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NAD(P)H oxidase isoform Nox2 plays a prosurvival role in human leukaemia cells

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Abstract
The mechanism involved in the prosurvival effect of interleukin-3 on the human acute myeloid leukaemia cell line M07e is investigated. A decrease in intracellular reactive oxygen species (ROS) content, glucose transport activity and cell survival was observed in the presence of inhibitors of plasma membrane ROS sources, such as diphenylene iodonium and apocynin, and by small interference RNA for Nox2. Moreover, IL-3 incubation stimulated the synthesis of Nox2 cytosolic sub-unit p47phox and glucose transporter Glut1. Thus, the inhibition of ROS generation by Nox inhibitors stimulated apoptosis showing that ROS production, induced by IL-3 via Nox2, protects leukaemic cells from cell death. Also incubation with receptor tyrosine kinase inhibitors, such as anti-leukaemic drugs blocking the stem cell factor receptor (c-kit), showed similar effects, hinting that IL-3 transmodulates c-kit phosphorylation. These mechanisms may play an important role in acute myeloid leukaemia treatment, representing a novel therapeutic target.

Keywords: *Reactive oxygen species, glucose transport, megakaryocytic cells, interleukin 3, apoptosis, NAD(P)H oxidase*

Abbreviations: *2-APB, aminoehtyl diphenyl borinate; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin; Ac-IETD-AMC, acetyl-Ile-Glu-Thr-Asp-7-amino-4-methyl coumarin; Ac-LEHD-AMC, acetyl-Leu-Glu-His-Asp-7-amino-4-methyl coumarin; AML, acute myelogenous leukaemia; CaMKII, Ca^{2+}/Calmodulin-Dependent Protein Kinase II; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; DOG, 2-deoxy-D-glucose; DPI, diphenyleneiodonium chloride; FCS, foetal calf serum; Glut1, glucose transporter 1; HE, hydroethidine; IL-3, interleukin-3; IMDM, Iscove's modified Dulbecco's medium; $InsP_3$, inositol 1,4,5-triphosphate; JNK, Jun N-terminal kinase; MAPK, Mitogen-Activated Protein Kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nox, NAD(P)H oxidase; PBS, phosphate buffered saline; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PMSF, phenylmethylsulphonyl fluoride; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SCF, stem cell factor; TK, tyrosine kinase; TLCK,* *N*-tosyl-lysine chloromethyl ketone; TPCK, *N*-tosyl-phenylalanine chloromethyl ketone; VEGFR2, vascular endothelial growth factor receptor 2.

Introduction

Large quantities of reactive oxygen species (ROS) are produced in phagocytes, mediating host defence against microorganisms, by the plasma membrane NAD(P)H oxidase (Nox), a well characterized multi-component enzyme with gp91phox and p22phox catalytic sub-units forming an integral complex called flavocytochrome b558 [1]. Recently, proteins

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belonging to the NAD(P)H oxidase family homologous to gp91phox have been shown to generate ROS in nonphagocytic cells [2,3].

A growing body of evidence indicates that ROS play a signalling role in physiological and pathophysiological processes, including proliferation [4], adhesion [5] and hypertension [6]. Although ROS have long been thought to promote cell death [7], recent data suggest that they may also play a pro-survival role through the activation of anti-apoptotic signalling pathways [8,9]. In particular, growth factors are known to stimulate ROS production in a variety of cell types through receptor-transducing pathways, although the detailed mechanism is poorly understood [10]. Growth factor-induced ROS production is believed to be necessary for optimal propagation of mitogenic signals in neoplastic proliferation [4] and for glucose transport activation [11]. Malignant cells are known to have accelerated metabolism, high glucose requirement and thus increased glucose uptake. In mammalian cells, glucose transport across the plasma membrane, 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 [12].

Recent data suggest that ROS inhibit apoptosis in smooth muscle cells [9], leukaemia cells [13] and prostate cancer cells [3,14]. The pro-survival effect of ROS can be mediated by anti-apoptotic redox-sensitive pathways [15]. Understanding the role of ROS as key mediators of tumour cell proliferation may provide opportunities for pharmacological intervention. Acute myelogenous leukaemia (AML) is a disease difficult to treat. Novel treatment strategies, including molecular targeted therapy, are currently being explored. The c-kit receptor represents a potential therapeutic target for AML treatment since it mediates proliferation and anti-apoptotic effects. In fact, this receptor is expressed in more than 10% of blasts, in 64% of *de novo* AMLs and 95% of relapsed AMLs [16].

In the human acute myeloid leukaemia cell line M07e, we have recently demonstrated that interleukin-3 (IL-3) induces ROS formation, positively affecting Glut1-mediated glucose uptake and cell survival, through different signalling pathways activation. Thus, ROS production induced by IL-3 can protect leukaemic cells from apoptosis, the effect being counteracted by antioxidants [15]. We also showed that, by treating with different antioxidants an acute leukaemic cell line (B1647) self-producing vascular endothelial growth factor (VEGF), ROS generation was crucially involved in the modulation of glucose transport (mediated by Glut1), which is frequently up-regulated in cancer cells [17]. Results proved that Nox2 and Nox4 are important in the regulation of glucose uptake on which cancer cells rely [18].

The present study seeks to identify the sources of ROS generation stimulated by IL-3 and whether ROS affect c-kit activation and apoptosis in an AML cell line, M07e. Our aim is to investigate whether IL-3 stimulates ROS generation by activating some isoforms of membrane NAD(P)H oxidase and whether the inhibition of ROS generation induces apoptosis in leukaemic cells. On the basis of the results here reported, we confirm that the pro-survival effect of ROS may become an important target in AML therapy.

