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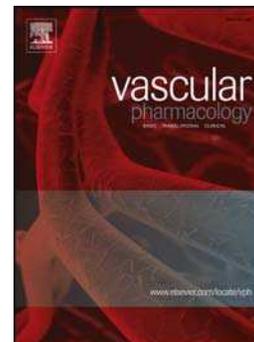
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Transendothelial glucose transport is not restricted by extracellular hyperglycaemia

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ABSTRACT

Endothelial cells are routinely exposed to elevated glucose concentrations post-prandially in healthy individuals and permanently in patients with metabolic syndrome and diabetes, and so we assessed their sugar transport capabilities in response to high glucose. In human umbilical vein (HUVEC), saphenous vein, microdermal vessels and aorta, GLUT1 (SLC2A1), GLUT3 (SLC2A3), GLUT6 (SLC2A6), and in microdermal vessels also GLUT12 (SLC2A12), were the main glucose transporters as assessed by mRNA, with no fructose transporters nor SGLT1 (SLC5A1). Uptake of ¹⁴C-fructose was negligible. GLUT1 and GLUT3 proteins were detected in all cell types and were responsible for ~60% glucose uptake in HUVECs, where both GLUT1 and GLUT3, but not GLUT6 siRNA knock-down, reduced the transport. Under shear conditions, GLUT1 protein decreased, GLUT3 increased, and ¹⁴C-deoxy-glucose uptake was attenuated. In high glucose, lipid storage was increased, cell numbers were lower, ¹⁴C-deoxy-glucose uptake decreased owing to attenuated GLUT3 protein and less surface GLUT1, and trans-endothelial transport of glucose increased due to cell layer permeability changes. We conclude that glucose transport by endothelial cells is relatively resistant to effects of elevated glucose. Cells would continue to supply it to the underlying tissues at a rate proportional to the blood glucose concentration, independent of insulin or fructose.

1. Introduction

The human endothelium is a large organ consisting of monolayers of endothelial cells lining all blood vessels in the body. Its many functions include regulating blood flow and maintaining normal vascular tone through production of NO, endothelin-1 and other factors [1-3], control of blood clotting and inflammation, and formation of a selective barrier between blood and tissues for distribution and exchange of nutrients, metabolites and gasses [4]. Endothelial cells in different vessels or organs are finely tuned to their specific functions [5-7]; for example, the permeability of the endothelial monolayer ranges from tightly controlled in blood brain barrier [8] to leaky in fenestrated endothelium of sinusoidal tissue in liver [9, 10]. One of the characteristic features of endothelial cells is their reliance on glycolysis for energy production [11-13], with sustained glucose consumption being critical for endothelial cell viability especially during angiogenesis [14, 15]. Thus glucose uptake and metabolism is critical for healthy endothelial physiology, and endothelial glucose transporters are especially important in the brain, where glucose can only cross the blood brain barrier via this mechanism [16]. On the other hand, dysregulated glucose transport and metabolism is linked to pathophysiological states [17-19]; increased GLUT expression is associated with carcinogenesis and tumour progression [20-22] and both hypo- and hyperglycaemia can lead to increased endothelial stress and inflammation, contributing to cardiovascular disease [23-25]. Despite the critical functional role of glucose in the endothelium, our understanding of its uptake and utilization by endothelial cells remains limited. As a hydrophilic molecule, glucose requires transporters in order to cross the plasma membrane. Glucose transporters (GLUTs) of the SLC2A family are transmembrane proteins classified in three groups (reviewed in [26-28]). Type I (GLUT1-4 and 14) and II (GLUT5, 7, 9 and 11) are largely present on the plasma membrane and typically enable glucose (Type I) or fructose (Type II and GLUT2) flux in/out of the cells. Type III (GLUT 6, 8, 10, and 12) have

an intracellular localization signal but some have also been reported on the plasma membrane [29, 30]. GLUTs have various affinities and specificities for different sugars, but glucose transport is most typically associated with Type I GLUT transporters. The K_m for 2-deoxy-glucose uptake ranges from 1.4 for GLUT3 to 4.6 for GLUT4, 6.9 for GLUT1 and 11.2 mM for GLUT2 (reviewed in [31]). Of those, GLUT1 is broadly expressed in most tissues including endothelium and is considered the universal glucose transporter to supply cells with glucose. Low affinity GLUT2 is involved in regulating glucose transport in tissues exposed to high glucose concentrations such as intestine and liver, or pancreas, where GLUT2 functions as a glucose sensor to promote insulin release. High affinity/high capacity GLUT3 expression in the brain is critical for maintaining steady supply of glucose to neurons and GLUT4 trafficking mediates glucose uptake in insulin-responsive tissues [32]. Type II GLUTs have affinity for fructose but some can also transport glucose. GLUT5 transports fructose in the small intestine, with GLUT2 also contributing to the fructose flux. The roles of the more recently discovered Type III GLUT transporters are more elusive. Other transporters known to mediate glucose uptake are Sodium-Glucose Linked Transporters (SGLT) of the SLC5 family [27], which actively co-transport glucose and Na^+ against a glucose gradient. Several studies reported SGLT1 (or SLC5A1) in microendothelial cells of brain or heart capillaries [33-35], but its presence in other endothelial cells has not been examined. To determine the capacity and flexibility for glucose transport regulation in the endothelium, especially for cells exposed to high glucose concentrations encountered in metabolic syndrome, diabetes and post-prandially, we have quantified the expression of GLUT transporters in endothelial cells derived from different vascular beds and elucidated how glucose uptake is related to GLUT expression after physiological (shear) and pathophysiological (hyperglycaemic) stress.

2. Materials and methods

2.1. Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs, Lonza Sales Ltd, Switzerland) were cultured in EGM-2 growth medium supplemented with EGM-2 bullet kit (Lonza) and used between passage 3-6. For mixed shear stress application, cells were seeded at a density of $4-5 \times 10^4$ cells/cm² in 6-well plates for 3 h and then incubated for 24 h on an orbital shaker (Grant Bio Orbital Shaker, model PSU-10i; Fisher Scientific, UK) at 210 rpm, producing laminar shear stress on the plate periphery [36]. To study long-term effects of high glucose concentration, confluent HUVECs were maintained for 6 days in standard medium (5.5 mM D-glucose), or with additional 20 mM D-glucose. In some cases 20 mM L-glucose or mannitol were used as an osmotic control. Unless specified otherwise, the medium was changed every other day. Saphenous Vein Endothelial Cells (SVECs) were isolated from saphenous vein segments obtained from patients undergoing coronary bypass surgery, and without a diagnosis of diabetes. The study conformed to the principles outlined in the Declaration of Helsinki and local ethical approval (CA/01/040) and informed, written patient consent were obtained. Confluent cells at passage 6 from three patients were used. They were maintained in complete endothelial medium, which consisted of M199 medium (Sigma-Aldrich, Dorset, UK), 20% FCS (Labtech International Ltd, Uckfield, UK), 1% Glutamine, 100 U/mL penicillin-streptomycin, 20 mM HEPES (all Life Technologies, Thermo Fisher Scientific, Paisley, UK), 15 µg/mL endothelial cell growth supplement, 1 mM pyruvate (both Sigma-Aldrich), and 5 U/mL heparin (LEO Laboratories Ltd., Hurley, UK). Human aortic (HAoEC) and microdermal (HMDEC) endothelial cells were obtained as cell pellets in RNAlater buffer from PromoCell GmbH, Germany. Half of the material was used for mRNA isolation and the remainder was processed for protein detection.

The immortalized human myoblasts LHCN-M2, which were used as a positive control in testing the insulin-induced glucose uptake, were a kind gift from Dr Vincent Mouly [37]. They were cultured on 0.2% gelatin-coated plates in DMEM (Invitrogen, Thermo Fisher Scientific) containing 10% FBS, 10% newborn calf serum (Invitrogen), 10 ng/ml EGF (Peprotech EC Ltd, London, UK), 1 ng/ml bFGF (Gibco, Thermo Fisher Scientific) and 0.4 µg/ml Dexamethasone (Sigma-Aldrich). Differentiation was induced by exposing confluent cells to 2% horse serum in DMEM for 4 days.

