Glucose regulates insulin mitogenic effect by modulating SHP-2 activation and localization in JAr cells.

Giuseppe Bifulco*, Costantino Di Carlo*, Matilde Caruso§, Francesco Oriente§, Attilio Di Spiezo Sardo*, Pietro Formisano§, Francesco Beguinot§ and Carmine Nappi*

*Dipartimento di Ginecologia, Ostetricia e Fisiopatologia della Riproduzione Umana and §Dipartimento di Biologia e Patologia Molecolare e Cellulare "L. Califano", Università degli Studi di Napoli "Federico II", Naples, Italy

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Corresponding author:
Dr. Giuseppe Bifulco
Via Pansini 5 - 80131 Napoli
ITALY
Tel.: +39-81-7462979
Fax: +39-81-7463865

E-mail: giuseppebifulco@hotmail.com
ABSTRACT

Glucose effect on cell growth has been investigated in the JAr human choriocarcinoma cells. When JAr cells were cultured in the presence of 6 mM glucose (LG), proliferation and thymidine incorporation were induced by serum, epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I), but not by insulin. At variance, at 25 mM glucose (HG), proliferation and thymidine incorporation were stimulated by insulin, serum, EGF and IGF-I, to a comparable extent, while basal levels were 25% lower than those in LG. HG culturing also enhanced insulin-stimulated insulin receptor (IR) and insulin receptor substrate 1 (IRS1) tyrosine phosphorylations while decreasing basal phosphorylations. These actions of glucose were accompanied by an increase in cellular tyrosine phosphatase activity. The activity of SHP-2, in HG-treated JAr cells, was 400% of that measured in LG-treated cells. SHP-2 co-precipitation with IRS1 was also increased in HG-treated cells. SHP-2 was mainly cytosolic in LG-treated cells. However, HG culturing largely redistributed SHP-2 to the internal membrane compartment, where tyrosine phosphorylated IRS1 predominantly localizes. Further exposure to insulin rescued SHP-2 cytosolic localization, thereby preventing its interaction with IRS1. Antisense inhibition of SHP-2 reverted the effect of HG on basal and insulin-stimulated IR and IRS1 phosphorylation as well as that on thymidine incorporation. Thus, in JAr cells, glucose modulates insulin mitogenic action by modulating SHP-2 activity and intracellular localization.
INTRODUCTION

Glucose controls a variety of cellular functions. Changes in the extracellular concentrations of glucose may determine either increased cell proliferation or cell death (1-3). For instance, beta-cell mitogenesis is stimulated by glucose through a protein kinase C-dependent mechanism (4,5). At variance, in human endothelial cells, high glucose concentrations activate JNK signalling and trigger apoptosis (6). Previous evidence indicates that glucose modulates the transduction pathways of many growth factors (7-13). However, the mechanisms responsible for glucose control of growth signalling have not been extensively investigated.

Chronic abnormalities in glucose concentration in the extracellular fluids may lead to impaired tissue homeostasis (14). High blood glucose levels in pregnant women with diabetes often causes placenta hypercellularity, resulting in larger and heavier placentas (15). There is evidence that placenta growth is regulated by glucose as well as by growth factors (16-20). In vitro, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1) and transforming growth factor α (TGFα) stimulate proliferation of cultured human cytотrophoblast cells and their ability to produce human chorionic gonadotropin and progesterone (21). Insulin also induces proliferative responses in placenta, likely due to the activation of Insulin Receptor Substrate (IRS)/Mitogen Activated Protein Kinase (MAPK) pathway (20). In several cell types, insulin signalling is regulated by glucose at different levels (7,22). But, whether and how glucose affects insulin mitogenic signalling in placenta is unclear.

In the present work, we have addressed this issue by investigating glucose regulation of insulin action in the JAr human choriocarcinoma cell line. We show that, in this placenta cell model, glucose regulates insulin signalling via the IRS1/MAPK pathway and mitogenesis by modulating
the activity and subcellular localization of the SHP-2 tyrosine phosphatase.
MATERIALS AND METHODS

Materials - JAr and BeWo cell lines were purchased from the American Type Culture Collection (Rockville, MD). Media and sera for cell culture were purchased from Sigma (St. Louis, MO). Protein A-Sepharose beads were from Pierce (Rockford, IL). Radiochemicals, western blot and ECL reagents were from Amersham Pharmacia Biotech (Priscataway, NJ). Monoclonal phosphotyrosine and monoclonal IRS1 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY) and the mAb3IR antibody from Oncogene Science (Manhasset, NY). The P-MAPK antibody was purchased from New England Biolabs (Beverly, MA). MAPK, SHP-2, PTP1B and LAR antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The SHP-2 sense and antisense oligonucleotides have been previously described and characterized (23), and were synthesized by PRIMM s.r.l (Milan, Italy). Lipofectamine and Optimem were purchased from Life Technology, Inc. All other reagents were from Sigma (St. Louis, MO).