Materials and methods

Materials

Phloretin, 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), hydroethidine (HE), Hoechst-33342, diphenyleneiodonium chloride (DPI), LY-294002, chelerythrine, wortmannin, 2-APB (aminoethyl diphenyl borinate), 4-hydroxy-3-methoxy-acetophenone (apocynin), 2-deoxy-glucose (DOG), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Trypan Blue, Igepal CA-630, orthovanadate, U-73122, PD98059, H_2O_2, piceatannol, phenylmethylsulphonyl fluoride (PMSF), *N*-tosyl-lysine chloromethyl ketone (TLCK), *N*-tosyl-phenylalanine chloromethyl ketone (TPCK) and mouse monoclonal anti-serum against tubulin were from Sigma (St Louis, MO). Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco (Grand Island, NY) and foetal calf serum (FCS) was from Hyclone (Holland). Akt inhibitor [1-L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-methyl-3-*O*-octadecylcarbonate], W-7 and SU-5614 were from Calbiochem (San Diego, CA). Caspase-3, 8, 9 fluorogenic substrates were from Alexis (San Diego, CA). Interleukin-3 (IL-3) was from Biosource International (Camarillo, CA). PP2 was purchased from Tocris (Ellisville, MO). 2-deoxy-[2,6-^{3}H]-glucose was from Amersham (UK); nitrocellulose paper was from Schleicher and Schuell (Keene, NH). Anti-p47phox and anti-Rac1 were from Upstate (Lake Placid, NY). Imatinib mesilate (Gleevec or STI-571) was generously provided by Novartis (Basel, Switzerland). Goat polyclonal anti-sera against Glut1, Nox1, Nox4, anti-rabbit, anti-goat and anti-mouse IgG conjugated to horseradish peroxidase and Western Blotting Luminol Reagent were purchased from Santa Cruz (Santa Cruz, CA). Nox2 was from Millipore (Temecula, CA). Anti p67phox was kindly provided by Dr M. Geiszt. Anti-phospho-c-kit was from Cell Signaling Technology (Beverly, MA). All the other chemicals and solvents were of the highest analytical grade.

Cell culture

M07e cells, purchased from DSMZ, Braunschweig (Germany), are a human leukaemic megakaryocytic line whose proliferation is IL-3 or GM-CSF dependent. Cells were cultured as previously reported [11]. In brief, cells were grown in IMDM supplemented with 5% foetal calf serum (FCS) and 10 ng/mL IL-3. Since some 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 18–24 h under these conditions.

Cell viability

Viable cells were evaluated by the Trypan blue exclusion test. Cell viability was also assayed by the MTT assay [18]. Cells, pre-treated for 20 h with NAD(P)H inhibitors, receptor tyrosine kinase inhibitors or the solvent DMSO (highest dose 0.1%), 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 dissolved in 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 Victor^2, Perkin Elmer) at a wavelength of 570 nm.

Glucose transport assay

Glucose transport assay was performed as previously reported [19]. In brief, after incubation with different inhibitors, glucose uptake was assayed in PBS buffer, pH 7.2 (in the absence of glucose) by adding 2-deoxy-[2,6-^{3}H] glucose (15 kBq/assay) and 1 mM unlabelled 2-deoxy-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 2-deoxy-glucose was less than 20% of the extracellular sugar concentration, therefore glucose transport assay could be considered in zero-trans conditions. Sample radioactivity was measured by liquid scintillation counting.

SDS-PAGE and Western blot analysis

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. 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 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. Blots were washed with TBS/Tween and 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.

Preparation of RNA and reverse transcriptase PCR (RT-PCR) analysis

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Scotland) according to the manufacturer's recommendations. After extraction, the RNA concentration was determined spectrophotometrically, measuring the absorbance at 260 nm. PCR products were amplified by using specific primers from TIB Molbiol (Genova, Italy): NOX1, antisense 5′-GAACTCTTGGGGTAGGTGTG-3′ and sense 5′-CATCCACAAACAGGAAAACA-3′; NOX2, antisense 5′-CTCACCCTTTCAAAACCATC-3′ and sense 5′-ACGATGCGGATATGGATACT-3′. Primers for human NOX4 were designed by published sequences [20] as follows: antisense 5′-AGAGGAACACGACAATCAGCCTTAG-3′ and sense 5′-CTCAGCGGAATCAATCAGCTGTG-3′. RT-PCR was carried out using 'Access RT-PCR Systems' (Promega, Madison, WI). The reaction mixtures were kept for 45 min at 45°C, 2 min at 94°C, then cycled 35 times through a programme of 30 s at 94°C, 1 min at 50°C for NOX1 and NOX2 or 1 min at 58°C for NOX4 and 1 min at 72°C; finally, the reaction was incubated for an extra 7 min at 68°C. After RT-PCR, the DNA products were electrophoresed on 2% agarose gel and stained with GelRed (Biotium, Hayward, CA).

RNA interference

For transient siRNA transfection, M07e cells were nucleofected with Cell Line Nucleofector^{TM} Kit C (Amaxa Biosystems, Cologne, Germany) programme X-05 following the manufacturer's recommendations and instructions, with siRNA against Nox2 or non-specific control siRNA (final siRNA concentration 50 nM). Oligos were obtained from Sigma-Genosys (Suffolk, UK). Specific oligos with maximal knock-down efficiency were selected among three different sequences for each gene. Subsequently, cells were immediately suspended in complete medium with IL-3 and incubated in a humidified 37°C/5% CO_2 incubator. After 24–48 h cells were used for experiments: evaluation of Nox2 expression by Western blotting, detection of intracellular ROS levels and measure of cell viability.

