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Original Article

High glucose condition down-regulates the inhibitory G-protein subunit, Gαi, in pheochromocytoma PC12 cells

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Abstract

Introduction: G-proteins have an important role in the cell signaling of numerous receptors. The situation of G-proteins in health and disease and their critical role in the development of diabetic side effects is an interested scientific field. Here, the changes in the expression of G-protein subunits (Gαi, Gαs and Gβ) were evaluated in hyperglycemic situation of PC12 cells as a cellular model for the induction of diabetic side effect.

Methods: Rat pheochromocytoma PC12 cells were grown in normal or high-glucose (4X normal glucose) medium. Cell viability was determined by MTT assay and the generation of intracellular reactive oxygen species (ROS) studied using fluorescence spectrophotometry. RT-PCR and immunobloting were performed to evaluate the expression of specific G-protein subunits in the levels of mRNA and protein, respectively.

Results: In high glucose condition (100 mM glucose for 48h), the cell viability was significantly decreased and intracellular ROS increased. In addition, Gαi expression level was significantly decreased in hyperglycemic PC12 cells. However, the levels of Gαs and Gβ mRNAs and their proteins were not altered in high glucose-treated cells.

Conclusion: The results demonstrate that deregulation or disruption in the signaling of Gai coupled receptors can be occurred in hyperglycemic condition.

Keywords:
G-proteins;
Gene expression;
Hyperglycemia;
PC12 cells

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Introduction

Diabetes mellitus (DM) is a major human health problem which approximately 5% of population worldwide has this disease and the number may dramatically increase by the year 2035 due to population growth, ageing, unhealthy diet, overweight/obesity and sedentary lifestyle (Tsasis et al., 2016). Neuropathy is one of the chronic complications of DM and painful diabetic neuropathy is characterized by mechanical and thermal hyperalgesia (Todorovic, 2016). However, the pathophysiology of diabetes-induced side effects such as neuropathy has not been fully clarified and current therapeutic approaches are incapable to completely relief the neuropathy diabetic subjects.

Elevated blood glucose (hyperglycemia) has a critical role in the induction of diabetic side effects (Gibbons and Freeman, 2015). Apoptosis and neural damage

could be considered proposed as a possible mechanism for high glucose-induced neural dysfunction in both *in vitro* and *in vivo* studies (Kaeidi et al., 2013). In addition, diabetes-induced complication is associated with a continuous generation of reactive oxygen species (ROS) via glucose auto-oxidation and a failure in antioxidant defense (Rochette et al., 2014).

Heterotrimeric G proteins (Gα, Gβγ subunits) are one of the most important signaling cascades which recruited by G protein-coupled receptors (GPCRs) superfamily. The activated GPCR catalyzes exchange of GTP for GDP on the Gα subunit, as a result conformational changes takes place in the GPCR, which leads to dissociation of Gβγ dimer from Gα and thus activates multiple molecules of G proteins. Activated Gα and Gβγ proteins in turn bind to various effectors and promote cellular effects. There are over than one thousand GPCRs known in humans and animal till to date (Duc et al., 2015).

It has been reported that G protein signaling and functions are impaired in diabetes and under hyperglycemic conditions (Hashim et al., 2006). In addition, diabetic-induced alteration in G proteins and adenylyl cyclase activity has been demonstrated in several tissues (Gawler et al., 1987; Rodgers et al., 2001). Alteration in G proteins expression has been recognized in the spinal cord of streptozotocin-induced diabetic rats (Hajializadeh et al., 2010).

It is well known that changes in the resting levels of G-protein subunits could influence intracellular signaling pathways by altering the subtype of G-protein associated with a specific receptor and consequently altering the type of effectors activated upon release of G-proteins (Morris and Malbon, 1999). For example, it has been reported that dysregulation of calcium channels by G proteins is an important mechanism contributing to enhanced calcium influx in diabetes and related complications (Hall et al., 2001).

