Reactive oxygen species downregulate glucose transport system in retinal endothelial cells

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Fernandes R, Hosoya K, Pereira P. Reactive oxygen species downregulate glucose transport system in retinal endothelial cells. Am J Physiol Cell Physiol 300: C927–C936, 2011.—Retinal endothelial cells are believed to play an important role in the pathogenesis of diabetic retinopathy. In previous studies, we and others demonstrated that glucose transporter 1 (GLUT1) is downregulated in response to hyperglycemia. Increased oxidative stress is likely to be the event whereby hyperglycemia is transduced into endothelial cell damage. However, the effects of sustained oxidative stress on GLUT1 regulation are not clearly established. The objective of this study is to evaluate the effect of increased oxidative stress on glucose transport and on GLUT1 subcellular distribution in a retinal endothelial cell line and to elucidate the signaling pathways associated with such regulation. Conditionally immortalized rat retinal endothelial cells (TR-iBRB) were incubated with glucose oxidase, which increases the intracellular hydrogen peroxide levels, and GLUT1 regulation was investigated. The data showed that oxidative stress did not alter the total levels of GLUT1 protein, although the levels of mRNA were decreased, and there was a subcellular redistribution of GLUT1, decreasing its content at the plasma membrane. Consistently, the half-life of the protein at the plasma membrane markedly decreased under oxidative stress. The proteasome appears to be involved in GLUT1 regulation in response to oxidative stress, as revealed by an increase in stabilization of the protein present at the plasma membrane and normalization of glucose transport following proteasome inhibition. Indeed, levels of ubiquitinated GLUT1 increase as revealed by immunoprecipitation assays. Furthermore, data indicate that protein kinase B activation is involved in the stabilization of GLUT1 at the plasma membrane. Thus subcellular redistribution of GLUT1 under conditions of oxidative stress is likely to contribute to the disruption of glucose homeostasis in diabetes.

glucose transporter 1; oxidative stress; protein kinase B; proteasome; retinal endothelial cells; reactive oxygen species

REACTIVE OXYGEN SPECIES (ROS) such as hydrogen peroxide (H2O2) and superoxide anion are constantly produced intracellularly as a result of normal metabolic activity. Oxidant damage all forms of biomolecules, including DNA, proteins, and lipids (26), and are implicated in a variety of pathological conditions, including diabetes (38, 39, 42, 43). Between the main targets of the microvascular complications of diabetes is the retina. The production of ROS is high in the normal retina, and certain regions of the retina are prone to lipid peroxidation, creating an environment susceptible to oxidative damage. This may be even more accentuated in the diabetic retina, where hyperglycemia may lead to elevated production of oxidants by vascular endothelium. Endothelial production of ROS, especially superoxide as a result of autooxidation of glucose and/or glycoxidation (44), is an important mechanism of vascular dysfunction in diabetes (55). There is substantial evidence from animal and clinical studies for both impaired antioxidant and increased oxidative damage in the diabetic retinas (55).

Retinal endothelial cells composing the blood-retinal barrier (BRB) play a critical role in restricting the nonspecific transport of hydrophilic substances and facilitating the transport of essential molecules between the circulating blood and the retina (8, 51). Glucose transporters (GLUTs) comprise a family of intrinsic membrane glycoproteins that are responsible for glucose transport into the mammalian cells (3, 37). They are expressed in a tissue-specific manner. The glucose transport mediated by GLUTs is independent on ATP and occurs according to its concentration gradient. This transport is specific, saturable, and bidirectional (37). GLUT1 is the main isoform of glucose transporters in retinal endothelial cells and is characteristically expressed in blood-barrier tissues, such as the blood-ocular barriers (30). In the inner BRB, ~50% of total cellular GLUT1 resides in cytосolic stores (31). GLUT1 trafficking seems to be regulated in vitro by a variety of stimuli and agents, including serum, growth factors, and inhibitors of oxidative phosphorylation (14, 19, 24, 25, 59). The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway was shown to be a prominent pathway promoting both an increase in the trafficking of GLUT1 to plasma membrane (PM) and its activity (10, 21, 46, 57).

The ubiquitin proteasome pathway (UFP) is well known to selectively target abnormal proteins and many cellular regulatory proteins to proteasomal degradation (16). However, other functions have been ascribed to ubiquitin over the last decades. These include receptor endocytosis or protein sorting (22, 54). Our group has shown that the oxidative stress in retinal endothelial cells upregulates UPP and increases turnover of ubiquitin conjugates. The upregulation of UPP is most likely an important component of cell response to stress in conditions where oxidative stress is increased, such as diabetes (13).

The present study was undertaken to evaluate the effect of increased oxidative stress on subcellular distribution of GLUT1 as well as on the transporter activity in retinal endothelial cells. In this study, we have used the TR-iBRB cell line as a model of retinal endothelial cells. This is a conditionally immortalized endothelial cell line derived from rat that exhibits endothelial cell properties, expressing proteins such as von Willebrand factor, GLUT1, and vascular endothelial growth factor receptor-2 (20).

