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High glucose causes dysfunction of the human glomerular endothelial glycocalyx

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Singh A, Fridén V, Dasgupta I, Foster RR, Welsh GI, Tooke JE, Haraldsson B, Mathieson PW, Satchell SC. High glucose causes dysfunction of the human glomerular endothelial glycocalyx. *Am J Physiol Renal Physiol* 300: F40–F48, 2011. First published October 27, 2010; doi:10.1152/ajprenal.00103.2010.—The endothelial glycocalyx is a gel-like layer which covers the luminal side of blood vessels. The glomerular endothelial cell (GEnC) glycocalyx is composed of proteoglycan core proteins, glycosaminoglycan (GAG) chains, and sialoglycoproteins and has been shown to contribute to the selective sieving action of the glomerular capillary wall. Damage to the systemic endothelial glycocalyx has recently been associated with the onset of albuminuria in diabetics. In this study, we analyze the effects of high glucose on the biochemical structure of the GEnC glycocalyx and quantify functional changes in its protein-restrictive action. We used conditionally immortalized human GEnC. Proteoglycans were analyzed by Western blotting and indirect immunofluorescence. Biosynthesis of GAG was analyzed by radiolabeling and quantified by anion exchange chromatography. FITC-albumin was used to analyze macromolecular passage across GEnC monolayers using an established in vitro model. We observed a marked reduction in the biosynthesis of GAG by the GEnC under high-glucose conditions. Further analysis confirmed specific reduction in heparan sulfate GAG. Expression of proteoglycan core proteins remained unchanged. There was also a significant increase in the passage of albumin across GEnC monolayers under high-glucose conditions without affecting interendothelial junctions. These results reproduce changes in GEnC barrier properties caused by enzymatic removal of heparan sulfate from the GEnC glycocalyx. They provide direct evidence of high glucose-induced alterations in the GEnC glycocalyx and demonstrate changes to its function as a protein-restrictive layer, thus implicating glycocalyx damage in the pathogenesis of proteinuria in diabetes.

proteoglycans; glycosaminoglycan; heparan sulfate; proteinuria

THE GLOMERULAR CAPILLARY WALL is the primary site of sieving of proteins in the kidney and is known as the glomerular filtration barrier (GFB). It is selectively permeable, allowing the free passage of water and small solutes but remaining relatively impermeable to macromolecules including albumin (9). Disturbance of this function leads to albuminuria, a hallmark of a number of kidney diseases, including diabetic nephropathy (33). The permselective properties of the GFB are the result of the characteristics of its three components: podocytes, glomerular basement membrane (GBM), and fenestrated glomerular endothelial cells (GEnC) (17). The contribution of the GBM and podocytes to permselectivity is well described, but the importance of GEnC has only recently been more fully appreciated (3, 44), especially after communications between podocytes and GEnC have been shown to be crucial for the normal function of the GFB (11). Nevertheless, our understanding of the precise mechanisms underlying the function of the GFB, and in particular the role of GEnC, is still far from complete.

GEnC fenestrations are transcellular pores 60–100 nm in diameter and are essential for the high hydraulic conductivity across the GFB necessary for filtration (41). Owing to the large size of these fenestrae relative to the size of plasma proteins (albumin: $4 \times 4 \times 8$ nm), the "empty" GEnC appear inadequately equipped to offer resistance to plasma protein passage. Therefore, other components of the GFB, i.e., podocytes and the GBM, have been the primary focus of experimental investigations. Recently, however, we and others have demonstrated that GEnC possess on their luminal side a 200- to 400-nm-thick layer of the glycocalyx which covers the fenestrae and has an important barrier function (21, 23 37, 45).

The glycocalyx is a hydrated, gel-like structure formed by proteoglycan core proteins, glycosaminoglycan (GAG) chains, and sialoglycoproteins along with adsorbed plasma proteins and forms an exclusion zone to the capillary blood flow (34). Heparan sulfate (HS) and chondroitin sulfate represent the majority of the sulfated GAG chains in the endothelial glycocalyx. Studies on systemic (nonglomerular) vessels provide evidence for a role of the endothelial glycocalyx in inflammation (46), atherosclerosis (32), ischemia reperfusion injury (38), regulation of the cellular microenvironment (4), and permeability to macromolecules (20). We have previously demonstrated the contribution of the GEnC glycocalyx, particularly its GAG chains, to the macromolecular sieving properties of the intact GFB in animal models (17, 22, 23). More recently, we detailed the biochemical characteristics of the human GEnC glycocalyx in vitro (5) and confirmed the protein-restricting ability of the glycocalyx in cultured GEnC monolayers by manipulation of its structural components (45). In the latter study, we showed that removal of specific carbohydrate components from the GEnC glycocalyx lead to disruption of its albumin-restricting ability. This appeared to be a

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direct consequence of the specific damage to the GEnC glycocalyx, as the alternative pathways via the interendothelial junctions remained unaffected.

Damage to the systemic endothelial glycocalyx has been implicated in the initiation of vascular complications of diabetes (30, 31). This observation could be potentially relevant to the glomerular microcirculation in the pathogenesis of diabetic microalbuminuria (44). Therefore, in the current study we analyzed the effects of high glucose on biosynthesis of key proteoglycan components of the GEnC glycocalyx and used previously established in vitro models to assess endothelial junctional integrity and passage of albumin across GEnC monolayers. We aimed to test the hypotheses that a high-glucose milieu directly alters the structural components of the GEnC glycocalyx and that these alterations have implications for the barrier to albumin.

