Biochemical and Biophysical Research Communications 474 (2016) 64–70

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Prolonged exposure to insulin with insufficient glucose leads to impaired Glut4 translocation

Abdul Khalique [a, b], Rucha D. Sarwade [a, b], Poonam R. Pandey [a, b], M.V. Vijayakumar [b], Manoj K. Bhat [b], Vasudevan Seshadri [b, *]

[a] Biotechnology Department, Savitribai Phule Pune University, Pune 411007 India
[b] National Centre for Cell Science, Pune 411007 India

ARTICLE INFO

Article history:
Received 8 April 2016
Accepted 13 April 2016
Available online 20 April 2016

Keywords:
Insulin
Glucose transporter (GLUT4)
Diabetes

ABSTRACT

Insulin maintains glucose homeostasis by stimulating glucose uptake from extracellular environment to adipose and muscle tissue through glucose transporter (GLUT4). Insulin resistance plays a significant role in pathologies associated with type2 diabetes. It has been previously shown that hyperinsulinemia can lead to insulin resistance. In these studies very high levels of insulin was used to achieve insulin resistance. We hypothesized that one of the causes of type 2 diabetes could be insulin synthesis in the absence of glucose stimulation. We used CHO cell line, stably expressing Myc-GLUT4-GFP along with human insulin receptor to study the effect of hyperinsulinemia in the presence of low glucose (6.5 mM) or high glucose (20 mM). The insulin responsiveness of these cells was assessed by FRAP, FACS and subcellular fractionation. The results suggest that exposure of cells to insulin in low glucose conditions made these cells insulin resistant within 10 passages, while the same level of insulin in the presence of high glucose did not result in insulin resistance. These results clearly suggest that hyperinsulinemia combined with hypoglycaemia may lead to insulin resistance and may be one of the causes for the typ2 diabetes.

1. Introduction

Insulin acutely stimulate rapid uptake of glucose in muscle and adipose tissue in order to maintain normal glucose homeostasis. This glucose uptake is largely contributed by translocation of glucose transporter (GLUT4) from an intracellular pool to the plasma membrane. In the basal state, GLUT4 glucose transporters are sequestered to specific intracellular vesicles and have a low exocytic rate [8,18,27] as against GLUT1 [27] and transferrin receptors (TfnR) [22]. Insulin stimulates the translocation of this sequestered GLUT4 to plasma membrane where it docks, fuses and gets activated enabling glucose entry into the cell. GLUT4 transporters are constantly recycling between endosomes and the plasma membrane during this dynamic process [6]. Extensive research over last decade have shown that impairment in any of these steps can contribute to diabetes [16,26] and thus detailed understanding of GLUT4 trafficking is vital to design new therapies to ameliorate diabetic condition. Studies in animals as well as humans indicate that alterations in GLUT4 expression, trafficking, and/or activity occur in adipose cells and muscles in diabetes and other insulin-resistant states [9].

It is observed that GLUT4 is localized in several sites involved in recycling pathway including Trans-Golgi network (TGN) [19], clathrin coated vesicles and endosomes [5]. However, a vast majority of this 48 KDa transmembrane glucose transporter is primarily located in specialized tubulo-vesicular elements called GLUT4-containing small vesicles (GSVs). Insulin upon binding to its receptor in GLUT4 containing tissue, leads to a net shift in subcellular distribution of GLUT4 to plasma membrane. This membrane bound GLUT4 then facilitates rapid glucose uptake down the concentration gradient into the cells leading to 20–30 fold increase in glucose uptake in response to insulin. There are other proteins such as gp160 also known as insulin responsive aminopeptidase (IRAP) [11,17] which shows similar insulin responsiveness in adipocytes [22]. This differential function of insulin to GLUT4 and other recycling protein is most probably due to separation of GLUT4 from normal endosomal trafficking pathways to form separate compartment which readily transfer to cell membrane in response

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* Corresponding author.
E-mail addresses: seshadrv@yahoo.com, seshadriv@nccs.res.in, seshadrv@hotmail.com (V. Seshadri).

http://dx.doi.org/10.1016/j.bbrc.2016.04.066

to insulin. There have been several reports suggesting GLUT4 storage compartment is a separate entity from the endosomal recycling compartment [1]. GLUT4 is accumulated in TfnR negative, highly insulin responsive compartment which is depleted of GLUT4 after acute insulin stimulation [4]. GLUT1 and GLUT4 are localized to distinct cellular vesicles and although both are insulin responsive, the relative increase of GLUT4 to the surface is more in response to insulin as it is more efficiently sequestered in basal state [3].

