VEGF-induced ROS generation from NAD(P)H oxidases protects human leukemic cells from apoptosis

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Abstract. Vascular endothelial growth factor (VEGF) and reactive oxygen species (ROS) play critical roles in vascular pathophysiology and in hematological malignancies. VEGF is supposed to utilize ROS as messenger intermediates downstream of the VEGF receptor-2. NAD(P)H oxidase (Nox) family is a major source of cellular ROS and is implicated in increased ROS production in tumor cells. We previously demonstrated that B1647 cells, a human leukemic cell line, express Nox2 and Nox4, both at mRNA and protein level. We suggest here that the VEGF-induced increase in ROS can be related to Nox2 and Nox4 activities. Nox-derived ROS are involved in early signaling events such as the autophosphorylation of VEGF receptor-2, and in the modulation of glucose uptake, a cellular activity strictly bound to VEGF-induced leukemic cell proliferation, as shown by experiments with antioxidants and Nox inhibitors and siRNA. Nox-generated ROS are required to sustain B1647 cell viability and proliferation; in fact, antioxidants such as EUK-134 or Nox inhibitors and siRNA direct cells to apoptotic cell death, suggesting that manipulation of cellular Nox2 and Nox4 could affect survival of leukemic cells.

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Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin; AML, acute myelogenous leukemia; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorodihydrofluorescin diacetate; DOG, 2-deoxy-D-glucose; DPI, diphenyleneiodonium chloride; Glut1, glucose transporter 1; HE, hydroethidine; HS, human serum; IMDM, Iscove's modified Dulbecco's medium; $InsP_3$, inositol 1,4,5-triphosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nox, NAD(P)H oxidase; PBS, phosphate-buffered saline; PI3-K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SOD, superoxide dismutase; TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; VEGFR-2, vascular endothelial growth factor receptor 2

Key words: leukemia, vascular endothelial growth factor, NAD(P)H oxidases, reactive oxygen species, apoptosis

Introduction

Vascular endothelial growth factor (VEGF) and reactive oxygen species play critical roles in vascular pathophysiology (1). During embryonic development, hemopoietic and early endothelial cells (angioblasts) originate from a common precursor known as hemangioblast. Given this common root, several pathways are shared by hemopoietic and vascular cells. One of these pathways is mediated by VEGFR-2 (also known as KDR). This receptor is not exclusively expressed only by endothelial cells, but is present in many leukemic cells. Since VEGF is also expressed by leukemic cells, this fact could result in the rise of an autocrine loop supporting their survival and proliferation (2).

Reactive oxygen species (ROS) are implicated in both stimulation and inhibition of cell proliferation, apoptosis, and cell senescence (3,4). ROS trigger genetic programs associated with transformation, resulting in alteration of genes by manipulating the cell cycle and signal transduction (5). It is known that ROS are spontaneously generated in malignant cancer cells, but their origin and biological meaning remained obscured (6).

NAD(P)H oxidase (Nox) family is one of the major sources for cellular ROS (7,8). Nox enzymes are the structural homologues of phagocytic Nox (gp91phox/Nox2) and consist of both single (Nox1-Nox5) and dual oxidases (Duox1 and Duox2) (9). The phagocytic-like Nox2-based oxidase consists of a membrane-integrated flavocytochrome b558, composed of Nox2 and p22phox, and four cytosolic components (p47phox, p67phox, p40phox, and Rac) that associate with the flavocytochrome to form an active enzyme (10). Recent studies revealed some aspects of the functional relationships between Nox family genes and increased ROS production in tumor cells. Nox1-generated ROS are functionally required for oncogenic Ras transformation phenotype including anchorage-independent growth and tumorigenesis (11,12). Nox4 is integrated into the receptor systems for insulin-induced glucose transport

(13) and lipopolysaccharide-stimulated inflammation (14). Nox4 contributes to cell survival of pancreatic cancer cells, and this survival signaling seemed to be mediated by the impaired activities of AKT and its target ASK1 (15,16). Nox5 has also been implicated in cell viability of prostate cancer cells (17) and Barrett esophageal adenocarcinoma cells (18).

Accumulating evidence suggests the involvement of low levels of ROS generated by Nox enzymes as mediators in inflammation, apoptosis, cell growth, and angiogenesis in various types of human cancers. Thus, it would seem that Nox and downstream targets, including ROS, are necessary for tumor growth, angiogenesis, and potentially metastasis and, therefore, are attractive targets for therapeutic intervention in cancer development. In particular, several reports suggest that ROS may play a role in angiogenesis and in regulation of leukemic cells proliferation (19). Moreover, VEGF utilizes ROS as messenger intermediates downstream of VEGFR-2.

It has been reported that B1647 cells, a human leukemic erythromegakaryocytic cell line not requiring additional cytokines to proliferate, constitutively produce VEGF (20), a potent inducer of angiogenesis and a stimulator of endothelial cell proliferation, differentiation and survival, and express its tyrosine-kinase receptor, VEGFR-2 (21). Recently (22), we demonstrated that B1647 cells express Nox2 and Nox4, both at mRNA and protein levels, and a relatively high level of p47phox and Rac1, thus being characterized by high basal ROS production regulating glucose transport.

