Inhibition of ATM/Chk2 Pathway Ameliorates Hypoxia-induced Myocardium Injury

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Abstract. Background: Acute myocardial infarction (AMI) is a kind of acute myocardial ischemic disease based on atherosclerosis that has high global morbidity and mortality. Recent work suggests the possible presence of DNA damage response (DDR) in AMI. However, the evidence is insufficient, and it remains elusive whether DDR can be used as a therapeutic target of AMI. Methods: The inductions of DDR were confirmed in rat myocardial ischemia model and cell model. Then, the specific inhibitor or siRNA target Chk1, ATM and Chk2 were used in H9C2 and NRVMs to assess their protective effect under hypoxia. Results: The induction of phosphorylation of H2A.X was observed in a rat model, suggesting that DDR occurs in myocardial tissues. DDR was also induced in vitro in H9C2 and NRVMs hypoxia models by the upregulation of p-H2A.X, p-Chk1, and p-Chk2. Then, we used UCN-01, a specific Chk1 inhibitor, to block Chk1 phosphorylation and find it has no effect on p-H2A.X expression, MTT, LDH, and cell apoptosis after 6h hypoxia. However, KU-55933, an ATM inhibitor, reversed the hypoxia-induced phosphorylation of ATM, H2A.X, and Chk2, and it also reduced apoptosis of H9C2 and NRVMs. Conclusion: Our results suggested that DDR occurs during myocardial ischemia in vivo and oxygen depletion injury in vitro. Inhibition of the ATM/Chk2 pathway might play a cytotoxic role in guarding against myocardial cell injury.

Introduction

Acute myocardial infarction (AMI) is a kind of acute myocardial ischemic hypoxia disease based on atherosclerosis. It has a high morbidity and mortality rate on a global scale and is seriously harmful to human health[1]. Its representative characterization is vascular occlusion, which is usually caused by thrombosis or plaque rupture. Studies have shown that a cell hypoxia condition can activate the oxidative stress reaction, and the production of reactive oxygen species (ROS) can be used as an apoptosis trigger[2]. At the same time, the accumulation of ROS can cause DNA chain rupture, DNA mutation, and other forms of DNA damage[3-4]. In order to maintain the integrity and stability of the genome, the organism initiates a series of DNA damage responses (DDRs) [5].

DDR is a complex reaction process, and classical DDR pathways include the ATR/Chk1 and ATM/Chk2 pathways. ATR and ATM are members of the PIKK family, which causes a series of phosphorylation cascades by activating downstream cell cycle checkpoints Chk1 and
Chk2, mediating cell cycle arrest and DNA repair. When the repair fails, it can further activate P53 and other death signaling pathways, ultimately leading to cell apoptosis[6].

It has been reported that after MI, free DNA in blood circulation rises significantly. Therefore, it can be used as a sensitive detection index of myocardial cell injury, playing an important role in monitoring and assessing the disease. In the plaques of atherosclerosis patients, the level of ATM visibly rises and is related to the severity of disease[7]. It is not fully clear at present whether ischemia of the heart and the accumulation of ROS in the infarction tissues can initiate DDR and what role DDR may play.

Materials and Methods

Reagents and antibodies An H9C2(2-1) cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences in Shanghai, China and conserved in our laboratory and stored in liquid nitrogen. The following antibodies were obtained from the given sources: anti-Chk1, anti-phosphorylated Chk1(ser296), anti-Chk2, anti-phosphorylated Chk2(Thr68), anti-H2A.X, anti-phosphorylated H2A.X(ser139), and anti-phosphorylated ATM(ser1981) from Cell Signaling Technology; anti-GAPDH from Bioworld; anti-Hif1-α from Abcam; KU-55933 from Sigma(SML1109); and UCN-01 from ENZO(ALX-380-222-MC25). LDH kit was purchased from Roche, and MTT kit and Annexin V-PI apoptosis detection kit were purchased from Key GEN Biotech.

MI model in rats Male Wistar rats 6 to 8 weeks old were purchased from Charles River and maintained in the animal facility of Henan University in a12h-light/12h-dark pattern with free access to food and water. Rats were divided into a ligated rat group and a control group used as a sham operation group. The animal experiments performed in this study were pre-approved by the Animal Experimentation Committee of Henan University.

