Brief communication

Demonstration of a visual cell-based assay for screening glucose transporter 4 translocation modulators in real time

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Insulin-stimulated translocation of glucose transporter 4 (GLUT4) to cell membrane leading to glucose uptake is the rate-limiting step in diabetes. It is also a defined target of antidiabetic drug research. Existing GLUT4 translocation assays are based on time-consuming immunoassays and are hampered by assay variability and low sensitivity. We describe a real-time, visual, cell-based qualitative GLUT4 translocation assay using CHO-HIRc-myc-GLUT4eGFP cells that stably express myc- and eGFP-tagged GLUT4 in addition to human insulin receptor (HIRc). GLUT4 translocation is visualized by live cell imaging based on GFP fluorescence by employing a cooled charge-coupled device camera attached to a fluorescent microscope. This video imaging method and further quantitative analysis of GLUT4 on the cell membrane provide rapid and foolproof visual evidence that this method is suitable for screening GLUT4 translocation modulators.

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Studies have shown that glucose uptake into adipocytes and muscle cells by insulin-regulatable glucose transporter 4 (GLUT4) is the rate-limiting step in diabetes (Pessin and Saltiel 2000; Bryant *et al.* 2002; Kahn *et al.* 2006). However, an insulin mimetic agent or an agent that enhances glucose uptake is still an elusive target of pharmaceutical research. In view of the availability of a large number of natural products and synthetic compounds or derived products for antidiabetic drug research, it is important to have simple and efficient *in vitro* systems and methods for GLUT4 translocation assay that facilitate rapid screening.

Investigations on insulin- or insulin-mimetic-drugs-stimulated GLUT4 translocation generally employ indirect methods such as western blot analysis of plasma membrane (PM) fractions, photoaffinity labelling, binding assay using cytochalasin B and qualitative assessment by immunofluorescence or immunoelectron microscopy (Suzuki and Kono 1980; Kozka *et al.* 1991; Smith *et al.* 1991). These time-consuming and relatively less sensitive techniques are hampered by imprecise and inefficient detection. Although immunofluorescence detection of the epitope tag (generally myc or HA) within the first exofacial loop of GLUT4 has been used, it is useful only to obtain still pictures of the PM-associated GLUT4 distribution in fixed and non-permeabilized cells (Kanai *et al.*1993; Dawson *et al.* 2001). Direct visualization of GLUT4 trafficking by tagging of GLUT4 with green fluorescent protein (GFP) and its expression in limited cell lines has made detection of GLUT4 movement by confocal microscopy relatively easy (Dobson *et al.* 1996; Dawson *et al.* 2001; Liu *et al.* 2009). Of

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Abbreviations used: CHO, Chinese hamster ovary; eGFP, enhanced green fluorescent protein; FSE, fenugreek seeds extract; GLUT4, glucose transporter 4; HA, hemagglutinin; HIRc, human insulin receptor; IR, insulin receptor; PM, plasma membrane; TIRF, total internal reflection fluorescence

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late, total internal reflection fluorescence (TIRF) microscopy has been employed to examine GLUT4 translocation in real time (Lampson *et al.* 2001; Chen and Saltiel 2007). TIRF microscopy does not permit assessment of its trafficking from perinuclear regions in the cell. Neither confocal nor TIRF microscopy is user-friendly so as to be utilized for quick and foolproof screening of a large number of GLUT4 translocation modulating compounds or natural products and their fractions.