The main aim of the present investigation was to better identify the connection between ROS sources and the VEGF autocrine loop present in these cells, bringing the study one step closer to a physiologically relevant situation. Therefore, this report focuses on the roles of Nox2 and Nox4, and VEGFR-2 regulation on leukemic cell survival.

Materials and methods

Materials. Phloretin, 2',7'-dichlorofluorescin-diacetate (DCFH-DA), hydroethidine (HE), diphenyleneiodonium chloride (DPI), 4-hydroxy-3-methoxy-acetophenone (apocynin), Hoechst-33342, 2-deoxy-D-glucose (DOG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Protein G-Agarose, trypan blue, Igepal CA-630, orthovanadate, H_2O_2, phenyl-methylsulfonyl fluoride (PMSF), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), LY-294002, wortmannin, superoxide dismutase (SOD) and mouse monoclonal antiserum against tubulin were from Sigma (St. Louis, MO, USA). Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco (Grand Island, NY, USA) and human serum (HS) was from Cambrex Bioscience. The synthetic superoxide/peroxide scavenger EUK-134 was provided by Cayman Chemical (Ann Arbor, MI, USA). Caspase-3 fluorogenic substrate was from Alexis (San Diego, CA, USA). VEGF was from Biosource International (Camarillo, CA, USA), 2-deoxy-D-[2,6-^{3}H]-glucose from Amersham (UK) and nitrocellulose review from Schleicher and Schuell (Keene, NH, USA). Rac1 inhibitor (NSC23766) and (Z)-3-[(2,4-dimethyl-3-(ethoxycarbonyl)pyrrol-5-yl)methylidenyl]indolin-2-one VEGF receptor 2 kinase inhibitor I (V2) were from Calbiochem.

Anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase, anti-Nox4 antibody and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine (P-Tyr-102) and anti-VEGF receptor 2 were from Cell Signaling Technology (Beverly, MA, USA). Anti-Nox2 was from Millipore (Temecula, CA, USA). TRIzol reagent was from Invitrogen. Access RT-PCR System and 100 bp DNA ladder were purchased by Promega and GelRed™ by Biotium. All the other chemicals and solvents were of the highest analytical grade.

Cell culture. Human acute myeloid leukemia (AML) cells B1647 are cultured in IMDM supplemented with 5% human serum. The experimental model employed 16-24 h serum-depleted cells, as these conditions were more apt for focusing experiments on self-produced VEGF role, ruling out other growth factor effects.

Cell viability and proliferation. Viable cells were evaluated by the trypan blue exclusion test. Cell viability was also assayed by the MTT assay, since the reduction of tetrazolium salts is widely accepted as a reliable way to examine cell viability/proliferation. Cells were incubated with 0.5 mg/ml MTT for 4 h at 37˚C. At the end of the incubation, purple formazan salt crystals were formed and dissolved by adding the solubilization solution (10% SDS, 0.01 M HCl), then the plates were incubated overnight in humidified atmosphere (37˚C, 5% CO_2). The absorption at 570 nm was measured on a multi-well plate reader (Wallac Victor^2, Perkin-Elmer).

Glucose transport assay. After different treatments, glucose uptake was assayed as previously reported (23). In brief, 0.5 ml cell suspension (2×10^6 cells) in PBS buffer, pH 7.2 (glucose-free) was treated with 2-deoxy-D-[2,6-^{3}H] glucose (15 kBq/assay) and 1 mM unlabeled 2-deoxy-D-glucose. After a 1-min incubation at 37˚C, the uptake was stopped by adding phloretin (0.3 mM final concentration). The uptake was linear up to 3 min. Transported 2-deoxy-D-glucose was <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.

Since B1647 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 (up to 1 h) was considered constant.

Immunoprecipitation, 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. Anti-phosphotyrosine and Protein-G-Agarose were added to cell lysates. Samples were separated on 10% SDS-polyacrylamide gel using a Mini-Protean II apparatus (Bio-Rad Laboratories). Proteins were transferred electrophoretically to nitrocellulose membrane at 100 V for 60 min. Non-specific binding was blocked by incubating with Tris-buffered saline (TBS)/Tween,

pH 8.0, containing 5% non-fat dried milk for 1 h at room temperature. Nitrocellulose membranes were incubated overnight at 4˚C with primary antibodies, 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 and developed using Western Blotting Luminol Reagent.

Measurement of intracellular ROS. Cells ($1x10^6$/ml) were washed twice in PBS and incubated with 5 μM 2',7'-dichlorofluorescin-diacetate (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 oxidised to the fluorescent 2',7'-dichlorofluorescein (DCF). For the hydroethidine (HE) assay, cells ($1.0x10^6$/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).

Preparation of RNA and reverse transcriptase PCR (RT-PCR) analysis. Total RNA extraction was performed using TRIzol reagent (Invitrogen, Scotland, UK) 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): NOX2, antisense 5'-CTCACCCTTTCAAAACCATC-3' and sense 5'-ACGATGCGGATATGGATACT-3'. Primers for human NOX4 were designed by published sequences (24) as follows: antisense 5'-AGAGGAACACGACAATCA GCCTTAG-3' and sense 5'-CTCAGCGGAATCAATCAGC TGTG-3'. RT-PCR was carried out using Access RT-PCR Systems (Promega, Madison, WI, USA). The reaction mixtures were kept 45 min at 45˚C, 2 min at 94˚C, then cycled 35 times through a program of 30 sec at 94˚C, 1 min at 50˚C for 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, USA).

