is a primary cause of cardiomyocyte apoptosis [2–4]. However, antioxidants such as vitamin E [5], N-acetyl cysteine [6] and 7-monohydroxyethylrutoside [7] do not attenuate cardiac symptoms in animal models or patients suffering Dox-cardiomyopathy, indicating that our understanding of the pathogenesis of Dox-cardiomyopathy is incomplete, and other mechanisms may also be involved in Dox-induced cardiomyocyte apoptosis.

Enolase is a key enzyme that catalyzes the dehydratation of 2-phosphoglycerate to phosphoenolpyruvate in the catabolic glycolytic pathway. In mammals, the enzyme exists as three isoforms: α-enolase is widely distributed in almost all tissues, and β-enolase is preferentially expressed in muscle tissues, whereas γ-enolase is predominantly found in neuron and neuroendocrine tissues [8]. Recent studies revealed that, in addition to its innate catalytic function, α-enolase plays an important role in other biological/pathological processes, such as myogenesis [9], tRNA transport [10], K^+ channel regulation [11,12], and tumor cell migration [13].

Among the three enolase isozymes, α-enolase is the predominant isoform in the heart. Previous research revealed that α-enolase is upregulated in cardiovascular diseases including cardiac ischemia [14,15] and cardiac hypertrophy [16,17], reflecting energy remodeling of heart under pathological stimulus. In addition, Mizukami et al. reported that introduction of α-enolase protein into rat cardiomyocytes increased cell survival after ischemic hypoxia and reoxygenation [14]. In the study of Luo et al., the interaction between constitutive heat shock protein 70 and α-enolase possibly protected H9c2 myoblasts from H_2O_2-induced damage [18]. Furthermore, α-enolase was supposed to be a regulator of cardiac ATP-sensitive K^+ channel (K_{ATP}), which conveys cardioprotective

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[1] *Abbreviations used:* Dox, doxorubicin; Cyt *c*, cytochrome *c*; $\Delta\psi_m$, mitochondrial transmembrane potential; mPTP, mitochondrial permeability transition pore; VDAC, voltage dependent anion channel.

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effects by managing K^+ homeostasis in cardiomyocytes [12]. These evidences indicate that α-enolase involves in the regulation of cardiomyocyte survival and death. Nevertheless, the role of α-enolase in cardiomyocyte apoptosis, especially the related mechanisms, has remained largely unexplored.

Over the past decade, the compartmentalization of α-enolase has been identified, which may partly explain its multifaceted functions. In eukaryotes, enolase can bind to mitochondria [19]. However, little attention has been devoted to the potential role of α-enolase anchoring to mitochondria. Mitochondria are the organelles that control the intrinsic pathway of programmed cell death [20]. In cardiomyocytes, long lasting opening of the mitochondrial permeability transition pore (mPTP) and release of mitochondrial cytochrome *c* (Cyt *c*) are early indicators of Dox-induced apoptosis [21].

Hence, to investigate the putative role of α-enolase in Dox-induced cardiomyocyte apoptosis at the mitochondrial level, we first determined the colocalization of α-enolase and mitochondria in cardiomyocytes, and then examined the change of α-enolase content in the mitochondrial fractions after Dox treatment *in vitro* and *in vivo*. We hypothesized that the locating of α-enolase in mitochondria might stabilize mitochondrial membrane and inhibit activation of the mitochondrial cell death pathway. Thus, using the recombinant α-enolase protein and RNA interference technology, we found that mitochondrial α-enolase prevented Ca^{2+}-induced loss of mitochondrial transmembrane potential ($\Delta\psi_m$), swelling of matrix, and release of Cyt *c* in isolated mitochondria. We further identified that α-enolase associated with voltage dependent anion channel 1 (VDAC1), a critical regulator of the mitochondrial cell death pathway. These data indicate that α-enolase has the potential to regulate cardiomyocyte apoptosis by interacting with mitochondria.