METHODS

GEnC culture. We used a normal human conditionally immortalized GEnC line as described in detail previously (43). Briefly, primary culture GEnC were exposed to separate retroviral vectors transducing a temperature-sensitive mutant of SV40 large T antigen and the catalytic subunit of human telomerase. At the permissive temperature of 33°C the tsSV40LT transgene is activated, causing cell proliferation (without telomere shortening), while at 37°C the transgene is inactivated, rendering cells nonproliferative and quiescent. Conditionally immortalized GEnC were used for experiments after they were maintained at the nonpermissive temperature for 7 days. GEnC were cultured in endothelial growth medium-2 microvascular (EGM2-MV, Cambrex, Wokingham, UK) containing fetal calf serum (5%) and growth factors as supplied with the exception of VEGF.

High-glucose milieu. The endothelial medium used for culture contained 5.5 mM D-glucose. This was taken to represent a normal glucose concentration and provided an appropriate control concentration. To control for the osmotic effect of high glucose, an osmotic control was incorporated in the control media. This was done by addition of 20 mM L-glucose in all experiments except for GAG biosynthesis, where isosmolar mannitol was used. High-glucose medium contained 25.5 mM glucose and was made by adding 20 mM D-glucose to the basic endothelial medium (already containing 5.5 mM D-glucose). Cells were cultured under normal- or high-glucose conditions for 2, 7, and 14 days before they were used for experiments.

Phase-contrast microscopy. Morphology of GEnC monolayers was examined by phase-contrast microscopy after high glucose and compared with monolayers exposed to media containing normal glucose. All images were acquired using a digital camera (Nikon Coolpix E 4500; Nikon UK, Surrey, UK).

Metabolic labeling and extraction of proteoglycans. Metabolic labeling of GAG was achieved by adding 50 μCi/ml [S^{35}]sulfate and 20 μCi/ml D-[3-^{3}H]glucosamine (Amersham Biosciences). GEnC were cultured in normal- and high-glucose media for 12 days before radiolabeling with media changes three times per week. On *day 12*, both [S^{35}]sulfate and [3-^{3}H]glucosamine were added to the respective media for 48 h. Medium was collected and filtrated through a 0.22-μm filter (Millipore). Cells were extracted in RIPA lysis buffer containing 10% (vol/vol) Triton X-100, 10% (m/vol) SDS, 10% (m/vol) sodium deoxycholate, and Complete Mini Protease Inhibitor (Roche) in PBS, and filtrated through a 0.22-μm filter. Protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL).

Isolation of proteoglycans. A HiTrap DEAE FF (1 ml; Amersham Biosciences) column connected to a äKTA FPLC (Amersham Biosciences) was equilibrated with 6 M urea, 0.5 M NaOAc, pH 5.8, 5 μg/ml bovine albumin, and 0.1% Triton X-100 (equilibration buffer). The sample was applied to the column at 1 ml/min and subsequently merged with the column matrix for 15 min. The column was then washed successively with 10 ml of three different buffers, at 10 ml/min: *1*) equilibration buffer; *2*) 6 M urea, 10 mM Tris, pH 8.0, 5 μg/ml bovine albumin, 0.2% Triton X-100; and *3*) 50 mM Tris, pH 7.5. The bound proteoglycans were eluted with 4 M guanidine-HCl, 50 mM NaOAc, pH 5.8, 5 μg/ml bovine albumin, 0.2% Triton X-100, and collected in 1-ml fractions. All fractions were quantified by liquid scintillation counting on a Beta Counter LS6500 (Beckman Coulter) with Ready Safe cocktail (Beckman Coulter).

Immunofluorescence. Cells were seeded on glass coverslips and cultured for 24 h before exposure to either control or high-glucose media as above and fixed in 2% paraformaldehyde for 10 min. For VE-cadherin only, cells were permeabilized with 0.01% Triton X-100 for 10 min. Thereafter, coverslips were incubated directly in the blocking solution (5% FCS and 0.05% Tween 20 in PBS) followed by antibody incubation for HS (HepSS-1, US Biologicals, Swampscott, MA) or VE-cadherin (Santa Cruz Biotechnology). Primary antibody binding was detected using FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for VE-cadherin or Alexa Fluor 488 for HS (Molecular Probes). Nuclear staining was demonstrated using 4,6-diamidino-2-phenylindole (DAPI). Control cells were incubated with secondary antibodies only. Coverslips were examined using a Leitz DMRB fluorescence microscope (Leica, Solms, Germany). Fluorescence intensity of images from independent experiments (for HS) was quantified using Image J software (downloaded from National Institutes of Health website: http://rsbweb.nih.gov/ij/).

Cell survival assay. This was performed using a commercially available WST-1 assay kit (Roche, Germany). GEnC were cultured in 24-well plates in normal- and high-glucose media as previously described. Control GEnC were cultured in normal glucose. GEnC were incubated with 1:10 dilution of WST-1 reagent for 2 h. Two hundred microliters of media from each well was pipetted into a 96- well plate and analyzed on a spectrophotometer at 420–480 nm.

Western blotting. GEnC exposed to high glucose as above were lysed in Laemmli sample buffer, and protein concentrations were determined as above. Samples were separated by SDS-PAGE and were blotted onto nitrocellulose or polyvinylidene difluoride (Immobilon-P, Millipore) membranes. The membranes were blocked in 5% fat-free milk before incubation with antibodies to syndecan-1, syndecan-4, perlecan (all Zymed Laboratories, San Francisco, CA), glypican-1 (Santa Cruz Biotechnology), versican (R&D Systems, Morrisville, NC), and actin to confirm loading of comparable amounts of protein in each lane. After incubation with secondary antibodies, bands were detected by using Supersignal West Femto maximum sensitivity chemiluminescence substrate (Pierce) followed by imaging using the MultiDoc-It imaging System (UVP, Upland, CA).

