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News & Views

Glucose-Transport Regulation in Leukemic Cells: How Can H_2O_2 Mimic Stem Cell Factor Effects?

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ABSTRACT

In leukemic cells, glucose transport is activated by SCF and H_2O_2 through a common signal cascade involving Akt, PLCγ, Syk, and the Src family, in this order. An explanation can be provided by the phosphorylation of c-kit, the SCF receptor, elicited by either SCF or H_2O_2. Moreover, antioxidants prevent the SCF effect on glucose transport, confirming the involvement of H_2O_2 in the pathway leading to glucose-transport activation and suggesting a potential role for reactive oxygen species in leukemia proliferation. *Antioxid. Redox Signal.* 8, 271–279.

GLUCOSE TRANSPORT REGULATION

Glucose uptake into cells is mediated by a family of glycosylated membrane proteins (Gluts) able to facilitate passive hexose transfer across the plasma membrane. The mechanisms by which external and internal signals regulate glucose transport have been extensively studied, although the final events responsible for short-term regulation of glucose uptake are still under debate. A large amount of information deals with transport regulation by insulin (15). In the cascade of events leading to the multiple effects of insulin, this hormone activates its receptor tyrosine kinase and stimulates a number of downstream signaling factors. Among the major end points of insulin is the stimulation of glucose uptake into several tissues via translocation of Glut1 and Glut4 from the cytoplasm to the plasma membrane (13). However, the precise steps between insulin-receptor activation and Glut translocation have not been entirely delineated (5). Some groups have provided a possible link between activation of PKB/Akt and the stimulation of Glut4 translocation (9). Conversely, several studies have found a lack of correlation between PKB/Akt and glucose transport in insulin-responsive cells. In the most striking example, cultured murine adipocytes, synthesis of a dominant-negative Akt that suppresses 80% of the endogenous kinase activity does not impair insulin-stimulated glucose transport (12).

In a previous work, we showed that in the leukemic cell line M07e glucose transport, mediated by insulin-independent Glut1, is activated by a short incubation with H_2O_2 or by some cytokines, such as SCF, GM-CSF, and TPO (7).

REACTIVE OXYGEN SPECIES, CYTOKINES, AND GLUCOSE TRANSPORT

Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide produced during cellular redox reactions, can act as intracellular messengers under physiologic conditions (23). We previously showed that incubation of M07e cells with growth factors produced an increase in the intracellular ROS content and that the synthetic antioxidant EUK-134, a catalase and superoxide dismutase mimetic able to penetrate into the cells, can modulate biosignaling pathways (8). To confirm data previously obtained with EUK-134, experiments have been performed using agents that remove ROS as ebselen, a glutathione peroxidase mimetic, or a highly lipophilic hydroxylamine probe [bis(1-hydroxy-

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2,2,6,6-tetramethyl-4-piperidinyl)decandioate], which is able to give a fast reaction with the majority of radical species involved in oxidative stress (24). As shown in Fig. 1, both compounds attenuate the activation of glucose transport due to SCF in M07e cells, confirming the effects obtained with EUK-134. Data do not allow discrimination of the roles of different ROS, but they suggest that both peroxides and radical species could be involved in glucose-transport modulation. We also demonstrated that a possible ROS source, linked somehow with glucose-transport activity, could be a NAD(P)H oxidase similar to the one present in phagocytic cells (25).

In M07e cells, inhibitors of tyrosine kinases (such as genistein and tyrphostin A23) or PLC (U-73122) abolish glucose-transport enhancement due to SCF and H_2O_2 and prevent translocation of Glut1, the major isoform expressed in this cell line. These data suggest that both stimuli could share at least some signaling pathways leading to glucose-uptake activation, involving protein tyrosine kinases and PLC; H_2O_2 could act by increasing the level of tyrosine phosphorylation through the inhibition of tyrosine phosphatases and mimicking the regulation role of endogenous ROS (21).

SCF AND H_2O_2 AFFECT GLUCOSE-TRANSPORT REGULATION THROUGH TYROSINE PHOSPHORYLATION

SCF and its receptor, c-kit, are critical in the survival and development of stem cells involved in hematopoiesis, pigmentation, and reproduction. Interaction of SCF with c-kit rapidly induces receptor dimerization and increases in autophosphorylation activity (18). We investigated whether changes in the total tyrosine phosphorylation pattern occurred in M07e cells exposed to SCF or to a low dose (50 μ*M*) of hydrogen peroxide for 15 min. Western-immunoblotting analysis after immunoprecipitation with anti-phosphotyrosine antibodies in the presence of orthovanadate showed that both SCF and H_2O_2 produced a similar increase in the phosphorylation pattern in M07e cells, although the H_2O_2 effect was by far more marked and generalized, regarding several protein bands in the range from 25 to 130 kDa (Fig. 2).

