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Chlorogenic acid attenuates glucotoxicity in H9c2 cells via inhibition of glycation and PKC α upregulation and safeguarding innate antioxidant status

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ABSTRACT

A series of cardiovascular complications associated with hyperglycemia is a critical threat to the diabetic population. Here we elucidate the link between hyperglycemia and cardiovascular diseases onset, focusing on oxidative stress and associated cardiac dysfunctions. The contribution of advanced glycation end products (AGE) and protein kinase C (PKC) signaling is extensively studied. For induction of hyperglycemia, H9c2 cells were incubated with 33 mM glucose for 48 h to simulate the diabetic condition in *in vitro* system. Development of cardiac dysfunction was confirmed with the significant increase of lactate dehydrogenase (LDH) release to the medium and associated decrease in cell viability. Various parameters like free radical generation, alteration in innate antioxidant system, lipid peroxidation, AGE production and PKC α -ERK axis were investigated during hyperglycemia and with chlorogenic acid. Hyperglycemia has significantly enhanced reactive oxygen species (ROS- 4 fold) generation, depleted SOD activity (1.3 fold) and expression of enzymes particularly CuZnSOD (SOD1) and MnSOD (SOD2), increased production of AGE (2.18 fold). Besides, PKC α dependent ERK signaling pathway was found activated (1.43 fold) leading to cardiac dysfunction during hyperglycemia. Chlorogenic acid (CA) was found beneficial against hyperglycemia most probably through its antioxidant mediated activity. The outcome of this preliminary study reveals the importance of integrated approach emphasizing redox status, glycation and signaling pathways like PKC α - ERK axis for control and management of diabetic cardiomyopathy (DCM) and potential of bioactives like CA.

1. Introduction

The prevalence of diabetes is steadily increasing worldwide [1]. Diabetes can lead to complications of multiorgan dysfunction and increase the chance of dying prematurely. But more than 60% of deaths in diabetic patients are due to CVD and associated problems [2]. Diabetic cardiomyopathy (DCM) constitutes structural and functional abnormalities of the myocardium without coronary artery disease or hypertension [3]. Relationships between glucose levels and CVD are remarkably inconsistent [4]. The complexity of the problem is so great that we have not yet unraveled the knot. However, there is much evidence of the occurrence of oxidative stress during hyperglycemia [5,6]. Oxidative stress results when the rate of oxidant production exceeds the rate of oxidant scavenging. Increased glucose flux enhances both oxidant production and impairs antioxidant defenses via multiple interacting pathways [7]. Some of these changes are in the activity of protein kinase C (PKC) [8], advanced glycation end products (AGEs) production [9], and enhanced sorbitol pathway [10,11]. PKC has received special attention in the pathogenesis of cardiomyopathy due to its important role in the intracellular signaling pathway for regulating cardiac myocyte development, inotropic function and cellular growth [12]. Previous studies have also indicated that PKC α, the major isozyme is a necessary mediator of cardiomyocyte hypertrophic growth through an ERK1/2-dependent signaling pathway [13]. Some studies have made a putative hypothesis regarding the connection between ROS and ERK1/2 activation [14]. There is no report on the functional hazards of cross talk between AGE, PKC, ERK and weakened antioxidant defense system during hyperglycemia. This is very much

Abbreviations: DCM, diabetic cardiomyopathy; CVD, cardiovascular diseases; LDH, lactate dehydrogenase; ROS, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, glutathione; AGE, advanced glycated end product; PKC, protein kinase C; ANP, atrial natriuretic peptide; CA, chlorogenic acid; FBS, fetal bovine serum; DCFH-DA, 2, 7 dichloro dihydro fluorescein diacetate; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; HRP, horseradish peroxidase; BCA, bicinchoninic acid; RIPA, radioimmunoprecipitation assay; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HG, High glucose

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required for target identification for therapeutic intervention. Based on this, efforts are made in this study to understand precisely the contribution of these pathways in the genesis of diabetic cardiomyopathy in *in vitro* model.

Extensive research has led to the discovery of various drugs for control and management of diabetes. Unfortunately, this is still an incurable disease, and the prevalence is increasing day by day. So the risk for development of other complication is also increased. This creates an urgent need for exclusive therapeutics for CVD as classical anti-diabetic drugs are not much effective to control and manage the heart issues [15]. Recently much heed has been given to bioactive from medicinal plant in search of therapeutics against hyperglycemic cardiomyopathy. There are ample examples of the natural product derived cardiovascular therapeutics such as reserpine, diltiazem, statins, etc. [16]. Affordability, minimum adverse effects and tolerability to prolonged use necessitates for better therapeutics to the general public. In this scenario chlorogenic acid (CA), an ester of caffeic acid and (−)-quinic acid, abundant in our daily beverage coffee [17] and common fruits [18–20] with plenty of medicinal properties is an ideal choice. It also shows blood pressure lowering property [21]. In addition antibacterial, antioxidant, and anticarcinogenic activities [22,23] also its beneficial role in glucose and lipid metabolism [24] are mentioned in literature. There is report of cardioprotective properties of the extract containing CA from our group [25] also. So we are curious to check beneficial properties of CA against hyperglycemia induced alterations in H9c2 cells.

