Heparin affects cytosolic glucose responses of hyperglycemic dividing mesangial cells

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Running Title: Hyperglycemia induces Hyaluronan Synthesis by Rat Mesangial Cells

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SUMMARY

Mesangial expansion underlies diabetic nephropathy, leading to sclerosis and renal failure. The glycosaminoglycan heparin inhibits mesangial cell growth, but the molecular mechanism is unclear. Here, rat mesangial cells (RMCs) were growth arrested in the G0/G1 phase of cell division, stimulated to divide in normal glucose (5.6 mM) or high glucose (25.6 mM) with or without heparin, and analyzed for glucose uptake. We observed that RMCs entering the G1 phase in normal glucose with or without heparin rapidly cease glucose uptake. RMCs entering G1 in high glucose sustained glucose uptake for the first 3 h, and high-glucose exposure of RMCs only in the first 8 h of G1 induced the formation of an extracellular monocyte adhesive hyaluronan matrix after cell division was completed. Moreover, a low heparin concentration in high-glucose conditions blocked glucose uptake by 1 h into G1. Of note, the glucose transporter 4 (glut4) localized on the RMC surface at G0/G1 and was internalized into G1 cells in normal glucose conditions with or without heparin within 30 min. We also noted that in high-glucose conditions, glut4 remains on the RMC surface for at least 2 h into G1 and is internalized by 4 h without heparin and within 1 h with heparin. These results provide evidence that the influx of glucose in hyperglycemic dividing RMCs initiates intermediate glucose metabolism leading to increased cytosolic UDP-sugars and induces abnormal intracellular hyaluronan synthesis during the S phase of cell division.

Introduction

Mesangial expansion is the principal glomerular lesion in diabetic nephropathy (DN) that reduces the area for filtration and leads eventually to sclerosis and renal failure (1,2). However, the mesangial extracellular matrix (ECM) expansion and sclerosis are preceded by a phenotypic activation and transient proliferation of the glomerular mesangial cells, followed by a prominent glomerular infiltration of monocytes and macrophages (3,4). Glomerular monocytes and macrophages have been prominently identified in DN in both animal models (4) and diabetic humans (5), and appear to have a key role in the induction of mesangial matrix expansion, hypercellularity, and the onset of proteinuria (6,7). Previous studies (8) as well as our own (9,10) have shown that there is a significant increase in hyaluronan matrix in glomeruli during the first week after induction of diabetes in rats by streptozotocin, which is coincident with glomerular monocyte/macrophage influx (4). There is compelling evidence for a causal link between increased glomerular hyaluronan matrix and monocyte/macrophage accumulation, and that in diabetic glomeruli, the structure of the hyaluronan matrix can mediate monocyte adhesion and activation, thereby contributing directly to the sclerotic process.

Hyaluronan is a linear glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid with alternating β-1,4 and β-1,3 glycosidic bonds. It is a major, ubiquitous component of extracellular matrices. The number of repeat disaccharides in a completed hyaluronan molecule can reach 20,000
or more, a molecular mass of >8 million Da, and a length of >20 µm. The formation of the monocyte-adhesive hyaluronan matrix by rat renal mesangial cells (RMCs) is a multi-phase process that requires: 1) a PKC signaling pathway(s) activated in RMCs dividing in hyperglycemic glucose, 2) synthesis and accumulation of hyaluronan in intracellular compartments (13-24 h from G0/G1) that initiates an endoplasmic reticulum (ER) stress/autophagic response, and 3) cyclin D3-mediated formation of the abnormal extracellular monocyte-adhesive hyaluronan matrix after completion of cell division (24-48 h from G0/G1) (10). Understanding these cellular and molecular events will provide significant insights into the mechanisms controlling cellular responses to hyperglycemia that initiate the progression of DN.

Heparin is a highly sulfated, highly polyanionic glycosaminoglycan with a repeating disaccharide that contains a hexuronic acid (either glucuronic acid or iduronic acid) and glucosamine (either N-acetylated or N-sulfated). It is synthesized as a proteoglycan (serglycin) that is found in mast cells. Heparin has been shown to inhibit mesangial cell growth in both experimental renal disease models (11-15) and in cell culture (16,17). Particularly pertinent to this study, heparin has been shown: 1) to prevent albuminuria and mesangial expansion and sustain glomerular function in STZ treated diabetic rats (11,12); and 2) to suppress mesangial cell proliferation and matrix expansion in experimental mesangio-proliferative glomerulonephritis (18). However, the molecular and cellular mechanism(s) underlying the beneficial roles of heparin in DN are still unknown.