Measurement of intracellular ROS

Cells (1×10^6/mL) were washed twice in PBS and incubated with 5 μM DCFH-DA for 20 min at 37°C. DCFH-DA is a small non-polar, non-fluorescent molecule that diffuses into the cells where it is enzymatically deacetylated by intracellular esterases to the polar non-fluorescent compound, that is oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) [21]. For the hydroethidine (HE) assay, cells (1.0×10^6/mL) were washed twice in PBS and incubated with 10 μM HE for 20 min at 37°C, in the dark. The fluorescence of oxidized probes was measured on a multiwell plate reader (Wallac Victor^2, Perkin Elmer).

Chromatin condensation assay

Cells were washed and fixed with formaldehyde, then stained with Hoechst 33342 (5 μg/mL) for 5 min at 25°C. The nuclei were visualized using a fluorescence Olympus IX50 microscope.

Caspase assay

Ac-DEVD-AMC, Ac-IETD-AMC and Ac-LEHD-AMC were used as fluorescent protease substrates for caspase-3, caspase-8 and caspase-9, respectively. After different treatments, cell lysates were incubated with specific substrates at 37°C for 15 min. The activity of caspase-3, -8, or -9 was determined following the cleavage of fluorogenic substrates excited at 370 nm by measuring the emission at 455 nm.

Statistical analysis

Data sets were analysed with Student *t*-test, comparing cytokine-treated samples vs controls and inhibitor-untreated vs inhibitor-treated samples.

Results

Effect of Nox inhibitors and anti-leukaemic compounds on M07e cells viability

NAD(P)H oxidase family components have been identified in many cellular systems, but their physiological roles and molecular nature in eukaryotes are still unclear [22]. To investigate the presence of NAD(P)H oxidase components, RT-PCR and Western blot were performed. The presence of Nox2 mRNA is shown in Figure 1A. Western blot analysis of cell lysates confirmed the expression of Nox2 and demonstrated that M07e cells express the p67phox and p47phox sub-units and the small GTPase-protein Rac1 (Figure 1B). Then, we investigated the role of Nox-derived ROS induced by IL-3 in neoplastic proliferation. Since ROS can act as pro-survival factors [15], we investigated the effect of exogenous ROS and inhibitors of Nox sources on leukaemic cell viability, evaluated by Trypan blue exclusion test (data not shown) and by the MTT assay (Figure 2A). Intracellular ROS production was evaluated with DCFH-DA assay (Figure 2B): similar results were obtained with HE probe (data not shown), as reported in Maraldi et al. [15]. The presence of IL-3 in cell culture induced an increase in cell proliferation and ROS content, as expected. More interestingly, incubation with 50 μM H_2O_2 for 4 h, in the absence of IL-3, did not show cytotoxic activity; on the contrary, cell viability was increased by ~20%, suggesting a pro-proliferative effect of exogenous hydrogen peroxide in this leukaemic cell

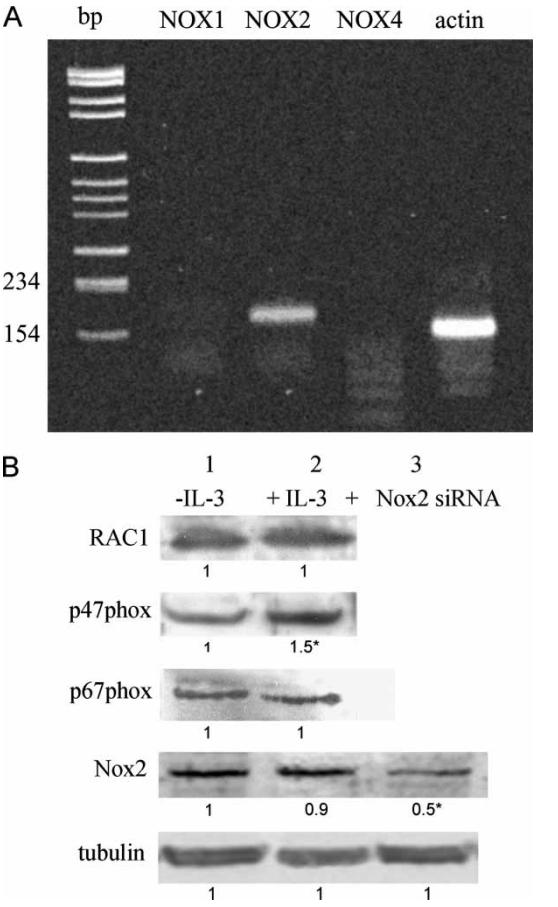

Figure 1. NAD(P)H oxidase expression in M07e cells. (A) RT-PCR of mRNA derived from M07e cells, using specific primers for NOX1, NOX2, NOX4 and actin. RT-PCR was performed as described in the Material and methods section. The data shown are representative of three independent experiments. (B) Representative immunoblots showing Rac1, p67phox, p47phox and Nox2 expression in M07e cells grown with or without IL-3 (lanes 1 and 2). Lane 3 shows the effect on Nox2 expression level after 48 h of Nox2 siRNA treatment; electroporate sample was considered as control and the not specific control did not give a significant effect (data not shown). Forty micrograms of protein per lane were electrophoresed and immunoblotted, as described in the Materials and methods section. The data shown are representative of three independent experiments. Relative amounts determined by scanning densitometry are in arbitrary units, normalized against tubulin values and compared to the corresponding band from IL-3-deprived cells, except for Nox2 with siRNA where silencing was compared to IL-3-treated cells. $^*p < 0.0001$, significantly different from control.

