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Nox-generated ROS modulate glucose uptake in a leukaemic cell line

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Abstract
The discovery of superoxide-generating enzymes homologues of phagocytic NAD(P)H oxidase, the Nox family, has led to the concept that reactive oxygen species (ROS) are 'intentionally' generated with biological functions in various cell types. In this study, by treating an acute leukaemic cell line with different antioxidants, ROS generation was shown to be crucially involved in the modulation of glucose transport (mediated by Glut1), which is frequently up-regulated in cancer cells. Then, this study tried to elucidate ROS source(s) and mechanisms by which ROS are involved in Glut1 activity regulation. Results prove that Nox2 and Nox4 are the candidates and that phosphorylation processes are important in the regulation of glucose uptake on which cancer cells rely. On the whole, data suggest that both Glut1 and Nox homologues may be considered new potential targets in the treatment of leukaemia.

Keywords: *NAD(P)H oxidase, reactive oxygen species, Glut1, antioxidants, tyrosine kinases, acute leukaemia*

Abbreviations: *CAPE, caffeic acid phenethyl ester; DOG, 2-deoxy-D-glucose; DPI, diphenylene iodonium; IL-3, interleukin-3; IMDM, Iscove's modified Dulbecco's medium; NHS, normal human serum; PBS, phosphate buffered saline; PI3-K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulphonyl fluoride; ROS, reactive oxygen species; RS, reactive species; TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.*

Introduction

Evidence has been accumulated that stimulation of a variety of cell surface receptors, including those for hematopoietic cytokines [1], insulin [2] and angiotensin II [3], induces generation of reactive oxygen species (ROS) such as O_2^{-} and H_2O_2 [1], which function as second messengers involved in critical ligand-mediated regulation of protein kinase activation, gene expression and proliferative responses [1–3]. Much remains to be learned about the specific intracellular targets of oxidant signalling and the full physiological significance of the redox modifications.

The discovery of a group of ROS-generating NAD(P)H oxidases, the Nox family, with unique tissue localization in non-immune cells, and the finding that the activity or level of some of these is regulated by growth factors, cytokines and calcium signals has reinforced the idea that ROS play biologically important roles, which vary depending on the cell type [4]. The prototypical well-known phagocytic NAD(P)H oxidase, a superoxide-producing enzyme complex, consists of at least six components: gp91phox/Nox2, p22phox, p40phox, p47 phox, p67phox and Rac. In recent years, a family of gp91phox/Nox2 homologues, Nox1, 2, 3, 4 and

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5 [5,6], and two related proteins, called Duox1 and Duox2, having Nox-homologue regions with peroxidase activity, have been discovered [4]. Their expression levels are dependent on cell type and environmental context and recent findings suggest that different types of Nox can occur in the same tissue [7]. On the other hand, the physiological significance of this co-existence is as yet poorly understood.

We previously showed that there is a relationship between the basal level of intracellular ROS and glucose transport activity in B1647 cells, a human leukaemic erythro-megakaryocytic cell line not requiring additional cytokines to proliferate. Compared with M07e cells, a growth factor-dependent human leukaemic erythro-megakaryocytic line, B1647 cells show a 4.8-fold greater hexose transport activity, due to a higher Glut1 content in the plasma membrane, and an intracellular ROS level significantly higher (1.4-fold) [8]. It has been very recently demonstrated that some leukaemic cell lines of the erythro-megakaryocytic phenotype constitutively produce vascular endothelial growth factor (VEGF) [9], a potent inducer of angiogenesis and a stimulator of endothelial cell proliferation, differentiation and survival, and that B1647 cells self-produce VEGF and express its tyrosine-kinase receptor, VEGFR-2 [10].

A high glucose uptake is a characteristic of cancer cells in order to satisfy energy demand for aberrant proliferation, indeed many cancer cells over-express Glut isoforms and a wide expression of Glut1 in various human cancers has been reported [11]. Furthermore, the accelerated cell cycle activity observed in neoplastic cells appear to be related to a constitutively elevated intracellular ROS level [3].

We hypothesized that, in B1647 cells, the source of ROS involved in the regulation of Glut1 could be mostly represented by one or more components of the Nox family. In the present study, to investigate ROS generation site we evaluated the effect of some ROS scavengers and NAD(P)H oxidase inhibitors on Glut1 activity and ROS intracellular level, and we also performed Western blot analysis and RT-PCR (reverse transcriptase PCR) in order to verify the presence of Nox family members. Then, we tried to elucidate mechanisms by which ROS are involved in the regulation of glucose uptake mediated by Glut1. Since ROS can regulate activities of redox-sensitive enzymes, including protein phosphatases and some kinases [12], we evaluated the effect of tyrosine kinase inhibitors on glucose uptake and ROS generation in B1647 cells. Furthermore, we investigated whether a family of non-receptor tyrosine kinases, Src, and phosphatidylinositol-3-kinases (PI3-K), were involved both in the regulation of Nox-generated ROS and Glut1 activity.

Materials and methods

Chemicals

Phloretin, 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), 2-deoxy-D-glucose (DOG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Trypan Blue, orthovanadate, phenylmethylsulphonyl fluoride (PMSF), N-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), Igepal CA-630, agarose, Protein G, diphenylene iodonium (DPI), apocynin and wortmannin were purchased from Sigma-Aldrich (St. Louis, MO). STI-571 (Imatinib mesylate, Glivec) was kindly provided by Novartis (Basel, Switzerland). Rac1 inhibitor (NSC23766) and PP2 were from Calbiochem (San Diego, CA). 2-Deoxy-D-[2,6-^{3}H]-glucose and nitrocellulose paper (Hybond) were from Amersham (GE Healthcare Europe GmbH, Milan, Italy); Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco (Milan, Italy) and normal human serum (NHS) from Cambrex Bioscience (Verviers, Belgium). LY294002 and tyrphostin A23 were from Alexis (Lausen, Switzerland). Anti-p47phox and anti-Rac1 were from Upstate (Lake Placid, NY). Anti-Phospho-Tyrosine (P-Tyr-102) was from Cell Signalling Technology (Beverly, MA). Anti-rabbit and anti-goat IgG conjugated to horseradish peroxidase, anti-Nox1, anti-Nox2, anti-Nox4, anti-Glut1 and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bis (1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate, a radical probe, was kindly provided by Professor G. F. Pedulli. TRIzol Reagent was from Invitrogen (Paisley, Scotland, UK). Access RT-PCR System and 100pb DNA Ladder were purchased from Promega (Madison, WI, USA) and GelRed™ from Biotium (Rome, Italy).

