# Changes of mitochondrial pathway in hypoxia/reoxygenation induced cardiomyocytes apoptosis

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**Abstract:** The role of mitochondrial apoptotic pathway in cardiomyocytes subjected to hypoxia/reoxygenation(H/R) was studied. Cultured cardiomyocytes from neonatal Sprague-Dawley rats were exposed to hyoxia/reoxygenation, the apoptotic cardiomyocytes were stained with Annexin-V-FITC, Hoechst 33342 and TUNEL assay. Mitochondrial transmembrane potential of cardiomyocytes was assessed by JC-1 under fluorescence microscope, the expressions of bcl-2, bax, cytochrome c, apoptosis-induced factor (AIF), and caspase-3 were tested by western-blot. Our data showed apoptosis of cardiomyocytes was significantly increased during H/R, accompanied by translocation of bax to mitochondria, release of cytochrome c and AIF to cytosol. The results indicate that the mitochondrial-mediated apoptotic pathway is initiated as a result of H/R.

**Key words:** Cardiomyocytes - Apoptosis - Mitochondrial transmembrane potential - Hypoxia - Reoxygenation

## Introduction

Reperfusion has the potential to introduce additional injury which is not evident at the end of ischaemia. Much evidence shows that apoptosis increases during reperfusion in ischemic heart [1-4]. It is therefore important to identify the mechanisms which mediate the induction and propagation of the apoptotic process. Numerous pathways are involved in the process of apoptosis, the roles of mitogen-activated protein kinase (MAPK) family[5-7] and JAK-STAT signaling pathway [8-10] in modulation of myocardial apoptosis during reperfusion have been extensively demonstrated.

As mitochondria comprise approximately 30% of the total intracellular volume within a mammalian cardiomyocyte [11,12], they can have notable influence on cardiomyocyte energy production and, ultimately, the health of an individual cell. Some studies [13,14] showed the structure and function of mitochondria within the cardiomyocytes were injured during reperfusion. In order to clarify the changes of mitochondrial apoptotic pathway during ischemia/reperfusion, we choose the in vitro hypoxia/reoxygenation (H/R) model. In this

study, cultured cardiomyocytes were subjected to H/R to induce apoptosis, and mitochondrial pathway during the apoptosis process was specifically investigated.

## Materials and methods

Neonatal Sprague-Dawley rats were obtained from the Medical Institute Animal Center of Zhejiang University for conducting the proposed study. The experiments were approved by the Animal Care and Use Committee of Zhejiang Province Medical Institute and were in compliance with the "Guide for the Care and Use of Laboratory Animals" as published by the US National institutes of Health (National Institutes of Health publication no.85-23, revised 1996).

**Cell culture.** Primary culture of cardiomyocytes was prepared by the method originally described by Simpson [15] with minor modifications. Briefly, the hearts from neonatal Sprague-Dawley rats were minced and dissociated with 0.125 % trypsin (Gibco, USA) and 0.1% collagenase type II (Worthington, USA). After incubation of dispersed cells on 25 $cm^2$ flask for 60 min in normoxic incubator (95% air/ 5%$CO_2$), unattached viable cells were collected and seeded into 25 $cm^2$ flask ($2 \times 10^6$ cell/dish) or 24-well plates ($2 \times 10^5$ cells/well) and incubated. The cells were then incubated with DMEM supplemented with 20% fetal calf serum plus 0.1 mmol/L 5-bromo-2-deoxyuridine (Sigma, USA) for 72 hours to prevent low-level nonmyocardial cell proliferation, and then replaced with DMEM plus 20 % calf serum.

**H/R.** To mimic hypoxia, the cardiomyocytes were incubated at 37°C in a modular incubator chamber for 24 hours, where normal air was replaced by 95% $N_2$/5% $CO_2$. Then cardiomyocytes were moved into normoxic incubator (95% air/5%$CO_2$) for 3 hours to mimic reoxy-