Cell culture and transfection and cell subfractionation - JAr, BeWo and 3T3 were routinely grown at 37°C with 95% air, 5% CO2 in DMEM medium, supplemented with 10% fetal bovine serum (FBS). Transient transfection experiments were performed with the Lipofectamine method according to the manufacturer’s instruction. Briefly, 50-80% confluent JAr cells were washed twice with Optimem and incubated for 8 hours with 10 µg of SHP-2 antisense oligonucleotides or with an equal amount of control oligonucleotides. The medium was replaced with DMEM supplemented with 10% fetal calf serum and the cells were further incubated for 6 hours before being assayed. Cells were incubated in Hepes buffer (HB) containing 12.5 mM Hepes, pH 7.4, 120 mM NaCl, 6mM KCl, 1.2 mM MgSO4, 1mM CaCl2, 1mM Na2HPO4 and 20 mM Hepes, supplemented with either 6 mM or 25 mM glucose for 48 hours. Insulin (100 nM) was then added in the last 10 min of incubation, where indicated. Cells were washed two times with HB and homogenized in 20 mM...
Hepes, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 1 mM phenylmethlysulfonyl fluoride, 1 μM leupeptin, pH 7.4. Subcellular fractions were prepared by a modification of the methods of Simpson (24). Briefly, the cells were washed with ice-cold buffer A (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 255 mM sucrose, 1 mM PMSF, 10 mM NaF, 100 μM Na3VO4, 1 mM NaP2O7, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and broken in ice-cold buffer A by passing through a 27-gauge needle and centrifuged at 800 x g for 5 min at 4°C to pellet nuclei. Supernatants were centrifuged at 16,000 x g for 20 min at 4°C. Pellets were applied on a 1.12 M sucrose cushion and centrifuged at 212,000 x g for 70 min for preparation of the plasma membrane fraction. Supernatants were further centrifuged at 212,000 x g for 20 min for preparation of the internal membrane (pellet) and cytosol (supernatant) fractions (24). Purity of the subcellular fractions was assessed by western blot analysis of cytochrome c oxidase (internal membrane), tubulin (cytosol) and by determination of 5'-nucleotidase activity (plasma membrane) according to (25), (26), and (27), respectively.

3[H]thymidine incorporation – For this assay, the cells were plated in 24-well plates (10^5 cells/well) in DMEM supplemented with 10% FBS. Upon 12h, the medium was substituted with glucose free DMEM supplemented with 0.25% BSA for 16h. The cells were then incubated in the medium supplemented with 6 mM (LG) or 25 mM (HG) glucose with or without insulin (100 nM), IGF-1 (100 nM), EGF (100 ng/ml), FBS (10%) for the indicated times. In control experiments the cells were incubated in glucose free DMEM containing 6 or 25mM sucrose or fructose. 5 μCi/ml 3[H]-thymidine were added and the incubation was prolonged for 4 more hours. The cells were then lysed in 1N NaOH and radioactivity was counted by liquid scintillation counting.

Cell proliferation and DNA fragmentation assay – For proliferation assay, JAr cells were plated in
24-well multiplates (2 x 10⁴ cells/plate) in DMEM supplemented with 10% FBS. 12 h later, this medium was replaced with serum-free DMEM containing 0.25% BSA. Upon 12 additional hours, the medium was substituted with DMEM containing either 6 or 25 mM glucose and growth factors for the indicated times. For DNA fragmentation analysis, JAr cells were deprived from serum and glucose for 12 h and then incubated for 48 h in DMEM containing 6 or 25 mM glucose in the absence or the presence of 100 nM insulin and 25 ng/ml TNFα. Cells (2 x 10⁶) were lysed in 0.5 ml lysis buffer (10 mM Tris, pH 7.5, 0.6% SDS, 10 mM EDTA). The RNase solution was added to a concentration of 15 µg/ml, and cell lysates were incubated for 20 min at 37°C. NaCl was then added to 1 M (final concentration) followed by incubation at 4°C for 2 h. Samples were centrifuged at 14,000 x g (4°C for 30 min), and supernatant DNA was extracted by phenol-chloroform and ethanol precipitation at -20°C overnight. Upon centrifugation at 14,000 x g, the DNA pellet was air dried and dissolved in 20 µl TE buffer (10 mM Tris, 10 mM EDTA). Identical amounts of DNA were electrophoresed on 1.5% agarose gels containing 0.5 µg/ml ethidium bromide and visualized by UV light as described in (28).

**Insulin receptor binding and insulin degradation** - Insulin receptor binding was performed by radioreceptor assay as described in (29). For this assay, JAr cells were deprived from serum and glucose for 16 h and then incubated in DMEM supplemented with 6 or 25 mM glucose for 48, 96 or 128 h. The cells were then incubated for 12 h at 4°C in 1 ml of binding buffer (pH 7.8, 25 mM Tris-HCl, 120 mM NaCl, 1.2 mM MgSO₄, 2.5 mM KCl, 2% BSA, 1 mg Bacitracin) containing 20,000 cpm ¹²⁵[I]-Insulin. Non specific binding was determined in the presence of 8.5 x 10⁻⁵ insulin and was subtracted from total bound radioactivity to yield specific binding. Binding data were analysed using the LIGAND program for curve fitting and parameter estimation (30). Insulin degradation
was determined by TCA precipitation as described in (31).

