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Signal processes and ROS production in glucose transport regulation by thrombopoietin and granulocyte macrophage-colony stimulation factor in a human leukaemic cell line

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Abstract
In M07e cells, a human megakaryocytic leukaemia line, reactive oxygen species (ROS) are generated in response to cytokines acting as intracellular messengers to modulate glucose transport. The aim of this work was to study the signal cascade involved in the acute glucose transport activation in cells exposed to growth factors, such as granulocyte macrophage-colony stimulation factor (GM-CSF) and thrombopoietin (TPO), to better understand some aspects of the aberrant proliferation in leukaemia. Results confirm ROS involvement in modulation of glucose transport in this cell line. Furthermore, GM-CSF and TPO produced changes in Glut1 phosphorylation and specific inhibitors employed to identify protein kinases involved in Glut activation by these cytokines proved that Akt, PLCγ, Syk and the Src family take part in signal transduction leading to Glut1 activation.

Keywords: *Reactive oxygen species, glucose transport, megakaryocytic cells, cytokines, tyrosine phosphorylation*

Abbreviations: *DOG, 2-deoxy-D-glucose; BHA, butylated hydroxyanisole; BAPTA-AM, 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethylester); [Ca^{2+}]c, cytosolic Ca^{2+} concentration; ER, endoplasmic reticulum; GM-CSF, granulocyte macrophage-colony stimulation factor; IL-3, interleukin-3; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate buffered saline; PI3K, phosphatidylinositol 3-kinase; PIC, piceatannol; PKB, protein kinase B; PLC, phospholipase C; PMSF, phenylmethylsulphonyl fluoride; ROS, reactive oxygen species; SCF, stem cell factor; SOD, superoxide dismutase; TK, tyrosine kinase; TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; TPO, thrombopoietin; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPases; TG, thapsigargin.*

Introduction

Malignant cells are known to have accelerated metabolism, high glucose requirements and increased glucose uptake. Transport of glucose across the plasma membrane of mammalian cells, mediated by facilitative glucose transporter (Glut) proteins, is the first rate-limiting step for glucose metabolism. In human studies, high levels of Glut1 expression in tumours have been associated with poor survival [1].

Various carcinogens may partly exert their effect by generating reactive oxygen species (ROS) during their metabolism. Elevated levels of ROS, down-regulation of ROS scavengers and antioxidant enzymes are associated with various human diseases including

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cancer. In fact, ROS affect proliferation, apoptosis, senescence and cellular processes implicated in the development of cancer [2].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is involved in growth and maturation of haematopoietic cells and in regulating host defence functions. The receptor for GM-CSF is composed of two sub-units, α (αGMR) and β (βGMR), and is found also in myeloid progenitors, mature granulocytes and mononuclear phagocytes [3]. It has long been known that cytokines like IL-3 and GM-CSF modulate glucose uptake in haematopoietic cells [3]. This stimulation is believed to be part of their function as survival factors by enhancing the availability of substrate for energy generation required for cell metabolism [4]. Little is known about the signal transduction pathway leading to this effect. Dhar-Mascareno et al. [3] examined the function of phosphatidylinositol 3-kinase (PI3K) in GM-CSF-stimulated glucose uptake in *Xenopus* oocytes and in human cells. Their results identify the early events in the stimulation of glucose uptake by GM-CSF as involving local H_2O_2 generation and requiring PI3-kinase activation.

Thrombopoietin (TPO) and its receptor (c-Mpl) are the major regulators of megakaryocyte and platelet production and play a critical and non-redundant role in haematopoietic stem cell biology. TPO signals through the Jak-STAT, Ras-Raf-MAPK and PI3K pathways and promotes survival, proliferation and polyploidization in megakaryocytes. Like other members of the haematopoietic cytokine receptor family, the signalling region does not encode a tyrosine kinase domain, tyrosine phosphatases or other enzymatic functions. Therefore, all the signalling events initiated by TPO binding to Mp1 must be due to homodimerization and recruitment of signalling molecules to docking sites on the signalling complex [5].

We previously showed that in megakaryocytic leukaemic M07e cells, expressing mainly Glut1 isoform, the presence of growth factors, such as SCF (stem cell factor), GM-CSF, TPO and IL-3, induced an activation of glucose transport, by decreasing the transporter apparent K_m for the substrate [6]. Moreover, cytokine treatment increased the intracellular ROS content, affecting biosignalling pathways that can be modulated by a catalase/superoxide dismutase mimetic compound [7]. We suggested that Glut1 activation by exogenous H_2O_2 and SCF is modulated through transporter translocation and that inhibitors of tyrosine kinases or phospholipase C (PLC) abolish this enhancement preventing Glut1 translocation. These data suggested that both stimuli could share at least some signalling pathways leading to glucose uptake activation, involving protein tyrosine kinases: in fact, exogenous H_2O_2 could act by increasing the level of tyrosine phosphorylation through the inhibition of tyrosine phosphatases, mimicking the regulation role of endogenous ROS [8].

In the present study, we investigated the signalling pathways involved in the acute stimulation of glucose transport by GM-CSF and TPO in M07e cells. To elucidate the role of ROS in this signalling process, we undertook experiments with antioxidants. The results here reported point to a central role for PLC, Akt and Src in signalling for increased glucose uptake and suggest that ROS generation after cytokine receptor activation is a key signalling event.