We previously reported that cytokine, particularly SCF, or H_2O_2 addition to M07e cells caused the maximal stimulation of glucose transport within 15–20 min (7). Figure 3 shows that not only SCF, but also H_2O_2 is able to increase the phosphorylation of c-kit: this fact can explain why H_2O_2 mimics the SCF effect on glucose-transport modulation, as previously observed (7).

It is well known that SCF binding to c-kit, a receptor tyrosine kinase, rapidly increases receptor autophosphorylation and activates downstream signal components (18). In M07e cells, treatment with imatinib mesylate, an anti-leukemic drug, results in a selective inhibition of c-kit tyrosine kinase and its effects, including activation of downstream target proteins (29). Thus, we tested the effects of imatinib mesylate up to 10 μ*M* on glucose-transport enhancement, observing that 10 μ*M* imatinib mesylate is able to block selectively the activation by both SCF and H_2O_2 (Fig. 3B).

To better understand the effect of imatinib mesylate on the signal transduction induced after SCF or H_2O_2 treatment, we compared the phosphorylation pattern obtained in the presence of imatinib or genistein, a nonspecific tyrosine kinase inhibitor able to remove glucose-transport activation in M07e cells (7). Figure 2 shows that both the inhibitors diminish the phosphorylation increase induced by SCF and hydrogen peroxide. These results suggest that the activation of the SCF receptor, c-kit, is crucial for the signal pathways elicited by both stimuli and explain the ability of H_2O_2 to mimic the action of SCF on glucose transport, previously reported (7, 21).

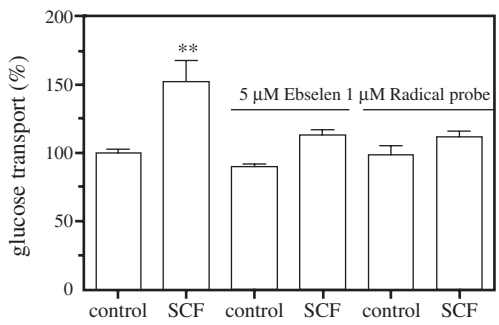

FIG. 1. Effect of ebselen or hydroxylamine radical probe on DOG uptake in M07e cells. Cells were incubated in PBS at 37°C in the absence or presence of 5 μ*M* ebselen or 1 μ*M* radical probe [bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate] for 10 min and then treated with SCF (5 ng/ml) for an additional 10 min. DOG uptake was measured over 5 min as described in the Material and Methods section. Results are expressed as means ± SD of two independent experiments, each performed in duplicate. $^{**}p < 0.005$, significantly different from control.

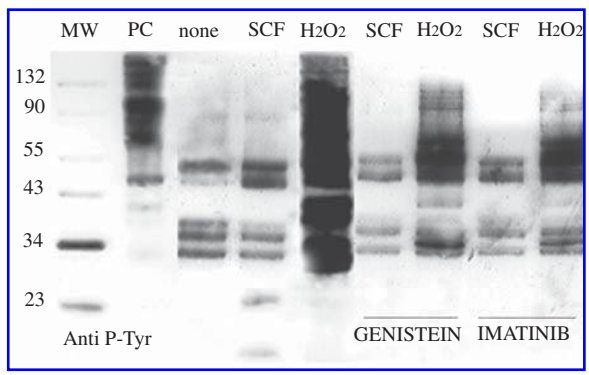

FIG. 2. Tyrosine phosphorylation by SCF and H_2O_2 in M07e cells: effect of imatinib mesylate and genistein. Cells pretreated with 10 μ*M* imatinib mesylate and 5 μ*M* genistein were incubated in PBS at 37°C in the absence or presence of SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates, positive control (PC), and standard molecular weight (MW) were electrophoresed, transferred, and immunoblotted with the indicated antibody, as described in Material and Methods.

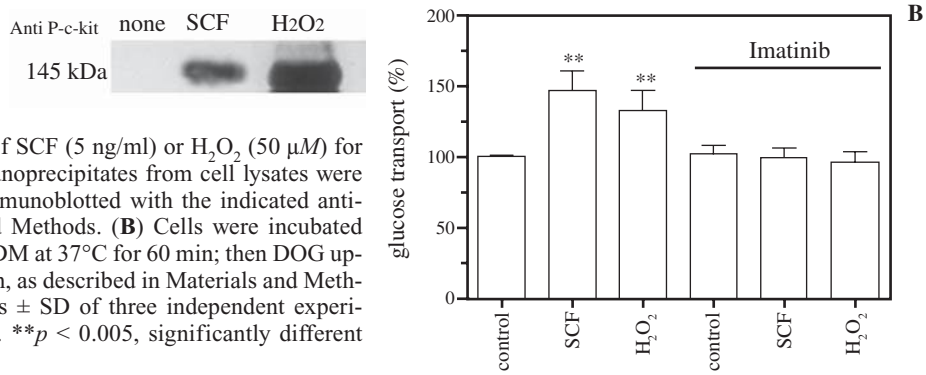

FIG. 3. c-Kit tyrosine phosphorylation by SCF and H_2O_2 in M07e cells: effect of imatinib mesylate on DOG uptake. (**A**) Cells were incubated in PBS at 37°C in the absence or presence of SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred, and immunoblotted with the indicated antibody, as described in Materials and Methods. (**B**) Cells were incubated with 10 μ*M* imatinib mesylate in IMDM at 37°C for 60 min; then DOG uptake was measured in PBS over 5 min, as described in Materials and Methods. Results are expressed as means ± SD of three independent experiments, each performed in duplicate. ***p* < 0.005, significantly different from control.

