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Enhancement of Glucose Transport in Rat Thymocytes by Different Radical Sources

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This study demonstrates that oxidative stress induced in rat thymocytes by the hydrophilic 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), the lipophilic cumene hydroperoxide (CumOOH) and the freely diffusible H_2O_2 is associated with an activation of facilitative glucose transport rate, mediated by GLUT1, the major transporter in this cell type.

We compared the effects of the three tested radical sources on the kinetic transport parameters, showing that the transport rate enhancement in the treated cells can be ascribed to an increase in the V_{max} value, apart from the site of generation of the oxidative stress.

The enhancement of glucose transport by the three oxidants in thymocytes was significantly attenuated both by protein tyrosine kinase inhibitors as genistein and tyrphostin A23 and by U73122, a phospholipase C inhibitor. Genistein and U73122 reversed also the cited increase of V_{max} values.

It is concluded that the stimulation of glucose transport in response to different oxidants is mediated, at least in part, through reactive oxygen species (ROS)-induced stimulation of protein tyrosine kinase and phospholipase C pathways.

Keywords: Thymocytes; Glucose transport; Reactive oxygen species; Redox signaling; Tyrosine kinase; Phospholipase C

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; CumOOH, cumene hydroperoxide; DMSO, dimethyl sulfoxide; DOG, 2-deoxy-D-glucose; GLUT, glucose transporter; PBS, phosphate-buffered saline; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline

INTRODUCTION

Reactive oxygen species (ROS) have been traditionally considered undesirable molecules responsible for cell damage. Recently, it has been recognized that ROS can play a role in the regulation of relevant cellular responses by virtue of redox control of functional proteins. Their involvement has been suggested as signaling intermediates for cytokines and growth factors.[1–3] Low doses of these agents have been implicated in the triggering of apoptosis, typically accompanied by a depletion of intracellular reduced glutathione.[4–6]

Lymphocytes represent a known target for ROS: H_2O_2 can alter the immune response of lymphocytes, inhibiting the proliferation of human T cells without cellular damage.[7] H_2O_2-induced oxidative stress affects T cell responses rapidly increasing tyrosine phosphorylation either through activation of tyrosine kinases,[8] or through the inhibition of protein tyrosine phosphatases.[9]

Evidences have been presented suggesting that in thymocytes both inhibitory and mitogenic signals elicited by antigens could involve the intracellular production of oxygen radicals, with relevant implications for the role of the redox regulation of immune functions.[10]

On the other hand, it has been shown that glucose depletion in rat thymocytes results in an increased generation of endogenous ROS, affecting cell proliferation. In mitogen-stimulated rat thymocytes, H_2O_2 affects glucose metabolism by suppressing glycolytic enzyme induction.[11]

Previously, we investigated the effect of oxidative stress on biochemical and functional parameters of rat thymocytes, showing that free radicals generated outside the cell or in the membrane rapidly stimulate

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facilitative glucose transport activity, suggesting the existence of a common activation pathway, independent on the site of radical generation. Our data ruled out a role of *de novo* transporter synthesis in the free radical-induced stimulation.[12]

Glucose transporters are a family of highly homologous glycoproteins, expressed in a tissue-specific manner, and acutely regulated by many stresses as viral infections, heat shock[13] and hypoxia.[14] Specifically, H_2O_2 has been reported to stimulate glucose uptake in many cell types[15,16] although the mechanisms underlying this activation are not completely understood.

The aim of the present study was to compare the effect of different radical sources such as the hydrophilic, thermolabile azocompound 2,2′-azo-bis(2-amidinopropane)dihydrochloride (AAPH), cumene hydroperoxide (CumOOH) and hydrogen peroxide on glucose uptake and transport kinetic parameters, in order to evaluate whether the short-term activation could be related to an increased transporter affinity or to a translocation of transporter molecules to the plasma membrane. Furthermore, since the potential pathways elicited by ROS in signal transduction cascades can be explored by studying the action of specific inhibitors, as a first step we studied the effect of tyrosine kinase inhibitors as genistein and tyrphostin, and U73122, a phospholipase C inhibitor.