RNA interference. For transient siRNA transfection, B1647 cells were nucleofected with Cell Line Nucleofector™ Kit C (Amaxa Biosystems, Cologne, Germany) program X-05 following the manufacturer's instructions, with siRNA against Nox2 and Nox4 or non-specific control siRNA (final siRNA concentration 50 nM). Oligos were obtained from Sigma-Genosys (Suffolk, UK). Specific oligos with maximal knockdown efficiency were selected among three different sequences for each gene. Subsequently, cells were immediately suspended in complete medium and incubated in a humidified 37˚C/5% CO_2 incubator. After 24-48 h cells were used for experiments: evaluation of Nox expression by Western blotting, detection of intracellular ROS levels and measure of cell viability.

Chromatin condensation assay. Cells were washed and fixed with formaldehyde, then stained with Hoechst 33342 (5 μg/ml) for 5 min at 25˚C. Nuclei were visualized using a fluorescence Olympus IX50 microscope.

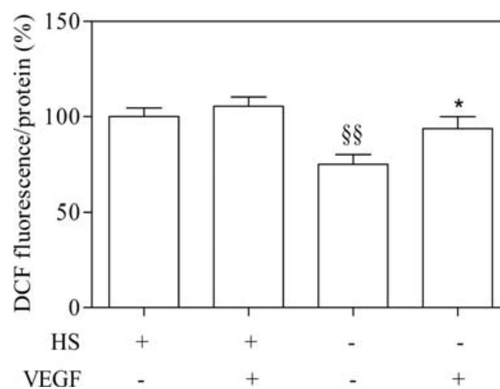

Figure 1. Effect of VEGF on ROS production in B1647 cells. Cells deprived or not of serum were treated for 30 min with 50 ng/ml VEGF. ROS content was measured in PBS with DCFH-DA probe as described in Materials and methods. The data shown are representative of three independent experiments. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. *$P<0.05$, significantly different from -VEGF sample; §§$P<0.01$, significantly different from +HS sample.

Caspase assay. Ac-DEVD-AMC was used as fluorogenic substrates for caspase-3. After different treatments, cell lysates were incubated with specific substrates at 37˚C for 15 min. The activity of caspase-3 was measured following the cleavage of fluorogenic substrates excited at 370 nm by measuring the emission at 455 nm.

Statistical analysis. Statistical analysis was performed with Student's unpaired t-test. Results are the mean of at least three independent experiments. Differences were considered significant at $P<0.05$.

Results

VEGF effect on ROS level in B1647 cells. At first, we observed that the intracellular ROS level, measured with the oxidant probe 2,7-dichlorodihydrofluorescin diacetate, is modulated by the starvation treatment. In fact, the absence of human serum causes a slight decrease in DCF fluorescence (Fig. 1), indicating that cellular ROS sources are controlled by different mechanisms, including VEGF self production. The VEGF effect on ROS concentration is more evident when the growth factor is added, as shown in Fig. 1, VEGF induces a weak but detectable increase on DCF fluorescence only in starved cells. Therefore, all the experiments were performed on serum-depleted cells, in order to better evaluate the role of self-produced VEGF, ruling out other growth factors effects.

Nox2 and Nox4 as ROS sources in B1647 cells. In B1647 cells, it was previously shown, by using treatment with inhibitors, Western blotting and PCR, that the Nox family is a major source of ROS (22). Here, the contribution of Nox4 and Nox2 was assessed through siRNA experiments. Three siRNAs were used to block the Nox effect, the first targeting the Nox2 isoform (siNox2), the second targeting Nox4 (siNox4), while scrambled siRNA was used as a negative control (Fig. 2A). Both mRNA and protein expression were strongly reduced in the cell line, when transfected with 50 nmol/l of Nox2 and Nox4 siRNA for up to 48 h (Fig. 2A), compared