Here we report that exposure of retinal endothelial cells to sustained oxidative stress results in a decrease of glucose...
transport activity due to increased internalization of GLUT1. We also show that this effect is not due to an oxidative modification of the protein present at the PM; rather, oxidative stress seems to increase the rate of GLUT1 internalization by a proteasome-dependent mechanism involving inactivation of Akt.

MATERIALS AND METHODS

Antibodies and reagents. The rabbit polyclonal anti-GLUT1 antibodies were obtained from FabGennix (Frisco, TX), Santa Cruz Biotecnology (Santa Cruz, CA), or Chemicon (Boston, MA). The monoclonal anti-ubiquitin antibodies were obtained from Covance (Emeryville, CA). The rabbit polyclonal anti-hemagglutinin antibody was obtained from Zymed Laboratories (South San Francisco, CA). The rabbit polyclonal antibodies anti-Akt and anti-phosphorylated Akt were obtained from Cell Signaling Technology (Danver, MA). The mouse anti-actin antibody was obtained from Boehringer Mannheim (Mannheim, Germany). The rabbit polyclonal caveolin-1 antibody was obtained from Abcam (Cambridge, MA). Unless otherwise noted, all other reagents were from Sigma-Aldrich (St. Louis, MO), except MG-132, which was obtained from Calbiochem (Darmstadt, Germany).

Exposure of retinal endothelial cells to oxidative stress. TR-iBRB cells (conditionally immortalized rat retinal endothelial cells) were developed by us (Toyama Medical and Pharmaceutical University, Japan). The cell line was maintained at 33°C under 5% CO2 and was shown to exhibit properties of retinal capillary endothelial cells (20). Cells were cultured in collagen-coated (0.5 mg/ml) dishes in DMEM with low glucose (5.5 mM), supplemented with 15 μg/ml endothelial cell growth factor, 10% bovine serum albumin, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Exposure to oxidative stress was accomplished by incubating cells with glucose oxidase (15 mU/ml), which produces a constant flow of H2O2 in the medium. Cell incubations were performed in a serum- and phenol red-free medium, supplemented with D-glucose (1,500 mg/l). Cells were exposed to 15 mU/ml glucose oxidase at 37°C for 4 h. This reaction was stopped by washing the plates twice with cold KRP solution containing 0.2 mM phloretin to quench 2-Dog uptake. The cells were then lysed in 0.4 ml 0.1% SDS/0.1 M NaOH. An aliquot of 350 μl was taken for H counting. The protein concentration was quantitated in the remaining aliquot via a Pierce BCA assay (Pierce, Rockford, IL).

Total membranes and cell lysate preparations. Proteins were extracted from retinal endothelial cells with buffer [10 mM Tris-HCl (pH 7.4) containing 0.5% sodium deoxycholate (DOC) and 1% Triton X-100] supplemented with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 2 mM phenylmethylsulfonyl fluoride, and 2 mM iodoacetamide. The cells were lysed by sonication, and the lysates were centrifuged at 14,000 g for 15 min at 4°C. The resulting supernatants were then used for protein quantification, followed by denaturation of the sample with Laemmli buffer.

Subcellular fractionation. TR-iBRB cells were washed two times with PBS and collected in HES buffer [255 mM sucrose, 20 mM HEPES (pH 7.4), 1 mM EDTA, 2 mM PMSE, and protease inhibitors] at 4°C, and the cells were immediately homogenized using a Potter-Elvehjem homogenizer (20 strokes). The homogenates (one 10-cm-diameter dish/condition) were subjected to subcellular fractionation as described previously (53) to isolate PM, high- and low-density microsomes (HDM and LDM) with minor modifications. Briefly, the homogenate was centrifuged at 14,000 g for 20 min. The resulting supernatant was centrifuged at 41,000 g for 20 min, yielding a pellet of HMD. The supernatant of this spin was centrifuged at 180,000 g for 75 min, yielding a pellet of LDM. The pellet obtained from the initial spin was resuspended in HES buffer, layered on a 1.12 M sucrose cushion, and centrifuged at 100,000 g for 20 min, yielding a pellet of PM. The protein concentration was measured using the BCA reagent (Pierce) with BSA as the standard.

Western blotting. For the Western blot analysis, 10–40 μg proteins were loaded per lane on SDS-PAGE. Following electrophoresis and transfer to polyvinylidene fluoride (PVDF) membranes (GE Healthcare Bio-Sciences, Uppsala, Sweden), the blots were incubated in Tris-buffered saline [20 mM Tris, 137 mM NaCl (pH 7.6)] containing 0.1% Tween (TBST) and 5% nonfat milk for 1 h. The membranes were washed one time with ice-cold PBS and then incubated with 15 μg/ml glucose oxidase at 37°C for 4 h. Uptake of 2-deoxy-D-glucose in KRP supplemented with 1% BSA, for 5 min at 37°C. We have found that 2-Dog uptake is linear for at least 10 min in TR-iBRB cells (data not shown). Nonspecific uptake of 2-deoxy-1Hglucose was assessed by the addition of cytochalasin B (20 μM), and the results were corrected for these values. The reaction was stopped by washing the plates twice with cold KRP solution containing 0.2 mM phloretin to quench 2-Dog uptake. The cells were then lysed in 0.4 ml 0.1% SDS/0.1 M NaOH. An aliquot of 350 μl was taken for H counting. The protein concentration was quantitated in the remaining aliquot via a Pierce BCA assay (Pierce, Rockford, IL).