Materials and methods

Materials and reagents

GM-CSF was provided by Behring Marburg (Germany); TPO by Amgen Thousand Oaks (CA, USA). Phloretin, 2-deoxy-D-glucose (DOG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Trypan Blue, genistein, Igepal CA-630, orthovanadate, Protein G, butylated hydroxyanisole (BHA), U-73122, bisindolylmaleimide, N-Acetyl-L-cysteine, α-tocopherol, SB203580, PD98059, H_2O_2, piceatannol, phenylmethylsulphonyl fluoride (PMSF), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and monoclonal Anti-B-tubulin 1 were from Sigma (St. Louis, MO). Akt Inhibitor [1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-methyl-3-O-octadecylcarbonate] was from Calbiochem (San Diego, CA). PP2 was purchased from Tocris (Ellisville, MO). 2-Deoxy-D-[2,6-^{3}H]-glucose was from Amersham (UK); nitrocellulose paper was obtained from Schleicher and Schuell (Keene, NH). Goat polyclonal antiserum against Glut1, antirabbit, antigoat and antimouse IgG conjugated to horseradish peroxidase and Western Blotting Luminol Reagent were purchased from Santa Cruz (Santa Cruz, CA). Anti-Phospho-Akt (Ser473), Anti-Phospho-Src Family (Tyr416), Anti-Phospho-PLCγ1 (Tyr783) and Anti-Phospho-Tyrosine (P-Tyr-102) were from Cell Signaling Technology (Beverly, MA). All the other chemicals and solvents were of the highest analytical grade.

Cell culture

M07e cells are a human leukaemic megakaryocytic line whose proliferation is IL-3 or GM-CSF dependent. Cells are cultured as previously reported [6]. In brief, cells grow in IMDM supplemented with 5% foetal calf serum and 10 ng/mL IL-3. Since all the experiments were performed with growth factor-deprived cells, the day before each experiment M07e cells were washed twice in PBS, pH 7.2, suspended in IMDM with 5% FCS and without IL-3 and maintained for 18 h under these conditions. The total cell number was determined using a Burker haemocytometer; viable cells were evaluated by the

Trypan blue exclusion test. Cell viability was also assayed by the MTT uptake assay. Cells were incubated with 0.5 mg/mL MTT for 4 h at 37°C. At the end of the incubation, purple formazan salt crystals were formed and dissolved by adding the solubilization solution (10% SDS, 0.01 M HCl), then the plates were incubated overnight in humidified atmosphere (37°C, 5% CO_2). The absorption of converted dye was measured on a multiwell plate reader (Wallac Victor2, Perkin Elmer) at a wavelength of 570 nm.

Glucose transport assay

After an incubation with GM-CSF (10 ng/mL) or TPO (40 ng/mL) and/or inhibitors, glucose uptake was assayed in PBS buffer, pH 7.2 (in the absence of glucose) by adding 2-deoxy-D-[2,6-^{3}H] glucose (0.4 μCi/assay) and 1 mM unlabelled 2-deoxy-D-glucose to 0.5 mL cell suspension (2×10^6 cells). After a 5 min incubation at 37°C, the uptake was stopped by adding phloretin (0.3 mM final concentration). The uptake was linear up to 10 min. Transported DOG was less than 20% of the extracellular sugar concentration, therefore glucose transport assay could be considered in zero-trans conditions [9]. Sample radioactivity was measured by liquid scintillation counting.

Since M07e cells were deprived of medium components and maintained in PBS during glucose transport measurements, their viability was followed (not shown). No significant decrease of viable cells was observed up to 2 h at 37°C, thus the number of viable cells during time intervals of experiments (up to 1 h) was considered constant.

The lowest concentrations of all the tested inhibitors affecting the glucose transport stimulation, but not the basal uptake and viability, were chosen.

Kinetic parameters were calculated using different concentrations of unlabelled 2-deoxy-D-glucose and the cited amount of radioactive glucose analogue. Non-linear regression analysis of the V vs [substrate] data was used for curve fitting and for calculating the apparent K_m and V_{max} values.

Immunoprecipitation

M07e cells (15×10^6 per experimental condition) were placed in PBS, incubated with GM-CSF (10 ng/mL) or TPO (40 ng/mL) for 15 min, then pelleted. Cells were lysed with a lysis buffer (1% Igepal, 150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, 0.1 mM PMSF, 0.1 mM TLCK, 0.1 mM TPCK, 1 mM orthovanadate and protease inhibitor cocktail, pH 8.0) in ice for 15 min. Lysates containing equal amounts of protein (1 mg) were incubated overnight with 2 μg affinity-purified monoclonal anti-phosphotyrosine. Then, samples were incubated with protein G-Agarose for 1 h at 4°C and then pelleted. Pellets were washed five times with lysis buffer, treated with sample reducing buffer containing 4% β-mercaptoethanol (final concentration) and then boiled for 3 min.

SDS-PAGE and Western blot analysis

Immunocomplexes and cell lysates were separated on 10% SDS-polyacrylamide gel using a Mini-Protean II apparatus (Bio-Rad Laboratories). Proteins were transferred electrophoretically to supported nitrocellulose membrane at 100 V for 60 min. Non-specific binding to membrane was blocked by incubating in Tris-Buffered Saline (TBS)/Tween, pH 8.0, containing 5% non-fat dried milk for 1 h at room temperature. Then, the nitrocellulose membranes were incubated overnight at 4°C with primary antibodies of different enzymes. Blots were washed with TBS/Tween and then incubated for 30 min at room temperature with secondary antibodies in TBS/Tween containing 5% non-fat dried milk. Membranes were washed with TBS/Tween and developed using Western Blotting Luminol Reagent.