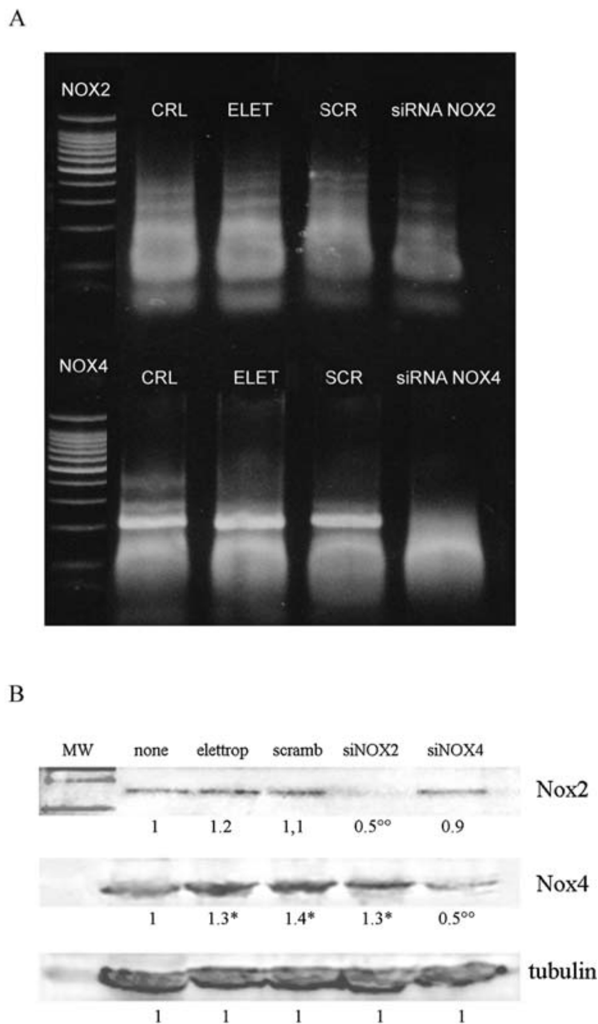

Figure 2. Silencing Nox2 and Nox4 in B1647 cells. (A) RT-PCR of mRNA derived from B1647 cells, after 48 h of Nox2 siRNA treatment; using specific primers for NOX2 and NOX4. RT-PCR was performed as described in Materials and methods. Electroporate sample was considered as control and the non-specific control (scrambled) did not give a significant effect. The data shown are representative of three independent experiments. (B) Representative immunoblots showing Nox2 and Nox4 expression level in B1647 cells after 48 h of Nox2 or Nox4 siRNA treatments. Electroporate sample was considered as control and the non-specific control (scrambled) did not give a significant effect. Forty micrograms of protein per lane were electrophoresed and immunoblotted, as described in Materials and methods. The data shown are representative of three independent experiments. Relative amounts determined by scanning densitometry are in arbitrary units. *P<0.05, significantly different from control;°°P<0.01, significantly different from electroporate.

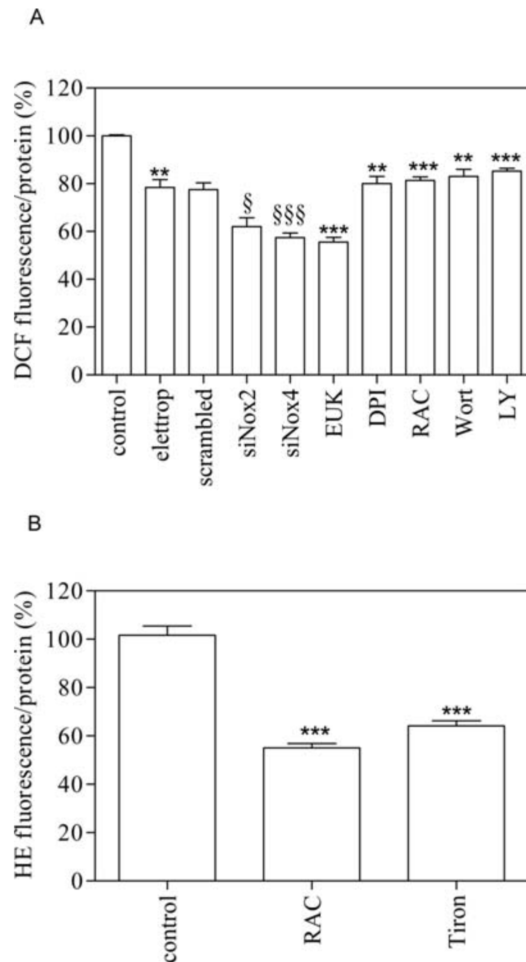

Figure 3. Effect of NAD(P)H oxidase siRNA and inhibitors on ROS content. (A) Silenced for Nox2 and Nox4, electroporated and scrambled samples were obtained as described for Fig. 2. Cells deprived of serum for 16 h were incubated for 16 h in IMDM with 150 μM EUK-134 and for 30 min with 10 μM DPI, 300 μM Rac1 inhibitor, 1 μM wortmannin and 25 μM LY294002. ROS content was measured in PBS with DCFH-DA probe as described in Materials and methods. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. **P<0.01; ***P<0.0001 significantly different from control. siRNA samples were compared to electroporate sample and the non-specific control (scrambled) did not give a significant effect; §P<0.05, §§§P<0.0001 significantly different from electroporate. (B) Cells were washed twice in PBS and incubated with 5 mM HE for 10 min at 37˚C, in the presence or absence of 300 μM Rac1 inhibitor or 1 mM Tiron for 20 min. The fluorescence of oxidized HE was determined by a multiwell plate reader, at λ_{exc} 480 nm and λ_{em} 586 nm. For each experiment four independent cell preparations were used with four parallel samples. ***P<0.0001, significantly different from control.

with those treated with the scrambled siRNA preparation, indicating that Nox siRNAs effectively repressed the Nox expression. Furthermore, siNox2, used as a specificity control, did not affect Nox4 protein expression and vice versa (Fig. 2B).