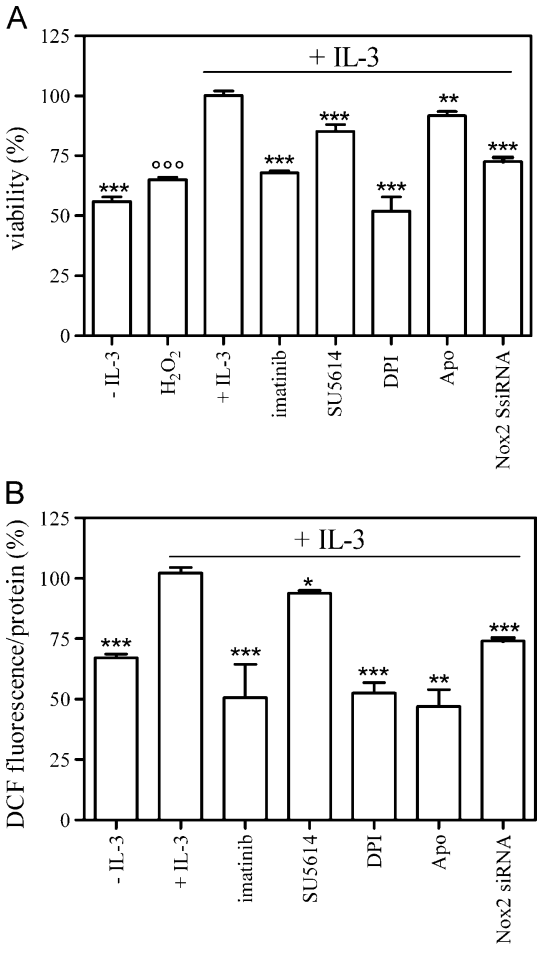

Figure 2. Effect of NAD(P)H oxidase inhibitors and anti-leukaemic drugs on M07e cells. (A) Cells deprived of IL-3 for 24 h were incubated for 4 h with 50 μM H_2O_2 while cells grown in the presence of IL-3 were incubated for 24 h in IMDM with 10 μM DPI, 1 mM apocynin, 100 μM imatinib and 10 μM SU5614. Nox2 silencing was performed as described in the Material and methods section. Cell viability was measured with MTT assay as described in the Material and methods section. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. ***$p < 0.0001$; **$p < 0.01$ significantly different from sample with IL-3. °°°$p < 0.0001$ significantly different from sample without IL-3. (B) ROS content of cells treated as described in (A), was measured in PBS with DCFH-DA probe as described in the Materials and methods section. Results are normalized against cellular protein content (fluorescence/protein) and expressed as means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$ significantly different from samples with IL-3.

line. Furthermore, the incubation for 18 h with IL-3 determined an increase in the p47phox sub-unit concentration (Figure 1B).

In order to confirm the role of hydrogen peroxide in the modulation of cell viability, we performed experiments using different Nox inhibitors such as diphenyleneiodonium (DPI), an inhibitor of flavoprotein centres, and apocynin, an inhibitor of NAD(P)H oxidase assembly; 10 μM DPI and 1 mM apocynin resulted in a dramatic reduction of both cell survival and ROS content (Figure 2A and B). Furthermore, treatment with siRNA selective for Nox2 showed an effect similar to the one obtained with the tested inhibitors (Figure 2A and B), confirming the Nox2 involvement in ROS production in M07e cells. Western blot analysis demonstrated the efficiency of Nox silencing after 48 h (Figure 2B). All these data indicate a positive role of Nox-generated ROS in leukaemic cell proliferation.

In myeloid leukaemia clinical trials, imatinib mesylate (also known as STI-571 or Gleevec), a potent inhibitor of receptor tyrosine kinases (RTKs) such as the PDGFβ-receptor and c-kit, has shown encouraging results and has become a paradigm for targeted cancer therapeutics [23]. Results reported in the literature indicate that another RTK inhibitor, SU5614, inhibits the growth of cell lines such as Kasumi-1, M07e and UT-7 [24]. The pre-treatment of M07e cells with imatinib or SU5614 for 24 h resulted in an obvious reduction of cell survival of 30% and 20%, respectively. Interestingly, the treatment with anti-leukaemic compounds generated a decrease of ROS concentration in M07e grown in the presence of IL-3 (Figure 2B).

Induction of apoptosis by Nox inhibitors and anti-leukaemic compounds

To characterize cell death induced by Nox inhibitors and anti-leukaemic compounds, typical apoptotic features were examined. Caspases play a central role in mediating various apoptotic responses and are activated in a sequential cascade of cleavages. The activation of an effector caspase, such as caspase-3, is executed by initiator caspases, such as caspase-9 and caspase-8, through proteolytic cleavage after a specific internal Asp residue, to separate the large and small sub-units of the mature caspase. To detect the enzymatic activity of caspases, three fluorogenic substrates were used. As illustrated in Figure 3A, the presence of IL-3 for 24 h decreased caspase activity. Moreover, incubation of cytokine-deprived cells with exogenous H_2O_2 induced a significant attenuation of caspase activity, confirming the positive role of low doses of H_2O_2 in cell survival. Furthermore, incubation with Nox inhibitors and anti-leukaemic compounds, which decrease ROS concentration, resulted in a significant stimulation of caspase-3-like activity (Figure 3B).

To confirm these data, observations of apoptotic nuclei were performed. All tested compounds, even if in a different extent, determined an increase in chromatin condensation and fragmentation (Figure 3C).