The ischemic heart rat model was established by ligating the left anterior descending (LAD) coronary artery for 6h. Briefly, the animals were anesthetized, and positive pressure ventilation was provided by a ventilator. A 2-cm transverse incision was made between the third and fourth intercostal spaces. The LAD coronary artery was ligated between the pulmonary cone and the left auricle.

Cell isolation and culture NRVMs (Neonatal Rat Ventricular Myocytes) were separated from the hearts of newborn Wistar rats within three days according to methods that have previously been described in detail [8]. The NRVMs were cultured for 24-48h, and cell confluence needed to reach approximately 80% of the area of the culture flask before experimentation.

Immunohistochemistry Myocardial tissues were collected and fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 4μm. The tissue sections were then deparaffinized, rehydrated, and incubated with 3% H2O2 at 37°C for 20 min in order to block the endogenous peroxidase activity. Antigen retrieval was conducted by microwave for 15 min in 0.01 M sodium citrate, pH 6.0. Then, the tissues were incubated sequentially with 5% BSA for 1h at room temperature and the anti-p-H2AX antibody (1:200 dilution, Cell Signaling, USA) in 4°C for one night. After incubation with goat anti-rabbit IgG(BA1054, Boster) as a secondary antibody, the color was developed using the AEC staining method. The nuclei were counter-stained with hematoxylin, and the glass coverslips were sealed with neutral balsam.
**TUNEL** Myocardial tissues were collected and fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 4μm. Slices were put into a drying oven for 4h at 60°C. Then, they were dehydrated with 100% xylene I, 95% xylene II, 90% xylene III, and 70% ethanol for 5 min each and then with ddH2O three times. 50μl of TUNEL reaction liquid was added to the tissues, which were then incubated in the dark for 1h at 37°C in an incubator. They were then washed three times with PBS, and 50μl of converter-POD was added at 37°C to the incubator for 30min. Finally, the nuclei were counter-stained with hematoxylin, and the glass coverslips were sealed with neutral balsam.

**Flow cytometry** The apoptosis rate was measured by flow cytometry (FCM) analysis using an AnnexinV-PI(KGA108) apoptosis detection kit. After being treated with hypoxia at different time points, the cells were washed with PBS, suspended by trypsinization which did not contain EDTA, harvested by centrifugation (2,000 rpm, 5 min) and re-suspended in 500μl of binding buffer. Then, 5μl of annexin V and 5μl of PI were added to the binding buffer and incubated for 15 min in the dark at room temperature. Afterward, the apoptosis rate was assessed by FCM analysis, and 10,000 cells were recorded for each analysis.

**Western Blotting** The cells were lysed with RIPA, and the protein concentration of cell lysate was detected by using a BCA reagent (CW0014, CWBIO). Equal amounts of protein samples(60μg) were separated by SDS-polyacrylamide gel electrophoresis, then transferred onto a nitrocellulose filter membrane and incubated sequentially in a blocking buffer for 1.5h. The primary antibodies used were from Cell Signaling (p-H2A.X, H2A.X, p-Chk2, Chk2, p-Chk1, Chk1, and p-ATM, all at a 1:1,000 dilution) or Bioworld(GADPH,1:5,000). Appropriate secondary antibodies (Boster) associated with horseradish peroxidase were used following the incubation. Finally, the antigens on the blots were revealed by using an enhanced chemiluminescence kit from Pierce.

**MTT assay** Cell viability was detected by MTT assay, according to the manufacturer’s instructions(KGA312). Cells were plated in 96-well plates for 24h at 37°C and then subjected to the hypoxia treatments. Afterward, 50μl of MTT mixture was added to each sample and incubated in a 37°C incubator for 4h. Then, the supernatant fluid was removed and 150μl of DMSO was added per well and shaken for 10-15 min at room temperature in the dark. The optical density of each sample was measured at 550 nm using an automatic microplate reader(Multiskan Ascent).

**LDH detection** Cell injury was assessed using an LDH kit(Roche,11644793001). Cells were plated in 96-well plates and cultured overnight. After hypoxia treatment, 100μl of supernatant fluid was moved to a new 96-well plate, and an equal volume of LDH mixture was added per well, which reacted in 20 min at room temperature away from light. Next, OD values were detected at a 492-nm wavelength.