To test whether Nox2 and Nox4 mediate intracellular ROS production, ROS generation (mainly superoxide and H_2O_2, a dismutated metabolite of Nox-derived superoxide) was measured in Nox siRNA-transfected cells after addition of the fluorescent oxidant indicator dye 2,7-dichlorodihydrofluorescin diacetate. Transfection with siNox4 reduced DCF fluorescence by ~30%, while siNox2 had a slighter effect on the ROS level (Fig. 3A). These observations were confirmed by data obtained with DPI, a flavoprotein-dependent oxidase inhibitor that under these experimental conditions is considered a Nox inhibitor (25), which caused a similar ROS level decrease, although not as dramatic as the one obtained with the ROS scavenger EUK-134 (a catalase and superoxide dismutase mimetic compound).

As the Noxes play a vital role in generating ROS, some of their known upstream regulators were examined with the aim to better understand the involved regulatory mechanisms. Rac1 inhibitor (NSC23766), a cell-permeable pyrimidine compound that specifically and reversibly inhibits Rac1

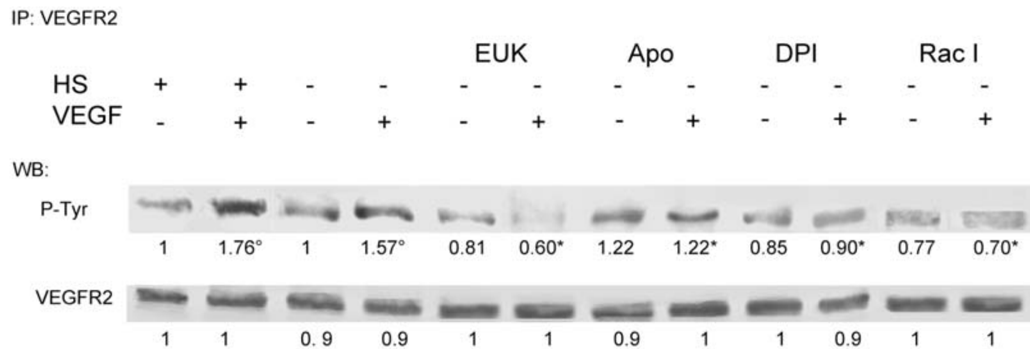

Figure 4. Effect of NAD(P)H oxidase inhibitors and EUK-134 on VEGFR-2 phosphorylation level. Cells deprived or not of serum were treated for 30 min with 50 ng/ml VEGF. In the presence or absence of VEGF, starved cells were incubated for 16 h in IMDM with 150 μM EUK-134, 1 mM apocynin, 10 μM DPI and 300 μM Rac1 inhibitor. Immunoprecipitation with anti-VEGFR-2 was performed as described in Materials and methods. Samples were electrophoresed, immunoblotted and revealed for anti-phosphotyrosine or with anti-VEGFR-2. A representative blot is shown. Results were obtained considering three independent Western blot experiments. Relative amounts determined by scanning densitometry are in arbitrary units. $^{*}P<0.05$, significantly different from starved sample treated with VEGF; $^{\circ}P<0.05$, significantly different from the corresponding sample without VEGF.

GDP/GTP exchange activity, and phosphatidylinositol 3-kinase (PI3-K) inhibitors wortmannin and LY294002 were able to decrease the ROS content (Fig. 3A), suggesting that the presence of VEGF self-produced by the cells could be linked to ROS production by the Nox system activation.

Data obtained with DCF have been supported by the evaluation of superoxide level, measured using the probe hydroethidine (HE). In fact, Rac1 is a cytosolic component, that associates with the flavocytochrome to form an active enzyme; superoxide production is decreased ~50% by Rac1 inhibitor as well as in presence of tiron, a superoxide selective scavenger (Fig. 3B). The role of Rac1 in Nox4-mediated superoxide generation appears controversial (26,27), but the similar effect on ROS and superoxide production by Rac1 inhibitor, compared to the less specific modulation by DPI and tiron, could suggest that ROS sources in these cells upon starvation are both Nox2 and Nox4 isoforms, although Nox2 in a slighter manner. In fact, Nox2 and Nox4 could play different roles in the generation of ROS in B1647 cells depending on self-produced VEGF or/and serum presence.

Effect of ROS on VEGFR-2 phosphorylation in B1647 cells. In order to check the ROS connection with the VEGF pathway, immunoprecipitation experiments with anti-VEGFR-2 were performed. After SDS-PAGE separation, samples were revealed for anti-phosphotyrosine antibody. Fig. 4 shows that VEGF increases VEGFR-2 phosphorylation in a similar way in the presence or absence of serum. These data suggest that self-produced VEGF maintains the receptor in a phosphorylation state that can be increased by a short, exogenous VEGF treatment, but is not dependent on serum presence. The phosphorylation is strongly quenched by EUK-134, indicating a role for ROS in VEGF modulation. The effect of EUK-134 has been compared with Nox inhibitors such as apocynin, DPI and Rac1 inhibitor (Fig. 4). Also in these cases, VEGF-receptor phosphorylation increase is limited, suggesting that, in cells exposed to VEGF, ROS source involved in VEGFR-2 activation could be identified in Nox activity. The lack of activity of treatments on starved cells could be due to the self-produced VEGF that causes a constitutive phosphorylation state of VEGFR-2, inducing a downstream Nox activation.