MATERIALS AND METHODS

Chemicals

Standard chemical compounds, CumOOH, genistein, phloretin, H_2O_2 in water (30% wt/wt solution), 2-deoxy-D-glucose (DOG) and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical (St Louis, MO, USA). AAPH was purchased from Polysciences (Warrington, PA, USA) and stored as a 0.5 M solution in water at −20°C in the dark. U73122 and tyrphostin A23 were from Alexis Biochemicals (San Diego, CA, USA). 2-Deoxy-D-[2,6-^{3}H]-glucose was from Amersham Pharmacia (UK). Nitrocellulose paper (BA 83) was obtained from Schleicher and Schuell (Keene, NH, USA). Rabbit polyclonal antiserum against rat GLUT1 and GLUT4, goat polyclonal antiserum against GLUT3, antirabbit and antigoat IgG conjugated to horseradish peroxidase, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were of the highest available purity grade.

Cell Preparation

Thymocytes were prepared from male Wistar 2–3 month old rats (Harlan-Nossan, Correzzana MI, Italy). Thymus glands were washed in PBS, cut in small pieces and filtered through nylon gauze. Thymocytes were pelleted by centrifugation at 800*g* for 5 min, suspended in PBS and counted. Typically, 1.5–2.0 × 10^9 cells were harvested from each gland.

Cell Viability Evaluation

The total cell number was determined using a Burker haemocytometer; the viable cells were evaluated by the Trypan blue exclusion test.

Glucose Transport Assay

In order to evaluate the glucose transport rate, cells (50×10^6/ml) were suspended in PBS, treated with inhibitors, when needed, and with oxidants, and were incubated with a mixture of 2-deoxy-D-[2,6-^{3}H]glucose (0.4 μCi/assay) and 1.0 mM unlabeled glucose analogue for 10 min at 37°C. Under these conditions, the uptake was linear for at least 20 min. After this time, DOG transport was stopped by adding phloretin, a potent inhibitor of glucose transport (final concentration 0.2 mM). The cells were pelleted at 4000*g* for 1 min and washed with PBS. Sample radioactivity was measured by liquid scintillation counting.

For the determination of kinetic parameters V_{max} and K_m, the transport activity was assayed in the same way, using different concentrations of unlabeled DOG (0.1–2.1 mM) and the cited amount of radioactive glucose analogue. Non linear regression analysis of the V vs. [substrate] data was used for curve fitting and for calculating the apparent K_m and V_{max} values (Prism, GraphPad Software, San Diego, CA, USA).

Preparation of Crude Membranes

Crude membranes were prepared according to Ahmed *et al.*[17] with slight modifications. Thymocytes (50×10^6) were washed in PBS and suspended in hypotonic homogenization buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA and 1 mM PMSF). Resuspended cells were sonicated and cellular debris were removed by centrifugation at 900*g* for 10 min at 4°C. The supernatant was freezed-thawed twice, centrifuged at 110,000*g* for 75 min and the crude membranes obtained were solubilized in 10 mM Tris–HCl, pH 7.4, containing 0.5% Triton X-100 and 1 mM PMSF for 1 h at 4°C. Insoluble material was removed by centrifugation for 5 min in a microfuge and the solubilized membranes were stored at −80°C.

SDS-PAGE and Western Blot Analysis

Solubilized cell membrane preparations (40 μg of protein/lane) were added to Laemmli sample buffer

and boiled for 3 min. Proteins 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 by incubating in TBS (Tris-buffered saline)/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 rabbit polyclonal antiserum against rat GLUT1 and GLUT4 and goat polyclonal antiserum against GLUT3 at 1:1000 dilution. Blots were washed with TBS/Tween and then incubated for 30 min at room temperature with anti-rabbit or anti-goat IgG conjugated to horseradish peroxidase diluted 1:2000 in TBS/Tween containing 5% non-fat dried milk. Membranes were washed with TBS/Tween and developed using Western Blotting Luminol Reagent.