**IRandIRS1 phosphorylation**—For IR phosphorylation studies, JAr cells were deprived from serum and glucose for 12 h and then incubated in DMEM supplemented with 6 or 25 mM glucose for 48 h. Cells were then stimulated with 100nM insulin for 10 min and solubilized in 1% Triton X-100, 50 mM Hepes, pH 7.5, 150 mM NaCl, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride. The insoluble material was separated by ultracentrifugation at 100,000 x g for 1 h at 4°C. The supernatant was applied to a wheat germ agglutinin (WGA)-Sepharose column pre-equilibrated with buffer containing 0.1% Triton X-100, 50 mM Hepes, pH 7.5, 150 mM NaCl and the protease inhibitors described above. The column was washed using the same buffer, and bound glycoproteins were eluted in the same buffer containing 0.3 M N-acetylglucosamine. For IRS1 phosphorylation studies, the cells were lysed in 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% Glycerol, 10 mM EDTA, 10 mM Na$_4$P$_2$O$_7$, 1 mM Na$_3$VO$_4$, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride (TA buffer) containing 1% Triton X-100. Cell extracts were then blotted with IR, IRS1 or phosphotyrosine antibodies or precipitated with IR and IRS1 specific antibodies followed by western blotting with phosphotyrosine antibody as described in (32).

**MAPK phosphorylation and PTPase assay**—JAr cells were incubated in 6 or 25 mM glucose and stimulated with insulin as described above. The cells were then lysed in TA buffer supplemented with 0.1% Triton-X-100 (TAT). Lysates were separated on 12% SDS-PAGE, transferred on nitrocellulose filter and then blotted with phospho-MAPK or MAPK antibodies according to (33). Alternatively, to determine protein tyrosine phosphatase activity (PTPase) the cells were incubated with or without 2 mM sodium vanadate in phosphate-free buffer for 20 min at 37°C, according to
Incubation with 100 µM pervanadate instead of 2mM vanadate yielded identical results. The cells were lysed in 0.5% Triton-X-100 and then immunoprecipitated with IR, IRS1 and SHP-2 specific antibodies. Pellets were resuspended in 50 mM Hepes, pH 7.0 and reactions initiated by the addition of 20 mM p-nitrophenyl phosphate (pNPP) at 37°C for 15 min. Reactions were stopped with 1N NaOH and release of p-nitrophenol was spectrophotometrically quantitated at 410 nm. PTPase (vanadate-sensitive activity) was expressed as the difference between the total phosphatase activity (measured in the absence of vanadate) and the vanadate-resistant activity.

**PTPs IRS1 association** – JAr cells were incubated in 6 or 25 mM glucose and stimulated with insulin as described above. Cells were then lysed with TAT buffer and extracts incubated with sepharose-bound IR or IRS1 antibodies at 4C for 3 h. The beads were washed with HNT buffer (TA containing 0.1% Triton X-100) and bound proteins released by heating at 65°C for 5 min with SDS sampling buffer (4% SDS, 10% glycerol, 100 mM Tris pH 6.8, 1 mM EDTA, 10 mM dithiothreitol, 8M urea). Released proteins blotted with SHP-2, PTP1B or LAR antibodies and revealed by ECL and autoradiography.
RESULTS

Glucose action on cell proliferation in JAr cells. We investigated growth factor effect on DNA synthesis in JAr cells cultured in the presence of either 6 mM (LG) or 25 mM glucose (HG). At 6 mM glucose, IGF-1 (100 nM), EGF (100 ng/ml), and FBS (10%) caused a time-dependent increase of thymidine incorporation in JAr cell nuclei (Fig. 1, panel A). At variance, insulin (100 nM) had no effect in stimulating thymidine incorporation. Culturing the cells in HG determined a 25% decrease in thymidine incorporation as compared to LG (p<0.05) (Fig. 1, panel C). However, in HG cells, insulin increased thymidine incorporation by 350% upon 96h of incubation (p<0.01) (Fig. 1, panel B). The permissive effect of glucose on insulin-induced thymidine incorporation was time-dependent and not mimicked by either sucrose (6-25 mM) (Fig. 1, panels A, B) or fructose (data not shown). In addition, HG culturing of the cells induced EGF-, IGF-1-, and FBS-dependent DNA synthesis 12h earlier compared to LG (Fig. 1, panels A, B).

Glucose modulation of insulin action was dose-dependent (Fig. 1, panel D). A significant increase in insulin-stimulated thymidine incorporation became detectable upon incubation of the cells with 18 mM glucose achieving a maximum at 25 mM glucose. As with thymidine incorporation, insulin effect on cell proliferation was only observed when the cells were cultured with HG and not with LG (Fig. 2, panel A,B). Also, raising glucose concentration from 6 to 25 mM, decreased basal cell proliferation by 20% (p < 0.05) (Fig. 2, panel C). Again, no significant difference of EGF-, IGF-1- and FBS-induced cell proliferation was observed at 6 and 25 mM glucose (Fig. 2, panels A,B). Thus, glucose specifically enabled insulin mitogenic effect in JAr cells and slightly reduced basal proliferation.

In different cell types, glucose affects apoptosis (35,36). However, no DNA laddering was observed in JAr cells cultured with LG or HG, either in the absence or in the presence of insulin (data not shown).
Glucose action on insulin mitogenic signalling. To explore the mechanism of glucose action on insulin mitogenesis, we investigated insulin binding and early signalling events in JAr cells cultured with different glucose concentrations. Based on Western blotting with IR Ab, IR protein content was almost identical in LG- and HG-cultured JAr cells (Fig. 3A, top panel). Binding levels and Kds also did not show significant differences in cells maintained in LG and in HG (Table I).

