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Is Nox the source of ROS involved in Glut1 activity in B1647 cells?

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ABSTRACT The discovery of a family of superoxide-generating enzymes, homologues of phagocyte oxidase, has led to the concept that ROS are "intentionally" generated with biological functions in various cell types. We have recently shown that, in two leukaemic cell lines (M07e and B1647), there is a correlation between ROS and an important physiological activity, like glucose uptake, which is up-regulated in leukaemic cells. In this study, we tried to elucidate the sources of ROS generation and the mechanisms by which ROS are involved in the regulation of glucose uptake in B1647 cells. In particular, we investigated the presence and the role of a member of the NAD(P)H oxidase family (Nox). Data obtained in the presence of Nox inhibitors suggest that ROS involved in glucose uptake could be generated by this membrane-bound enzymatic complex. The effects of tyrosine kinase inhibitors and antioxidants show the importance of phosphorylation processes in the regulation of glucose uptake. PI3-kinase seems to be involved in ROS generation, possibly through Rac, which binds to Nox. The activation of tyrosine kinase receptor by vascular endothelium growth factor (VEGF), produced by an autocrine pathway in this cell line, seems to be an important step of this pathway.
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KEY WORDS

reactive oxygen species
glucose transport
NAD(P)H oxidase
leukaemic cell line

Reactive oxygen species (ROS), conventionally thought as cytotoxic and mutagenic, are now considered intracellular mediators of growth, senescence and apoptosis, when present at low level. The discovery of a family of superoxide-generating enzymes, homologues of phagocyte NAD(P)H oxidase (Nox2), has led to the concept that ROS are "intentionally" generated with biological functions in various cell types (Lambeth 2004).

We have recently shown (Fiorentini et al. 2004) that, in two leukaemic megakaryocytic cell lines (M07e and B1647), there is a correlation between ROS and an important physiological activity, like glucose uptake, which is up-regulated in leukaemic cells. Glut1, the transporter isoform present in these cell lines, is responsible for the basal glucose uptake in many cell types and is subjected to acute regulation by several metabolic and oxidative stresses.

In this study, we tried to elucidate the sources of ROS generation and the mechanisms by which ROS are involved in the regulation of glucose uptake in B1647 cell line, established from bone marrow cells of a patient with acute myelogenous leukaemia. In particular, we investigated the presence and the role of a member of the NAD(P)H oxidase family (Nox).

Materials and Methods

Chemicals

4-hydroxy-3-methoxyacetophenone (apocynin), capsaicin, diphenylene iodonium chloride (DPI), 2-deoxy-D-glucose (DOG) and phloretin, were purchased from Sigma Chemical (St. Louis, MO, USA). 2-Deoxy-D-[2,6-^{3}H]-glucose was from Amersham (UK); Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco (Grand Island, NY, USA); normal human serum (NHS) were from Hyclone (Holland). WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) and PMS (1-methoxy-5-methylphenazinium methylsulfate) were purchased by Dojindo (Japan). All other chemicals were of the highest available purity grade.

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Cells and cell cultures

B1647 erythro-megakaryocytic cell line, established from bone marrow cells obtained from a patient with acute myelogenous leukaemia, is maintained in IMDM supplemented with 5% normal human serum (Bonsi 1997). These cells do not need growth factor addition for proliferation.

Cell viability evaluation

Total cell number was determined using a Bürker haemocytometer; the viable cells were evaluated by the Trypan blue exclusion test. In order to verify the absence of effect on cell viability and proliferation of tested inhibitors, MTT assay was used (Mosmann 1983).

Measurement of trans-plasma membrane electron transport (t-PMET)

t-PMET was measured by a simple dye reduction assay using

the cell-impermeable tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) (Berridge and Tan 1998). In the presence of the intermediate electron acceptor PMS (1-methoxy-5-methylphenazinium methylsulfate), WST-1 is reduced to its soluble formazan at the cell surface via reducing equivalents derived from intracellular NAD(P)H, as described by Berridge (2005). Briefly, exponentially growing cells were washed twice, resuspended in PBS to a density of 10^6 cells/mL and incubated with WST1/PMS solution (final concentration 500 μM WST-1, 20 μM PMS). Dye reduction was monitored as the absorbance at 450 nm – 580 nm in a dual wavelength Jasco V-555 UV/Vis spectrophotometer in the presence or absence of inhibitors (DPI, apocynin, capsaicin) preincubated for 20 min at 37°C.