Protein-bound carbonyl determination. Protein carbonyl formation was used as an indicator of oxidized proteins. TR-iBRB cells were treated with 15 mU/ml glucose oxidase for 37°C. The KRP was removed and replaced with 0.1 mM unlabeled 2-Dog and 0.5 μCi/ml labeled glucose, 2-deoxy-D-[2-14C]glucose in KRP supplemented with 1% BSA, for 5 min at 37°C. We have found that 2-Dog uptake is linear for at least 10 min in TR-iBRB cells (data not shown). Nonspecific uptake of 2-deoxy-1Hglucose was assessed by the addition of cytochalasin B (20 μM), and the results were corrected for these values. The reaction was stopped by washing the plates twice with cold KRP solution containing 0.2 mM phloretin to quench 2-Dog uptake. The cells were then lysed in 0.4 ml 0.1% SDS/0.1 M NaOH. An aliquot of 350 μl was taken for H counting. The protein concentration was quantitated in the remaining aliquot via a Pierce BCA assay (Pierce, Rockford, IL).

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Western blotting. For the Western blot analysis, 10–40 μg proteins were loaded per lane on SDS-PAGE. Following electrophoresis and transfer to polyvinylidene fluoride (PVDF) membranes (GE Healthcare Bio-Sciences, Uppsala, Sweden), the blots were incubated in Tris-buffered saline [20 mM Tris, 137 mM NaCl (pH 7.6)] containing 0.1% Tween (TBST) and 5% nonfat milk for 1 h. The membranes were then incubated with 1:4,000 dilution of affinity-purified rabbit polyclonal anti-GLUT1 (FabGennix), 1:2,500 dilution of affinity-purified rabbit polyclonal anti-GLUT1 (Chemicon), 1:1,000 dilution of mouse monoclonal anti-ubiquitin (P4D1; Covance), 1:1,000 dilution of rabbit polyclonal caveolin-1 antibody (Abcam), 1:250 dilution of rabbit polyclonal anti-hemagglutinin (Zymed Laboratories), 1:2,000 dilution of mouse monoclonal anti-actin (Boehringer Mannheim), 1:1,000 dilution of rabbit polyclonal anti-phosphorylated Akt (Cell Signaling Technology), or 1:2,000 dilution of rabbit polyclonal anti-Akt (Cell Signaling Technology) for 1 h in TBST containing 0.5% nonfat milk. After five washes with TBST, blots were incubated with horseradish peroxidase-conjugated secondary antibody. The proteins were visualized with enhanced chemiluminescence reagents according to the manufacturer’s protocol (GE Healthcare Bio-Sciences). In some experiments, the intensity of the bands was quantitated with a scanning densitometer.

Protein-bound carbonyl determination. Protein carbonyl formation was used as an indicator of oxidized proteins. TR-iBRB cells were treated with 15 mU/ml glucose oxidase for several time points. The cells were washed one time with ice-cold PBS and lysed in Tris-HCl buffer with 1% Nonidet P-40, pH 7.6, supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). For derivatization of carbonyl-containing proteins, equal amounts of pro-
teins were mixed with an equal volume of 10 mM 2,4-dinitrophenyl-hydradine (DNPH) in 10% trifluoroactic acid and incubated at room temperature for 15 min. The reaction was stopped by precipitation of proteins with 20% trichloroactic acid. The pellet was washed with ethylacetate-ethanol (1:1) to remove free DNPH. Next, the pellet was solubilized with Laemmli buffer. For Western blot, samples were resolved by SDS-PAGE using 12% gels and transferred to PVDF membranes. The membrane was probed with antibody to dinitophenylhydradine derivatives. Levels of carbonyl were quantified by densitometry analysis of all bands of the blot.

**Transfection of TR-iBRB cells by electroporation.** Trypsinized TR-iBRB cells were resuspended in a small volume of OptiMem supplemented with 10% FBS. The constitutively active Akt (pCMV6-Myr-Akt-HA) expression vector (7.5 μg) was added to a 400-μl aliquot of cell suspension (1.9 × 10^6 cells). Transfection by electroporation was performed at 960 μF and 200 volts. Approximately 24 h after electroporation, the cells were used for measurement of protein expression by Western Blotting.