We have earlier reported the development of CHO-HIRc cells overexpresssing GLUT4 tagged with myc and eGFP (enhanced GFP), designated as CHO-HIRc-mycGLUT4eGFP cells, for confocal-microscopy-aided GLUT4 translocation assay in fixed cells (Vijayakumar *et al.* 2005). Further, we investigated the application of CHO-HIRc-mycGLUT4eGFP cells in screening GLUT4 translocation modulating compounds or natural products, by live cell imaging based on GFP fluorescence by employing a cooled charge-coupled device camera attached to a simple fluorescent microscope. The present study takes advantage of the following properties of CHO-HIRc-mycGLUT4eGFP cells for live cell imaging of GLUT4 translocation:

(i) CHO cells overexpressing the insulin receptor (CHO-HIRc) exhibit a significantly high level of GFP fluorescence in addition to being insulin sensitive (Perfetti *et al.* 1997).
(ii) In the basal state, the exogenously overexpressed GLUT4 chimera (mycGLUT4eGFP) in CHO-HIRc cells is mainly sequestered intracellularily at perinuclear region (Guilherme *et al.* 2000).
(iii) The mycGLUT4eGFP chimera remains intact, and the junction between the two proteins is not susceptible to proteolysis to generate native GFP (Dobson *et al.* 1996).
(iv) Insulin-stimulated translocation of mycGLUT4-eGFP-associated vesicles to the cell surface PM can be visualized by live cell imaging based on the movement of GFP fluorescence (Oatey *et al.* 1997).
(v) The myc tag provides additional and precise information on GLUT4 translocation by immunofluorescence detection *or ELIZA* (Wang *et al.* 1998; Baus *et al.* 2010).
(vi) There is no requirement of differentiation.

The protein, mycGLUT4eGFP, which is constitutively expressed under the cytomegalovirus (CMV) promoter translocates to the PM upon insulin stimulation (Bose *et al.* 2002; Jiang *et al.* 2002). GFP fluorescence is detected mostly in the perinuclear region of CHO-HIRc-mycGLUT4eGFP cells with a scattered distribution throughout the cytosol (figure 1A). The PM was devoid of any significant fluorescence. Insulin-stimulated cells were positive for distinct membrane-associated GFP fluorescence as well as myc staining with the monoclonal antibody 9El0 (figure 1B, C and D).

To visualize GLUT4 translocation in real time, live cell image analysis of CHO-HIRc-mycGLUT4eGFP cells treated with 100 nM insulin was performed. Images were captured at one frame per minute. Image-Pro Plus 5.0 software–aided image analysis based on the increase in GLUT4-associated GFP fluorescence on the PM revealed that insulin treatment caused sudden movement of GLUT4 vesicle to the PM. The movement of GLUT4 vesicles was visible 2 min after insulin stimulation and these movements were sustained (figure 2A and supplementary movie 1). In order to examine whether the GLUT4 translocation is induced specifically by insulin, the cells were pre-treated with individual pharmacological inhibitors of insulin action (cytochalasin B, genistein or wortmannin) for 30 min (Lawrence *et al.* 1992; Smith *et al.* 1993; Hausdorff *et al.* 1999). Pre-treatment with cytochalasin B or genistein caused localization of GFP fluorescence on restricted areas of the PM and the morphology of wortmannin treated cells was similar to that of control cells (supplementary figure 1). Further, movement of GFP upon insulin stimulation was low in cells treated with any of the pharmacological inhibitors, indicating that these inhibitors abrogate the insulin-induced GLUT4 translocation (supplementary movies 2, 3 and 4).

Quantitative analysis of these videos using Image-Pro Plus AMS software was performed, which was based on the increase in mean fluorescence density on the membrane. This analysis showed that insulin enhanced the GLUT4 translocation by 2.6-fold in 2 min which further increases to 3.2-fold by 25 min. In the presence of pharmacological inhibitors, GLUT4 translocation increased by only 1- to 1.2-fold over the basal level in 0–15 min (figure 3A). Glucose uptake studies, which measured the rate of ^{14}C-2-deoxy glucose uptake in CHO-HIRc-mycGLUT4eGFP cells, showed that Insulin in a dose-dependent manner enhanced glucose uptake in this *in vitro* cell model, and EC_{50} for insulin was calculated to be 1 nM (supplementary figure 2A). Insulin-stimulated glucose uptake was abrogated by pretreating the cells with genistein or wortmannin (supplementary figure 2 B).

