International Journal of Cardiology 168 (2013) 270–280

Contents lists available at ScienceDirect

International Journal of Cardiology

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

Diallyl trisufide (DATS) suppresses high glucose-induced cardiomyocyte apoptosis by inhibiting JNK/NFκB signaling via attenuating ROS generation ☆

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ARTICLE INFO

Article history:
Received 18 November 2011
Received in revised form 2 June 2012
Accepted 15 September 2012
Available online 13 November 2012

Keywords:
Hyperglycemia
Cardiomyocytes
Nuclear factor-κB (NF-κB)
Reactive oxygen species (ROS)
Diallyl trisulfide (DATS)
Apoptosis

ABSTRACT

Background: Hyperglycemia is an important risk factor for cardiovascular diseases no matter if it resulted from type I or type II diabetes mellitus. High glucose-induced generation of reactive oxygen species (ROS) can lead to diabetic cardiomyopathy. In our previous study, we showed that NADPH oxidase-related ROS-induced apoptosis is mediated via the JNK-dependent activation of NF-κB in cardiomyocytes exposed to high glucose (HG).
Objective: In this study, we investigated the mechanisms governing the anti-apoptotic effect of diallyl trisulfide (DATS) on HG-exposed cardiac cells both in vitro and in vivo.
Methods: H9c2 cells were incubated with media containing 5.5 or 33 mM of glucose for 36 h in the presence or absence of DATS.
Results: We found that DATS treatment led to a dose-dependent decrease in ROS levels as well as protein levels of p22phox, gp91phox, phosphorylated JNK, and phosphorylated c-Jun. In addition, DATS inhibited the HG-induced activation of caspase 3 as well as the nuclear translocation of NF-κB. Similar results were observed in HG-exposed neonatal primary cardiomyocytes and streptozotocin-treated diabetic rats. Echocardiographic data showed that DATS administration led to a marked increase in fractional shortening and cardiac output.
Conclusion: DATS appears to suppress high glucose-induced cardiomyocyte apoptosis by inhibiting NADPH oxidase-related ROS and its downstream JNK/NF-κB signaling, and may possess the potential on the therapy of diabetic cardiomyopathy.

1. Introduction

Hyperglycemia, a major feature of type I and type II diabetes mellitus, can cause oxidative stress, which is associated with a variety of diabetic organ damage, including cardiovascular diseases. Cardiac cells are susceptible to oxidative stress, which can induce cardiomyocyte apoptosis and decrease cardiac function under high glucose conditions, leading to diabetic cardiomyopathy [1].

Studies have demonstrated that NADPH oxidase-related reactive oxygen species (ROS) are involved in the detrimental effect of high glucose-induced cell damage [2]. In endothelial cells and cardiomyocytes, NADPH oxidase isoforms generate reactive oxygen species as modulators of redox-sensitive signaling pathways [3,4]. Over activation of NADPH oxidase, however, can result in loss of the redox balance, thereby leading to abnormal ROS production. Structurally, NADPH oxidase comprises multiple subunits. Cytochrome b_{558}, the membrane-bound component, consists of the subunits p22phox and gp91phox, which are responsible for enzyme activity. The enzyme complex also includes at least four cytosolic subunits, p47phox, p67phox, p40phox, and the small guanosine triphosphatase Rac1, which translocate to the cell membrane and associate with the membrane-bound components forming an active complex [5]. NADPH oxidase inhibitors, such as apocynin, inhibit the formation of the oxidase complex and its subsequent activation by preventing the association of p47phox with the membrane-bound components [6].

Garlic oil has been shown be a potent antioxidant and to have cardioprotective effects [7]. Garlic oil comprises three organosulfur

Abbreviations: DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, diallyl trisulfide; DCF, dichlorofluorescein; DCFH-DA, 2′,7′-Dichlorofluorescein diacetate; DM, diabetes mellitus; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HDAC-1, histone deacetylase-1; HG, high glucose; IκB, inhibitor κ B; IκK, inhibitor IκB kinase; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein-kinases; MI-R, myocardial ischemia–reperfusion; MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide; NAC, N-acetyl cysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-κB; NG, normal glucose; ROS, reactive oxygen species; SAPKs, stress-activated protein kinases; STZ, streptozotocin; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end labeling.

☆ This work was supported by the National Science Council, Taiwan (NSC99-2320-B-039-033-MY3) and China Medical University (DOH101-TD-B-111-004 and CMU98-S-07).

