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Cardioprotection with 8-O-acetyl shanzhiside methylester on experimental myocardial ischemia injury

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ABSTRACT

8-O-acetyl shanzhiside methylester (ND01) was isolated from the leaves of *Lamiophlomis rotata* (*Benth.*) *Kudo*. In this study, we investigated the anti-myocardial ischemia and reperfusion (I/R) injury effects of ND01 in vivo and elucidated the potential mechanism in vitro. The results indicated that ND01 significantly attenuated hypoxia-induced cytotoxicity in a concentration-dependent manner in H9c2 cells. Treatment of H9c2 cells with ND01 9 μM blocked TNF-α-induced nuclear factor kappaB (NF-κB) phosphorylation by blocking High-mobility group box1 (HMGB1) expression. Treatment of rats with ND01 10 mg/kg, (i.v.) protected the animals from myocardial I/R injury as indicated by a decrease in infarct volume, improvement in hemodynamics and reduction of myocardial damage severity. Treatment with ND01 also lowered serum levels of pro-inflammatory factors and reduced High mobility group box-1 protein (HMGB1) and phosphorylated NF-κB expression in ischemic myocardial tissue. Additionally, continuos i.v. of ND01 14 days attenuated cardiac remodeling. These protective effects suggested that ND01 might be due to block of myocardial inflammatory cascades through an HMGB1-dependent NF-κB signaling pathway.

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1. Introduction

Primary myocardial ischemia and reperfusion (I/R) therapies, including percutaneous coronary intervention (PCI) and thrombolysis, are the standard of care for the treatment of acute coronary syndromes. Prompt restoration of blood flow to ischemic myocardium limits infarct size and reduces mortality. Paradoxically, however, the return of blood flow can also result in additional cardiac damage and complications as reperfusion injury. Effective therapies to reduce or prevent reperfusion injury have proven elusive. Despite an improved understanding of the pathophysiology of this process and encouraging preclinical trials of multiple agents, most of the clinical trials to prevent reperfusion injury have been disappointing (Bolli et al., 2004; Cannon, 2005). Despite these problems, adjunctive therapies to limit reperfusion injury remain an active area of investigation.

Previous studies have indicated that nuclear factor kappaB (NF-κB) plays a key role in inflammatory response during myocardial I/R injury (Frantz et al., 2007). Suppression of NF-κB activation diminishes myocardial I/R damage and potentially offers myocardial protection (Kim et al., 2009). High mobility group box-1 protein (HMGB1) has been described as a key cytokine to play an extracellular role involving cellular activation and proinflammatory responses (Lotze and Tracey, 2005; Yang et al., 2005). HMGB1 may be signal through the receptor for advanced glycation end products (RAGE) (Huttunen and Rauvala, 2004), and promote the chemotaxis and production of cytokines in a process that involves the activation of NF-κB, which is a ubiquitously expressed transcription factor that plays a pivotal role both in inflammatory response and cell survival, and regulates a vast number of genes including those encoding cytokines, death and survival proteins, intercellular adhesion molecules, cyclooxygenase-2 and inducible nitric oxide synthase (Małek et al., 2007). Inhibiting HMGB1 blocks inflammatory response and may be a therapeutic target during myocardial I/R injury (Jiang et al., 2012).

8-O-acetyl shanzhiside methylester (ND01), an iridoid glucoside is isolated from the leaves of *Lamiophlomis rotata* (*Benth.*) *Kudo*., which is a Chinese folk medicinal plant in Xi-zang (Tibet). For thousands years, *L.* rotata has been used as one of the traditional drugs with the effects of alleviating pain, detumescence, hemostasis, reinforcing marrow and promoting blood circulation to remove blood stasis (Yi et al., 1997). Previous studies indicated that ND01 attenuated apoptosis and ameliorated mitochondrial energy metabolism in rat cortical neurons exposed to oxygen–glucose deprivation (Jiang et al., 2010b), increased angiogenesis and improved functional recovery after stroke (Jiang et al., 2011). The aim of our study is to investigate the effects of AX in a rat model

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of myocardial I/R and its potential cardioprotective mechanism in cultured H9c2 cells as well as in intact rats.

2. Materials and methods

2.1. Materials

8-O-acetyl shanzhiside methylester (ND01, Purity > 99.0%. It was obtained from State Key Laboratory of Long-acting and Targeting Drug Delivery Technologies, Yantai, PR China). Troponin T (Tn-T) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Maisha Biology Company (Shanghai, PR China). Tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) ELISA kits were purchased from Xitang Biology Technology Company (Shanghai, PR China). Polyclonal rabbit anti-mouse HMGB1, NF-κB and phosphor-NF-κB antibodies were purchased from Biosynthesis Biotechnology Company (Beijing, PR China).

2.2. Animals

Adult male Sprague–Dawley rats (250–280 g, body weight) were housed individually under constant temperature (22 ± 2 °C) and humidity with a 12 h light/dark cycle and had free access to chow and water. The experimental designs, including all procedures, were conducted in accordance with the Animal Care Guidelines of the Animal Experimental Committee of Binzhou Medical University.