It is widely accepted view that GLUT4 translocation to plasma membrane is the major mechanism of increased glucose uptake [7,20]. In many cases of type 2 diabetes, cells expressing insulin receptor become non responsive to insulin [23]; however in pre-diabetic stage serum insulin level are increased. We believe that at the stage of pre-diabetic condition, prolonged exposure GLUT4 expressing cells to insulin without corresponding increased glucose can make these cells resistant to insulin, which might cause type-2 diabetes.

Insulin production is mainly regulated by glucose in mammals. In the initial phase of glucose stimulation, insulin biosynthesis is regulated mainly at the translation level. Previously we have shown that insulin biosynthesis is increased by PDI (protein disulfide isomerase) during glucose stimulation, where it binds to 5′UTR of insulin mRNA to increase insulin translation [14]. There are many reports which suggest that several pathological and physiological conditions causing cellular stress, resulting in increased PDI expression including, ER stress caused by UPR [2] and during hypoxia in neuroblastoma cells [21]. We speculate that beta cell stress may lead to increased expression of PDI resulting in high level of insulin without increase in blood glucose. This high insulin production may leads to hyperinsulinemia condition. Furthermore, obesity and metabolic syndrome also linked with deregulation of insulin secretion resulting in hyperinsulinemia [13]. In addition, hyperinsulinemia in low glucose is causing impairment of insulin induced tyrosine kinase activity in non diabetic sand rats [12], and immune suppression in zebrafish leading to insulin resistance [15]. Based on all the above finding we hypothesized that this chronic exposure of high insulin (hyperinsulinemia) to insulin sensitive cells without corresponding increase of glucose may cause GLUT4 expressing cells (adipocytes or skeleton muscles) to become insulin resistant. We tested this hypothesis using Glut4 and insulin receptor expressing CHO cells.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary cell line (CHO), stably expressing myc-GLUT4-GFP and insulin receptor has been previously described [25]. The cells were maintained in F12K medium (HiMedia, AT106) supplemented with 10% FBS (SIGMA), 100 U/ml of penicillin, and 100 mg/ml streptomycin at 37 °C in humidified CO_2 incubator. For experiment, cells were maintained with insulin or without insulin (SIGMA 16634) in low (6.5 mM) and high glucose (20 mM) F12K media for several passages. Before insulin response assay the cells were incubated in serum free DMEM (HiMedia) medium lacking glucose for 4 hrs to establish basal state.

2.2. FRAP (fluorescence recovery after photobleaching)

The FRAP analysis was performed using laser scanning confocal microscope (TCS SP5, Leica), with a plane apochromat 100× objective. CHO cells were trypsinized and 0.5×10^6 cells were seeded onto glass cover slips, and incubated at 37 °C in humidified CO_2 incubator. 24 hr later, the culture media was changed to serum and glucose free media and further incubated for 4hr to establish basal condition. Cover slips were then transferred to FRAP chamber, washed with 1× PBS and 500 µl HEPES were added. Cells were visualized under confocal microscope for GFP expression, then small area on the membrane was chosen for photo bleaching by 488 blue argon laser and repeated photo bleaching of delineated region was done using 100% intensity of 488 nm argon laser. Samples were maintained at 37 °C. Images were taken prior to Photobleaching and immediate after Photobleaching and every 5 s thereafter. Recovery of the GFP fluorescence was measured. To see the GLUT4 dynamics in response to insulin, 50 ng/ml (8.6 nM) purified insulin was added to the same cover slips and allowed it to spread uniformly for 5 min. Time of recovery for GFP fluorescence to plasma membrane was noted and graph was plotted between time (sec) and 50% recovered GFP fluorescence intensity for treated and untreated cells.

2.3. Subcellular membrane fractionation and immunoblotting

For subcellular fractionation, differential ultracentrifugation was used as described previously (2). Briefly, CHO cells were incubated overnight in DMEM without glucose and serum free media at 37 °C in CO_2 incubator to establish basal condition and stimulated with 50 ng/ml (8.6 nM) insulin for 30 min. The cells were washed twice with 1XPBS, scraped with cell scraper and homogenized in ice cold HES buffer (20 mM HEPES pH7.5, 1 mM EDTA, 255 mM Sucrose, 1× Protease Inhibitor cocktail, 1 mM PMSF). The homogenates were centrifuged at 20000 g, 4 °C for 20 min. The pellets were resuspended in 2 ml HES buffer, layered on to 1 ml 1.15 M sucrose in HES buffer and ultra centrifuged at 100000 g for 20 min at 4 °C in swing bucket rotor. Plasma membrane fraction was collected from the interface by careful aspiration, resuspended in HES buffer and isolated by centrifugation at 41000 g for 20 min at 4 °C. For high and low density microsome (HDM and LDM), supernatant from first step was centrifuged at 180,000 g for 75 min at 4 °C and pellet was resuspended in HES buffer. All fractions were resuspended in equal volume of HES buffer and stored in −20 °C until used. For detection of GLUT4 distribution in all fractions, equal amount were loaded onto 10%SDS PAGE, transferred to PVDF membrane and probed with GFP antibody (SC-9996). The same blot was stripped and re-probed with N-Cadherin (ab18203) and transferrin receptor (H-68.4, SC-65882) antibodies that served as controls for membrane and endosomes respectively.