2.2. Quantification of mRNA by droplet digital PCR

RNAqueous® Total RNA Isolation Kit (Ambion™, Life Technologies) was used to isolate mRNA from samples harvested in RNAqueous lysis buffer and stored at -80°C for up to several months. The mRNA was then reverse-transcribed into cDNA with GoScript kit (Promega UK Ltd, Southampton, UK). Negative controls without cDNA were generated in parallel by omitting reverse transcriptase from the mix. Since the GLUT1 probe also detected genomic DNA, samples for GLUT1 detection were digested with TurboDNase (Life Technologies) prior to the reverse transcription to remove genomic contamination. All these steps were performed using the standard recommended manufacturer's protocols and guidelines for digital PCR [38] were followed as appropriate. FAM-labelled gene-specific probes were from Bio-Rad Laboratories: GLUT1 (SLC2A1) (qHsaCEP0050995), and Life Technologies: GLUT2 (SLC2A2; Hs01096905_m1), GLUT3 (SLC2A3; Hs00359840_m1), GLUT4 (SLC2A4; Hs00168966_m1), GLUT5 (SLC2A5; Hs01085390_m1), GLUT6 (SLC2A6; Hs01115485_m1), GLUT7 (SLC2A7; Hs01013553_m1), GLUT8 (SLC2A8; Hs00205863_m1), GLUT9 (SLC2A9; Hs00417125_m1), GLUT10 (SLC2A10; Hs00229205_m1), GLUT11 (SLC2A11; Hs00368843_m1), GLUT12 (SLC2A12; Hs01547015_m1), GLUT14 (SLC2A14; Hs03044883_m1), and eNOS (Hs01574659_m1). Traditionally, a housekeeping gene such as GAPDH is used for a reference in mRNA quantification. As the digital PCR determines the absolute number of mRNA molecules in the reaction, use of stably expressed reference gene is not essential but is still highly recommended to account for variations in the sample preparation or to provide comparison between different conditions. Multiplexing conditions in digital PCR require that the reference gene expression levels fall in the range compatible with the target gene abundance to allow for simultaneous detection and droplet analysis; GAPDH has

dramatically higher expression levels than GLUT transporters and so it was unsuitable. Instead the Ribosomal Protein L37 (RPL37) was selected as a control, since its expression levels were better-matched to the target genes and in addition were found to be similar between the different cells. The VIC-labelled control gene-specific probe was from Life Technologies: RPL37 (Hs03044965_g1). Samples corresponding to 5-15 ng mRNA were analyzed for specific gene expression using QX100 Droplet Digital PCR system (Bio-Rad Laboratories Ltd, Herts, UK). First, the reaction mix consisting of the cDNA, PCR Supermix for probes and the gene-specific fluorescent probes was dispersed into oil droplets using QX-100 Droplet Generator. The droplets with the reaction mix were then loaded into C1000 Touch thermal cycler and incubated for 10 min at 95 °C, followed by 40 cycles of 0.5 min at 94 °C and 1 min at the primer-specific optimized temperature and finished by 10 min incubation at 98 °C. Products were kept at 12 °C before analysis by QX100 Droplet Reader. QuantaSoft software (Kosice, Slovakia) was used to analyze the data and determine the number of copies present in the mix, which were then converted to copies per ng of mRNA, assuming 1:1 RT PCR efficiency.

2.3. Protein detection by Simple Western

HUVECs or SVECs were harvested by scraping in ice-cold PBS containing protease inhibitors, centrifuged at 500 x g and cell pellets stored at -80 °C until analysis. For analysis, pellets were dissolved in 60 mM octyl glucoside, 20 mM Tris and 150 mM NaCl, pH 7.4 (with protease inhibitors) on ice for 20 min with intermittent agitation, and clarified by 10 min centrifugation at 14,000 x g at 4 °C. Protein concentration was determined from A_{280} measured on a Nanodrop ND1000 spectrophotometer (Labtech International). Proteins in the lysate were detected and quantified by Simple Western analysis using WES, an automated capillary-based system with quantitative immunodetection (ProteinSimple, Bio-Techne, US) [39, 40] by following manufacturer's instructions. Lysates were combined with Mastermix denaturing buffer and 0.1x Sample Diluent buffer to obtain 0.1 mg/ml (for GLUT1 detection) or 0.2 mg/ml (for GLUT3 detection) protein concentration. To avoid GLUT aggregation, sample denaturation prior to the analysis was performed by 15 min incubation at 37 °C. Samples and manufacturer-provided reagents as well as primary antibodies were dispensed in the supplied microplate and subjected to automated capillary electrophoresis followed by immunodetection with bioluminescence as the readout. Standard manufacturer's protocol was followed except that the primary antibody incubation time was increased to 60 min. Compass software (ProteinSimple, Bio-Techne) was used to analyze the results. Multiplexing with Na,K-ATPase antibodies provided control for normalization. Linear range of detection was confirmed for sample concentrations between 0.025-0.4 mg/ml for all antibodies both with and without multiplexing (Supplementary figure 1). The optimized antibody dilution was 1:50 for GLUT1 (Rabbit Anti-Glucose Transporter GLUT1 antibody [EPR3915] (ab115730), lot number GR106925-16, from Abcam, Cambridge, UK) and Na, K-ATPase (Rabbit Na, K-ATPase Antibody #3010, lot number 4, from Cell Signaling Technology, New England Biolabs (UK) Ltd, Hitchin, UK), or 1:100 for Glut3 (Rabbit Anti-Glucose Transporter GLUT3 [EPR10508(N)] antibody - N-terminal (ab191071), lot number GR177395-4, Abcam). N-linked glycan chains were removed from the glycoproteins by 10 min treatment at 37 °C with Rapid PNGase (New England Biolabs), using lysates diluted with water so that the final concentration of octyl glucoside was below 5 mM to prevent inhibition of PNGase activity. Under these conditions, 1 μ l of the enzyme was sufficient to digest lysate with 10 μ g total protein with minimal losses or aggregation of GLUT1 or GLUT3.

2.4. Cell surface biotinylation

Biotinylation was performed using EZ-Link Sulfo-NHS-SS-Biotin and Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific, Pierce, UK). Manufacturer's protocol was applied, except 60 mM octyl-glucoside, 150 mM NaCl, 20 mM Tris pH 7.4 buffer with protease inhibitors was used for cell lysis and the elution step was carried out by 20 min incubation with 50 mM DTT in 1x ProteinSimple Sample Diluent or Bicine-CHAPS buffer (both from ProteinSimple, Bio-Techne) at 37 °C. Both biotinylated and total GLUT1 and GLUT3 were quantified by the Simple Western and multiplexing with Na, K-ATPase was used as a control.

2.5. Deoxy-glucose uptake assay

Cells were washed with glucose-free buffer (2.5 mM MgCl₂, 1 mM CaCl₂, 0.14 M NaCl, 20 mM HEPES, 3.5% NaHCO₃, pH 7.3), incubated for 10 min at 37 °C in glucose-free RPMI medium and then supplemented for 10 or 20 min with 0.55 mM deoxy-glucose containing 0.1 µCi/ml 2-[¹⁴C(U)]-deoxy-D-glucose (PerkinElmer, Seer Green, UK). Radioactive medium was rapidly removed, cells were washed three times with cold PBS, lysed in 1M NaOH and radioactivity in the cell lysate measured by scintillation counting. Standard calibration curve was constructed using the medium with known amounts of deoxy-glucose and the radioactive tracer to allow converting the measured radioactivity to nmol of deoxy-glucose. Henceforth the combined uptake of ¹⁴C-deoxy-glucose and cold deoxyglucose is referred to as deoxy-glucose uptake. Cells that were seeded in parallel at the same density were trypsinized and counted and the deoxy-glucose uptake was expressed as fmol/cell. A similar protocol was used for fructose and glucose uptake, using [¹⁴C(U)]-fructose (Moravek Biochemicals, Hartmann Analytics GmbH, Germany) and D-[¹⁴C(U)]-glucose (PerkinElmer), respectively. To estimate GLUT-independent deoxyglucose uptake, 10 µM Cytochalasin B was included in the medium during determination of uptake with radiolabelled sugars.

2.6 Insulin-induced glucose uptake assay

Confluent HUVECs or differentiated LHCN-M2 cells in 6-well plates were starved for 4 hours in serum- and glucose-free RPMI or DMEM medium, respectively. Cells were subsequently treated with 100 nM insulin or a vehicle for 20 min. Glucose uptake was measured after 1 h exposure to 0.55 mM glucose containing 1 µCi/ml 2-[¹⁴C(U)]-glucose (PerkinElmer) in the same medium, using the protocol described above. The uptake was normalized to cellular protein in each well, estimated by Bradford assay (Bio-Rad) using 5 µl aliquots of the 1M NaOH lysate.

2.7. Transfection

90%-confluent HUVECs in 12-well plates were transfected with 10 nM siRNA specific for GLUT1 (S12926), GLUT3 (s12933) or GLUT6 (s22075) or a negative control (AM4613) (all from Ambion, Life Technologies) using Lipofectamine 2000 (Life Technologies). The transfection complexes were prepared according to the manufacturer's instructions, applied to the cells for 4 h and then replaced with the standard culturing medium. After two days, the cells were used for deoxy-glucose transport experiments or mRNA or protein isolation. The viability of GLUT1, GLUT3 or GLUT6 siRNA transfected cells was comparable to the control cells. Transfecting the cells with combined GLUT siRNA decreased their viability by ~30%. The measured activities were normalized to cell numbers.

2.8. Monolayer permeability measurement

Endothelial cells were plated at 2x10⁵ cells/well on the upper side of 0.4 µm polycarbonate membrane insert in 12-well Transwell plates (Corning Life Sciences, UK). After 1 day, control cells were maintained in normal medium containing 5.5 mM glucose whilst high glucose-treated cells had 20 mM glucose added to the luminal medium in the upper chamber to achieve a final concentration of 25.5 mM. The abluminal medium in the lower compartment remained at 5.5 mM glucose for all cells. The medium was changed every day for 6 days, then the monolayer transendothelial electrical resistance (TEER) was measured using a Millicell ERS voltohmmeter (Millipore (UK) Ltd, Watford, UK) according to the manufacturer's instructions. The resistance of empty wells was measured at the same time to assess the relative contribution of the polycarbonate membranes and subsequently subtracted to estimate the cell monolayer resistance. To measure permeability for small molecules, cells were washed and equilibrated in wash buffer. FITC-labelled 4-kD dextran (Sigma) was applied to the luminal side to the final concentration of 1 mg/ml. After 10 and 20 min, the amount of FITC-dextran on the abluminal side was determined from the fluorescence of the abluminal media measured by a PheraStar plate reader (BMG

LABTECH, Germany) and calibration curve constructed for known FITC-dextran concentrations. The apparent permeability P was determined as $P=(dC_i/t)/(A \times C_o)$ where C_o is the initial luminal concentration of the solute, A is the area of the well, t is the time interval and dC_i is the change of the abluminal solute concentration during the time t .