A semi-quantitative evaluation of the slabs was performed by using a Fluor S image analyser.

Statistical analysis

Data sets were performed with the unpaired Student *t*-test comparing cytokine-treated samples vs controls and inhibitor-untreated vs inhibitor-treated samples. Data are expressed as means ± SD. In Table I, the

Table I. Effect of U-73122, thapsigargin and BAPTA-AM on the kinetic parameters of DOG uptake. M07e cells were pre-treated with 10 nM thapsigargin for 45 min in IMDM or 5 mM BAPTA-AM for 10 min in PBS or 5 μM U-73122 for 5 min and then with GM-CSF (5 ng/mL) or TPO for 15 min, when indicated. Glucose uptake was determined over 1 min using a range of increasing 2-DOG concentrations. Data were analysed using a non linear regression method. Values are the means ± SD of at least four experiments. Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls's multiple comparison test.

Treatment	K_m (mM)	V_{max} (nmol/2×10^6 cell min)
None	2.98 ± 0.50	1.04 ± 0.15
U-73122 (5 μM)	3.15 ± 0.59	1.07 ± 0.14
Thapsigargin (10 nM)	1.65 ± 0.40**	1.13 ± 0.16
BAPTA-AM (5 μM)	2.94 ± 0.55	1.08 ± 0.25
GM-CSF (10 ng/mL)	1.72 ± 0.30**	1.14 ± 0.25
GM-CSF + U-73122	2.38 ± 0.10°	1.20 ± 0.10
GM-CSF + thapsigargin	1.30 ± 0.38	1.06 ± 0.16
GM-CSF + BAPTA-AM	3.38 ± 0.41°°	1.00 ± 0.11
TPO (40 ng/mL)	1.83 ± 0.38**	1.04 ± 0.24
TPO + U-73122	2.96 ± 0.20°°	0.95 ± 0.48
TPO + thapsigargin	1.42 ± 0.55	0.95 ± 0.22
TPO + BAPTA-AM	3.18 ± 0.31°°	1.01 ± 0.15

** $p < 0.001$; significantly different from control; ° $p < 0.05$; significantly different from treated with cytokine only. °° $p < 0.001$; significantly different from treated with cytokine only.

statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls' multiple comparison test. Differences were considered significant for $p < 0.05$.

Results

Effect of antioxidants on glucose uptake stimulated by GM-CSF and TPO in M07e cells

We previously observed that in M07e cells low doses of exogenous H_2O_2 as a well as some cytokines were able to activate glucose transport [6]. In order to confirm the role of ROS in the modulation of Glut1 activity, we performed experiments using different ROS scavengers such as α-tocopherol (α-T), N-acetylcysteine (NAC) and butylhydroxyanisol (BHA). Figure 1 shows that the lipophilic antioxidant α-tocopherol does not affect the glucose uptake, while NAC and BHA substantially attenuate the activation of glucose transport due to GM-CSF and TPO, confirming ROS involvement in the regulation of glucose transport.

Effect of GM-CSF and TPO on Glut1 phosphorylation in M07e cells

We investigated whether changes in the tyrosine phosphorylation pattern occurred in M07e cells exposed to GM-CSF and TPO for 15 min. Western-immunoblotting analysis after immunoprecipitation with anti-phosphotyrosine antibodies in the presence of orthovanadate showed that both cytokines produced a similar increase in the phosphorylation pattern in M07e cells, regarding several protein bands in the range 25–130 kDa (data not shown).

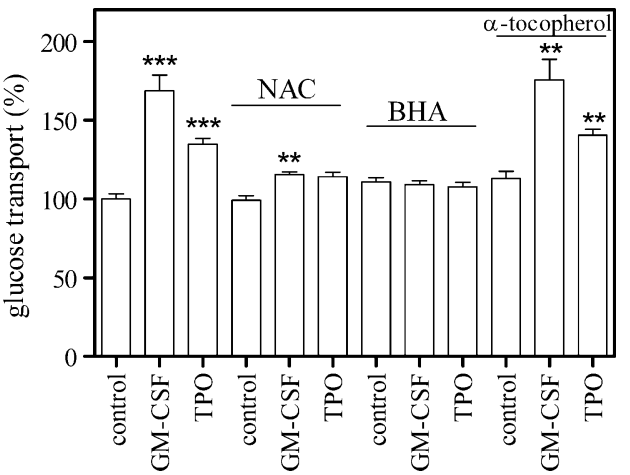

Figure 1. Effect of ROS and antioxidants on glucose uptake in M07e cells. Cells were incubated at 37°C in the absence or presence of 20 μM α-tocopherol in IMDM for 24 h, 5 μM NAC or 1 μM BHA in PBS for 10 min and then treated with TPO (40 ng/mL) and GM-CSF (10 ng/mL) for an additional 10 min. DOG uptake was measured over 5 min as described in the Materials and methods section. Results are expressed as means ± SD of three independent experiments, each performed in duplicate. *** $p < 0.0001$, ** $p < 0.005$, significantly different from control.