**Immunofluorescence** The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100. Then they were incubated sequentially with 3% BSA for 1h at room temperature and the anti-p-H2A.X antibody (1:300 dilution, Cell Signaling, USA) in 4°C for one night, followed by incubation with the goat anti-rabbit IgG secondary antibody. The nuclei were counter-stained by DAPI. After washes, the coverslips were mounted on slides and observed with a fluorescence microscope.

**Caspase 3/7 activity** Cells were put into 96-well plates and cultured overnight. Then they were treated with 100μl of PKRB 6h for hypoxia, and then the same volume of Caspase 3/7 activity reaction buffer was added and incubated for 30min at room temperature without light. OD values were detected using the chemiluminescence method.
**Hoechst staining** Apoptotic morphological changes in the nuclear chromatin of the NRVMs were detected using Hoechst 33342 staining. NRVMs were plated on a 3.5-cm dish (2.0 × 10⁵ cells/well). After treating with hypoxia at different times, the cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS, then incubated with Hoechst 33342 staining for 30 min in the dark at room temperature. After being washed with PBS three times, the NRVMs stained with Hoechst 33342 were imaged with a fluorescence microscope (Eclipse Ti, Nikon).

**Statistical analysis** Statistical analyses were carried out using GraphPad Prism 5.0 for a single-factor analysis of variance (one-way ANOVA), and all the data were expressed as \( \bar{x} \pm s \). Probability values (P-values) < 0.05 were considered statistically significant.

**Results**

**DDR Participated in Myocardial Ischemia Injury in Rats**

We used a common rat model of myocardial infarction to examine DDR in heart tissue. Rats were randomly divided into a control group and a 6h MI group. As Fig. 1A shows, HE results indicate that, compared with the control group, cardiac muscle fibers were swollen, arranged in disorder, and even broken in the 6h myocardial infarction group. In order to further verify whether the model was established successfully, TUNEL was used to detect the apoptosis of myocardial cells after MI. As shown in Fig. 1B, positive staining of cells was significantly higher in the MI group, proving that the MI model was successful.

Immunohistochemical results showed that p-H2AX was low in sham-operated myocardial tissue and was up-regulated following ischemia. p-H2AX positive cells were significantly increased in the infarction group compared to the sham control (Fig. 1C), which implies the induction of DDR following ischemia. Together, these results indicate the induction of DDR during myocardial ischemia.

![Figure 1](image)

**Figure 1. DDR participated in myocardial ischemia injury in rats.**

**DDR Was Induced in Cardiac Hypoxic Damage in Vitro**

In order to study the mechanism of DDR in myocardial ischemia, an in vitro model was used, in which H9C2 was put into a hypoxia workstation with 1% oxygen at different time points. With the hypoxia time prolonged, cellular proliferative capacity was gradually reduced, as determined by MTT (Fig. 2B) and LDH, and the apoptosis rate increased (Fig. 2A, D). Western Blot was used to detect the expression of Hif1-α, and Fig. 2C shows that the
expression of Hif1-α increased gradually. All these results suggest that the hypoxia model of H9C2 was successful, and it can be used for further experiments.

Next, we detected the expression of the DNA damage response protein. Western Blot results showed that phosphorylation of H2A.X, Chk1, and Chk2 was induced at 1h, reached peak level at 6h, and remained elevated after 12h (Fig. 2E). Immunofluorescence showed almost no signals of p-H2A.X in the nuclei of normal cells. However, after hypoxia, p-H2A.X accumulated to form nuclear foci and, with time prolonged, the number of positively stained nuclei increased (Fig. 2F).

![Figure 2. DDR is induced in cardiac hypoxic damage in vitro.](image)

**Blocking the ATM/Chk2 Pathway Can Protect H9C2 from Hypoxia Injury**

H9C2 was treated in different concentrations of ATM inhibitor KU-55933 and hypoxia for 6h. Western Blot showed that, compared with a pure oxygen 6h group, the expression of p-ATM did not change significantly in drug concentrations of 5μM and 10μM. In 20μM, however, there was a certain degree of reduction, which also occurred most obviously in 30μM. We found that, in the 30μM group, phosphorylation levels of ATM, H2A.X, and Chk2 were significantly lower, showing that the DDR had been alleviated (Fig. 3A). We also used immunofluorescence to detect p-H2A.X and found the same results, which showed that the nuclear foci formation of p-H2A.X was significantly reduced(Fig. 3B). FCM results showed that, compared with the 6h hypoxia group, with the concentration of KU-55933 increased, the cell apoptosis rate had a tendency to decrease. It had a statistically significant difference in 20μM and decreased most apparently in 30μM(Fig. 3C).