2.9. Deoxy-glucose transport across the monolayer

HUVEC cells were plated in Transwell plates and maintained in normal or 25.5 mM luminal glucose as described above. Cells were washed with the buffer on both luminal and abluminal side and equilibrated in glucose-free RPMI for 10 min. RPMI with 5 mM deoxy-glucose and 0.5 μ Ci/ml 2- 14 C(U)-deoxy-D-glucose was then applied to the luminal side and the amount transported across the monolayer was measured after 10, 20, 30 or 60 min as the radioactivity in the abluminal medium.

2.10. Lipid droplet quantification by Oil red O

Confluent HUVECs were maintained in normal 5.5 mM glucose or 25.5 mM glucose-containing medium for 5 days. For comparison, confluent HUVECs were exposed for 5 days to 30 μ M docosahexaenoic acid (DHA) or 0.025% ethanol vehicle. The cells were washed in PBS with Ca^{2+} and Mg^{2+} , fixed in 10% paraformaldehyde and washed with PBS. Air-dried monolayers were incubated with oil red O, washed extensively and air-dried again. The incorporated oil red O was solubilised in isopropyl alcohol and quantified as A_{514} using a PheraStar plate reader (BMG Labtech, Aylesbury, UK). HepG2 cells (ATCC HB-8065, LGC Promochem, Teddington, UK) were used for comparison since they are known to contain high concentrations of lipid droplets due to their prominent lipid metabolism [41].

2.11. Statistics and data analysis

Test and control data which were obtained in pairs were compared using independent Student t-tests. ANOVA with Bonferroni post-hoc analysis was used for sets with multiple samples. Differences with $p < 0.05$ (*) were considered significant. All results were obtained from at least 2-3 independent experiments. All data are presented as mean \pm SEM unless stated otherwise. The n indicates the number of biological replicates. Data were analyzed and presented using Origin software (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. GLUT transporter expression in endothelial cells

The expression profile of glucose transporters in human umbilical vein endothelial cells (HUVECs) was assessed using ddPCR, providing absolute mRNA quantification. In agreement with previous studies [42], HUVECs were found to express GLUT1 and GLUT3, and moderate amounts of GLUT6, GLUT8 and GLUT 10 (>100 copies/ng mRNA) as well as small amounts of GLUT 9, 11 and 12 (<100 copies/ng mRNA) (Figure 1A). SGLT1 or GLUT2, 4, 5, 7 and 14 were not detected (<0.8 copy/ng mRNA), indicating lack of fructose transporters and insulin-responsive transporters. Saphenous vein endothelial cells (SVECs), aortic endothelial cells (HAoECs) and dermal microvessel endothelial cells (HDMECs) had a comparable GLUT mRNA expression profile with the exception of notably high levels of GLUT12 (>2000 copies/ng mRNA) in HDMECs (Figure 1A). GLUT1 and GLUT3 proteins were both expressed as glycosylated forms in HUVECs as confirmed by molecular weight shift after PNGase F treatment (Figure 1B, C). In agreement with the mRNA quantification, SVECs expressed higher amounts of GLUT1 and GLUT3 than HUVECs with lower amounts in HAoECs and HDMECs (Figure 1D, E, Figure 2). Cell surface biotinylation confirmed the presence of GLUT1 and GLUT3 on the plasma membrane (Table 1).

3.2. Uptake of monosaccharides in HUVECs

The ability of HUVECs to take up monosaccharides was assessed using the radioactive tracer assay with 14 C-labelled 2-deoxy-D-glucose and 14 C-labelled fructose. HUVECs efficiently took up 2-deoxy-D-glucose, but fructose uptake was relatively low (Figure 3A), reflecting the absence of potential fructose transporters. The SGLT inhibitor phloridzin had no effect on glucose uptake (Figure 3B), consistent with

the absence of SGLT mRNA. In addition, glucose uptake in HUVECs was not affected by short-term insulin treatment, while the same treatment significantly increased uptake in GLUT4-expressing differentiated muscle cells (Figure 3C). In order to assess the role of the individual GLUTs in the transport, knock-down of GLUT1, GLUT3 or GLUT6 was performed resulting in > 70% decrease in mRNA (Figure 3 D, E, F). For GLUT1 and GLUT3, the knock-down was also confirmed on a protein level (Figure 3G). The active uptake of deoxy-glucose in cells with down-regulated GLUT1 or GLUT3 was decreased by 35 and 28%, respectively (Figure 3H), and in a double GLUT1/GLUT3 knock-down, total deoxy-glucose transport decreased by ~62% (Figure 3H), indicating that both GLUT1 and GLUT3 contribute substantially to glucose uptake. This was in contrast to GLUT6 knock-down, which either individually or in combination with GLUT1 and GLUT3 did not further impair the deoxy-glucose transport. Of the total uptake, 13.4% was not inhibited by Cytochalasin B and therefore was unrelated to GLUTs (Figure 3H).

3.3. The effect of shear stress on GLUT transporter expression in HUVECs

Shear stress generated by blood flow regulates many endothelial cell functions. In order to determine its relevance for GLUT expression in HUVECs, cells were subjected to shear stress for 24 h and GLUT mRNA was quantified by ddPCR. In cells grown under shear stress, increase in eNOS mRNA, a positive control, was observed together with changes in GLUT12 mRNA (decrease to $44\pm 4\%$; $p=0.012$) and GLUT3 mRNA (increase to $142\pm 16\%$; $p=0.011$) (Figure 4A), accompanied by 2.1-fold increase in GLUT3 protein (Figure 4B). On the other hand, even in the absence of mRNA changes, GLUT1 protein levels were $24\pm 6\%$ lower in the cells under flow (Figure 4B), consistent with the overall reduction of deoxy-glucose uptake in shear stress-exposed cells (Figure 4C).

3.4. The effect of elevated glucose levels on GLUT transporter expression and function in HUVECs

Endothelial cells are normally exposed to ~5 mM glucose concentration in fasting blood but this is elevated in diabetes, metabolic syndrome and post-prandially [43]. Growth of HUVECs in 25.5 mM glucose for 5-6 days moderately decreased cell viability (Figure 5A) in agreement with previous reports [44, 45], but not when L-glucose was used for osmolarity control (Figure 5A). Elevated glucose can lead to accumulation of lipid droplets in HUVECs [46] and although very low basal level of oil red O staining was observed in HUVECs in 5.5 mM glucose, there was a small increase in cells grown in the presence of 25.5 mM glucose (Figure 5B). DHA (30 μ M) treatment resulted in a more prominent increase in oil red O staining (Figure 5B) as expected [47]. For comparison, extensive basal lipid droplet staining was detected in HepG2 cells which have prominent lipid storage [41]. Except for the slight decrease in cell numbers, HUVECs exposed to elevated glucose were indistinguishable from cells grown in normal medium, and with the exception of slightly increased GLUT10 levels, their GLUT transporter mRNA levels were comparable to those in control cells (Figure 5C). No differences were observed in cells cultured with 20 mM L-glucose supplementation (Figure 5D). GLUT1 [48-51] and GLUT3 [52, 53] function is strongly affected by posttranscriptional and posttranslational regulation. Since cells grown in chronically high glucose transported ~20% less deoxy-glucose than control cells (Figure 5E), we have further investigated cell surface and total protein levels of GLUT1 and GLUT3. High glucose-exposed cells contained lower total levels of GLUT3, but not GLUT1 protein (Figure 6A, B). In addition, there was ~25% less GLUT1 on their cell surface, while the plasma membrane levels of GLUT3 remained the same (Figure 6C, D). The cell surface levels of Na, K-ATPase were comparable in all treatments (Figure 6E, F). The combined effect of GLUT1 and GLUT3 protein changes could account for the decrease in the deoxy-glucose uptake in the cells exposed to high glucose levels.

3.5. Effect of high glucose on HUVECs transendothelial transport

Endothelial cells line blood vessels in a polarized manner forming a barrier between blood on the luminal side and surrounding tissues on the abluminal side. To mimic that function, HUVECs were grown on Transwell inserts and developed intercellular junctions resulting in transendothelial electrical resistance

(TEER; 47.4 ± 6.1 , 44.4 ± 4.3 and 22.0 ± 3.9 Ohm/cm² in three separate experiments with 4-6 replicates each). There was a decrease of ~25% in TEER in cells grown in high luminal glucose (Figure 7A), the monolayers became more permeable to 4-kDa dextran when grown in 25.5 mM glucose (Figure 7B), and deoxy-glucose was transported across the cell monolayer with small but significant differences (increase of ~11%, $p=0.004$ for basolateral transport at 20 min; Figure 7C, D), indicating changes in barrier tightness.