Role of ROS and VEGFR-2 on glucose transport regulation in B1647 cells. Glucose transport has been considered a model for cellular activity strictly bound to VEGF-induced cell proliferation. In B1647 cells, where glucose transport activity is quite high, we previously observed that EUK-134 decreased glucose uptake rate (28). Upon starvation, cells show a lower glucose transport rate; incubation with VEGF is able to increase this activity only under this experimental condition (Fig. 5A), suggesting that the increase of the constitutive phosphorylation state, due to VEGF treatment, is related to the sugar uptake modulation. The effect of EUK-134 and superoxide dismutase (SOD) has been compared with the pretreatment with Nox inhibitors such as apocynin and DPI in B1647 cells upon starvation. Antioxidants and Nox inhibitors induce a similar decrease in sugar uptake (Fig. 5B). Nox activation pathway was confirmed using Rac1 inhibitor and wortmannin, inhibitors of proteins related to the activation of Noxes.

The involvement in glucose transport regulation of VEGF produced by cells and therefore the constitutive VEGFR-2 activation are supported by the parallel effect of VEGF receptor-2 kinase inhibitor (V2), a specific inhibitor of KDR (Fig. 5).

Role of ROS and VEGFR-2 in B1647 cell viability. To explore the functional role of ROS-generating enzymes in leukemic cells, we first examined whether proliferation/viability requires ROS production. To verify whether inhibition of ROS sources affected the cell survival, a cell viability assay was performed after treatment with the antioxidant EUK-134, Nox pathway specific inhibitors or siRNA. When B1647 cells were serum starved for 24 h, their viability decreased by 35% (Fig. 6A). Exogenous VEGF addition slightly increased proliferation in both conditions. When cells were pre-incubated with the antioxidant EUK-134, cell viability was efficiently reduced. Co-treatment with VEGF limited the EUK-134 effect, completely restoring the viability to the initial level only in the case of serum starved cells, indicating a close connection between VEGF effect and ROS formation in the absence of serum growth factors. Furthermore, V2 treatment negatively affected only serum starved cell viability, confirming that autocrine VEGF pathway is necessary to sustain growth in cells when deprived of serum stimuli, which, in turn, may

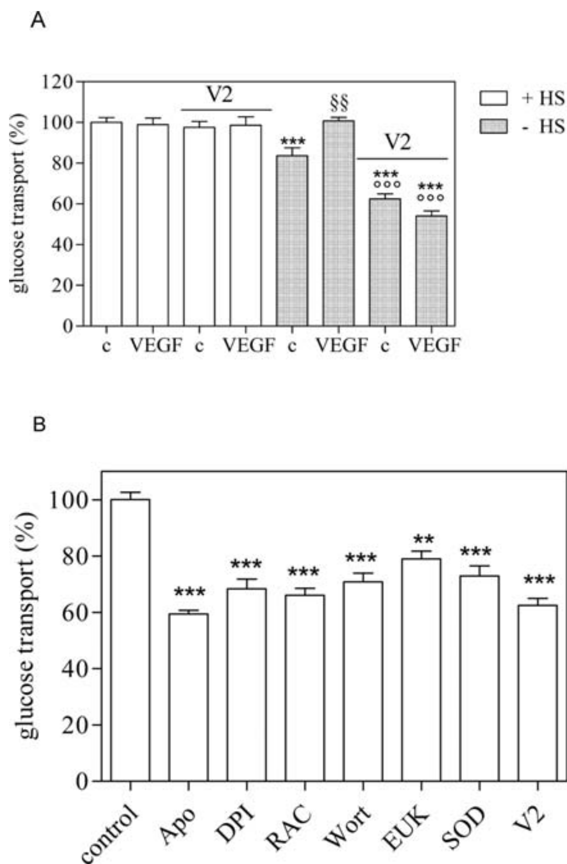

Figure 5. Effect of NAD(P)H oxidase and VEGFR-2 inhibitors on glucose uptake in B1647 cells. (A) Cells deprived or not of serum for 16 h were incubated at 37˚C for 30 min with 50 ng/ml VEGF and treated with 10 μM V2. DOG uptake was measured in PBS over 1 min as described in Materials and methods. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. ***P<0.0001, significantly different from control with serum;°°°P<0.0001, significantly different from control without serum and V2; §§P<0.01, significantly different from control without serum and VEGF. (B) Cells deprived of serum for 16 h were incubated at 37˚C for 16 h in IMDM with 150 μM EUK-134, and for 30 min with 1 mM apocynin, 10 μM DPI, 300 μM Rac1 inhibitor, 1 μM wortmannin, 250 U/ml SOD and 10 μM V2. 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 triplicate. ***P<0.0001;**P<0.01 significantly different from control.