Measurement of transendothelial protein passage. GEnC were seeded on polycarbonate supports (0.4-μm pore size, 0.5-cm^2 surface area) in tissue culture inserts (1-cm diameter; Nalge Nunc International, Rochester, NY) and treated with high-glucose or control media as above. We analyzed the transendothelial passage of albumin after 2, 7, and 14 days of incubation in high-glucose media. Transendothelial permeability to macromolecules was assessed by measuring passage of FITC-labeled BSA (Sigma) across the monolayer using tissue culture inserts as described previously (45). Briefly, the medium in the insert was replaced with 500 μl of serum-free medium (SFM) containing 0.5 mg/ml FITC-labeled BSA; that in the well was replaced with 500 μl of SFM containing 0.5 mg/ml unlabeled BSA (Sigma). At 1, 2, and 3 h, 100-μl aliquots were removed and replaced with 100 μl of SFM containing unlabeled BSA (0.5 mg/ml). The fluorescence of the aliquots was measured as above, and the amount of FITC-BSA passing through the monolayer was calculated by reference to a set of standard dilutions.

Measurement of transendothelial electrical resistance. Transendothelial electrical resistance (TEER) was measured using an automated bioimpedance-sensing system (ECIS; Applied Biophysics) as described previously (45). GEnC were seeded in 8-well, 10-electrode well arrays (8W10E) supplied by the manufacturer. The resistance is reported in ohms, and the measurement from each well, at a given time point, is an average of the recordings from 10 electrodes.

RNA isolation. GEnC were cultured under normal- and high-glucose conditions, and experiments were performed after 2, 6, and 48 h of treatment. Total RNA was extracted from cells using an RNeasy Minikit with DNase I treatment, according to the manufacturer's protocol (Qiagen, Hilden, Germany). The RNA quality was determined with an Agilent 2100 bioanalyzer and RNA Nano LabChip kit (Agilent Technologies, Waldbronn, Germany). The RNA concentration was determined with a Nanodrop 1000 Spectro-photometer (ThermoFisher Scientific).

RT and real-time PCR. Reverse transcription of RNA was performed with TaqMan Reverse Transcription Reagents (P/N: N8080234, Applied Biosystems, Foster City, CA). Samples not treated with retro-transcriptase were included to exclude genomic DNA contamination. The RT step was carried out at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min on a GenAmp PCR System 2700 (Applied Biosystems).

The mRNA levels of target genes were quantified by real-time PCR using TaqMan Universal PCR Master Mix (P/N: 4304437, Applied Biosystems) with the ABI Prism 7900 sequence detection system (Applied Biosystems). The standard curve method for relative quantification was used to calculate the difference in gene expression between the groups. Human β-actin (P/N: 4310881E, Applied Biosystems) was used as an endogenous control to normalize target mRNA values for differences in the amount of total RNA added to each reaction. All samples were analyzed in triplicate real-time PCR and carried out in a mix of 100 ng of cDNA. Four inventoried human TaqMan Gene Expression Assays (P/N: 4331182, Applied Biosystems) that contain a predeveloped primer/probe mix were utilized in this study. The following gene expression assays were evaluated: exostosis 1 (exostoses 1; Hs00609163_m1); exostoses 2 (EXT-2; Hs00181158_m1); *N*-deacetylase/*N*-sulfotransferase (NDST-1; Hs00155454_m1); and chondroitin sulfate synthase 1 (CHSY1; Hs00208704_m1).

Statistical analyses. The Graph Pad Prism-4 statistical software package (Graph Pad Software San Diego, CA) was used for all analyses, including SE and ANOVA. $P < 0.05$ was taken to indicate statistical significance.

RESULTS

High-glucose reduces biosynthesis of GEnC-associated GAG chains. Analysis of incorporated [3-^{3}H]glucosamine into GEnC GAG chains revealed that exposure to high glucose for 14 days caused a consistent reduction across the full range of fractions, separated according to anionic charge (Fig. 1*A*, *top*). These differences were confirmed to be significant when cumulative data were analyzed from all the fractions, showing a 57% reduction in incorporated [3-^{3}H]glucosamine after high glucose (Fig. 1*A*, *bottom*). This result suggests a reduction in biosynthesis of both sulfated and nonsulfated GAG chains (as glucosamine is a component of the disaccharide chain of GAG "backbone"). Analysis of S^{35} incorporated into the GEnC GAG chains revealed similar reduction with high glucose (Fig. 1*B*, *top*), which was confirmed by data pooled from all isolated

