Resolution of the direct interaction with and inhibition of the human GLUT1 hexose transporter by resveratrol from its effect on glucose accumulation

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Cancer cells produce energy predominantly via the anaerobic degradation of glucose. This process, which occurs even in the presence of high concentration of oxygen, assures the high rate of proliferation of cancer cells (67). Recent evidence indicates that the rapid breakdown of glucose through both the glycolytic and pentose phosphate pathways provides not only a quick source of energy but also an immediate supply of metabolites intermediates that will finally feed the biosynthetic pathways (13, 24, 61). Consistently, the consumption of glucose in cancer cells is significantly enhanced compared with normal cells, indicating that glucose plays a key role in the survival of cancer cells (12, 20, 25). Indeed, cancer cells are sensitive to glucose deprivation, as limiting the supply of glucose prevents cell proliferation and induces apoptosis (4, 36, 37, 51, 72). To assure the uptake of high amounts of glucose, cancer cells upregulate the expression of the glucose carrier GLUT1, a membrane protein that facilitates the transport of glucose across the plasma membrane (30, 71). Thus the inhibition of GLUT1 arises as a promising strategy to prevent the proliferation of cancer cells.

GLUT1 resides mainly in the plasma membrane where it moves glucose to either side of the membrane depending on the direction of the glucose gradient (15). The kinetic properties of the glucose transport have been extensively studied, as red blood cells have provided a good experimental system due to the high expression of this carrier in their plasma membrane (14, 15). Likewise, these investigations have revealed a set of different natural and artificial inhibitors of GLUT1, which do not share structural similarities with glucose (6, 9, 34, 36, 64). These blockers differ not only in their structure but also in their mode of action. For instance, cytochalasin B binds to the cytosolic face of the transporter and competitively inhibits the exit of glucose from cells (6, 17). On the other hand, phloretin binds to the extracellular face GLUT1 and competitively blocks the uptake of glucose (6, 27). Despite the wide variety of known GLUT1 inhibitors, most of them show low biological stability or they cannot reach the serum concentration required to exert a significant effect in vivo, which makes necessary the identification of novel blockers that can reach inhibitory concentrations at the level of plasma membranes.

Resveratrol (3,5,4′-tri-hydroxystilbene) is a phytoalexin produced naturally by plants of human consumption (e.g., grapes and peanuts), which exhibit a variety of physiological effects of clinical and therapeutic interest. For instance, resveratrol prevents the proliferation of cancer cells, reduces the risk of cardiovascular diseases, and displays anti-diabetic properties (2, 3, 5, 7, 8, 16, 33, 45, 49, 53, 59, 70). The exact mechanism of action of resveratrol remains unclear, but in vivo and in vitro studies have suggested that this compound somehow affects glucose metabolism (7, 10, 33, 41, 58, 66) perhaps by inhibiting GLUT1 activity. Resveratrol is structurally similar to several flavonoids that have been identified as efficient inhibitors of GLUT1 (62, 63), and the available data show that resveratrol causes a competitive inhibition of facilitated transport of glucose in leukemic cells (42) and hinders glucose metabolism in human ovarian cancer cells (29). However, those studies were performed measuring the uptake of 2-deoxy-d-glucose (2DG) during 5–10 min, which does not give a clear distinction between cellular trapping of 2DG induced by its intracellular phosphorylation (11, 56, 68) and GLUT1-
mediated glucose transport, which normally occurs within 1 min (22, 65).

We studied the effect of resveratrol on both the transport and the accumulation of glucose and glucose analogs in HL-60 and U-937 human leukemic cells and in human erythrocytes. We show here that resveratrol decreased both sugar uptake and metabolic accumulation into leukemic cells. Furthermore, we provide evidence of the inhibition of glucose transport by a direct interaction between resveratrol and GLUT1.

EXPERIMENTAL PROCEDURES

Materials. D-glucose, sodium bisulfite, sodium phosphate dibasic anhydrous, sodium chloride, potassium chloride, magnesium chloride, potassium phosphate monobasic, EDTA, HEPES, dimethylsulfoxide, dithiothreitol, and Tris-base were obtained from Sigma Chemical. All radioisotopes (36.2 Ci/mmol 2-[1,2-3H]dideoxy-D-glucose; 86.7 Ci/mmol 3-O-methyl-[3H]mehtyl-D-glucoside; and 20 Ci/mmol [4,6-[3H]cytochalasin B) were from American Radiolabeled Chemicals. HyClone RPMI 1640 cell culture media and fetal bovine serum were from Thermo Fisher.