Cells and cell cultures

B1647 erythro-megakaryocytic cell line, established from bone marrow cells of a patient with acute myelogenous leukemia, is maintained in IMDM supplemented with 5% NHS. These cells do not require growth factor addition for their proliferation [10].

Cell viability assay

Cell viability was assayed by Trypan Blue exclusion test and MTT assay. For Trypan Blue staining, B1647 cells were incubated with 0.2% Trypan Blue for 5 min at room temperature and counted with a hemocytometer. For the MTT assay, cells were incubated with 0.5 mg/mL MTT for 4 h at 37°C. The formed formazan salt crystals were dissolved with the solubilization solution (10% SDS, 0.01 M HCl). The plates were incubated overnight (37°C, 5% CO_2). The

absorption was measured on a multiwell plate reader (Wallac Victor^2, Perkin Elmer) at a wavelength of 570 nm.

Measurement of intracellular ROS

Cells (1.0×10^6/mL) were treated with antioxidants and inhibitors for 30 min, washed twice in PBS, pH 7.2, and incubated with 5 μM DCFH-DA for 20 min. DCF fluorescence was measured with a multiwell plate reader (Wallac Victor^2, Perkin Elmer), at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

Glucose transport assay

Cells (4.0×10^6/mL) in PBS, pH 7.2, were treated with a mixture of 2-deoxy-D-[2,6-^{3}H]glucose (15 kBq/assay) and 1.0 mM unlabeled glucose analogue for 1 min at 37°C ensuring that experiments were run under *zero-trans* conditions [13]. The uptake was stopped with phloretin (final concentration 0.3 mM), a potent glucose transport inhibitor. Cells were pelleted at 4000 *g* for 1 min and washed with PBS. Sample radioactivity was measured by liquid scintillation counting.

Immunoprecipitation

B1647 cells (15×10^6 per experimental condition) were washed in PBS and 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

Cells lysates were obtained from 15×10^6 cells in ice-cold lysis buffer (150 mM NaCl, 1% Igepal, 50 mM Tris, 5 mM EDTA, pH 8.0) in the presence of proteases inhibitors (100 μM each TPCK, PMSF, TLCK and Proteases inhibitor cocktail, Sigma) [13] and protein concentrations of the supernatants were determined by using the Bradford method [14]. Immunocomplexes and cell lysates proteins were separated on 10% SDS-polyacrylamide gel using a Mini-Protean II apparatus (Bio-Rad Laboratories) and transferred electrophoretically to nitrocellulose membranes at 100 V for 60 min. Proteins were detected by using primary antibodies against p47phox, Rac1, Nox4, Nox2, Glut1 and Phospho-Tyrosine at 1:1000 dilution according to standard Western blotting procedure and membranes were developed using Western Blotting Luminol Reagent.

Reverse transcription-polymerase chain reaction

One microgram of total cellular RNA isolated by TRIzol reagent was reverse transcripted to cDNA and PCR-amplified in a total volume of 50 μL with 50 pmoles each of forward and reverse primers. The primer sequences used are detailed in Table I. Positive control primers were designed to the housekeeping gene human β-actin (sense 5′-CCA ACC GCG AGA AGA TGA-3′ and antisense 5′-CCA GAG GCG TAC AGG GAT AG-3′). The conditions were 35 cycles of denaturation at 94°C (30 s), annealing at the temperatures listed in Table I (1 min) and extension at 68°C (2 min), followed by a further 7 min extension. PCR products were electrophorased on 2% agarose gel precasted with GelRed 1 × TBE (Tris-Borate-EDTA buffer). Gel was imaged using 300 nm transillumination and

Table I. Features of the primers used for reverse transcriptase PCR.

Primers (5′ →3′)	T_{ann} (°C)	PCR product length (bp)
Nox1 sense 5′-CCCCTTTGCTTCTATCTTGA-3′ antisense 5′-TTGCCTAATTCCTCCATCTC-3′	49°C	168
Nox2 sense 5′-CTCACCCTTTCAAAACCATC-3′ antisense 5′- ACGATGCGGATATGGATACT-3′	58°C	169
Nox4 sense 5′-CTCAGCGAATCAATCAGCTGTG-3′ antisense 5′AGAGGAACACGACAATCAGCCTTAG-3′	58°C	285
Nox5 sense 5′-CCTTCTAGTTGCGCTTTTGC-3′ antisense 5′-CCATCTTCTCCTGCAATGGT-3′	60°C	232

photographed with a Polaroid 667 on black-and-white print films.

Statistical analysis

Data sets were performed with the unpaired Student *t*-test comparing treated samples vs controls. Data are expressed as means ± SD. Differences were considered significant for $p < 0.05$.

Results

For experiments in which B1647 cells were incubated with inhibitors or antioxidants, alone or in combination, Trypan Blue exclusion test and MTT assay were performed in order to control that the effect of different compound additions was due to a direct action on specific targets and not to changes in viability and proliferation (not shown) [15].