2.4. Fluorescence activated cell sorter (FACS)

To quantitate the amount of GLUT4 receptor on plasma membrane in response to insulin in response to insulin, flow cytometry using fluorophore conjugated GLUT4 or extracellular tag antibody was done. We followed the FACS as described earlier (3). Briefly, overnight serum starved cells were trypsinized and 1×10^6 cells were plated in serum free medium for 2 h at 37 °C for recovery. Then, 50 ng/ml (8.6 nM) of insulin was added to these cells and incubated 30 min then transferred to 15 ml falcon tube. The cells were then fixed in 1% PFA for 20 min at RT in dark. Cells were collected by centrifugation at 250 g for 5 min, washed with 1XPBS and transferred to 1.5 ml centrifuge tube. Primary (mouse anti-Myc antibody; SC-40, Santacruz) followed by secondary antibody (anti-mouse alexa fluor 647; A-21236, Invitrogen) at 1:10 dilution was added to each tube except one where only secondary antibody was added as a unstained control and incubated for 30 min at RT. Data was acquired in FACS CantoII for 10,000 cells. Analysis was done using (FACS) FLOWJO software V10 (Ashland, USA).

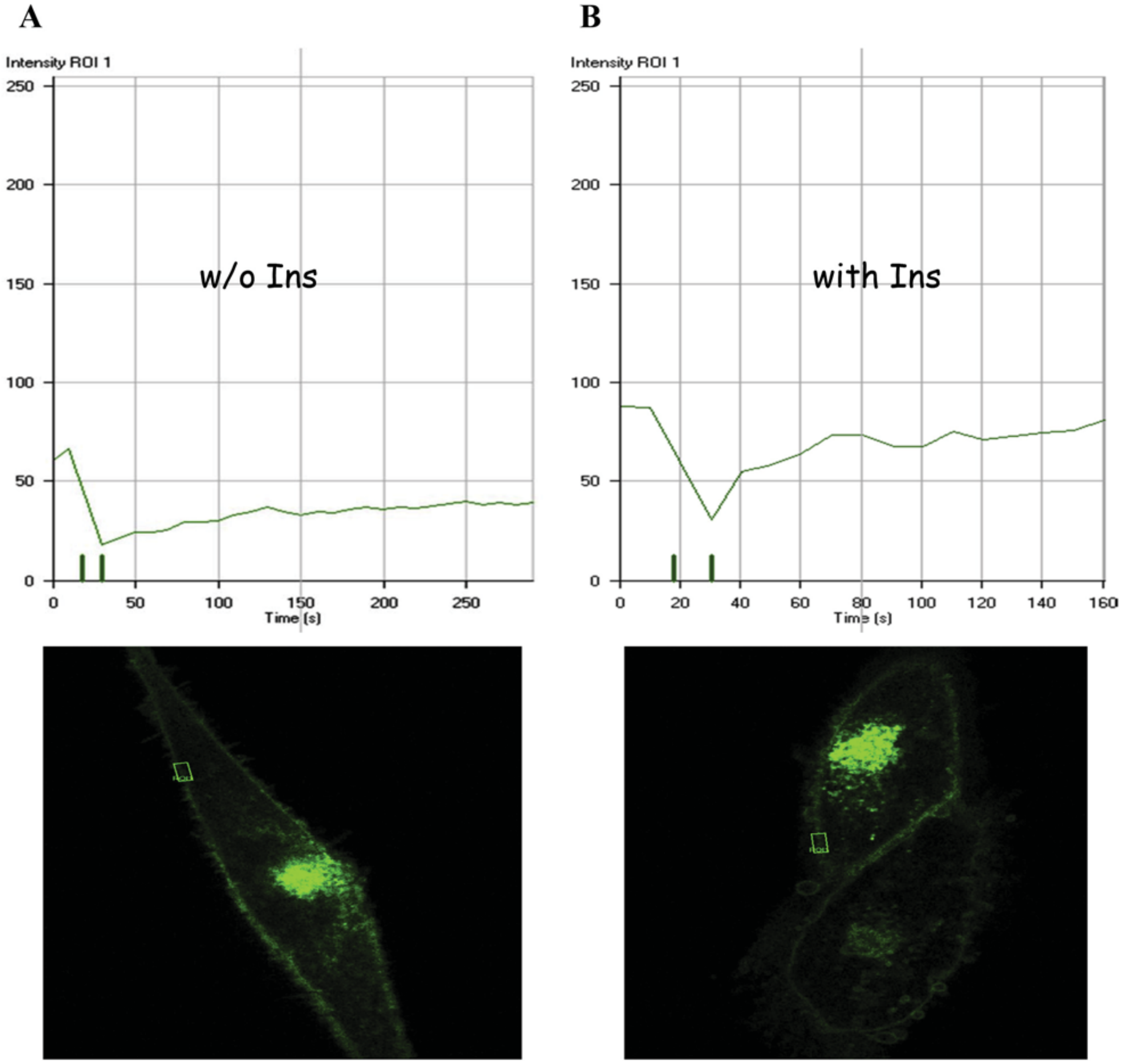

Fig. 1. CHO cells expressing Myc-GLUT4-GFP is insulin responsive. Insulin responsiveness of Glut4-CHO cells was assessed by FRAP analysis. A small region of the cell membrane was photobleached using full intensity of argon laser and GFP fluorescence recovery time to the photobleached area (before and after adding the insulin) was analysed in the presence (left panels) and absence (right panels) of insulin. The graph was plotted between GFP fluorescence intensity in the photo-bleached area and time (sec).

2.5. *Statistical analysis*