With respect to the critical role of G-protein subunits in the induction of receptor signaling at the cellular level in physiological condition and their contribution in the pathobiology of diabetes mellitus, the present study was designed to determine the changes in the expression levels of G-proteins in high glucose-treated PC12 cells as an approved *in vitro* research model of diabetes and its related side effects.

Materials and methods

Chemical and reagents

Cell culture reagents, penicillin–streptomycin solution, trypsin EDTA, fetal bovine serum (FBS) and heat-inactivated horse serum (HS) were obtained from Biosera Co. (East Sussex, UK). Culture plates and dishes were acquired from SPL Lifesciences Inc. (Gyeonggi-Do, South Korea). 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT), 2,7-dichlorofluorescein diacetate (DCFH-DA), d-(+)-glucose powder and primary monoclonal anti-β-actin antibody were purchased from Sigma (St. Louis, MI, USA). Primary polyclonal primary antibodies [Gαs (K-20): sc-823, Gαi (C-10): sc-262 and Gβ (T-20): sc-378] were obtained from Santa Cruz Biotechnology, Inc. (Delaware Ave., Santa Cruz, USA).

Cell culture

Rat pheochromocytoma PC12 cells were obtained from National Cell Bank of Iran (NCBI)-Pasteur Institute of Iran (Tehran, Iran). Cells were grown with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 5% HS and penicillin-streptomycin (100 U/ml and 100 µg/ml). They were maintained at 37 °C in a 5% CO2 incubator. The cells were plated at the density of 5000 per well in a 96-well plates for the MTT and intracellular ROS assay. Control cells were grown in DMEM with 25 mM glucose and high glucose-treated group grown in DMEM with 100 mM glucose. For mRNA and protein extraction, PC12 cells were grown in 6-well plates and permitted to attach and grow for 24 h. Then the cells were incubated with control or high glucose medium for 48 h.

Cell viability analysis

PC12 cells viability was estimated by the reduction of 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) to formazan (Denizot and Lang, 1986). MTT was dissolved in phosphate buffered saline (PBS) and added to the wells (0.5 mg/ml). After 2h the media were completely removed and 100 µl dimethyl sulfoxide (DMSO) was added to each well, and the absorbance were assessed by spectrophotometry at 490 nm with an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). Each experiment was

Table 1: the sequence of primers

Name	sequence	product length (bp)
Gαi/o forward:	5'-TAC AGC AAC ACC ATC CAG TC-3'	350
Gαi/o reverse:	5'-AAG TGG GTT TCT ACG ATG CC-3'	
Gas forward:	5'-AGT TCC AGG ACA AAG TC-3'	518
Gas reverse:	5'-GGA TGA TGT CAC GGC CAG TC-3'	
Gβ forward:	5'-CCG CTG CGT TCC TCC TGG GT-3'	612
Gβ reverse:	5'-CCA GAG ATT GCA GTT GAA GT-3'	
β-actin forward:	5'-CCC AGA GCA AGA GAG GCA TC-3'	830
β-actin reverse:	5'-CTC AGG GG AGC AAT GAT CT-3'	

performed six independent times. The results were expressed as percentages of control.

Measurement of intracellular reactive oxygen species production

Intracellular levels of ROS were determined with DCFH-DA probe and fluorescence spectrophotometry. The cells were incubated with 1 mM DCFH-DA in the dark (10 min at 37 °C). Then, the wells were washed (three times) with PBS and analyzed immediately on the fluorescence plate reader (FLX 800, BioTek, USA). The fluorescence intensity was determined at an excitation of 485 nm and an emission of 538 nm. Results were expressed as fluorescence percentage of control cells (Pasban-Aliabadi et al., 2013).

mRNA analysis

Total cellular RNAs were isolated by a modification of the guanidine isothiocyanate–phenol–chloroform method using RNX+ reagent and a semiquantitative RT-PCR method was used. Briefly, the RT-PCR reaction was performed using Oligo-dT primer and M-MuLV reverse transcriptase (Fermentas GMBH, Germany). Each PCR reaction was performed using selective forward and reverse primers for β-actin (as an internal standard), Gαi/o, Gαs and Gβ subunits (Table 1).