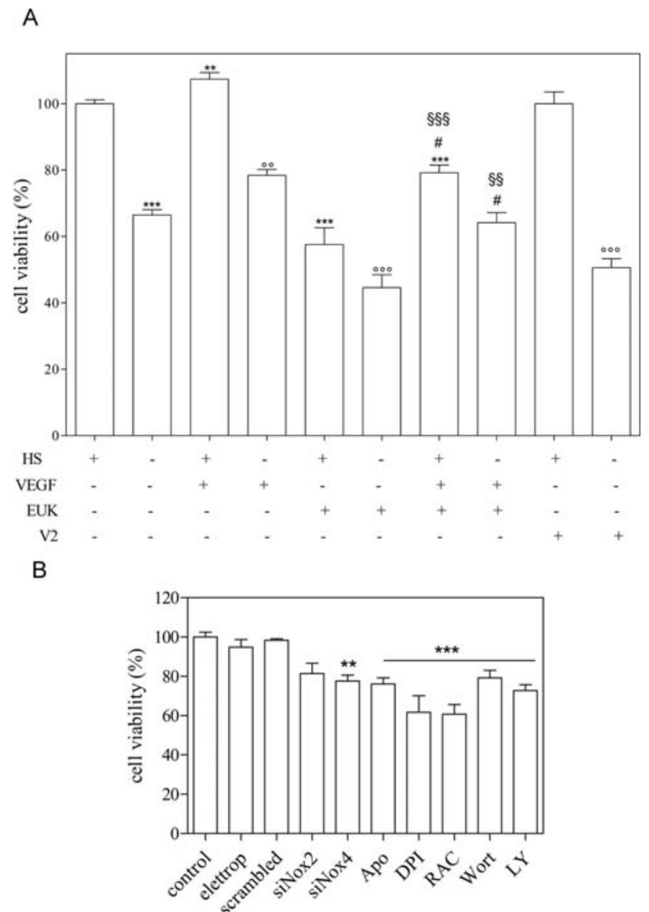

Figure 6. Effect of NAD(P)H oxidase and VEGFR-2 inhibitors on cell viability of B1647 cells. (A) Cells deprived or not of serum for 24 h were incubated at 37˚C for 24 h with 50 ng/ml VEGF and at the same time treated with 150 μM EUK-134 or with 10 μM V2. Cell viability was measured with MTT assay as described in Materials and methods. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. ***P<0.0001, **P<0.010, significantly different from control sample with serum;°°°P<0001; °°P<0.010, significantly different from the control sample without serum; §§§P<0001; §§P<0.010 significantly different from the corresponding sample without EUK-134; #P<0.05 significantly different from the corresponding sample without VEGF. (B) Cells deprived of serum for 24 h were incubated at 37˚C for 24 h with 1 mM apocynin, 10 μM DPI, 300 μM Rac1 inhibitor, 1 μM wortmannin and 25 μM LY294002. Silenced samples for Nox2 or Nox4 were obtained as described in Fig. 2. Cell viability was measured with MTT assay as described in Materials and methods. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. ***P<0.0001; **P<0.01 significantly different from control, except for siRNA samples that were compared to the electroporate sample and the non-specific control (scrambled) did not give a significant effect.

overcome the VEGFR-2 block by modulating ROS sources and phosphorylation pathways in a more complex way.

To probe the functional role of Nox in acute leukemia, viability test was performed in the presence of Nox pathway inhibitors. Because all the treatments blocked proliferation of B1647 cells (Fig. 6B), we next examined whether suppression of the Nox4 activity by siNox4 induces dysregulation of cell proliferation. The data indicate that Nox-generated ROS are required to sustain growth of this AML cell line and show that manipulation of Nox2, Nox4 and of known regulatory factors of the Nox pathway can affect survival of B1647 cell line.

Induction of apoptosis by Nox and VEGFR-2 inhibitors. To characterize cell death induced by Nox and VEGFR-2 blockers, typical apoptotic features were examined. Caspase-3 plays a central role in mediating various apoptotic responses and is activated in a sequential cascade of cleavages. As illustrated in Fig. 7A, V2 treatment induced a similar caspase-3 activation to the one obtained with the large spectrum antioxidant EUK-134. Moreover, incubation of serum-deprived cells with direct Nox inhibitors, such as apocynin and DPI, or with inhibitors of Nox regulatory factors, such as PI3-K and Rac1 inhibitors, which also decrease ROS concentration, resulted in a significant stimulation of caspase-3 activity. To confirm these data, observations of apoptotic nuclei were performed. All the tested compounds determined a significant increase in the amount of cells showing chromatin condensation and fragmentation (Fig. 7B). Overall, this study demonstrates