**Immunocytochemistry and confocal microscopy.** The evaluation of subcellular distribution of the proteins in cells was performed in cultures of endothelial cells by immunocytochemistry. TR-iBRB cells were plated on collagen-coated cover slips. The cells were treated with glucose oxidase (15 mM/ml) in the absence or presence of the proteasome inhibitor, MG-132 (20 μM), at 37°C for 4 h. The culture medium was removed, and the cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. Cells were then permeabilized for 10 min in 1% Triton X-100 in PBS, pH 7.4, and blocked with 10% goat serum for 20 min. Primary antibodies were diluted in PBS containing 0.02% BSA (PBS/BSA). The primary antibodies were then added, and the cells were incubated for 1 h at room temperature. After incubation, the cells were extensively washed with PBS/BSA solution. Specimens were subsequently incubated with secondary antibodies, diluted in PBS/BSA for 1 h. The cover slips were washed before mounting with Glycergel Dako mounting medium (Dako, Glostrup, Denmark). The cells were visualized by confocal microscopy, using a confocal image system MRC600 (Bio-Rad), connected to a fluorescence microscope Nikon Optiphot-2.

**Quantitative analysis of gene expression by RT-PCR.** Total RNA was isolated from TR-iBRB cells using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. Samples of 4 μg of RNA were subjected to reverse transcription, with random hexadeoxynucleotide as a primer, in 20 μl of a reaction mixture using Superscript II. PCR reactions were run at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C denaturing for 15 s and 60°C annealing for 1 min. The expression levels of GLUT1 mRNA were amplified for 18S rRNA.

The real-time PCR analysis was conducted on an ABI Prism 7000 (Applied Biosystems, Foster City, CA) quantitative PCR system using SYBR Green PCR master mix supplied by Bio-Rad according to the manufacturer’s instructions. PCR reactions were run at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C denaturing for 15 s and 60°C annealing for 1 min. The expression levels of GLUT1 mRNA were normalized for the 18S rRNA.

**Biotinylation of PMs.** The cell-surface proteins of TR-iBRB cells were biotinylated as previously described (12). Briefly, cells treated with glucose oxidase in the absence or presence of the proteasome inhibitor, MG-132, were washed twice in PBS, pH 7.4, containing 0.5 mM MgCl2·6H2O and 1 mM CaCl2·2H2O and then incubated in 3 ml of ice-cold solution containing 1 mg/ml freshly added NHS-SS-biotin (Pierce Biotechnology). After 30 min of gentle swirling at 4°C, cells were washed two times with PBS, pH 7.4, containing 0.5 mM MgCl2·6H2O, 1 mM CaCl2·2H2O, and 100 mM glycine. The cells were scraped in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, and 0.1% SDS; pH 7.5) supplemented with protease inhibitor cocktail (Roche Diagnostics), 2 mM PMSF, and 2 mM iodoacetamide. After 10–20 min on ice, homogenates were centrifuged at 16,000 g for 10 min. The protein content of the supernatants was determined, and the same amount of protein was transferred to 1.5-ml Eppendorf microfuge tubes containing 200 μl of Neutavirdin (Pierce). After 2 h of incubation at 4°C, the beads were washed four times with RIPA buffer. The resulting pellets were resuspended in 150 μl of 2% Laemmli buffer and incubated for 30 min at 37°C. The beads were pelleted, and the proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and blotted against GLUT1 antibodies.

**Immunoprecipitation.** After treatment, cells were washed twice with PBS, harvested in 100 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.5% SDS, 2 mM iodoacetamide, 2 mM PSF, and protease inhibitor cocktail), and incubated on ice for 30 min. Following centrifugation at 16,000 g for 10 min, supernatants were transferred to new tubes, and 5 μg of anti-GLUT1 antibody (Santa Cruz Biotechnology) were added, and incubation proceeded at 4°C for 2 h. The beads were extensively washed with lysis buffer, and immunoprecipitated proteins were eluted in Laemmli buffer and resolved by SDS-PAGE. Western blot analysis was performed using anti-GLUT1 and anti-ubiquitin antibodies.

**Statistical analysis.** Results are expressed as means ± SE of at least three independent experiments. Statistical analysis was performed by GraphPad Prism 5.0 Software (GraphPad Software) using one-way ANOVA with Bonferroni’s or Dunnett’s correction to compare the control and treatment results. For comparison between two groups, the unpaired t-test was used. A difference was considered to be statistically significant when P < 0.05.

**RESULTS**

**Oxidative stress leads to an accumulation of oxidized proteins in TR-iBRB cells.** To study the effect of oxidative stress on regulation of GLUT1 in retinal endothelial cells, we first incubated the TR-iBRB cells with glucose oxidase for 4 h in a serum- and phenol red-free medium containing high glucose concentration (25 mM), and the concentration of H2O2 produced in the medium was measured by a colorimetric assay. Addition of 15 μM/ml glucose oxidase to TR-iBRB cells resulted in a steady-state concentration of H2O2 in the medium of ~40 μM, after ~2 h of incubation (Fig. 1A). The rate of degradation of H2O2 was 6.46 nmol/min. The activity of this enzymatic system remained relatively constant after 4 h, leading to the formation of H2O2 and, subsequently, to intracellular superoxide anion formation, as revealed by increased DHE staining in TR-iBRB cells that were previously subjected to oxidative stress (Fig. 1B). In this study, we chose to use levels of protein carbonyls as an indicator of oxidized proteins. As shown in Fig. 1C, cell lysates obtained after treatment with glucose oxidase show a clear and time-dependent increase in levels of carbonyl groups.