We also examined if this new assay would be useful in assessing the efficacy of fenugreek seed extract (FSE), a natural product with reported insulin-signalling and insulin-activating properties (Vijayakumar *et al.* 2005). As a negative control, cells were treated with PBS. Live cell imaging revealed that FSE-induced GLUT4 translocation was slow in comparison with insulin and the translocation of GLUT4 to PM was detected only after 20 min of treatment (figure 2B, supplementary movie 5). In contrast, the vehicle control for FSE (PBS) had no effect on GLUT4 translocation to the PM (figure 2C, supplementary movie 6). Quantitative

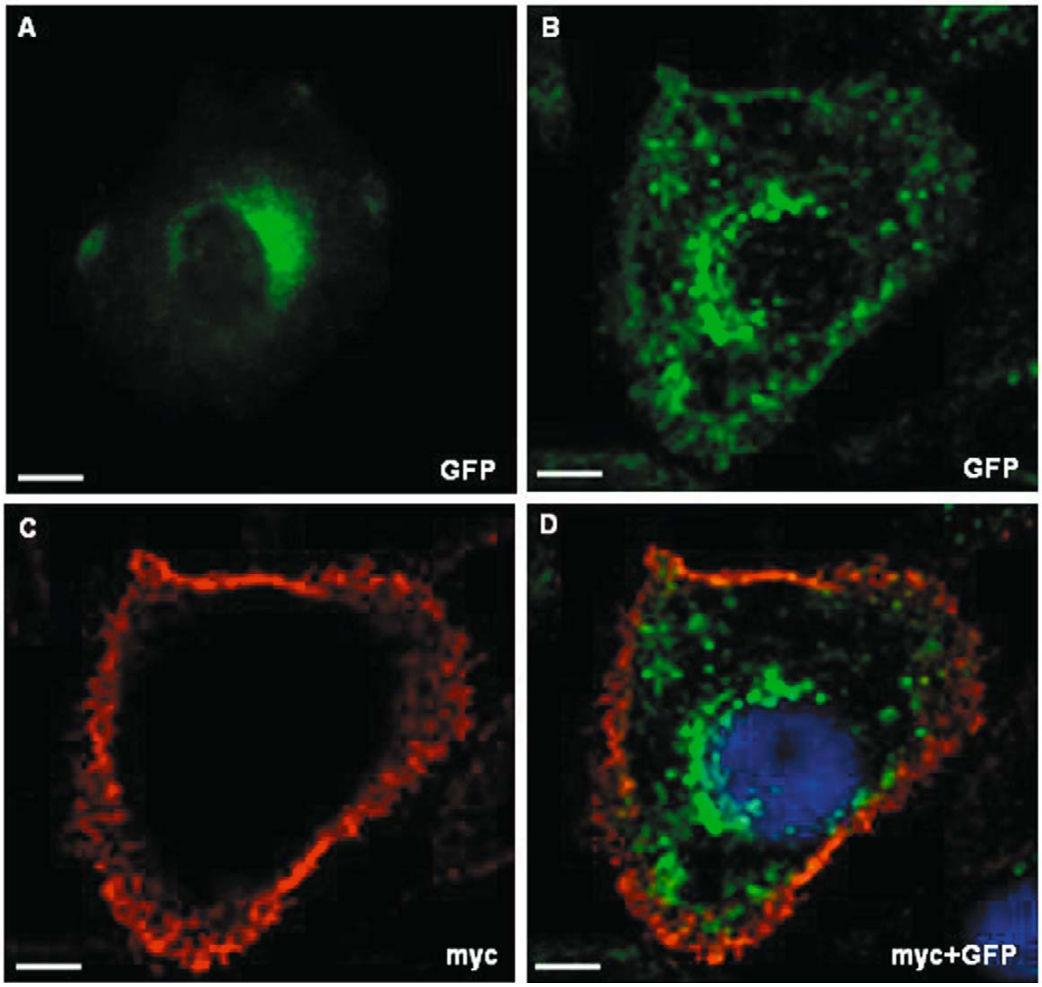

Figure 1. Confocal microscopy images of the CHO-HIRc-mycGLUT4eGFP cell. The GLUT4 chimera, mycGLUT4eGFP, is localized at the perinuclear region and translocates to the plasma membrane upon insulin stimulation. Images are visualized based on GFP fluorescence or by immunostaining for myc in nonpermeabilized cells. The blue colour indicates nucleus stained by 4′,6-diamidino-2-phenylindole (DAPI) present in the mounting medium. (**A**) Basal, (**B** and **C**) stimulated with 100 nM insulin and (**D**) superimposed image of (**B**) and (**C**) Scale bars 20 μM.

analysis of these videos showed that FSE enhanced GLUT4 translocation by 1.5-fold over the basal level or that mediated PBS by 25 min, and these effects were sustained up to 35 min (figure 3B).

