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Small molecule antidepressant amitriptyline protects hypoxia/reoxygenation-induced cardiomyocyte apoptosis through TrkA signaling pathway

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ABSTRACT

Hypoxia/reoxygenation (H/R) induced cardiomyocytes apoptosis is a major factor leading to cardiovascular diseases. In this study, we investigated the protective effect of small molecule antidepressant amitriptyline (AMP) in regulating H/R-induced apoptosis in neonatal mouse cardiomyocyte in culture. Cardiomyocytes of C57BL/6J mice were treated with H/R condition *in vitro*. Various concentration of AMP was added into culture 2 h prior to H/R conditioning. Cardiomyocyte apoptosis was evaluated by TUNEL assay. AMP induced downstream signaling pathway proteins, including tropomyosin receptor kinase A receptor (TrkA), phosphor-TrkA (p-TrkA), protein kinase B (Akt) and phosphor-Akt (p-Akt) were probed by western blot. TrkA phosphorylation was then blocked by K252a to investigate whether TrkA was functionally involved in the protection of AMP in H/R-injured cardiomyocyte. We found that H/R condition induced significant cardiomyocyte death and apoptosis, whereas AMP pretreatment considerably rescued cardiomyocyte death and apoptosis. Western blot analysis showed AMP activated TrkA signaling pathway through the phosphorylation of TrkA/Akt proteins. We also found that application of K252a inhibited the phosphorylation of TrkA/Akt signaling pathway, and subsequently abolished the protective effect of AMP in H/R-induced apoptosis in cardiomyocyte. Thus, our study revealed that AMP, through the activation of TrkA/Akt signaling pathway, plays a protective role in regulating H/R-induced apoptosis in cardiomyocyte.

1. Introduction

Coronary heart disease is one of the leading causes of death, accounting for ~13% of all premature mortality in the Untied States (Writing Group et al., 2016). Of several direct causes of coronary heart disease, myocardial infraction, or sudden ischemic death of myocardial tissue is the leading candidate, causing adverse events in near one million patients every year in the states (Frangogiannis, 2015; Writing Group et al., 2016). As post-infraction reperfusion is key component for reestablishing blood flow and normal cardio function, ischemia and immediate reperfusion (I/R) induced-injury often occurs, causing irreversible apoptosis or cell death among cardiomyocytes (Eltzschig and Eckle, 2011; Ferdinandy et al., 2007; Widgerow, 2014). During the past decades, although several signaling pathways, including reactive oxygen species (ROS), inflammatory, or calcium circulating pathways, had been shown to play important roles in I/R-inducing cardiomyocyte apoptosis (Muntean et al., 2016; Neri et al., 2015; Saxena et al., 2016; Webster, 2012), the exact molecular mechanism underlying myocardial fraction-induced cardiac damage is still unclear.

Tropomyosin receptor kinase A receptor (TrkA) belongs to a family of tyrosine kinases receptors (TrkA/TrkB/TrkC) that regulates neural growth and development in both central and peripheral nervous system (Huang and Reichardt, 2003; McMahon et al., 1994). In heart and during the process myocardial infraction, TrkA signaling pathway was found to be activated by nerve growth factor (NGF), thus rendering protective effect on ischemic damage induced cardiomyocyte apoptosis (Caporali et al., 2008; Meloni et al., 2010).

Amitriptyline (AMP) is a FDA-approved tricyclic antidepressant back in 1961. Yet, its exact molecular mechanisms in human or animal is unclear. Recent study demonstrated that AMP might act as small

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molecular TrkA agonist, thus easily penetrate brain/blood barrier to have potent neurotrophic effect in various disease models (Ho et al., 2008; Jang et al., 2009; O'Neill et al., 2016). Inspired by those research data, we hypothesized in this study that, AMP might have a protective effect in hypoxia/reoxygenation (H/R)-induced cardiac damage and apoptosis, through the activation of TrkA receptors, in cardiomyocytes. Thus, we utilized an *in vitro* H/R injury model to test this hypothesis by treating cultured neonatal mouse cardiomyocytes with AMP prior H/R conditioning. Cardiomyocyte viability, apoptosis, as well as the activities of TrkA signaling pathways were then examined in our study. Moreover, we applied k252a, a TrkA agonist, in the culture of AMP-treated cardiomyocyte to examine whether TrkA signaling pathway was directly involved in the process of AMP-induced apoptosis protection in H/R injured cardiomyocyte. Our results will undoubtedly help to broaden our understanding on the underlying mechanisms of ischemic injury or myocardial infraction in human patients.

2. Materials and methods

2.1. Ethic approval

All protocols were approved by the Clinical Research and Ethics Committees at Zhongshan Hospital of Fudan University in Shanghai, and the first people's hospital of Wenling in Wenling, China.