Upon blotting with phosphotyrosine Abs (pTyr), extracts of cells cultured in 6 mM glucose showed a 95 kDa band corresponding to IR β subunit (Fig. 3A, middle panel). The intensity of this band increased by 35% upon exposure of the cells to insulin. Cell culturing in the presence of 25 mM glucose decreased basal IR tyrosine phosphorylation by 30% compared to LG cells (p< 0.05), but resulted in a 300% increase in insulin-dependent phosphorylation (p<0.01). Similar changes were observed by analysing anti-phosphotyrosine blots of IR precipitates of partially purified receptor preparations from LG and HG cultured cells (Fig 3A, bottom panel and bargraph). At variance with the IR, neither glucose nor insulin induced any change in IGF1 receptor (IGF-1-R) phosphorylation (Fig. 3, panel B). IGF1 stimulated to comparable extent IGF-1-R phosphorylation both in LG- and HG-cultured cells. In addition, culturing JAr cells in the presence of LG or HG caused no change in their ability to degrade insulin (Fig. 3, panel C). This finding suggested that the differences in insulin action observed in LG and in HG were not due to changes in the ability of the cells to degrade insulin.

To further address the molecular mechanism of glucose effect on insulin action, we next investigated phosphorylation of IRS1 in the JAr cells. As shown in Fig. 4 (panel A), insulin-independent IRS1 phosphorylation was 50% lower in HG- as compared to LG-treated cells (p< 0.05). However, insulin increased IRS1 phosphorylation by 400% of control in HG-cultured cells (p< 0.01) but elicited no significant effect in LG cells. The differences in IRS1 phosphorylation in cells cultured in LG and HG were accompanied by similarly-sized differences in MAPK
phosphorylation (Fig. 4, panel B). There were no changes in IRS1 and MAPK protein levels in LG and HG cells (Fig 4, panel C).

**Effect of glucose on protein-tyrosine phosphatase activity.** We next investigated whether glucose effect on IR and IRS1 phosphorylation is mediated by changes in cellular tyrosine phosphatase (PTPase) activity. As shown in Table II, exposure to HG increased PTPase activity in JAr cell extracts by almost 300% (p<0.01). Insulin, inhibited PTPase activity in LG cells by about 30% (not statistically significant) and almost completely abolished the effect of HG (p<0.05). PTPase activity was also measured in IR and IRS1 immunoprecipitates from the cells. Neither glucose nor insulin affected the IR-co-precipitated PTPase, however. At variance, IRS1-associated PTPase activity was 350% higher in HG than in LG cells (p < 0.01) and was blocked by insulin stimulation.

To verify whether specific PTPase(s) are involved in glucose regulation of IR signalling we evaluated the expression of SHP-2, PTP1B and LAR in JAr cells. All of these PTPases are involved in insulin signalling (37-39) and, as shown in Fig. 5 (panel A), all are expressed in the JAr cells. Neither glucose nor insulin caused any change in the expression levels of these PTPases. Interestingly, in HG cultured cell extracts but not in those from LG cells, SHP-2 co-precipitated with IRS1 (Fig. 5, panel B). Further exposure of HG-treated cells to insulin completely abolished IRS1-SHP-2 co-precipitation. No IR-SHP-2 co-precipitation was detected either in HG or in LG cells. LAR and PTP1B association with IRS1 was barely detectable in JAr cells and was not modified by glucose or insulin treatment (Fig. 5, panel C). As shown in Fig. 6 (panel B), phosphatase activity in SHP-2 immunoprecipitates was increased by 400% upon exposure of the cells to HG. Same as in the IRS1 precipitates, insulin stimulation of the cells completely inhibited PTPase activity in the SHP-2 precipitates.

To prove SHP-2 involvement in glucose action on insulin mitogenesis, we have transiently
transfected SHP-2 antisense oligonucleotides (SHP-2-AS) in the JAr cells. As shown in Fig. 6 (panel A), the antisense reduced SHP-2 expression by >70% (p<0.001). Consistently, SHP-2 activity was barely detectable in SHP-2 precipitates from cells transfected with SHP-2-AS but unmodified by control oligonucleotides (Fig. 6, panel A). Also, the SHP-2-AS completely inhibited SHP-2-IRS1 co-precipitation in extracts from HG-treated cells (Fig. 7A, top panel). In HG-cultured cells, block of SHP-2 expression increased insulin-independent IR and IRS1 phosphorylation, respectively, by 30% and 50% (p< 0.01). The effect of the antisense was accompanied by a 300% decrease in insulin-stimulated phosphorylations of IR and IRS1. SHP-2-AS treatment of HG cells, also returned basal and insulin stimulated thymidine incorporation to levels comparable to those observed in LG cells (Fig. 7B). SHP-2-AS had no effect on basal and insulin-dependent IR and IRS1 phosphorylation (Fig. 7A, middle and bottom panel, lanes E-F vs lanes A-B) as well as thymidine incorporation (Fig. 7B) in LG-exposed cells.