Taq DNA polymerase (Roche, Germany) used for DNA amplification and the reactions were incubated at 94 °C for 5 min, followed by 25 thermal cycles (45 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C). There was a linear relation between the number of PCR cycles and PCR products as well as between the initial amount of cDNA template and PCR product. PCR products were quantified by densitometry using Lab Works analyzing software (UVP, UK). The possibility of the presence of contaminating genomic DNA was ruled out and the identity of the amplified DNA has been confirmed by digestion with the restriction endonucleases (Esmaeili-Mahani et al., 2008).

Immunoblot analysis

Rat pheochromocytoma cells were homogenized in a buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1% SDS, 0.1% Na-deoxycholate and 1% NP-40] containing protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, aprotinin and sodium orthovanadate). After centrifugation (14,000 g, 15 min at 4 °C), protein concentrations of the supernatant were determined by Bradford method. Total protein (40 µg) were electrophoresed on a 9% SDS–PAGE gel and transferred to polyvinylidene difluoride membrane. After overnight blocking (at 4 °C) with 2% non-fat dried milk in Tris-buffered saline with Tween 20 (TBST), the membranes were probed with G protein subunit antibodies (1:1000) for three hours at room temperature. After washing in TBST, the blots were incubated (60 min at 25 °C) with a horseradish peroxidase-conjugated secondary antibody. The blotting complexes were detected using the ECL system and exposed to Lumi-Film chemiluminescent detection film (Roch, Germany). Lab Work analyzing software (UVP, UK) was used to analyze the intensity of the expression. β-actin immunoblotting was used

to control for loading. The immunoblot experiments for each protein were performed 4 independent times.

Statistical analysis

The results are expressed as mean±SEM. The differences in mean MTT, intracellular ROS and averages for tested mRNAs and proteins between experimental groups were determined by un-paired student t-test. *P*< 0.05 was considered significant.

Results

Analysis of control and high glucose-incubated PC12 cell viability

As shown in Fig. 1, 48h treatment with 100 mM glucose significantly induced cell toxicity and reduced cell viability. Hyperglycemic condition had about 50% reducing effect on relative cell viability.

Measurement of intracellular ROS in PC12 cells

The intracellular ROS levels were measured in control and hyperglycemic PC12 cells. Exposure of cells to 100 mM glucose led to an increase in ROS level as compared to control cells (Fig. 3).

The effect of high glucose medium on the levels of G protein subunits mRNA

PCR products showed a single band of the expected size: 830 base pair (bp) for β-actin, 350 bp for Gαi, 612 bp for Gβ and 518 bp for Gαs. High glucose-

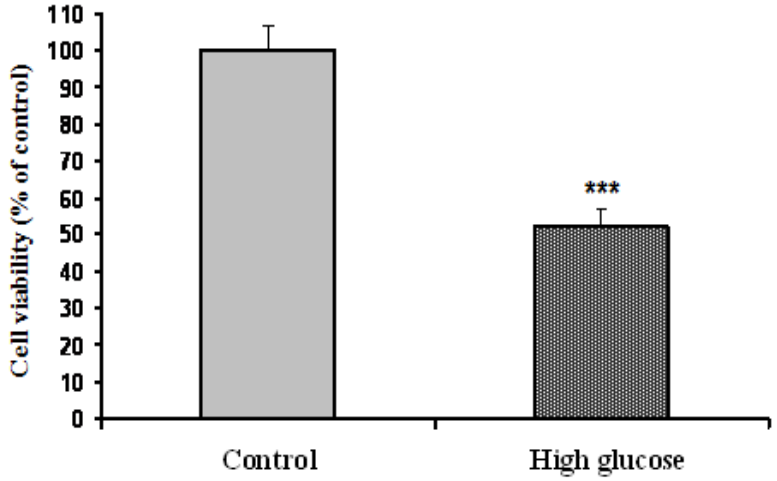

Fig.1. Effect of high-glucose medium on PC12 cell viability which was determined by MTT assay. The cells were incubated with control (25 mM glucose) or high-glucose (100 mM) medium for 48h. Data are expressed as mean±SEM; n = 6 wells for each group; ****P*< 0.001 compared to control cells.

