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Involvement of caspases and their upstream regulators in myocardial apoptosis in a rat model of selenium deficiency-induced dilated cardiomyopathy☆

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ABSTRACT

Keshan disease is an endemic dilated cardiomyopathy (DCM) which is closely related with selenium-deficient diet in China. In the previous study, we reported that the low selenium status plays a pivotal role in the myocardial apoptosis in the DCM rats, however, the underlying mechanism remains unclear. The present study aimed to determine whether the intrinsic, extrinsic pathways and the upstream regulators were involved in the myocardial apoptosis of selenium deficiency-induced DCM rats. Therefore, the rat model of endemic DCM was induced by a selenium-deficient diet for 12 weeks. Accompanied with significant dilation and impaired systolic function of left ventricle, an enhanced myocardial apoptosis was detected by TUNEL assay. Western blot analysis showed remarkably increased protein levels of cleaved caspase-3, caspase-8, caspase-9, and cytosolic cytochrome *c* released from the mitochondria. In addition, the immunoreactivities of p53 and Bax were significantly up-regulated, while the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-X_L were down-regulated. Furthermore, appropriate selenium supplement for another 4 weeks could partially reverse all the above changes. In conclusion, the intrinsic, extrinsic pathways and the upstream regulators such as p53, Bax, Bcl-2, and Bcl-X_L were all involved in selenium deficiency-induced myocardial apoptosis.

Introduction

Selenium is an indispensable trace element for human health [1]. During the nutrient metabolism in the body, selenium is incorporated into selenocysteine which is the 21st amino acid and the component of selenoproteins. As a constituent of selenoproteins, selenium mainly functioned as the structural and catalytic center in their wide biological effects such as the antioxidant, anticancer, anti-inflammatory effects and immune response regulation. Besides, selenium is also required in the production of active thyroid hormone and the regulation of sperm motility [2]. Some of the selenoproteins are antioxidant enzymes which all function in a selenium-dependent manner such as glutathione peroxidases, thioredoxin reductases and iodothyronine deiodinases [3,4]. All the three selenoproteins mentioned above are best known for their biological functions in oxidoreductions, redox signaling and antioxidant defense, and in turn the involvement of the pathogenesis of oxidative stress-related diseases [5,6].

Growing scientific evidence supports that dietary selenium deficiency is linked to some adverse consequences for disease susceptibility and the maintenance of optimal health [7]. Low selenium status may exacerbate the progression of some diseases such as HIV infection and cancer [2,8]. Moreover, severe selenium deficiency may also contribute to the etiology of some human diseases such as Keshan disease [9]. As an endemic dilated cardiomyopathy (DCM), Keshan disease only occurs in China and most patients mainly

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distribute to 2953 townships in 16 provinces from the northeast to the southwest of Chinese mainland where the oil and the grains are in severe selenium deficient status [10,11]. As a consequence, the level of hair, urine and blood selenium of the residents in the epidemic area is also relative lower than in the non-epidemic area [12]. Appropriate supplement of sodium selenite could not only reduce the morbidity of Keshan disease, but also improve the prognosis [13]. Altogether, the low content of selenium in the environment may be the pivotal etiological factor of Keshan disease.

Multifocal degeneration and loss of cardiomyocytes accompanied with replacement fibrosis, which was observed in the myocardium specimen of Keshan disease patients, was reported to lead to ventricle dilation, acute or chronic congestive heart failure [14]. However, Zhong et al. [15] showed that the myocardial apoptosis rate was significantly enhanced in Keshan disease patients in a comparative *in vivo* study. Therefore, the apoptotic cell death may play a role in triggering the loss of cardiomyocytes in the pathogenesis of Keshan disease. However the underlying molecular mechanism remains unclear.

In an earlier study, we showed that endoplasmic reticulum (ER) stress was induced and involved in selenium deficiency-induced cardiac dysfunction [16]. Further study revealed that 4-phenylbutyric acid (4-PBA), a chemical chaperone decreasing ER stress response signaling, could alleviate the myocardial apoptosis in selenium-deficient rats [17]. In addition to ER stress, accumulating evidence suggested that oxidative stress was involved in selenium deficiency-induced apoptosis [18]. The impaired catalytic capacity of glutathione peroxidases to degrade H_2O_2 may be the crucial factor which leads to the increased level of oxidative stress [19,20].

