Biomedicine & Pharmacotherapy 104 (2018) 52–59

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Protective effects of echinacoside against anoxia/reperfusion injury in H9c2 cells via up-regulating p-AKT and SLC8A3

Min Chen[1], Xiaodong Wang[1], Bo Hu, Jian Zhou, Xin Wang, Wei Wei, Hua Zhou*

Department of Cardiovascular Medicine, Shanghai East Hospital, Tongji University School of Medicine, 150 Jimo Road, Pudong New Area, 200120, Shanghai, China

ARTICLE INFO

Keywords:
Echinacoside
anoxia/reperfusion (A/R)
Apoptosis
Ca^{2+}SLC8A3
p-AKT

ABSTRACT

Echinacoside is a natural ingredient with various pharmacological activities. In this study, we investigated the protective effects of echinacoside on cardiomyocytes (rat H9c2 cells) in an anoxia/reperfusion (A/R) model. Further, the regulatory function of sodium-calcium exchanger protein 3 (SLC8A3/NCX3) as well as the protein kinase B (AKT) signaling were studied. The present results indicated that echinacoside protected against A/R-induced apoptosis in a dose manner, which was characterized by a decrease in the apoptosis and caspase 3 protein levels in H9c2 cells. Further, Ca^{2+} uptake were dose-dependently reduced in H9c2 cells by echinacoside under A/R conditions. Whereas, relative mRNA expression of SLC8A3 and protein levels of SLC8A3 and p-AKT showed opposite tendency. On the one hand, the A/R-induced abnormalities in H9c2 cells were remarkably ameliorated by activated p-AKT and over-expression of SLC8A3 but aggravated by inhibited p-AKT, and the aggravated effection were ameliorated by echinacoside. Moreover, protein levels of SLC8A3 were positively regulated by p-AKT signaling. On the other hand, apoptosis and Ca^{2+} uptake as well as protein levels of caspase 3 were significantly increased by SLC8A3 silencing in H9c2 cells under normoxic conditions, and this symptom was remarkably reversed by echinacoside or Nimodipine (an antagonis of Ca^{2+}) treatment. Collectively, echinacoside has showed a cardioprotective effect against A/R treatment in a dose dependent manner in vitro, and this cardioprotective effect was potentially achieved via up-regulating p-AKT and SLC8A3.

1. Introduction

Coronary heart disease (CHD) is one of major manifestations of cardiovascular disease, and 3.8 million men and 3.4 million women die of the disease each year [1]. Although reperfusion is essential for saving CHD and maintaining myocardial viability after continuous ischemia, the actual process of reperfusing ischemic myocardium can itself paradoxically induce injury - a process termed myocardial reperfusion injury, and such damage attenuates the benefits of myocardial reperfusion [1,2]. Ischemia (anoxia)/reperfusion (I(A)/R)-induced cardiac injury is due to two primary mechanisms, apoptosis and necrosis; both of them are accelerated by reperfusion [3,4]. Previous findings on the molecular mechanisms involved in A/R reveal that cardioprotection is achieved by activating the prosurvival and anti-apoptotic kinase signaling cascades phosphatidylinositol 3-kinase/ protein kinase B (PI3K/AKT) during reperfusion [5,6]. Although the myocardium injury caused by I/R can be modulated by preconditioning particularly [7], the inadequacy of the available animal models of I/R injury with regard to representing the wide spectrum of comorbidities in patients with CHD (such as diabetes, hypertension, hyperlipidemia, and pre-existing coronary artery disease) as well as other coexisting factors (such as advanced age and other medical therapy) [8]. Currently, there is no effective clinical drugs to reduce I(A)/R-induced cardiac injury [9]. Therefore, new therapeutic methods are urgently needed.

I/R is also associated with the increase of intracellular calcium concentration [10]. Ca^{2+} is a vital and multipurpose secondary messenger in cells, and it carries crucial information to almost all processes important to the life activity and function of cells. For instance, Ca^{2+} couples excitation to contraction, hormone secretion, gene transcription, and regulates enzyme activity via protein phosphorylation-dephosphorylation that relates to varied life activities [11,12]. On the other hand, prolonged high Ca^{2+} activates nucleases that cleave DNA and degrade cell chromatin, and finally to cell lysis [13]. Nimodipine, a calcium channel blocker (CCB), is mainly used for the treatment of acute subarachnoid hemorrhage. It is highly selective for L-subtype calcium channels [14].