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0167-5273/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ijcard.2012.09.080

compounds, namely diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). Liu et al. [8] reported that the antioxidant potential of these organosulfur compounds is related to the number of sulfur atoms and is in the order DATS > DADS > DAS. In addition to the hypolipidemic effects by decreasing fatty acid synthesis and lipid accumulation [9], DATS has been shown to attenuate oxidative stress-induced liver injury in mice [12] and suppress oxidized LDL-induced adhesion molecule expression in vascular endothelial cells [10]. Studies have also provided evidence that DATS can inhibit LPS-induced activation of NF-κB in the lung [11] and in macrophages [8]. Stent coated with DATS improved the damage of endothelial function in the coronary by increasing the eNOS expression to raise the NO level [12]. Furthermore, using myocardial ischemia–reperfusion injury (MI-R) animal model, DATS treatment could reduce infarct size, increased nitric oxide bioavailability, reversed myocardial contractile function [13]. In our previous study we demonstrated that garlic oil dose-dependently ameliorated hyperglycemia-induced cardiac dysfunction and apoptosis [14]. However, the mechanisms underlying the protective effects of DATS against hyperglycemia-induced cardiac damage are not known.

Excessive production of ROS induced by high glucose has been documented to be an important mechanism in the pathogenesis of diabetic cardiomyopathy [15]. In a previous study using apocynin, and JNK and NF-κB small interfering RNAs, we found that ROS-induced apoptosis was mediated via the JNK-dependent activation of NF-κB in cardiomyocytes exposed to high glucose [16]. In the present study, we investigated the mechanisms governing the anti-apoptotic effect of DATS in cardiac cells exposed to high glucose in vitro and in vivo.

2. Materials and methods

2.1. Cell culture and treatments

H9c2 cell lines purchased from American Type Culture Collection (ATCC), with passages between 8 and 10 were used in this study. Cells were cultured in Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM pyruvate in humidified air (5% CO_2) at 37 °C. During the experiment period, H9c2 cells were cultured in minimal essential medium with glucose at normal concentrations (5.5 mM) for 12 h, then followed by the administration of high glucose (33 mM) for 36 h, and treated with DATS, and then the cells were extracted for analysis. DATS (100 mg) was purchased from LKT Laboratories Company (Minnesota, USA), and its purity was examined to be a minimum of 98% by high-performance liquid chromatography. The specificity of the inhibitions of ROS and NADPH oxidase was by adding N-acetyl cysteine (NAC, 500 μM) and by adding apocynin (100 μM), respectively.

2.2. Cardiomyocyte culture

The preparation of primary cardiomyocytes was performed as described previously [16]. Briefly, ventricles from one- to two-day-old Wistar rats were pooled, and the ventricular cells were dispersed by a digestion solution at 37 °C. Ventricular cardiomyocytes were isolated and cultured in DMEM containing 10% fetal bovine serum, 100 g/ml penicillin, 100 g/ml streptomycin, and 2 mM glutamine. After 3–4 days, cells were incubated in a serum-free essential medium overnight before treatment with the indicated procedures.

2.3. Animal model and treatments

Male Wistar rats (four-week old) were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). The animals were maintained under a 12-h light–dark cycle and ambient temperature was kept at 25 °C. Animals were given free access to water and standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA). Room conditions and experimental procedures were followed according to the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan. After one week of acclimatization, diabetes was induced by the injection of streptozotocin (STZ, 65 mg/kg body weight in a citrate buffer, pH 4.5) into a lateral tail vein. After three days of injection, glycemia was measured with the Accu-Check Compact kit (Roche Diagnostics Gmbh, Mannheim, Germany). Only animals whose hyperglycemia had been successfully induced were randomly separated into two groups and fed 40 mg/kg body wt DATS or vehicle (corn oil, 2 ml/kg body wt) every other day for 16 days. The dose of DATS used in the current study was in accordance with the previous work that rats received garlic oil (100 mg/kg every other day for 16 days) showing a significantly improved cardiac function of the diabetic rats, and DATS was analyzed as 40% of the garlic oil constituents [14]. The other normoglycemic control animals were fed corn oil (2 ml/kg body wt). Sixteen days after treatment, all animals were anesthetized and the echocardiography was performed. Then, they were sacrificed and hearts were removed for further analysis.