Since early data suggested an increased phosphorylation of Glut4 in adipose cells during glucose transport activation by insulin [10], we investigated if cytokines affected the phosphorylation of Glut1, the major transporter expressed in M07e cell line [6]. Under experimental conditions used, there was a significant change in the tyrosine phosphorylation of Glut1, suggesting that these cytokines may affect Glut1 affinity, as shown in Table I, by increasing the transporter phosphorylation (Figure 2A and B).

Role of the tyrosine kinase Src family and Syk on glucose uptake activation induced by GM-CSF and TPO

In order to identify some of the steps connecting GM-CSF and TPO stimulation to Glut1 activation, we tested the effect of tyrosine kinase (TK) inhibitors on glucose transport, an approach often reported in the literature [11]. Results shown in Figure 3A demonstrate that the pre-treatment of M07e cells with PP2, a Src TK inhibitor [12], was able to significantly reduce the glucose transport stimulation induced by the tested stimuli. The hypothesis that Src TK family can be involved in glucose transport modulation by cytokines is confirmed by immunoblotting experiments, since the incubation with both

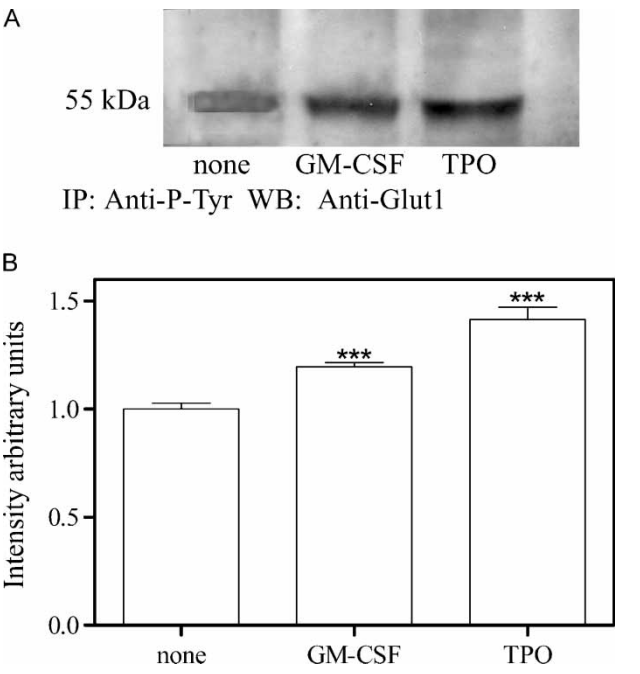

Figure 2. Effect of TPO and GM-CSF on Glut1 phosphorylation in M07e cells. (A) Cells were incubated in PBS at 37°C in the absence or presence of TPO (40 ng/mL) or GM-CSF (10 ng/mL) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred and immunoblotted with Anti-Glut1, as described in the Materials and methods section. (B) Results of scanning densitometry analysis performed on three independent autoradiographs are presented. Relative amounts (means ± SD) of the band showed in panel B are expressed as fold increase compared to control cells not exposed to the stimuli. *** $p < 0.0001$, significantly different from control.

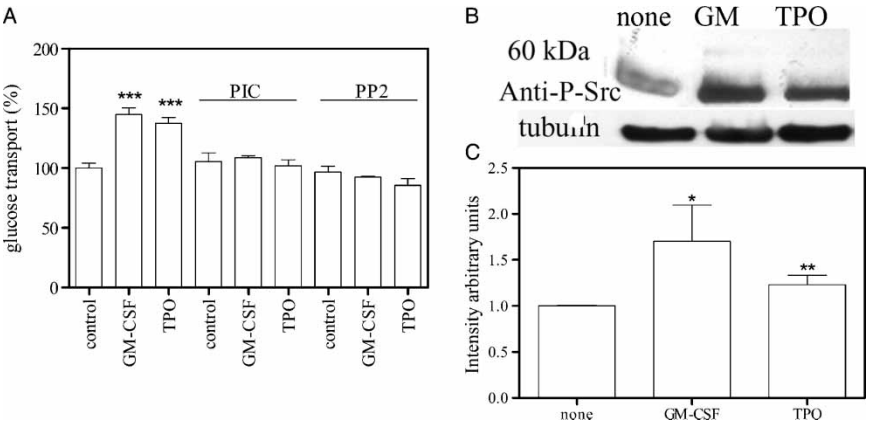

Figure 3. Effect of PP2 and piceatannol on DOG uptake in M07e cells stimulated by GM-CSF and TPO; role of Src tyrosine kinase family. (A) Cells incubated with 100 nM PP2 or 1 μM piceatannol (PIC) in PBS at 37°C for 10 min were exposed to TPO (40 ng/mL) or GM-CSF (10 ng/mL) for 10 min. Then DOG uptake was measured in PBS over 5 min as described in the Materials and methods section. Results are expressed as means ± SD of three independent experiments, each performed in duplicate. *** $p < 0.0001$, significantly different from control. (B) Cells were incubated in PBS at 37°C in the absence or presence of TPO (40 ng/mL) or GM-CSF (10 ng/mL) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred and immunoblotted with Anti-P-Src and Anti-tubulin antibody, as described in the Materials and methods section. (C) Results of scanning densitometry analysis performed on three independent autoradiographs are presented. Relative amounts (means ± SD) of the indicated bands are in arbitrary units and compared to the corresponding from cells not exposed to the stimuli. ** $p < 0.005$, * $p < 0.05$, significantly different from control.

the stimuli caused an increase in Src phosphorylation (Figure 3B and C).

