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Allicin protects against myocardial apoptosis and fibrosis in streptozotocin-induced diabetic rats☆

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ABSTRACT

To evaluate the cardioprotective effect of allicin (AL) on myocardial injury of streptozotocin (STZ)-induced diabetic rats and to further explore its underlying mechanisms. Hyperglycemia was induced in rats by single intraperitoneal injection of STZ (40 mg/kg). Three days after STZ induction, the hyperglycemic rats (plasma glucose levels ≥16.7 mmol/l) were treated with AL by intraperitoneal injection at the doses of 4 mg/kg, 8 mg/kg, and 16 mg/kg daily for 28 days. The fasting blood glucose levels were measured on every 7th day during the 28 days of treatment. The body weight, blood glucose, and parameter of cardiac function were detected after 4 weeks to study the cardioprotective effects of AL on diabetic rats *in vivo*. The apoptotic index of cardiomyocytes was estimated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The expressions of Fas, Bcl-2, CTGF, and TGF-β_1 protein were studied by immunohistochemistry. Laser scanning confocal microscopy technique was utilized to observe the effects of AL on intracellular calcium concentration ($[Ca^{2+}]_i$) in rat ventricular cardiomyocytes. AL at the doses of 4 mg/kg, 8 mg/kg, and 16 mg/kg significantly reduced blood glucose levels in a dose-dependent manner and increased body weight as well compared with the model group. Hemodynamic parameters including left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and maximum rate of left ventricular pressure rise and fall (+dp/dtmax and −dp/dtmax) were significantly restored back to normal levels in AL-treated (8 mg/kg and 16 mg/kg) rats compared with diabetic model rats. AL markedly inhibited cardiomyocyte apoptosis induced by diabetic cardiac injury. Further investigation revealed that this inhibitory effect on cell apoptosis was mediated by increasing anti-apoptotic protein Bcl-2 and decreasing pro-apoptotic protein Fas. Additional experiments demonstrated AL abrogated myocardial fibrosis by blocking the expressions of CTGF and TGF-β_1 protein. AL shows protective action on myocardial injury in diabetic rats. The possible mechanisms were involved in reducing blood glucose, correcting hemodynamic impairment, reducing Fas expression, activating Bcl-2 expression, decreasing intracellular calcium overload, inhibiting the expressions of TGF-β_1 and CTGF, and further improving cardiac function.

Introduction

Diabetes has appeared as a main threat to people's health all over the world. The number of adults affected with diabetes is estimated to increase from 135 million in 1995–300 million by 2025 globally (King et al. 1998). The fundamental harm of diabetes is that it can cause various complications, of which cardiovascular complications are the most common and leading cause of diabetes-related morbidity and mortality (Potenza et al. 2011), which accounts for 80% of the mortality in the diabetic population (Amos et al. 1997). The morbidity of cardiac disease in patients with diabetes is 2–4 folds higher than that in subjects without diabetes, among those, myocardial infarction incidence is about 10 folds higher.

Abbreviations: AL, allicin; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; CTGF, connective tissue growth factor; TGF-β_1, transforming growth factor-beta1; $[Ca^{2+}]_i$, intracellular calcium concentration; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dp/dtmax, maximum rate of left ventricular pressure rise; −dp/dtmax, maximum rate of left ventricular pressure fall; MET, metformin; HR, heart rate; PBS, phosphate buffered saline; ROS, reactive oxygen species.

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Epidemiological studies found that the risk for chronic heart failure and sudden cardiac death increased significantly (Yeung et al. 2007). A large number of animal experiments showed that hypertrophy, fibrosis, apoptosis, and inflammation existed in the early stages of experimental diabetic myocardial injury (Fiordaliso et al. 2007; Poornima et al. 2006). The important events in diabetic cardiomyopathy are fibrosis and apoptosis which have been described in human (van Heerebeek et al. 2008). These pathological changes in diabetes ultimately result in the development of heart failure (Mizushige et al. 2000). Therefore, blocking fibrosis and apoptosis can inhibit the progression of heart failure efficiently. Although the features of diabetic heart disease have been identified, the effective treatment strategies have not been elucidated.

Large amount of resources were spent in all countries in the treatment of diabetes . Many kinds of anti-diabetic medicines have been developed for patients; most of them are chemical agents. Up to now, complementary and alternative medicines used by consumers are escalating rapidly. It is estimated that out-of-pocket expenditures for herbal therapies are at more than 5 billion dollars per year in the United States alone (Eisenberg et al. 1998). This tendency is now spreading rapidly worldwide. Allicin (AL) has been known as one of popular herbal medicine today. Previous researches revealed many beneficial and health-related biological properties of garlic, including antioxidant, antibacterial and antiparasite activities, reducing serum lipid levels as well as inhibition of platelet aggregation (Chung 2006; Hasan et al. 2006; Ginter and Simko 2010). The main property of AL for therapeutic effects is antioxidant activity against oxidative damage in cardiovascular diseases (Banerjee et al. 2003). In addition, it has also been confirmed that AL can protect ischemia-reperfusion myocardial injury (Shi et al. 2005). AL is also the most commonly used alternative medicine for diabetic patients (Ryan et al. 2001). However, there is very limited information about the effect of AL on diabetic heart functions and related mechanisms. This study aimed to investigate the effect of AL on diabetic cardiac function, myocardial fibrosis, and apoptosis, further to clarify the potential mechanisms.