4. Discussion

One of the key endothelial functions is to ensure delivery of glucose to the underlying tissues, some of which, including muscle and adipose tissue, are insulin responsive. It is not clear to what extent the endothelium can regulate the transfer of sugars from the blood to the tissues, and it is also uncertain how the rate of transfer is affected by pathophysiological high glucose levels, as found in diabetes, metabolic syndrome and post-prandially. In addition, glucose metabolism in endothelial cells has recently gained increased importance based on its emerging role as one of the driving forces in determining cell fate during angiogenesis (reviewed in [15] and [54]). Extensive glycolysis is a hallmark of endothelial metabolism with GLUT transporters being critical for the glucose uptake. Here we have found HUVEC GLUT transporter mRNA expression qualitatively and quantitatively similar to that in other endothelial cells from capillaries (HDMEC), veins (SVEC) and arteries (HAoEC). The notable exception was very high expression of GLUT12 detected in HDMEC, suggesting a microcirculation-specific function possibly related to the reported GLUT12 insulin responsiveness [55] or distinct physiological characteristics [30, 56]. The relative protein levels of two of the most abundant transporters, GLUT1 and GLUT3, correlated well with the mRNA expression, and both transporters were glycosylated in all cells examined. Based on the apparent molecular weight of the band, GLUT1 in the aortic ECs was more extensively glycosylated with possible implications for its stability or activity, as reported in overexpression studies in CHO cells [50, 51]. GLUT1 is highly abundant in the endothelium [57] and was also quantified by mass spectrometry as the most abundant plasma membrane (PM) transporter in immortalized endothelial cell line hCMEC/D3, at 74.4 ± 4.4 fmol GLUT1/ μ g protein in PM fraction [58]. The same study detected 7.26 fmol GLUT1/ μ g PM protein in HUVECs, while the levels of other GLUT transporters were below detection, confirming the dominant position of GLUT1 in endothelial cells. On the other hand, our study, as well as others [59, 60], identified additional GLUT transporters as relevant for glucose uptake in the endothelium. In particular GLUT3, even if less abundant at the protein level, could significantly contribute to endothelial glucose uptake because of its rapid turnover and high affinity for glucose, especially relevant in hypoglycemic conditions. Similar to GLUT1, GLUT3 is upregulated by HIF-1 and is important for endothelial tube formation in HUVECs [59], and therefore it could participate in hypoxia-driven vasculogenesis. Our observation that deoxy-glucose uptake decreased when either GLUT1 or GLUT3 was knocked down and was further reduced in GLUT1/GLUT3 double knock-down cells confirms involvement of both transporters in the uptake. On the other hand, GLUT6 knock-down even slightly increased deoxy-glucose uptake, suggesting that its primary function is distinct from supplying glucose across the plasma membrane; this could be related to the presence of intracellular localization signal. Up to 38% of the active glucose uptake was mediated by other GLUTs; based on the mRNA levels, the other contributors could be GLUT6, 8 or 10. Several previous studies reported protein, mRNA or both for GLUT4, GLUT5 or SGLT1 in cells or cell lines [33-35 and 61] which were derived from microendothelium, raising a possibility that additional transporters may be present in microvasculature. In agreement, even though GLUT4 mRNA was absent in HUVECs or quite low (<2 copies/ng mRNA) in SVECs and HAoECs, the dermal microendothelial cells contained almost 40 copies/ng mRNA. This suggests specific differences in macro- and microvasculature which could affect the regulation of glucose transport and metabolism, in particular the presence of GLUT4 and GLUT12 implies sensitivity of glucose uptake to insulin signalling. Tightly regulated transport in the microendothelium of the blood-brain barrier could be reflected by further variations in the presence of glucose transporters.

Endothelial cells in the human vasculature rely on a steady supply of glucose to fuel their largely glycolytic metabolism [11-13]. However, both chronically exceeding the physiological concentration of 5 mM and the inability to control large post-prandial glucose spikes ultimately lead to endothelial dysfunction. This is driven by increased intracellular glucose concentration with consequences ranging from increased flux of sugars through the polyol and hexosamine pathways, elevated formation of advanced glycation end products (AGE) and increased pro-inflammatory signalling via Receptor for AGE (RAGE)/NF κ B pathways to diacylglycerol-driven PKC activation and subsequent eNOS impairment, and, most importantly, all-encompassing increased mitochondrial superoxide production (reviewed in [62]). The glucose uptake into the cells ultimately relies on the presence and functionality of glucose transporters. Therefore, we have examined how glucose transporters in HUVECs respond to shear stress, a physiological force acting on the endothelium *in vivo*, and how the transporters are affected by pathological increases in glucose concentration. A recent microarray analysis suggested that in endothelial cell/astrocyte co-cultures of a blood-brain barrier model, shear stress alters mRNA expression of many transporters including some GLUTs [61], but the gene expression was not validated and the impact on the glucose transport activity was not examined. In HUVECs studied here, shear stress application only mildly affected mRNA levels of GLUT3 and GLUT12 but in addition, GLUT1 protein levels were significantly reduced, correlating well with the observed decrease in deoxy-glucose transport. Lower glucose uptake is consistent with the reports of shear stress-induced shift from predominantly glycolytic to more oxidative metabolism in endothelial cells [61, 63 and 64] that has been documented as a decrease in glycolytic enzyme levels and a concomitant decline in lactate production. Chronically elevated glucose levels, which damage endothelium in conditions of uncontrolled diabetes, can lead to increased apoptosis and lower cell viability and proliferation in cell culture [44, 45] (Figure 5A). The glucose uptake in the endothelium under hyperglycaemia has been studied in various models with results showing either no effect or some impairment of the uptake. Hyperglycaemia in the rat model did not affect glucose uptake in the brain [65], and likewise 2d exposure of human aortic EC to increased glucose concentrations had no effect on glucose uptake [66]. However, similar treatment was found to decrease glucose uptake in bovine aortic EC [67]. Bovine brain and rat heart endothelial cells exposed to 30 mM glucose for 1-3 days reduced their glucose uptake while bovine retinal endothelial cells remained quite unresponsive to increased glucose concentration even after 3 days [60], suggesting differences in the response depending on the species or cell origin. We have observed a moderate decrease in deoxy-glucose uptake in the chronically treated mature endothelial monolayers as a result of downregulation of total GLUT3 and cell surface GLUT1, while no major changes in GLUT mRNA expression were detected. Absence of mRNA changes suggests that direct epigenetic modulation, documented for various genes in hyperglycaemia [68], was not relevant for GLUTs in our study. Alternatively, post-translational regulations may involve changes in palmitoylation [69] or glycosylation, which could modify GLUT localization, stability or activity directly or via interactions with other molecules [70, 71]. Another mechanism could involve decrease in the activity of AMPK [72, 73], a known modulator of GLUT1 function. However, the modest downregulation of HUVEC glucose uptake would not be enough to compensate for the several-fold glucose overload indicating that the cells do not have a tight regulation to limit their intracellular glucose concentration, perhaps as a result of high reliance on glucose for their metabolism. Since one of important functions of the endothelial monolayer is allowing sufficient supply of glucose to the surrounding tissues, we have also examined glucose transport across the monolayer. Tightly regulated glucose transport across the blood-brain barrier is known to be mediated by GLUT transporters [74, 75]. However, most of the glucose transendothelial transport in other tissues occurs via the paracellular route [76, 77], even though the specific endothelial contribution to glucose utilization in peripheral tissues is not well established. We have found that the glucose permeability in HUVEC monolayers exposed to high glucose remained high, even slightly increased compared to the control cells, suggesting that they lack mechanisms that would limit the amount of glucose passing across the monolayer. While beneficial under physiological conditions, this could lead to increased amounts of glucose reaching the tissues in conditions of uncontrolled diabetes potentially exacerbating its detrimental effects.

In summary, we have used HUVECs as a model of mature endothelium in the macrovasculature and detected a variety of glucose transporters some of which could have novel functions. The expression levels were comparable to those in other endothelial cells from macrovasculature, but some notable differences were observed for microendothelial cells. The HUVEC transcriptional profile was consistent with absence of fructose uptake and lack of insulin responsiveness, although the exact role of insulin in endothelial glucose uptake has been controversial and could be related to the cell origin [66, 78]. Our study indicates that GLUT1 together with GLUT3 function as the major route responsible for at least 60% of the active glucose uptake in HUVECs and possibly in other endothelial cells, with some overlap in transport activities. In general, the GLUT mRNA expression was quite stable even in the presence of different stresses; but at least for GLUT1 and GLUT3, posttranscriptional or posttranslational regulation contributed to controlling their function, especially in conditions of elevated glucose concentration. On the other hand, the modulation of function was relatively modest, suggesting that regarding sugar uptake or transport across the monolayer, mature endothelium is relatively unresponsive to increases in glucose concentration.

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Conflict of interest

ST, AK and KEP declare that they have no conflicts of interest with the contents of this article, but GW has recently, or currently, received other research funding from Nestle and Florida Department of Citrus, and conducted consultancy for Nutrilite, USA, and Suntory, UK.

