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Cytokine Stimulation Promotes Increased Glucose Uptake Via Translocation at the Plasma Membrane of GLUT1 in HEK293 Cells

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ABSTRACT

Interleukin-3 (IL-3) and granulocyte/macrophage colony-stimulating factor (GM-CSF) are two of the best-characterized cell survival factors in hematopoietic cells; these factors induce an increase in Akt activity in multiple cell lines, a process thought to be involved in cellular survival. It is known that growth factors require sustained glucose metabolism to promote cell survival. It has been determined that IL-3 and GM-CSF signal for increased glucose uptake in hematopoietic cells. Interestingly, receptors for IL-3 and GM-CSF are present in several non-hematopoietic cell types but their roles in these cells have been poorly described. In this study, we demonstrated the expression of IL-3 and GM-CSF receptors in HEK293 cells and analyzed their effect on glucose uptake. In these cells, both IL-3 and GM-CSF, increased glucose uptake. The results indicated that this increase involves the subcellular redistribution of GLUT1, affecting glucose transporter levels at the cell surface in HEK293 cells. Also the data directly demonstrates that the PI 3-kinase/Akt pathway is an important mediator of this process. Altogether these results show a role for non-insulin growth factors in the regulation of GLUT1 trafficking that has not yet been directly determined in non-hematopoietic cells. J. Cell. Biochem. 110: 1471–1480, 2010.

KEY WORDS: IL-3; GM-CSF; GLUT1 TRANSLOCATION

Dependence on extracellular growth factors is one mechanism by which multicellular organisms regulate the growth and survival of their constituent cells [Raff, 1992]. When growth factors are withdrawn, cells undergo programmed cell death.

Interleukin-3 (IL-3) and granulocyte/macrophage colony-stimulating factor (GM-CSF) are two of the best-characterized cell survival factors. IL-3 and GM-CSF belong to a family of cytokines that regulate the proliferation, survival, and differentiation of hematopoietic cells via interaction with specific cell-surface receptors. The receptors for these cytokines consist of a ligand-specific α subunit (GMRα and IL-3Rα) and a common β subunit (βc) [Gearing et al., 1989; Hayashida et al., 1990].

GM-CSF receptors have been identified on most myeloid progenitors and on mature monocytes, neutrophils, eosinophils, basophils, dendritic cells, and tumor cell lines [Brosnan et al., 1993; Rivas et al., 1998]. IL-3 receptors are present on early hematopoietic progenitor cells, on certain committed myeloid progenitors, on eosinophils and basophils and in brain [Tabira et al., 1998]. Binding of IL-3 or GM-CSF to their receptor leads to the stimulation of multiple signal transduction pathways, including the Jak/STAT pathway, the Ras/Raf/mitogen-activated protein kinase pathway, the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (PKB) pathway, and the protein kinase C (PKC) pathway [de Groot et al., 1998]. It is known that IL-3 and GM-CSF induce an increase in PKB/Akt activity in multiple cell lines, a process thought to be involved in cell survival. Akt has been shown to phosphorylate and inactivate several proapoptotic mediators such as BAD, caspase 9, and the Forkhead transcription factor [Datta et al., 1999]. However, the prosurvival function of activated Akt is dependent in part on its stimulatory effect on glucose metabolism as Akt-mediated cell survival is decreased in medium containing limiting levels of glucose. Extensive work has documented that IL-3 and GM-CSF inhibit the activation of proapoptotic factors. However, the molecular mechanisms by which these factors promote cell survival are less understood. It has been reported that growth factors require sustained glucose metabolism to promote cell survival.

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Hematopoietic cells normally utilize glucose at a low rate until stimulated by a specific growth or mitogenic factor, such as IL-3, which can promote dramatic increases on glucose uptake and consumption [Bentley et al., 2003]. Also, IL-3 increases glucose transport in myeloid cells [Berridge and Tan, 1995]. Similarly, it has been demonstrated that GM-CSF signaling increased uptake of glucose and vitamin C in human host defense cells [Vera et al., 1998] and mouse bone marrow cells [Scott et al., 1998].

Glucose uptake is controlled via a family of facilitative glucose transporters (GLUTs). In hematopoietic cells, cell surface localization of the GLUT1 is a cytokine-controlled process essential to support the metabolism and survival of these cells. GLUT1 synthesis is cytokine-dependent with loss of GLUT1 mRNA and surface protein levels when cells are cytokine-deprived [Rathmell et al., 2000; Vander Heiden et al., 2001; Bentley et al., 2003]. Also in these cells, IL-3 maintained the GLUT1 transporter on the cell surface [Wieman et al., 2007].

Previously, we have shown that non-hematopoietic cells, such as bovine spermatozoa, express functionally active low- and high-affinity GM-CSF receptors that signal for increased transport of glucose and vitamin C [Zambrano et al., 2001; Rauch et al., 2004] and that this increase is GLUT-dependent [Rauch et al., 2004]. Interestingly, receptors for GM-CSF and IL-3 are also present in several non-hematopoietic cell types, such as placental trophoblasts, endothelial cells, oligodendrocytes, male germ cells, neurons, and some tumors [Gearing et al., 1989; Baldwin et al., 1993; Guillaume et al., 1993; Rivas et al., 1998; Zambrano et al., 2001, 2007].

In this work, we evaluated the expression of IL-3 and GM-CSF receptors in HEK293 cells and determined the effect of these cytokines on glucose transport. Our data indicated that HEK293 cells express receptors for IL-3 and GM-CSF and signaled for increased glucose uptake in these cells. This increase involves the subcellular redistribution of GLUT1. Additionally, the data demonstrated that the PI 3-kinase/Akt pathway is an important mediator of this process. With these results we suggest that the subcellular redistribution of GLUT1 is a general mechanism induced by IL-3 and GM-CSF to support viability and cell growth in non-hematopoietic cells.

MATERIALS AND METHODS

CELL CULTURES

The HEK293 cell line (ATCC® CRL-1573TM) was grown using DMEM-F12 (United States Biological, Swampscott, MA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 50 U/ml penicillin, 50 mg/ml streptomycin, 50 ng/ml amphotericin B, and 2 mM L-glutamine (Nalgene, Rochester, NY).