SCF AND H_2O_2 AFFECT COMMON SIGNALING STEPS LEADING TO GLUCOSE-TRANSPORT ACTIVATION

To identify some of the steps connecting c-kit activation by SCF or H_2O_2 to Glut1 modulation, we tested the effect of TK inhibitors on glucose transport, an approach often reported in the literature (14). Results shown in Fig. 4A demonstrate that the pretreatment of M07e cells with PP2, an Src TK inhibitor (19), was able to significantly reduce the glucose-transport stimulation induced by the tested stimuli. The hypothesis that Src TK family is involved in the glucose-transport modulation by SCF is confirmed by immunoblotting experiments, because the incubation with both the stimuli caused an increase of Src phosphorylation (Fig. 4B and C).

Because 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 (3), we investigated whether Syk TKs are involved in the glucose-transport activation using a Syk inhibitor, piceatannol (19). The results shown in Fig. 4A suggest that Syk TKs also participate in the glucose-uptake increase due to the cytokine and H_2O_2 treatment.

It has been reported that Akt it is rapidly activated by growth factors and plays an important role in mediating the effects of insulin on glucose utilisation in skeletal muscle and adipocytes (9). Therefore, we preliminarily tested the effect

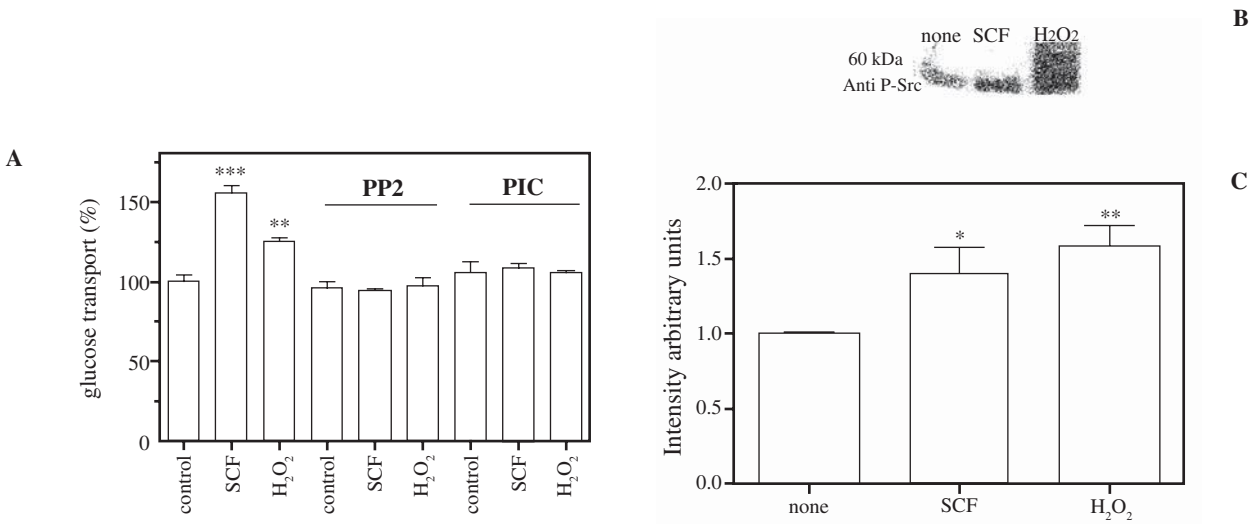

FIG. 4. Effect of PP2 and piceatannol on DOG uptake in M07e cells stimulated by SCF and H_2O_2: role of Src tyrosine kinase family. (**A**) Cells incubated with 100 n*M* PP2 or 1 μ*M* piceatannol (PIC) in PBS at 37°C for 10 min were exposed to SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min, and then DOG uptake was measured in PBS over 5 min, as described in Materials and Methods. Results are expressed as means ± SD of three independent experiments, each performed in duplicate. ****p* < 0.001,***p* < 0.005, **p* < 0.05, significantly different from control. (**B**) Cells were incubated in PBS at 37°C in the absence or presence of SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred, and immunoblotted with the indicated antibody, as described in Materials and Methods. (**C**) Results of scanning densitometry analysis performed on three independent autoradiographs. Relative amounts (means ± SD) of the indicated bands are in arbitrary units and compared with the corresponding from cells not exposed to the stimuli. ***p* < 0.005, **p* < 0.05, significantly different from control.

of different concentrations of an Akt inhibitor (10) on the glucose uptake activated by SCF, and we identified the inhibitor concentration (25 μ*M*) able to abolish the SCF-activating effect without affecting the basal glucose uptake. The Akt inhibitor pretreatment caused a significant decrease in the glucose uptake activated by both the stimuli (Fig. 5A). Western blot experiments with anti-phospho-Akt showed a significant Akt activation (Fig. 5B and C), confirming the involvement of this kinase in the signal transduction induced by SCF and H_2O_2. Therefore, these data suggest a role of Akt in the regulation of Glut1 activity.