FOOTNOTES

The abbreviations used are: AGE, Advanced Glycation End Products; DHA, Docosahexaenoic Acid; eNOS, endothelial Nitric Oxide Synthase; GLUT, Glucose transporter; HAoEC, Human Aortic Endothelial Cell; HDMEC, Human Dermal Microendothelial Cell; HUVEC, Human Umbilical Vein Endothelial Cell; Na, K-ATPase, Sodium Potassium ATPase; PNGase F, Peptide N-Glycosidase F; PM, Plasma Membrane; RAGE, Receptor for Advanced Glycation End Products; SGLT, Sodium-Glucose Linked Transporter; SVEC, Saphenous Vein Endothelial Cells; TEER, Transendothelial Resistance

Figure legends

Fig 1. Glucose transporter expression in endothelial cells. GLUT and SGLT1 mRNA expression in human endothelial cells from umbilical vein (HUVEC), saphenous vein (SVEC), microdermal vessels (HMDEC) and aorta (HAoEC) was quantified using ddPCR with RPL37 as a reference gene (A). Simple (quantitative automated capillary) Western was used to detect GLUT1 (B) and GLUT3 (C) protein with and without deglycosylation by PNGase F. The full arrow designates the glycosylated, undigested GLUT1 or GLUT3, while the dashed arrow indicates the protein in which the N-glycosylation was removed by PNGase F, resulting in a decrease of the apparent molecular mass. The total protein levels of GLUT1 (D) and GLUT3 (E) in HUVECs, SVECs, HDMECs and HAoECs were detected by Simple Western relative to Na, K-ATPase expression levels. For both mRNA and protein analysis of HUVECs and SVECs, n=3. For HDMEC and HAoEC samples, replicates from the same biological material rather than separate biological samples were tested and the error bars represent SD.

Fig 2. Lane and electropherogram view for detection of GLUT1 and GLUT3 protein in different endothelial cells by Simple Western. Optimized concentration of GLUT1 (A) or GLUT3 (B) antibody was used to detect GLUT1 and GLUT3 in HUVEC, SVEC, HDMEC and HAoEC samples, Na, K-ATPase (labelled by *) was detected as a control. Protein concentration of the applied lysate was 0.1 (SVEC) or 0.2 mg/ml (HUVEC, HDMEC and HAoEC) for GLUT1 and 0.2 (HUVEC and SVEC) or 0.4 (HDMEC and HAoEC) mg/ml for GLUT3 and was within the linear range of detection (0.025-0.4 mg/ml).

Fig 3. Sugar uptake in HUVECs. The uptake of deoxy-glucose (closed bars) and fructose (open bars) in HUVECs was measured using ¹⁴C-labelled monosaccharides and normalized to the number of cells from wells seeded in parallel (n=10 for both) (A). Similarly, glucose uptake in HUVECs was determined using ¹⁴C-glucose in the presence or absence of 100 μM phloridzin (n=6) (B). Short-term insulin treatment increased glucose uptake in muscle cells, but not in HUVECs (n=9) (C). To establish the role of GLUT1 and GLUT3 in deoxy-glucose uptake, specific siRNA was transfected into HUVECs to knock down GLUT1 (n=3) (D), GLUT3 (n=4) (E), GLUT6 (n=3) (F) or a combination of these (n=3) (E, F). Down-regulation of GLUT1 and GLUT3 protein was confirmed by Simple Western (G); the detected protein levels are representative of three independent experiments, with a knock-down efficiency about 90% for GLUT1 and 70% for GLUT3. The deoxy-glucose uptake in control and GLUT siRNA-transfected cells was measured after 10 min incubation with the ¹⁴C-labelled monosaccharide (H) (n=10 for GLUT1, n=12 for GLUT3 and GLUT6, n=9 for GLUT1/GLUT3 knock-down and n=8 for the triple knock-down). To inhibit GLUT-mediated uptake, 10 μM cytochalasin B was included during the 10-min incubation (n=8) (H).

Fig4. The effect of shear stress on glucose transporter expression and function in HUVECs. GLUT mRNA expression in HUVECs grown in the presence (closed bars) or absence (open bars) of shear stress for 24 h was determined by dd PCR using RPL37 as a reference gene and eNOS as a positive control known to be upregulated by shear stress (A); n=3 except for GLUT3 and GLUT12 (n=5). GLUT1 and GLUT3 protein levels in control (open bars) and shear stress-exposed (closed bars) cells were analyzed by Simple Western and normalized to Na, K-ATPase expression (B) (GLUT1 n=10, GLUT3 n=6). Deoxy-glucose uptake in cells exposed to shear stress was compared to the control cells using the ¹⁴C-deoxy-glucose assay with normalization to the number of cells (C) (n=6).

Fig 5. The effects of chronic exposure of HUVECs to increased D-glucose levels on cell health, viability and glucose transport. Cell numbers were determined for HUVECs cultured for 5 days in 5.5 mM D-glucose, 25.5 mM D-glucose or 5.5 mM D-glucose with added 20 mM L-glucose (A). Neutral lipids were quantified with Oil red O staining and extraction in cells cultured for 5 days in 25.5 mM glucose or 30 μM DHA (closed bars) and compared to control conditions (open bars) (B); n=9, except for DHA control where n=6. HepG2 cells are also shown for comparison (n=6). GLUT mRNA expression

in cells grown for 6 days in 5.5 mM (open bars) or 25.5 mM glucose (closed bars) was quantified by ddPCR (C) (n=3, except for GLUT6 and GLUT10 where n=5). Control experiment with cells cultured for 6 days in normal (5.5 mM D-glucose; open bars) or with 20 mM L-glucose (grey bars) supplemented medium did not show any differences in mRNA expression of selected GLUTs (D). Deoxy-glucose uptake in HUVECs cultured in media with different glucose or mannitol concentrations was measured using ^{14}C -deoxy-glucose uptake with normalization to the number of cells (E).

Fig 6. GLUT1 and GLUT3 protein expression changes in HUVECs cultured in the presence of 25.5 mM glucose. Total protein levels of GLUT1 (A) and GLUT3 (B) in HUVECs grown in 5.5 or 25.5 mM glucose were measured by Simple Western and normalized to Na, K-ATPase expression. Cells were also exposed to extracellular biotinylating agent and relative cell surface levels of GLUT1 (C) and GLUT3 (D) were determined as the biotinylated fraction of the total GLUT1 or GLUT3 amount. As a control, cell surface levels of Na, K-ATPase were determined in the same samples used for GLUT1 (E) or GLUT3 (F) quantification. For all samples n=5.

Fig 7. Chronic exposure of HUVECs grown on permeable supports to increased glucose concentrations affects glucose transport as well as endothelial permeability. TEER was measured for HUVECs cultured for 6 days on 12-well Transwell filters with 5.5 or 25.5 mM luminal glucose (A) (n=16). Subsequently, the permeability to 4-kDa FITC-labelled dextran applied on the luminal side was determined from fluorescence increase between 10-20 min in the abluminal medium (B) (n=12). The transport of deoxy-glucose was studied in HUVECs grown for 6 days on 12-well Transwell filters in the presence of 5.5 mM or 25.5 mM glucose on the luminal side (C). ^{14}C -deoxy-glucose was applied to the luminal side and the amount of radioactivity in the cells and in the luminal and abluminal medium was measured after 10, 20, 30 and 60 min (n=9). Significant increase of deoxy-glucose transport was observed at 20 min in cells cultured in 25.5 mM glucose (closed bars) compared to the control (open bars) (D). In all experiments, the abluminal glucose concentration during cell culturing was 5.5 mM and the medium was changed daily.

Table 1: Cell surface-biotinylated GLUT1 and GLUT3 fraction quantified by Simple Western and corresponding value for biotinylated Na, K-ATPase determined from the same samples

Detected	Biotinylated GLUT %	Biotinylated Na, K-ATPase %
GLUT1	6.05±0.60	5.53±0.50
GLUT3	2.95±0.83	5.91±0.33