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Cells were grown on coverslips coated with poly-L-lysine and fixed with Histochoice (Sigma–Aldrich).

Cells were cultured in the absence or presence to IL-3 (6 nM) or GM-CSF (6 nM) for different time periods (0, 30, 60, or 90 min). Cultured cells were incubated in PBS containing 1% BSA and 0.3% Triton X-100 overnight with the following antibodies: anti-GLUT1 (1:300 USBiological), anti-GLUT3 (1:100 USBiological), anti-IL-3Rα, anti-GM-CSFRα, and anti-βc (1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed and incubated with anti-rabbit, anti-goat IgG-Alexa 488 (1:300, Invitrogen, Carlsbad, CA) and propidium iodide (1.7 μg/ml, Sigma Chemical Co, St. Louis, Mo), and subsequently washed and mounted. Cells were examined using an inverted Olympus FluoView 1000 confocal microscope.

UPTAKE ANALYSIS

Cultures were selected under the microscope to ensure that plates showing uniformly growing cells were used. Uptake assays were performed in 400 μl of incubation buffer (IB, 15 mM Hepes pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$) containing 1–1.2 μCi of D-3-*o*-[methyl^{3}H]-glucose (OMG, 26.2 Ci/mmol, Dupont NEN, Boston, MA). Uptake was halted by washing cells with ice-cold IB containing 0.2 mM $HgCl_2$. Cells were lysed in 200 μl of lysis buffer (10 mM Tris–HCl pH 8.0, 0.2% SDS), and the incorporated radioactivity was measured by liquid scintillation spectrometry. The Michaelis constant, K_m, and V_{max} were calculated with the Michaelis–Menten equation (single rectangular hyperbolas were fitted to the data using nonlinear regression). Linear and nonlinear regressions were calculated using SigmaPlot v9.0 software. In all experiments, OMG concentration used was 0.5 mM. Statistical comparison between two or more groups of data was performed using analysis of variance (ANOVA, followed by Bonferroni post-test).

WESTERN BLOT ANALYSIS

Cells were cultured in the absence or presence of IL-3 (6 nM) or GM-CSF (6 nM) for different time periods (0, 30, 60, or 90 min). Afterwards, cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μM leupeptin, and 1 μg/ml pepstatin) and the protein concentration was determined by Bradford Assay. Protein extracts were resolved by SDS–PAGE (60 μg per lane) on a 10% polyacrylamide gel and transferred into immobilon (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk the membranes were incubated with primary antibodies; actin (Sigma Chemical Co), GLUT1, GLUT3, IL-3Rα, GM-CSFRα, and βc in a 1% BSA in PBS overnight at 4°C, at a 1:1,000 dilution. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma Chemical Co) for 1 h at room temperature. The antibody blots were developed by chemiluminescence (Amersham, Arlington Heights, IL).

BIOTINYLATION CELL-SURFACE PROTEINS

Cells (1×10^8) were incubated for 1 h in IB in either the presence or absence of 6 nM recombinant IL-3 or GM-CSF. Photolabeling was performed using Sulfo-NHS-SS-Biotin (Thermo Scientific, Rockford, IL) as described by the manufacturer. Briefly, cells were washed three times with ice-cold PBS (pH 8.0) to remove any contaminating proteins, then resuspended at a concentration of 2×10^7 cells/ml in PBS (pH 8.0) and then 80 μl of 10 mM Sulfo-NHS-SS-Biotin per milliliter of reaction volume was added. The cells were incubated at room temperature for 30 min and washed three times with ice-cold PBS (pH 8.0). The labeled cells were homogenized in 200 μl of buffer A with Triton X-100 and then precipitated by continuous mixing

with 40 μl of Immobilized NeutrAvidin™ Protein (Thermo Scientific, Rockford, IL) for 2 h at room temperature. The precipitates were then washed four times in 0.3% (w/v) Triton X-100 and twice in PBS. The resin-bound complex was boiled in SDS–PAGE sample buffer. Samples were resolved on 10% (w/v) SDS–PAGE, and Western blotting was performed with either affinity-purified anti-GLUT1 or anti-GLUT3. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma Chemical Co) for 1 h at room temperature. The antibody blots were developed by chemiluminescence (Amersham).

STATISTICAL ANALYSIS

Data were expressed as the mean ± SE of the values from the number of experiments performed in triplicate as indicated in the corresponding figures. Data and histograms were evaluated statistically by using the Student's *t*-test, with $P < 0.05$ and $P < 0.001$ considered significant.

RESULTS

EXPRESSION OF IL-3 AND GM-CSF RECEPTORS IN HEK293 CELLS

The expression of both α and β subunits of IL-3 and GM-CSF receptors in HEK293 cells was analyzed using specific antibodies. Immunofluorescence analysis using anti-IL-3Rα, GM-CSFRα, and IL-3/GM-CSFRβ (Fig. 1A) antibodies showed clear positive immunostaining in these cells. Confirming the results of the immunofluorescence studies, immunoblotting of proteins extracted from HEK293 cells demonstrated the presence of protein bands reacting with the antibodies. The specific antibodies reacted with bands with apparent molecular weight of 42, 80, and 120 kDa for IL-3Rα, GM-CSFRα, and IL-3/GM-CSFRβ, respectively (Fig. 1B).

EXPRESSION OF FACILITATIVE GLUCOSE TRANSPORTERS, GLUT1 AND GLUT3, IN HEK293 CELLS

Using specific primers for each transporter, GLUT1 (Fig. 2A, panel a) and GLUT3 (Fig. 2A, panel b), expression in HEK293 cells was shown by RT-PCR. Expression of transporters was verified by immunofluorescence analysis. For GLUT1 and GLUT3, staining was localized to the membrane and cytoplasm (Fig. 2A, panels c and d). These results were confirmed by immunoblotting assays as shown (Fig. 2A, panels e and f).