Materials and methods

Induction of experimental diabetes

Diabetes mellitus was induced in male Wistar rats (200–250 g, provided by the Experimental Animal Center of Harbin Medical University, Grade II) with a single intraperitoneal injection (i.p.) of streptozotocin (STZ, Sigma Chemical Co., USA), freshly prepared solution in 0.1 mol/l citrate buffer (pH 4.5) and 40 mg/kg after overnight fast. Tail vein blood glucose was measured 3 days later and those with plasma glucose levels $\geq$16.7 mmol/l (2 times continuously) were considered to be diabetic. The control group only received an injection of same amount of citrate buffer. From the model establishment, AL (Xuzhou Ryen Pharma. Co., Ltd., China, 15 mg/ml) 4 mg/kg, 8 mg/kg, and 16 mg/kg were given by i.p. in AL groups, and metformin (MET, Sigma Chemical Co., USA) was given by intragastric administration at the dose of 100 mg/kg daily for 28 consecutive days.

Hemodynamic measurements

After 28 days, under the anesthesia with pentobarbital sodium (40 mg/kg), a fluid-filled cannula was inserted into the right carotid artery and then advanced into the left ventricle. Heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and maximum rate of left ventricular pressure rise and fall (+dp/dtmax and −dp/dtmax), all of which reflect cardiac performance and they were measured by a pressure transducer interfaced with BL-420E organism function experiment system.

Detection of apoptosis

The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay was used to assess cardiomyocyte apoptosis, according to the instructions in the reagent kits (Roche Molecular Biochemicals). These sections were visualized by an Olympus visible light microscope at 400× magnification. TUNEL-positive cardiomyocytes were carefully evaluated under double-blind conditions. In each visual field, the number of positive cell nuclei was calculated among 200 myocardial cell nuclei. The rate of apoptotic cell nuclei is defined as apoptotic positive cell nuclei/total cell nuclei in the field × 100.

Immunohistochemistry

Rat hearts of each group were immediately excised. All of the hearts were cut into 4-mm sections, and then fixed in paraformaldehyde (4%, w/v) for 30 min. After being washed in phosphate buffered saline (PBS), the sections were incubated with the primary antibody (either anti-Bcl-2 or anti-Fas antibodies, Boshi Microbiological Engineering Ltd., Wuhan, China; either anti-CTGF or anti-TGF-β_1 antibodies, Santa Cruz, CA, USA) at the dilution of 1:500 for an overnight incubation at 4 °C. The sections were then incubated with secondary antibodies (1:1000) conjugated with FITC for 1 h. Following a washing step with PBS, the slides were examined with a fluorescent microscope.

Cell isolation

Single Wistar rat ventricular cardiomyocytes were isolated using the methods as described previously in detail. Briefly, adult Wistar rats were anaesthetized by injecting sodium pentobarbitone. The hearts were quickly removed and washed with cool and oxygenated Tyrode solution. The hearts were then cannulated on a Langendorff perfusion apparatus and retrogradely perfused via the aorta with normal Tyrode solution. Finally, the heart was perfused with normally Ca^{2+}-free Tyrode solution, followed by perfusion with the same solution containing collagenase II (7.8 mg/50 ml) and bovine serum albumin (7.8 mg/50 ml). The ventricular tissue was minced after it was softened and single cell was obtained by gentle aspiration using glass pipette. Only Ca^{2+}-tolerant, quiescent, and rod-shaped cardiomyocytes were selected for intracellular calcium measurement.

Ca^{2+} fluorescence measurements

Fluorescence measurements in cardiomyocytes have been described previously. Briefly, single cardiomyocytes were attached to the coverslips of chamber and incubated with a working solution containing 10 μmol/l Fluo-3/AM (acetoxymethyl ester form, Molecular Probes) and 0.03% Pluronic F-127 at 37 °C for 45 min. Then the fluorescent intensity was detected by an inverted Olympus confocal imaging system (Fluoview-FV300, Japan) equipped with a 40× objective, 488 nm argon ion laser for excitation and 530 nm for emission at room temperature. Increases in $[Ca^{2+}]_i$ were expressed as the ratio of fluorescence intensity of Fluo-3/AM over baseline (FI/FI_0).

Statistic analysis

All data were expressed as mean ± SD and were analyzed using SPSS 13.0 software. Statistical comparisons among multiple groups were performed by analysis of variance (ANOVA). If significant effects were indicated by ANOVA, SNK-q (Student–Newman–Keuls) test was used to evaluate the