**Oxidative stress induces a selective decrease in GLUT1 at the PM of retinal endothelial cells.** To establish whether GLUT1 is regulated in response to oxidative stress, TR-iBRB cells were exposed to glucose oxidase (to generate H2O2), and the total levels of both protein and mRNA for GLUT1 were detected. Data show that exposure of cells to oxidative stress does not induce significant changes in the total amount of GLUT1 protein (Fig. 2A). Conversely, exposure to glucose oxidase resulted in a decrease in GLUT1 mRNA levels as determined by real-time RT-PCR (Fig. 2B). To examine...
whether oxidative stress induces alterations in subcellular distribution of this glucose transporter, biotinylation of cell surface proteins and membrane subcellular fractionation assays were performed, and the amount of GLUT1 protein at the cell surface was evaluated by Western blot analysis. Oxidative stress induced by incubation with glucose oxidase leads to a decrease in GLUT1 content at the PM (from 100% to 54.7 ± 15%) as shown by the biotinylation assay in Fig. 3A. Consistently, subcellular fractionation assays show that incubation in the presence of glucose oxidase leads to a decrease in GLUT1 abundance at the PM (from 100% to 67.6 ± 6.1%) with a parallel increase in HDM and LDM (from 100% to 121 ± 6.9%) fractions (Fig. 3B).

To investigate GLUT1 internalization, membrane surface proteins were first biotinylated, and then cells were incubated in the absence or presence of glucose oxidase for different periods of time. Cells were subsequently permeabilized with Triton X-100 to remove the intracellular stores of GLUT1, the biotinylated proteins were finally purified with neutravidin, and GLUT1 was immunodetected by Western blotting. Data show that the half-life of GLUT1 at the PM is of ∼430 min under oxidative stress while in control conditions there is not a significant decrease for the duration of the experiments (Fig. 3C), that is, there is a decrease of ∼50% on the levels of GLUT1 present at the PM following exposure to oxidative stress for 4 h.

**UPP is involved in downregulation of GLUT1 at the PM following oxidative stress.** We reported previously a down-regulation of GLUT1 in retinas of diabetic rats by a proteasome-dependent mechanism (11). To study the involvement of the UPP in downregulation of GLUT1 at the cell surface following oxidative stress, immunocytochemistry, biotinylation, and immunoprecipitation assays were performed. Confocal immunofluorescence data revealed that there is a decrease in GLUT1 present at the PM when the cells are subjected to oxidative stress (Fig. 4A). When the proteasome is inhibited with MG-132, there is a stabilization of the GLUT1 present at the PM (Fig. 4, A and B). This immunofluorescence data was confirmed by biochemical data showing that inhibition of the proteasome leads to an accumulation of biotinylated GLUT1 at the PM (Fig. 4B).

Having established that oxidative stress induces an alteration in subcellular distribution of GLUT1, decreasing the availability of the transporter at the PM, we proceeded by assessing the impact of the oxidative stress in the activity of GLUT1 and further establishing the effect of proteasome incubation on...
GLUT1-dependent glucose transport. Data shown in Fig. 5A indicate that exposure of cells to oxidative stress (15 mU/ml glucose oxidase for 4 h) results in a decrease in cell viability. TR-iBRB cells treated with 15 mU/ml glucose oxidase for 4 h showed a significant decrease in MTT reduction from 100% to 77.9 ± 9.3% (Fig. 5A). Under these conditions, we observed a significant decrease in 2-DOG uptake (to 35.8 ± 3.7%). When the cells were incubated with glucose oxidase in the presence of the proteasome inhibitor, there is a significant decrease in MTT reduction (to 69.8 ± 10.0%) compared with the control (Fig. 5A). Because incubation of the proteasome leads to a stabilization of GLUT1 at the PM, we hypothesized that oxidative stress leads to a destabilization of GLUT1 present at the PM by interfering with proteasome-dependent regulation of GLUT1. Therefore, we analyzed the glucose transport evaluated by 2-DOG uptake in cells subjected to oxidative stress when the proteasome was inhibited. Proteasome inhibition leads to a significant increase in 2-DOG uptake (to 124.3 ± 18.6%) compared with the control. Furthermore, MG-132 significantly prevents the decrease in 2-DOG uptake induced by oxidative stress (119.2 ± 10.0%). This suggests that the proteasome is involved in oxidative stress-induced GLUT1 redistribution (Fig. 5B). To further confirm the involvement of the proteasome in GLUT1 regulation under oxidative stress, we investigated whether GLUT1 is ubiquitinated. Data shown in Fig. 6 show that GLUT1 immunoprecipitates cross-react modestly with ubiquitin antibodies, particularly on cells exposed to oxidative stress. The staining profile of the bands on Fig. 6 that cross-react with ubiquitin antibodies are consistent with monoubiquitinated or dimonoubiquitinated forms of GLUT1, since their molecular weights are consistent with the addition of one or two molecules of ubiquitin. Interestingly, a band of 54 kDa may reflect coprecipitation of GLUT1 with an unknown ancillary protein that is ubiquitinated.