The statistical significance of the data was assessed by *t*-test using mean ± standard deviation in Sigma plot 12.0 (systat software Inc., CA, USA). Statistical data significance levels were represented as $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) significant, very significant and highly significant respectively.

3. Results

3.1. *Myc-GLUT4-GFP translocates to plasma membrane upon insulin stimulation in CHO cells*

Translocation of GLUT4 transporter from intracellular compartment to the cell surface in response to insulin is well characterized in adipocytes and muscle cells. To study the effect of prolonged exposure of insulin on GLUT4 translocation we used Myc-GLUT4-GFP construct, stably expressing in CHO cells and responsive to insulin [24,25]. The Myc tag fused is at the N-terminus end of GLUT4 in such a way that it is exposed to extracellular side of cell surface which can be detected by fluorescent labelled myc antibodies whereas GFP is fused at C-terminus of GLUT4, exposing it to the cytoplasmic side of the plasma membrane. The detailed information of this construct generation and transfection to CHO fibroblast cells to generate stable cell line, expressing GLUT4 protein is previously reported [25]. Insulin responsive GLUT4 trafficking to cell membrane was assessed by FRAP (Fluorescence Recovery after Photobleaching) and subcellular fractionation. Glut4-CHO cells were seeded on cover slips and a small region of plasma membrane was selected and photo bleached and time for GFP fluorescence recovery at photo bleached area was assessed. GFP fluorescence recovery was assessed in the presence or absence of insulin stimulation in order to measure the insulin responsive GLUT4 translocation. Time taken to recover 50% fluorescence