In many cases the Src family seems to operate together with Syk tyrosine kinases, being a general hypothesis that activation of Src family kinases precedes activation of Syk kinases [13]. We investigated whether also Syk TKs are involved in glucose transport activation by using a Syk inhibitor, piceatannol [14,15]. The results shown in Figure 3A suggest that Syk TKs participate in the glucose uptake increase due to cytokine treatment.

Role of Akt and PLCγ on glucose uptake activation induced by GM-CSF and TPO

It has been reported that Akt is rapidly activated by growth factors and plays an important role in mediating the effects of insulin on glucose utilization in skeletal muscle and adipocytes [16]. The Akt inhibitor pre-treatment caused a significant decrease in the DOG uptake activated by both stimuli, as shown in Figure 4A. Experiments with anti-phospho-Akt showed a significant increase in Akt phosphorylation (Figure 4B and C), confirming the involvement of this kinase in the signal transduction induced by GM-CSF and TPO. Therefore, data here reported suggest a role of Akt in the regulation of Glut1 activity.

Akt binds to and phosphorylates phospholipase C-γ1 in response to growth factors mediated receptors tyrosine kinases [17]. To investigate the involvement of PLCγ in the signal transduction by GM-CSF and TPO, the effects of these stimuli on Glut1 activity and PLCγ phosphorylation were evaluated. Figure 4B and C show that the incubation with both the stimuli produced an increase in the PLCγ phosphorylation. U-73122 prevents the glucose transport activation induced by these cytokines without affecting the basal activity (Figure 4A).

We previously observed that GM-CSF and TPO activated Glut1 by increasing predominantly the affinity for glucose [6]. Table I shows that U-73122 prevents the variations of the K_m values caused by GM-CSF and TPO, confirming a role of this enzyme in the transport regulation.

Calcium modulation on glucose transport kinetic parameters

The activation of PLC and the subsequent production of inositol 1,4,5-triphosphate ($InsP_3$) can stimulate the release of Ca^{2+} from endoplasmic reticulum. We previously observed that intracellular Ca^{2+} could be involved in conveying signals towards acute Glut1 activation in M07e cells [9]. In fact, a $[Ca^{2+}]_c$ increase obtained by depleting intracellular stores with thapsigargin (TG), a specific inhibitor of sarcoplasmic/endoplasmic Ca^{2+}ATPases (SERCA) [18,19], induced glucose transport activation.

To better understand the mechanism involved in this TG effect, we determined the kinetic parameters, K_m and V_{max}, for glucose uptake in the presence of GM-CSF and TPO. The data reported in Table I show that TG alone induced a further, significant increase in the affinity of Glut1 for the substrate without affecting the V_{max}. In the presence of TG, K_m values already affected by cytokines further decreased when compared to the values obtained in the presence of cytokines alone.

We previously showed that the calcium chelator acetoxymethyl ester BAPTA (BAPTA-AM) completely abolished the activation of glucose uptake