Effect of Nox inhibitors and anti-leukaemic compounds on glucose uptake in M07e cells

In M07e cells, we observed that low doses of exogenous H_2O_2 as well as IL-3 were able to activate glucose

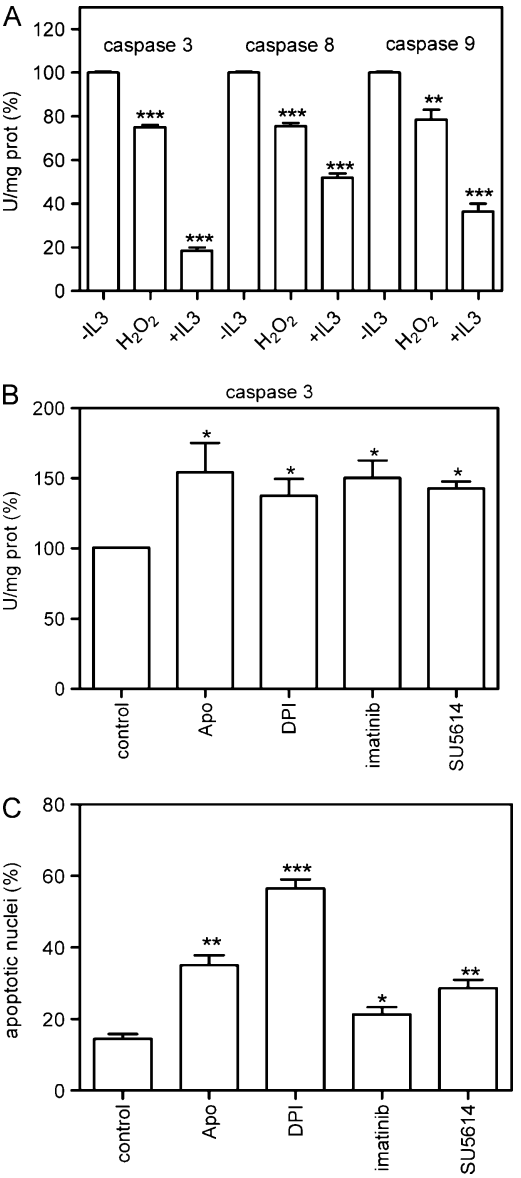

Figure 3. Caspase activation by NAD(P)H oxidase inhibitors and anti-leukaemic drugs in M07e cells. (A) Cells grown in the presence of IL-3 or cells deprived of IL-3 for 24 h were incubated in the presence or absence of 50 μM H_2O_2 for 24 h. Caspase 3, 8 and 9 activity were measured on cell lysates, 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; **$p <0.01$ significantly different from control. (B) Cells grown in the presence of IL-3 were incubated for 24 h in IMDM in the presence of 10 μM DPI, 1 mM apocynin, 100 μM imatinib and 10 μM SU5614. Caspase 3 activity was measured on cell lysates, as described in the Materials and methods section. Results are expressed as means ± SD of three independent experiments, each performed in duplicate. *$p <0.05$ significantly different from control. (C) Apoptotic nuclei of cells, treated as shown in (A), were measured in PBS with Hoechst 33342 probe (5 μg/mL) as described in the Materials and methods section. Results are expressed as percentage of cells containing apoptotic nuclei ± SD. ***$p <0.0001$; **$p <0.01$; *$p <0.05$ significantly different from control.

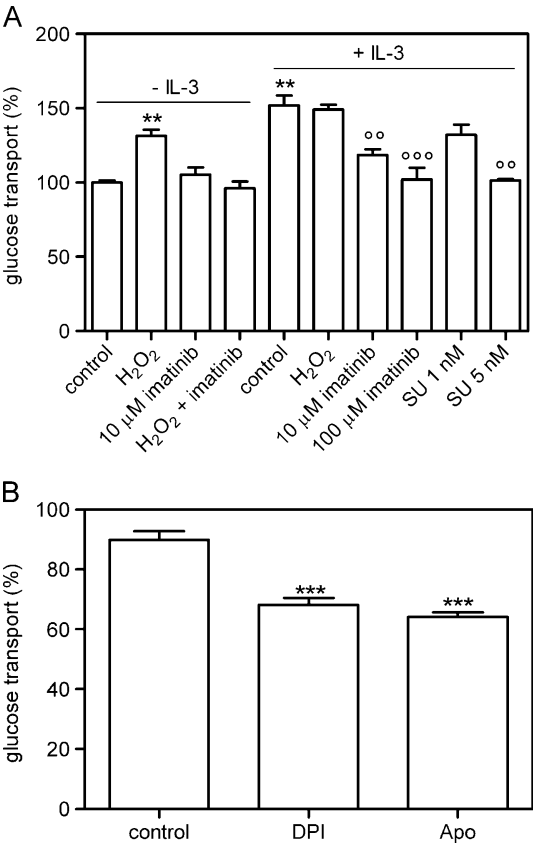

Figure 4. Effect of NAD(P)H oxidase inhibitors and anti-leukaemic drugs on glucose uptake in M07e cells. (A) Cells deprived or not of IL-3 for 18 h were incubated at 37°C with 50 μM H_2O_2 for 15 min, 10–100 μM imatinib and 1–5 μM SU5614 for 30 min. DOG uptake was measured in PBS over 5 min as described in the Materials and methods section. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. **$p <0.01$ significantly different from control without IL-3. °°°$p <0.0001$; °°°$p <0.01$ significantly different from control with IL-3. (B) Cells grown in the presence of IL-3 were incubated for 24 h in IMDM in the presence of 10 μM DPI and 1 mM apocynin. 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.

transport in cytokine-deprived M07e (Figure 4A). The treatment with the anti-leukaemic drug imatinib (10 μM) did not affect the basal glucose uptake of IL-3 deprived cells, but prevented the activation due to hydrogen peroxide. The addition of H_2O_2 to cells grown with IL-3 did not cause any glucose uptake modulation, while imatinib and SU5614 substantially attenuate, in a concentration-dependent manner, the activation of glucose transport, taking the transport value back to IL-3 deprived sample.