In order to further verify the influence of ATM inhibitor KU-55933 on apoptosis, TUNEL was used to detect the apoptosis cells in H9C2. Results showed that the number of positive-staining nuclei obviously declined in the 30μM group after a lack of oxygen for 6h, compared with the 6h hypoxia group(Fig. 3D). In accordance with previous results, compared
with the 6h hypoxia group, LDH OD values gradually declined with an increase in drug concentration, most significantly in 30μM, showing that KU-55933 can reduce cell damage induced by hypoxia(Fig. 3E).

Caspase activity can be used as an important criterion for evaluating apoptosis. As shown in Fig. 3F, compared with the 6h hypoxia group, Caspase3/7 activity visibly decreased in the 20μM KU-55933 group, and most significantly in 30μM, suggesting that blocking the ATM/Chk2 pathway can reduce the apoptosis caused by hypoxia and has a protective effect on H9C2.

Figure 3. Blocking the ATM/Chk2 pathway can protect H9C2 from hypoxia injury.

**Discussion**

In this study, we first established a MI model in rats and detected its morphology with the HE staining method. The myocardial apoptosis number visibly increased, as detected by TUNEL. This proved that the MI model had been successfully established.
Cells undergoing an oxygen deficit often lead to oxidative stress reactions and the accumulation of ROS. It has been reported that an increasing amount of ROS can cause a variety of forms of DNA damage such as DNA strand breaks and DNA mutation. p-H2A.X, the 139th serine phosphorylation protein of histone H2A.X, is widely considered to be the molecular signature of DNA damage[9-10]. In this study, we have established an MI or hypoxia/reperfusion model in vivo and in vitro and detected p-H2A.X, the marker of DNA damage. Results showed that when myocardial ischemia occurred, the expression of p-H2A.X significantly increased, illustrating the presence of DDR in myocardial tissues.

DDR is a complex reaction process. It can regulate the downstream cycle checkpoint kinases Chk1 and Chk2 by precepting the phosphorylation of ATR and ATM, and it triggers a series of phosphorylation cascades, mediating cell cycle arrest, repair, or apoptosis[11]. Accordingly, it is more inclined toward the inhibition of DNA repair in DDR caused by oxygen deficiency in tumor cells, and this effect also serves to promote the genetic instability of tumor cells[12]. At the same time, the threshold value of the resistance to DNA damage is not the same for different cells; therefore, they have different sensitivities to apoptosis induced by DDR[13]. As a result, there is a lack of sufficient evidence for what role DDR plays in the myocardial anoxic injury in myocardial cells and for whether blocking the DDR pathway can alleviate it.

A recent study finds that the phosphorylation levels of ATM and H2A.X visibly increase in the plaques of atherosclerosis patients, which is related to the severity of the disease. Previous results also found that the ATM/Chk2 pathway is activated and Chk2 phosphorylation levels increase in myocardial ischemia. Therefore, we applied KU-55933, an ATM inhibitor, to block another classic pathway and explore its role in myocardial injury induced by hypoxia. Results demonstrates that inhibiting ATM can alleviate the DDR induced by hypoxia. In order to further explore the influences on cell hypoxia injury, we used FCM, TUNEL, and Caspase3/7 activity to detect cell apoptosis. Previous study has reported that ATM knockout mice show reduced apoptosis of the myocardial infarction area, reduced heart inflammation, and improved heart function after ischemia[14-15]. Meanwhile, it is interesting to observe that in the ATM knockout group, apoptosis in the border area increases. It is reasonable that silenced ATM can reduce apoptosis. However, DNA damage in the border area of infarctions is mild, and DNA repair is the main function, so knocking out ATM can increase apoptosis.

In conclusion, DDR is a complex reaction process, participating in myocardial anoxic injury and playing an important role in regulating repair and apoptosis. Inhibition of the ATM/Chk2 pathway can significantly improve myocardial cell injury induced by hypoxia. Thus, it offers a new way of treating MI.

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References