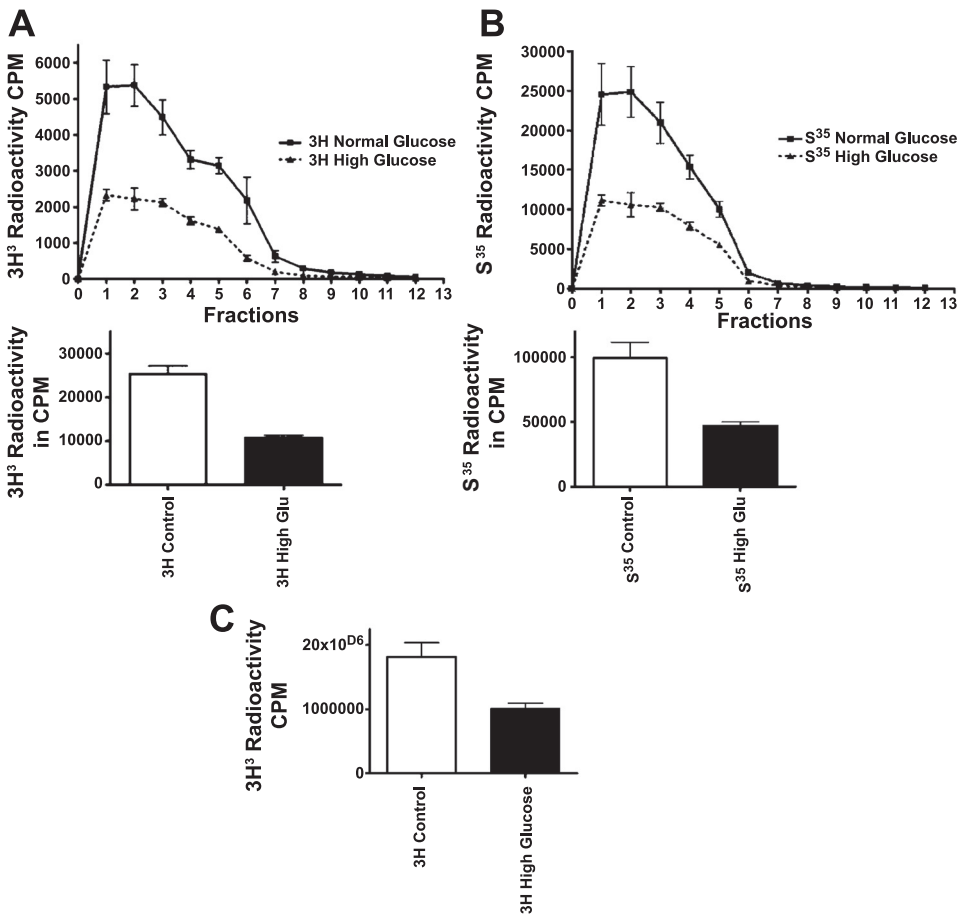


Fig. 1. *A*, *top*: graph showing profiles of [3-^{3}H]glucosamine activity in the glycosaminoglycan (GAG) fractions isolated by anion exchange chromatography from glomerular endothelial cells (GEnC) cultured under normal- vs. high-glucose conditions. Results show a stunted profile after treatment with high glucose for 14 days ($n = 5$; $P < 0.05$). *Bottom*: chart showing comparison of cumulative [3-^{3}H]glucosamine fractions (area under the curve) obtained from the data in the *top* graph ($n = 5$; $P < 0.05$). These results signify a marked reduction in the biosynthesis of total (sulfated plus nonsulfated) GAG chains on GEnC surface after exposure to high glucose. *B*, *top*: graph showing profiles of the radioactive S^{35} activity in the fractions containing isolated GAG from GEnC cultured under normal- vs. high-glucose conditions. Results show a stunted profile (similar to [3-H^3]glucosamine) after treatment with high glucose for 14 days ($n = 5$; $P < 0.05$). *Bottom*: chart showing comparison of cumulative S^{35} activity in the fractions (area under the curve) obtained from the data in the *top* graph ($n = 5$; $P < 0.05$). These results signify a marked reduction in the biosynthesis of GEnC-associated sulfated GAG chains after high glucose. *C*: chart showing comparison of the cumulative [3-^{3}H]glucosamine GAG fractions in the conditioned media obtained from GEnC cultured under normal- vs. high-glucose conditions. Results show marked reduction in the [3-^{3}H]glucosamine in the conditioned media from GEnC cultured under high glucose ($n = 5$; $P < 0.05$) This result confirms reduced biosynthesis of secreted GAG chains.

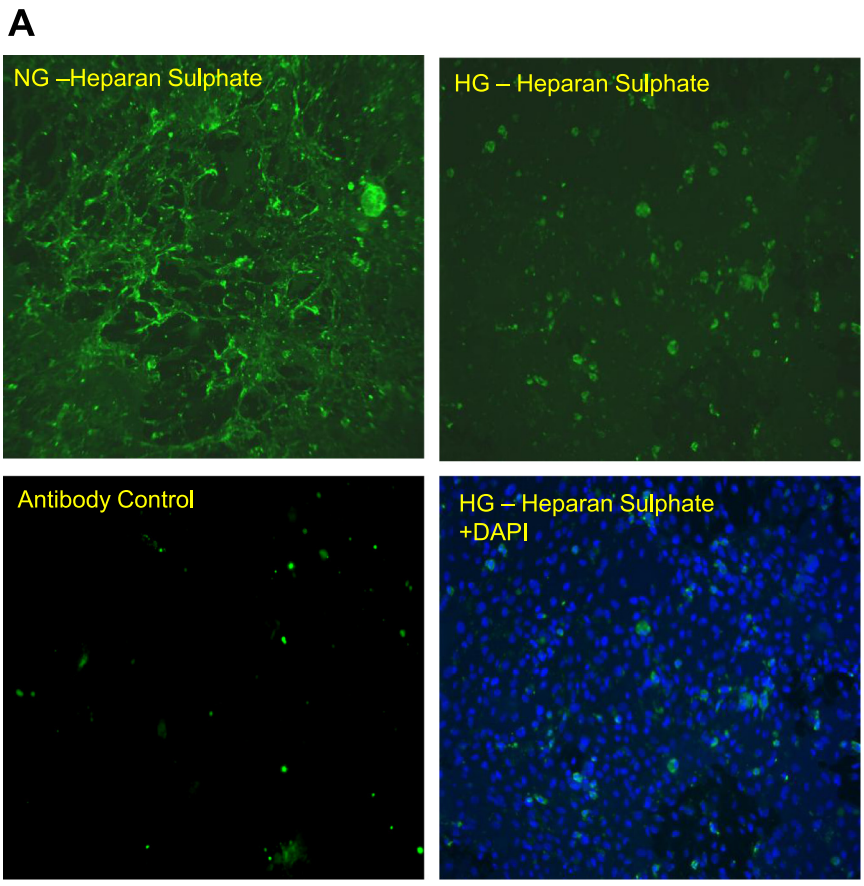
fractions. This confirmed a significant reduction in the biosynthesis of sulfated GAG chains by 52% (Fig. 1*B*, *bottom*).