Materials and methods

Primary cultures of neonatal rat cardiomyocytes and cultures of H9c2 cardiac myoblasts

Primary cultures of neonatal rat cardiomyocytes (NRCMs) were prepared as described previously [22,23]. Briefly, myocardial tissues from 1- to 3-day-old Sprague–Dawley rats were digested with 0.08% trypsin solution at 37 °C for 8–12 times. Cells were gathered by centrifugation for 5 min at 800*g*. Then cells were plated in Dulbecco's modified Eagle's medium (DMEM) with 10% new born calf serum (NBCS) for 1 h to reduce the non-myocytes. Then the purified cardiomyocytes were cultured in DMEM supplemented with 10% NBCS and 0.1 mM 5-bromodeoxyuridine. Before each experiment, cells were placed in DMEM containing 1% NBCS for 16–18 h. Then Dox was added to medium, and cultures were further incubated for the indicated time. H9c2 rat cardiac myoblasts were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cell culture media were changed every 2 days. Before Dox stimulation, H9c2 cells were cultured in DMEM containing 1% FBS for serum starvation.

Animal models, echocardiography and morphometric measures

Wistar rats (male, weighing 280–320 g, SPF grade, certification no. 44008500000530) were supplied by the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). The investigation 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). The animal experiments were approved by the Research Ethics Committee of Sun Yat-Sen University.

Cardiomyopathy was induced by intraperitoneal injection of a single dose of Dox (15 mg/kg). Rats given normal saline (NS) were regarded as the vehicle control group. The total number of rats was 7 per group. After 7 days, two-dimensionally guided M-mode echocardiography was performed using a Technos MPX ultrasound system (ESAOTE, Italy) equipped with an 8.5-MHz imaging transducer. For morphometric measures, the transverse sections of the hearts were fixed with neutral buffered formalin (10%), embedded in paraffin, cut into 5 μm cross sections and stained with hematoxylin–eosin dye (H&E) [22].

In situ detection of DNA fragmentation

To detect DNA fragmentation in situ, nick-end labeling was performed using an TdT-mediated dUTP nick end labeling (TUNEL) situ apoptosis detection kit (Keygen Biotech, China). Briefly, the sections of fixed myocardium or slides of cells were permeabilized with 20 μg/ml proteinase K or 1% Triton X after deparaffinizing, and then treated with 3% hydrogen peroxide (H_2O_2) for 15 min to inactive endogenous peroxidases; sections or slides were then processed for TUNEL staining according to manufactory instruction. Cells were counted in 10 of visual fields containing more than 50 cells; the percentage of apoptotic cardiomyocytes was obtained by dividing the number of cells with TUNEL-positive staining by the total number of cells.

Western blot analysis

Rabbit anti-α-enolase polyclonal antibody (diluted 1:1500, Proteintech Group, USA), anti-caspase3 polyclonal antibody (diluted 1:1000, Cell Signaling Technology, USA), anti-bcl2 polyclonal antibody (Beyotime, diluted 1:500), anti-bax polyclonal antibody (diluted 1:1000, Epitomics, USA), anti-VDAC1 polyclonal antibody (diluted 1:1000, Proteintech Group, USA), and mouse anti-Cyt *c* monoclonal antibody (diluted 1:200, Beyotime, China) were used as primary antibodies. Mouse anti-α-tubulin monoclonal antibody (diluted 1:10,000, Sigma, USA) served as loading control of the whole cell lysis. And anti-VDAC1 polyclonal antibody also served as loading control of the mitochondrial lysis. Briefly, proteins were separated by SDS–PAGE gel electrophoresis, and then transferred to PVDF membranes (Millipore, USA). The membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP)-labeled second antibodies at room temperature. Immunoreactive bands were detected with enhanced chemiluminescent substrate (Pierce, USA).

Immunofluorescence assay

NRCMs plated on coverslips were stained with 50 nM Mitotracker Red CMXRos (Invitrogen, USA) for 30 min at 37 °C and then fixed with 4% paraformaldehyde for 15 min at room temperature. Permeabilization was performed with 0.1% Triton-X-100 followed by blocking with 10% goat serum solution at room temperature for 1 h. The cells were further incubated with primary antibodies of α-enolase (diluted 1:100, Proteintech Group, USA) overnight at 4 °C, and then incubated with Alexa Fluor 488-labeled secondary antibodies (diluted 1:1000, Invitrogen, USA) for 1 h at room temperature. Nuclei were stained with 5 μg/ml of DAPI (Invitrogen, USA). After washed with PBS, the coverslips were inspected with a laser confocal microscope (Zeiss 710). For colocalizaion measurement of α-enolase and VDAC1, the fixed NRCMs were incubated with primary antibodies of α-enolase (diluted 1:100) and VDAC1 (diluted 1:50, Santa Cruze, USA). Then Alexa Fluor 488-labeled and Cy3-labeled secondary antibodies were used to mark α-enolase and VDAC1, respectively.