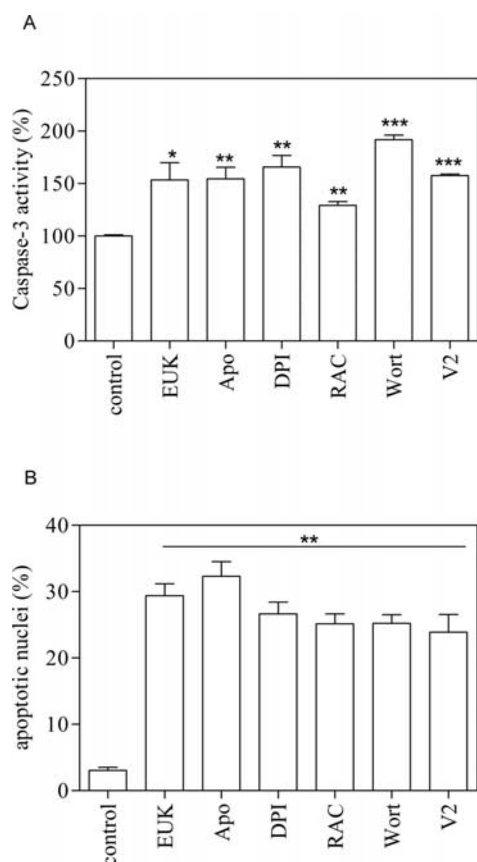

Figure 7. Apoptosis induction by NAD(P)H oxidase and VEGFR-2 inhibitors in B1647 cells. (A) Cells deprived of serum for 24 h were incubated at 37˚C for 24 h with 150 μM EUK-134, 1 mM apocynin, 10 μM DPI, 300 μM Rac1 inhibitor, 1 μM wortmannin and 10 μM V2. Caspase 3 activity was measured on cell lysates, as described in Materials and methods. Results are expressed as means ± SD of three independent experiments, each performed in duplicate. $^{***}P<0.0001$; $^{**}P<0.01$; $^{*}P<0.05$, significantly different from control. (B) Apoptotic nuclei of cells, treated as shown in (A), were measured in PBS with Hoechst 33342 probe (5 μg/ml) as described in Materials and methods. Results are expressed as percentage of cells containing apoptotic nuclei ± SD. $^{**}P<0.01$, significantly different from control.

that the Noxes, mainly Nox2 and Nox4, play a prosurvival role in B1647 cells.

Discussion

Vascular endothelial growth factor (VEGF) is not only a key angiogenic growth factor, but also a pivotal regulator in hematological malignancies. Current studies indicate that VEGF stimulates the proliferation and self-renewal of AML progenitors (29). The mechanisms of signal transduction by VEGF receptors have received growing attention, especially in relation to the possibility to inhibit cancer cell growth. A role for ROS, particularly hydrogen peroxide, as mediators in signal transduction by human VEGFR-2 was described (19). A major source of ROS not only in endothelial, but also in cancer cells is the NAD(P)H oxidase (Nox) family (30). NAD(P)H oxidase is activated by various growth factors, including VEGF, and ROS derived from this oxidase are involved in VEGFR-2 autophosphorylation, and in several redox signaling pathways leading to induction of transcription factors and genes involved in angiogenesis (31).

In endothelial cells, ROS stimulate induction of VEGF, which in turn increases ROS through activation of NAD(P)H oxidases involved in VEGFR-2 autophosphorylation, although the mechanisms by which the Noxes are activated remain unclear (1). Evidence suggests that VEGFR-2-mediated signaling is temporally and spatially controlled and that NAD(P)H oxidases are localized within discrete subcellular compartments, which is required for localizing ROS production and activation of specific redox signaling events, such as cell proliferation and migration. The signaling properties of ROS are due, in part, to reversible oxidative inactivation of redox-sensitive target proteins including protein tyrosine phosphatases (PTPs) (31). ROS effects may result in oxidation of cysteine residues on receptor and non-receptor protein kinases and phosphatases (32).

We have previously demonstrated that NAD(P)H oxidase-derived ROS are required for proliferation of acute leukemia cells, including self-producing VEGF cells (22,28,33-39). However, significant work remains to be performed to define the role of each Nox isoform and its regulatory subunits, and to identify molecular targets of oxidase-derived ROS involved in proliferation/viability of leukemic cells. Thus, the goal of this study is to better understand the extent to which VEGF signaling is coupled to NAD(P)H oxidase activity. The acute leukemia B1647 cell line was transfected with short interfering RNA against isoforms Nox2 and Nox4 or treated with Nox inhibitors or antioxidants and VEGF, then assayed for ROS production, signaling modulation, glucose transport, and cell viability. ROS decrease abrogated the proliferative effect of the growth factor VEGF. Thus, ROS generated via a NAD(P)H oxidase-dependent pathway play a prosurvival role in leukemic cells, which is mediated at least in part by Nox2 and Nox4. In fact, selective Nox silencing demonstrated that VEGF-induced ROS production is due to Nox2 and Nox4 activities, strengthening the results obtained with inhibitors. However, data suggested that Nox4 plays the major role in ROS generation. 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 antiapoptotic effect of ROS is yet to be determined. Further silencing experiments using/implying short interfering RNA against VEGF could support our hypothesis.

Here, we show that Nox2 and Nox4 activities are both required for some, but not all, downstream effects of ROS in leukemic cells. We suggest that NAD(P)H oxidase activity may play a role in maintaining the receptor in the phosphorylated state. The therapeutic modulation of ROS could selectively influence the effect of VEGF on leukemia progression.

Since dietary antioxidants appear to be effective for treatment of tumor angiogenesis, understanding the molecular mechanisms of their action should provide basis to design more effective anti-angiogenic drugs targeted to various cancers (31). In the last decade, we have demonstrated that different antioxidant treatments can modulate the signaling in leukemic cells, reducing some patho-physiological activities and the proliferation capability of these cells (22,33). Thus, the development of specific inhibitors of NAD(P)H oxidases and redox signaling components (kinases, phosphatases) as

well as understanding the mechanism by which dietary antioxidants inhibit hyper-proliferation could offer useful therapeutic strategies not only for treatment of various angiogenesis-dependent pathophysiologies (32), but also of blood cancer such as AML.

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