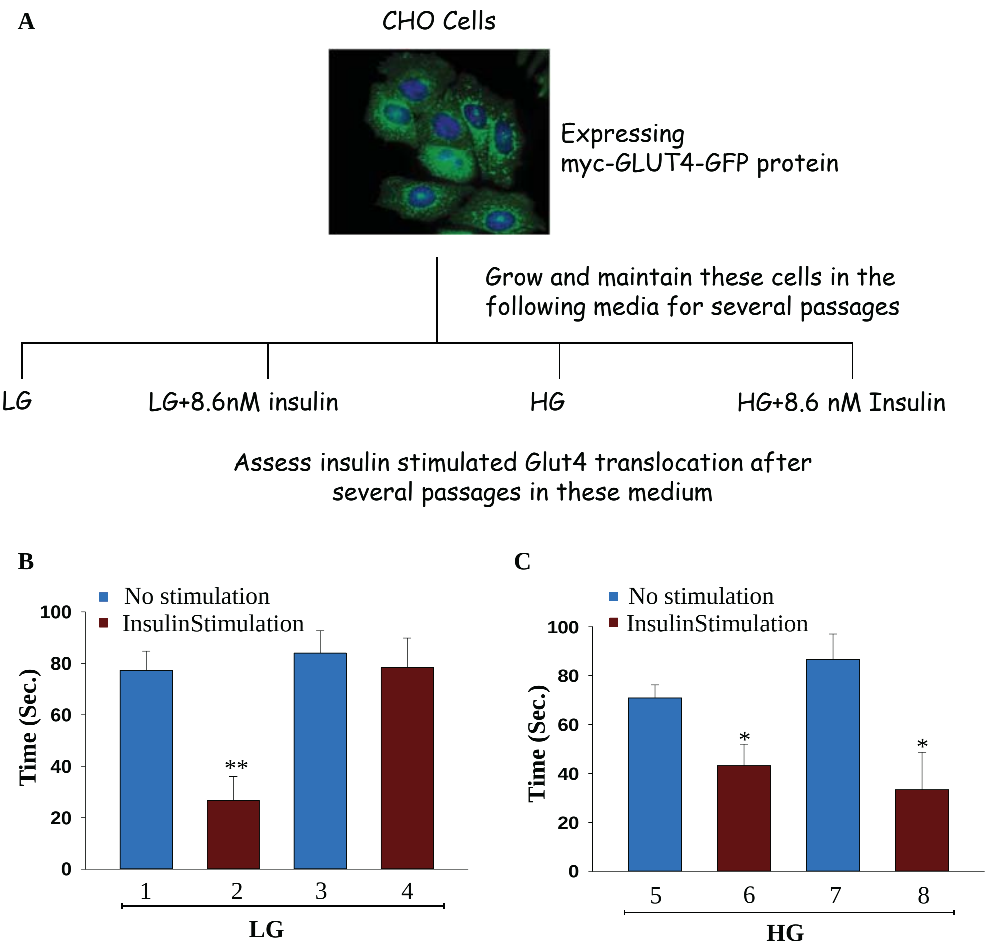

Fig. 2. Insulin responsiveness of CHO cells is reduced upon prolonged exposure to insulin in low glucose containing medium. Schematic representation of experimental design to assess the insulin responsiveness of the Glut4-CHO cells exposed to insulin **(A)**. CHO cells expressing Myc-GLUT4-GFP was maintained in a media with or without insulin in low or high glucose for several passages. FRAP analysis of Myc-GLUT4-GFP expressing CHO cells after 10 generation of insulin exposure in low glucose or high glucose medium was performed. The GFP fluorescence recovery time for each was recorded before (Blue bars) or after (Brown bars) insulin (8.6 nM) stimulation and graph was plotted for the time (sec) to recover 50% of GFP fluorescence and represents an average of three biological repeat experiments. The recovery time for the cells maintained in low glucose medium without insulin (bars 1 and 2) and with insulin (Bars 3, 4) is shown in the top panel **(B)**, while The recovery time for the cells maintained in high glucose medium without insulin (bars 5 and 6) and with insulin (Bars 7, 8) is shown in the lower panel **(C)**. Statistical analysis was done by *t*-test where * indicates $P<0.05$ and ** indicates $P<0.01$.

intensity after Photobleaching is rapid after addition of insulin compared to basal level, suggesting GLUT4 exocytosis rate is increased upon insulin stimulation (Fig. 1), suggesting a robust insulin responsive Glut4 translocation in these cells. In addition, subcellular fractionation of cytoplasm and plasma membrane was performed using sequential ultracentrifugation after stimulation by insulin followed by immunoblotting with GLUT4 antibody further confirming that GLUT4 translocation to plasma membrane from cytoplasmic vesicles is increased in response to insulin compared (Fig. S1). Both the experiment suggests that CHO cells expressing GLUT4 receptor are highly insulin responsive and can be monitored by GLUT4 trafficking in vivo and in-vitro.

3.2. Prolonged exposure of insulin to insulin sensitive cells in low glucose cause resistance

Insulin stimulates glucose uptake in adipocytes and skeletal muscle cells primarily by stimulating the transport of glucose transporter type 4 (GLUT4) to plasma membrane from specialized GLUT4 storage vesicles (GSV). We tested the effect of hyperinsulinemia and or hyperglycemia on the insulin responsiveness of these cells. The Glut4 expressing CHO cells were maintained in medium containing different levels of insulin and glucose. The schematic of the experimental procedure is shown in Fig. 2A. After 10 passages the cells were tested for insulin responsiveness by FRAP (Fluorescence recovery after Photobleaching), subcellular fractionation of GLUT4 containing vesicles and FACS.