Protein concentration was determined using the Bradford method.[18]

RESULTS

Since there is evidence that, in addition to GLUT1, other glucose transporter subtypes as GLUT3 may contribute to glucose uptake in lymphoid cells,[19,20] we determined the transporter subtypes present in thymocytes membranes by Western blotting. Figure 1 shows that GLUT1 is the major isoform expressed in rat thymocytes, whereas GLUT 3 and GLUT 4 were not detected. Therefore, changes in glucose transport activity and in its kinetic parameters can be assigned to a single transporter isoform, i.e. GLUT1.

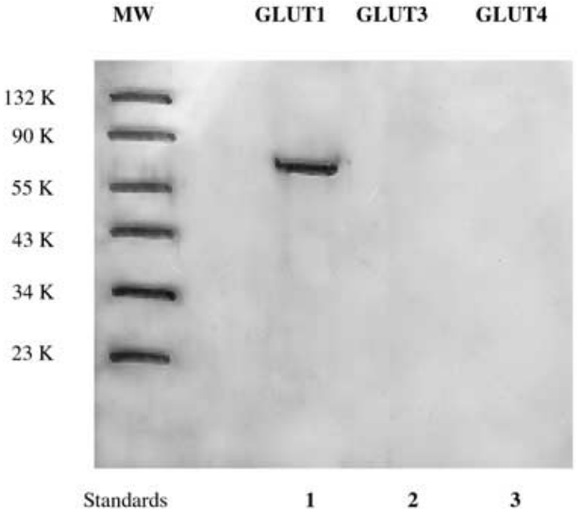

FIGURE 1 Western blot analysis of crude membranes prepared from thymocytes. Crude membrane preparation and blotting procedure are described in the "Material and Methods" section. Each lane contained 40 μg of protein.

Enhancement of Glucose Transport in Response to AAPH, CumOOH and H_2O_2

We previously studied the effects of two radical sources, AAPH and CumOOH on thymocyte viability and chose their concentrations in order to expose cells to low levels of oxidative stress, but being able to induce the activation of glucose transport.[12] It has been shown that also hydrogen peroxide stimulates glucose uptake in several cell lines expressing different GLUT isoforms.[15,21] In order to determine the concentration-response relationship in thymocytes, these cells were exposed to 0.025–0.400 mM H_2O_2 for different time intervals. In Fig. 2, the effect of different concentrations of H_2O_2 on glucose transport after 15 min incubation is reported. In the following experiments, we used 0.05 mM H_2O_2, a concentration that gives a relevant transport activation without significantly affecting the cell viability up to 60 min (data not shown).

The effect of hydrogen peroxide on glucose transport has been compared with that evoked by AAPH and CumOOH. Figure 3 shows that all the tested oxidants induce a similar transport enhancement. The different activation time courses determined at 37°C in PBS can be related to the chemical properties of the three radical generators. In fact, AAPH releases radicals at a constant rate in a temperature-dependent manner in the aqueous medium,[22] while H_2O_2 diffuses freely across

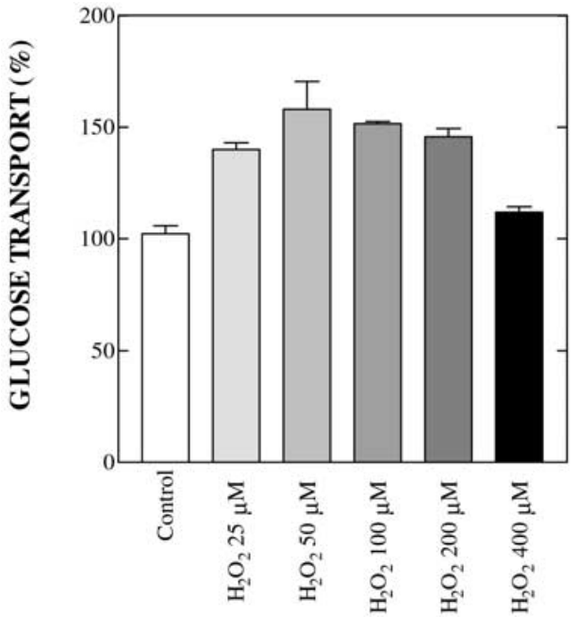

FIGURE 2 Effect of different concentrations of H_2O_2 on glucose transport in rat thymocytes. Glucose uptake of cells incubated in PBS at 37°C was determined in the absence and presence of increasing concentrations (0.025–0.400 mM) of H_2O_2 for 15 min. DOG transport was measured as described in the "Materials and Methods" section. Results are expressed as means ± SE of three independent experiments, each performed in duplicate.