The expression of GLUTs was further established by functional assays using radiolabeled OMG (Fig. 2B). OMG transport was linear for the first 20 s, with a plateau reached at 60 s (Fig. 2B, panels a and b). The initial velocity for OMG transport was 5.7 ± 0.7 nmoles/10^6 cells/min (Fig. 2B, panels c and d). The lines in the plots (Fig. 2B, panels a and b), correspond to exponential single curve using two parameters (a, R:0.9972) and linear regression (b, R:0.9954). The line in the plot (Fig. 2B, panel c) corresponds to hyperbola single rectangular (R: 0.9931) and corresponded to K_m: 1.3 ± 0.4 mM and V_{max}: 12.2 ± 1 nmoles/10^6 cells/min. From Eadie–Hofstee plot (Fig. 2B, panel d) corresponds to linear regression (a, R:0.9442) obtained the following parameters: K_m: 1.7 ± 0.5 mM and V_{max}: 12.3 ± 2.4 mmoles/10^6 cells/min.

EFFECTS OF IL-3 AND GM-CSF ON OMG TRANSPORT IN HEK293 CELLS

IL-3 and GM-CSF have been reported to stimulate glucose uptake in hematopoietic cell lines. In this study, we evaluated the effects of

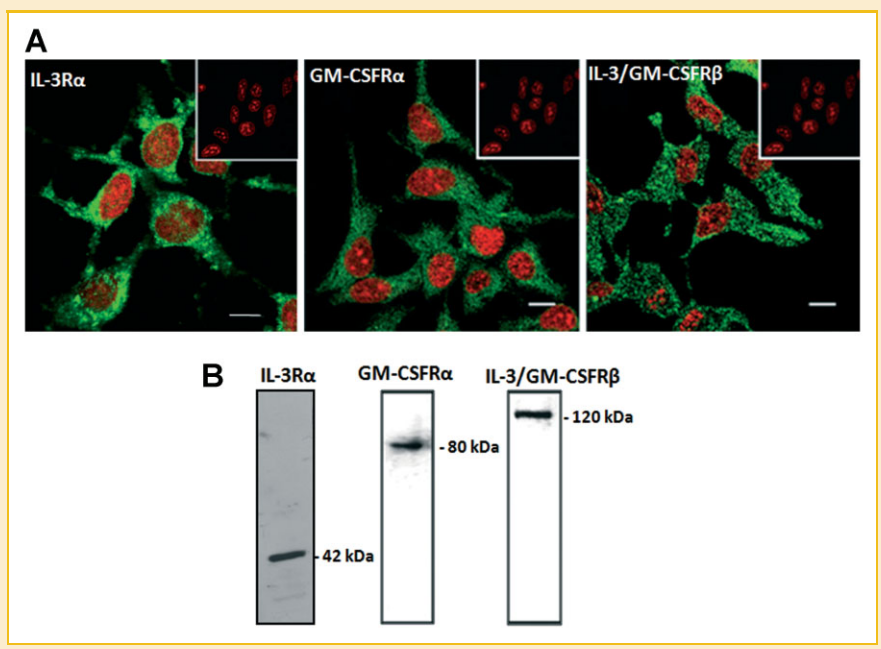

Fig. 1. Expression of IL-3 and GM-CSF receptors in HEK293 cells. A: Immunofluorescence analysis for IL-3rα subunit (green), GM-CSFrα subunit (green), and IL-3/GM-CSFrβ subunit (green) in cultured HEK293 cells. Nuclei were stained with propidium iodide (red). The inset is the negative control without primary antibodies. Scale bar, 10 μm. B: Western blot analysis for IL-3rα subunit, GM-CSFrα subunit, and IL-3/GM-CSFrβ subunit in total proteins from HEK293 cells.

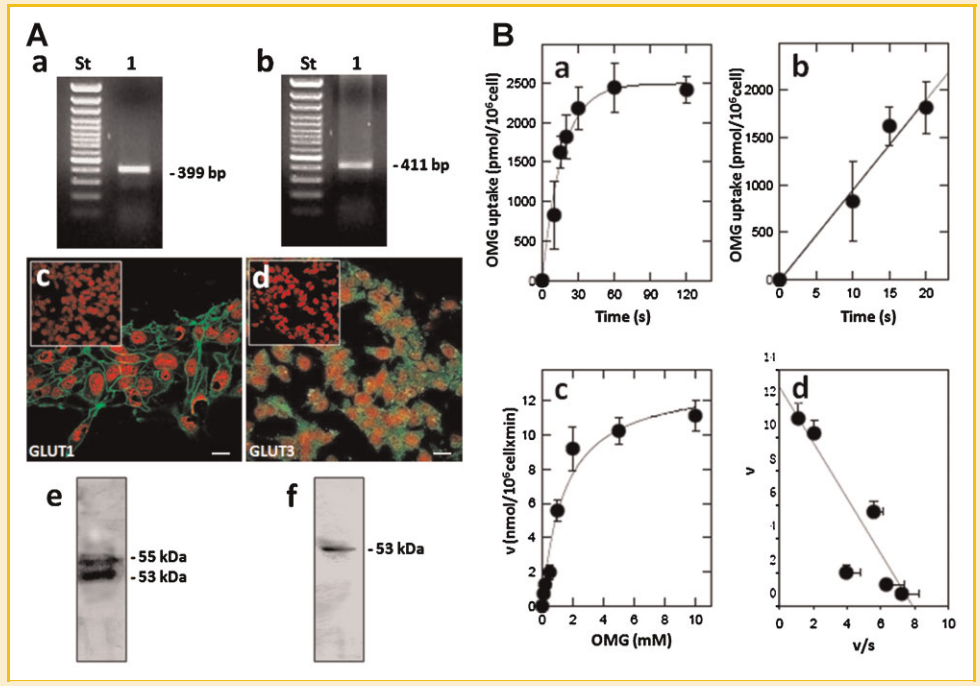

Fig. 2. Expression and functional analysis of GLUT1 and GLUT3 in HEK293 cells. A: RT-PCR for GLUT1 (a) and GLUT3 (b) from HEK293 cells mRNA (line 1); DNA 100 bp standard (St). Immunofluorescence analysis for GLUT1 (c) and GLUT3 (d), nuclei were stained with propidium iodide (red). Scale bar, 10 μm. Inset is the negative control. Western blot analysis for GLUT1 (e) and GLUT3 (f) in total proteins from HEK293 cells. B: Functional analysis. Time course for uptake of 0.5 mM OMG at 20°C (a, b). The lines in the plots correspond to exponential single using two parameters (a, R:0.9972) and linear regression (b, R:0.9954). Dose-dependent curve of OMG uptake by HEK293 cells, using a 15 s uptake assay at 20°C (c). The line in the plot corresponds to hyperbola single rectangular (R:0.9931). Eadie–Hofstee plot from data obtained in c (d). The line in the plot is a linear regression (a, R:0.9442). Data correspond to the mean ± SD of four samples.