For the FRAP experiment a small area in the plasma membrane was photobleached and fluorescence recovery in presence and absence of insulin stimulation was recorded. Graph was plotted for the time taken to recover 50% GFP fluorescence and a reduce time indicates a faster translocation and a good insulin response. As expected the cells maintained in medium without insulin showed a robust insulin responsive Glut4 translocation in both high and low glucose growth conditions (Fig. 2B and C the first two bars), whereas cells maintained in insulin and low glucose medium showed a complete loss of insulin responsive Glut 4 translocation (Fig. 2B bars 3 and 4), however the cells maintained in insulin and High glucose conditions still retained insulin responsive Glut4 translocation (Fig. 2C bars 3 and 4). Further, low glucose condition alone in the absence of insulin did not affect the insulin responsiveness of CHO cells (Fig. S2). This data suggest that long time insulin exposure in low glucose conditions leads to insulin resistance however cells exposed to similar levels of insulin but with

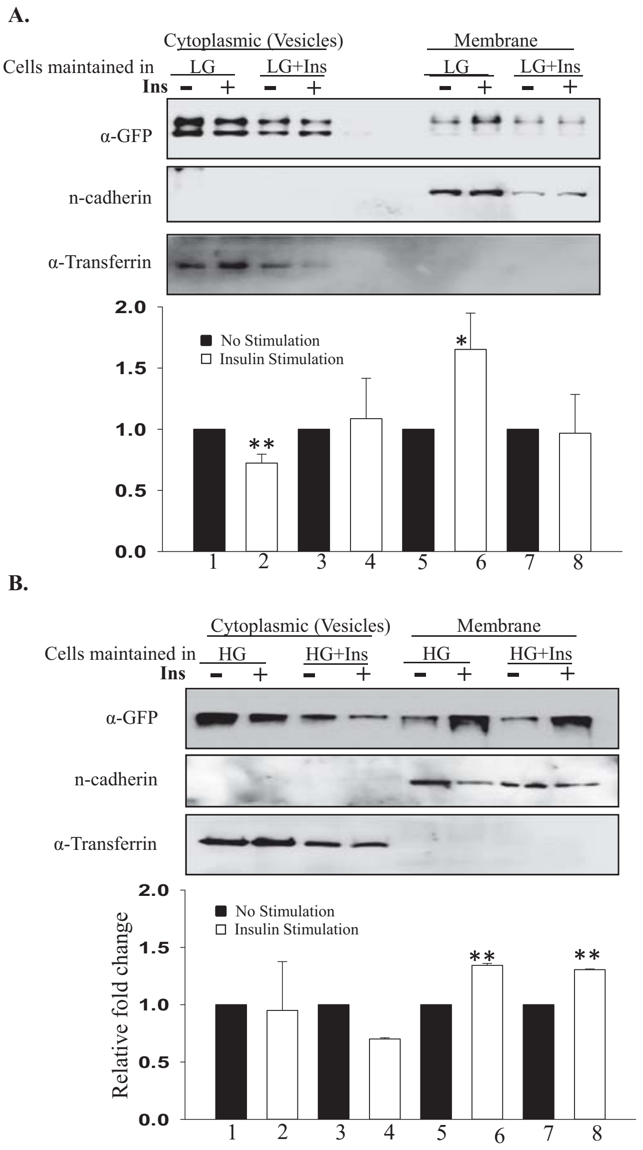

Fig. 3. Glut4 translocation to the plasma membrane is reduced in cells maintained in medium containing insulin and low glucose. Glut4-CHO cells were lysed and subcellular fractionation was performed through sequential ultracentrifugation. Vesicle and membrane fractions were analyzed for GLUT4 by western blot using GFP antibody. The blot was stripped and probed with N-Cadherin and Transferrin receptor antibody as fraction markers. The normalized band intensity was plotted for each sample and is represented as bar graph below the western blots. Normalized Glut4 levels in cytoplasmic vesicle fraction (1–4) and membrane fractions (5–8) for cells maintained in low glucose media is indicated **(A)**. Similarly for the Glut4-CHO cells maintained in high glucose medium the results are indicated **(B)**. The relative band intensity was calculated from three independent experiments after normalization with N-cadherin and transferrin receptor for each fraction. Statistical significance was calculated by *t*-test using systat 3.5 where P˂0.05 (*), P˂0.01 (**).

higher levels of glucose are still insulin responsive.