In order to evaluate whether NAD(P)H oxidases could be a ROS generation site involved in the regulation of glucose transport, we tested the effect of 10 μM DPI and 1 mM apocynin on glucose transport activity. Figure 4B shows that inhibitors of NAD(P)H oxidases, while decreasing ROS production, also inhibited glucose uptake activation, suggesting that ROS, produced by Nox, can contribute to the regulation of glucose transport and thus to cell proliferation [21].

IL-3 and H_2O_2 affect common signalling steps leading to glucose transport activation

We recently showed that in M07e cells, the treatment with IL-3 or exogenous H_2O_2 generated an increase in tyrosine phosphorylation of several kinases, such as Src, Akt, p42/44 and JNK, whereas both stimuli did not affect p38. Only the presence of a less specific stimulus, such as H_2O_2, produced a CaMKII phosphorylation increase, indicating that these kinases are not involved in IL-3 triggered cascade [15]. In order to identify some of the steps connecting receptor activation by IL-3 to Glut1 modulation and cell proliferation, we tested the effect of enzyme inhibitors on glucose transport. Results shown in Figure 5 demonstrate that pre-treatment of M07e cells with 0.1 μM PP2, a Src tyrosine kinase (TK) inhibitor [25], was able to significantly reduce glucose transport stimulation induced by IL-3. Being a general hypothesis that activation of Src family kinases precedes the activation of Syk kinases [26], we investigated whether also Syk TKs are involved in the glucose transport activation using 1 μM piceatannol, a Syk inhibitor [25]. The results shown in Figure 5 suggest that also Syk TKs participate in glucose uptake activated by IL-3.

Numerous studies have related the activation of glucose transport to signalling pathways involving phosphoinositide 3-kinase (PI3K). In a murine mast cell line, Bentley et al. [27] showed that IL-3 stimulation of glucose transport and transporter translocation were prevented by PI3K inhibitors. In order to evaluate the role of PI3K in M07e cells, we followed a similar approach, choosing 25 μM LY-294002 and 1 μM wortmannin as PI3K inhibitors [28]. Under these experimental conditions, both the inhibitors negatively regulated glucose transport (Figure 5).

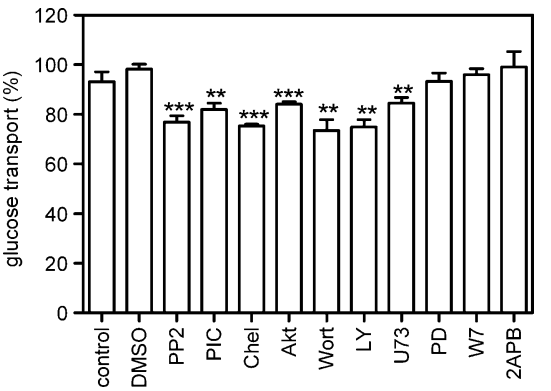

Figure 5. Signal cascade activated by IL-3 involved in glucose transport modulation. Cells grown in presence of IL-3 were incubated for 24 h in IMDM with 100 nM PP2, 1 μM piceatannol, 25 μM chelerythrine, 100 μM Akt inhibitor, 1 μM wortmannin, 25 μM LY-294002, 5 μM U-73122, 50 μM PD98059, 25 μM W7 and 25 μM 2-APB for 30 min. 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. ***$p < 0.0001$; **$p < 0.01$ significantly different from control.

It has been reported that the PI3K/Akt pathway 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 [29]. Cell pre-treatment with 100 μM Akt inhibitor caused a significant decrease in glucose transport induced by IL-3 (Figure 5). Akt binds to and phosphorylates phospholipase C (PLC)-γ1 in response to growth factors [30]. We previously observed [31] that PLCγ is involved in the modulation of glucose transport in M07e cells exposed to stem cell factor (SCF) and H_2O_2. Data here reported show that the PLCγ inhibitor U-73122 decreases the glucose uptake due to IL-3 (Figure 5).

As far as protein kinase C (PKC) is concerned, the cytokine-enhanced glucose uptake was affected by 50 μM chelerythrine, a PKC inhibitor [32] (Figure 5). Taken together, these results indicate that IL-3 increases glucose transport activity probably through a PI3K/Akt/PKC-dependent mechanism. To better understand the signal transduction in glucose transport modulation by IL-3, we also evaluated the effect of 50 μM PD98059, a p42/44 inhibitor [33]. We did not observe any change in glucose transport activity (Figure 5), even using PD98059 concentrations up to 0.1 mM.

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+} and calmodulin could be involved in conveying signals towards acute Glut1 activation in M07e cells [19]. The effects of W-7, a calmodulin antagonist [34], and of 2-APB, a compound described as a membrane-permeant inhibitor of $InsP_3$-induced Ca^{2+} release [35], were tested. Pre-incubation of M07e cells with 25 μM 2-APB or 25 μM W-7 had no effect on the basal level of transport, providing evidence against the involvement of an intracellular Ca^{2+} response.