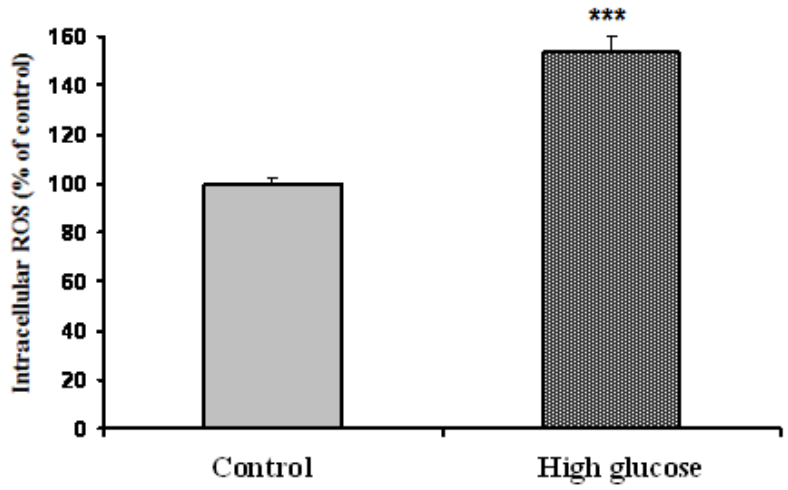

Fig.2. Effect of high glucose treatment (100 mM for 48h) on the production of intracellular ROS. Data are expressed as mean±SEM; n = 6 wells for each group. ****P*< 0.001 compared to control cells.

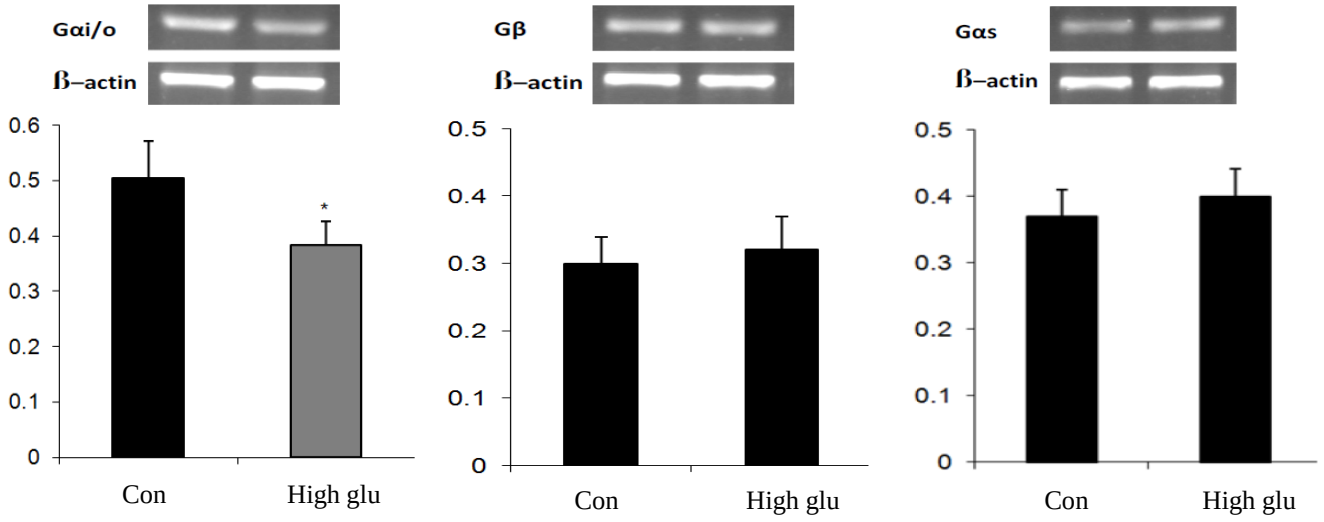

Fig.3. Effect of high glucose medium on Gαi, Gαs and Gβ mRNA expression in PC12 cells. β-actin was used as an internal control. Data are expressed as mean±SEM; n = 4 wells for each group. *P< 0.05 versus control cells.

