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Activation of an apoptotic signal transduction pathway involved in the upregulation of calpain and apoptosis-inducing factor in aldosterone-induced primary cultured cardiomyocytes

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ABSTRACT

In this study, aldosterone (ALD)-induced apoptosis of cardiomyocyte was evaluated based on the previous studies, and the roles of calpain signaling were clarified. Primary cultured rat cardiomyocytes were injured by ALD (0.01–10 μM) for varying time periods. Then, the effects of ethylene glycol tetraacetic acid (EGTA) (0.5 mM), calpeptin (2.5 μM), and spironoclactone (10 μM) were evaluated on cardiomyocytes activated by ALD. Cardiomyocytes that were injured by ALD were assayed by the MTT and LDH leakage ratio. Apoptosis was evaluated by a TUNEL assay, annexin V/PI staining, and caspase-3 activity. The expression of cleavage of Bid (tBid), calpain and apoptosis-inducing factor (AIF) was evaluated by western blot analysis. ALD increased calpain expression and caspase-3 activity and promoted Bid cleavage. It also induced the release of AIF from mitochondria into the cytosol. The upregulation of calpain, tBid and caspase-3 activity were further inhibited by treatment with EGTA in the presence of ALD. Additionally, AIF levels in the cytosol decreased due to EGTA but not due to calpeptin. This was also accompanied by a significant decrease in apoptosis. Furthermore, treatment with spironoclactone not only attenuated the pro-apoptotic effect of ALD but reversed the ALD-induced increase of calpain and AIF levels.

1. Introduction

Heart remodeling consists of cardiomyocyte hypertrophy, cardiac fibroblast proliferation and cardiomyocyte loss. Accumulating evidence suggests that the loss of functional cardiomyocytes due to apoptosis plays a crucial role in the transition from heart remodeling to heart failure (Dickhout et al., 2011). Apoptosis has been recognized as a major mechanism in the development of cardiovascular diseases (Kang and Izumo, 2003). Aldosterone (ALD) is an important factor involved in cardiovascular diseases promoted by endothelial dysfunction and fibrosis; it is secreted from not only the zona glomendosa of the adrenal cortex but also extra-adrenal tissues such as the heart and blood vessels. Moreover, recent studies provided plenty of evidence suggesting that apoptosis may be a key factor in the pathological progress of cardiomyocyte injury induced by ALD *in vivo* and *in vitro* (Häfner et al., 2012; Mano et al., 2004; Tan et al., 2004).

It is well known that a disturbance of Ca^{2+} homeostasis as a stimulus is implicated in various cell apoptotic events (Graidist et al., 2007; Griffiths et al., 2010), and evidence also indicated that ALD-mediated actions may cause various intracellular responses associated with the elevation of intracellular Ca^{2+} through both L-type Ca^{2+} channels (LTCC) and T-type Ca^{2+} channels (TTCC) (Ferron et al., 2011; Gustafsson and Gottlieb, 2008; Lalevée et al., 2005) with persistent increase in Ca^{2+} influx eventually leading to organ failure by reducing the number of functional cells. Recent studies have suggested that the elevation of intracellular Ca^{2+} can activate several critical enzymes (e.g., calmodulin, calcineurin, calpain). Proteolysis of various substrate proteins by calpains in distinct cellular locations leads to their altered function in cells, and this makes calpains important regulators of cellular signaling mechanisms in almost every aspect of biological activities. Calpains also play pivotal roles in the signaling pathway resulting in mitochondrial dysfunction and lead to cell apoptosis (Ajiro et al., 2008; Selimovic et al., 2011; Shaerzadeh et al., 2011; Vindis et al., 2005). One of classical substrates cleaved by calpains, Bid, is a pro-apoptotic BH3-only member of the Bcl-2 family. Cleavage of Bid (tBid) promotes the pro-apoptotic effect of Bax by cleaving the loop region in Bcl-xL to change an anti-apoptotic molecule into a pro-apoptotic molecule (Nakagawa and Yuan, 2000). Bypassing the Bcl-2 family, calpain cleavage of pro-caspase also leads to

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activation of these proteases (Blomgren et al., 2001) as in the case of ionizing irradiation, which indicates that calpain activation may be involved in many apoptotic pathways. It presently seems that the roles of calpain in apoptosis have not been elucidated, the involvement of which is also limited to certain cell types and to specific stimuli (Mani et al., 2009). Thus, the calpain-dependent signal transduction pathways need to be explored. In addition, studies indicated that intracellular Ca^{2+} overload results in apoptosis-inducing factor (AIF) release from mitochondria to the cytosol and then into nuclei, thereby exerting its pro-apoptotic effects (Lee et al., 2006). However, whether AIF release from mitochondria to the cytosol is dependent on or independent of calpain remains controversial. Recent studies reported that the translocation of AIF due to intracellular calcium overload induces cell apoptosis (Vindis et al., 2005; Zhang and Bhavnani, 2006). However, Polster and his colleagues researched the effect of calpain and found that calpain induced cleavage and the release of AIF from isolated mitochondria (Nakagawa and Yuan, 2000). The aim of the present study was to explore the role of the calcium signaling pathway in ALD-induced injury of cardiomyocytes, with a particular focus on the stimulation of the calpain signaling pathway and the promotion of AIF release. To our knowledge, this is the first example of experimental data showing that ALD-induced cardiomyocyte apoptosis involves stimulating the calpain signaling pathway and promotes the release of AIF from mitochondria into the cytosol.

2. Materials and methods

2.1. Animals

All chemicals and reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA), unless stated otherwise. Sprague–Dawley (SD) rats between 1 and 3 days old were provided by the experimental animal center of Guiyang Medical University.