both IL-3 and GM-CSF on HEK293 cells using uptake of the non-metabolized analog, OMG. Cells were deprived of the cytokines for 1 h and then further incubated for 30 min with a range of IL-3 (Fig. 3A) or GM-CSF (Fig. 3B) concentrations.

The addition of IL-3 to cytokine-deprived cells induced an increase in OMG uptake, with maximum stimulation occurring at 6 nM IL-3 (Fig. 3A). Similarly, the addition of GM-CSF to cytokine-deprived cells induced an increase in OMG uptake at 2 nM GM-CSF (Fig. 3B).

To investigate the time course of the responses to IL-3 and GM-CSF withdrawal and re-addition, cells were incubated for a further 90 min in the presence of 6 nM IL-3 or 2 nM GM-CSF. Addition of IL-3 caused an increase in the rate of OMG transport at 30 min (Fig. 3C). After a 30 min treatment, there was a significant increase in the rate of transport (1.8-fold). Also, addition of GM-CSF caused an increase in the rate of OMG transport at 10 min. After a 10 min treatment, there was a significant increase in the rate of transport (2.8-fold), after a 30 min the increase was 3.5-fold (Fig. 3D).

EFFECTS OF IL-3 AND GM-CSF ON GLUT1 AND GLUT3 EXPRESSION LEVELS

Total cellular levels of GLUT1 and GLUT3 were measured in the following a 1-h period of IL-3 deprivation and subsequent incubation in the absence or presence of 6 nM IL-3 (Fig. 4A) or 2 nM GM-CSF (Fig. 4D) for periods of 30, 60, and 90 min. Cell lysates were immunoblotted using isoform-specific affinity-purified antibodies raised against GLUT1 and GLUT3 (Fig. 4A,D). The levels of each immunodetected protein, analyzed by densitometry (Fig. 4B,C,E,F) did not differ significantly between cells incubated in the absence or presence of IL-3 or GM-CSF.

EFFECTS OF IL-3 AND GM-CSF ON THE SUBCELLULAR DISTRIBUTION OF GLUT1 AND GLUT3

To investigate if the turnover of cell surface transporters might be regulated by IL-3 or GM-CSF in HEK293 cells, the effects of IL-3 or GM-CSF withdrawal and re-addition on the subcellular distribution of GLUT1 and GLUT3 transporters were qualitatively analyzed by immunofluorescence using confocal microscopy.

Immunofluorescence analysis using a GLUT1-specific antibody revealed that after a 1-h period of IL-3 deprivation, GLUT1 was located both intracellularly and to a lesser extent on the cell surface (Fig. 5A). The re-addition of IL-3 to deprived cells caused a substantial decrease in the intensity of this intracellular staining for GLUT1 and increased staining for this transporter at the cell surface at 30 min (Fig. 5B). Similar analysis using a GLUT3-specific antibody showed that this transporter was mainly located in intracellular structures during IL-3 deprivation and re-addition of IL-3 to deprived cells (Fig. 5A). The effect of GM-CSF on the subcellular distributions of GLUT1 and GLUT3 was analyzed using immunofluorescence analysis. Results using a GLUT1-specific antibody revealed that after a 1-h period of GM-CSF deprivation GLUT1 was located on cell surface and intracellular compartment.

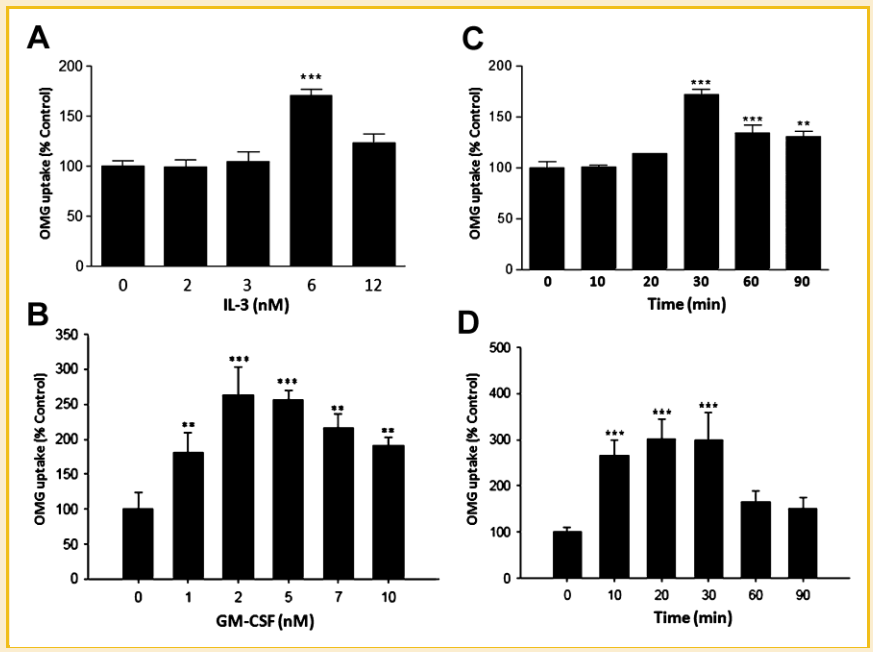

Fig. 3. Effect of IL-3 and GM-CSF on OMG transport in HEK293 cells. A: Cells were washed for 1 h to remove cytokines from culture medium and then incubated for further 30 min with a range of IL-3 concentration (0–12 nM). Transport of 0.5 mM OMG was measured at 20°C for 15 s. B: Cells were incubated for further 30 min with a range of GM-CSF concentrations (0–10 nM). Transport of 0.5 mM OMG was measured at 20°C for 15 s. C: Cells were incubated at the time points indicated in presence of 6 nM IL-3. Transport of 0.5 mM OMG was measured at 20°C for 15 s. D: Cells were incubated at the time points indicated in presence of 2 nM GM-CSF. Transport of 0.5 mM OMG was measured at 20°C for 15 s. Results are the mean ± SE of three experiments, in each of which measurements were performed in triplicate. The asterisks indicate statistically different values from the control, $^{**}P < 0.01$, $^{***}P < 0.001$.