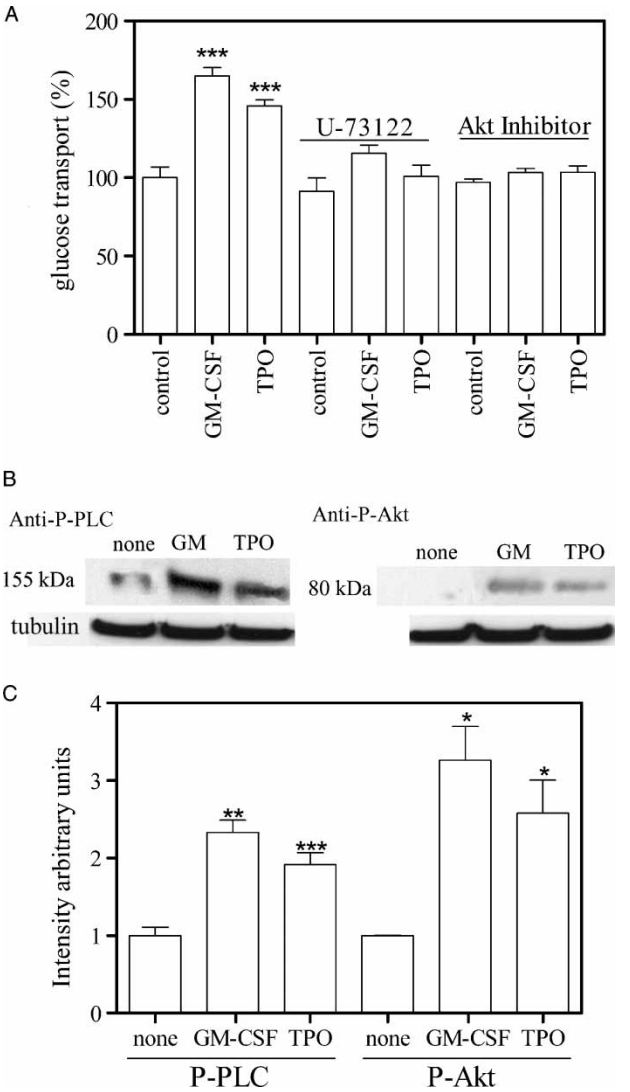

Figure 4. Effect of U-73122 and Akt inhibitor on DOG uptake in M07e cells stimulated by cytokines. (A) Cells incubated with 5 μM U-73122 or 25 μM Akt inhibitor in PBS at 37°C for 10 min were exposed to TPO (40 ng/mL) or GM-CSF (10 ng/mL) for 10 min. Then DOG uptake was measured in PBS over 5 min, as described in the Materials and methods section. Results are expressed as means ± SD of three independent experiments, each performed in duplicate; *** $p < 0.0001$, significantly different from control. (B) Cells were incubated in PBS at 37°C in the absence or presence of TPO (40 ng/mL) or GM-CSF (10 ng/mL) for 15 min. Cell lysates were electrophoresed, transferred and immunoblotted with Anti-P-Akt antibody, as described in the Materials and methods section. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred and immunoblotted with the indicated Anti-P-PLC and Anti-tubulin antibody, as described in the Materials and methods section. (C) Results of scanning densitometry analysis performed on three independent autoradiographs are presented. Relative amounts (means ± SD) of the indicated bands are in arbitrary units and compared to the corresponding from cells not exposed to the stimuli. *** $p < 0.0001$, ** $p < 0.005$, * $p < 0.05$, significantly different from control.

induced by GM-CSF, TPO [9]. To further investigate the effect of BAPTA on glucose transport activation, the kinetic parameters were determined in the presence of the cytokines. As reported in Table I, 5 μM BAPTA did not affect the kinetic parameters in untreated cells, but it abolished the changes in K_m caused by both the stimuli.

Role of PKC and MAP kinases on glucose transport activation in M07e cells

Because membrane translocation of conventional protein kinase C (PKC) isoforms, such as PKC-β, follows changes in the intracellular Ca^{2+} concentration and localization [20], we evaluated whether PKC is involved in glucose transport activation due to GM-CSF and TPO. The cytokine-enhanced uptake was not affected by 10 μM bisindolylmaleimide, a PKC inhibitor [21], whereas at higher concentration the basal glucose transport was decreased (data not shown). Taken together, these results indicate that GM-CSF and TPO increase glucose transport probably through a PKC-independent mechanism. We evaluated also the effect of MAP kinase inhibitors, such as SB203580 [22] and PD98059 [23], p38 and p42/44 inhibitors, respectively. Using concentrations up to 100 μM, we did not observe any change in glucose transport acute activation due to growth factors. Higher inhibitor concentrations inhibited basal and activated glucose uptake in the same manner (data not shown).

Identification of the order in the phosphorylation cascade induced by GM-CSF and TPO and involved in the modulation of glucose uptake

To clarify the activation order in the enzymes involved in the signal transduction leading to glucose uptake activation, we evaluated the effect of some inhibitors on the phosphorylation of PLCγ, Src and Akt induced by GM-CSF and TPO. At first, we pretreated M07e cells with PP2, a Src inhibitor, without observing any modulation in the phosphorylation of PLCγ and Akt (data not shown). On the other hand, in the presence of U-73122, a PLCγ inhibitor, a significant decrease in the band intensity of P-Src was found, suggesting that the activation of PLCγ is upstream of the Src phosphorylation (Figure 5A and B). Moreover, incubation with the Akt inhibitor affected the PLCγ phosphorylation increase induced by the tested cytokines (Figure 5C and D), indicating that the Akt involvement occurs earlier than the PLCγ one.

Taking together the results obtained using enzyme inhibitors, we suggest that the phosphorylation order upstream Glut1 activation by GM-CSF and TPO can be: Akt, PLCγ and Src.

Discussion

Haematopoietic cytokines stimulate the proliferation of precursor cells and promote the survival and function of mature cells. These processes require