To confirm that insulin or FSE promoted translocation of GLUT4 chimera to the PM, fixed and non-permeabilised CHO-HIRc-myc-GLUT4eGFP cells were stained with the monoclonal antibody 9El0, which recognizes myc epitope present in the GLUT4 chimera. As shown in figure 4A, the distribution of GLUT4 in the basal state was similar to that shown in figure 1A. Insulin treatment indeed promotes an increase in immunoreactive chimera on the PM in parallel with increased GFP fluorescence (figure 4B). Likewise, dose-dependent increase in fluorescence on the PM by FSE was comparable to that induced by insulin (figure 4C and D).

The data presented here clearly demonstrate that CHO-HIRc-mycGLUT4eGFP cells would be a useful *in vitro* model for live cell imaging of GLUT4 translocation in real time. Established *in vitro* models, such as 3T3-L1, L6 and C2C12 cells used for GLUT4 translocation assay, are relatively accurate although they may not exactly represent insulin sensitive muscle and adipose cells (Liu *et al.* 2009). Requirements for differentiation, a single largest variable and, the presence of GLUT1 makes screening with the above-mentioned cells difficult, especially in tedious pharmaceutical drugs screening (Kuri-Harcuch and Green 1978; Mehra *et al.* 2007). Although the existence of a complete machinery required for GLUT4 translocation in the CHO cell line is debatable, a significant number of studies have shown that CHO cells overexpressing the insulin receptor and GLUT are sensitive to insulin (Kanai