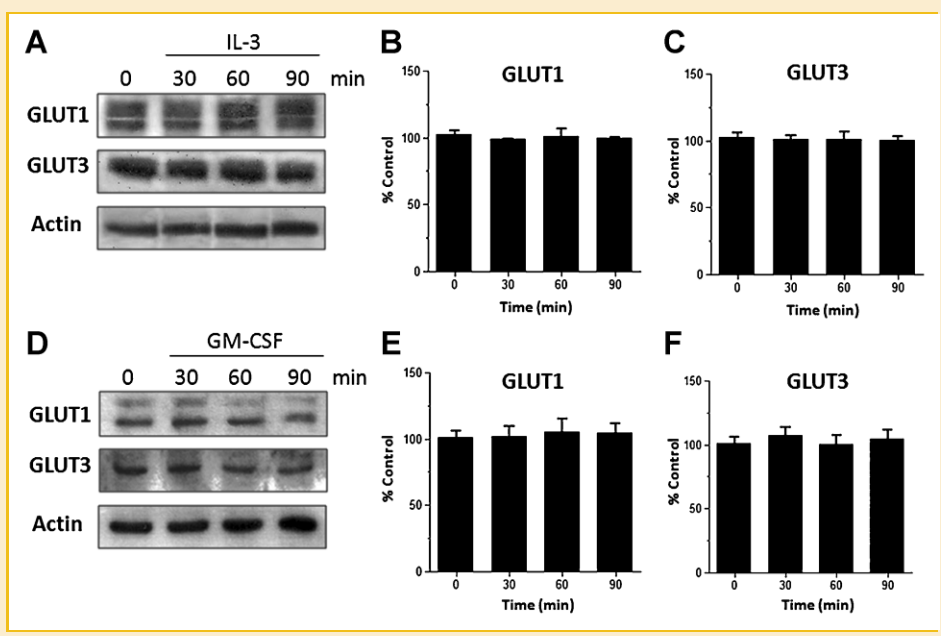

Fig. 4. Effect of IL-3 and GM-CSF on total levels of GLUT1 and GLUT3 in HEK293 cells. A: HEK293 cells were washed for 1 h and then incubated in the absence (0 min) or presence (30, 60, 90 min) of 6 nM IL-3. B: Normalized densitometry scans of effect of IL-3 on GLUT1 levels. C: Normalized densitometry scans of effect of IL-3 on GLUT3 levels. D: HEK293 cells were washed for 1 h and then incubated in the absence (0 min) or presence (30, 60, 90 min) of 2 nM GM-CSF. E: Normalized densitometry scans of effect of GM-CSF on GLUT1 levels. F: Normalized densitometry scans of effect of GM-CSF on GLUT3 levels. Results shown are representative of three independent experiments.

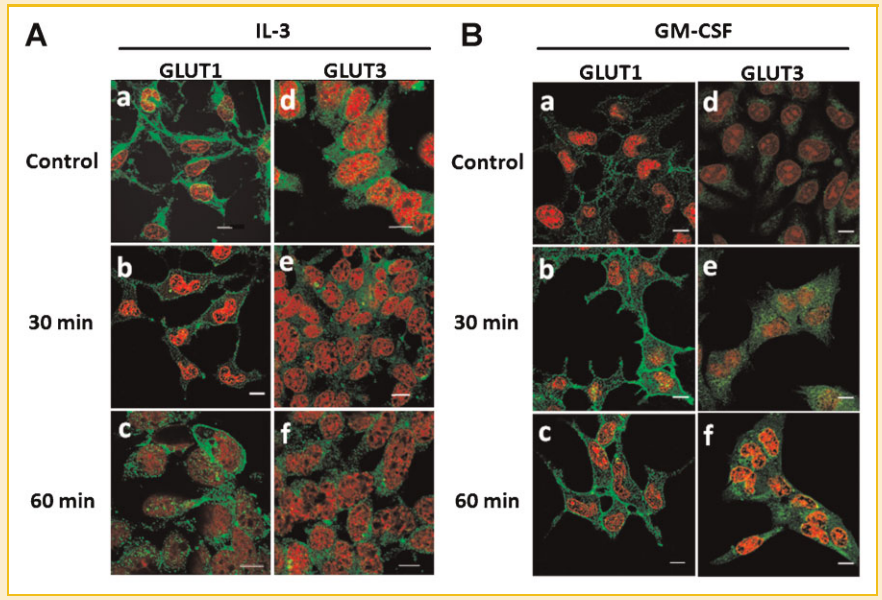

Fig. 5. Effect of IL-3 and GM-CSF on the subcellular distribution of GLUT1 and GLUT3. A: Cells were washed over a period of 1 h and then incubated for 30 or 60 min in the presence (b, c, e, and f) or absence (a and d) of 6 nM IL-3. Cells were immunolabeled with affinity-purified anti-GLUT1 (a–c) or anti-GLUT3 (d–f) and visualized using immunofluorescence microscopy. B: Cells were washed over a period of 1 h and then incubated for 30 or 60 min in the presence (b, c, e, and f) or absence (a and d) of 2 nM GM-CSF. Cells were immunolabeled with affinity-purified anti-GLUT1 (a–c) or anti-GLUT3 (d–f) and visualized using immunofluorescence microscopy. Each image is representative of three independent experiments. Scale bar, 10 μm.