To confirm whether the decrease in 2-DOG uptake in response to glucose oxidase is indeed related to the increased oxidative stress, we preincubated the cells with two antioxidants, α-tocopherol and N-acetylcysteine (NAC), and then the cells were exposed to glucose oxidase. α-Tocopherol significantly protects the cells from MTT reduction (Fig. 5A). However, α-tocopherol was only able to recover minimally the 2-DOG uptake when the cells were incubated with glucose oxidase (Fig. 5B). Additionally, cell viability was restored when the cells were incubated with NAC (Fig. 5A). NAC was able to revert the 2-DOG uptake (from 35.8 ± 3.7 to 65.3 ± 9.9%) induced by glucose oxidase (Fig. 5B).

It was previously shown that PI3K/Akt, besides its function as critical regulators of cell survival and proliferation (48), are important regulators of protein synthesis that may affect glucose uptake through regulation of GLUT1 activity and/or trafficking (57). Based on the results showing that sustained oxidative stress reduces 2-DOG uptake, due to the subcellular redistribution of the protein, we next assessed whether the PI3K/Akt signaling pathway is involved in that process. Glucose oxidase consistently activated Akt (increasing P-Akt), peaking at 60 min and then decreasing over 4 h (Fig. 7A). Furthermore, we showed that glucose oxidase induces a decrease in activation of Akt without changing the total levels of Akt (Fig. 7B). This decrease in Akt-P is reverted when the proteasome is inhibited (Fig. 7B). These data suggest that oxidative stress induces a destabilization of GLUT1 present at the PM. This decrease is concomitant with a decrease in glucose transport and appears to involve proteasome-dependent activation of Akt. To evaluate whether the constitutively active form of Akt is important to restore GLUT1 activity in PM, we transfected the cells with the constitutively active form of Akt (Myr-Akt) and then exposed the cells to glucose oxidase. We observed an increase in GLUT1 present at the cell surface when the cells overexpressing the active form of Akt were treated with glucose oxidase (Fig. 7C).

**DISCUSSION**

Increasing evidence suggests that oxidative stress plays a key role in the pathogenesis of diabetes mellitus (58). Hyperglycemia may lead to both impaired antioxidant protection (40) and increased production of ROS by vascular endothelium (9, 27, 41). The effect of the altered redox balance in endothelial cells is likely to contribute to the onset of endothelial dysfunction. For example, Brownlee (5) reported that excessive production of superoxide by mitochondria electron chain may stimulate several biochemical pathways, such as the activation of protein kinase C, polyol, and advanced glycation pathways, all of which were shown to contribute to the pathophysiology of diabetes complications.

GLUT1 is the main glucose transporter responsible for glucose uptake by capillary endothelial cells that comprise the BRB (29). Furthermore, in the inner retina, a substantial portion (∼50%) of total cellular GLUT1 resides in cytosolic stores (31). Because this intracellular pool of GLUT1 is not in contact with the cell surface, it is not available for glucose transport. However, in response to appropriate stimuli, glucose transporters such as GLUT1 may undergo a subcellular redistribution that increases its availability at the PM. In this study, we investigated the effects of sustained oxidative stress on
GLUT1 as well as on glucose transport activity. Because ROS have a short half-life (45), we used a ROS-generating system that continuously produces a steady-state concentration of H$_2$O$_2$ over a period of 4 h. We found that glucose transport by retinal endothelial cells decreased in response to oxidative stress. The mechanism did not involve changes in the total amount of GLUT1 protein; rather, oxidative stress seems to trigger alterations in subcellular distribution of GLUT1 presumably because of inactivation of Akt, by a proteasome-dependent mechanism.

Our results clearly indicate that exposure of retinal endothelial cells to moderate sustained oxidative stress (40 mU/ml) did affect the rate of glucose transport, without significantly changing the levels of GLUT1 protein. Rather, oxidative stress seems to trigger alterations in subcellular distribution of GLUT1 presumably because of inactivation of Akt, by a proteasome-dependent mechanism.