It has been reported that Akt binds to and phosphorylates phospholipase C-γ1 in response to growth factor–mediated receptors tyrosine kinases (27). We have recently shown (21) that PLCγ is involved in the modulation of glucose transport in M07e cells exposed to SCF and H_2O_2, because PLCγ participates to the mechanism of Glut1 intracellular redistribution: the PLCγ inhibitor U-73122 was able to prevent the Glut1 translocation to the plasma membrane and consequently the glucose-uptake increase. To confirm the involvement of PLCγ in the signal transduction by these stimuli, the effects of SCF and H_2O_2 on PLCγ phosphorylation were evaluated. Figure 5B and C shows that the incubation with both the stimuli produced an increase in the PLCγ phosphorylation, as expected.

ARE AMPK AND MAPK KINASES INVOLVED IN GLUCOSE-TRANSPORT REGULATION IN M07E CELLS?

In Clone 9 cells expressing only the Glut1 isoform, hexose uptake is markedly stimulated in response to hypoxia or inhibition of oxidative phosphorylation, conditions that would be expected to cause a stimulation of AMP-activated protein kinase (AMPK) activity (1). The exposure of these cells to 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR), an adenosine analogue that stimulates AMPK activity, resulted in a marked increase in the rate of glucose transport, accompanied by activation of AMPK. The researchers concluded that stimulation of AMPK is associated with enhancement of Glut1 activity and that this response is mediated by activation of Glut1 transporters preexisting in the plasma membrane.

Therefore, to see whether this association exists in the cell system under investigation, we pretreated M07e cells with different concentrations of AICAR, but we did not find any significant effect on glucose transport, ruling out any potential role for AMPK (Fig. 6A).

To better understand the signal transduction in glucose-transport modulation by SCF, we also evaluated the effect of MAP kinases inhibitors [*i.e.*, SB203580 (16) and PD98059