2.2. Cardiomyocytes culture

Culture of neonatal mouse cardiomyocytes was performed according to a previously published method with small modification (Yue et al., 2012). Briefly, postnatal 1-day-old C57BL/6J neonatal mice were quickly killed by decapitation. Chest was opened and heart was immediately transferred into an ice-cold petri dish containing Hanks' balanced salt solution (HBSS, PH=7.4, MilliporeSigma, USA). A surgical scissors with fine tips was used to chop heart tissues into small pieces, followed by treatment of 0.1% trypsin-EDTA (ThermoFisher Scientific, USA) for 20 mins at 37 °C. After adding serum to stop trypsinization, and then centrifugation (150*g*, 15 min, 4 °C), supernatant was removed. Cell pellet was resuspended in culture medium containing DMEM/F12 solution (ThermoFisher Scientific, USA), 10% fetal bovine serum (FBS, ThermoFisher Scientific, USA) and penicillin (100 U/ml) and streptomycin (100 μg/ml) (ThermoFisher Scientific, USA). Cell culture was maintained at 37 °C for 4 h to enrich cardiomyocyte population. After that, floating cardiomyocytes were re-collected and re-plated in 6-well plate in culture medium at 37 °C with 95% O_2/5% CO_2.

2.3. Hypoxia/reoxygenation condition and amitriptyline

The method to introduce hypoxia and reoxygenation condition in cardiomyocyte culture was performed according to a previously published method with small modification (Yue et al., 2012). Briefly, when cardiomyocyte culture reached confluence of 70–80%, it was incubated in culture medium without serum for 12 h to initiate serum starvation treatment. The culture was transferred to a hypoxia chamber flushed with a mixed gas of 95% N_2 and 5% CO_2 for 4 h. Then, cardiomyocytes were transferred to a regular tissue-culture incubator at 37 °C with 95% O_2/5% CO_2 for 8 h for reoxygenation. For amitriptyline (AMP, MilliporeSigma, USA) treatment, various concentration of AMP (0nM–5 uM) was added into cardiomyocyte culture 2 h prior hypoxia condition.

2.4. Viability assay

Cardiomyocyte viability was evaluated using a trypan blue staining method as described in previous publicaiton (Lopez et al., 2007). Briefly, cardiomyocyte culture was treated with 0.1% trypsin-EDTA for 20 mins at 37 °C. After adding serum to stop trypsinization, and then centrifugation (150*g*, 15 mins, 4 °C), supernatant was removed. Cardiomyocyte pellet was resuspended in culture medium mixed with 0.2% trypan blue medium (MilliporeSigma, USA) for 10 min at 37 °C. Then, dead cardiomyocytes (trypan blue stained) and healthy cardiomyocytes (trypan blue un-stained) were counted using a Z1 Coulter cell counter (Beckman Coulter, USA). Relative viability was measured as the percentage of healthy cardiomyocytes against all (dead and healthy) cardiomyocyte population.

2.5. TUNEL assay

Apoptosis was evaluated using a previously published method with small modification (Yue et al., 2012). Briefly, cardiomyocyte culture was fixed by 4% paraformaldehyde (MilliporeSigma, USA) for 20 min at room temperature. It was treated with 0.3% TritonX-100 (MilliporeSigma, USA) for 1 h at room temperature, and then a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (MilliporeSigma, USA). DAPI (MilliporeSigma, USA) staining was used to identify cardiomyocyte nuclei. The percentage of healthy cardiomyocyte was characterized by counting TUNEL-negative cells, and normalizing them against DAPI-positive cells.

2.6. Western blot assay

Cardiomyocyte culture was collected and lysed using a RIPA buffer (MilliporeSigma, USA). Protein product was separated on 10% SDS-PAGE and immunoblotted with antibodies against TrkA (1:1000, Cell Signaling Technology, USA), phosphor-TrkA (1:200, Cell Signaling Technology, USA), Akt (1:1000, Cell Signaling Technology, USA) and phosphor-Akt (1:1000, Cell Signaling Technology, USA) for 12 h at 4 °C. The secondary peroxidase-conjugated antibodies were applied for 2 h at room temperature. Beta-actin was used as control protein for western blot assay. Blots were visualized and semi-quantified using an enhanced chemiluminescence system (Pierce, USA).

2.7. Statistical analysis

Statistical analysis was conducted by Student's *t*-test using a SPSS software (SPSS, Version 13.0, USA). For each assay, data were averaged from at least three biological repeats and presented as mean +/- S.E.M. $P < 0.05$ is termed as statistic difference.

3. Results

3.1. Amitriptyline rescued cardiomyocyte death induced by hypoxia/reoxygenation

It is well known that hypoxia/reoxygenation (H/R) condition caused cell death in cardiomyocytes both *in vitro* and *in vivo* (Li and Jackson, 2002; Yue et al., 2012). In this study, we firstly used a previously published H/R condition to introduce cell death in cultured neonatal mouse cardiomyocytes (Yue et al., 2012). As shown in Fig. 1, a condition of 4 h hypoxia treatment and 8 h reoxygenation caused death in approximate 40% cardiomyocyte (Fig. 1, Control *vs*. 0 AMP, $*P < 0.05$). To examine the possible protective effect of amitriptyline (AMP), AMP was added into cardiomyocyte culture 2 h prior to hypoxia treatment. After H/R treatment, analysis of viability assay showed, low concentrations, from 1 nM to 20 nM, of AMP pre-treatment had no effect on H/R-induced cardiomyocyte death. However, at concentrations between 50 and 200 nM, AMP significantly rescued H/R-induced cardiomyocyte death (Fig. 1, compared to 0 AMP, $**P < 0.05$). Then, at much higher concentrations, (> 500 nM), the protective effect of AMP disappeared, probably due to high-dosage-induced toxicity.

As we showed moderate concentrations of AMP, between 50 and 200 nM, could rescue H/R-induced cardiomyocyte death, the following