The re-addition of GM-CSF to deprived cells induced an increased staining for GLUT1 at the cell surface at 30 min that was sustained after 60 min (Fig. 5B). Similar analysis using a GLUT3-specific antibody showed that this transporter was mainly located in intracellular structures during GM-CSF deprivation; the re-addition of GM-CSF to deprived cells did not alter the subcellular distribution of GLUT3.

Biotinylation reagent, Sulfo-NHS-SS-Biotin, was used to confirm that re-addition of IL-3 or GM-CSF induce a redistribution of GLUT1 in cytokine-deprived cells. Sulfo-NHS-SS-Biotin becomes covalently linked to exofacial proteins, including GLUTs, only when these transporters are present at the cell surface. Precipitation of biotinylated proteins using Immobilized NeutrAvidin Protein, followed by quantitative Western blotting for GLUT1 and GLUT3 isoforms revealed that cell surface levels of GLUT1 present in IL-3-treated cells were fourfold higher than those in IL-3-deprived cells (409% ± 20% compared with 100% ± 10%, mean ± SE, n = 3; Fig. 6A). In contrast, the cell-surface labeling of GLUT3 did not alter significantly after IL-3 re-addition.

Similarly, cell-surface levels of GLUT1 present in GM-CSF-treated cells were twofold higher than those in GM-CSF-deprived cells (192% ± 8% compared with 100% ± 10%, mean ± SE, n = 3; Fig. 6B). The cell-surface labeling of GLUT3 did not alter significantly after GM-CSF re-addition.

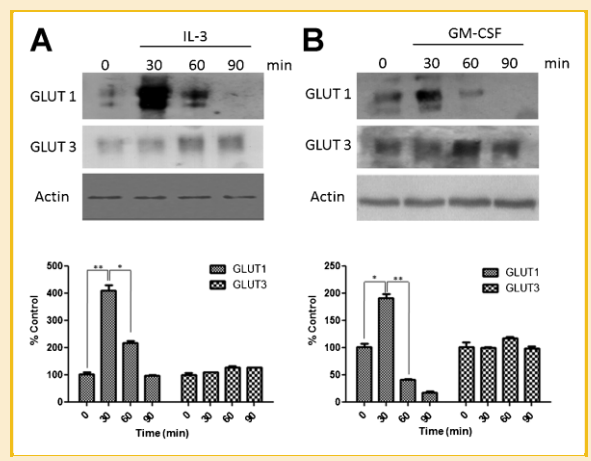

Fig. 6. Effect of IL-3 and GM-CSF on the presence of GLUT1 and GLUT3 at the surface of HEK293 cells. A: Cells were incubated for the time indicated either in the absence (0 min) or presence (30, 60, and 90 min) of 6 nM IL-3. Samples were incubated with biotinylation reagent. Biotinylated proteins were subjected to Western blotting using GLUT1 or GLUT3-specific antibodies. The histogram (bottom) shows the mean extent of labeling ± SE in these experiments measured by densitometry, relative to that in IL-3-deprived cells (100%), using actin as loading control B. Cells were incubated for the time indicated either in the absence (0 min) or presence (30, 60, and 90 min) of 2 nM GM-CSF. Samples were incubated with biotinylation reagent. Biotinylated proteins were subjected to Western blotting using GLUT1 or GLUT3-specific antibodies. The histogram (bottom) shows the mean extent of labeling ± SE in these experiments measured by densitometry, relative to that in GM-CSF-deprived cells (100%). Results shown are representative of three independent experiments. The asterisks indicate statistically different values, $^{*}P < 0.05$, $^{**}P < 0.001$.

PI 3-KINASE/Akt PATHWAY ACTIVATION BY IL-3 AND GM-CSF INDUCE GLUCOSE TRANSPORT

IL-3 and GM-CSF have been reported to induce the activation of PI 3-kinase/Akt pathway in hematopoietic cell lines and it has been suggested that this pathway is involved in regulation of glucose