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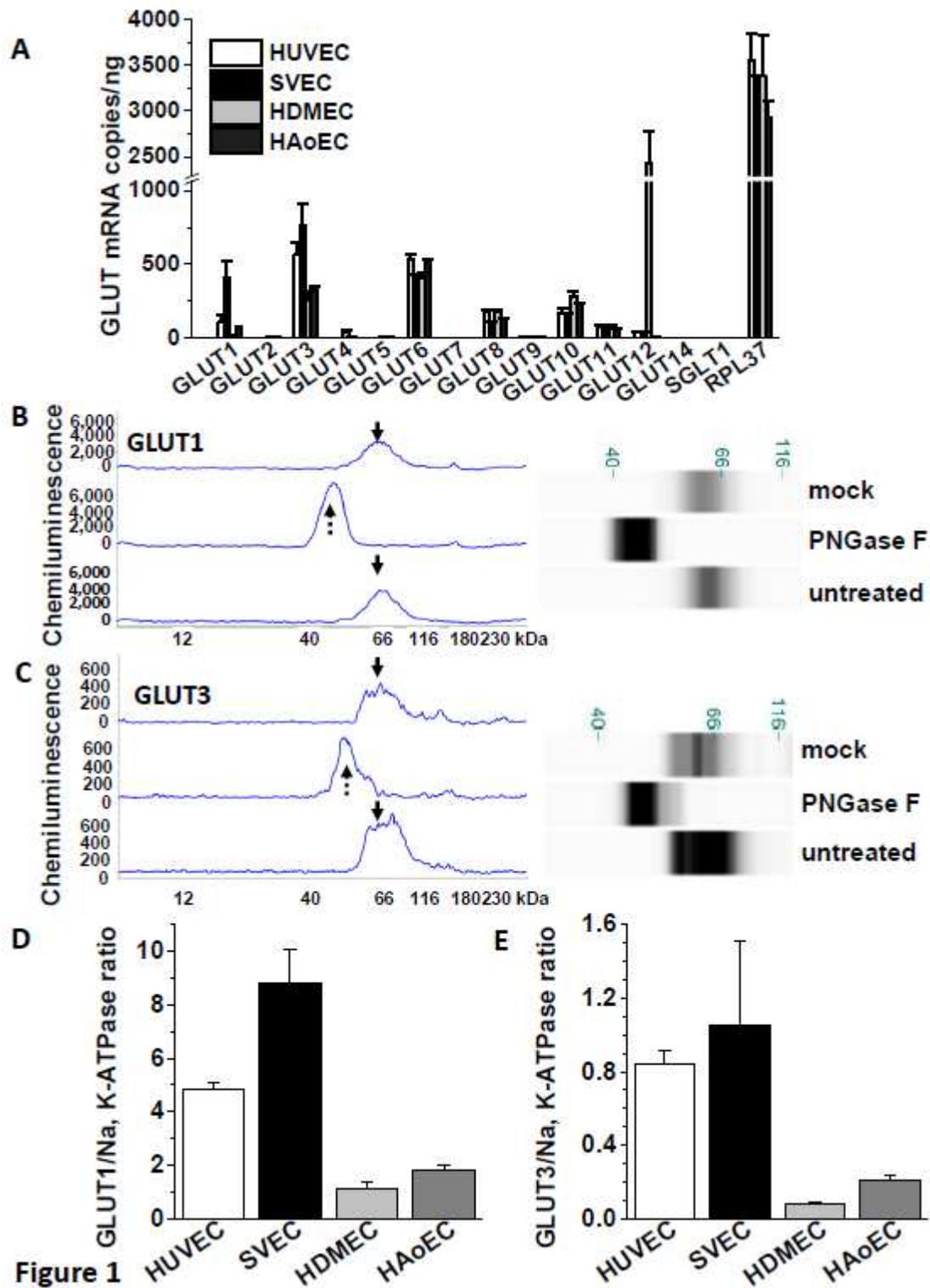
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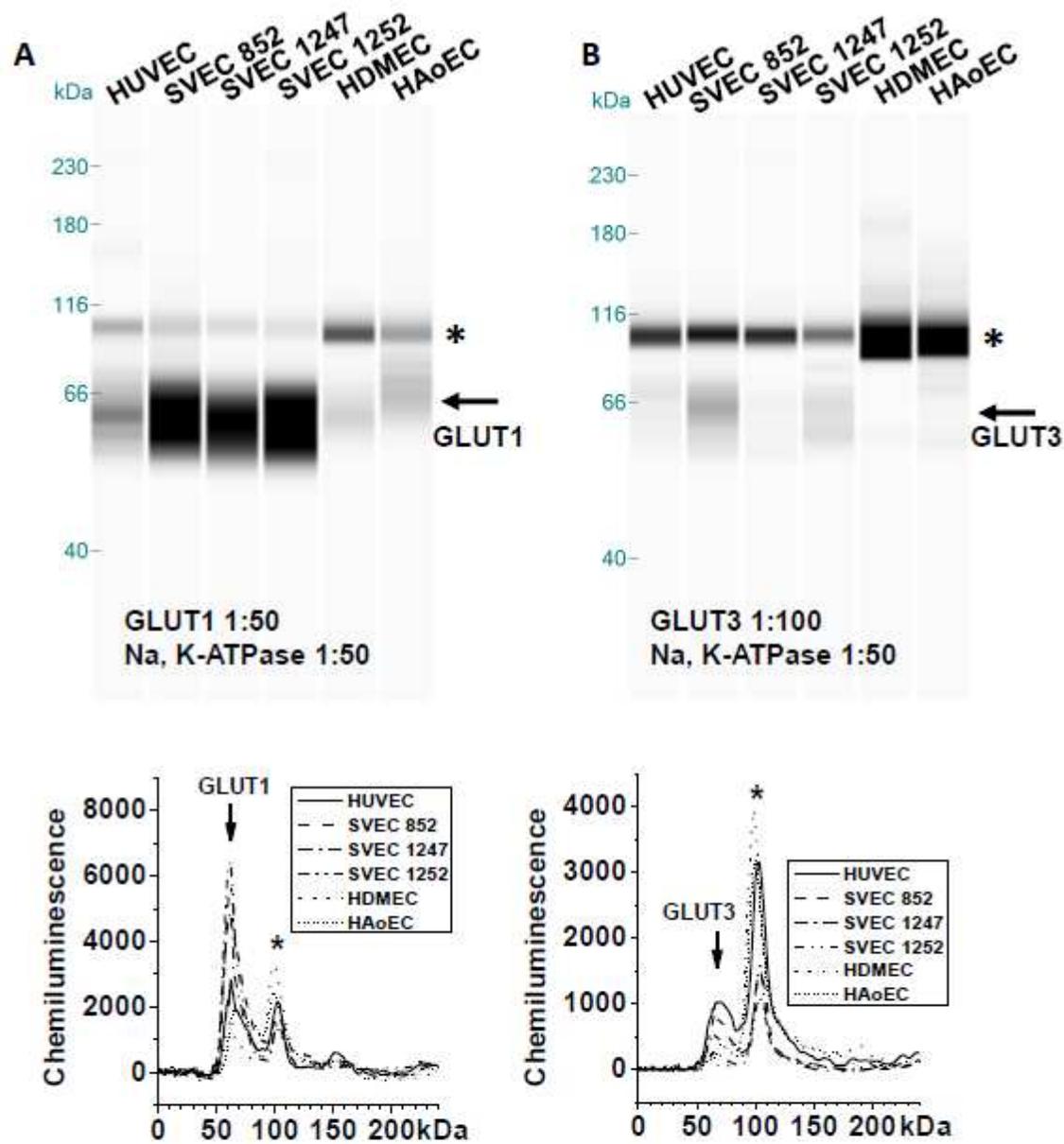