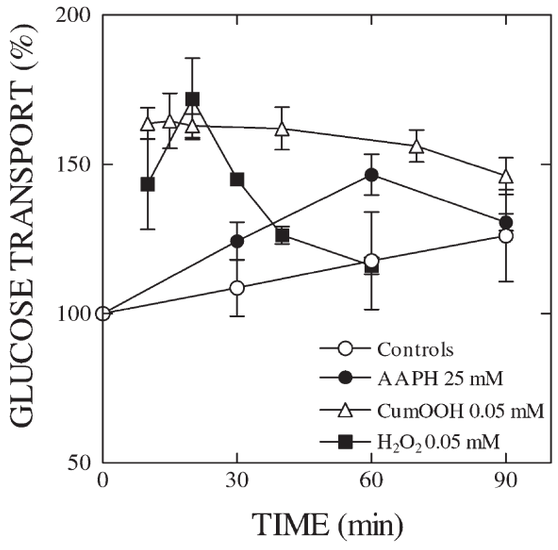

FIGURE 3 Effect of AAPH, CumOOH and H_2O_2 on glucose transport in rat thymocytes. Glucose uptake of cells incubated in PBS at 37°C was determined in the absence (○) and in the presence of 25 mM AAPH (●), or 0.05 mM CumOOH (△), or 0.05 mM H_2O_2 (■). DOG transport was measured as described in the "Materials and Methods" section. Results are expressed as means ± SE of five independent experiments, each performed in duplicate.

the cell membrane. CumOOH, because of its lipophilic characteristics, partitions immediately in the phospholipid bilayer, where is expected to remain localized.[23] For these reasons, the highest transport stimulation by AAPH was reached slowly (45–60 min); in the same time interval, untreated cells showed a slight stimulation, as previously observed by Pasternak *et al.* in BHK cells deprived of glucose.[24] On the other hand, H_2O_2 and CumOOH elicited an immediate increase in glucose transport.

The acute enhancement of glucose transport evoked by AAPH, CumOOH and H_2O_2 in thymus cells was further investigated by measuring the kinetic parameters, K_m and V_{max}, of hexose uptake. Cells were treated with AAPH for 60 min, with CumOOH or H_2O_2 for 15 min before measuring DOG uptake at increasing substrate concentrations. Analysis of the results, summarized in Table I, shows that all the compounds significantly increase V_{max}; the highest effect was obtained with H_2O_2, while AAPH and CumOOH caused a similar change. A 2.7-fold increase in V_{max} value for transport stimulation by H_2O_2 was consistent with the observed activation (Fig. 3), in spite of a significant rise in the K_m value. A slight, not significant rise in the K_m was observed also in the cells treated with AAPH or CumOOH.

Effect of Genistein on the Stimulation of DOG Uptake in Response to AAPH, CumOOH and H_2O_2

To investigate whether the changes in GLUT1 activity observed after the addition of the three

TABLE I Effect of AAPH, CumOOH and H_2O_2 on the kinetic parameters of DOG uptake

Treatment	K_m (mM)	V_{max} (nmol/25 × 10^6 cells/min)
None	1.65 ± 0.16	1.23 ± 0.12
AAPH (60 min)	2.17 ± 0.25	1.93 ± 0.12*
CumOOH (15 min)	1.77 ± 0.19	2.05 ± 0.20*
H_2O_2 (15 min)	2.87 ± 0.39*	3.27 ± 0.34*