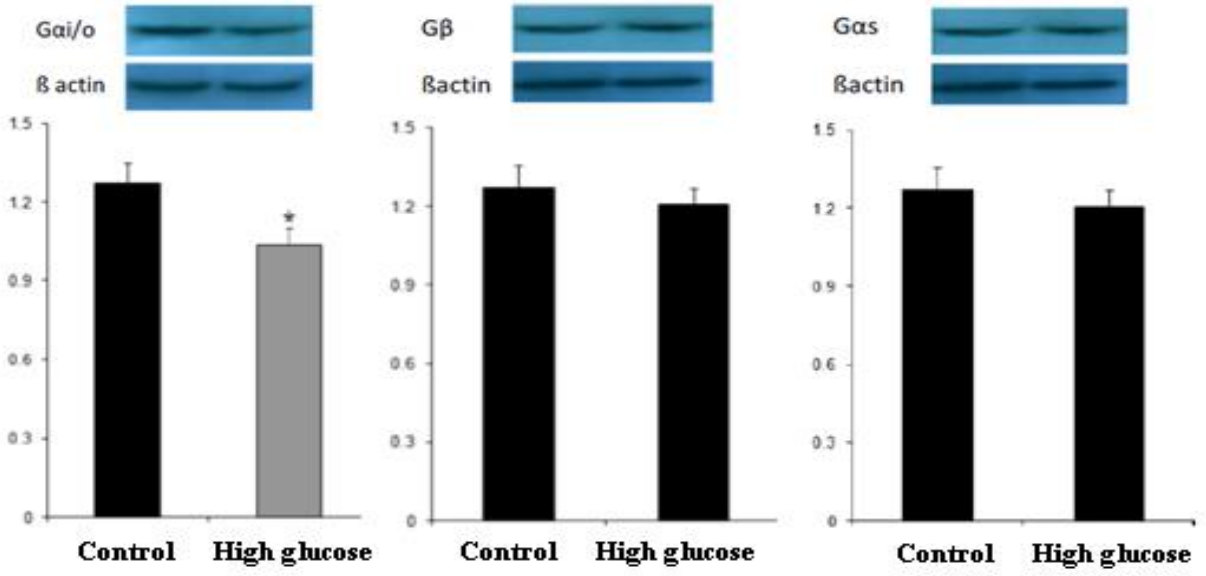

Fig.4. The average band density ratio of Gαi, Gαs and Gβ proteins in control and high glucose-treated PC12 cells. G proteins levels were assayed by western blotting. Each value in the graph represents the mean±SEM band density ratio for each group. β-actin was used as an internal control. *P< 0.05 versus control cells.

treated cells showed a significant (P<0.05) decrease in Gαi levels. However, in hyperglycemic PC12 cells, levels of Gαs and Gβ mRNA were close to those in control cells (Fig. 3). It means there were no changes in the mRNA levels of Gαs and Gβ following the incubation with 100 mM glucose.

Western blot analysis of G protein subunits in control and high glucose-treated PC12 cells

Immunoblot analysis of the cells extract stained with anti-Gαi, Gαs and Gβ revealed bands with predicted sizes according antibodies' datasheet (Fig. 4). Gαi protein was significantly (P<0.05) decreased in the high glucose-treated PC12 cells, while Gαs and Gβ proteins did not changed.