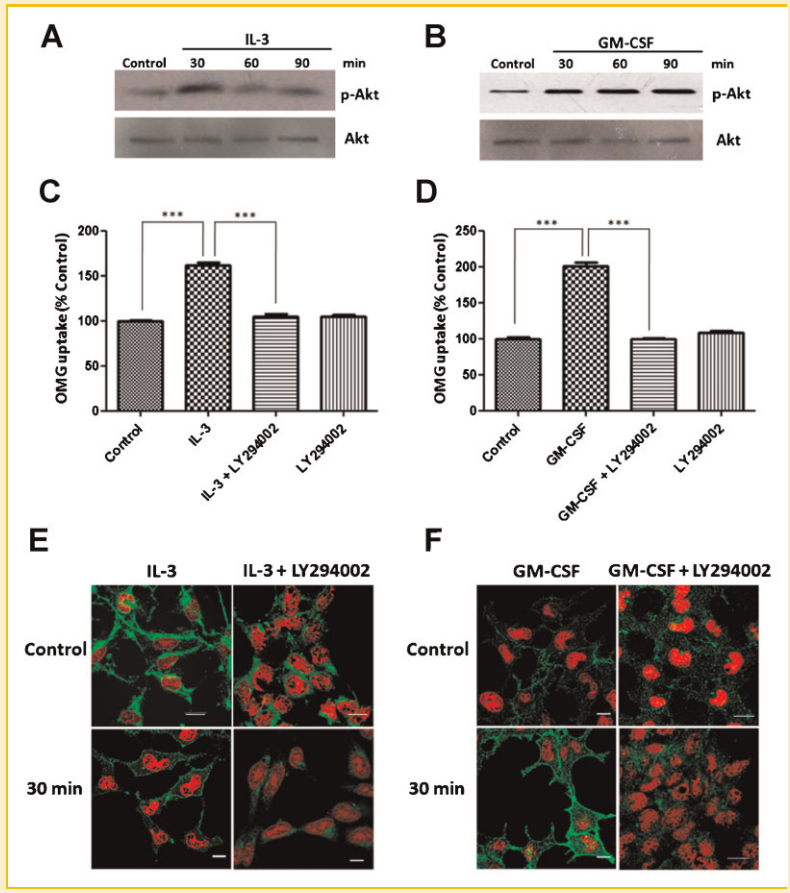

Fig. 7. Involvement of PI 3-kinase/Akt pathway in IL-3 or GM-CSF-induced stimulation of glucose transport. A: Western blot analysis shows Akt phosphorylation in HEK293 cells treated with 6 nM IL-3 for the indicated times. B: Western blot analysis shows Akt phosphorylation in HEK293 cells treated with 2 nM GM-CSF for the indicated times. C: IL-3-depleted cells were incubated for 30 min with or without 6 nM IL-3 in the presence or absence of 50 μm LY294002 and then assessed by OMG transport assay. D: GM-CSF-depleted cells were incubated for 30 min with or without 2 nM GM-CSF in the presence or absence of 50 μm LY294002 and then assessed by OMG transport assay. Results shown are representative of three independent experiments. The asterisks indicate statistically different value, $^{***}P < 0.001$. E: IL-3-depleted cells were incubated for 30 min with or without 6 nM IL-3 in the presence or absence of 50 μm LY294002 and then assessed by immunofluorescence analyses. F: GM-CSF-depleted cells were incubated for 30 min with or without 2 nM IL-3 in the presence or absence of 50 μm LY294002 and then assessed by immunofluorescence analyses.

transport in those cells. To demonstrate that the PI 3-kinase/Akt pathway is activated by IL-3 and GM-CSF in HEK293, Western blot analyses were carried out using specific antibodies against p-Akt and total Akt. As shown in Figure 7A, treatment with 6 nM IL-3 led to increased phosphorylation of Akt at 30 min. Also treatment with GM-CSF led to increased phosphorylation of Akt at 30 min and this phosphorylation remained elevated for 90 min (Fig. 7B).

To determine whether Akt activation participates in IL-3-induced increase in OMG uptake in HEK293, we used a specific inhibitor of PI 3-kinase, LY2940002, which is highly selective for PI 3-kinase inhibition. HEK293 cells were pretreated with 50 μM LY2940002 for 30 min prior to addition of 6 nM IL-3. As shown in Figure 7C, pre-treatment of cells with LY2940002, totally abolished the increase in OMG uptake induced by IL-3. The same results were obtained when cells were pre-treated with 50 μM LY2940002 for 30 min prior to addition of 2 nM GM-CSF (Fig. 7D). These data strongly suggested that IL-3 and GM-CSF-mediated GLUT1 translocation was regulated by PI 3-kinase/Akt pathway. Additionally, immunofluorescence analysis demonstrated the direct relationship between PI 3-kinase and GLUT 1 re-localization. Figure 7 (panels E and F), shows that pre-treatment with 50 μM LY2940002 inhibited GLUT1 re-localization. These cells present a cytoplasmic pattern of GLUT1 in the presence of LY2940002.

DISCUSSION

In this study, we demonstrated that IL-3 and GM-CSF receptors were expressed in HEK293 cells and led to increased glucose uptake. These increases involved the subcellular redistribution of GLUT1 transporter and it was dependent on PI 3-kinasee/Akt activation.

The cytokines, IL-3 and GM-CSF are important regulators which exhibit pleiotropic activities. They are expressed in hematopoietic cells as well in several non-hematopoietic cell types [Morstyn and Burgess, 1988; Farrar et al., 1989; Nicola, 1989; Zambrano et al., 2001; Vilanova et al., 2003; Rauch et al., 2004], and in both hematopoietic and non-hematopoietic cells, it has been suggested

that they have an important role in cell survival [Kamegai et al., 1990; Zambrano et al., 2007].

Here, we demonstrated the expression of α and β subunits of both IL-3 and GM-CSF receptors in HEK293 cells. We have indicated in previous work that IL-3 and GM-CSF receptors are expressed in different non-hematopoietic cells, but the role of these receptors are yet unclear. IL-3 receptor is expressed in male germ cells, mouse testicular Leydig cells, the central cholinergic neurons, and in both the hippocampus and cerebral cortex [Tabira et al., 1998; Rauch et al., 2004; Zambrano et al., 2007]. Also, GM-CSF receptor is expressed in placental trophoblasts, endothelial cells, oligodendrocytes, neurons in the central and peripheral nervous system and male germ cells [Bussolino et al., 1989; Gearing et al., 1989; Baldwin et al., 1993; Vilanova et al., 2003; Rodriguez-Gil et al., 2007], and some tumors [Spielholz et al., 1995; Rivas et al., 1998]. However, it is important to clarify if these receptors have similar mechanisms to insulin increasing glucose uptake or other nutrients such as vitamin C, relevant in cell metabolism and survival. In general, growth factors play critical roles to promote cell metabolism, sustain viability, and allow cell growth. These factors control cellular growth and survival by regulating nutrient uptake from the extracellular milieu. In hematopoietic cells, growth factors such as IL-3 can provide critical signals to promote glucose uptake [Rathmell et al., 2001; Bentley et al., 2003]. GM-CSF signaling for increased uptake of glucose and vitamin C has been demonstrated in human host defense cells [Vera et al., 1998] and in mouse bone marrow cells. IL-3 also increases glucose transport in myeloid cells [Berridge and Tan, 1995]. This shows that GM-CSF and IL-3 exhibit overlapping biological activities in hematopoietic cells due to a similar pattern of receptor expression.

Our results demonstrated that HEK293 cells expressed functional facilitative GLUTs, specifically GLUT1 and GLUT3 as previously suggested [Castro et al., 2008]. According to Eadie–Hofstee plot, we observe only one functional component but because glucose affinities of isoforms 1 and 3 are very similar, the observe K_m may correspond to GLUT1 and GLUT3 function [Maher et al., 1996; Vannucci et al., 1997]. Interestingly, here we demonstrated that IL-3 and GM-CSF induced a clear increase in glucose uptake by HEK293 cells. The addition of 6 nM IL-3 to cytokine-deprived cells induces an increase in glucose uptake at 30 min. Also, the addition of 2 nM GM-CSF induced an increase in glucose uptake at 10 min.