Effect of ROS scavengers and Nox inhibitors on Glucose transport

To confirm the previously observed inhibitory effect on glucose uptake of EUK-134 [8], a SOD and catalase-mimetic able to penetrate inside the cells, and to obtain more information about reactive species (RS) involved in glucose uptake modulation, we tested the effect of several RS scavengers on this function. In particular, we used ebselen, a glutathione peroxidase mimetic and a lipoxygenase inhibitor [16], caffeic acid phenethyl ester (CAPE), which has been demonstrated to have antioxidant and anti-inflammatory effects and to be a H_2O_2 scavenger and an inhibitor of nitric oxide synthase [17], and a radical probe (bis1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate), which quantitatively and instantaneously reacts with oxygen-centred radicals (including superoxide) to yield the parent nitroxide [18]. B1647 cells were treated with 20 μM ebselen, 25 μM CAPE or 20 μM radical probe for 20 min before measuring Glut1 activity. Pre-treatments caused a glucose uptake inhibition by 20%, 40% and 25%, respectively; moreover, the simultaneous incubation of this cell line with the three RS scavengers caused a cumulative effect on DOG uptake, decreasing the transport activity by ~75% (Figure 1A). The same pre-treatments induced also a decrease in DCF fluorescence, pointing out the scavenging of reactive species by the added compounds, the effectiveness of CAPE and the cumulative antioxidant effect obtained with the simultaneous incubation with the three RS scavengers (Figure 1B). These data undoubtedly prove that a correlation does exist between RS level and Glut1 activity.

In order to evaluate whether one or more members of the Nox family could be the ROS generation site involved in maintaining the high glucose uptake in B1647 cells, we used 10 μM DPI, an inhibitor of flavoprotein centres demonstrated to be a potent Nox inhibitor at this concentration [19], 1 mM apocynin, an inhibitor of the complex, preventing the assembly of the NAD(P)H oxidase cytosolic subunit p47phox to the membrane subunits [20], and 200 μM Rac1 inhibitor (NSC23766), a cell-permeable pyrimidine compound that specifically and reversibly inhibits Rac1 GDP/GTP exchange activity [21]. A 30 min pre-incubation of B1647 cells with each of these compounds caused a decrease in glucose uptake by ~30–40% (Figure 1C). DPI and Rac1 inhibitor decreased also the intracellular ROS/RNS level by ~40% (Figure 1D). Fluorescence measurements could not be performed in the presence of apocynin due to its interference with DCF [22]. These data confirm Rac1 importance for the activation of NAD(P)H oxidase [4,12], which, in turn, is involved in Glut1 activity modulation in the leukaemia cell line under study. Moreover, when cells were simultaneously incubated with apocynin and antioxidants, i.e. by both blocking the potential RS source and directly scavenging RS, an additive inhibitory effect on glucose uptake occurred (Figure 1C): this is in agreement with the suggested role of Nox-generated RS in maintaining high Glut1 activity in B1647 cell line.

Presence of Nox isoforms

To further substantiate these results, we searched for p47phox subunit and Rac1. Western immunoblotting analysis of cell lysates demonstrated that B1647 cell line effectively expresses p47phox subunit and the small GTPase-protein Rac1 (Figure 2A). Since the presence of p47phox and Rac1 is not sufficient to implicate NAD(P)H oxidase, we performed RT-PCR in order to test the expression of Nox1, Nox2 (gp91phox), Nox4 and Nox5, the isoforms that could be present in hematopoietic cell lines as reported in the literature [7]. B1647 cells were found to express Nox2 and Nox4 isoforms (Figure 2B), but not Nox1 or Nox5 mRNA; these data were also confirmed by Western blot analysis (Figure 2C).

Modulation of tyrosine phosphorylation levels by Nox inhibitors and effect of tyrosine kinase inhibitors on Glut1 activity

Recent experimental evidence shows that ROS can regulate activities of redox-sensitive enzymes. Among intracellular ROS targets are protein tyrosine phosphatases (PTPs), which are inactivated through the reversible oxidation of their catalytic cysteine(s), hence allowing a sustained phosphorylation level [12]. Western blot analysis of B1647 cell lysates revealed with antiphosphotyrosine antibody showed that, after incubation with the antioxidant EUK-134, a decrease in tyrosine phosphorylation occurred (not