To address the tissue-specificity of glucose action on SHP-2 activity and insulin signalling, we have further compared SHP-2 activity in the choriocarcinoma BeWo cells, and in 3T3 fibroblasts. As shown in Fig. 8, HG exposure of BeWo cells increased SHP-2 activity, similarly as in JAr cells. Also, in the BeWo, as well as in the JAr cells, insulin blocked glucose activation of SHP-2. By contrast SHP-2 activity was identical in 3T3 cells whether cultured in HG or in LG and increased upon insulin exposure by 500 % of control both upon LG and HG treatment. In addition, in LG-cultured BeWo cells, insulin failed to increase DNA synthesis. HG culturing determined a 20% reduction of thymidine incorporation compared to LG, but allowed a > 300% increase in DNA synthesis. By contrast, insulin induced similar increases in thymidine incorporation in 3T3 cells both in the presence of LG and of HG and basal levels of thymidine incorporation were not modified by glucose in these cells.
Glucose effect on SHP-2 intracellular localization. To further investigate the mechanism of SHP-2 activation by glucose, we compared the intracellular localization of SHP-2 and IRS1 in JAr cells. To this end, we prepared internal membrane (IM) and cytosolic fractions (Cy) from JAr cells. The purity of the different fractions is shown in Table III. As shown in Fig. 9 (panel A), in LG-cultured cells, SHP-2 was largely cytosolic either in the absence or in the presence of insulin. At variance, HG culturing led to a prominent SHP-2 localization in the internal membrane (IM) compartment of the cells. Insulin exposure of HG-cultured cells determined translocation of SHP-2 back to the cytosol, however. Different from SHP-2, IRS1 was equally distributed in the cytosol and IM fractions of HG- as well as LG-treated cells. Upon insulin exposure, however, about 80% of IRS1 translocated from the cytosol to the IM fraction both in LG and in HG cells. Based on anti-phosphotyrosine blotting, phosphorylated IRS1 predominantly localized (> 85%) in the IM fraction (Fig. 9, panel C). Identical results were also obtained in the BeWo cells (data not shown).

Thus, in JAr and BeWo cells glucose induces translocation of SHP-2 to the IM of the cells where most tyrosine phosphorylated IRS1 localizes. Insulin stimulation of the cells simultaneously re-localizes SHP-2 to the cytosol and promotes IRS1 phosphorylation.
DISCUSSION

We have investigated glucose regulation of cell growth in the JAr human choriocarcinoma cell line. These cells maintain in culture many features characteristic of human first trimester trophoblast, including chorionic gonadotropin and progesterone secretion (21). Because of these features, JAr cells have been widely used to investigate glucose and insulin action in placenta (19,20). We found that insulin does not induce proliferation of JAr cells when these are maintained in low glucose medium. This finding is consistent with previous reports showing that, in vivo, insulin is a weak mitogenic factor in placenta when glycemic control is achieved (17,40). As recently reported by Weiss et al. (19), chronic exposure of JAr cells to high glucose concentrations slightly inhibited proliferation. In addition, we now show that high glucose induces insulin proliferative effect in JAr cells. The effect of glucose on insulin-induced mitogenesis was dose-dependent and specific for insulin since IGF-1, EGF and serum stimulated cell proliferation to the same extent both at low and at high glucose concentrations. Glucose permissive action on insulin mitogenic effect also occurred in the BeWo choriocarcinoma cell line, suggesting that high glucose concentrations might contribute to placenta hypercellularity in vivo as well.

Glucose co-operation with growth factor signalling has already been described in different cell types (5, 41). For instance, in insulinoma cells, glucose induces a dose-dependent increase of nerve growth factor effect on insulin secretion, but the mechanisms remain elusive (5, 41). However, the data in the present report show, for the first time, that extracellular glucose also controls insulin mitogenic action. Our study shows that glucose does not affect insulin receptor (IR) number or affinity in the JAr cells. Still, chronic exposure to high glucose concentrations slightly reduced the basal activation state of the IRS1/MAPK cascade but significantly increased insulin-stimulated signalling along this pathway. Thus, at least in part, glucose potentiates insulin mitogenic signalling in JAr cells by inducing the IRS1/MAPK pathway. We envisioned two possible mechanisms for
this glucose regulatory effect. Firstly, in JAr cells, glucose may affect PKC activity. These Ser/Thr kinase phosphorylate several insulin signalling molecules and inhibit their subsequent tyrosine phosphorylation and function (42,43). Hence, our previous work in muscle cells showed that glucose rapidly causes reverse translocation of PKC alpha from the plasma membrane to the cytoplasm. Reverse translocation of PKC alpha reduces PKC alpha-IR association and IR ser/thr phosphorylation and acutely activates the insulin signalling system in these cells (42). A similar mechanism is unlikely to account for glucose effect on insulin mitogenic signalling in the JAr cells, however. In fact, treatment of JAr cells with the PKC inhibitor bisindolylmaleimide does not affect IR and IRS1 phosphorylation in the presence of either low or high glucose concentrations (data not shown). Alternatively, in the JAr cells, glucose may regulate the action of tyrosine phosphatase(s) (PTPases) on key elements of the insulin signalling pathway (37,44). Consistent with this hypothesis, we show here that the activity of the SHP-2 PTPase is increased in extracts of JAr as well as in those from BeWo placenta cells cultured in high glucose medium. In addition, we have shown that SHP-2 but not PTP1B or LAR, coprecipitated with IRS1 in JAr cell extracts. SHP-2-IRS1 association only occurred in HG cultured cells. Importantly, antisense block of SHP-2 expression in HG cells returned IRS1 phosphorylation and insulin mitogenesis to levels comparable to those of cells maintained in low glucose medium indicating that SHP-2 mediates the permissive effect of glucose on insulin signalling through the IRS1/MAPK pathway.