Erythrocyte isolation and preparation of membranes. Blood was obtained from units (containing dextrose, adenine, and sodium citrate as anticoagulant) provided by the Blood Bank Unit of the Valdivia Regional Hospital. All procedures were approved by the Ethics Committees from the Valdivia Regional Hospital and the Universidad Austral de Chile. Erythrocytes were suspended in HEPES saline (15 mM HEPES pH 7.3, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, and 0.8 mM MgCl2) and washed by centrifugation in a clinical centrifuge, and the cell pellet was resuspended in HEPES saline. Before resuspension, the white layer on top of the erythrocytes, containing neutrophils, macrophages, and lymphocytes, was removed by aspiration with a pipette. This process of centrifugation-resuspension was repeated three times to obtain a clear supernatant. The washed red cells were maintained in HEPES saline until use. To prepare unsealed ghosts from human red cells, after the final wash, the erythrocytes were lysed by hypotonic stress by 5 vol addition of 5 mM sodium phosphate buffer containing 0.5–2 M-sorbitol and 500 mM NaCl, and washed with 10 mM sodium phosphate, pH 7.4. This centrifugation-suspension procedure was repeated three times to obtain erythrocyte membranes devoid of cytoplasm. These cleaned membranes were suspended in 10 mM sodium phosphate, pH 7.4, subsequently treated with a basic solution (25 ml of 10 mM NaOH, 2 mM EDTA, and 0.2 mM DTT) for 45 s, and washed with and finally kept in 10 mM phosphate buffer, pH 7.4.

Cell lines. Human leukemic HL-60 (ATCC CCL240) and U-937 (ATCC CRL1593.2) cells were obtained from ATCC (Manassas, VA) and were maintained in culture in RPMI 1640 without l-glutamine and supplemented with 10% fetal bovine serum and antibiotics. Cell number was determined in a Neubauer chamber. Cell viability was assayed by trypan blue exclusion and was always >95%.

Infinite-cis net sugar efflux experiments (Sen-Widdas assays). This method is based on that described by Sen and Widdas (52). Previously washed erythrocytes were incubated at 30°C for at least 1 h in 100 mM D-glucose. The exit rate of D-glucose was recorded using a Perkin Elmer LS-50 spectrophotometer with temperature controlled at 30°C and equipped with a magnetic stirrer. The excitation and emission wavelengths were both set to 650 nm with 5-nm slits in both cases. The assay was started by adding 5 µl of cells loaded with d-glucose (50–60 × 10⁶ cells/ml), over 2.5 ml of HEPES saline pH 7.4 with or without inhibitor, in a 3 ml-quartz cuvette. Concentrated stock solutions of resveratrol (50 mM) in dimethylsulfoxide, were diluted in saline HEPES, pH 7.4, at concentrations calculated to maintain final concentration of dimethylsulfoxide <0.5%. The exit times were determined through nonlinear fitting of the data point of light scattering against time to an three-parameter exponential model: y = yo + A × [1-exp(-k × t)], where yo is the scattered light at time 0, A is the amplitude of the variation in light scattering, and k corresponds to the rate constant. The exit time corresponds to the half-life of D-glucose exit, estimated as t1/2 = ln2/k.

Zero-trans and equilibrium exchange assays in human erythrocytes. Uptake assays were performed as previously described (46, 65). Briefly, human red cells were incubated at room temperature in incubation buffer containing radioactive OMG or 2DG (1 µCi) and adequate concentrations of the respective unlabeled compounds for the times indicated in the figures. For exchange assays, cells were equilibrated with the desired concentrations of cold substrate at least 30 min before performs the transport assays.

Hexose uptake in leukemic cells. For uptake assays, the HL-60 or U-937 cells were suspended in PBS buffer (10 mM Na2HPO4·2 H2O, 2 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) for at least 60 min to eliminate intracellular free glucose, washed by centrifugation, and resuspended at 0.5–2 × 10⁶ cells/ml in the same buffer. Uptake assays were performed in a final volume of 0.2 ml of PBS buffer containing 0.5–2×10⁶ cells, 0.2–1 µCi of 2DG, and a final concentration of 0.1–20 mM 2DG. The mixture was incubated for 40 s or 40 min at room temperature, and uptake was stopped by adding 10 vol of cold phosphate-buffered saline (4°C) containing 10 µM HgCl2 (stopping solution). The cells were collected and washed twice by centrifugation in cold stopping solution. Samples were processed for scintillation spectrometry as described above. When appropriate, resveratrol was added to the uptake assays, or either the cells were preincubated in their presence.