In order to further confirm this phenomenon, we performed subcellular fractionation of GLUT4 expressing CHO cells. The cells were lysed in hypotonic buffer and sequential ultra-centrifugation was done as described in materials and method. The fractions were resolved on SDS-PAGE, transferred to PVDF membrane and probed for GFP (Glut4), Transferrin receptor (endosomal) and N-Cadherin (Plasma membrane) antibody. We find an increased GLUT4 translocation to the membrane in response to insulin in cells which were maintained in medium lacking insulin (Fig. 3A and B). On the other hand, cells which were maintained in presence of insulin and low glucose for 10 passages, showed no significant change in GLUT4 levels on membrane to cytoplasmic vesicles in response to insulin (Fig. 3A). In contrast, cells which were maintained in insulin and high glucose medium throughout 10 passages, showed increase insulin stimulated membrane associated GLUT4 (Fig. 3B). The transferrin receptor (endosomal protein) did not show any change in cytoplasmic to membrane translocation in response to insulin and is mainly located in intracellular compartment (Fig. 3A and B).

Similar results were also observed by FACS analysis, where Glut4-CHO cells was stimulated by insulin and stained with anti-Myc antibody (sc-40, santacruz) without permeabilizing the cells. The total plasma membrane associated GLUT4 was estimated by FACS and mean fluorescence intensity (MFI) was calculated and plotted for cells grown in different medium (Fig. S3). An increased MFI indicates an increased Plasma membrane associated Glut4 suggesting a good insulin responsiveness. The Relative Mean fluorescence intensity (MFI) of cells which was maintained in low glucose and no insulin media were increased significantly in response to insulin (Fig. 4A left panel) compare to those cells which were maintained in insulin and low glucose (Fig. 4A right panel). However, cells which were maintained in high glucose and in presence or absence of insulin didn't show insulin resistance (Fig. 4B). The relative increases in MFI from multiple experiments are averaged and depicted in Fig. 4C. In addition, we assessed whether prolonged exposure to insulin and glucose affects the total levels of GLUT4 protein. We prepared protein lysates from cells, after 5 and 10 passages of insulin exposure in presence and absence of insulin and assessed Glut4 expression levels by western blot using GFP. We find no significant change in GLUT4 protein levels, suggesting GLUT4 protein biosynthesis is not affected by long term insulin exposure but only its translocation to the membrane (Fig. S4). Altogether, these results strongly suggest that increased level of insulin exposure to insulin responsive cells (Adipocytes and skeleton muscles) in low glucose condition leads to insulin resistance.

4. Discussion and conclusion

Insulin is the main regulatory hormone which maintains glucose homeostasis in the blood [10] by acting on insulin sensitive cells (adipocytes and skeleton muscle cells) to internalize glucose by GLUT4 transporters from the extracellular space. Insulin is preferentially produced and secreted by β cells of the pancreas in response to glucose elevation in the blood. At short period of glucose stimulation, insulin is mainly regulated at the translation level. Previously, we have identified PDI as an insulin 5′UTR trans-acting protein that increases insulin production [14].

PDI expression is up-regulated in various cellular stress conditions including ER stress or metabolic disorders. This increased level of PDI may results in high insulin production even in absence of glucose. Moreover, during metabolic disorders and obesity insulin secretion pathways are affected which leads to increased insulin levels without corresponding increase of plasma glucose. So we believe that prolonged higher levels of insulin exposure of the responder cells (adipocytes and skeleton muscle cells) in the absence of increased glucose may result in insulin resistance. We used CHO cell line which was stably expressing human insulin receptor and GLUT4 glucose transporter [25]. We maintained the cells till 10 passages in presence of insulin (8.6 nM) and low (6.5 mM) or high (20 mM) glucose. The cells then again test for insulin responsiveness through GLUT4 translocation by FRAP, FACS and subcellular fractionation. The results suggest that prolonged exposure of insulin in low glucose reduces the sensitivity for insulin compare to control cells which was maintained in absence of