*$P < 0.01$; significantly different from control. Thymocytes were treated with 25 mM AAPH for 60 min, with 0.05 mM CumOOH or 0.05 mM H_2O_2 for 15 min; AAPH was removed by centrifugation before glucose transport evaluation. Glucose uptake was determined over 10 min intervals using a range of 2-DOG concentrations (0.066–2 mM). Values are the means ± SE of three experiments performed in duplicate. Data were analysed using a non linear regression method.

tested radical sources could be mediated by tyrosine kinases, thymocytes were treated with increasing concentrations of genistein, a selective, natural inhibitor of protein tyrosine kinase activities[25] (data not shown). Genistein, at a concentration (10 μM) that does not alter the transport activity in untreated cells, was able to extensively attenuate the stimulation of glucose transport induced by all the tested oxidants, as shown in Fig. 4.

Furthermore, genistein brought back V_{max} of the treated cells to values similar to that of control cells, as shown in Table II. The inhibitor at the concentration used had no direct effect on K_m and V_{max} of the controls, ruling out a direct inhibition of glucose transport (see "Discussion" section).

We also tested the effect of a synthetic tyrosine kinase inhibitor, tyrphostin A23, which is not able to directly affect glucose uptake.[26,27] Results shown in Fig. 4 indicate that 50 μM tyrphostin almost

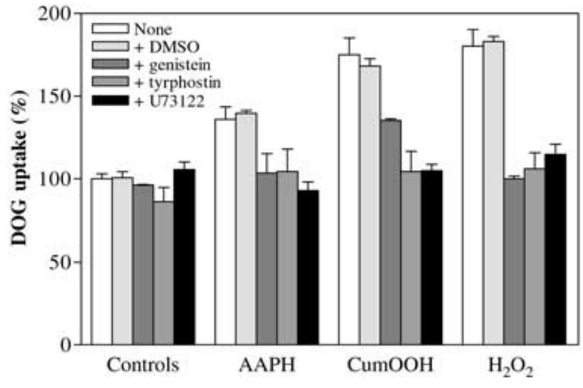

FIGURE 4 Effect of genistein, tyrphostin and U73122 on the stimulation of DOG uptake in response to AAPH, CumOOH and H_2O_2. Thymocytes were treated with 25 mM AAPH for 60 min, or 0.05 mM CumOOH or 0.05 mM H_2O_2 for 15 min; when indicated, cells were pretreated for 5 min with 10 μM genistein, or with 50 μM tyrphostin A23, or 5 μM U73122. AAPH was removed by centrifugation before glucose transport evaluation. Glucose uptake was determined over 10 min intervals, as described in the "Materials and Methods" section. Control experiments were run including DMSO in the same amount used as solvent for the inhibitors. Values are the means ± SE of at least three experiments performed in duplicate.

TABLE II Effect of genistein and U73122 on glucose transport kinetic parameters after treatment with AAPH, CumOOH and H_2O_2

Treatment	Inhibitor pre-treatment		K_m (mM)	V_{max} (nmol/25 × 10^6 cells/min)
	Genistein	U73122		
None†	−	−	1.65 ± 0.16	1.23 ± 0.12
AAPH† (60 min)	−	−	2.17 ± 0.25	1.93 ± 0.12**
AAPH (60 min)	+	−	1.95 ± 0.10	1.40 ± 0.17
AAPH (60 min)	−	+	2.15 ± 0.20	1.17 ± 0.10
CumOOH† (15 min)	−	−	1.77 ± 0.22	2.05 ± 0.21**
CumOOH (15 min)	+	−	1.53 ± 0.27	1.47 ± 0.19
CumOOH (15 min)	−	+	1.68 ± 0.16	1.29 ± 0.20
H_2O_2† (15 min)	−	−	2.87 ± 0.50**	3.27 ± 0.34**
H_2O_2 (15 min)	+	−	2.57 ± 0.47*	1.55 ± 0.34
H_2O_2 (15 min)	−	+	2.66 ± 0.18**	1.73 ± 0.13*

*$P < 0.05$; **$P < 0.01$; significantly different from control. † Data from Table I reported for comparison. Thymocytes were pretreated, when indicated, with 10 μM genistein or 5 μM U73122 for 5 min, then added with 25 mM AAPH for 60 min, or 0.05 mM CumOOH or 0.05 mM H_2O_2 for 15 min. AAPH was removed by centrifugation before glucose transport evaluation. Glucose uptake was determined over 10 min using a range of 2-DOG concentrations (1.0–2.1 mM). Values are the means ± SE of three experiments performed in duplicate. Data were analysed using a non linear regression method.

abolished the stimulation of glucose transport, in agreement with the results obtained with genistein.