Cytochalasin B binding to protein-stripped membranes. The experiments were done with alkali-treated membranes from washed red cells (57). D-Glucose-displaceable binding of cytochalasin B to functional glucose transporter was estimated from the difference between cytochalasin B bound in the presence of 500 mM D-sorbitol and 500 mM D-glucose. The amount of specifically bound cytochalasin B was estimated by the quantity of radioactive ligand associated with the membrane pellet (64).

Data analysis. Statistical analyses and curve fitting were done with SigmaPlot 11 (Systat Software). All data are presented as means with error bars (means ± SE). In figures, n indicates the number of independent experiment performed. Differences between means were analyzed using Student’s t-test for paired or unpaired data wherever appropriate with a P value of <0.05 taken as statistically significant. Inhibition and saturation data were analyzed by nonlinear regression. Scatchard and Eadie-Hofstee plots are used to visually display the results. Final figures for publication purposes were prepared on Canvas 12 (ACD Systems of America).

RESULTS

Effect of resveratrol on glucose transport in HL-60 and U-937 cells. 2DG is a substrate of facilitative glucose transporters, which after crossing plasma membrane accumulates intracellularly as 2-deoxy-D-glucose-6-phosphate (11, 56, 68). Discrimination between transport and accumulation is usually accomplished experimentally by carrying out very short uptake assays. Two components were observed when the time course of the uptake of 5 mM 2DG was measured in the HL-60 cells (Fig. 1, A and B). The amount of cell-associated radioactivity increased rapidly at short incubation times, and the rate of increase was linear for the first minute of incubation. Thereafter, the rate of incorporation decreased and a second component was observed that was linear for at least 60 min. We interpreted these results as indicating that the initial linear phase of incorporation of radioactivity by the HL-60 cells

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represented the transport of 2DG, with the second linear component representing the phosphorylation and intracellular accumulation of phosphorylated 2DG. Therefore, at extended incubation periods, as those used by Park (42), the rate-limiting step of 2DG uptake was the cellular trapping and accumulation of the phosphorylated sugar, and it was no longer possible to measure transport separately and as distinct from accumulation. Dose-response experiments examining uptake at 40 s indicated that transport of 2DG reached saturation at millimolar concentrations of 2DG with an apparent $K_m$ for transport of 3.2 ± 1.4 mM and a $V_{\text{max}}$ value of 2.2 ± 0.6 nmol $\times 10^6$ cells$^{-1}$ $\times$ min$^{-1}$ (Fig. 1C). Uptake of 5 mM 2DG was blocked by resveratrol with an IC$_{50}$ value of 30 µM (Fig. 1D). To elucidate the nature of the interaction of resveratrol with the glucose transporter, we measured the uptake of different concentrations of 2DG in the presence of fixed variable concentrations of resveratrol. The Eadie-Hofstee plot of these data shows that the inhibition was noncompetitive as evidenced by the common slope of the regression lines (Fig. 1E). A secondary plot of these data ($1/V_{\text{max}}$ as a function of resveratrol concentration) permitted us to obtain by extrapolation a $K_i$ value of 122 µM (not shown).

Two components were also observed when the time course of the uptake of 5 mM 2DG was measured in the U-937 cells (Fig. 2, A and B). The amount of cell-associated radioactivity increased rapidly at short incubation times, and the rate of increase was linear for the first minute of incubation. Thereafter, the rate of incorporation decreased and a second component was observed that was linear for at least 60 min. We interpreted these results as indicating that the initial linear phase of incorporation of radioactivity by the U-937 cells represented the transport of 2DG, with the second linear component representing the phosphorylation and intracellular accumulation of phosphorylated 2DG. Dose-response experiments examining uptake at 40 s indicated that transport of 2DG reached saturation at millimolar concentrations of 2DG with an apparent $K_m$ for transport of 3.6 ± 1.2 mM and with a $V_{\text{max}}$ value of 3.5 ± 1.4 nmol $\times 10^6$ cells$^{-1}$ $\times$ min$^{-1}$ (Fig. 2C). Uptake of 5 mM 2DG was blocked by resveratrol with an IC$_{50}$ value of 52.4 µM (Fig. 2D). The inhibition was also mixed noncompetitive, as evidenced by the similar slopes and varying intercept of the Eadie-Hofstee plots of substrate saturation curves determined in the presence of fixed variable concentrations of resveratrol (Fig. 2E). A $K_i$ value of 100 µM was calculated from a secondary plot of these data (not shown).