So experiments are planned to investigate the alterations in H9c2 cells during hyperglycemia emphasizing the cross talk between oxidative stress, glycation, and PKC α - ERK axis for identification of probable biochemical targets for future drug development and protective effect of CA.

2. Materials and methods

D-glucose and metformin were purchased from SRL (India). Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin antibiotics and fetal bovine serum (FBS) were from Gibco (USA).3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RIPA buffer and 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) were from Sigma Aldrich (St Louis, MO, USA).CA (99% purity) was from Natural Remedies Pvt. Ltd. (Bangalore, India).All antibodies were from Santa Cruz (USA). All other chemicals used were of analytical grade.

2.1. Cell culture

H9c2 embryonic rat heart-derived cell line from American Type Culture Collection (ATCC), USA, were grown in low glucose DMEM supplemented with 10% FBS and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) under a humidified atmosphere with 5% CO_2 at 37 °C. In order to rule out the effect of changes in osmolarity on vital parameters of cell function, mannitol containing group was included in the study. Parameters like cell viability, ROS generation and PKCα - ERK pathway were studied with mannitol group.

2.2. Induction of hyperglycemia and treatment with CA

After 50% confluence, H9c2 cells were incubated with 33 mM glucose for 48 h. All the parameters were studied after 48 h of incubation of cells with glucose (33 mM) in presence or absence of various concentrations of CA (10 μM or 30 μM) or metformin (1 mM).

Experimental group consists of control (5.5 mM glucose, C), high glucose (33 mM glucose, HG), high glucose + metformin 1 mM (Met), high glucose + CA 10 μM (CA1), high glucose + CA 30 μM (CA2).

2.3. Evaluation of cell viability

Cell viability was determined by MTT assay. Briefly, 100 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Thus the formazan crystals formed were dissolved in DMSO. Then the plates were read after 20 min in a microplate reader (Biotek Synergy 4, USA) at 570 nm and percentage of cell viability were calculated [26].

2.4. Determination of LDH leakage

LDH release was measured using LDH cytotoxicity assay kit (Clontech, USA). Briefly, 100 μL of medium was collected from cultured cells and was added with 100 μL of LDH reaction solution containing NAD+, lactic acid, iodonitrotetrazolium (INT) and diaphorase. The mixture was then incubated with gentle shaking for 30 min at room temperature, and the absorbance was read at 490 nm.

2.5. Detection of intracellular ROS

Intracellular ROS levels were determined using DCFH-DA as probe [27]. DCFH-DA is cleaved intracellularly by nonspecific esterase and turn to high fluorescence upon oxidation by ROS. After respective treatments, cells were washed with phosphate buffer saline (PBS, pH 7.4) and then incubated with DCFH-DA (20 μM) for 20 min at 37 °C in a humidified atmosphere of 5% CO_2. After incubation, cells were washed with phosphate buffer (pH 7.4). Fluorescence imaging was done (Ex. 488 nm; Em. 525 nm) to visualize the ROS generation with a spinning disk fluorescent microscope.

2.6. Estimation of TBARS

Lipid peroxidation was estimated for all experimental groups with TBARS estimation kit (Himedia, India).After respective treatments, the cells were collected along with culture medium and sonicated for 5 s. 100 μL of sample and standard were added to labeled tubes. To that 100 μL of sodium dodecyl sulfate (SDS) and 4 mL of coloring reagent were added. Then tubes were boiled for 1 h and were placed in an ice bath for 10 min to stop the reaction. After incubation, it was centrifuged for 10 min at 1600 × *g* at 4 °C and incubated at room temperature for 30 min. From this 150 μL of samples were transferred to plate and absorbance was read at 530 nm in a plate reader.

2.7. Estimation of protein carbonyl content

Protein carbonyl content was determined using assay kit (Cayman, USA). Briefly, after respective treatments, cells were collected and homogenized on ice in 1–2 mL of cold buffer (50 mM phosphate buffer, pH 6.7 containing 1 mM EDTA). After centrifugation at 10,000 × *g* for 15 min at 4 °C, the supernatants were collected. 200 μL of the sample (supernatant) was transferred to 2 mL plastic tubes. 800 μL of DNPH was added to the sample tubes and 800 μL of 2.5 M HCl to the control tube. All the tubes were kept in the dark for 1 h. 1 mL of 20% TCA was added to each tube and vortexed. The samples were centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatant was removed. The pellet was resuspended in 1 mL of 60% TCA. Then it was incubated on ice for 5 min and then centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 1 mL of (1:1) ethyl acetate/ethanol mixture. It was then vortexed well and centrifuged at 10,000 × *g* for 1 min at 4 °C. The protein pellet was resuspended in 500 μL of guanidine hydrochloride and vortexed. Again it was centrifuged at 10,000 × *g* for 10 min at 4 °C to remove any leftover debris. 220 μL of supernatant was taken, and the absorbance was read at 370 nm using a multimode plate reader.