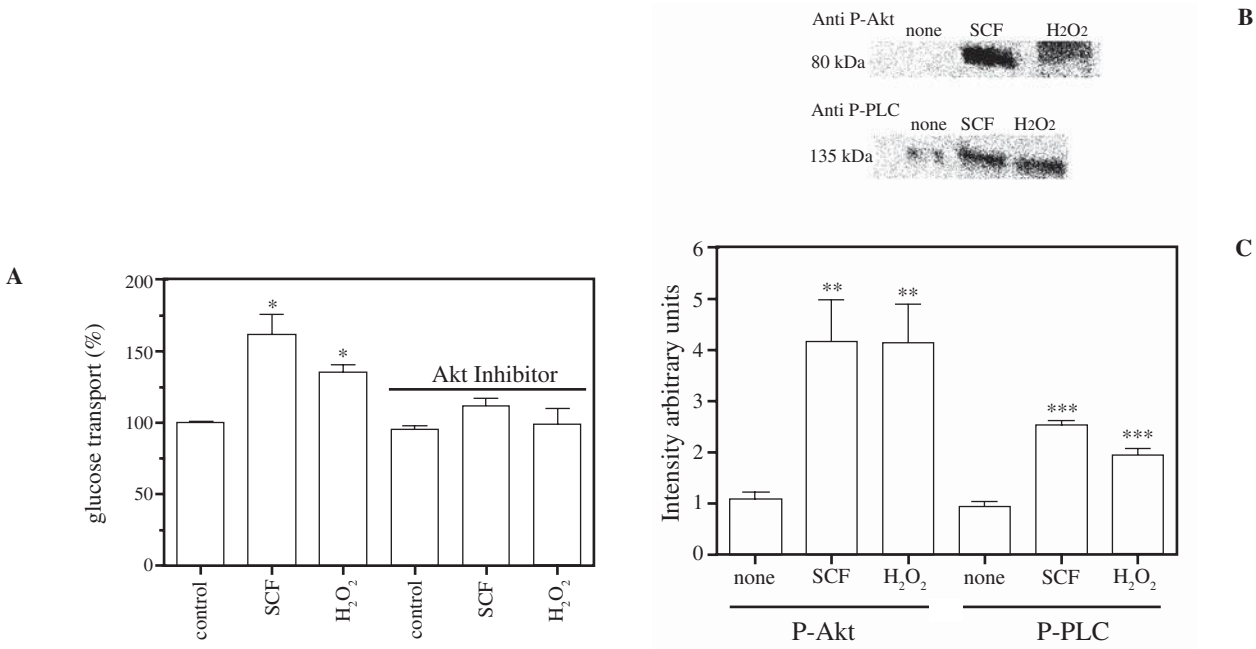

FIG. 5. Effect of SCF and H_2O_2 on Akt and PLCγ phosphorylation linked to DOG-uptake stimulation. (**A**) Cells incubated with 25 μ*M* Akt inhibitor in PBS at 37°C for 10 min were exposed to SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min, then DOG uptake was measured in PBS over 5 min, as described in Materials and Methods. Results are expressed as means ± SD of three independent experiments, each performed in duplicate; *$p < 0.05$, significantly different from control. (**B**) Cells were incubated in PBS at 37°C in the absence or presence of SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred, and immunoblotted with the indicated antibody, as described in Materials and Methods. (**C**) Results of scanning densitometry analysis performed on three independent autoradiographs. Relative amounts (means ± SD) of the indicated bands are in arbitrary units and compared with the corresponding from cells not exposed to the stimuli. ***$p < 0.001$, **$p < 0.005$, *$p < 0.05$, significantly different from control.

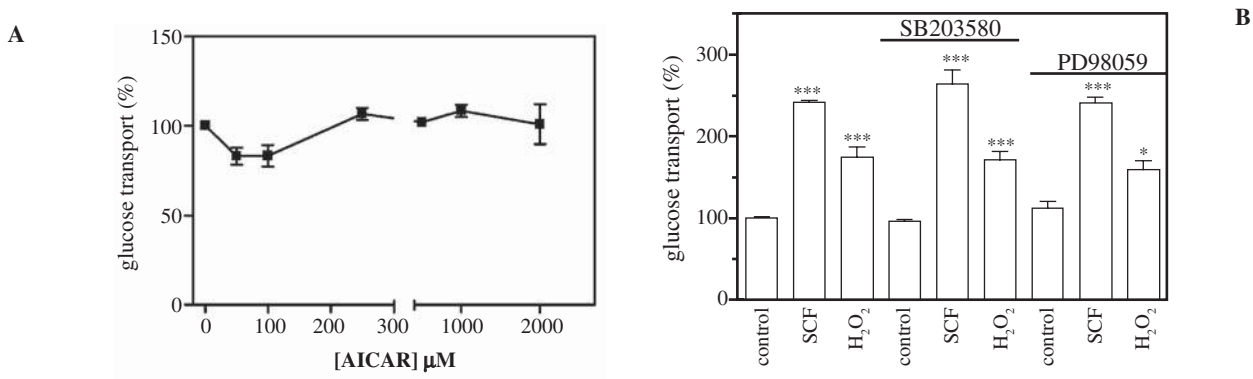

FIG. 6. Effect of AICAR, SB203580, and PD98059 on DOG uptake in M07e cells. (**A**) Cells were incubated with AICAR (0.05–2 m*M*) in IMDM at 37°C for 40 min, and then DOG uptake was measured in PBS over 5 min, as described in Materials and Methods. Results are expressed as means ± SD of three independent experiments, each performed in duplicate. (**B**) Cells incubated with 25 µ*M* SB203580 or 50 µ*M* PD98059 in IMDM at 37°C for 30 min were exposed to SCF (5 ng/ml) or H_2O_2 (50 µM) for 15 min, and then DOG uptake was measured in PBS over 5 min, as described in Materials and Methods. Results are expressed as means ± SD of three independent experiments, each performed in duplicate. ***$p < 0.001$, **$p < 0.005$, *$p < 0.05$, significantly different from control.

(11), p38 and p42/44 inhibitors, respectively]. Even with different concentrations, we did not observe any change in glucose transport acute activation due to the growth factor or H_2O_2 (Fig. 6B).

A SUGGESTED HYPOTHESIS ABOUT THE ORDER IN THE PHOSPHORYLATION CASCADE INDUCED BY SCF AND H_2O_2 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 control, we evaluated the effect of some inhibitors on the phosphorylation of PLCγ, Src, and Akt, because c-kit seems to be the first step in the pathway induced by SCF and H_2O_2. At first, we pretreated M07e cells with PP2, the Src inhibitor, and we did not observe any modulation in the phosphorylation of these enzymes (data not shown). Conversely, in the presence of U-73122, the PLCγ inhibitor, we saw a significant decrease in the band intensity of P-Src, suggesting that the activation of PLCγ is upstream from the Src phosphorylation (Fig. 7A and B). Moreover, the incubation with the Akt inhibitor affected the PLCγ phosphorylation increase induced by SCF and hydrogen peroxide, as shown in Fig. 7C and D, indicating that the Akt involvement occurs earlier than that of the PLCγ.