Effect of resveratrol on glucose trapping in HL-60 and U-937 cells. Dose-response studies of trapping in HL-60 cells using 40-min uptake assays revealed the presence of one component that is saturated at ~5 mM 2DG, with an apparent $K_m$ of 0.6 ± 0.4 mM and a $V_{\text{max}}$ of 0.32 ± 0.12 nmol $\times 10^6$ cells$^{-1}$ $\times$ min$^{-1}$ (Fig. 3A). Thus the kinetic analysis of 2DG uptake demonstrated the presence of one functional activity involved in the transport in the HL-60 cells, with a second, higher affinity component associated with intracellular trapping.

With the use of U-937 cells, dose-response studies of trapping employing 40-min uptake assays also revealed the pres-
ence of one component that is saturated at ~5 mM 2DG, with an apparent $K_m$ of $1.1 \pm 0.4$ mM and a $V_{\text{max}}$ of $0.91 \pm 0.21$ nmol $\times 10^6$ cells$^{-1} \times$ min$^{-1}$ (Fig. 3D). This $K_m$ value is therefore not significantly different from the $K_m$ value for trapping obtained for HL-60 cells. Thus the kinetic analysis on these cells corroborated the presence of one functional activity involved in the transport of 2DG in leukemic cells, with a second, higher affinity component associated with intracellular trapping. We next analyzed the effect of resveratrol on the trapping of 2DG by the U-937 cells. Uptake experiments by 40 min at 1 mM substrate indicate that resveratrol inhibited, in a dose-dependent manner, the trapping of 2DG with an IC$_{50}$ of 55 $\mu$M (Fig. 3E). The character of the inhibition by resveratrol was mixed competitive (Fig. 3F), and a $K_i$ value of 90 $\mu$M was calculated by linear regression in a secondary plot as described above (not shown).

Effects of resveratrol on hexose transport in human erythrocytes. Human erythrocytes offer a simple system to study the effect of resveratrol on glucose transport mediated by the hexose transporter GLUT1. This carrier constitutes close to 2–3% of the total membrane protein in red blood cells, and most of their kinetic properties have been characterized by using this experimental system. To test whether resveratrol affects the functionality of GLUT1, we preloaded the erythrocytes with D-glucose and measured the rate of D-glucose exit out of the cells in the presence of different concentrations of resveratrol. Under infinite-cis conditions, the phytoalexin decreases D-glucose exit out of human erythrocytes in a dose-dependent manner, with 50% inhibition (IC$_{50}$) observed at ~24 $\mu$M (Fig. 4A), suggesting that resveratrol alters the ability of GLUT1 to move glucose across the membrane. We validated the viability of our experimental system by measuring the effect of known blockers of GLUT1 on D-glucose exit. As expected, cytochalasin B and phloretin inhibited the exit of the hexose with IC$_{50}$ of 0.5 and 1.0 $\mu$M, respectively, whereas the transport was insensitive to cytochalasin E (40).

We wondered if the phytoalexin also affected the binding of D-glucose to the transporter. There are two described binding sites for D-glucose in the transporter, one facing the extracellular compartment (external site) and one facing the cytosol (internal site). To test the effect of resveratrol in the binding of D-glucose to the external site, we measured the exit of the hexose from D-glucose preloaded red blood cells, but in contrast to the infinite-cis exit assay described above, we added increasing concentrations of D-glucose in the extracellular compartment. When the results are presented in a Sen-Widdas plot (Fig. 4B), the dissociation constant of glucose for the external binding site can be determined from the intercept on the abscissa (82). Interestingly, the kinetic analysis indicated that resveratrol did not affect the affinity of D-glucose for the external site, as judged by the lack of displacement of the intercept along the x-axis in the presence of resveratrol. On the other hand, resveratrol decreased the $V_{\text{max}}$ for D-glucose exit (notice the change in the slope), suggesting that the phytoalexin did not compete with D-glucose for the external binding site. A secondary plot of the apparent $V_{\text{max}}$ values for glucose as a function of resveratrol concentration shows that the inhibition was linear and permits to extrapolate a $K_i$ value of 45 $\mu$M for resveratrol (not shown). As controls we used two known inhibitors that interact with the transporter at either the internal face (cytochalasin B) or the external face (phloretin) (17, 27), where only the latter was able to modify the $K_i$ value for D-glucose at the external binding site (40, 43). Thus our results indicate that resveratrol does not affect the affinity of D-glucose for the external binding site, suggesting that the phytoalexin does not interact with the external face of the transporter.
To test if the inhibitory activity of resveratrol on glucose exit involved its interaction with GLUT1, we analyzed the effect of resveratrol on the binding of radiolabeled cytochalasin B to the hexose transporter present in purified human erythrocyte ghosts. This assay has been widely used to test the interaction of molecules with GLUT1 since the binding of cytochalasin B is prevented by the binding of other molecules, including glucose, to the transporter (21, 23, 55). Increasing concentrations of resveratrol efficiently inhibited the binding of cytochalasin B to the purified erythrocyte ghosts with an IC50 of 33 μM (Fig. 4C), suggesting that the phytoalexin interacts with GLUT1. Furthermore, resveratrol displaced the previously bound cytochalasin B in a competitive manner (Fig. 4D), which confirms a direct interaction between resveratrol and the transporter. Thus our data suggest that resveratrol blocks glucose transport by interacting with GLUT1.