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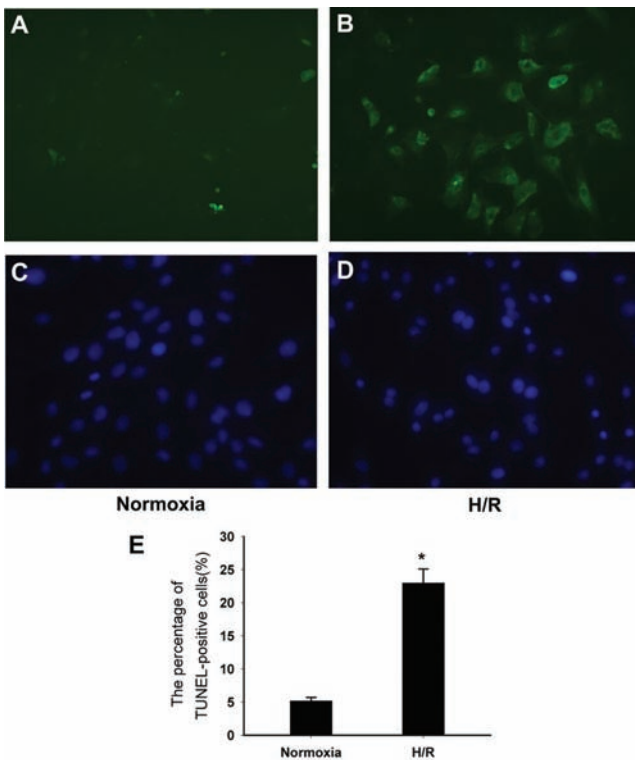
**Fig. 1**. H/R induced the apoptosis of cardiomyocytes. A-B, apoptotic cells were detected by Annexin V-FITC staining for labeling early-stage apoptotic cells (green). C-D, Hochest33342 staining of cardiomyocytes, apoptotic cells were characterized by nuclear shrinkage with condensed chromatin structure. E, quantification of apoptotic cardiomyocytes mesusred by TUNEL assay. The fraction of apoptotic cells was determined in 5 random microscopic fields totally at least 1000 cells/group. Results are representative of three independent experiments. Original magnification ×200 (A-D). Data are shown as mean±SD,* p<0.01 vs. normoxia group.

genation process. Normoxia group was incubated under standard cell culture conditions (95% air/5%$CO_2$).

**Apoptosis assessment.** We used 3 methods to determine apoptosis of cardiomyocytes. Annexin V-FITC apoptosis detection kit (BioVision, USA) and Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL,Roche Diagnostic,Germany) assays were performed according to the manufacturer's protocol. Nuclear staining with the chromatin dye Hochest 33342 (Sigma, USA) was performed. Briefly, cells were fixed for 1hour in 4% paraformaldehyde at room temperature, and then exposed to 5 μg/ml Hoechst 33342 for 30 min at 37°C in the dark. Cells were observed by using a fluorescence microscope.

**Mitochondrial transmembrane potential assessment.** Mitochondrial transmembrane potential was assessed using the lipophilic cationic probe JC-1 (BioVision, USA), a sensitive fluorescent dye. Briefly, cardiomyocytes were incubated with 10 μM JC-1 for 15 min at 37°C in the dark and monitored by using a fluorescence microscope. Red fluorescence is attributable to a potential-dependent aggregation in the mitochondria. Green fluorescence, reflecting the monomeric form of JC-1, appeared in the cytosol after mitochondrial membrane depolarization.

**Western blot analysis**. Preparation of mitochondrial and cytosolic fractions was achieved using mitochondria/cytosol fractionation kit (BioVision,USA) according to the manufacturer's protocol.
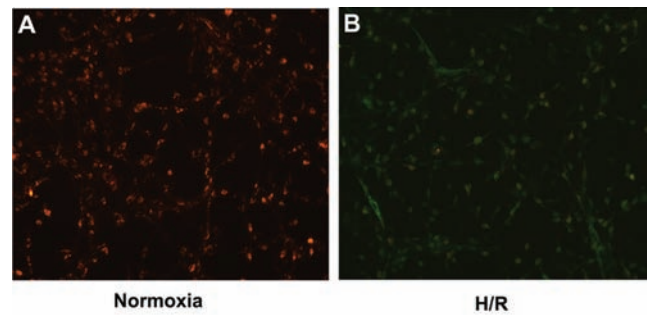


**Fig. 2**. H/R reduced the mitochondrial membrane potential of cardiomyocytes. Mitochondrial membrane potential was determined using the potential-sensitive fluorescent probe JC-1. Normal cultured cardiomyocytes contained red fluorescent mitochondria in the cytoplasm (A), cardiomyocytes subjected to H/R showed green fluorescence (B), indicating the loss of mitochondrial membrane potential. Results are representative of three independent experiments. Original magnification ×200 (A-B).