Na^{+}/Ca^{2+} exchanger (NCX) proteins remove Ca^{2+} in cells to maintain cellular Ca^{2+} homeostasis [15]. In cardiomyocytes, Na-Ca

* Corresponding author.
E-mail address: zhzju0560@126.com (H. Zhou).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.biopha.2018.04.188
Received 27 December 2017; Received in revised form 25 April 2018; Accepted 29 April 2018

exchanger is widely distributed over the cell surface [16] and characterized as a high-capacity (k_{cat} ~2500 s^{-1}) and low-affinity (K_m ~10–20 μM) system which can regulate cellular Ca^{2+} concentration at a wide dynamic range within short moment [17–19]. Towards the NCX family, all exchangers were regulated by intracellular Ca^{2+}. Further, depletion of cellular ATP inhibited NCX1 (SLC8A1) and NCX2 (SLC8A2) but, interestingly, does not affect the activity of NCX3 [20]. As a non-ATP dependent sodium-calcium exchanger, the potential function of NCX3 during the process of A/R is still unclear.

Echinacoside is the main bioactive compound isolated from *Cistanche* and *Echinacea* plants [21]. In China, extractive of different parasitic *Cistanche* plants' stem served as a tonic from earliest times to the present day [22]. In US and Europe, extracts of *Echinacea* has been widely used as an herbal supplement to remedy infections and common cold [23,24]. Echinacoside also has exhibited multiple biological activities and pharmacological activities, including anti-apoptosis, neuroprotection, hepatoprotection, immunomodulation, anti-senescence, anti-diabetes and promotion of bone formation [25–32], whereas, no report has invesigated the cardioprotection of echinacoside under A (I)/R, and the detailed molecular mechanisms remain unclear.

Thus, this study has investigated the protective effects of echinacoside on rat H9c2 cells in an A/R model. Moreover, the regulatory function of non-ATP dependent sodium-calcium exchanger protein 3 (SLC8A3) as well as the AKT signaling in H9c2 cells were studied.

2. Materials and methods

2.1. Materials

Echinacoside was purchased from Aladdin (Shanghai, China). Nimodipine was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). LY294002 was purchased from Selleckchem (Houston, TX, USA). Insulin-like growth factor 1 (IGF-1) was purchased from PeproTech (Rocky Hill, NJ, USA). Primary antibodies of SLC8A1, SLC8A3 and Caspase3 were purchased from Abcam Biotech (Cambridge, MA, USA). Antibodies of AKT, p-AKT and GAPDH were purchased from Cell Signaling Technologies (Beverly, MA, USA). Antibody of SLC8A2 was purchased from Bioss (Beijing, China). Annexin V-FITC apoptosis detection kit was purchased from Beyotime Biothech (Shanghai, China).

2.2. Cell culture

H9c2 embryonic rat heart-derived cells were obtained from Shanghai Institute of Biochemistry and Cell Biology (SIBCB, Chinese Academy of Sciences, Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, Utah, USA) with 10% fetal bovine serum (FBS, Gibco Company, USA) and 100 U/ml penicillin, and incubated at 37 ℃ under normoxic conditions. H9c2 cells at log phase were used in subsequent experiments.

2.3. A/R protocol

To mimic the I/R, an A/R model in vitro was established according to a previous report with minor modifications [33], H9c2 cells at log phase were incubated with a glucose-free DMEM (Gibco Company, USA) at 37 ℃ for 2 h in a hypoxic chamber (1% O_2, 5% CO_2 and 94% N_2). Oxygen was monitored continuously with an oxygen electrode (Controls Katharobic, Philadelphia, PA, USA) inside the chamber. There was no significant change in medium pH after been exposure to hypoxia. During the reoxygenation period, the medium was substituted with maintenance medium (DMEM with 10% FBS), and cells were incubated at 37 ℃ under normoxic conditions (DMEM with 10% FBS at 37 ℃ with 5% CO_2) for 6 h.