Because resveratrol prevented the binding of cytochalasin B to the internal surface of the transporter, but it did not affect the binding of glucose to the external binding site, we reasoned that the phytoalexin either (1) interacts with GLUT1 at the endofacial binding site for glucose, or (2) binds to the transporter at a site independent of both the exofacial and endofacial glucose binding sites. If the first case is true, resveratrol should act as a competitive blocker of the transport under conditions when the endofacial site is accessible for the substrate: equilibrium exchange and zero-trans exit assays. In contrast, if resveratrol interacts with GLUT1 independently of the glucose binding sites, it should behave as a noncompetitive inhibitor of glucose transport under equilibrium exchange and zero-trans entry and exit conditions. We, therefore, performed equilibrium exchange and zero-trans entry and exit assays using the nonmetabolizable OMG as substrate to avoid its intracellular accumulation. The results are presented as Eadie-Hofstee graphs (Figs. 4, E–G), where the intercept on the ordinate corresponds to the Vmax value and the slope represents the negative value of Km for substrate at that particular resveratrol concentration.

Equilibrium exchange assays (equal substrate concentrations inside and outside) are recognized as an unambiguous test to differentiate between competitive and noncompetitive inhibitors of glucose transport (18). Under those conditions, the effect of different concentrations of resveratrol using OMG saturation curves resulted in a set of converging lines on the ordinate (Fig. 4E), indicating that resveratrol did not affect the Vmax values. The slopes, however, varied in the presence of the phytoalexin, showing that the Km increased in the presence of the inhibitor (Km; 19.3 μM). This behavior, unaffected Vmax and increased Km, is typical of competitive inhibitors, suggesting that resveratrol acts as a competitive inhibitor of the glucose exchange in red blood cells. As with the exchange assays, the zero-trans efflux assays revealed that resveratrol acts as a competitive blocker of the internal glucose binding site (Ki; 24.9 μM), as Vmax was unaltered and Km values were increased in the presence of resveratrol (Fig. 4F). Consistent with the Sen-Widdas assays using D-glucose under infinite-cis conditions (Fig. 4B), we observed that resveratrol decreased the Vmax for OMG uptake without altering the Km value for OMG transport under zero-trans entry conditions (Ki; 29.9 μM), a behavior
that is expected for a mixed noncompetitive inhibitor (Fig. 4G). This indicates that resveratrol is unable to compete with OMG for binding to the external site and suggesting that the competitive behavior occurs through the internal glucose binding site. Taken together, the kinetic data suggest that resveratrol hinders hexose transport in different experimental settings with essentially the same affinity. Most significantly, the kinetic data and the cytochalasin B binding analysis provide evidence of a direct interaction of resveratrol with the cytosolic surface of the GLUT1 transporter.