Acute stimulation of HG-cultured cells with insulin prevented SHP-2-IRS1 co-precipitation. Thus, in JAr cells, insulin causes the release of the SHP-2-IRS1 association induced by the chronic exposure to HG levels. We propose that the SHP-2 association is responsible for the reduced IRS1 phosphorylation levels we have observed in HG-cultured JAr cells. Subsequent exposure of these cells to insulin releases the association and enables IRS1 to undergo tyrosine phosphorylation more effectively than in LG cells, which feature higher basal levels of IRS1 phosphorylation. Enhanced
phosphorylation of IR/IRS1 in HG-cultured cells, in turn, more actively conveys insulin signal through the MAPK/mitogenic pathway. Thus, by fostering SHP-2 association, glucose maintains IRS1 in a low phosphorylation state. Insulin then release the association enabling IRS1 to achieve higher levels of phosphorylation by IR kinase. The changes in IRS1 phosphorylation determined by HG exposure were paralleled by similar changes in the phosphorylation of IR kinase. At variance with IRS1 however, no glucose- and insulin-dependent IR-SHP-2 co-precipitation was detectable in the JAr cells. SHP-2 association with the IR may be weaker than with IRS1, preventing co-immunoprecipitation. In addition, previous work by Solow et al. evidenced that IRS1 may determine the activation state of the IR through a PTPase-dependent mechanism (45). Thus, HG effects on IR tyrosine phosphorylation may also reflect the regulation of IRS1 activity by SHP-2.

SHP-2 is a cytosolic PTPase (46). However, in the present report, we show that exposure of JAr cells to high glucose concentrations determines SHP-2 translocation from the cytosol to the internal membrane compartment. This compartment also hosts most of the tyrosine phosphorylated IRS1 present in the insulin-unstimulated cells, so that the SH2 domain of SHP-2 may bind IRS1 phosphotyrosine. Subsequent stimulation of the cells by insulin reverted SHP-2 translocation and re-localized most cellular SHP-2 in the cytosolic fraction. Thus, chronic exposure of JAr cells to high glucose levels fosters SHP-2-IRS1 co-localization. Insulin relocalizes SHP-2 in a compartment of the cells distinct from the one which hosts tyrosine phosphorylated IRS1, and prevents SHP-2-IRS1 association. The sequence(s) of SHP-2 necessary for glucose and insulin control of SHP-2 localization are currently under investigation in our laboratory.

Previous studies evidenced that SHP-2 plays an important role in transducing insulin mitogenic signals (37,47). Consistent with these reports, we describe that antisense inhibition of SHP-2 expression in JAr cells led to a significant inhibition of insulin-induced DNA synthesis. Interestingly, however, we have also found that HG exposure slightly inhibits DNA synthesis, but
increases SHP-2 activity. Thus, in JAr cells, SHP-2 activation may not be sufficient to elicit growth stimulatory responses. Hence, when the cells are stimulated with insulin in the presence of high glucose levels, coincidence of SHP-2-IRS1 dissociation and induction of MAPK and DNA synthesis suggests that SHP-2 might play a regulatory rather than a direct causal role in JAr cell proliferation, by controlling the phosphorylation state of signalling molecules. In 3T3 cells, the expression of a catalytically inactive SHP-2 causes a reduction in MAPK phosphorylation and insulin mitogenesis with no effect on IR and IRS1 phosphorylation (47). At variance, in JAr cells SHP-2 modulates the insulin mitogenic signalling cascade by acting at the level of IR and IRS1 phosphorylation. It appears therefore that the function of SHP-2, as well as that of glucose, feature tissue-specificity. Regulation of SHP-2 also appears to involve different mechanisms in different cell types. In fact, previous evidence indicates that insulin stimulates SHP-2 activity in fibroblasts (47). Here, we show that both the JAr and BeWo placenta cells feature glucose but not insulin activation of SHP-2. Glucose itself impinges on molecular mechanisms in a tissue-specific fashion. For example, it stimulates MAPK activation in INS-1 cells (5, 41) and activates PKC in many other cell types (4, 42,43).

In conclusion, in the present report, we have shown that glucose exerts a permissive action on insulin mitogenesis in placenta cells. Glucose-induced activation and intracellular de-localization of SHP-2 is a key mechanism controlling insulin signal transduction through the IRS1/MAPK pathway.
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FIGURE LEGENDS

**Fig. 1. Effect of glucose on $^3$HThymidine incorporation in JAr cells.** A,B - JAr cells were cultured in media supplemented with 6mM (LG) or 25 mM glucose (HG) in the absence or presence of 100nM insulin or 100nM IGF1 or 100ng/ml EGF or 10% FBS for the indicated times. Alternatively, the cells were cultured in the presence of 6 or 25 mM sucrose. $^3$HThymidine incorporation into DNA was then determined as described under “Materials and Methods”. For clarity, values measured in the absence of insulin are detailed in panel C. D - Cells were cultured with indicated concentrations of glucose for 48h and then stimulated with 100 nM insulin followed by determination of $^3$HThymidine incorporation as above. Each value is the mean ± S.D. of triplicate determinations in five independent experiments. Statistical significance was assessed by unpaired Student’s t test analysis.