Figure 2

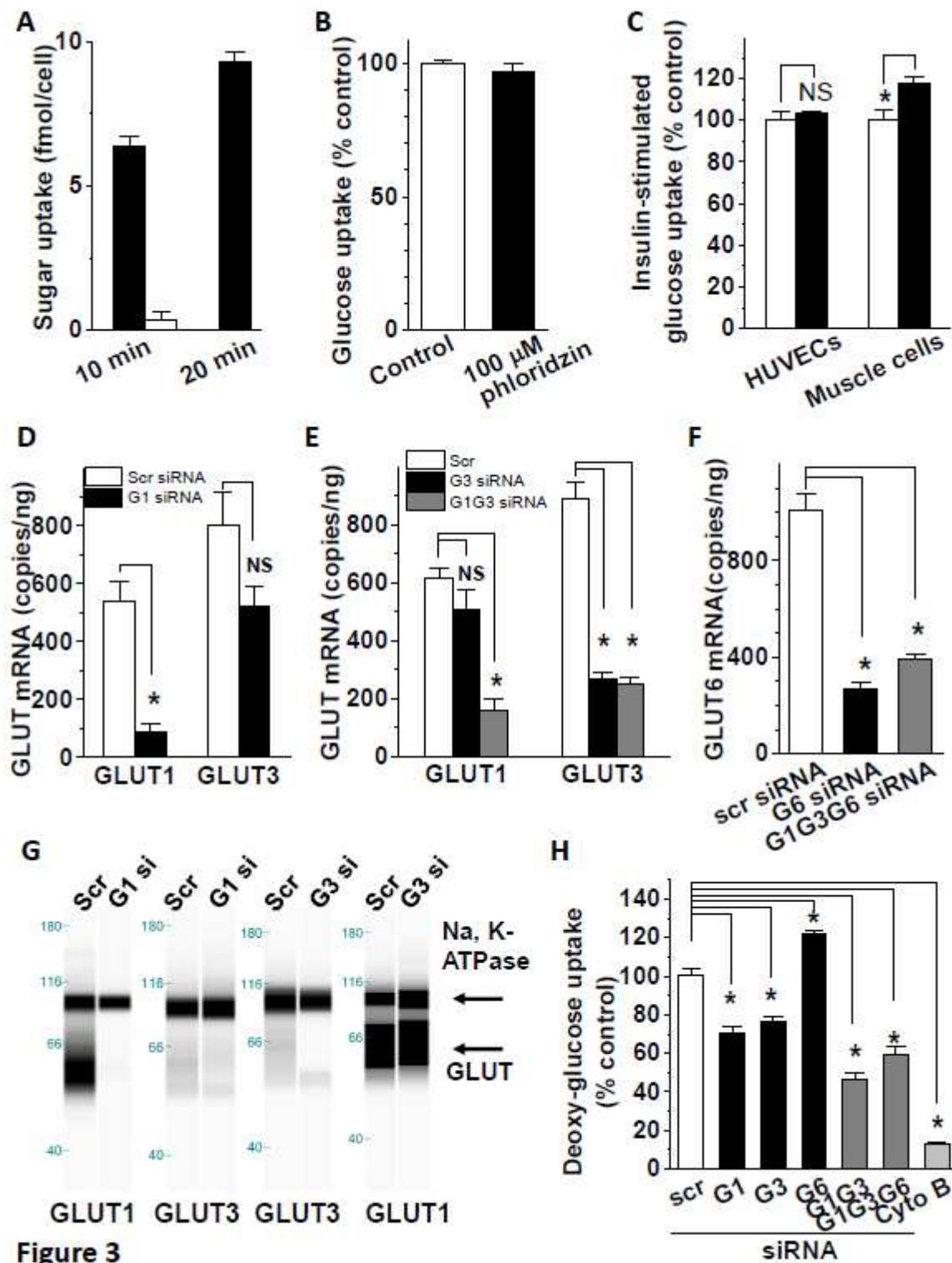


Figure 3

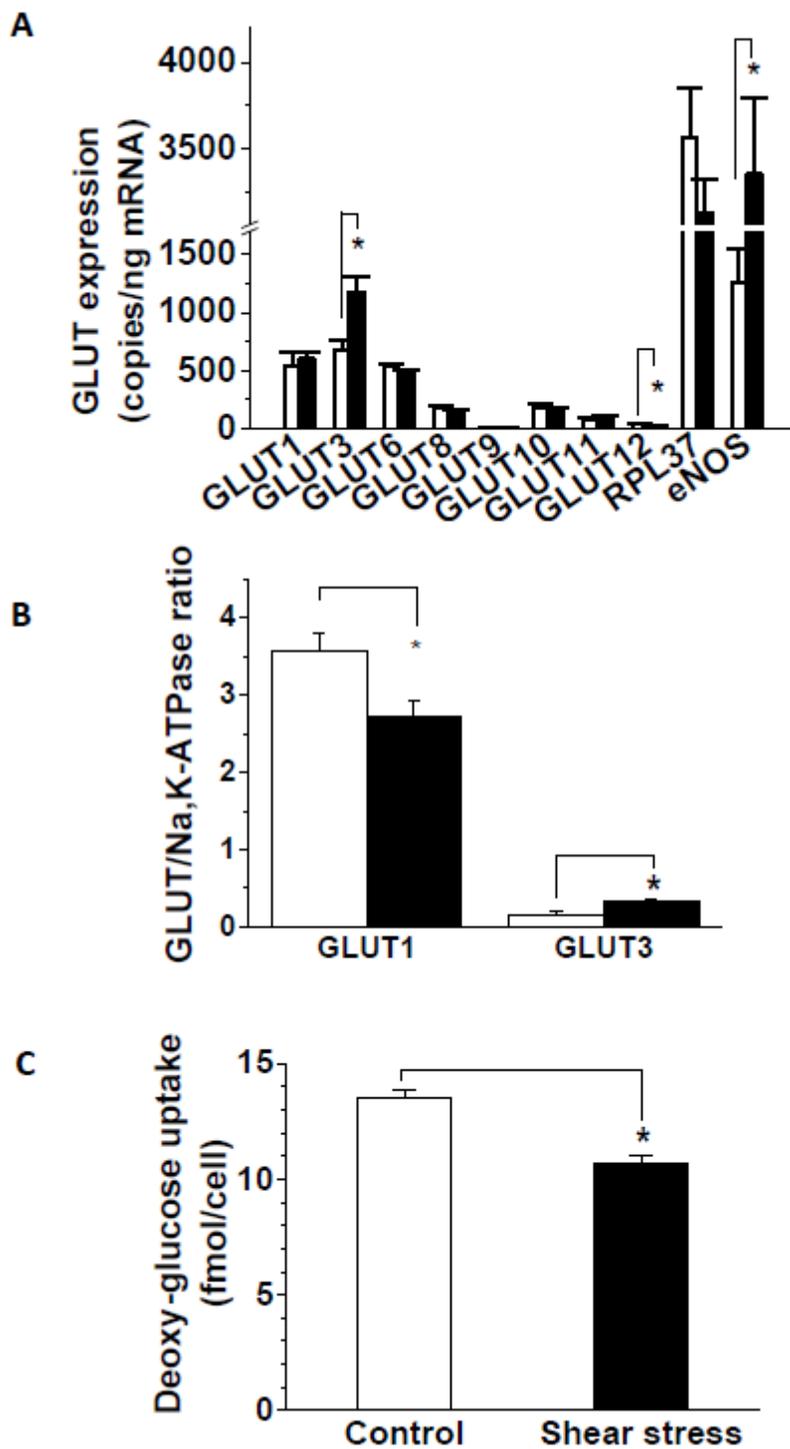
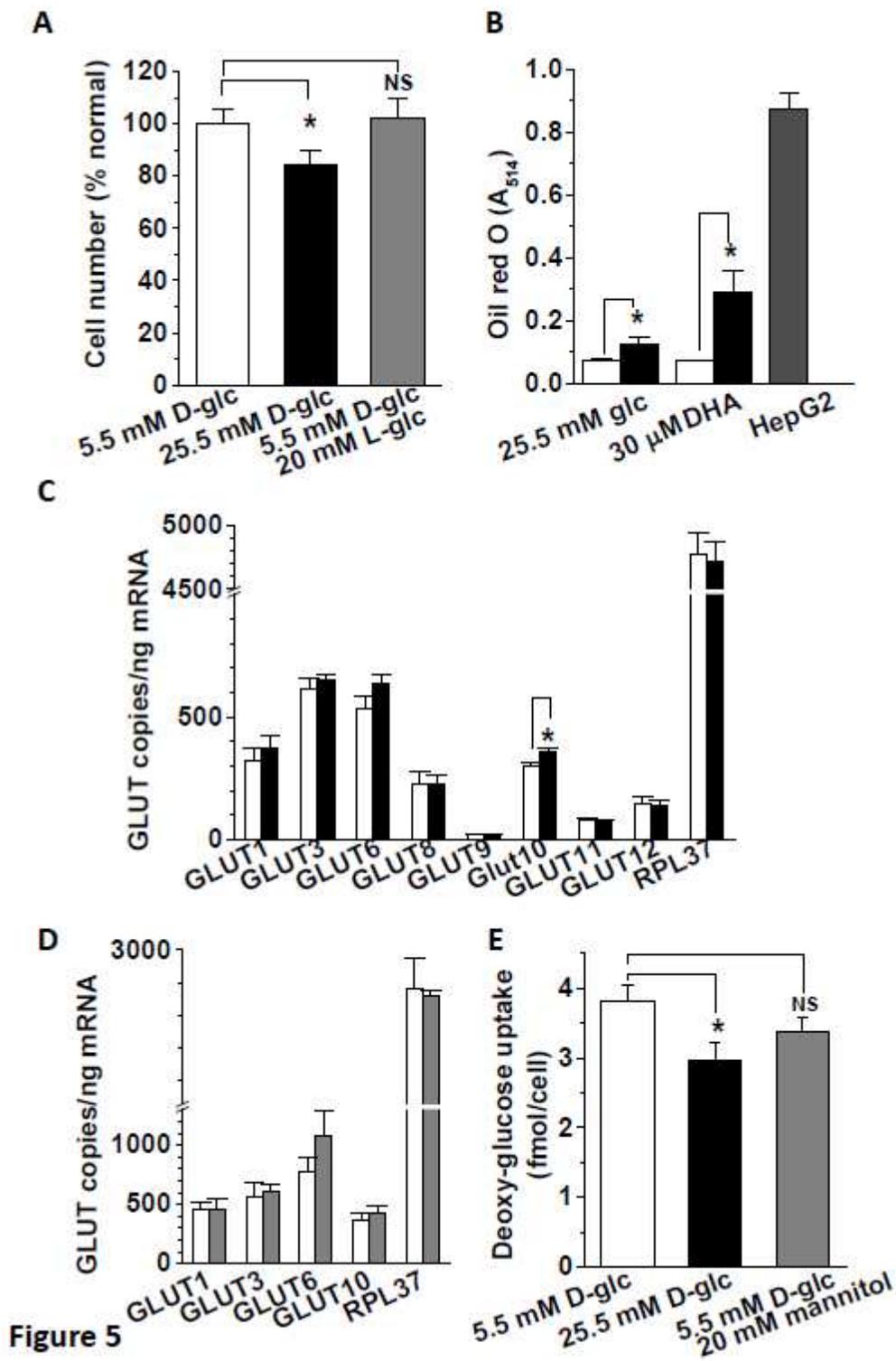