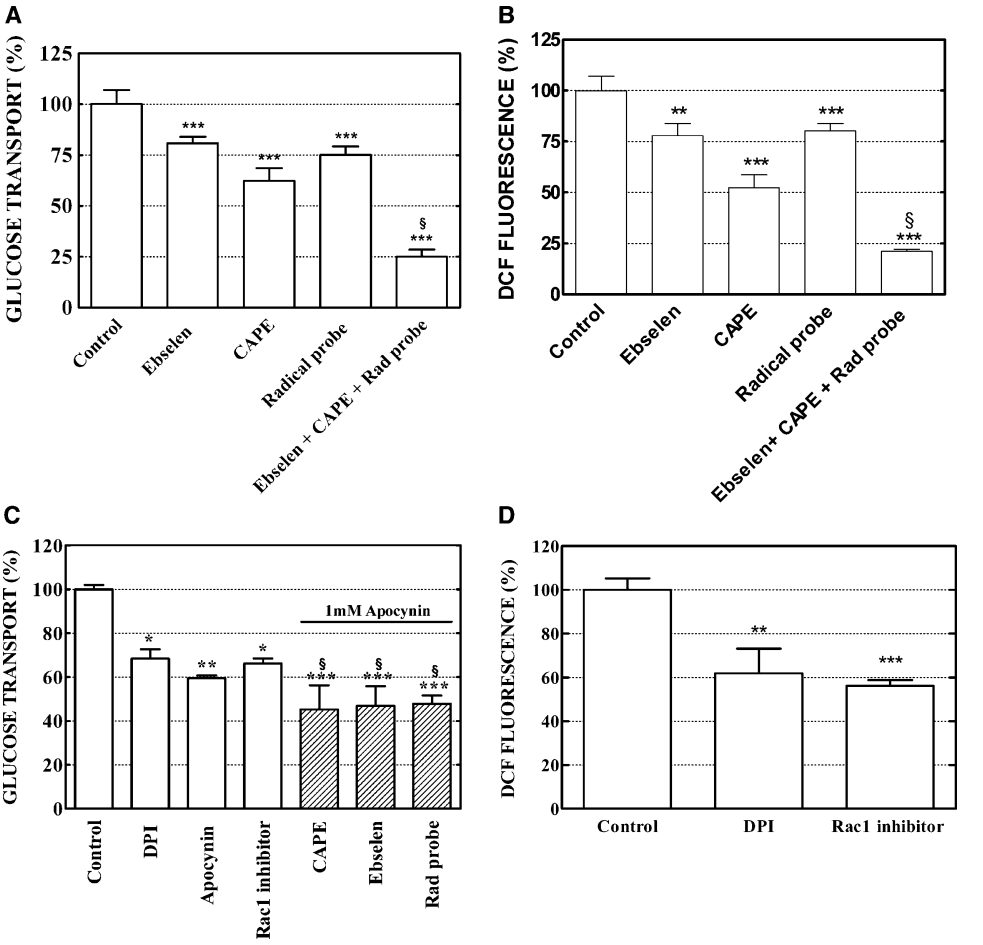

Figure 1. Effect of antioxidants and NAD(P)H inhibitors on glucose transport and intracellular ROS level in B1647 cell line. (A) Cells (4×10^6/mL) were incubated in PBS at 37°C in the absence or presence of 20 μM Ebselen; 25 μM CAPE or 20 μM radical probe, for 30 min. DOG uptake was measured as described 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. $^{\S}p < 0.0001$ significantly different from cells treated with only one antioxidant. (B) Cells (1×10^6/mL) pre-incubated or not with 20 μM Ebselen; 25 μM CAPE and/or 20 μM radical probe for 30 min as described in the Materials and methods section were washed in PBS, labelled with 5 μM DCFH-DA for 20 min and analysed by means of a multiwell plate reader, at λ_{exc} 485 nM and λ_{em} 535 nM. Values are the means ± SD of four independent experiments. ***$p < 0.0001$, **$p < 0.01$, *$p < 0.05$ significantly different from control. $^{\S}p < 0.0001$ significantly different from control and cells treated with only one antioxidant. (C) Cells (4×10^6/mL) in PBS in the presence or absence of inhibitors (10 μM DPI, 1 mM apocynin, 200 μM Rac1 inhibitor) were pre-incubated for 20 min at 37°C. Other cells (striped bars) were simultaneously pre-incubated with 1 mM apocynin and antioxidants (25 μM CAPE, 20 μM ebselen or 20 μM radical probe). DOG uptake was measured as described in the Materials and methods section. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. ***$p < 0.0001$, **$p < 0.01$, *$p < 0.05$ significantly different from control. $^{\S}p < 0.0001$ significantly different from cells treated with apocynin. (D) Cells pre-treated or not with 10 μM DPI or 200 μM Rac1 inhibitor for 20 min in PBS were labelled with 5 μM DCFH-DA for 20 min. Values are the means ± SD of four independent experiments. ***$p < 0.0001$, **$p < 0.01$ significantly different from control.

shown), indicating that intracellular ROS are involved in this process by means of protein kinase activation and/or, more likely, inhibition of protein tyrosine phosphatases. Consequently, since EUK-134 also decreased Glut1 activity, as previously shown [8], we suggest that ROS are involved in phosphorylation processes, which maintain the high activity of glucose transport in B1647 cells. Similar results regarding both tyrosine phosphorylation (not shown) and Glut1 activity (Figure 1A) were also obtained with the antioxidant ebselen, confirming the above reported suggestion. Moreover, a significant decrease in tyrosine phosphorylation level occurred when cells were preincubated with NAD(P)H oxidase inhibitors (DPI, apocynin and Rac1 inhibitor), as reported in Figure 3A. In order to underline the important role played by tyrosine phosphorylation in Glut1 activity modulation, we evaluated the effect of two different tyrosine kinase activity inhibitors on glucose transport. In particular, we used 10 μM tyrphostin A23, a broad spectrum protein tyrosine kinase inhibitor, and 10 μM Imatinib, an anti-cancer drug designed to block specific constitutively activated tyrosine kinases. Indeed, Imatinib is a specific inhibitor of the Bcr-Abl protein tyrosine kinase that competes with ATP for its specific binding site in the

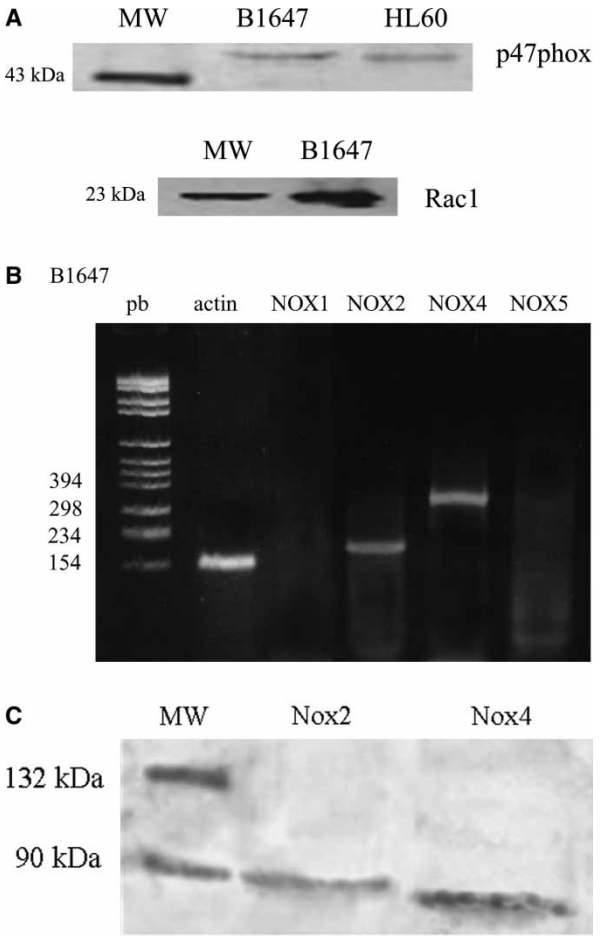

Figure 2. Rac1, p47phox subunit and Nox expression in B1647 cell line. (A) Representative immunoblots showing p47phox and Rac1 expression in B1647 cells. HL60 cell line was taken as a p47phox-positive control. Corresponding bands migrating at ~47 kDa and 21 kDa, respectively, are shown. (B) Expression of Nox1, Nox2, Nox4 and Nox5 catalitic subunit of the Nox family obtained by RT-PCR: total RNA was isolated, reverse transcribed and amplified by PCR. All the amplicons were visualized on a GelRed™ 2% agarose gel. Further experimental details are reported in the Materials and methods section. (C) Cell lysates were electrophorased, transferred and immunoblotted with Anti-Nox2 or Anti-Nox 4 antibodies, as described in the Materials and methods section.

kinase domain [23]. Results in Figure 3B show that after a 20 min pre-incubation both inhibitors significantly decreased DOG uptake in B1647 cells. Interestingly, the same cell pre-treatments caused also a decrease in intracellular ROS level (Figure 3C).