**Fig. 2. Effect of glucose on JAr cell proliferation.** JAr cells were seeded at 2 x $10^4$ cells/well in LG (A) or HG (B) media in the absence or the presence of 100nM insulin or 100nM IGF1 or 100ng/ml EGF or 10% FBS for the indicated times. The cells were then trypsinized and counted with a Coulter counter. For clarity, values measured in the absence of insulin either in LG or in HG media are detailed in panel C. Each value is the mean ± S.D. of triplicate determinations. Statistical significance was assessed by t test analysis.

**Fig. 3. Effect of glucose on the insulin receptor tyrosine phosphorylation and total content in JAr cells.** A - JAr cells were maintained in LG or HG media for 48 h, stimulated with 100nM insulin for 5 min and solubilized as described under “Materials and Methods”. Cell lysates were directly blotted with insulin receptor antibodies (IR) or phosphotyrosine antibodies (PTyr), or partially
purified by WGA chromatography, followed by IR precipitation and pTyr immunoblotting. Blotted proteins were revealed by ECL and autoradiography. Representative experiments are shown. Quantification of blots with WGA purified receptors is shown in the bar graph. Bars represent the mean value ± S.D. of four independent experiments. B - LG or HG cells were stimulated with 100 nM insulin or 100 nM IGF-1, lysed, and precipitated with IGF-1-receptor Abs and blotted with pTyr Abs. Blots were revealed by ECL and autoradiography. The autoradiographs shown are representative of three independent experiments. C - JAr cells were incubated with either 6 mM or 25 mM glucose for the indicated times. The cells were then incubated with $^{125}$I-insulin at 4C for 12 h, rinsed, and further incubated at 37C for 30 or 60 min as indicated. Insulin degradation was evaluated by TCA precipitation of the extracellular medium, as described under Materials and Methods. Bars are the means ± S.D. of duplicate determinations in three independent experiments.

Fig. 4. Effect of glucose on IRS1 and MAPK phosphorylations. JAr cells were incubated in LG or HG media in the absence or the presence of 100nM insulin as outlined in the legend to Figure 4. Cell lysates were either immunoprecipitated with IRS1 and then immunoblotted with phosphotyrosine Ab (A), or immunoblotted with P-MAPK Ab (B). Detection was achieved by ECL and autoradiography. Bars represent the mean ± S.D. of values from four (IRS1) and five (P-MAPK) independent experiments. Representative autoradiographs are shown in the insets. For control, filters were blotted again with IRS-1 and MAPK antibodies (panel C).

Fig. 5. Glucose effect on SHP-2, LAR and PTP1B protein expression and IRS1 association in JAr cells. JAr cells were incubated in LG or HG media in the absence or the presence of 100nM insulin as indicated. Cells were solubilized and cell lysates were blotted with SHP-2, LAR and PTP1B antibodies (panel A). Alternatively, the lysates were immunoprecipitated with IRS-1 or IR
antibodies followed by blotting with SHP-2 (panel B), LAR or PTP1B antibodies (panel C). Blots were revealed by ECL and autoradiography. The autoradiographs shown are representative of three (panel A), four (panel B), and two (panel C) experiments.

Fig. 6. Antisense inhibition of SHP-2 in JAr cells. JAr cells were transiently transfected with SHP-2 sense (S) or antisense (AS) oligonucleotides. The cells were solubilized, immunoprecipitated with SHP-2 antibodies, and further blotted with SHP-2 antibodies (A). Filters were revealed by ECL and autoradiography. The autoradiograph shown is representative of three independent experiments. Alternatively, the cell lysates were precipitated with SHP-2 antibodies and SHP-2 activity measured in the precipitates as described under Materials and Methods. Bars represent mean values ± S.D. of four independent experiments. C stands for untrasfected cells.

Fig. 7. Effects of SHP-2 antisense on IRS1-SHP-2 association, IRS1 and IR phosphorylation and $^3$HThymidine incorporation in JAr cells. A - JAr cells were transiently transfected with SHP-2 sense (S) or antisense (AS) oligonucleotides and then stimulated with 100 nM insulin. Cells lysates were immunoprecipitated with IRS-1 or IR antibodies followed by blotting with SHP-2 or phosphotyrosine (pTyr) antibodies, as indicated. Blots were revealed by ECL and autoradiography. The autoradiographs shown are representative of three independent experiments. B - Alternatively, oligonucleotide transfected and untrasfected cells (c) were stimulated with insulin and assayed for $^3$HThymidine incorporation as described under Materials and Methods. Bars represent the mean ± S.D. of triplicate determinations in five independent experiments.

Fig. 8. Glucose and insulin action on SHP-2 activity and thymidine incorporation in BeWo and 3T3 cells. BeWo and 3T3 cells were cultured in either LG or HG media. The cells were then
stimulated with insulin for 10 min and SHP-2 activity was assayed as described under Materials and Methods (A). Alternatively, the cells were incubated with 100 nM insulin and then assayed for thymidine incorporation as outlined in the legend to Fig. 8 (B). Bars represent the mean ± S.D. of duplicate determinations in three (panel A) and four (panel B) independent experiments.