Previously, we have shown that non-hematopoietic cells, such as bovine spermatozoa, expressed GM-CSF receptors that signal for increased transport of glucose [Zambrano et al., 2001]. Interestingly IL-5, which belongs to the IL-3/GM-CSF receptor family, failed to induce an increase in hexoses uptake in bull spermatozoa. Experiments using HL-60 eosinophils that express functional IL-3 and IL-5 receptors confirmed that IL-3, but not IL-5, signaled for increased hexose uptake [Rauch et al., 2004]. GM-CSF has been shown to enhance glucose uptake in melanoma cells [Spielholz et al., 1995] and *Xenopus laevis* oocytes [Ding et al., 1994]. In all of the above cases, the mechanism induced by these cytokines resulting in glucose uptake has been poorly described.

It has become clear that growth factors play prominent roles in regulating glucose uptake and metabolism [de Groot et al., 1998; Rathmell et al., 2000; Plas et al., 2001; Vander Heiden et al., 2001; Rathmell et al., 2003]. Normally, when cells are withdrawn from growth factors, GLUT1 is internalized and degraded in lysosomes, leading to decreased glucose uptake and metabolism prior to cell death [Edinger et al., 2003; Wieman et al., 2007]. In many cell types, including lymphocytes, glucose transport into the cell is determined by the level of the GLUT1, present on the cell surface [Chakrabarti et al., 1994]. The expression of GLUT1 has been shown to be controlled by both cytokine- and T-cell receptor-mediated survival signals in lymphocytes [Rathmell et al., 2000]. GLUT1 trafficking can be regulated, because insulin signaling has been shown to affect surface GLUT1 levels in hematopoietic cells [Plas et al., 2001; Rathmell et al., 2003].

Overexpression of GLUT1 can significantly delay the onset of apoptosis in response to growth factor withdrawal, indicating that intracellular glucose availability is an important determinant in the commitment to programmed cell death [Vander Heiden et al., 2001].

In non-hematopoietic cells, the translocation of the GLUT4 to the cell surface is downstream of signals from cell-surface receptors [Rea and James, 1997]. Although growth factor-stimulated synthesis of GLUT1 has been shown to affect surface GLUT1 levels in hematopoietic cells [Plas et al., 2001; Rathmell et al., 2003] a role for non-insulin growth factors in regulation of GLUT1 activity and trafficking has not yet been directly determined. Here, we have shown that IL-3 and GM-CSF caused translocation of intracellular GLUT1 transporters to the cell surface, whereas a second transporter isoform, GLUT3, remained predominantly intracellular. It is important to highlight that there is no change in GLUT1 and GLUT3 expression in cells treated with IL-3 or GM-CSF. We had similar results using cycloheximide to check the novo protein synthesis (data not shown) These finding are consistent with other studies where glucose stimulation has been investigated in IL-3-dependent murine hematopoietic cells, where IL-3 stimulates GLUT1 translocation from an intracellular compartment to the cell surface [Bentley et al., 2003]. Similar effects of IL-3 in maintaining cell-surface protein levels have been reported for transporters of other nutrients, including amino acids, iron, and low-density lipoproteins [Edinger and Thompson, 2002].

A variety of oncogenes, including Akt [Thompson and Thompson, 2004] promote glucose metabolism. Akt has been shown to phosphorylate and inactivate several proapoptotic mediators such as BAD, caspase 9, and the Forkhead transcription factors [Datta et al., 1999; Testa and Bellacosa, 2001]. The prosurvival function of activated Akt is dependent in part on its stimulatory effect on glucose metabolism because Akt-mediated cellular survival is decreased in medium containing limiting levels of glucose [Gottlob et al., 2001; Plas et al., 2001].

Several mechanisms may promote cytokine-mediated regulation of GLUT1 trafficking; PI 3-kinase and its downstream effector kinase Akt/PKB have a well-established role on GLUT4 vesicule trafficking to the cell membrane in response to insulin [Welsh et al., 2005].

IL-3 and GM-CSF rapidly activate PI 3-kinase in a tyrosine phosphorylation-dependent manner. There is a direct link between IL-3-stimulated βc serine phosphorylation, PI 3-kinase activation, and hematopoietic cell survival [del Peso et al., 1997; Stomski et al., 1999; Guthridge et al., 2000]. The data presented in this paper showed that IL-3-induced activation of PI 3-kinase/Akt pathway in

HEK293 cells and Akt phosphorylation was sustained for 30 min. GM-CSF also induced the activation of this pathway and Akt phosphorylation was sustained for over 90 min in HEK293 cells. It is important to emphasize that both IL-3- and GM-CSF-induced activation of Akt phosphorylation was fast (in 5 min) and this increase was sustained over a period of time in HEK293 cells (data no shown).

Additionally, IL-3 and GM-CSF-induced transport of GLUT1 was prevented by the PI 3-kinase inhibitor LY294002, indicating that PI 3-kinase was important in IL-3- and GM-CSF-induced glucose uptake.

These finding are consistent with other studies that have demonstrated that the translocation of GLUT4 and GLUT1 to the adipocyte plasma membrane also stems from stimulation of PI 3-kinase activity by insulin [Arai et al., 2002; Khan and Pessin, 2002].

The downstream effectors of PI 3-kinase/Akt involved in regulation of GLUT1 trafficking remain unclear. It has been suggest that in hematopoietic cells, IL-3 regulated GLUT1 through PI 3-kinase and its downstream effector, Akt via activation of mTOR, inactivation of GSK3, and other pathways to control GLUT1 activity, recycling, and internalization [Wieman et al., 2007].

In the present study, we have shown that IL-3 and GM-CSF are able to maintain GLUT levels at the cell surface in HEK293 cells. Here, we showed that the growth factors, IL-3 and GM-CSF, promote glucose uptake in part by redistribution of GLUT1 on the cell surface in non-hematopoietic cells. Also the data directly demonstrated that the PI 3-kinase/Akt pathway is an important mediator of this process. Altogether these results strongly suggest that the subcellular redistribution of GLUTs could be a general mechanism induced by IL-3 and GM-CSF to support viability and cell growth.

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