Glucose transport assay

To evaluate glucose transport rate, cells (4 x 10^6/ml) were suspended in PBS and treated with a mixture of 2-deoxy-D-[2,6-^{3}H]glucose (0.5 μCi/assay) and 1.0 mM unlabeled glucose analogue for 1 min at 37°C under conditions where the uptake was linear at least for 10 min. After this time, the uptake was stopped by adding phloretin (final concentration 0.3 mM), a potent inhibitor of glucose transport. Cells were pelleted at 4,000 x g for 1 min and washed with PBS. Sample radioactivity was measured by liquid scintillation counting.

Statistical analysis

Each experiment was repeated three times with at least three parallel samples. Data are expressed as mean ± SEM. Statistical analysis was performed with Graph Pad Prism 4.

Results

Human phagocyte flavocytochrome b_{558} is an integral membrane protein composed of two polypeptides (gp91phox and gp22phox), that serves as the electron transferase of the NAD(P)H oxidase complex, generating superoxide anion when assembled with cytosolic subunit (p40phox, p47phox, p67phox and Rac). Homologues of human gp91phox have been identified in a variety of tissues. Recently, these novel proteins (Nox family) have been shown to play unique roles in development and signal transduction (Bokoch and Knaus 2003; Lambeth 2004). Progress in the study of Nox protein expression has been impeded because of paucity of immunological probes (Baniulis et al. 2005). Therefore, in order to evaluate the presence of a trans-plasma membrane electron transport (t-PMET) responsible of ROS generation in a leukaemic cell line, we used a simple dye reduction assay developed by Berridge and co-workers (1998). This method is based on the cell-impermeable tetrazolium salt (WST-1) that, in the presence of the intermediate electron acceptor PMS, is reduced to its soluble formazan at the cell surface via reducing equivalents derived from intracellular NADH.

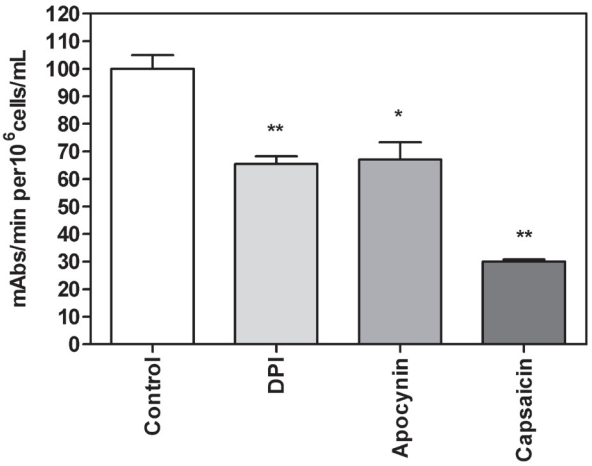

Figure 1. Effect of DPI, Apocynin and Capsaicin on t-PMET activity in B1647 cell line. Different from the control at level of significance of $p<0.05$ (*) and $p<0.01$ (**).

The physiological electron acceptor for t-PMET is oxygen (Scarlett et al. 2004).

By using inhibitors, such as, diphenylene iodonium, apocynin and capsaicin, we tried to investigate the structure of t-PMET in the B1647 cell line. In particular, diphenylene iodonium (DPI) is considered a non specific NAD(P)H oxidase inhibitor as it is capable of inhibiting additional flavin-dependent enzymes (Shen et al. 2006; Li and Trush 1998); apocynin (4-hydroxy-3-methoxy-acetophenone) is a specific NAD(P)H oxidase inhibitor that blocks the assembly of cytosolic subunit p47phox to the membrane complex (Dodd-O and Pearse 2000) and capsaicin, a homovanillic acid derivative (8-methyl-N-vanillyl-6-noneamide), a ubiquinone analogue (Herst et al. 2004), that inhibits CoQ redox cycling thought to be involved as electron shuttle in t-PMET (Scarlett et al. 2004). Treatment of B1647 cells with 10 μM DPI, 1 mM apocynin or 100 μM capsaicin for 20 min caused a significant decrease of WST-1/PMS reduction by 35%-40%-60%, respectively, as shown in Figure 1.

Since we demonstrated that there is a link between ROS generation and glucose uptake, in this leukaemic cell line (Fiorentini 2004; Prata 2004), we tested the effect of DPI, apocynin and capsaicin on glucose transport in order to investigate if t-PMET, a model of Nox, could be the ROS source involved in this process. Data reported in Figure 2 show that pretreatment of B1647 cells with the tested compounds resulted in a decreased glucose uptake by approximately 40%, 50% and 80%, respectively. The inhibitor effect of DPI, apocynin and capsaicin either on t-PMET or on glucose uptake revealed that this enzymatic complex seems to be crucial for ROS generation involved in glucose transport.