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This is in contrast to what was previously reported in other cell types such as in 3T3-L1 adipocytes or L6 myotubes (28), where the authors observed an increase in glucose uptake under oxidative stress associated with increased expression of GLUT1 mRNA and protein. The contribution of the subcellular redistribution of existing transporters to the increased glucose transport observed was relatively minor compared with the increased GLUT1 protein synthesis observed in the response to oxidative stress. There are, however, some substantial differences in the time approaches. The authors observed a glucose transport stimulation beginning only after 6 h of continuous exposure to 20 mU/ml of glucose oxidase. This is consistent with GLUT1 gene induction. More recently, other authors have shown downregulation of glucose transport in response to high glucose in vascular endothelial cells within 36–48 h (1). The authors associate the findings to arachidonic acid metabolites, products of lipid peroxidation and calreticulin-induced destabilization of GLUT1 mRNA, rendering it susceptible to degradation. Consequently, the cell content of GLUT1 protein and its abundance at the PM are reduced, resulting in downregulation of glucose uptake (47). These studies suggest an entirely different mechanism that involves ROS-induced lipid peroxidation and generation of a second messenger that activates peroxisome proliferator-activated receptor-δ. Moreover, in the present study, the content of GLUT1 mRNA is decreased without significant changes on the total protein content, suggesting a possible deregulation of the GLUT1 subcellular trafficking induced by oxidative stress.

In this study, we observed that exposure of TR-iBRB endothelial cells to oxidative stress for 4 h leads to a rapid inter-

**Fig. 3.** Exposure of TR-iBRB cells to GO decreases the amount of GLUT1 and its half-life in the plasma membrane (PM). A and B: TR-iBRB cells were exposed to GO (15 mU/ml) for 4 h. A: NHS-SS-biotin (1 mg/ml) was present during the last 30 min of incubation. The cells were incubated at 4°C and subsequently permeabilized with Triton X-100, and the biotinylated proteins were purified by neutravidin precipitation. GLUT1 and β-actin were immunodetected by Western Blotting. ***P < 0.001, significantly different from GLUT1 at the PM in control cells (one-way ANOVA with Bonferroni’s multiple-comparison test). B: subcellular fractionation was performed; proteins were separated by SDS-PAGE and transferred to PVDF membranes; and GLUT1 and caveolin-1 were immunodetected using antibodies directed against GLUT1 and caveolin-1. ***P < 0.001, significantly different from GLUT1 at the PM in control cells. **P < 0.01, significantly different from GLUT1 at the high-density microsome (HDM) + low-density microsome (LDM) in control cells (one-way ANOVA with Bonferroni’s multiple-comparison test). C: TR-iBRB cells were incubated with NHS-SS-biotin (pulse) and then were treated with GO (15 mU/ml) for different times. The cells were then permeabilized with Triton X-100, and the biotinylated proteins were purified by neutravidin precipitation. GLUT1 was immunodetected by Western Blotting.

**A**

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**B**

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<th>Caveolin-1</th>
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**C**

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GLUT1 as well as on glucose transport activity. Because ROS have a short half-life (45), we used a ROS-generating system that continuously produces a steady-state concentration of H$_2$O$_2$ over a period of 4 h. We found that glucose transport by retinal endothelial cells decreased in response to oxidative stress. The mechanism did not involve changes in the total amount of GLUT1 protein; rather, oxidative stress seems to trigger alterations in subcellular distribution of GLUT1 presumably because of inactivation of Akt, by a proteasome-dependent mechanism.

This is in contrast to what was previously reported in other cell types such as in 3T3-L1 adipocytes or L6 myotubes (28), where the authors observed an increase in glucose uptake under oxidative stress associated with increased expression of GLUT1 mRNA and protein. The contribution of the subcellular redistribution of existing transporters to the increased glucose transport observed was relatively minor compared with the increased GLUT1 protein synthesis observed in the response to oxidative stress. There are, however, some substantial differences in the time approaches. The authors observed a glucose transport stimulation beginning only after 6 h of continuous exposure to 20 mU/ml of glucose oxidase. This is consistent with GLUT1 gene induction. More recently, other authors have shown downregulation of glucose transport in response to high glucose in vascular endothelial cells within 36–48 h (1). The authors associate the findings to arachidonic acid metabolites, products of lipid peroxidation and calreticulin-induced destabilization of GLUT1 mRNA, rendering it susceptible to degradation. Consequently, the cell content of GLUT1 protein and its abundance at the PM are reduced, resulting in downregulation of glucose uptake (47). These studies suggest an entirely different mechanism that involves ROS-induced lipid peroxidation and generation of a second messenger that activates peroxisome proliferator-activated receptor-δ. Moreover, in the present study, the content of GLUT1 mRNA is decreased without significant changes on the total protein content, suggesting a possible deregulation of the GLUT1 subcellular trafficking induced by oxidative stress.