Furthermore, incorporation of [3-^{3}H]glucosamine were analyzed in the GAG fractions isolated from the cell supernatant to test whether the previously observed reduction in cell-associated GAG was due to increased cleavage of GAG from the GEnC surface. The results from the supernatant followed a similar trend with a 43% overall reduction, again consistent with a reduction in biosynthesis (Fig. 1*C*).

High glucose reduces expression of GEnC surface HS GAG. Analysis of GEnC surface HS GAG by immunofluorescence revealed reduction after exposure to high glucose (Fig. 2*A*). Quantification of the fluorescence intensity confirmed a 50% reduction relative to the controls (Fig. 2*B*).

High glucose does not alter expression of proteoglycan core proteins. Expression levels for proteoglycan core proteins syndecan-1, syndecan-4, glypican-1, versican, and perlecan were analyzed on cell lystes from GEnC by Western blotting after exposure to high glucose for the same 14-day period as in the above experiments. Densitometry of each band, corresponding to the molecular weight of individual proteoglycans core proteins from different experiments, demonstrated no significant differences between GEnC cultured under normal- and high-glucose conditions (Fig. 3).

High glucose does not alter GEnC morphology and survival. Phase-contrast microscopy revealed no significant changes in the morphology GEnC monolayers after exposure to high glucose for 14 days (Fig. 4*A*). High glucose also did not cause



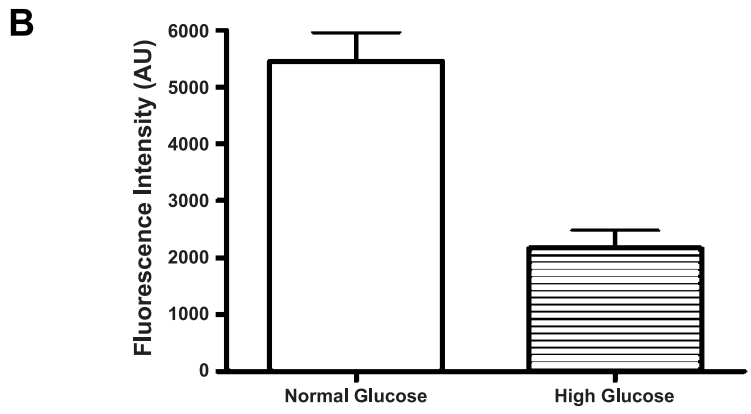


Fig. 2. *A*: images of immunofluorescence microscopy after labeling with anti-heparan sulfate (HS) antibody demonstrating differences in fluorescence labeling between GEnC cultured under normal (NG)- vs. high-glucose (HG) conditions. Nuclei of cells treated with high glucose were costained with DAPI to provide an estimation of cell numbers. Control cells treated with secondary antibody only do not show significant fluorescence. Magnification ×100. *B*: bar graph showing comparison of the fluorescence intensity of heparan sulfate quantified (using Image J software) from images derived from 3 independent experiments. Results show a significant reduction ($n = 3$; $P < 0.05$) in HS GAG expression after treatment with high glucose.

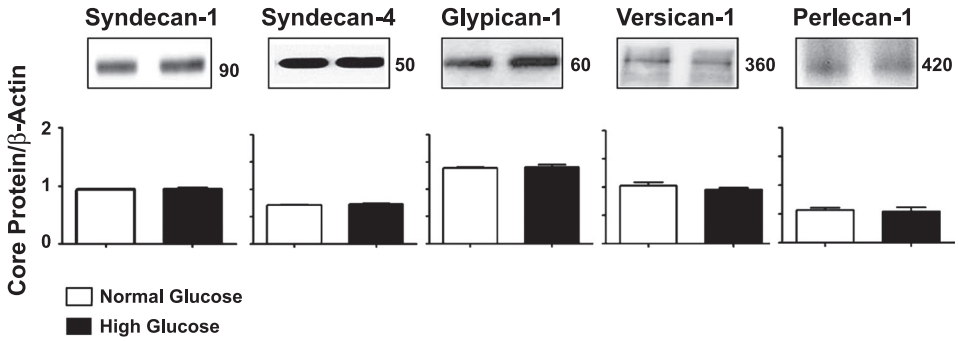


Fig. 3. Expression of proteoglycan core proteins by Western blotting of lysates derived from GEnC cultured under normal-glucose (incorporating osmotic control) or high-glucose conditions for 14 days. *Top*: representative blots showing individual proteoglycan core proteins bands. For each core protein, the band on the *left* represents control and that on the *right* represents high-glucose conditions. Numbers indicate molecular mass (in kDa) corresponding to the standards lane (not shown). *Bottom*: densitometry of each band (each core protein blot repeated 4 times) as the ratio of intensity of core protein/β-actin showing no significant differences after high glucose [$n = 4$; P = not significant (ns)].

significant alterations in GEnC survival compared with cells cultured under normal conditions (Fig. 4*B*).

High glucose increases passage of albumin across GEnC monolayers. Next, we investigated whether the observed reduction in GEnC glycocalyx GAG affected passage of albumin. Exposure of GEnC monolayers to high glucose over 14 days lead to a significant increase in the passage of labeled albumin across monolayers compared with controls (Fig. 5). Earlier time points of 2 and 7 days were tested and did not show any significant changes (data not presented).

High glucose does not affect interendothelial cell junctions. Immunofluorescence showed maintenance of a confluent GEnC monolayer and preservation of the junctional distribution of the key adherens junction protein VE-cadherin, after exposure to high glucose (Fig. 6*A*). Similarly, there was no effect on the expression levels of VE-cadherin protein by Western blotting (Fig. 6*B*).