According to data reported in literature (Droge 2002; Chiarugi and Cirri 2003; Dusting et al. 2005), we hypoth-

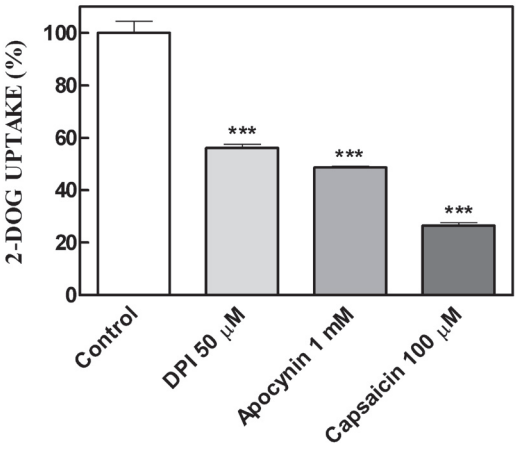

Figure 2. Effect of DPI, Apocynin and Capsaicin on glucose uptake, mediated by Glut1, in B1647 cell line. Different from the control at level of significance of $p<0.001$ (***).

esized that ROS could be generated by NOX after activation of tyrosine kinase receptors linked to growth factors. In this case the growth factor involved is VEGF, recently demonstrated to be produced by an autocrine pathway in B1647 cells (Bonsi et al. 2005). The small GTPase protein Rac1 seems to play a key role, since our preliminary results showed that Rac1 inhibitor (NSC23766) caused a significant decrease both in glucose uptake mediated by Glut1 and in intracellular ROS level. Moreover, since ROS may regulate activities of redox-sensitive enzymes, including protein phosphatases and, consequently, kinases (Chiarugi and Cirri 2003), by using antioxidants, we found that ROS are crucially involved in tyrosine phosphorylation, an important process that resulted important in the regulation of Glut1 activity in B1647 cells (not shown).

Discussion

Glucose transport regulation plays a key role in the aberrant growth of cancer cells, frequently characterized by high intracellular ROS level (Szatrowski and Nathan 1991; Toyokuni et al. 1995). Our previous results showed that ROS are involved in the modulation of Glut1 activity in B1647 cell line, where glucose uptake and basal level of intracellular ROS are higher than in normal cells (Fiorentini et al. 2004). Searching for the origin of ROS production, we focused on a family of membrane-localized NAD(P)H oxidase, Nox, homologus to the phagocytic one and present in various types of cells and tissues (Lambeth 2004).

The effects of DPI (a non specific NAD(P)H oxidase inhibitor), apocynin (a specific NAD(P)H oxidase inhibitor) and capsaicin (an ubiquinone analogue that inhibits CoQ redox cycling) on the trans-plasma membrane electron transport (t-PMET) revealed the involvement of flavoproteins, p47 phox subunit and CoQ cycle, respectively, in this NAD(P)H oxidase activity. Moreover, data obtained in the presence of the same inhibitors suggested that ROS involved in glucose uptake could be generated by this membrane-bound enzymatic complex. Data obtained in our laboratory (not shown) suggest a multistep pathway resulting in maintaining high Glut1 activity, in which phosphorylation process, modulated by ROS, plays a key role. In particular, PI3-kinase seems to be involved in ROS generation, possibly through Rac that, in turn, binds to NAD(P)H oxidase, as reported for other cell lines (Seshiah et al. 2002). An important step of this pathway should be also the activation of the tyrosine kinase receptor linked to vascular endothelium growth factor (VEGF), that is autoproduced in this cell line, as recently published (Bonsi et al. 2005). Moreover, VEGF has been reported to be induced by Nox1 in some tumor cells (Arbiser 2002); therefore, despite of the lack of specific Nox immunoprobe availability (Baniulis 2005), we can speculate that this isoform could be present in B1647 cell line. It's still remain unclear how ROS generated outside the cell penetrate inside and/or if different isoforms of Nox coexist in the same cell type, some of which producing ROS extracellularly and others intracellularly, as the simultaneous presence of multiple Nox proteins was demonstrated in one cell type, endothelial cells (Lassegue et al. 2001).

Acknowledgments

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