Effect of PI3-K and Src inhibitors on intracellular ROS level and on Glut1 activity and modulation of Glut1 phosphorylation level by PP2, apocynin and CAPE

To investigate the complex and still unclear signal transduction pathways linking ROS generation to Glut1 activity modulation in B1647 cells, we investigated whether phosphatidylinositol-3-kinase (PI3-K) and Src kinases, involved in the signalling cascade in many cell lines [24], were connected with ROS intracellular level and glucose transport in these cells. DCF fluorescence was blunted and a significant decrease of glucose uptake occurred when cells were pre-incubated for 20 min with PI3-K inhibitors, such as 100 nM wortmannin or 1 μM LY294002 (Figure 4A and B). To determine whether Src take part in ROS generation probably due to NAD(P)H oxidase activity and are involved in maintaining the high rate of glucose uptake in this cell line, we used PP2, a Src inhibitor. As shown in Figure 4A and B, the pre-incubation of the cells with 20 μM PP2 inhibited DOG uptake, but it did not reduce intracellular ROS level. Moreover, we verified that Glut1 phosphorylation decreased after 20 min-preincubation of the cells with 20 μM PP2, 1 mM apocynin or 25 μM CAPE, as shown in Figure 4C.

These data implicate a multi-step pathway resulting in maintaining the high Glut1 activity in B1647 cell line.

Discussion

A number of sources have been proposed for the origin of RS generation in tumour cells, but attention has recently turned to a family of membrane-localized NAD(P)H oxidases, homologous to the gp91phox component of the phagocytic enzymatic complex [4,6].

Relevant findings reported in this study are that Nox2 and Nox4 are co-expressed in the acute leukaemic B1647 cells and that ROS produced by NAD(P)H oxidase are directly involved in maintaining the high glucose uptake rate, essential for tumour cells in order to proliferate. High levels of Glut1 expression in tumours have been associated with poor survival [11], thus investigation on glucose transport regulation in an acute leukaemia is an important issue.

To achieve this aim, at first, we demonstrated the involvement of different reactive species in Glut1 activity modulation by using different antioxidants (see Figure 1). In order to throw a glance at RS involved in this process, we chose scavengers of different reactive species, not only directed against $O_2^{\cdot-}$ and H_2O_2.

Then, we identified NAD(P)H oxidase as a major source of ROS involved in this process by using Nox inhibitors and identifying Nox isoforms and subunits by RT-PCR and Western blotting techniques (Figure 2). These results are strengthened by data showing that the simultaneous pre-treatment of the cells with antioxidants and apocynin caused an additive effect on decreasing glucose uptake, due to both an inhibition of the ROS source and a direct ROS scavenging. Previous studies demonstrated that inappropriate activation or expression of Nox enzymes has been associated with neoplastic growth [25–28]; moreover, a plethora of

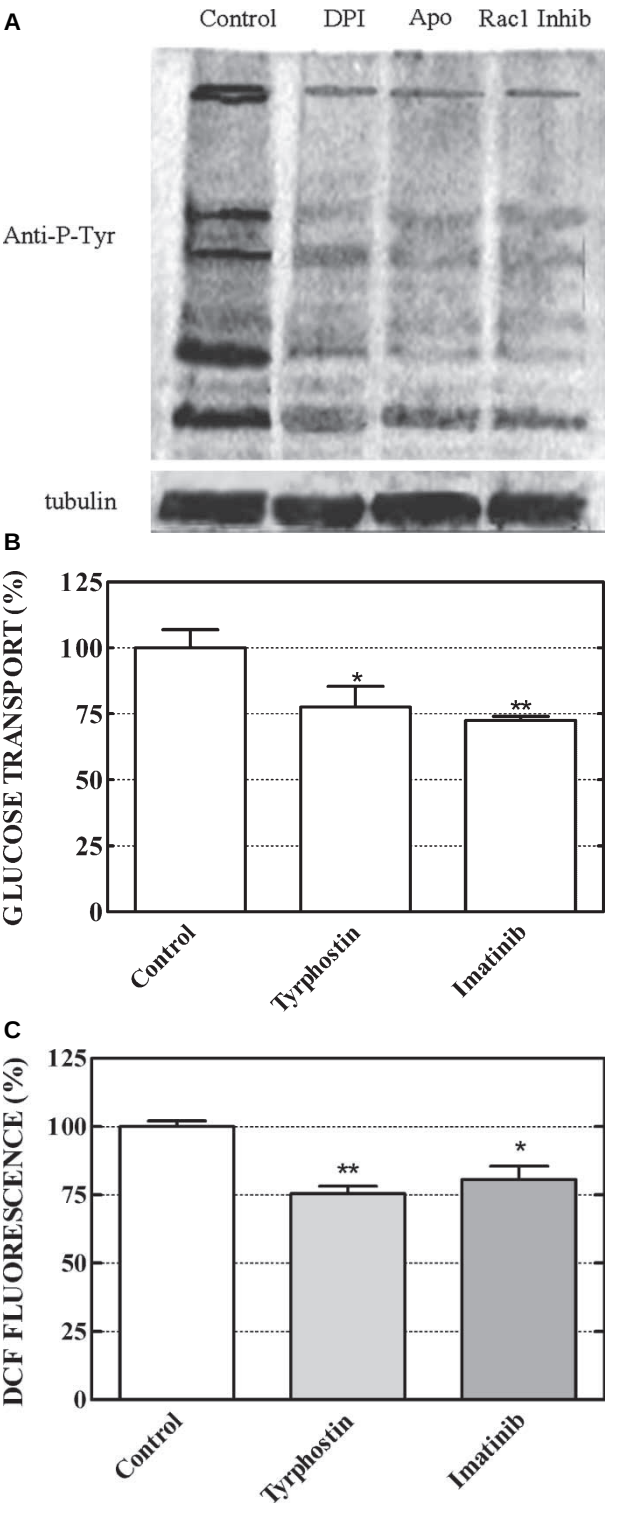

Figure 3 (*Continued*)

human tumour types expresses various Nox homologues [4] and Nox transfection into normal cells increases ROS production [5,25], transforms normal fibroblasts, and creates cell lines that are tumourigenic and angiogenic [25]. Conversely, Nox antisense oligonucleotides inhibit tumour cell proliferation [28].