**DISCUSSION**

A key consideration when studying the cellular uptake of sugars is to carry out the experiments using experimental conditions that faithfully differentiate the transport of the substrate from the intracellular trapping. It is known that the capacity of HL-60 cells to transport glucose exceeds its own ability to trap (phosphorylate) the hexose, and in a typical uptake assay the transport step of the uptake process is very rapid and can be detected only by using short uptake assays; at extended times, the rate-limiting step of uptake is the cellular phosphorylation, and it is no longer possible to assess transport separately and as distinct from trapping (65). Accordingly, we show here that in HL-60 cells the maximal rate of hexose uptake (2.2 nmol × 10⁶ cells⁻¹ × min⁻¹) surpasses by a factor of 7 the metabolic capability of these cells to accumulate substrate as indicated by the corresponding maximal rate of intracellular trapping (0.22 nmol × 10⁶ cells⁻¹ × min⁻¹). Although U-937 cells show higher maximal rates of both transport and trapping of glucose than HL-60 cells, a similar calculation revealed that their maximal transport rate exceeded by about fourfold the maximal rate of substrate accumulation.

Fig. 4. Resveratrol interaction with GLUT1 in human erythrocytes. A: dose dependence of the inhibition of net D-glucose exit rates from human erythrocytes by resveratrol. The solid line represents the nonlinear regression fit of the data to a 1-parameter hyperbolic inhibition model with a IC₅₀ value of 23.9 µM (n = 6). B: Sen-Widdas plot of the half-time of D-glucose exit at different concentrations of D-glucose in the external medium in the absence (•) or in the presence of 30 (▼) or 60 µM (■) resveratrol (n = 3). Solid lines correspond to linear regression fit for each curve. C: resveratrol displaces the cytochalasin B bound to the glucose transporter GLUT1 in human erythrocyte ghosts. The solid line represents the nonlinear regression fitting of the data to a 1-parameter hyperbolic inhibition model with a IC₅₀ value of 32.9 µM (n = 6). D: scatchard analysis of the binding of different concentrations of cytochalasin B to human erythrocyte membranes in presence of various fixed levels of resveratrol. A solid line corresponds to the linear regression fit of each curve. Since intersects on the abscissa in the inhibition model with a IC₅₀ value of 23.9 µM (n = 6).
Short uptake assays enabled us to carry out a detailed kinetic analysis of the transport of 2DG as distinct from the accumulation of the hexose. Both HL-60 and U-937 cell lines express mainly GLUT1 (48, 65), and only one saturable component of substrate transport was detected on either cell line, with $K_m$ values of 2.5–8.5 mM, as expected from the functional properties of GLUT1 (38, 39, 65). Resveratrol inhibited glucose transport in both cells, which demonstrated that the deleterious effect of the phytoalexin on glucose uptake could be validated in different human cells, providing convincing evidence that resveratrol affects the functional activity of GLUT1 in a manner independent of the cellular environment in which the transporter is expressed. Kinetic characterization of the mechanism of inhibition under zero-trans uptake conditions revealed that the phytoalexin behaves as a mixed noncompetitive inhibitor of hexose transport in both cell lines. The noncompetitive behavior suggests that resveratrol does not compete with 2DG by binding to the external glucose site of GLUT1.

A second, higher affinity component involved in the uptake of 2DG in the leukemic cells was detected only in long uptake experiments designed to assess the intracellular trapping of substrate. We failed to detect this high-affinity component in short uptake assays assessing transport, using a wide range of 2DG concentrations, from 100 µM to 20 mM. We interpreted these data as supporting the notion that the high-affinity component detected in these conditions is not involved in the transport of 2DG but is instead associated with its intracellular trapping. Competition experiments using 40-min assays revealed that although resveratrol completely blocked 2DG uptake in the leukemia cell lines under these conditions, the inhibition was noncompetitive. The noncompetitive character of the inhibitory action of resveratrol can be explained as if the phytoalexin is hindering directly the primary event responsible for the entry of 2DG into the cells, that is GLUT1 mediated 2DG transport; therefore, in long-term uptake studies resveratrol causes transport to become the rate-determining step, affecting accumulation in an only indirect manner (22, 65). 2DG is a known substrate for mammalian hexokinases, enzymes that use ATP to phosphorylate hexoses to the corre- sponding phosphorylated species at the sugar 6'-position. The fact that the $K_m$ value for trapping in the leukemic cells are in the range of $K_m$ values reported for 2DG enzyme-dependent phosphorylation is consistent with this interpretation (22, 26, 47).