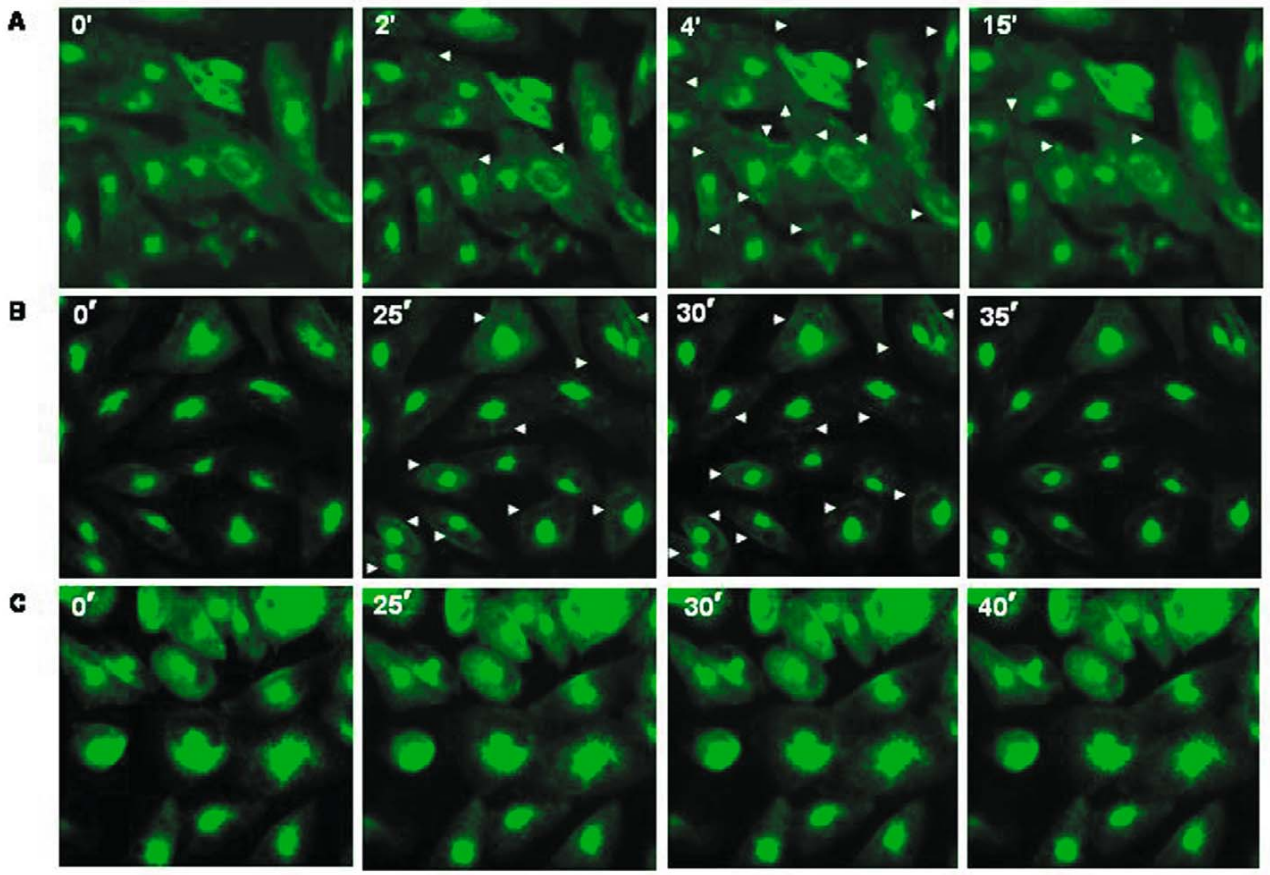

Figure 2. Video image analysis of GLUT4 translocation. CHO-HIRc-mycGLUT4eGFP cells were treated with insulin, natural product (FSE) or vehicle control of FSE (PBS). To confirm the specificity of insulin-induced GLUT4 translocation, cells were pre-treated with either 10 μM cytochalasin, 50 μM genistein or 100 nM wortmannin for 30 min, followed by treatment with 100 nM insulin. Images were captured at a magnification of 40× at the rate of one frame per min for 25–40 min using charge-coupled device camera attached to microscope (supplementary movies 1–6). Conversion of videos to individual frame was done using Image-Pro Plus 5.0 software. (**A**) 100 nM insulin, (**B**) FSE 25 μg/ml, (**C**) PBS. Arrow marks indicates the localization of GLUT4 chimera on the plasma memebrane. Legends on the figure indicate the time points at which images were captured after treatment. Representative images shown were compiled from four independent experiments.

