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

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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
that insulin biosynthesis is increased by PDI (protein disulfide
regulated mainly at the translation level. Previously we have shown
In the initial phase of glucose stimulation, insulin biosynthesis is
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 S’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 zebra fi sh 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 re-
ceptor 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 CO2 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 100x
objective. CHO cells were trypsinized and 0.5 × 10^6 cells were
seeded onto glass cover slips, and incubated at 37 °C in humidified
CO2 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 argon laser 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 immediately 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 CO2 incubator to establish basal condition and
stimulated with 50 ng/ml (8.6 nM) insulin for 30 min. The cells
were washed twice with 1X PBS, 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
to 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, 4 °C for
45 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 mem-
brane 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,
onight 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 1X PBS
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 FACS3 Cantoll for 10,000 cells. Analysis was done
using (FACS) FLOWJO software V10 (Ashland, USA).
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 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
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 hyper-insulinemia 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 (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.

**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.
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 ultracentrifugation 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 in 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. 3). 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) compared 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. 3). 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 glycoprotein transportor[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
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.

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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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