Control experiments with DMSO were also run, showing that the solvent had no effect on the basal and activated glucose uptake.

Role of Phospholipase C in the Stimulation of Glucose Transport in Response to AAPH, CumOOH and H_2O_2

It has been reported that, in a lectin-activated monocytic cell line, oxidative stress induces tyrosine phosphorylation of phospholipase C, a well characterized mediator of many signaling processes.[28] In Clone 9, a rat liver cell line, glucose transport activation has been suggested to be mediated through H_2O_2-induced stimulation of tyrosine kinases and phospholipase C.[15] To verify the involvement of phospholipase C in the regulation of glucose transport in thymocytes, we examined the effect of U73122, a potent inhibitor of this enzyme.[29,30]

Preliminary experiments were performed with different inhibitor concentrations, in order to choose a concentration unable to affect the basal glucose uptake. In the presence of 5 μM U73122, the stimulatory effect of all the tested oxidants was almost completely removed (Fig. 4). Cells treated with 5 μM U73122 alone exhibited no change in DOG uptake.

Table II shows that also the phospholipase C inhibitor was able to bring back the V_{max} values of the treated cells close to that of control cells, while it did non affect the kinetic values when added to untreated cells (data not shown).

DISCUSSION

A variety of signals have been reported to involve low-dose ROS generation, although there is no consensus on the specific species produced.[1] Toxic effects on glucose transport in cultured cells induced by differently located ROS generators have been reported, and it has been suggested that the lipid environment surrounding glucose transporters may be susceptible to localized damage induced by CumOOH.[31] However, a careful choice of the radical sources concentration and of the incubation time avoids damaging effects, showing instead a ROS regulation role in glucose transport.[12,16]

First, we investigated the role of the location of oxidative stress in the activation of glucose transport, choosing appropriate oxidants. AAPH has been extensively used in lipoperoxidation studies; it generates a constant free radicals flow in the aqueous environment outside the cells, lacking accessibility to the intact cells.[22] As a lipophilic source, we have chosen CumOOH, which has also been used in lipoperoxidation studies[23,32] and is expected to be localized in the cell membrane. We tested also the effect of the freely diffusible H_2O_2, since it has been reported that in lymphocytes exogenously added H_2O_2 induces a number of signaling effects, including tyrosine phosphorylation.[10] Furthermore, many studies have demonstrated that hydrogen peroxide stimulates glucose transport in different cell systems expressing various GLUT isoforms,[15] but the pathways mediating the stimulating effects are less-well understood.

Acute or "early" stimulation of glucose transport in response to a variety of stimuli occurs in the absence of any increase in the total number of GLUT transporters within the cell. In fact, in rat thymocytes we previously ruled out a relationship between transport activation and protein synthesis during the time intervals employed for measurements.[12]

The present study demonstrates that thymocytes express GLUT1 as the major transporter subtype, as shown by immunoblotting experiments. Furthermore, the affinity of the transporter for 2-deoxyglucose ($K_m = 1.65$ mM) in thymocytes is consistent

with the known K_m values found in other cell types expressing GLUT1.[33]

The patterns of glucose uptake stimulation are only slightly different in thymocytes treated with the tested oxidants, reflecting the fact that the azocompound decomposes at a slow rate, evoking a smooth rise in the transport activity. On the other hand, CumOOH and H_2O_2, administered in a single dose, diffuse freely in or through the membrane, respectively, inducing an immediate increase in the sugar transport rate. We observed the highest DOG uptake stimulation when the cells were exposed to 50 μM H_2O_2, although in the literature higher concentrations (up to 1000 μM) have been used to induce this effect.[15] In our experimental conditions, concentrations of H_2O_2 higher than 100 μM showed a decrease in the uptake stimulation in thymocytes, suggesting an early toxic effect of the oxidant, not yet relevant to the observed cell viability.