Taking together the results obtained using enzyme inhibitors, we suggest that the phosphorylation order downstream of c-kit activated by SCF and H_2O_2 can be as follows: Akt, PLCγ, and Src.

CONCLUSIONS AND OPEN QUESTIONS

Results here reported show that SCF stimulation of glucose transport was mimicked by a low concentration of H_2O_2, suggesting a key role for ROS in the signaling leading to Glut1 regulation. ROS scavenging by antioxidants prevented SCF-dependent transport activation (see Fig. 1). We previously observed that SCF and H_2O_2 share the capability to promote Glut1 translocation to the cell membrane (21). We show that both SCF and H_2O_2 lead to a c-kit–dependent increase in tyrosine phosphorylation and glucose transport. In particular, SCF and H_2O_2 treatment caused phosphorylation of the SCF receptor (see Fig. 3A). Taken together, these results suggest that the stimulation of glucose transport in response to H_2O_2 may be mediated through activation of the c-kit pathway.

Potentially, protein tyrosine phosphatase activity has been shown to be inhibited by H_2O_2 (26); furthermore, H_2O_2 treatment could cross-link PTP-1B to the RTKs through formation of disulfide bridges (22). Accessibility of substrate RTKs for protein tyrosine phosphatases may be an additional level of regulation.

Because SCF can increase intracellular ROS content in M07e (8) and H_2O_2 is able directly to activate c-kit, we speculate that SCF treatment could elicit a positive loop involving ROS increase and leading to glucose-uptake activation.

Further studies were performed to evaluate the signaling pathway(s) mediating the stimulatory effect of this cytokine and H_2O_2 on glucose transport using inhibitors of several kinases implicated in glucose-transport modulation.

After initiation of c-kit activity, tyrosine residues, mostly outside the kinase domain, become phosphorylated and function as docking sites for signal-transduction molecules with phosphotyrosine binding on Src homology 2 (SH2) domains (17). The Src family plays important roles in cell responses induced by growth factors, including growth, survival, and cancer formation and progression (6). Recently it was reported that hyperactivation of Lyn, a Src tyrosine kinase, with subsequent increased ROS production, may constitute one mechanism by which acute myeloid leukemia (AML) arises. G-CSF stimulation showed an increase in ROS production in a myeloid cell line, correlating with activation of the Src kinase Lyn and Akt. Inhibition of Lyn, PI3-kinase, and Akt, but