**Fig. 9. Glucose action on SHP-2 and IRS1 subcellular localization in JAr cells.** JAr cells were cultured in HG or LG media and stimulated with 100 nM insulin for 10 min. The cells were rinsed, homogenized, and internal membrane (IM) and cytosol (Cy) fractions prepared as described under Materials and Methods. Equal amount of proteins from these fractions and the total homogenate (T) were then immunoblotted with IRS-1 or SHP-2 antibodies (A). Aliquots of the IRS-1 precipitates were also blotted with phosphotyrosine antibodies (B). The autoradiographs shown are representative of four independent experiments.
Table I. $^{125}$I-insulin binding in JAr cells

|        | Kd $\text{nM}$ | % of binding |
|--------|----------------|--------------|
|        |                |              |
| LG     |                |              |
| 48h    | 2.1±0.15       | 16.1±1.2     |
| 96h    | 1.7±0.25       | 16.7±1.7     |
| 168h   | 1.8±0.20       | 16.5±1.5     |
| HG     |                |              |
| 48h    | 1.8±0.25       | 16.3±1.3     |
| 96h    | 1.6±0.15       | 17.2±2.0     |
| 168h   | 2.0±0.15       | 16.8±1.6     |

Binding experiments were performed on cells kept in the presence of 6 mM glucose (LG) or 25 mM glucose (HG) for 48, 96 and 168 hours. Data represent the mean ± S.D. of triplicate determinations in three independent experiments. Kds were determined by Scatchard analysis of binding data as described under Materials and Methods.

Table II. Tyrosine phosphatase (PTPase) activity in JAr cells

|        | TOTAL | IR | IRS1 |
|--------|-------|----|------|
|        | LG    | HG | LG   | HG   |
| - Ins  | 0.8±0.04 | 2.3±0.04 | 0.31±0.013 | 0.4±0.02 |
| +Ins   | 0.6±0.02 | 0.9±0.02 | 0.29±0.011 | 0.39±0.023 |

PTPase activity was measured from total cell lysates (50 µg protein) and from IR and IRS1 precipitates (500 µg proteins, as determined before the immunoprecipitation) using p-nitrophenylphosphate as substrate. The activity is expressed as nmol mg $^{-1}$ hr$^{-1}$ as described under Materials and Methods. Data are the means ± S.D. of three independent experiments.
JAr cells were homogenized and internal membrane (IM), cytosolic (Cy), and plasma membrane (PM) fractions obtained as described under Materials and Methods. The purity of the different fractions was assessed by Western blotting equal amounts of proteins with cytochrome c oxidase or tubulin antibodies, or by comparison of 5’-nucleotidase activity. Results are reported as % ± S.D. of the total levels/activity measured in the homogenate. N.D. stands for not detectable. The figures in this legend did not show significant difference whether the cells are cultured in LG or HG media.

|       | Cytochrome c oxidase (% of total amount) | Tubulin (% of total amount) | 5’-nucleotidase (% of total activity) |
|-------|-----------------------------------------|----------------------------|--------------------------------------|
| IM    | 96±1.5                                  | <1                        | N.D.                                 |
| CY    | <1                                      | 95±1.4                    | N.D.                                 |
| PM    | N.D.                                    | N.D.                      | 97±0.9                               |
Fig. 2

(A) LG

Cell proliferation (number of cells $10^4$)

Time (h) 0 48 96 168

FBS EGF IGF1 Insulin Basal

(B) HG

Cell proliferation (number of cells $10^4$)

Time (h) 0 48 96 168

FBS EGF IGF1 Insulin Basal

(C) LG vs. HG

Cell proliferation (number of cells $10^4$)

Time (h) 0 48 96 168

LG HG
Fig. 4

A

IRS1 phosphorylation (Arbitrary Units)

| Insulin | LG    | HG    |
|---------|-------|-------|
| -       | 100   | 0     |
| +       | 200   | 500   |

B

MAPK phosphorylation (Arbitrary Units)

| Insulin | LG    | HG    |
|---------|-------|-------|
| -       | 100   | 0     |
| +       | 200   | 300   |

C

IRS1

| Insulin | LG    | HG    |
|---------|-------|-------|
| -       |       |       |
| +       |       |       |

MAPK

| Insulin | LG     | HG     |
|---------|--------|--------|
| -       |       |        |
| +       |       |        |
### A

|SHP-2| LAR| PTP1B|
|-----|----|------|
| ![Image of SHP-2](Insulin: LG, HG; Present: +, Absent: -) | ![Image of LAR](Insulin: LG, HG; Present: +, Absent: -) | ![Image of PTP1B](Insulin: LG, HG; Present: +, Absent: -) |

### B

| IP: IRS1 | IP: IR |
|---------|-------|
| IB: SHP-2 | IB: SHP-2 |
| ![Image of IP: IRS1](Insulin: LG, HG; Present: +, Absent: -) | ![Image of IP: IR](Insulin: LG, HG; Present: +, Absent: -) |

### C

| IP: IRS1 | IP: IRS1 |
|---------|---------|
| IB: LAR | IB: PTP1B |
| ![Image of IP: IRS1](Insulin: LG, HG; Present: +, Absent: -) | ![Image of IP: IRS1](Insulin: LG, HG; Present: +, Absent: -) |
Fig. 6

A

IP: SHP-2
IB: SHP-2

C  S  AS  S  AS
LG  HG

B

Shp-2 activity (nmol/mg/hr)

Insulin
- +
C  C  S  S  AS  AS
LG  HG  LG  HG  LG  HG
Fig. 8
Glucose regulates insulin mitogenic effect by modulating SHP-2 activation and localization in JAr cells
Giuseppe Bifulco, Costantino Di Carlo, Matilde Caruso, Francesco Oriente, Attilio Di Spiezio Sardo, Pietro Formisano, Francesco Beguinot and Carmine Nappi

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