Protein (20-100 μg) prepared from the disposed cells was loaded per lane and electrophoresed in SDS-PAGE, and then transferred onto polyvinylidene difluoride (PVDF) Immobilon-P membrane (Bio-Rad, USA) using a transblot apparatus (Bio-Rad, USA). The membranes were blocked in 10mmol/L Tri-HCl (pH=8.0), 150 mmol/L NaCl and 0.05% Tween 20 (TBST) with 5% (w/v) non-fat milk at room temperature, followed by overnight incubation at 4°C with primary antibodies diluted in TBST (1:1000 for bcl-2, bax, caspase-3 and β-actin; Cell Signal, USA; 1:1000 for cytochrome c, BD Pharmagin,USA; 1:1000 for AIF, Santa Cruz, USA ). After washing with TBST, the membranes were incubated for 1 hour with an HRP-conjugated secondary antibody diluted 1:5000 in TBST, and the labeled proteins were detected by using the enhanced chemiluminescence reagents and exposed to the film (Kodak, USA).

**Statistical analysis**. Data were expressed as mean ±standard deviation (SD). Statistical significance between two groups was made by student t test using SPSS11.5. P < 0.05 was considered statistically significant.

## Results

### H/R induced cardiomyocyte apoptosis

We demonstrated exposure of cultured cardiomyocytes to H/R led to increased cell apoptosis, as assessed by three methods: Annexin V-FITC staining (Fig.1A-B), Hochest33342 staining (Fig.1C-D) and TUNEL assay, H/R increased the apoptotic rate by approximately 3.4-fold (Fig.1E, normoxia group: 5.2% ± 0.5%; H/R group: 23.0% ± 2.1%, p<0.01).

### H/R led to cardiomyocytes mitochondrial dysfunction

As shown in Fig. 2, normal cardiomyocytes exhibited red fluorescence (A), cardiomyocytes after H/R developed a diffuse green staining pattern (B), representative of reduced mitochondrial membrane potential.
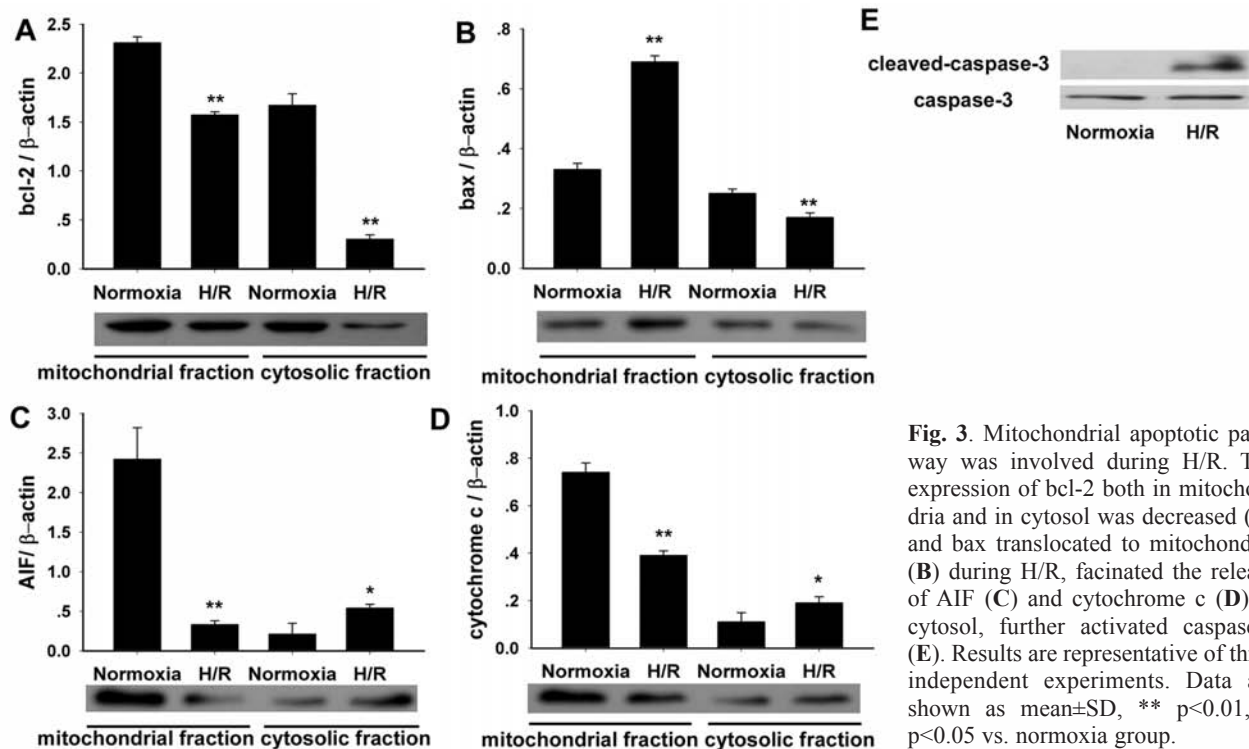
Fig. 3. Mitochondrial apoptotic pathway was involved during H/R. The expression of bcl-2 both in mitochondria and in cytosol was decreased (A) and bax translocated to mitochondria (B) during H/R, facinated the release of AIF (C) and cytochrome c (D) to cytosol, further activated caspase-3 (E). Results are representative of three independent experiments. Data are shown as mean±SD, ** $p < 0.01$, * $p < 0.05$ vs. normoxia group.