Despite compelling evidence showed that oxidative stress and ER stress were linked with the increased apoptotic cell death in the myocardium of Keshan disease patients only detected using TUNEL assay, it has not been determined whether caspases-dependent mechanism are involved. In this study, we aimed to illustrate the role of caspases and their upstream regulators in the myocardial apoptosis of selenium-deficient rats. Therefore, we raised selenium deficient rats and investigated the activity of caspase-dependent pathway. And then the impact of selenium supplement on caspases and their upstream regulators was analyzed.

Materials and methods

Animal model

All the animal experimental procedures and protocols described in the study were approved by the Institutional Animal Research and Ethics Committee of Xi'an Jiaotong University. All 36 3-week-old Sprague-Dawley rats were provided by the Animal Experimental Center of Xi'an Jiaotong University. After a week of adaptive feeding, the rats were equally and randomly divided into three groups. The rats of SD group (selenium deficiency, $n = 12$) were fed on selenium-deficient diets for 12 weeks, in which the content of selenium was below 0.02 mg/kg and the contents of all the other nutrients were at the standard level. The rats of the SS group (selenium supplement, $n = 12$) were fed on selenium-deficient diets for 12 weeks, followed by selenium-sufficient diets for 4 weeks which consisted of a mix of selenium-deficient diets and sodium selenite with a final concentration of 0.4 mg/kg. The rats of the control group were fed on standard diets for 12 weeks in which the content of selenium was approximate 0.1 mg/kg. The selenium-deficient and selenium-sufficient diets were produced by TROPHIC Animal Feed High-technology Co. (Nantong, Jiangsu, China) and the standard diets were offered by the Animal Experimental Center of Xi'an Jiaotong University.

Echocardiographic and hemodynamic studies

To evaluate the cardiac structure and function, echocardiographic and hemodynamic studies were performed by an investigator blind to the animal feed protocol, respectively. The echocardiographic study was firstly performed using an echocardiographic system which was equipped with a 12–4 MHz transducer (Philips iE33, Netherlands). And then, a PE-50 catheter was inserted into the right carotid artery and advanced into the left ventricle. Subsequently, a pressure-electricity transducer inline to PowerLab 4.12 system (AD instrument, Sydney, Australia), connected to the catheter, was run to record the cardiac pressure. A series of parameters were measured and calculated to identify whether the DCM model was successfully established, such as heart rate (HR), left ventricular end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), inter-ventricular septal thickness (IVST), posterior wall thickness (PWT), left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), ejection fraction (EF), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and first derivative of pressure (dp/dt).

Measurement of the whole blood selenium concentration

The blood samples were obtained immediately after the hemodynamic study and frozen at −80 °C for later analysis. The concentration of selenium was measured by a flameless atomic absorption spectrophotometry method, using a Z-5000 spectrophotometer (Hitachi, Japan) with a selenium cathode lamp (resonance line, 196.0 nm, Photron, Australia) following the routine procedures.

TUNEL staining

The terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to detect the myocardial apoptosis *in situ* following the manufacturer's instructions of *In Situ* Cell Death Detection Kit (Roche, Switzerland). Briefly, the fresh heart tissue was fixed in 40 g/L paraformaldehyde at 4 °C for 48 h. Five sections from each group were rinsed with PBS for three times and then were digested with Proteinase K working solution at 37 °C for 10 min. Subsequently, the sections were permeabilized in 0.25% Triton X-100 for 10 min and incubated with TUNEL reaction mixture for 90 min and DAPI (Boster, Wuhan, China) for 5 min at 37 °C in the absence of light. All the steps above were separated by sufficiently washing in PBS. Finally, the number of TUNEL-positive cells was counted in 5–10 high power fields for each slide ($n = 5$ for each group) using a fluorescence microscope (Leica, Germany).

Preparation of cytosolic and mitochondrial fractions

In order to analyze the expression of cytochrome *c* in cytosolic and mitochondrial fractions, cytosolic and mitochondrial proteins were isolated using mitochondria/cytosol fractionation kit, following the manufacturer's instruction. Briefly, 200 mg tissue samples were cut into pieces and lysed with 1 mL ice-cold cytosolic separation buffer containing 1 μL protease inhibitor, 5 μL phosphatase inhibitor and 1 μL dithiothreitol. The lysates were homogenized on ice and then centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was collected and centrifuged at 12,000 rpm for 30 min at 4 °C to obtain the cytosolic fraction (supernatant). The supernatant was collected and frozen at −80 °C for later analysis, whilst the deposition was incubated with 0.1 mL mitochondrial separation