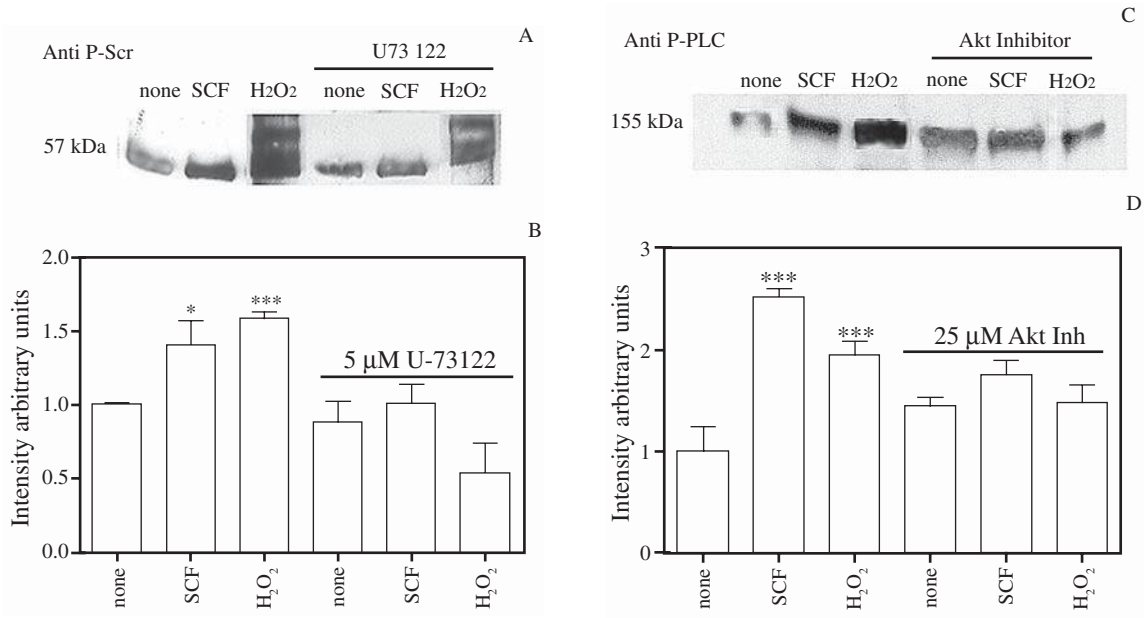

FIG. 7. Effect of U73122 and Akt inhibitor on Src and PLCγ phosphorylation in M07e cells. (**A**) Cells pretreated with 5 μ*M* U72122 were incubated in PBS at 37°C in the absence or presence of SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred, and immunoblotted with the indicated antibody, as described in Materials and Methods. (**B**) Results of scanning densitometry analysis performed on three independent autoradiographs. Relative amounts (means ± SD) of the indicated bands are in arbitrary units and compared with the corresponding from cells not exposed to the stimuli; ***$p < 0.001$, *$p < 0.05$, significantly different from control. (**C**) Cells pretreated with 25 μ*M* Akt inhibitor were incubated in PBS at 37°C in the absence or presence of SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electrophoresed, transferred, and immunoblotted with the indicated antibody as described in Materials and Methods. (**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 with the corresponding from cells not exposed to the stimuli; ***$p < 0.001$, **$p < 0.005$, significantly different from control.

not Erk1/2, abrogated G-CSF–induced ROS production. These studies suggest that one beneficial effect of the therapeutic targeting of Lyn-PI3K-Akt cascade is to block ROS production (30).

In this regard, we investigated the involvement of Src tyrosine kinases in glucose-transport stimulation (see Fig. 4A). SCF and H_2O_2 produced a significant increase in Src phosphorylation, indicating that this TK family is activated in the presence of both these stimuli (see Fig. 4B). To clarify whether this step is linked to glucose-transport modulation, we tested the effect of PP2, an Src inhibitor (19), on glucose uptake stimulated by SCF or H_2O_2. Because the inhibitor at low concentrations was able to affect the activation of glucose transport, we suggest that Src phosphorylation is relevant in this pathway.

Syk protein tyrosine kinase has been implicated in a variety of hematopoietic cell responses and also was found to be involved in oxidative and osmotic stress signaling in B cell lines (29). In M07e cells, piceatannol, a selective Syk inhibitor (19), was able to prevent the glucose-transport activation due to the incubation with SCF and H_2O_2, suggesting a role of Syk TK in the hexose-uptake modulation.

Several studies have demonstrated that Akt is rapidly activated in response to insulin and growth factors, has a key function in relaying the PI3K survival signal, and it acts as an antiapoptotic signaling kinase in a variety of cell types. The second step involves the Akt translocation from a mainly cytosolic location to the plasma membrane, where the kinase is activated by phosphorylation (9). Under our experimental conditions, incubation with SCF and hydrogen peroxide determined the phosphorylation of Akt. Moreover, an Akt inhibitor prevented the glucose-transport activation by the tested stimuli, hinting that this kinase is involved in the signaling pathway leading to the increased glucose transport, unlike PKC (21). Both PLCγ1 and Akt play significant roles in the intracellular signaling mechanism exploited by receptor tyrosine kinases, including epidermal growth factor receptor. Wang *et al*. (27) showed that the interaction between PLCγ1 and Akt resulted in the phosphorylation of PLCγ1 by Akt. We previously observed that the PLC inhibitor U-73122 prevents the glucose-transport stimulation in M07e cells (21). SCF induces a weak association between the SH2 domain of PLCγ and tyrosine 936 of human c-kit, as well as small increases in tyrosine phosphorylation of PLCγ (18). Furthermore, Src activation has been associated with tyrosine phosphorylated PLCγ (4). In samples treated with SCF and H_2O_2, an increase of the band intensity of phosphorylated PLCγ occurs (see Fig. 5B and C), confirming the involvement of PLCγ in glucose-transport activation by these stimuli.

We also investigated the involvement of AMPK, because stimulation of AMPK in skeletal and cardiac muscle by AICAR leads to an enhancement of glucose transport mediated by translocation of Glut4, raising the question whether an increase in AMPK activity might also be associated with a stimulation of transport mediated by Glut1 (1). However, the lack of AICAR effect on the rate of glucose transport in M07e cells ruled out the involvement of AMPK in the modulation of glucose uptake in M07e cells.

Mahadev *et al.* (20) showed that the insulin-stimulated oxidant signal affects downstream components of the insulin signaling pathway (*i.e.*, PI3-kinase and MAP kinase), and that glucose uptake in eosinophils is governed by specific mechanisms involving mobilization of Gluts through the activation of the MAP kinase pathway. Conversely, the role of MAP kinases in the short-term regulation of glucose activation was excluded by treating M07e cells with p38 or p42/44 inhibitors.

Further studies were performed to delineate the sequence of events leading from enhancement of tyrosine kinases to the observed glucose-transport stimulation in response to SCF and H_2O_2 (see Fig. 7). The analysis of the effect of several kinase inhibitors on Src, Akt, and PLCγ phosphorylation suggested that, after c-kit activation by SCF and H_2O_2, Akt phosphorylation can be one of the early steps leading to PLCγ involvement. Because Src inhibitor does not affect PLCγ and Akt activation, we conclude that Src involvement occurs later in the glucose transport–modulation cascade.