et al. 1993; Quon *et al.* 1994; Perfetti *et al.* 1997; Lampson *et al.* 2000). Live cell imaging of GLUT4 translocation by confocal microscopy is reported and it has been performed only in cells transfected transiently with GLUT4 chimera (Dobson *et al.* 1996; Oatey *et al.* 1997). Recently, Thermo Scientific (Waltham, MA, USA) has come up with a CHO-cell-line-expressing GLUT4 chimera of GFP (Product 023-01), and GLUT4 translocation in this cell line is measured by a redistribution assay involving the fixing and staining of the cells. However, a qualitative assay for GLUT4 translocation by video image analysis performed in differentiated cells or CHO cells in real time does not exist. In this study images were captured using a 40× objective rather than one with a higher magnification in order to increase the area of the field. This provides reliable and collective information about a significantly large number of cells (n>25), a highly desired parameter in any form of research. The cell-based GLUT4 translocation assay presented here is simple, economical, quick and sensitive enough to detect even active components in crude extract. Moreover, stimulation of GLUT4 translocation can be quantified using compatible software. In principle, this system would be useful in tracking the GLUT4 translocation modulating effects especially during the fractionation process in which a large number of fractions are to be tested and those retaining activity could be taken up for further isolation and purification until the active individual ingredients are obtained. Therefore, live cell imaging of a stable cell line of CHO-HIRc-mycGLUT4eGFP represents a simple and rapid assay for screening GLUT4-stimulating antidiabetic compounds or products.

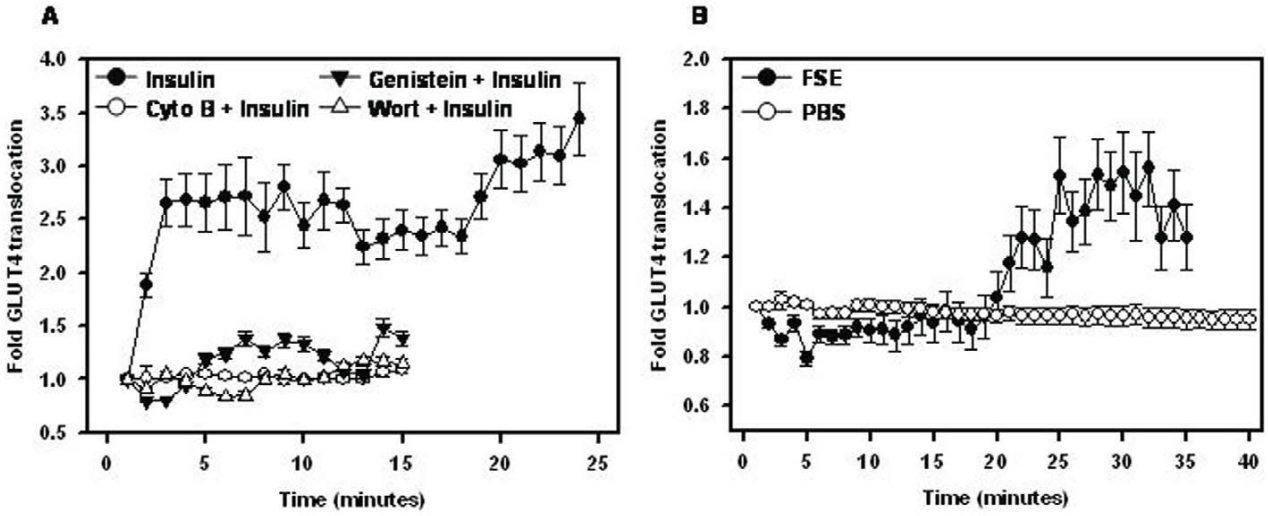

Figure 3. Quantitative analysis of GLUT4 translocation from video images. GLUT4 translocation was quantified on the basis of the increase in mean GFP fluorescence density in a defined area of the PM of cells using the Image-Pro Plus AMS software (MediaCybernetics, Silver Spring, MD, USA) The mean density data obtained versus frame was used to calculate fold GLUT4 translocation per minute. Ths standard deviation was obtained from 10 sample values. (**A**) Insulin-enhanced GLUT4 translocation by 2.6-fold in 2 min and to 3.2-fold by 25 min. Treatment with the pharmacological inhibitors increased fold GLUT4 translocation by only 1- to 1.2-fold over basal. (**B**) FSE enhanced the GLUT4 translocation by 1.5-fold over the basal or that mediated PBS by 25 min, and these effects were sustained up to 35 min.