The following step was to investigate how Nox-derived ROS could maintain the high glucose uptake rate in the leukaemic cell line under investigation. A well known mechanism by which ROS produced by Nox can partecipate in signal transduction pathways is the inhibition of protein phosphatases and activation of kinases [12] and data here reported demonstrate that phosphorylation processes sustained by ROS are very important for Glut1 activity in B1647 cells (see Figures 3 and 4). In fact, antioxidants and Nox inhibitors able to decrease glucose transport caused a decrease in protein phosphorylation level and in Glut1 phosphorylation, in particular. Although some information is available concerning the proximal signalling steps by which NAD(P)H oxidase is activated, an integrated picture remains elusive; it may include growth factor receptors, tyrosine kinases, PI3-kinase and Rac1. In some non-phagocytic cells, the activation of Nox by growth factor receptors is Src dependent and, in turn, PI3-K is activated [29]; in others, NAD(P)H oxidase activation is accomplished by PI3-K-dependent mechanisms not involving Src activation [30]. In HepG2 cells, it has been shown that PI3-K is required for oxidase activation by platelet-derived growth factor and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), the product of PI3-K, has been proposed to bind directly to p47phox [31] and to activate Rac [32]. Therefore, we investigated how Nox-derived ROS take part in the signal transduction pathway cascade that, by modifying the activity of redox sensitive enzymes including phosphatases and some kinases, leads to glucose transport modulation in B1647 cell line.

The whole of the data here reported suggests that the relationship between ROS generation and glucose uptake mediated by Glut1 could be explained according to a model (see Figure 5) creating a loop, where PI3-K produces PIP_3, which in turn activates Rac. GTP-bound Rac most likely binds to the NAD(P)H oxidase complex (presumably binding to p47phox and causing its phosphorylation) leading to

Figure 3. Effect of Nox inhibitors on protein phosphotyrosine level and effect of tyrosine kinase inhibitors on glucose transport and intracellular ROS level in B1647 cell line. (A) Cells, pre-treated with 10 μM DPI, 1 mM apocynin, 200 μM Rac1 inhibitor for 20 min, were lysated, electrophorased, transferred and immunoblotted with Anti-phosphotyrosine and Anti-tubulin antibody, as described in the Materials and methods section. (B) Cells (4×10^6/mL) were incubated in PBS at 37°C in the absence or presence of 10 μM Tyrfostin A23 and 10 μM Imatinib for 20 min. DOG uptake was measured as described in the Materials and methods section. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. $^{**}p < 0.01$, $^{*}p < 0.05$ significantly different from control. (C) Cells (1×10^6/mL) pre-incubated or not with 10 μM Tyrfostin A23 or 10 μM Imatinib for 20 min as described in the Materials and methods section were washed in PBS and labelled with 5 μM DCFH-DA for 20 min. Values are the means ± SD of four independent experiments, each performed in triplicate. $^{**}p < 0.01$, $^{*}p < 0.05$ significantly different from control.

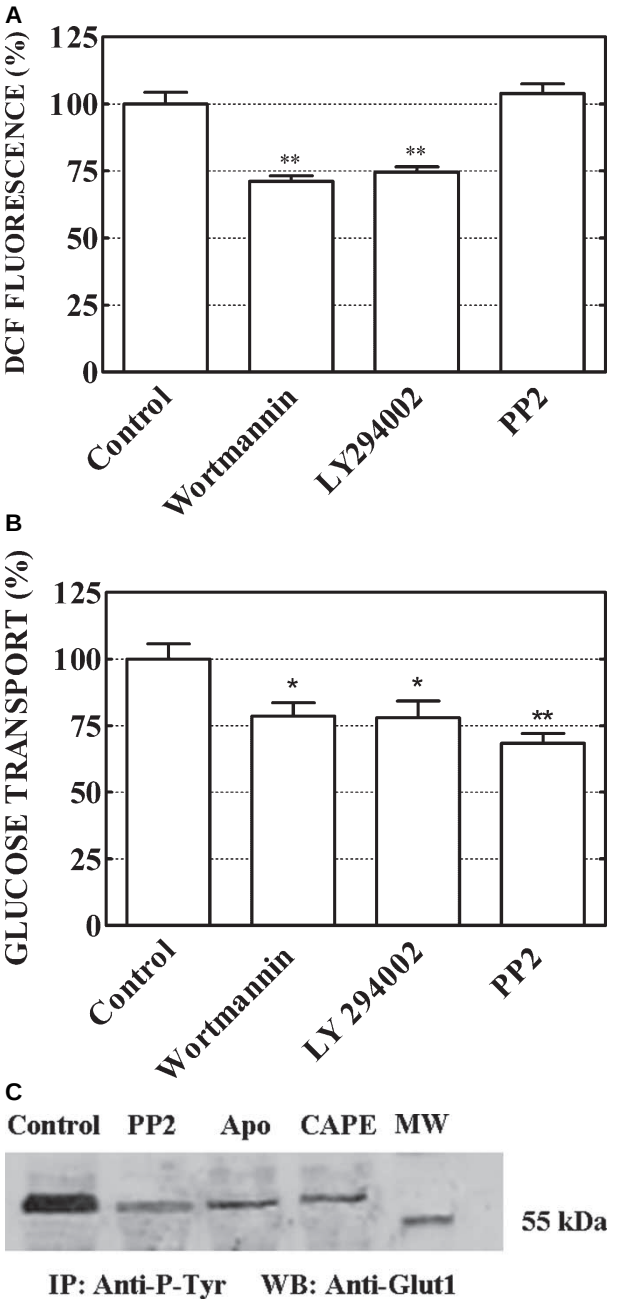

Figure 4 (*Continued*)