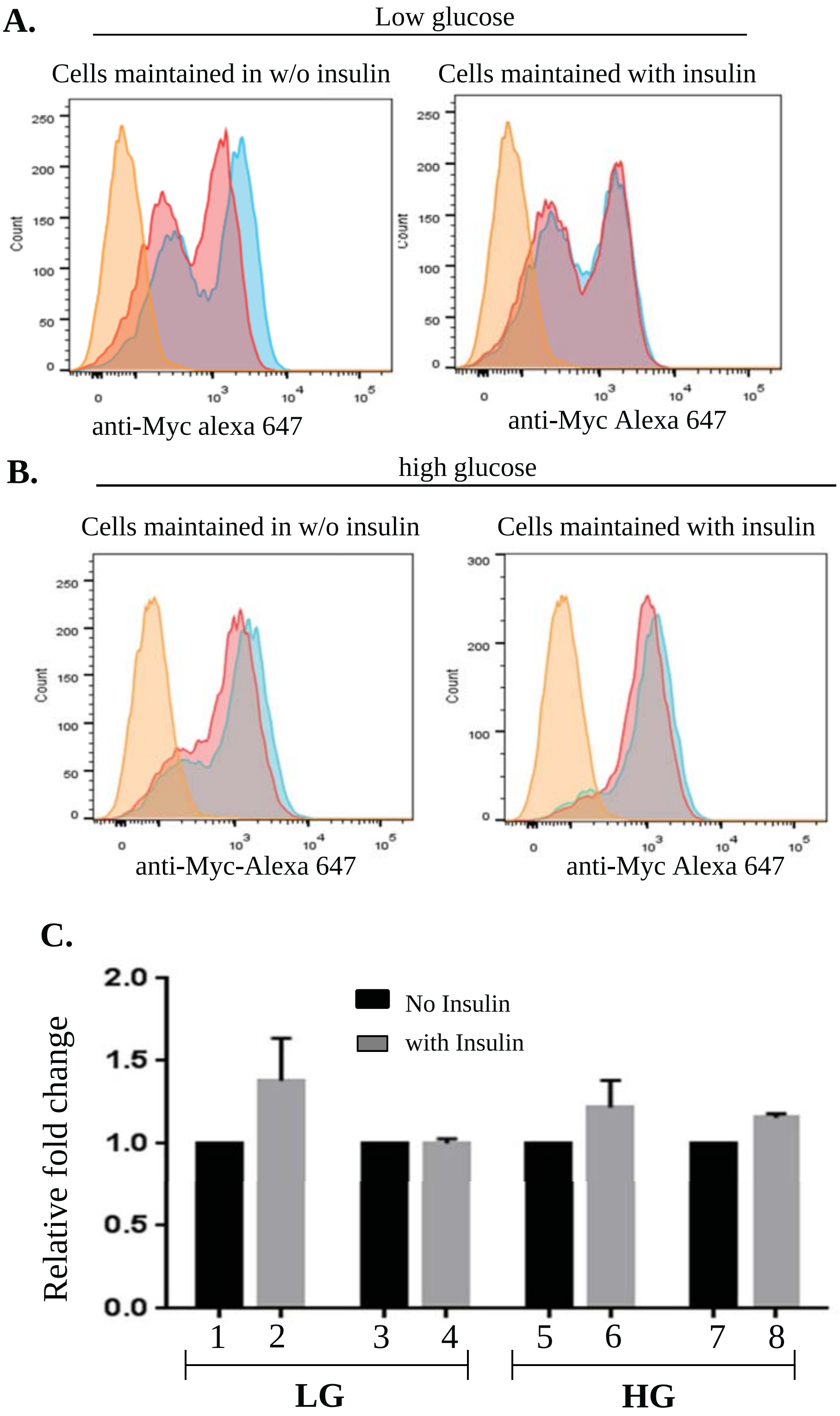

Fig. 4. FACS analysis of insulin responsive GLUT4 translocation to plasma membrane. The Glut4-CHO cells were fixed and stained with anti-myc mouse monoclonal antibody without permeabilization to monitor the membrane expression of Glut4. The Fluorescence intensity for the population of cells maintained in low glucose medium **(A)** and high glucose medium **(B)** was measured. Mean fluorescence intensity (MFI) was calculated using Flowjo software (V10) and Relative MFI was calculated by normalizing with no insulin MFI and graph was plotted between relative fold changes upon insulin stimulation for cell populations that were grown in indicated medium **(C)**. Statistical analysis was done using Sigma plot 12.0 (Systat software Inc., CA, USA).

insulin. In contrast prolonged exposure of insulin in high glucose did not show significant reduction of insulin responsiveness. However, the total GLUT4 protein expression is not affected in response to insulin. So it is very likely that insulin signalling pathway might be playing critical role in development of insulin resistance. Thus individuals with hyperinsulinemia may develop insulin resistance if they are also exposed to hypoglycaemia, however the insulin resistance can be averted if sufficient levels of glucose is provided to them at intervals.

Acknowledgement

This work has been funded by the grant to VS from NCCS (intramural) and DBT (BT/PR14109/BRB/10/812/2010). AK is supported by fellowship from University Grants Council, India. RSD and PP are supported by fellowship from Department of Biotechnology, India. The authors would like to acknowledge Mr Deepak Khuperkar, NCCS for help in conducting the FRAP experiments and Mr. Anil Sharma, NCCS for analysis of FACS data.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.04.066.

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