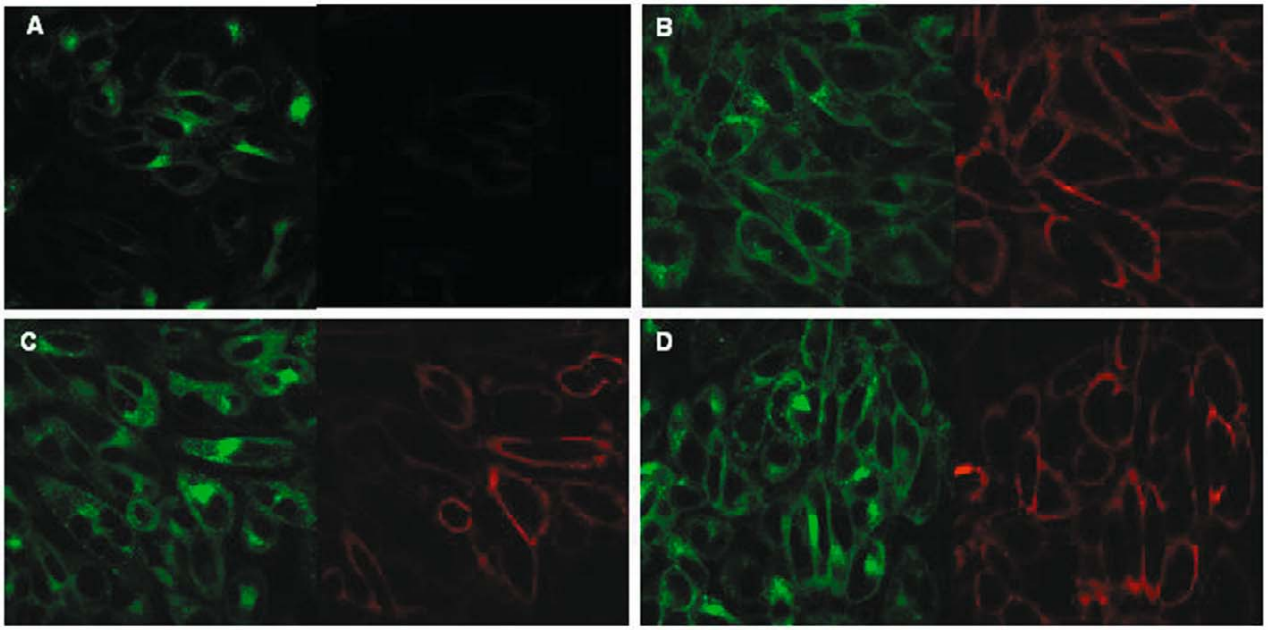

Figure 4. Confocal microscopy analysis of GLUT4 translocation in CHO-HIRc-mycGLUT4eGFP cells. GLUT4 translocation was visualized on the basis of GFP fluorescence or immunostaining of myc present in nonpermeabilized cells. (**A**) Basal, (**B**) 100 nM insulin, (**C**) 10 μg/ml FSE and (**D**) 25 μg/ml FSE. Insulin treatment increased immunoreactive chimera on the PM in parallel with increased GFP fluorescence. Dose-dependent increase in fluorescence on the PM by FSE was comparable to that induced by insulin. Representative image from three independent experiments are shown.

Expression, intracellular compartmentalization and insulin responsiveness of CHO-HIRc-mycGLUT4eGFP cells suggest that the exogenously expressed reporter molecule mimics endogenous GLUT4 in insulin-sensitive cells. In the present study, translocation of mycGLUT4eGFP vesicle to the cell surface was visualized by live cell

imaging based on the movement of GLUT4-associated GFP fluorescence to the PM. This method also provides optimal time point at which GLUT4 was translocated to the cell surface and is a foolproof visual tool useful for the screening of GLUT4-stimulating products. Because CHO-HIRc-mycGLUT4eGFP cells possess the basic machinery required for insulin stimulated GLUT4 translocation and glucose uptake, these cells will have application in pharmaceutical research and also in basic research.

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