2.4. In vivo cardiac function

Transthoracic echocardiograms were performed as described previously [14]. Briefly, rats were anesthetized with isoflurane mixed with O_2 at a flow rate of 5 psi 16 days after the DATS feeding by an echo machine (Vivid *i*, 10S transducer, GE Medical Systems, Milwaukee, Wisconsin, USA) at heart rates of 300–450 beats per minute, using a 4–11 MHz phase-array transducer. M-mode images were obtained in the parasternal long- and short-axis views of the left ventricle.

2.5. Lowry protein assay

Diluted bovine serum albumin (BSA) at 0, 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml as standards of the Lowry method for determining protein concentration uses Folin–Ciocalteu reagent for enhanced color development. Protein is first reacted with alkaline cupric sulfate in the presence of tartrate (2% Na–K tartrate: 1% $CuSO_4 \cdot 5H_2O$: 2% Na_2CO_3 in 0.1 N NaOH = 1:1:98) during a 10 minute incubation at room temperature. During this incubation, a tetradentate copper complex forms from four peptide bonds and one atom of copper. Following the incubation, a Folin phenol reagent is added. It is believed that the color enhancement occurs when the tetradentate copper complex transfers electrons to the phosphomolybdic/phosphotungstic acid complex. Finally OD value is read at 750 nm after 30 minute room temperature incubation.

2.6. Western blot analysis

Sample protein was separated in 12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific protein binding was prohibited by a blocking buffer (5% milk, 20 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20) and blotted with specific antibodies against α-tubulin, JNK, c-Jun, caspase 3, NFκB, HDAC1, p22phox, p91phox (Santa Cruz Biotechnology), phospho-IκK (Ser176) phospho-JNK (Thr183), phospho-c-Jun (Ser63) (Santa Cruz Biotechnology) and phospho-IκBα (Ser32) (cell signaling) in the blocking buffer at 4 °C overnight. After incubations with a secondary antibody for 2 h, the densitometry of immunoblots was analyzed by Fuji LAS 3000 imaging system. α-Tubulin was used as a loading control. Bolt quantification was normalized by the housekeeping protein blot intensity (alpha-tubulin or HDAC-1). For repeated blotting, nitrocellulose membranes were stripped with a Restore Western blot stripping buffer at room temperature for 30 min.

2.7. Reactive oxygen species production

Intracellular generation of ROS was examined by flow cytometry using peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes). DCFH-DA is formed by intracellular esterases to DCFH, which can be oxidized into the fluorescent dichlorofluorescein (DCF) by a proper oxidant, and then quantified by flow cytometry. Additionally, production of reactive free radicals of superoxide ($O_2^{\bullet -}$) was evaluated by chemical probes of dihydroethidium (DHE, Molecular Probes). DHE, a nonfluorescent membrane-permeable probe, reacts with $O_2^{\bullet -}$, to cause the release of membrane-impermeable ethidium cations. Results for the estimate of $O_2^{\bullet -}$ were repeated for 3 times for each treatment. The pictures of culture slides were taken immediately after mounted with coverslips by UV light microscopic observations. For every well, 8 areas were randomly chosen to be photographed and the integrated optical densities (IODs) of the images were counted.

2.8. DAPI staining and TUNEL assay

DAPI staining and TUNEL assay were performed as described previously [16]. Cells cultured on a 6 mm plate were fixed with a 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with PBS, cells were treated with permeation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4 °C. After washing with PBS, samples were first incubated with a terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) reagent containing terminal deoxynucleotidyl transferase and fluorescent isothiocyanate-dUTP. The cells were also stained with 1 μg/ml DAPI for 30 min to evaluate the cell nucleus by UV light microscopic observations (blue). Samples were analyzed in a drop of PBS under a fluorescence and UV light microscope, using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green). All morphometric measurements were observed by at least three independent individuals in a blinded manner.

2.9. Nuclear extraction

Separations of cytoplasmic and nuclear fractions were performed with the extraction reagent containing a membrane lysis buffer (10 mM Hepes (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol (DTT), proteinase inhibitor) and a nuclear lysis buffer (20 mM Hepes (pH 8.0), 1.5 mM MgCl2, 10 mM NaCl, 1 mM DTT, 0.2 mM EDTA, 0.25 M glycerol, proteinase inhibitor). Briefly, after the treatments, cells were resuspended in PBS