Effect of different inhibitors on intracellular ROS level in M07e cells

To analyse signal transduction pathways linking ROS generation to Glut1 activity modulation, we investigated whether PI3K, Src, Syk, MAP kinase, PLCγ and calcium release were connected with ROS intracellular level and glucose transport. When cells were pre-incubated for 30 min with the above mentioned inhibitors of PI3K, Syk and of PLCγ, DCF fluorescence (Figure 6) was blunted. Concomitantly, a significant decrease of glucose uptake occurred (Figure 5). On the other hand, Src, calcium release, calmodulin and p42/44 did not seem to take part in ROS generation involved in glucose uptake activation in this cell line, as shown

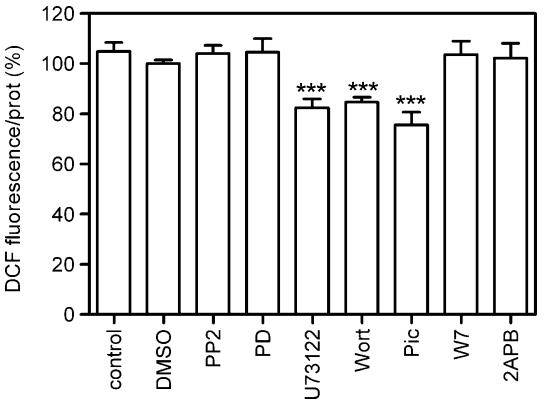

Figure 6. Signal cascade activated by IL-3 and involved in intracellular ROS increase. Cells grown in presence of IL-3 were incubated for 24 h in IMDM with 0.1 μM PP2, 1 μM piceatannol, 1 μM wortmannin, 5 μM U-73122, 50 μM PD98059, 25 μM W7 and 25 μM 2-APB for 30 min. ROS content was measured in PBS with DCFH-DA probe 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.

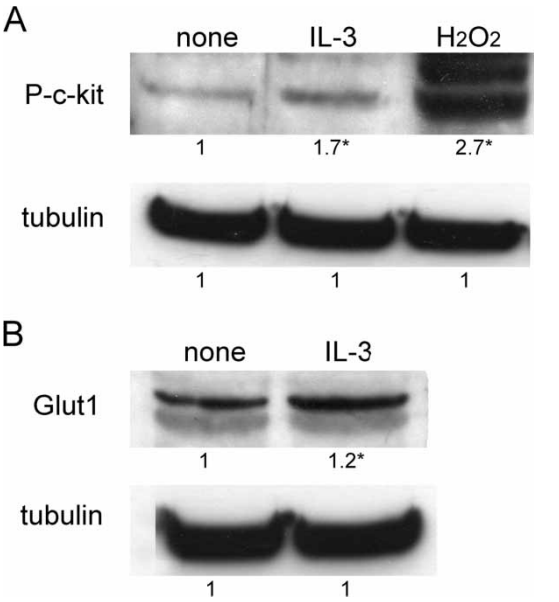

Figure 7. Effect of IL-3 on Glut1 content and c-kit phosphorylation. (A) Lysates from cells grown in the presence of IL-3 or cells deprived from IL-3 for 24 h were incubated in the presence or absence of 50 μM H_2O_2 for 15 min. Cell lysates were electrophoresed, transferred and immunoblotted with anti-P-c-kit and or-tubulin antibodies, as described in the Materials and methods section. (B) Lysates from cells grown in presence of IL-3 or cells deprived form IL-3 for 24 h were electrophoresed, transferred and immunoblotted with anti-Glut1 or anti-tubulin antibodies, as described in the Materials and methods section. The data shown are representative of three independent experiments. Relative amounts determined by scanning densitometry are in arbitrary units, normalized against tubulin values and compared to the corresponding bands from IL-3- deprived cells. *$p < 0.0001$, significantly different from control.

in Figure 6. These data implicate a multi-step pathway resulting in IL-3 modulation of Glut1 activity: Src and p42/44 appear to be downstream NAD(P)H oxidase activation while PI3K, PLC and Syk seem to regulate oxidase activity.

Effect of IL-3 on c-kit phosphorylation and Glut1 content

SCF and its receptor, c-kit, are critical in the survival and development of stem cells involved in hematopoiesis. Interaction of SCF with c-kit rapidly induces receptor dimerization and increases its autophosphorylation activity [36].

We previously showed that phosphorylation of c-kit is elicited by both SCF and H_2O_2 [37]. Figure 7A shows that also IL-3 is able to increase the c-kit phosphorylation: this result can explain why imatinib and SU5614, which both oppose c-kit activation, can affect glucose transport modulated by IL-3. These data suggest a phenomenon of transmodulation between IL-3 receptor and c-kit, leading to glucose transport increase and cell proliferation.

M07e cells express mainly Glut1 isoform [11]. Glucose uptake activation induced by the presence of IL-3 could depend on a higher intracellular content of the transporter Glut1, indicating a possible increased synthesis of Glut1 (Figure 7B).

Discussion

Reactive oxygen species (ROS) are generated in response to growth factors, cytokines, G-protein coupled receptor agonists or shear stress and can function as signalling molecules in non-phagocytic cells. However, it is poorly understood how freely diffusible ROS can activate specific signalling, so called redox signalling pathways. NAD(P)H oxidases are a prominent source of ROS and now recognized to have specific subcellular localizations and this targeting to specific compartments is required for localized ROS production. Recent observations have revealed that NAD(P)H oxidase-derived ROS production is related to cellular signalling and gene regulation by numerous growth factors, which promote cell proliferation, also in cancer cells. This involvement has become one of the hottest topics in free radical biology [38,39]. Understanding these mechanisms may provide insights into the NAD(P)H oxidases and redox signalling components as potential therapeutic targets for tumour progression.