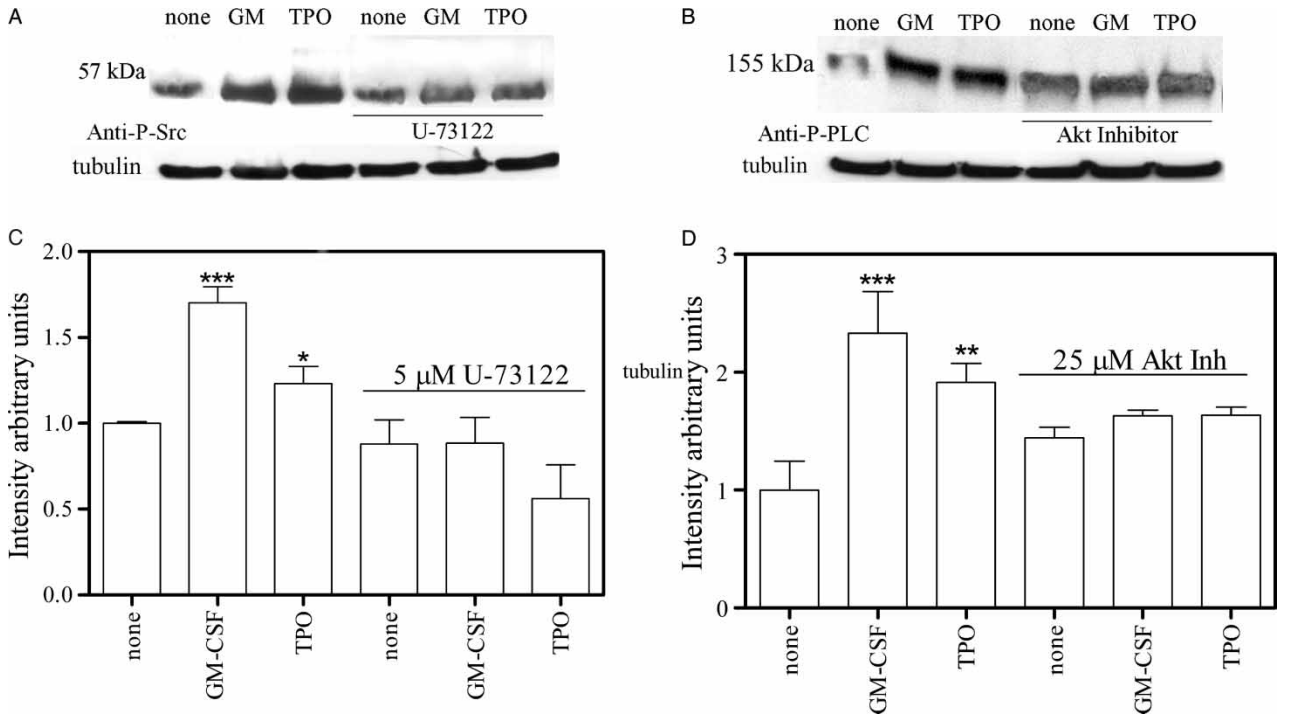

Figure 5. Effect of U73122 and Akt inhibitor on Src and PLCγ phosphorylation, respectively, in M07e cells. (A) Cells, pre-treated with 5 μM U72122 for 5 min, were incubated in PBS at 37°C in the absence or presence of TPO (40 ng/mL) or GM-CSF (10 ng/mL) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred and immunoblotted with the indicated antibody, as described in the Materials and methods section. (B) Results of scanning densitometry analysis performed on three independent autoradiographs are presented. Relative amounts (means ± SD) of the indicated bands are in arbitrary units and compared to the corresponding from cells not exposed to the stimuli; *** $p < 0.0001$, * $p < 0.05$, significantly different from control. (C) Cells pretreated with 25 μM Akt inhibitor were incubated in PBS at 37°C in the absence or presence of TPO (40 ng/mL) or GM-CSF (10 ng/mL) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred and immunoblotted with the indicated antibody, as described in the Materials and methods section. (D) Results of scanning densitometry analysis performed on three independent autoradiographs are presented. Relative amounts (means ± SD) of the indicated bands are in arbitrary units and compared to the corresponding from cells not exposed to the stimuli; *** $p < 0.0001$, ** $p < 0.005$, significantly different from control.

energy and cytokines such as IL-3, IL-1 and GM-CSF are known to enhance glucose transport in target cells [3,6], where the uptake can be acutely modulated by regulating the number of transporters on the cell surface and/or by altering their transport efficiency. GM-CSF, TPO and IL-3 can enhance the intrinsic efficiency of Glut1 as reflected by a decrease in the K_m value without a change in V_{max} [6,24]. The ability of GM-CSF and TPO to increase Glut1 affinity suggests that initial signalling from the receptors results in modulation of the intrinsic properties of the transporter, ruling out an effect on transporter translocation by these cytokines. This hypothesis is in agreement with the observed increase in Glut1 phosphorylation that could be responsible for the transporter affinity change. In fact, phosphorylation-dependent events have previously been shown to modulate Glut1 activity. Protein kinase C isozymes stimulate Glut1 by recruiting Glut1 transporter to the cell membrane [25] and the AMP-activated protein kinase stimulates Glut1 without affecting the total amount of Glut1 protein at the cell surface [26]. In some studies on adipocytes [27], a role of PKB in the regulation of Glut1 phosphorylation has been suggested. The SGK1 (serum- and glucocorticoid-inducible kinase)-dependent regulation of Glut1 phosphorylation may participate in the adjustment of cellular glucose uptake in *Xenopus laevis* oocytes [28]. At the moment, the lack of correlation between the degree of Glut1 phosphorylation and the growth factor's potency to stimulate glucose uptake is not yet clarified.

The effect of GM-CSF can be related to the nature of α sub-unit of its receptor devoid of RTK activity, transducing signals in a phosphorylation independent manner [29]. Similarly, the extracytoplasmic domain of the TPO receptor does not encode enzymatic function [3].

It is known that GM-CSF and TPO use reactive oxygen species as second messenger molecules in their signalling pathways [7,30] and that ROS in the form of hydrogen peroxide have a role in stimulating glucose uptake [7,31]. We recently showed that low doses of H_2O_2 as well as some cytokines are able to stimulate glucose transport activity in M07e cells. This activation was completely removed when cells were pre-treated with EUK-134, a catalase and superoxide dismutase mimetic [7].

Understanding the role of ROS as key mediators in signalling cascades may provide opportunities for

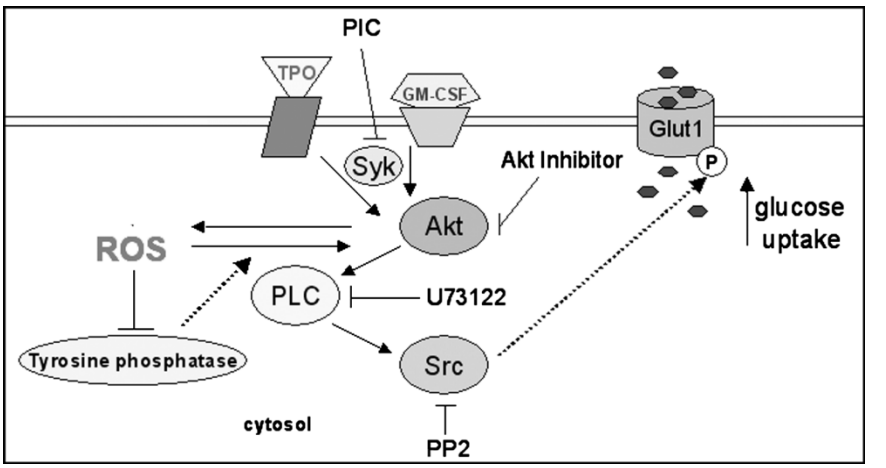

Figure 6. A model for signal transduction in Glut1 modulation. The figure shows the signal cascade induced by TPO and GM-CSF leading to Glut1 phosphorylation. In the suggested mechanism, Syk, Akt, PLC, Src and ROS, produced from plasma membrane, are involved.