ROS generation. Src appears to be downstream oxidase activation (see Figure 4A and B) and responsible for the phosphorylation of both Glut1 and growth factor receptor [3], even if the mechanism is not completely clear at this time. The growth factor receptor involved in signal cascade activation can be the tyrosine-kinase receptor VEGFR-2 stimulated by the autocrine production of VEGF, characteristic of B1647 cells [10]. To this regard, it has been very recently shown that VEGF stimulates ROS production *via* activation of gp91phox (Nox2)-based NADPH oxidase and that ROS are involved in VEGFR2-mediated signalling linked to endothelial cell migration and proliferation [33]. This analogy is not surprising as vascular and haematopoietic cells originate from a common precursor, the hemangioblast, during embryogenesis [34]. Moreover, Nox2, a critical component of endothelial NAD(P)H oxidase, and Nox4, most abundantly expressed in endothelial cell, where it is involved in basal- and Ang II-induced superoxide production [33], are also expressed in the acute leukaemic B1647 cell line.

A better understanding of the responsiveness of Nox homologues expression to VEGF in leukaemic cells is clearly required, nevertheless we suggest an integrated signalling mechanism whereby the autocrine VEGF production could lead to the activation of PI3-K and then of NAD(P)H oxidase (Nox2 and/or Nox4) and, in turn, to ROS generation. Data here presented demonstrate that Nox-derived ROS are involved in sustaining the high glucose uptake *via* a direct action on phosphorylation processes, which are also connected to Glut1 vesicle translocation from intracellular pool to the plasma membrane, as we previously showed [8]. Actually, phosphorylation events have been recently shown to modulate Glut1 activity in other cell types [35,36].

These results underline the cross-talk between the phosphorylation—dephosphorylation and redox regulation, two systems of cellular process modulation [37].

In conclusion, the strict dependence of the acute leukaemic B1647 cell proliferation on glucose uptake maintained by Nox-generated ROS suggests that both Glut1 and Nox homologues can be new targets for novel therapeutic strategies to be used to improve the

Figure 4. Effect of PI3-K and Src inhibitors on intracellular ROS level and on Glut1 activity and effect of PP2, apocynin and CAPE on Glut1 phosphorylation level in B1647 cell line. (A) Cells (1×10^6/mL) pre-incubated or not with PI3-K inhibitors (100 nM Wortmannin or 1 μM LY294002) or Src inhibitor (20 μM PP2) for 20 min as described in the Materials and methods section were washed in PBS and labelled with 5 μM DCFH-DA for 20 min. Values are the means ± SD of four independent experiments, each performed in triplicate. $^{**}p < 0.01$ significantly different from control. (B) Cells (4×10^6/mL) were incubated in PBS at 37°C in the absence or presence of 100 nM wortmannin or 1 μM LY294002 or 20 μM PP2 for 20 min. DOG uptake was measured as described in the Materials and methods section. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. $^{**}p < 0.01$, $^{*}p < 0.05$ significantly different from control. (C) Cells were incubated in PBS at 37°C in the absence or presence of 20 μM PP2, 1 mM apocynin (Apo) or 25 μM CAPE for 20 min. Anti-phosphotyrosine immunoprecipitates from cell lysates were electroforased, transferred and immunoblotted with anti-Glut1, as described in the Materials and methods section. $^{**}p < 0.001$, significantly different from control.

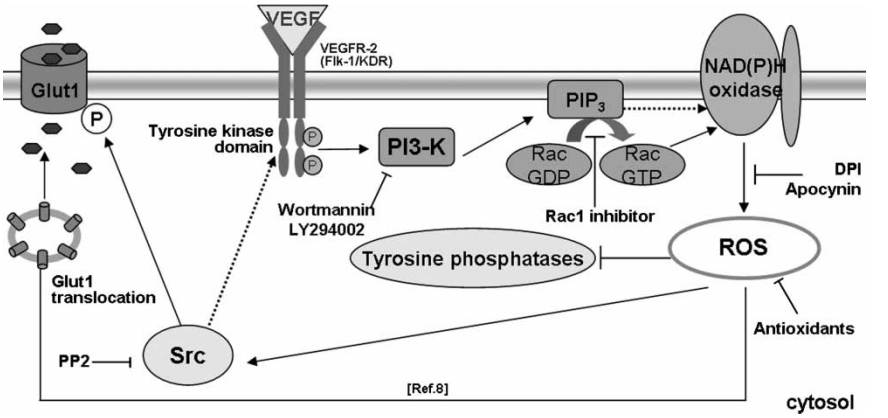

Figure 5. A model of relationship between Nox-derived ROS and Glut1 activity modulation. Phosphorylated VEGFR-2 activates PI3-K that, in turn, activates NAD(P)H oxidase. Generated ROS maintain high glucose uptake by both Src activation and Glut1 vesicles translocation.

cancer treatment. Indeed, a combination therapy including chemotherapeutic agents and kinase inhibitors, such as Imatinib, has already been tested [38,39].

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References

[1] Iiyama M, Kakihana K, Kurosu T, Miura O. Reactive oxygen species generated by hematopoietic cytokines play roles in activation of receptor-mediated signaling and in cell cycle progression. Cell Signal 2006;181:74–82.

[2] Goldstein BJ, Mahadev K, Wu X. Redox paradox: insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signaling targets. Diabetes 2005;4:311–321.

[3] Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. Circ Res 2002;91:406–413.

[4] Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004;4:181–189.

[5] Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD. Cell transformation by the superoxide-generating oxidase Mox1. Nature 1999; 401:79–82.

[6] Cheng G, Cao Z, Xu X, van Meir EG, Lambeth JD. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. Gene 2001;269:131–140.

[7] Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 2007;87:245–313.

[8] Fiorentini D, Prata C, Maraldi T, Zambonin L, Hakim G, Landi L. ROS contribution to the regulation of GLUT1 in two hemopoietic cell lines differing in cytokine sensitivity. Free Radic Biol Med 2004;37:1402–1411.