2.3. Cell culture

Myocardiac H9c2 cells, a clonal line derived from embryonic rat heart, were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were routinely cultured in Dulbecco's modified Eagle'smedium (DMEM) with D-glucose at 4.5 g/l, 20% FBS, 10,000 U/l penicillin, and 10 mg/l streptomycin, in an incubator with 5% CO_2, at 37 °C. The medium was changed every 2 days and when confluence was reached, the cells were subcultured by detaching with 0.25% trypsin–EDTA solution (Sigma.) and re-seeding into new plates at a ratio of 1:5, then incubated in DMEM containing 2% FBS. Cells were kept at 37 °C in a humidified 5% CO_2/95% air incubator.

2.4. In vitro hypoxia model

To mimic the hypoxia injury in vitro, cells were incubated in a hypoxia solution for 6 h. The hypoxia solution (Zhang et al., 2011) contained 0.9 mM NaH_2PO_4, 6.0 mM $NaHCO_3$, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 40 mM Natrium lacticum, 20 mM HEPES, 98.5 mM NaCl, 10.0 mM KCl (pH adjusted to 6.8) and bubbled with N_2 for 30 min before application. The pO_2 of the hypoxia solution was adjusted to reach a level of ⩽4.0 kPa. Hypoxic condition was produced by placing the plates of cultured cardiomyocytes in a hypoxic incubator (Kendro, Germany) and oxygen was adjusted to 1.0% and CO_2 to 5.0%. Prior to hypoxia, cells were pretreated with various concentrations (1, 3, 9 and 27 μM) of ND01 for 24 h. Normal culture (DMEM containing 2% FBS under 20% oxygen and 5% CO_2) served as a negative control, the hypoxia solution culture served as the control.

2.5. Cell viability assays

Cell viability was determined by a Microculture Tetrazolium (MTT) assay. Cells were seeded at a density of 8×10^3 cells/well in 96-well cell culture plates. Following exposure to hypoxia, 20 μl of the MTT solution (5 mg/ml) was added into each well and made the final concentration at 0.5 mg/ml, and then the plates were incubated for an additional 2 h and measured by absorbance at 490 nm in a microplate reader. The percent viability was defined as the relative absorbance of treated vs. untreated control cells.

2.6. TNF-α-stimulated H9c2 cell assays

For TNF-α-stimulated H9c2 cell line experiments in vitro, H9c2 cells (5×10^5) were pre-incubated with HMGB1 inhibitor glycyrrhizin (at a final concentration of 100 μM), NF-κB inhibitor Pyrrolidinedithiocarbamic acid (PDTC, 100 μM), or ND01 (9 μM) for 30 min and then incubated with TNF-α (20 ng/ml) for 30 min.

2.7. Western blot analysis

Cells were cultured for 24 h, then washed twice with ice cold PBS on ice and lysed in NP40 lysis buffer (Biosource, Camarillo, CA, USA) (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% NP-40 and 0.02% NaN_3) supplemented with 1 mM PMSF and 1 × protease inhibitor cocktail (Sigma, Saint Louis, MO, USA). Equal amounts of cell protein (50 μg) were separated by SDS–PAGE and analyzed by western blot using specific antibodies to NF-κB, phospho-NF-κB, HMGB1 and PCNA (as a loading control). Optical densities of the bands were scanned and quantified with a Gel Doc 2000 (Bio-Rad Laboratories Ltd.). Data were normalized against those of the corresponding PCNA bands. Results were expressed as fold increase over control.

2.8. Induction of myocardial I/R injury

Myocardial I/R operations were induced according to a previously procedure (Jiang et al., 2010a). Briefly, Rats were anesthetized with ketamine 100 mg/kg (i.m.) and xylazine 10 mg/kg (i.m.) and ventilated with room air using a rodent respirator. The chest was opened by middle thoracotomy. After pericardiotomy, a 4–0 black silk ligature was placed under the left aortic descending coronary artery, and the ends of the tie was threaded through a small vinyl tube to form a snare for reversible left aortic descending coronary artery occlusion. After 30 min of ischemia, the myocardium was reperfused by loosening the snare for 24 h.

A pilot study was conducted with four different doses of ND01 (2.5, 5, 10, or 20 mg/kg) to determine the dose dependent effect in acute I/R-treated rats. It was observed that ND01 post-treatment at doses of 5, 10 and 20 mg/kg significantly ($P < 0.05$) lowered elevated levels of CK-MB, LDH and cTnT in serum of acute I/R-induced rats after 240 min of experiment study. Hence, ND01 10 mg/kg was chosen for this study.

One hundred and eight rats were divided into three groups, all groups include three subgroups: (I) non-myocardial I/R rat, the silk suture crossed without ligation and the rat did not incur I/R; (II) I/R rats received saline alone); (III) I/R rats received intravenous injection (i.v.) of ND01 10 mg/kg, the animals of the first group and the third group is 10 in each subgroup, the second group is 16 in each subgroup and received drug treatment (i.v.) at the indicated dosage after reperfusion for 5 min. The third group is administered daily via i.v. of corresponding drug for continuous 14 days after I/R 24 h. ND01 was dissolved in sterilized saline to make stock solutions and appropriate dilutions according to the dosages required. The first group of animals was used for hemodynamics evaluation, infarct size and serum levels of Tn-T, TNF-α and IL-6 determination. The second group was used for histopathological and Western blots analysis. The third group was used to determine hemodynamics and analyze histopathological change.

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