Our results showing noncompetitive blocking of glucose transport into both leukemic cell lines are in contrast with those reported previously by Park (42), who analyzed the effect of resveratrol on the intracellular uptake of glucose and dehydroascorbic acid in U-937 and HL-60 cells. Their kinetic studies showed that glucose uptake was competitively inhibited by resveratrol; the $K_i$ values calculated were 85 and 89 µM in HL-60 and U-937 cells, respectively. These studies were performed, however, under long uptake experimental conditions (10 min) that fail to discriminate between substrate transport and trapping, raising the question of the identity of the rate-limiting step under those conditions. Of major concern is that the determination of the kinetic constants and the analysis of the character of the inhibition were performed using the Michaelis-Menten plot, which in our hands is not suitable for discriminating between competitive and noncompetitive mechanism using transport assays. We kinetically discriminated GLUT1 mediated 2DG transport from the metabolic accumulation process and employ the Eadie-Hofstee representation to illustrate the noncompetitive character of the inhibition of glucose entry into the cells.

The zero-trans entry $IC_{50}$ values for resveratrol determined for each leukemic cell were approximately two to three times lower than the corresponding $K_i$ values. These $IC_{50}$ values correspond to the apparent inhibition constants measured at fixed substrate concentrations, which are dependent on the level of saturation of the transporter when the inhibitor follows a mixed noncompetitive mechanism (18). Our kinetic analysis on human erythrocytes provides evidence that the resveratrol site lies on the internal surface of the transporter, which led us to propose that resveratrol binds to the inward-facing conformation of the carrier. The classical carrier model assumes that GLUT1 catalyzes glucose transport by a kinetic scheme comprising rapid binding of glucose to an external sugar site on the transporter, translocation of the sugar-transporter complex, release of sugar from the internal (endofacial) site, and, finally, a relaxation step of free carrier that regenerates the substrate exofacial binding site. Since it has been determined that this latter relaxation corresponds to the rate-limiting step, therefore under zero-trans entry assay conditions the equilibrium should be displaced towards the inward-facing conformation, and thus resveratrol binding shall be favored in the substrate presence.

Furthermore, we used kinetic assays in human red cells and binding assays in isolated erythrocyte membranes to directly define how resveratrol interacts with the GLUT1 transporter. We employed OMG as substrate under initial rate conditions, and the inhibition of glucose flux through GLUT1 was tested under a range of functional paradigms of glucose influx (zero-trans entry, equilibrium exchange) and efflux (infinite-cis, zero-trans exit). OMG is efficiently transported by GLUT1 but corresponds to a nonmetabolizable glucose analog that is not phosphorylated by hexokinases, and therefore, it is not trapped into cells. The transport of OMG by these cells occurred rapidly, being linear for at least 40 s with half the equilibrium concentration reached in ~5 min and equilibrium attained at ~20 min; the intracellular concentration of OMG never exceeded the respective extracellular concentrations from 1 to 20 mM OMG (65). Resveratrol in the red cells competitively obliterated GLUT1 activity in equilibrium exchange and in zero-trans exit transport experiments but acted as a noncompetitive inhibitor in zero-trans entry, in close agreement with the results from the leukemic cells. Assuming the traditional mobile carrier model for the transporter (35, 69), occupation of the endofacial substrate site reduces $V_{max}$ under zero-trans influx conditions because return of the substrate site to the outside is blocked, and there is no substrate inside to displace the inhibitor. Under equilibrium exchange and zero-trans efflux conditions, internal substrate competitively displaces the inhibitor and the transport $K_m$ is increased. These observations validate a direct interaction of resveratrol with the GLUT1 hexose transporter and indicate that the binding site for resveratrol is accessible only from the internal surface of the transporter. Further support for this interpretation is provided by the infinite-cis assays that demonstrated that resveratrol fully obliterated glucose exit out of the cells and did not compete with the external glucose site of the transporter.

Direct interaction of resveratrol with the GLUT1 carrier is also supported by the fact that no preincubation step with resveratrol was necessary to observe its inhibitory effect on the
transport of D-glucose, OMG, and 2DG transport across cell membrane in human erythrocyte and HL-60 or U-937 leukemic cell lines. This view is also consistent with the binding data indicating that resveratrol blocked the glucose-sensitive binding of cytochalasin B to GLUT1 present in human erythrocytes. Finally, our data using 2DG as substrate in two human leukemic cell lines also indicate that resveratrol affects the facilitated transport of hexoses as distinct from the trapping/accumulation of the transported substrate. The overall data are consistent with the concept that resveratrol interacts directly with GLUT1 by binding to an endofacial site and that this interaction overrides both the transport of hexoses across the plasma membrane and binding of cytochalasin B to the internal surface of the carrier. We postulate that resveratrol stabilizes a GLUT1 conformation that is refractory to bind either cytochalasin B and D-glucose.