TEER was used to test integrity of GEnC monolayers as the technique used provides sensitive quantification of the functional properties of interendothelial junctions (43). High glu-

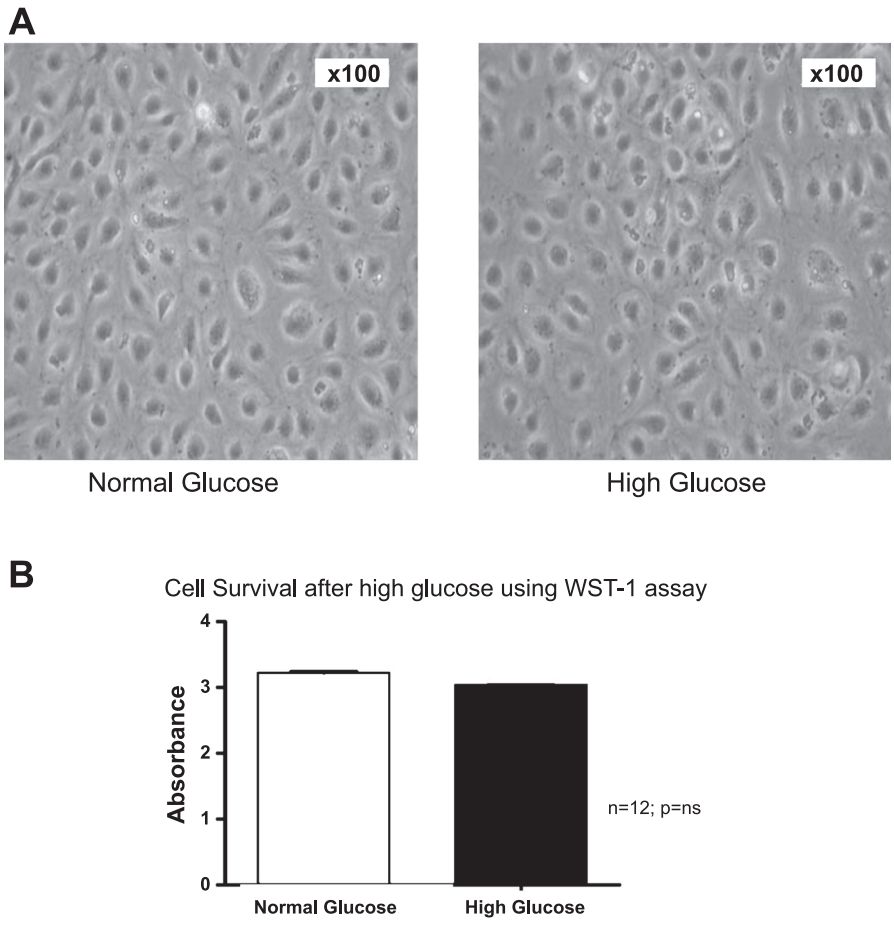


Fig. 4. *A*: phase-contrast microscopy images of conditionally immortalized human GEnC cultured for 2 wk under normal-glucose (*left*) and high-glucose (*right*). Comparison of these images shows no qualitative difference in the phenotype of cells cultured under these 2 conditions. *B*: bar graph showing measurement of absorbance in arbitrary units (Y-axis), which estimates amount of formazan produced by cells which is used as a surrogate for cell survival. Results show no significant differences between GEnC cultured under normal- vs. high-glucose conditions ($n = 12$; P = ns; t-test).

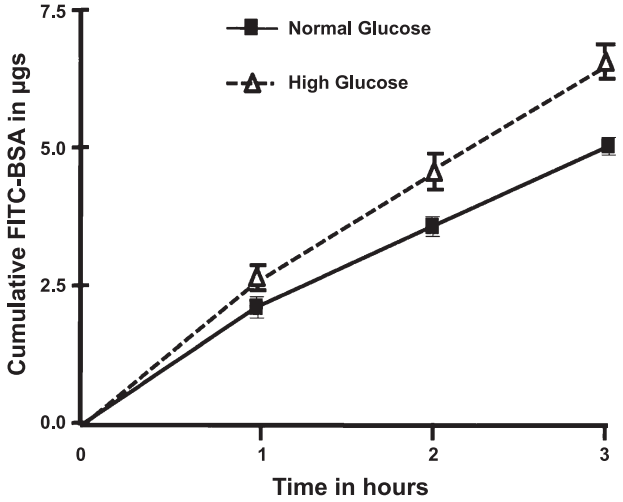


Fig. 5. Graph showing cumulative flux of FITC-BSA (Y-axis) across GEnC monolayers over time (X-axis). GEnC monolayers cultured under normal-glucose or high-glucose conditions for 14 days before performance of this experiment ($n = 12$; $P < 0.005$). These results show a significant increase in the passage of albumin across GEnC monolayers under high-glucose conditions.

cose did not cause significant changes in TEER recordings analyzed over 14 days (Fig. 7). These observations further confirm preservation of the GEnC monolayer and exclude significant effects of high glucose exposure on the contribution of cell-cell junctions to overall GEnC monolayer barrier properties.

Real-time PCR of GAG biosynthetic enzymes. Expression of EXT-1, EXT-2 (both involved in HS GAG biosynthesis), CHSY1 (involved in synthesis of chondroitin sulfate), and NDST-1 (catalyzes both the *N*-deacetylation and sulfation of GAG) was analyzed. Time course experiments after exposure of GEnC to high glucose for 2, 6, and 48 h were performed. We did not see any significant changes in the mRNA transcription of these enzymes with high glucose (data not shown).