In order to point out any difference in the action of the tested oxidants, we evaluated the possible changes in kinetic parameters of the transport activity. Our data show that the activation is related to a significant increase in the V_{max} values, with the highest effect (2.6-fold) due to H_2O_2.

The decrease in the affinity, shown as a small increase in K_m values for all the oxidant species used, but significant only for H_2O_2, could be caused by an alteration in the GLUT microenvironment. The increase in V_{max} values following the oxidative stress is consistent with the hypothesis of a translocation of GLUT molecules from intracellular pools to the cell surface.

There is a growing evidence that redox regulation might occur at multiple levels in the signaling pathways from plasma membrane to nucleus. Alterations in protein tyrosine phosphorylation levels are among the best established consequences of cell exposure to ROS.[34,35] It is documented that H_2O_2 causes stimulation of tyrosine kinases, leading to activation of signaling cascades.[28,36–39] On the other hand, ROS may modulate the function of protein tyrosine phosphatases, a class of enzymes opposing the action of tyrosine kinases in receptor signaling. In fact, tyrosine phosphatases contain a critical cysteine residue in their active site, a potential target for redox regulation.[34,40,41] The prolonged tyrosine phosphorylation observed in thymocytes stimulated by lectins is consistent with a role of oxygen radicals as physiological inhibitors of tyrosine phosphatases.[10] In fact, glutathione may be involved in redox regulation of signals through thiol/disulfide exchanges.[42]

Recently, it has been suggested the involvement of protein tyrosine kinases and phospholipase C in glucose transport stimulation in response to H_2O_2 in Clone 9, a rat liver cell line.[15] In order to investigate the ability of different oxidative species to cause tyrosine phosphorylation, we evaluated the effect of genistein, a powerful tyrosine kinase inhibitor, on the activation of glucose transport. The choice of genistein concentration is quite relevant, since it has been reported that in cells expressing GLUT1 genistein can directly inhibit the facilitative uptake of hexoses and dehydroascorbic acid.[27,43] However, in our experiments genistein up to 10 μM did not affect the basal DOG transport, nor the relative kinetic parameters, V_{max} and K_m (data not shown). On the other hand, 10 μM genistein completely abolished the activation by the oxidant species, bringing back the V_{max} close to the values found in untreated cells. These results were confirmed using tyrphostin A23, a compound belonging to a family of potent, selective tyrosine kinase inhibitors, having hydrophobic properties which allow them to transverse the cell membrane.[26] As previously reported in Ref. [27] tyrphostin is unable to directly interact with GLUT1.

On the basis of observations reported in the literature, we evaluated the role of phospholipase C, a well characterized mediator of many signaling pathways, whose function is affected by protein tyrosine kinases.[28,44,45] PLC has been also implicated in modulating GLUT4-mediated glucose transport in a phosphotyrosine-dependent manner.[46] The role of PLC has been investigated using the aminosteroid U73122, a known specific inhibitor of this enzyme. As in the case of genistein, U73122 is able to restore V_{max} significantly increased by oxidants, suggesting that phospholipase C is involved in the enhancement of glucose transport in response to radical sources.

In conclusion, radical-generating oxidants in different cell sites can show a specificity in the location of damages produced in thymocytes, with some species acting on lipophilic cell components and other species restricted to hydrophilic sites as previously shown.[12] However, the similar short-term effect of different oxidative stresses on glucose transport activation suggests that changes in the redox state of the cell are a common regulation pathway. Although significant work needs to be done in order to identify the direct targets of ROS, the enhanced glucose uptake in thymocytes treated with different radical generators can be considered an early mechanism of adaptation to oxidative stress.

Acknowledgements

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