Other GLUT1 effectors, including cytochalasin B, gossypol, and tyrphostin A47, have been also identified as endofacial-binding blockers of the GLUT1 hexose transporter by these same kinetic criteria (1, 43, 44). However, besides its interaction with the endofacial surface of the carrier, the precise nature of the binding of resveratrol and GLUT1 cannot be at present specified. Our data indicate that resveratrol interacts with sites on the transporter involved in the binding or transport of hexoses and that this interaction is also responsible for interfering with cytochalasin B binding. Several reports in the literature indicate that the endofacial cytochalasin B binding site can promiscuously interact with a variety of different chemical entities, including steroids and benzoic acid derivatives (19, 31, 32). There is room therefore for the possibility that binding to the same site may explain the inhibitory action of cytochalasin B, gossypol, tyrphostin A47, and resveratrol on glucose transport facilitated by GLUT1. Alternatively, on the basis of the obvious structural and chemical differences between these compounds, we favor the option that resveratrol may actually bind to an independent endofacial site whose occupancy renders the carrier refractory to bind cytochalasin B. We propose that resveratrol may elicit the displacement of bound cytochalasin B in a manner similar to the effect of phloretin or genistein, molecules that are known to interact with the external face of the carrier and behave as negative allosteric ligands of cytochalasin B binding (6, 27, 28, 43). In fact, we provided data suggesting that gossypol interacts with an endofacial site on the GLUT1 protein that is distinguishable from the cytochalasin B binding site (44). The interaction of gossypol with the endofacial surface of the GLUT1 transporter was also suggested by the requirement for a gossypol preincubation step in human erythrocytes, most likely due to a slow penetration of the compound across the red cell plasma membrane before interacting with the internal surface of the transporter (44). In the present case resveratrol was able to affect hexose transport when added along with the substrates in different experimental settings, including infinite-cis exit and zero-trans entry and exit transport assays. These observations suggest that resveratrol has the ability to move efficiently into and quickly equilibrate across plasma membranes.

The IC50 and Ki values for resveratrol inhibition for glucose exit from red blood cells are lower than the IC50 and Ki values for sugar entry into HL60 and U937 cells. The lesser resveratrol affinity on the leukemic cells could partially be due to the different experimental protocol employed or by the different cellular context on which GLUT1 is expressed and evaluated. Alternatively, this difference may be due to that HL60 and U937 cells may express other GLUT transporters and these GLUTs may bind resveratrol with lower affinity. Literature data shows that besides the abundant expression of GLUT1 no reactivity was observed with anti-GLUT2, -GLUT3, -GLUT4, or -GLUT5 antibodies in HL60 (65) or U937 cells (48). However, there are no data regarding the expression of GLUT6–12 on these cells, so we cannot dismiss the possibility that HL60 or U937 cells express some of these GLUT transporters and that these GLUTs may bind resveratrol with lower affinity.

Finally, we highlight that our results indicating that resveratrol directly inhibits glucose uptake are restricted to the functional activity of the hexose transporter GLUT1. The effect of the phytoalexin on other members of this family of carriers has not been satisfactorily probed, albeit some literature data suggest the interesting possibility that resveratrol may have different and even opposing effects on others forms of the GLUT carriers. For instance, Park (42) suggests that the human GLUT3 hexose transporter when overexpressed into Chinese hamster ovary cells is also sensitive to inhibition by resveratrol. In clear contrast, Breen et al. (10) found that in L6 rat skeletal muscle cells resveratrol elicited a significant increase in glucose uptake in the absence of insulin. Since GLUT4 is the major hexose carrier isomorph present on these cells, in basal and insulin-stimulated states, accounting for >90% of all glucose transporters (50, 54, 60), the authors favor the interpretation that resveratrol triggers an intrinsic activation of the GLUT4 transporter already present at the plasma membrane. Alternatively, they suggest that resveratrol may stimulate glucose transport indirectly by increasing the glucose gradient because of increased intracellular glucose metabolism. In our experiments using HL60 and U937 leukemic cells we did not see any change in the rate of glucose metabolism as a result of acute treatment with resveratrol. However, the possibility that an intrinsic difference could exist between muscular and leukemic cells regarding the rate of deoxyglucose trapping by phosphorylation and cellular accumulation cannot be rejected. Thorough kinetic experiments such as those described here for GLUT1 should be performed to test if resveratrol affects directly the functional activity of the GLUT4 hexose transporter.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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DIRECT INHIBITION OF GLUT1 BY RESVERATROL