Figure 4



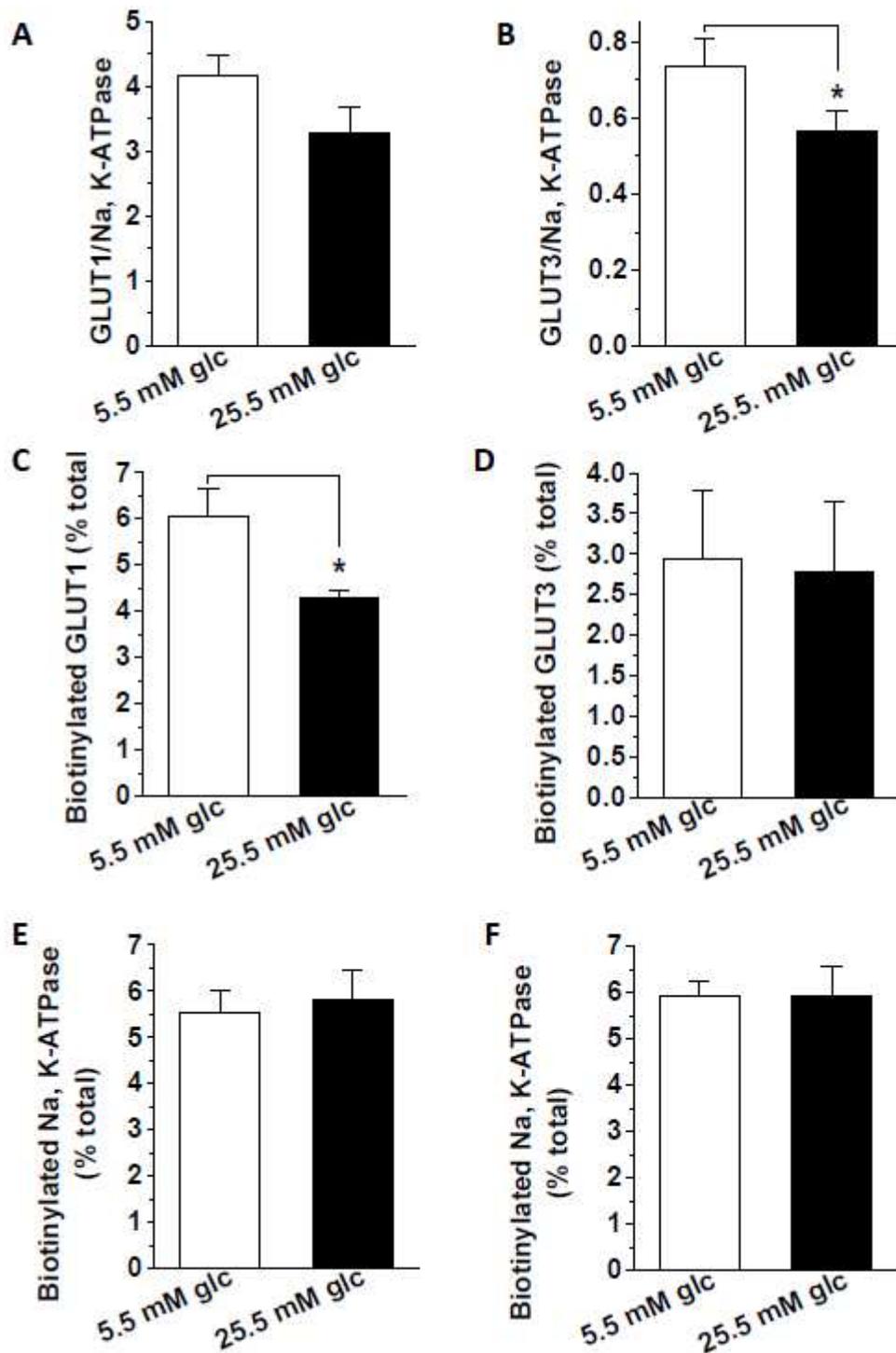


Figure 6

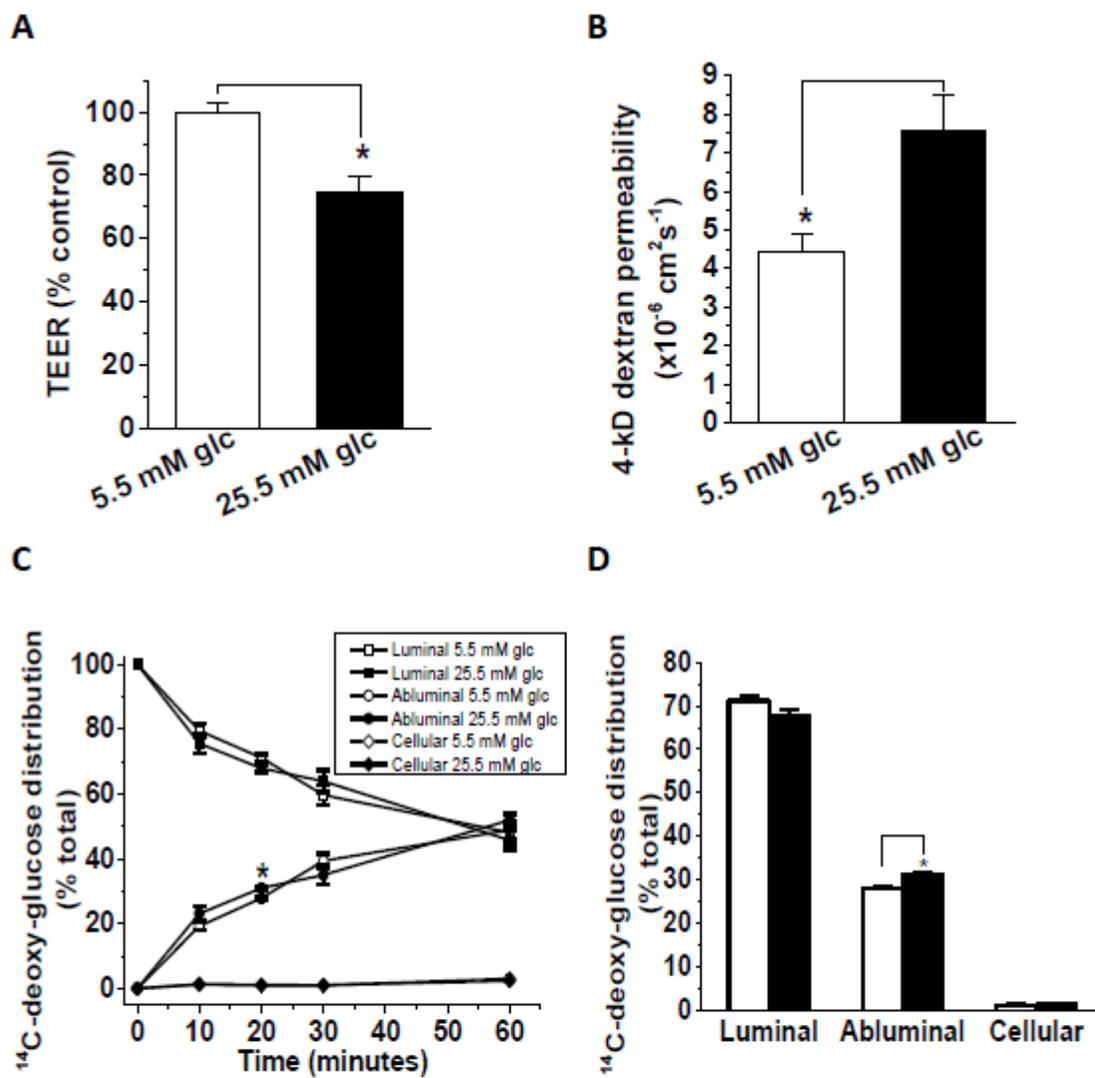
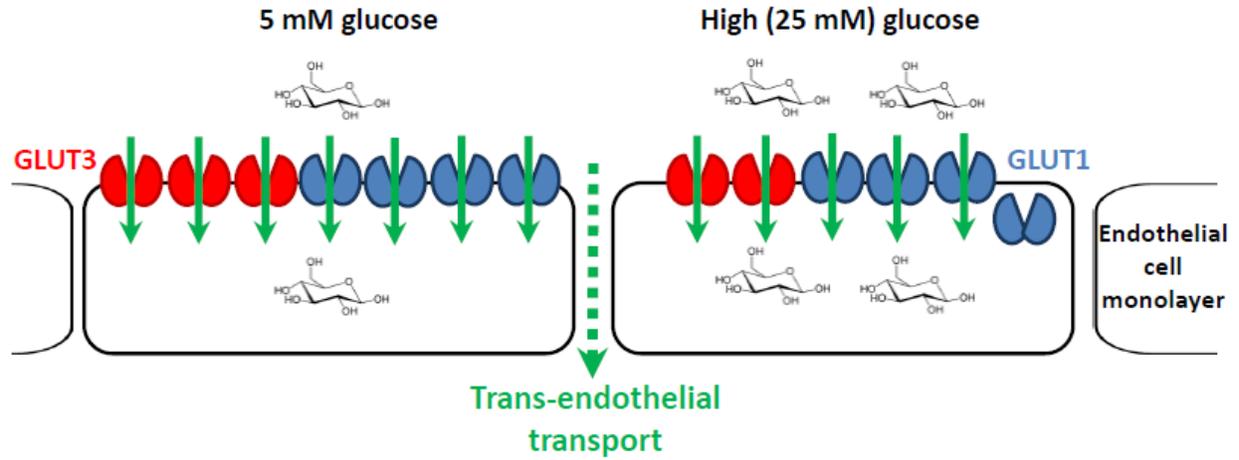


Figure 7



Graphical abstract

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