## Changes of mitochondrial pathway during H/R

Since H/R had an impact on the mitochondrial transmembrane potential, the mitochondrial apoptotic pathway was assessed. As shown in Fig. 3A-B, the level of bax in mitochondria increased by approximately 1-fold ($p < 0.01$) while the level of bcl-2 decreased by 0.3-fold ($p < 0.01$) during H/R, resulting an increased ratio of bax/bcl-2 in mitochondria.

Next, we investigated the distribution of cytochrome c and AIF both in mitochondria and cytosol, and found H/R promoted the release of AIF and cytochrome c from mitochondria to cytosol. The expression of AIF in cytosol increased to approximately 2.5-fold after H/R ($p < 0.05$, Fig. 3C), while cytochrome c increased to approximately 1.7-fold ($p < 0.05$, Fig.3D), and their expression in mitochondria decreased accordingly ($p < 0.01$). Cleaved-caspase-3 (17KD, Fig. 3E), the activated form of caspase-3, while not detected in normal cultured cardiomyocytes, was present as an intense band after H/R (Fig. 3E).

## Discussion

The importance of apoptosis in cell death following ischemia and reperfusion has been demonstrated. Prolonged periods of myocardial ischemia are related to an increased rate of necrosis, whereas, paradoxically, reperfusion leads to an enhancement in apoptosis [16,17]. Reperfusion restores oxygen and glucose supply which is essential for the survival of viable cells,

however, reperfusion also restores energy required for the completion of apoptosis and can accelerate the apoptotic process [16,17]. In our study we found cardiomyocyte apoptosis increased obviously after 24 hours of hypoxia and 3 hours of reoxygenation, that was in accordance with previous researches [18-20].

Cumulative evidence [21] indicates that the mitochondria play a pivotal role in cell death and cell survival. Mitochondria isolated from ischemic or ischemic/reperfused hearts have impaired function [22]: decreased adenine nucleotide content, reduced adenine nucleotide translocase activity, depressed respiratory chain complex activity, lower membrane potential, and decreased NADH dehydrogenase activity. Mitochondrial dysfunction results in mitochondrial $Ca^{2+}$ overload, free radical production, and declines in respiratory activity, ultimately, the induction of cell death, including necrosis and apoptosis [14]. In our study, we found mitochondrial membrane potential was reduced during H/R, which was partly caused by increased bax/bcl-2 ratio in mitochondria, and resulted in the release of cytochrome c and AIF.

Proapoptotic members like bax, which could induce apoptosis [23], are translocated from the cytosol to mitochondria facilitating the membrane permeabilization [24]. Bax translocation and permeabilization of membranes is blocked by antiapoptotic proteins like bcl-2, which are localized mainly in mitochondrial membranes where they block membrane permeabilization [24]. So an increased ratio of bax/bcl-2 leads to the formation of pores in the mitochondria, results in an efflux of small

molecules inside the mitochondria such as cytochrome c, AIF and other proapoptotic factors [25]. Mitochondria act as a crossover point between caspase dependent and -independent apoptotic pathways. AIF, which is located in the mitochondrial intermembrane space and is released to the cytosol and to the nucleus in response to death stimuli, is a key trigger of caspase-independent apoptosis [26,27]. Release of AIF results in generation of apoptotic phenotypes like chromatin condensation and phosphatidylserin exposure. Mitochondrial-mediated apoptosis involves, in part, the release of cytochrome c, when released from mitochondria into the cytosol, where it binds to apoptotic-protease activating factor-1 (Apaf-1) and activates the initiator caspase-9 which in turn activates the effector caspase-3 [28,29].

In summary, mitochondrial pathway participated in H/R induced cardiomyocyte apoptosis, however, more propound researches are needed to demonstrate whether it is really vital for cardiomyocyte apoptosis and whether it works the same in the ischemic/reperfused heart.

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