ROS are believed to inactivate protein tyrosine phosphatases, thereby establishing a positive feedback system promoting activation of specific redox signalling pathways involved in various functions. Additionally, ROS production may be localized through interactions of NAD(P)H oxidase with signalling platforms associated with caveolae/lipid rafts, endosomes and nuclei. The specificity of ROS-mediated signal transduction may be modulated by the localization of Nox isoforms and its regulatory sub-units within specific sub-cellular compartments [40]. For

example, cellular stimulation by TNFα induced NAD(P)H-dependent superoxide production in the endosomal compartment in fibroblasts and this ROS was required for IKK-mediated activation of NFkB. Moreover, TNFα and IL-1β signalling pathways can both utilize Nox2 to facilitate redox-activation of their respective receptors [41].

Here we demonstrated that Nox2 isoform is expressed in M07e leukaemia cells. This observation is consistent with data, published by several authors [42], indicating that Nox2 is expressed in hematopoietic stem cells.

In the present study, we found that a decrease in ROS concentration caused by NAD(P)H oxidase inhibitors, DPI and apocynin, or with anti-leukaemic drugs, imatinib and SU5614, stimulated apoptosis in leukaemia cells, while exogenous H_2O_2 promoted cell viability. Furthermore, ROS decrease abrogated the anti-apoptotic effect of the growth factor IL-3. Thus, ROS generated via a NAD(P)H oxidase-dependent pathway have a pro-survival role in leukaemia cells, which is mediated at least in part by Nox2. In fact, selective Nox2 silencing demonstrated that IL-3 induced ROS production is due to Nox2 activity, strengthening the results obtained with inhibitors. Moreover, we found that ROS inhibition is responsible for the presence of apoptotic features such as caspase activation and internucleosomal DNA fragmentation, although a detailed mechanism of the anti-apoptotic effect of ROS is yet to be determined. Depending on the cell type and the protein machinery recruited, redox unbalance is able to induce a bifurcated signalling system, which could result in opposite fates: apoptotic death or anti-apoptotic response [15].

In hematopoietic cell lines, IL-3 rapidly and transiently activates Rac, a Rho family GTPase. The cytokine-induced Rac activation is inhibited by a PI3K inhibitor, which also inhibits ERK activation [43]. In leukaemia cells, ROS generated by Nox4, at least in part, transmit survival signals through the Akt-PI3K pathway and their depletion leads to apoptosis. Nox1 constitutively binds the Rac guanine exchange factor βPix [44]. Growth factor stimulation causes the binding of Rac1 to Nox1 in a fashion analogous to the one previously described in neutrophils; other studies demonstrated that ROS act downstream Rac as specific biological effector molecules [45]. In M07e cells, we observed that PI3K, activated by Rac1, is involved in upstream ROS production, as well as PLCγ and Syk TK, while Src and p42/44 are activated after ROS generation. Moreover, Rac-stimulated ROS production can involve the direct oxidation of cysteine residues on proteins, which may affect their functions [44]. Oxidation of essential cysteines abolishes phosphatase activity and increases tyrosine phosphorylation of target proteins. For instance, MAP kinase phosphorylation was found to be dependent on H_2O_2 production. Furthermore, H_2O_2 also appears to promote tyrosine phosphorylation by activating protein tyrosine kinases, such as the Src family [46].

A primary function of growth factors, such as IL-3, is to regulate glucose uptake and metabolism [47]. Here we showed that IL-3 modulates glucose transport through the activation of a kinase cascade involving Src, Syk, PKC, PI3K, Akt and PLCγ. In the presence of the cytokine, this regulation eventually results in the increased synthesis of Glut1 transporter, as shown in Western blot experiments. Interestingly, both H_2O_2 and IL-3 induced an increase in the phosphorylation status of c-kit, the SCF receptor. The proliferative response to IL-3 has been shown to act synergistically with SCF, a cytokine known to promote the growth of leukaemic cells *in vitro* [48]. The receptor for IL-3 consists of a unique specific α-chain, IL-3Rα, which binds to IL-3 with low affinity, and the common β-chain (β_c) shared by GM-CSF. Accumulating evidence suggests a role of β_c in modulating signalling of other hematopoietic cytokines including SCF via c-kit; in fact, SCF is able to induce serine/threonine phosphorylation of β_c [49].

The receptor tyrosine kinase c-kit is essential for the development of normal hematopoietic cells and has been proposed to play a functional role in acute myeloid leukaemia. Binding of the c-kit ligand SCF initiates a signal transduction cascade that includes receptor autophosphorylation and subsequent phosphorylation of intracellular substrates. In addition to its role in normal hematopoiesis, c-kit is expressed in leukaemic blasts in $\sim$60–80% of AML patients [48].

SU5614 and imatinib are inhibitors of RTKs such as VEGFR-2 and FLT3 receptor [24], having structural and sequence similarity to c-kit. SCF and FLT3 ligands have been shown to inhibit apoptosis in several myeloid leukaemia cell lines established from patients with AML [48]. Consistently with this observation, SU5614 and imatinib induced apoptosis in M07e cells at concentrations similar to those required to decrease ROS content and proliferation in these cells. In contrast to human endothelial cells, the growth inhibitory activity in leukaemic cells was not determined by the expression of VEGF and its receptors, but by the expression of c-kit [24]. Therefore, we could speculate that a receptor transmodulation or receptor transphosphorylation occurs in the context of leukaemia cell growth responses to individual cytokines. Otherwise, an autocrine SCF production could take place, as shown in most AML cells [24].

In conclusion, we showed that IL-3-stimulated NAD(P)H oxidase activity could protect leukaemic cells from apoptosis. This mechanism may contribute to leukaemia resistance to apoptosis, indicating that

these enzymes could be targeted to stimulate leukaemic cell death.

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