DISCUSSION

Our previous work on glomerular permeability has demonstrated the importance of the GEnC glycocalyx as a significant contributor to the restriction of proteins offered by the GFB (16, 17, 22, 23). Recently, we have been successful in generating conditionally immortalized human GEnC (43) which have allowed detailed study and direct biochemical analysis of the GEnC glycocalyx. We have previously shown that conditionally immortalized cells express the glycocalyx in addition to the typical endothelial markers including fenestrations (43). Confirming our findings from animal models, the glycocalyx expressed by GEnC monolayers also demonstrated resistance to the passage of albumin, and its specific GAG components were shown to be crucial to maintain this function (45). In the current study, we have demonstrated a significant effect of high-glucose conditions on biosynthesis of GAG chains by GEnC, with particular downregulation of HS GAG. High glucose was also shown to increase passage of labeled albumin across monolayers. These functional changes are consistent with the observed the effects of high glucose on the GEnC glycocalyx.

Our results show marked reduction in incorporation of glucosamine, which forms the basic structure of both sulfated and nonsulfated GAG chains, following exposure to high glucose. Specific reduction in biosynthesis of sulfated GAG was supported by the reduction in S^{35} incorporation and reduction in HS GAG expression after exposure to high glucose. These findings imply regulation by catalytic enzymes involved in the biosynthesis of GAG. Therefore, to test this hypothesis, we analyzed mRNA transcription of four key enzymes involved in the biosynthesis of GAG but we found no changes in the expression of EXT-1, EXT-2, CHSY1, and NDST-1 after culturing GEnC in high glucose (data not shown). These results highlight the complexity of the mechanisms involved in the regulation of GAG, which is likely achieved by a subtle balance between enzymes involved in the process of biosynthesis and breakdown. This demands further and more comprehensive analysis in future studies to fully explore the ramifications of these observations.

GAG chains form an important part of the biophysical structure of the endothelial glycocalyx and play an important role in its function (34). Addition of sulfate groups to GAG chains confers high anionic density (12), which is important in GFB function (16). Previous studies have reported loss of GFB charge selectivity in diabetics, with microalbuminuria and corresponding loss of HS GAG and anionic charge from the GFB without any other structural abnormality (8, 27, 47). Historically, the site of this functional defect in the anionic charge was believed to be the GBM (47). Loss of HS GAG from the GBM has been reported in early stages of diabetic nephropathy (29). Ha et al. (15) showed reduction in perlecan mRNA and reduced sulfation of GAG by bovine GEnC after incubation in high glucose for over 3 mo. Although this study was designed to study the contribution of GEnC to the previously apparent loss of HS proteoglycans from the GBM in diabetic subjects, it bears relevance to our work and supports our findings. More recently, the contribution of the GBM to the glomerular permselective barrier especially via charge selectivity has been questioned (19). Studies in genetically modified mice with altered HS GAG chains (agrin and perlecan via a podocyte-specific promoter) specifically in the GBM showed loss of anionic charge but no evidence of proteinuria (6, 18). Since these alterations were induced via podocyte-specific genetic modifications, they are unlikely to have directly affected the structure and contribution of the GEnC glycocalyx. Therefore, alterations in GAG chains of the GEnC glycocalyx suggest an attractive alternate anatomic explanation for those situations where GFB charge selectivity is reduced or lost. This is supported by a recent study demonstrating a charge defect on luminal surface of glomerular capillaries from diabetic endothelial nitric oxide synthase knockout mice (25). Interestingly, in our study despite marked changes in GAG biosynthesis, high glucose did not alter the expression of proteoglycan core proteins, to which the GAG chains are bound. This suggests posttranscriptional regulation of proteoglycan GAG chains under conditions of high glucose.

Our results show an increased permeability of albumin across GEnC monolayers after exposure to high glucose, without any significant changes in TEER. TEER measures resistance to the passage of ions across a monolayer (28), and in confluent monolayers it may be used as an index of monolayer integrity, decreasing TEER suggesting loss of integrity of