Table 1
Primers used in Real-time Fluorogenic PCR Assays.

Gene name	Sequence (5'-3')	Description
SLC8A1	GGAGACCCAGAAGGAAATCAG	Forward
SLC8A1	TGACCCGAGACAAGCAATC	Reverse
SLC8A2	GCCTCTGGTTCCTCTACATTC	Forward
SLC8A2	GAGCAAGTGCCAACAAGTC	Reverse
SLC8A3	CACCCTCCACAGAAAGAC	Forward
SLC8A3	CCCACCTCAGACACAAAC	Reverse
GAPDH	GGAGTCTACTGGCGTCTTCAC	Forward
GAPDH	ATGAGCCCTTCCACGATGC	Reverse

2.4. Experiment groups

2.4.1. Protective effects of echinacoside on H9c2 cells under A/R conditions

H9c2 cells were divided into 5 groups: normoxic control group, A/R group, A/R + 5 μM echinacoside group, A/R + 10 μM echinacoside group and A/R + 20 μM echinacoside group. Echinacoside was dissolved in dimethyl sulfoxide (DMSO). Cells in echinacoside treatment groups were treated by different concentrations of echinacoside (5 μM, 10 μM and 20 μM) and cells in NC group and model group were substituted with equal amount of DMSO. After incubation for 24 h under normoxic conditions, H9c2 cells in latter 4 groups were incubated according to A/R protocol. H9c2 cells in NC group were incubated for 8 h under normoxic conditions. The cells were collected at the end of experiments.

Relative mRNA expressions of SLC8A1 (NM_001270772.1), SLC8A2 (NM_078619.1) and SLC8A3 (NM_078620.2) in each group were determined using real-time PCR. Protein levels of SLC8A1, SLC8A2, SLC8A3, AKT, P-AKT and Caspase 3 were determined by Western blotting. Concentration of Ca^{2+} and apotosis of H9c2 cells were measured using flow cytometry methods.

2.4.2. Effects of AKT signaling on H9c2 cells under A/R conditions

H9c2 cells were divided into 5 groups: normoxic control group, A/R model group, A/R + LY294002 group, A/R + IGF-1 group and A/R + IGF-1 + echinacoside group. In A/R + LY294002 group and A/R + IGF-1 group, LY294002 (2 μM) and IGF-1(100 ng/mL) were respectively added to the H9c2 cells and then, cells were incubated for 1 h under normoxic conditions. In A/R + IGF-1 + echinacoside group, echinacoside (20 μM) was added to the H9c2 cells and incubated for 24 h at normoxic conditions, then IGF-1(100 ng/mL) were added and cells were incubated for 1 h under normoxic conditions. H9c2 cells in normoxic control group and A/R model group were substituted for equal amounts of solvent (DMSO) and incubated for 1 h at normoxic conditions. Subsequently, cells in normoxic control group were incubated at normoxic conditions for 8 h. while cells in other 4 groups were incubated according to A/R protocol.

Protein expressions of SLC8A3 and Caspase3, concentration of Ca^{2+} as well as apotosis of H9c2 cells were measured at the end of experiments.

2.4.3. Effects of SLC8A3 over-expression on H9c2 cells under A/R conditions

H9c2 cells were divided into 4 groups: normoxic control group, A/R model group, A/R + empty plasmid control (EPC) group and A/R + SLC8A3 over-expression (OE) group. H9c2 cells in SLC8A3 A/R + OE group and A/R + EPC group were transduced with SLC8A3 over-expression and empty plasmid lentivirus respectively. And then, cells in each group were incubated at normoxic conditions for 48 h. Subsequently, H9c2 cells in latter 3 groups were incubated according to A/R protocol, H9c2 cells in NC group were incubated for 8 h under normoxic conditions.

Protein expressions of SLC8A3 and Caspase 3, concentration of Ca^{2+} as well as apotosis of H9c2 cells were determined at the end of