In this study, we observed that exposure of TR-iBRB endothelial cells to oxidative stress for 4 h leads to a rapid inter-
nalization of GLUT1, decreasing the glucose transport. The decrease observed in glucose transport can presumably be ascribed to a decrease in the half-life of GLUT1 present at the PM, as confirmed by biotinylation experiments. These data combined with those showing that GLUT1 accumulates in intracellular pools demonstrate that oxidative stress affects endothelial cell function and activates a trafficking pathway, resulting in the rapid endocytosis of GLUT1 present at the PM. Significantly, this process appears to be dependent on the UPP. Indeed, GLUT1 is likely to be mono- or diubiquitinated. This observation is not without precedent, since monoubiquitination was described to regulate the endocytic transport of proteins located at the PM, such as several ion channels and signal-transducing receptors (4, 18, 32, 33, 50, 52). Most of the proteins that are ubiquitinated at the PM are recognized by specific adaptors and targeted for lysosomal degradation. It was further shown that diubiquitin chains linked through Lys63 further enhance basal internalization rates of membrane proteins (15, 49).

Understanding the role of ROS as key mediators in signaling cascades may provide opportunities for pharmacological intervention. To investigate whether ROS could be a direct mediator of the effect on glucose transport, we treated the retinal endothelial cells with the antioxidants α-tocopherol and NAC. The lipophilic α-tocopherol, which concentrates at the PM, only minimally affected the regulation of glucose transport, suggesting that cytosolic ROS were involved in the modulation of glucose transport. Indeed, when NAC was used, a significant increase was observed in glucose uptake.

Fig. 4. Proteasome inhibition prevents the decrease of GLUT1 at PM following oxidative stress. A: TR-iBRB cells were cultured in cover slips and incubated with GO (15 μU/ml) in the absence or presence of MG-132 (20 μM) for 4 h. The cells were then fixed with paraformaldehyde (PFA), permeabilized with Triton X-100, and stained with antibodies against GLUT1, GO and proteasome inhibition lead to a subcellular redistribution of GLUT1. B: TR-iBRB cells were exposed to GO (15 μU/ml) for 4 h, either in the absence or presence of MG-132 (20 μM). Surface proteins were biotinylated as described before, and GLUT1 was immunodetected as described by Western blotting. *P < 0.05, significantly different from GLUT1 at the PM (one-way ANOVA with Bonferroni’s multiple-comparison test).

Fig. 5. Sustained oxidative stress induces cytotoxicity and downregulates glucose transport. Both proteasome inhibition and antioxidants restore glucose transport to basal levels. However, treatment with proteasome inhibitors is further associated with increased toxicity in cells exposed to oxidative stress. TR-iBRB cells were exposed to GO (15 μU/ml) for 4 h, in the absence or presence of MG-132 (20 μM) or in the absence or presence of the antioxidants α-tocopherol (5 μg/ml) and N-acetylcysteine (NAC, 5 mM). A: cell viability was determined by the MTT assay. The results were expressed as a percentage of control. Results are means ± SE of 3 independent experiments, each performed in duplicate or triplicate. ***P < 0.001, significantly different from control (one-way ANOVA with Dunnett’s multiple-comparison test). B: glucose transport was measured by 2-[3H]deoxyglucose (DOG) uptake, and the results were expressed as a percentage of control. Results are means ± SE of 3 independent experiments, each performed in duplicate or triplicate. **P < 0.001, significantly different from control. *P < 0.05, significantly different from GO (one-way ANOVA with Bonferroni’s multiple-comparison test).
We hypothesized that generated intracellular ROS could take part in a pathway cascade that, by modifying the activity of redox-sensitive enzymes, including kinases, leads to decreased glucose transport and glucose transporter translocation to intracellular pools. It has been recently reported that the Akt pathway may be critical for GLUT1 docking at the PM to promote optimal glucose transport (7).

Thus we investigated whether Akt was involved in modulation of glucose uptake upon oxidative stress. Consistently, acute treatment of cells with H2O2 was shown to activate Akt (17). Surprisingly, we observed that sustained oxidative stress induced an inactivation of Akt. Consistently, we also observed that, when retinal endothelial cells overexpress the constitutively active form of Akt (Myr-Akt), GLUT1 is stabilized at the cell surface upon oxidative stress. However, Akt seems to be transiently activated, being maximal at 60 min and then decreasing over 4 h.

In addition to the well-known role of the UPP in protein degradation, recently new functions have been identified for ubiquitination, such as in receptor endocytosis or protein sorting. It is currently accepted that ubiquitination serves a regulatory function much like phosphorylation (23, 56). Our study shows that the activation of Akt induced by proteasome inhibitors leads to stabilization of GLUT1 at the PM and increased glucose transport in endothelial cells.

Our group and other authors have shown that retinal GLUT1 abundance decreases in experimental diabetes and, following exposure of retinal endothelial cells, to elevated concentrations of glucose (2, 11). We show that decreased abundance of GLUT1 is likely to be associated with its increased degradation most likely by a ubiquitin-dependent mechanism.

The results from the present study indicate that the ubiquitin proteasome system regulates glucose transport into the endothelial cells in response to oxidative stress. The molecular mechanisms involved appear to involve inactivation of Akt without changing the total levels of the protein. The modulation of glucose transport is complex, and multiple pathways are likely to be involved. Further studies are warranted to elucidate...
the signaling events that are associated with the oxidative regulation of GLUT1 as well as to the role of the ubiquitin-proteasome system in this process.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