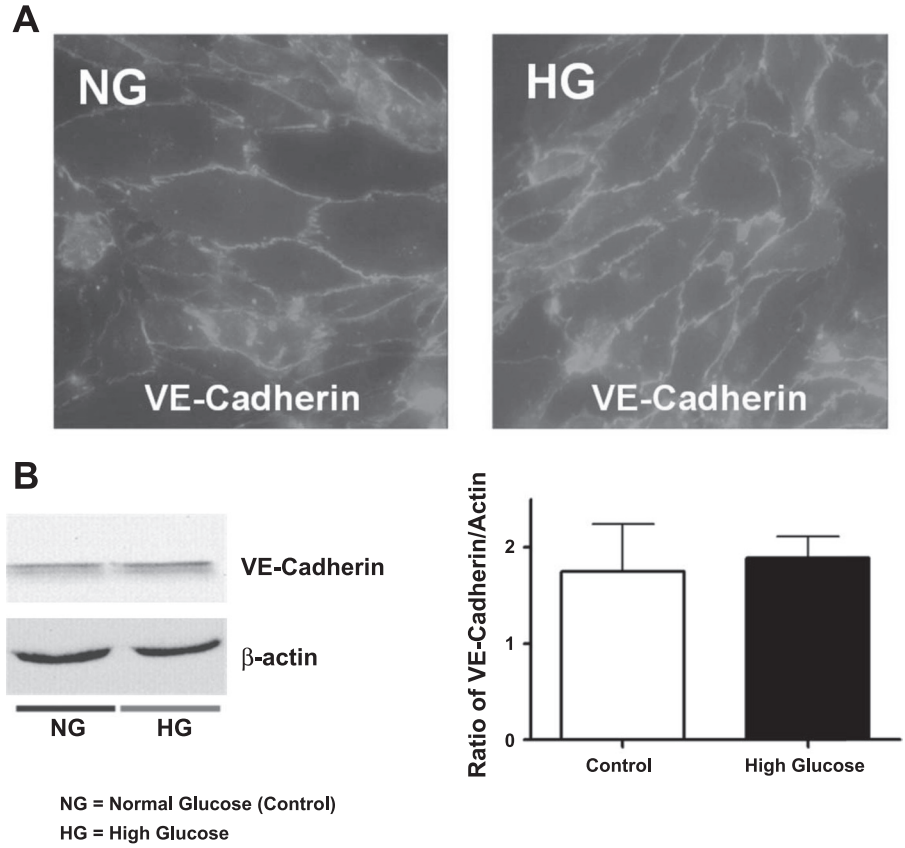


Fig. 6. *A*: immunofluorescence images showing distribution of VE-cadherin under normal (5.5 mM D-glucose with 20.5 mM L-glucose)- and high-glucose (25.5 mM D-glucose) conditions showing no significant changes in the morphology of endothelial junctions ($n = 4$ separate experiments). *B*: expression of VE-cadherin by Western blotting (representative blot). Densitometry shows no quantitative change after high glucose ($n = 4$, $P =$ ns).

interendothelial junctions through modification of junctional proteins or as a result of cellular contraction (40, 43). This was also supported by the phase-contrast microscopy showing preserved GEnC monolayer morphology and lack of adverse effect on cell survival under high-glucose conditions. Insignificant or undetectable change in TEER therefore implies an absent or minimal contribution from increased endothelial junctional pathways to the increased passage of albumin. This was also confirmed by no alterations in the major endothelial adhesion molecule VE-cadherin (10) under high-glucose conditions.

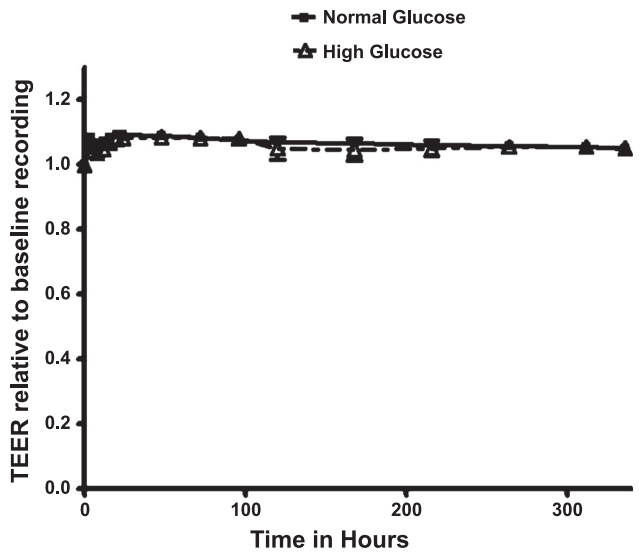


Fig. 7. Graph showing transendothelial electrical resistance (TEER) recordings of GEnC monolayers under normal- and high-glucose conditions. TEER (Y-axis) is shown as a ratio of baseline recording vs. time (X-axis). Results show no changes in the TEER recordings during 14 days of exposure to high glucose ($n = 4$; $P =$ ns). This confirms the integrity of endothelial monolayers and no significant alterations in endothelial junctions.

Passage of albumin across GEnC monolayers was measured using an established in vitro model (28, 40, 45). This finding is consistent with our previous observations where similar changes in albumin passage were detected after specific removal of HS GAG from the GEnC glycocalyx (45). While we recognize inherent limitations of in vitro models (in particular, that measurements in endothelial monolayers do not indicate absolute permeability coefficients in intact animals or humans) (1), we have previously found a comparable relationship between enzymatic glycocalyx removal and increased albumin passage in vitro and in vivo (23, 45). Furthermore, the sensitivity of the models used to detect changes in albumin passage and TEER, comparable to in vivo effects of the same the mediators (e.g., thrombin, cAMP, VEGF), has been extensively reported by us and others (13, 24, 39, 40, 43).

Antecedent generalized endothelial dysfunction is thought to explain why albuminuria is a powerful predictor of cardiovascular risk and associated mortality in patients with diabetes mellitus as well as in the general population (2, 7, 14, 35, 36). However, exactly which aspect or aspects of endothelial dysfunction are pathophysiologically responsible is not yet clear (2). The evidence strongly suggesting the vascular endothelium as a common denominator implies that glomerular and systemic endothelia are similarly vulnerable to diabetic damage

and that glomerular endothelial dysfunction contributes to diabetic GFB dysfunction (44). It is therefore important to ask how dysfunction of the glomerular endothelium may be linked to disruption of the GFB.

Our study shows that high glucose modulates the structure of the GEnC glycocalyx by altering the biosynthesis of sulfated GAG chains, particularly the HS GAG. These changes are significant for GEnC barrier function and cause an increase in macromolecular passage across GEnC monolayers. The underlying mechanism leading to the observed changes in the GEnC glycocalyx is still unclear. Role of mediators known to be upregulated by high glucose (e.g., endothelial nitric oxide synthase, ANG II, and NAPDH oxidase) in the regulation of the GEnC glycocalyx cannot be excluded. Nevertheless, these findings are important and suggest a novel mechanism by which diabetes, through hyperglycemia, may cause dysfunction of the GFB. In a recent study, Nieuwdorp et al. (30) show a reduction in the systemic glycocalyx volume in diabetic humans and further that these changes in the systemic glycocalyx paralleled microalbuminuria, suggesting that systemic changes in the glycocalyx coincide with dysfunction of the GFB. Our study provides further evidence to suggest that structural modulation of the GEnC glycocalyx induced by high glucose has functional implications.

These observations have further implications beyond the kidney, suggesting that endothelial glycocalyx dysfunction may be the link between cardiovascular disease and proteinuria through contributing to the pathophysiology of atherosclerosis in systemic vessels (46) as well as to GFB dysfunction. Further dissection of the molecular mechanisms of the findings presented will lead to novel preventative and therapeutic strategies in systemic vascular disease in diabetes as well as in nephropathy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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