We recently hypothesised that a homologue of the phagocytic NAD(P)H oxidase could be a potential ROS source, linked somehow with Glut1 activity in M07e cells (25). However, the ROS involved in the signaling pathway is still under scrutiny. Thus, further understanding of the mechanisms of the short-term glucose-transport regulation will require a composite knowledge of the source, chemical nature, and the downstream targets of the ROS that are generated only transiently at the very beginning of the signaling process. Current work is in progress in that direction.

Malignant cells are known to have accelerated metabolism, high glucose requirements, and increased glucose uptake. Stimulation of glucose uptake appears to play an important role in the suppression of apoptosis in normal hemopoietic cells (2). Abrogating ROS production could be an interesting approach in the therapeutic targeting of signaling cascade leading to glucose-transport activation.

ABBREVIATIONS

AICAR, 5-aminoimidazole-4-carboxamide-ribonucleoside; AMPK, AMP-activated protein kinase; DOG, 2-deoxy-D-glucose; 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, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SCF, stem cell factor; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

APPENDIX

Notes

1. Materials and Methods

SCF was provided by Amgen (Thousand Oaks, CA). Phloretin, 2-deoxy-D-glucose (DOG), genistein, SB203580, PD98059, H_2O_2, 4-amino-5-imidazole-carboxamide riboside (AICAR), piceatannol, phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-lysine chloromethyl ketone (TLCK) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Sigma (St. Louis, MO). Akt Inhibitor [1-L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-methyl-3-*O*-octadecylcarbonate] and ebselen were from Calbiochem (San Diego, CA). PP2 was purchased from Tocris (Ellisville, MO). 2-Deoxy-D-[2,6-^{3}H]-glucose was from Amersham (U.K.); nitrocellulose paper was obtained from Schleicher and Schuell (Keene, NH). Antirabbit and antimouse IgG conjugated to horseradish peroxidase, and Western Blotting Luminol Reagent were purchased from Santa Cruz (Santa Cruz, CA). Anti-Phospho-c-kit (Tyr719), Anti-Phospho-Akt (Tyr326), Anti-Phospho-Src Family (Tyr416), Anti-Phospho-PLCγ1 (Tyr783), and Anti-Phospho-Tyrosine (P-Tyr-102) were from Cell Signaling Technology (Beverly, MA). Imatinib mesylate (STI-571) was a kind gift from Novartis (Basel, Switzerland). Hydroxylamine probe [bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate] was provided by Prof. G.F. Pedulli. All the other chemicals and solvents were of the highest analytic grade.

2. Cell culture

M07e cells are a human leukemic megakaryocytic line whose proliferation is IL-3 or GM-CSF dependent. Cells are cultured as previously reported (7). In brief, cells grow in IMDM supplemented with 5% fetal calf serum and 10 ng/ml IL-3. Because 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 hemocytometer; viable cells were evaluated by the Trypan blue exclusion test.

3. Glucose-transport assay

After an incubation with SCF (5 ng/ml) or H_2O_2 (50 μ*M*) 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 m*M* unlabeled 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 m*M* final concentration). The uptake was linear up to 10 min. Transported DOG was <20% of the extracellularsugar concentration; therefore glucose-transport assay could be considered in zero-trans conditions. Sample radioactivity was measured by liquid scintillation counting.

Because M07e cells were deprived of medium components and maintained in PBS during glucose-transport measurements, their viability in this buffer 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 (≤1 h) was considered constant.

4. Immunoprecipitation

M07e cells (15×10^6 per experimental condition) were placed in PBS, incubated with SCF (5 ng/ml) or H_2O_2 (50 μ*M*) for 15 min in the presence of 1 m*M* orthovanadate, and then pelleted. Cells were lysed with a lysis buffer (1% Igepal, 150 m*M* NaCl, 50 m*M* Tris-Cl, 5 m*M* EDTA, 0.1 m*M* PMSF, 0.1 m*M* TLCK, 0.1 m*M* TPCK, 1 m*M* orthovanadate, and SIGMA 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 5 times with lysis buffer, treated with sample reducing buffer containing 4% β-mercaptoethanol (final concentration), and then boiled for 3 min.

5. SDS-PAGE and Western blot analysis

Immunocomplexes 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. Nonspecific binding to membrane was blocked by incubating in Tris-buffered saline (TBS)/Tween, pH 8.0, containing 5% nonfat dried milk for 1 h at room temperature. Then the nitrocellulose membranes were incubated overnight at 4°C with primary antibodies directed to the phosphorylated forms 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% nonfat dried milk. Membranes were washed with TBS/Tween and developed using Western Blotting Luminol Reagent.

A semiquantitative evaluation of the slabs was performed using a Fluor S image analyzer.

6. Statistical analysis

Student's *t* test was performed comparing cytokine/H_2O_2-treated samples with controls and inhibitor-untreated with inhibitor-treated samples. The *p* values are reported in figures only for the former data analysis.

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