2.2. Cell culture and treatment

Primary cultures of cardiomyocytes were prepared from Sprague–Dawley rats between 1 and 3 days old. The isolated cardiomyocytes were minced and sequentially digested in phosphate-buffered saline (PBS) containing 0.08% trypsin at 37 °C for 10 min. This digestion exposure was repeated 5 times in turns. Cells obtained from each digestion exposure were suspended in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) containing 10% fetal calf serum (Kangzhou Sijiqing Biological Engineering Material Co., Ltd.) and centrifuged for 10 min at 1000 rpm. Pellets were resuspended and combined in the culture medium and precultured for 2 h to deplete fibroblasts, and the resulting suspension was collected. The purity of cardiomyocyte cultures were >97% as assessed by immunocytochemical staining using cardiac muscle sarcomeric α-actinin antibody (Boster Biotechnology, China). During the final incubation in serum-deprived DMEM, cardiomyocytes treated with 0.2% DMSO (Amersco, USA) served as a vehicle, and cardiomyocytes were treated with ALD (0.01–10 μM; Fluka, Switzerland) for 24 h or treated with 10 μM ALD for the indicated time periods (0–24 h). To evaluate the roles of calcium and calpain, myocytes were stimulated with 10 μM ALD after treatment with either EGTA (0.5 mM; Amresco, USA); a calcium chelator, calpeptin (2.5 μM, Calbiochem, USA); an inhibitor of calpain, or spironolactone (Spiro) (10 μM, National Institute for the Control of Pharmaceutical and Biological Products, China); a nonselective mineralocorticoid receptor (MR) antagonist for 1 h. All drugs were freshly constituted in 0.2% DMSO, and there was no significant effect on cardiac myocytes (data not shown).

2.3. Analysis of cell viability

Cell viability was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (American Type Culture Collection).

2.4. Lactate dehydrogenase release

Lactate dehydrogenase (LDH) release was measured as a biomarker of cell injury. After treatment, the culture medium was collected and centrifuged for 10 min at 1000 rpm, and the LDH activity in the supernatant was measured as the extracellular LDH activity. Alternatively, the cell lysate was collected by scraping and centrifuging for 10 min at 1000 rpm, and the supernatant LDH activity was measured as the intracellular LDH activity. LDH activity was measured by determining the absorbance at 405 nm using a spectrophotometer (722N, Shanghai Instrument Factory, China). LDH release was expressed as the percentage of extracellular LDH activity out of the total LDH activity (extracellular LDH activity plus intracellular LDH activity).

2.5. Detection of apoptotic cells

We used two methods to determine apoptosis of cardiomyocytes. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL, KeyGEN, Nanjing, China) assays were performed according to the manufacturer's protocol. Apoptotic cells were stained brown, and normal cells were stained purple-blue. The percentage of TUNEL-positive cells was determined by counting at least 200 cells in 10 randomly selected fields. The annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit (KeyGEN, Nanjing, China) was used according to the manufacturer's instructions. The apoptotic cell membranes gave off green fluorescence and the nucleoli gave off a red fluorescence observed with a fluorescence microscope (BX.51, OLYPUS, Japan). The apoptotic cell counts were expressed as a percentage of the total number of cells giving off fluorescence.

2.6. Caspase-3 activity assay

Relative caspase-3 activity was determined using the Caspase-3 colorimetric assay kit (KeyGEN, Nanjing, China). According to the manufacturer's protocol, cell lysates were incubated with lysis buffer (1 mol/L DTT) and labeled caspase-3 substrate DEVD-*p*NA for 48 h at 37 °C. Cleavage of a substrate was quantified by measuring the absorbance at 405 nm using a microplate reader (ELX800, GE, USA).

2.7. Western blot analysis

After the treatments were completed, the cells were washed once in PBS and lysed in lysis buffer (Beyotime, Jiangsu, China). The protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Jiangsu, China). For western blot analysis, 20 μg of protein was separated on an 8–12% SDS–polyacrylamide gel and then transferred to a PVDF membrane. After blocking the membrane with Tris-buffered saline-Tween 20 (TBST, 0.1% Tween 20) containing 5% non-fat dried milk for 2 h at room temperature, the membranes were washed twice with TBS-T and incubated with primary antibodies for 2 h at room temperature or overnight at 4 °C. The following primary antibodies were used: rabbit anti-AIF (1:500, CAT. # sc-5586, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-calpain (1:500, CAT. # sc-30064, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-Bid (1:500, CAT. # sc-6291, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed 3 times with TBS-T for 10 min, followed by incubation for 1.5 h at room temperature with horseradish peroxidase (HRP) – conjugated secondary antibodies (1:8,000, Boster Biotechnology, China). After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Boster Biotechnology, China). β-actin (1:300, Boster Biotechnology, China) was used as the standard for equal loading of the protein samples. The band intensities were quantified using a Photo-Image System (Image quant400, GE Healthcare).

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) at least 3 independent experiments. The data were analyzed by a one-way ANOVA, and LSD test in Post hoc method was applied to analyze the difference between every two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Time- and dose-dependent effects of ALD on the apoptotic pathway and cytotoxicity

To evaluate the time- and dose-dependent reactions of ALD-induced injury of cardiac myocytes, equal numbers of cells were treated with 0.01–10 μM ALD for 24 h or with 10 μM ALD for 0–24 h. Cardiomyocytes treated with 1 and 10 μM ALD had significantly decreased cell viability (Fig. 1A) and increased cardiomyocyte apoptosis (Fig. 2A) compared with the vehicle. The higher concentration of ALD treatment had a strong detrimental effect, whereas the lower dose group showed a weaker detrimental effect on the myocytes, which demonstrated the dose-dependence of the effect. As previously reported, no significant difference was found in the number of annexin-V/PI-stained cells between 24 and 48 h of ALD exposure Malmqvist et al., 2003.