Discussion

Diabetes is a major public health problem, serious, costly but yet manageable disease. Although hyperglycemia is a major factor in the development of diabetic complications, the pathogenic mechanisms

have not yet been fully clarified. Hyperglycemia induces cellular oxidative stress and then mitochondrial damage occurs due to excess formation of ROS and reactive nitrogen species (Obrosova et al., 2005).

Oxidative stress is a major contributor for the induction of diabetes-induced side effects. It has been demonstrated that hyperglycemia can produce oxidative stress by of superoxide and hydroxyl radicals, which in turn elicit direct toxic effects on nervous system (Tangvarasittichai, 2015). There are numerous established therapeutic approaches for the prevention of diabetic side effects, while currently there is only one method, just strict glycemic control. Chronic oxidative stress and hyperglycemia appear to be deleterious factors leading to insulin resistance and impaired glucose tolerance and ultimately leading to diabetic complication. Normally, antioxidant defense mechanisms neutralize cellular ROS production which is considered as the physiological state for the prevention of any oxidative damage. The imbalance of the ROS production and antioxidants defense system in hyperglycemic situations caused oxidative stress brings to tissue damage, dysfunction and failure of various organs especially the eyes, kidneys, nerves, heart and blood vessels.

Activated nuclear factor (NF)-κB and its related kinases have been identified in uncontrolled hyperglycemia which participates in diabetes-induced side effects (Obrosova, 2009). In addition, it has been shown that experimentally-induced diabetes leads to the changes in G proteins and adenylate cyclase function (Gawler et al., 1987; Rodgers et al., 2001).

Previously, it has been shown that Gαi protein did not change in the diabetic rat's spinal cord (Chen et al., 2002) and dorsal root ganglion (Hall et al., 2001). However, in this *in vitro* study, we observed a decreased in the expression of Gαi mRNA and protein in high glucose-treated PC12 cells. However, the changes in other G protein subunits could not be observed. The results are in accordance globally with our previous findings in an *in vivo* model of diabetic rats (Hajializadeh et al., 2010), just with an exception that there was difference in Gαi mRNA level in *in vitro* (decreased) and *in vivo* (increased) models of hyperglycemia.

Such discrepancy reveals that high glucose uses different mechanism for the reduction of Gαi protein in *in vitro* and *in vivo* models. The decreased in Gαi protein levels is due to the reduction of its transcription in PC12 cells, while Gαi degradation is responsible for its decreasing level in diabetic rats.

It has been reported that Gαi but not Gαs content, significantly decreases in hyperglycemic blood vessels smooth muscle cells (Hashim et al., 2006) which is in accordance with present results. In addition, Li and colleagues showed that high glucose-induced decreased levels of Gαi and associated signaling in A10 vascular smooth muscle cells may be attributed to the enhanced oxidative stress due to augmented levels of peroxynitrite. In such situation hyperglycemia activates transcription factor NF-κB which in turn increases nitric oxide (NO) synthase expression and NO production and finally decreases Gαi signaling (Li et al., 2008).

The data suggest that hyperglycemia promotes the down-regulation of Gαi gene or accelerates the degradation of Gαi protein. It has been demonstrated that the levels of proteins are determined by many factors including transcriptional regulation, as well as translational and post-translational modification (Campagnoni et al., 1991; Morris and Malbon, 1999). The causality of the decrease in Gαi mRNA and protein requires further studies. Irrespective of the mechanisms by which it occurs, the changes observed in inhibitory G-protein expression could have an important functional role in the signal transduction of G-protein-coupled receptors in hyperglycemic situation as well as diabetes.

Conclusion

Taken together, it seems that the changes in this key regulatory G protein in high glucose situation may be expected to lead to pleiotropic effects diabetes. In addition, high glucose-induced oxidative stress can be attributed in the regulation of Gαi in PC12 cells. The effects of high glucose situations are not completely similar in different tissues and experimental models. However, *in vitro* and *in vivo* animal models cannot be considered as full approved pathologic models for diabetes and the experimental results needs to be finalized in clinical studies.

Conflict of interest

There is no conflict of interest.

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