[9] Hiramatsu A, Miwa H, Shikami M, Ikai T, Tajima E, Yamamoto H, Imai N, Hattori A, Kyo T, Watarai M, Miura K, Satoh A, Itoh M, Imamura A, Mihara H, Katoh Y, Nitta M. Disease-specific expression of VEGF and its receptors in AML cells: possible autocrine pathway of VEGF/type1 receptor of VEGF in t(15;17) AML and VEGF/type2 receptor of VEGF in t(8;21) AML. Leuk Lymphoma 2006;47:89–95.

[10] Bonsi L, Pierdomenico L, Biscardi M, Marchionni C, Gavazzi S, Fossati V, Ghinassi B, Alviano F, Rondelli D, Franchina M, Bagnara GP, Grossi A. Constitutive and stimulated production of VEGF by human megakaryoblastic cell lines: effect on proliferation and signaling pathway. Int J Immunopathol Pharmacol 2005;18:445–455.

[11] Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 2005;202:654–662.

[12] Chiarugi P, Buricchi F. Protein tyrosine phosphorylation and reversible oxidation: two cross-talking posttranslation modifications. Antioxid Redox Signal 2007;9:1–24.

[13] Maraldi T, Rugolo M, Fiorentini D, Landi L, Hakim G. Glucose transport activation in human hematopoietic cells M07e is modulated by cytosolic calcium and calmodulin. Cell Calcium 2006;4:373–381.

[14] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.

[15] Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu Rev 2005;11:127–152.

[16] Shi H, Liu S, Miyake M, Liu KJ. Ebselen induced C6 glioma cell death in oxygen and glucose deprivation. Chem Res Toxicol 2006;19:655–660.

[17] Ozen S, Akyol O, Iraz M, Sogut S, Ozugurlu F, Ozyurt H, Odaci E, ldirim Z. Role of caffeic acid phenethyl ester, an active component of propolis, against cisplatin-induced nephrotoxicity in rats. J Appl Toxicol 2004;4:27–35.

[18] Paolini M, Valgimigli L, Marchesi E, Trespidi S, Pedulli GF. Taking EPR 'snapshots' of the oxidative stress status in human blood. Free Radic Res 2003;37:503–508.

[19] Henderson LM, Chappel JB. NADPH oxidase of neutrophils. Biochim Biophys Acta 1996;1273:87–107.

[20] Miller AA, Drummond GR, Sobey CG. Novel isoforms of NADPH-oxidase in cerebral vascular control. Pharmacol Ther 2006;111:928–948.
[21] Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. Proc Natl Acad Sci 2004;101:7618–7623.
[22] Gu Y, Xu YC, Wu RF, Souza RF, Nwariaku FE, Terada LS. TNFalpha activates c-Jun amino terminal kinase through p47(phox). Exp Cell Res 2002;272:62–74.
[23] Knight LA, Di Nicolantonio F, Whitehouse PA, Mercer SJ, Sharma S, Glaysher S, Hungerford JL, Hurren J, Lamont A, Cree IA. The effect of imatinib mesylate (Glivec) on human tumour-derived cells. Anticancer Drugs 2006;17:649–655.
[24] Kuan YH, Lin RH, Lin HY, Huang LJ, Tsai CR, Tsao LT, Lin CN, Chang LC, Wang JP. Activation of phosphoinositide 3-kinase and Src family kinase is required for respiratory burst in rat neutrophils stimulated with artocarpol A. Biochem Pharmacol 2006;71:1735–1746.
[25] Arbiser JL, Petros J, Klafter R, Govindajaran B, McLaughlin ER, Brown LF, Cohen C, Moses M, Kilroy S, Arnold RS, Lambeth JD. Reactive oxygen generated by Nox1 triggers the angiogenic switch. Proc Natl Acad Sci 2002;99:715–720.
[26] Cai H, Griendling KK, Harrison DG. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. Trends Pharmacol Sci 2003;24:471–478.
[27] Geiszt M. NADPH oxidases: new kids on the block. Cardiovasc Res 2006;71:289–299.
[28] Arnold RS, Shi J, Murad E, Whalen AM, Sun CQ, Polavarapu R, Parthasarathy S, Petros JA, Lambeth JD. Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. Proc Natl Acad Sci 2001;98:550–555.
[29] Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of nox family proteins in human atherosclerosis. Circulation 2002;105:1429–1435.
[30] Bae YS, Sung JY, Kim OS, Kim YJ, Hur KC, Kazlauskas A, Rhee SG. Platelet-derived growth factor-induced H2O2 production requires the activation of phosphatidylinositol 3-kinase. J Biol Chem 2000;275:10527–10531.
[31] Kanai F, Liu H, Field SJ, Akbary H, Matsuo T, Brown GE, Cantley LC, Yaffe MB. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. Nat Cell Biol 2001;3:675–678.
[32] Han J, Luby-Phelps K, Das B, Shu X, Xia Y, Mosteller RD, Krishna UM, Falck JR, White MA, Broek D. Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. Science 1998;279:558–560.
[33] Ushio-Fukai M. VEGF signaling through NADPH oxidase-derived ROS. Antioxid Redox Signal 2007;9:731–739.
[34] Choi K, Kennedy M, Kazarov A, Papadimitriou J, Keller G. A common precursor for hematopoietic and endothelial cells. Development 1998;125:725–732.
[35] Maraldi T, Prata C, Fiorentini D, Zambonin L, Landi L, Hakim G. Signal processes and ROS production in glucose transport regulation by thrombopoietin and granulocyte macrophage-colony stimulation factor in a human leukaemic cell line. Free Radic Res 2007;41:1348–1357.
[36] Palmada M, Boehmer C, Akel A, Rajamanickam J, Jeyaraj S, Keller K, Lang F. SGK1 kinase upregulates GLUT1 activity and plasma membrane expression. Diabetes 2006;55:421–427.
[37] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 4th ed.: Oxford University Press Inc., New York; 2007. p 187.
[38] Pardanani A, Tefferi A. Imatinib targets other than bcr/abl and their clinical relevance in myeloid disorders. Blood 2004;104:1931–1939.
[39] Johnson DH. Targeted therapies in combination with chemotherapy in non-small cell lung cancer. Clin Cancer Res 2006;12:4451–4457.

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