pharmacological intervention. To investigate whether ROS could be a common mediator of the effect due to TPO and GM-CSF, we treated M07e cells with antioxidants. Whereas treatment with NAC or BHA almost abolished the acute stimulatory action of GM-CSF and TPO, the lipophilic α-tocopherol, located into the membrane, did not affect the glucose uptake regulation, suggesting that cytosolic ROS were involved in the increase of glucose transport due to these growth factors.

We hypothesized that GM-CSF and TPO generate ROS taking part in a pathway cascade which, by modifying the activity of redox-sensitive enzymes including kinases and phosphatases, leads to glucose transport modulation [3].

Then, we investigated whether some kinases, such as Akt, Src, Syk, PKC, MAPK, known to play an important role in cytokine-regulated cells, were involved in glucose uptake modulation. Evidence exists that acute treatment of cells with hydrogen peroxide activates Akt [32]. This kinase affects glucose uptake and glycolysis in murine blastocysts by inducing the translocation of Glut1 and Glut4 to the plasma membrane [33]. Also in M07e cells, Akt seems to be involved in the cytokine regulation of glucose uptake (see Figure 4).

As far as PLCγ is concerned, Wang et al. [17] showed that the interaction between PLCγ and Akt resulted in the phosphorylation of PLCγ by Akt. Prasad and Ismail-Beigi [34] suggested that stimulation of protein tyrosine kinase activity mediates, at least in part, the enhancement of glucose transport in response to H_2O_2, since PLC activity increases after H_2O_2 exposure and the PLC stimulation causes the enhancement of glucose transport in response to H_2O_2. Data here reported support the involvement of PLCγ in glucose transport stimulation by GM-CSF and TPO through a mechanism involving the affinity increase of Glut1.

We have recently demonstrated that PLC-dependent calcium release from endoplasmic reticulum plays a role in glucose transport stimulation by cytokines [17]. To better understand this effect, we analysed Glut1 kinetic parameters in the presence of molecules affecting intracellular calcium concentration. Table I shows that transport affinity was significantly raised by elevation of cytosolic Ca^{2+} concentration due to thapsigargin. Moreover, intracellular Ca^{2+} chelation by BAPTA decreased Glut1 affinity stimulation due to GM-CSF and TPO. These results suggest that in M07e cells calcium level controls glucose transport by affecting the transporter affinity.

Since membrane translocation of conventional PKC isoforms, such as PKC-β, follows changes in the intracellular concentration and localization of Ca^{2+} [20] and members of the PKC superfamily play key regulatory roles still poorly defined [35] in glucose transport, we tested a general PKC inhibitor on glucose uptake stimulation. Nevertheless, no alteration of the cytokine effect occurred, indicating that these kinases do not take part to the signal cascade under investigation. As far as acute glucose transport modulation is concerned, MAPK inhibitor did not prevent the cytokine stimulation, suggesting that a role of MAP kinases in the short-term regulation of glucose activation could be excluded.

As known, the Src family plays important roles in cell responses induced by growth factors, including cell growth, survival, cancer formation and progression. Recently, it has been reported that hyper-activation of Lyn, a Src tyrosine kinase, with augmented ROS production, may constitute one mechanism by which acute myeloid leukaemia (AML) arises [36]. Moreover, in vascular smooth muscle cells insulin-like growth factor induces Src activation through ROS [37]. In this regard, we investigated the involvement of Src tyrosine kinase

family in Glut1 stimulation. Glucose transport assays and immunoblotting experiments show that these kinases are involved in GM-CSF- and TPO-induced pathway in M07e cell line.

Syk protein tyrosine kinase has been implicated in a variety of haematopoietic cell responses and was also found to be involved in oxidative and osmotic stress signalling in B cell lines [38]. In M07e cells, piceatannol, a selective Syk inhibitor [14,15], prevented the glucose transport activation due to the incubation with cytokines, suggesting a role of Syk TK in the hexose uptake modulation.

Further studies were performed to delineate the sequence of events leading from enhancement of protein tyrosine kinases to the observed stimulation of glucose transport in response to GM-CSF and TPO. The analysis of the effect of several kinase inhibitors on Src, Akt and PLCγ phosphorylation indicated that, before Glut1 phosphorylation, Akt activation can be an early step leading to PLCγ activation. Src inhibitor has no effect on PLCγ and Akt activation, suggesting that Src involvement occurs later in the glucose transport modulation cascade (see Figure 6). Since data here reported suggest that ROS generation is a key signalling event, it is likely that studies elucidating the mechanisms of redox control of growth factor signalling will rapidly emerge in the next years and understanding of such regulation should help in the development of therapeutic strategies against cancer.

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