Our previous studies (19-21) support our proposal for a receptor for heparin on the surface of quiescent, growth arrested G0/G1 RMCs based on the observations that: 1) binding of heparin to RMCs is specific, rapid (5-10 min), saturable (within 60 min) and reversible; 2) Scatchard analysis of heparin binding indicates a single class with ~6.6 x 10^6 binding sites per cell (K_d = 1.6 x 10^-8 M) in quiescent cells; 3) surface-bound heparin can be internalized and degraded; 4) the affinity and number of heparin binding sites are affected by the stage of RMC growth; and 5) heparin acts at the RMC surface to affect both PKC-dependent and independent pathways.

RMCs can be growth arrested by serum starvation for 48 h. At this time the cells are quiescent because: 1) less than 10 % of the cells express PCNA, a marker for cell proliferation, and this number increases to ~60 % at 16 h after serum stimulation; 2) serum starved RMCs incorporate very little [3H]thymidine into DNA during one hour of labeling while a transient burst of DNA synthesis is observed at 16 h after serum stimulation; 3) no c-fos mRNA is detected in starved RMCs by Northern analysis while a transient expression of c-fos mRNA is observed between 0.5 - 1 h after serum stimulation; 4) ~75 % of starved RMCs are arrested in G0/G1 phase using flow cytometry analysis and progress to S phase by 18 h after serum stimulation; and 5) the G1/S boundary is reached at ~12 h, and cell cycle is completed at ~24 h after cells re-entered G1. Therefore, after 48 h of serum-starvation, RMCs are quiescent at G0/G1 and re-enter the cell cycle with good synchrony upon serum stimulation (19-21). Therefore, this serum-starved RMC model is an excellent one to investigate the responses to high glucose during a synchronized cell cycle and to what extent heparin impacts on the high glucose induced responses. This study provides evidence for the critical role of glucose uptake during the first 4 hours of G1 phase in the responses of the RMCs to high glucose in the absence or presence of heparin.

Results

The minimal exposure time that yields the RMC responses to high glucose – Our previous studies have shown that the serum starved, near confluent mesangial cell (RMC) cultures stimulated to divide in 25.6 mM glucose (high glucose) and 10% FBS for 72 h form an extruded monocyte-adhesive hyaluronan matrix after division (9,10). In order to determine the minimal exposure time that yields maximal monocyte binding in response to high glucose concentration, serum starved, near confluent RMC cultures were treated with 10% FBS to stimulate cell division in normal glucose (5.6 mM) or high glucose for 72 hours, or with high glucose for 8 hours that was changed to normal glucose and continued to 72 hours. The cultures were cooled to 4° C, and U937 cells, a myeloid cell line used to monitor monocyte adhesion (22), were added for 1 hour.
Figure 1 shows examples of the numbers of bound U937 cells for the 3 culture conditions. As shown previously, the cultures in high glucose for 72 hours bound significantly more U937 cells than in the normal glucose cultures (9,10). The cultures that were changed to normal glucose at 8 hours also showed significantly more U937 cell binding than the normal glucose cultures, but also significantly less than in the 72 h high glucose cultures (Fig. 1). Parallel cultures were analyzed for hyaluronan contents by FACE analyses (9,10). Interestingly, the significant increases in hyaluronan contents compared with the normal glucose cultures were the same for both the 72 hour and the 8 hour high glucose treated cultures (Fig. 2). These results provide evidence that the RMCs exposed to high glucose for only 8 hours into G1 are already re-programmed to initiate the intracellular hyaluronan synthesis, which does not start until the cells enter into S phase (19-21), and that the structure of the extracellular matrix extruded after completing cell division is monocyte-adhesive, albeit not as much as observed in the continuous 72 hour high glucose cultures.

Dividing RMCs block glucose transport – To determine the uptake of glucose by the RMCs at the initial stage of cell cycle, RMC cultures were stimulated to divide from G0 in normal and high glucose with or without heparin. The fluorescent labeled glucose analog, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), that enters through glucose transporters and is retained in cells, was added to the cultures at time 0, and cultures analyzed for fluorescence at 1, 2, 3 and 4 hours. The fluorescence for the 2-hour cultures is shown in figure 3, and the measured fluorescence in cultures at each time point are shown in the bar graphs. Panel A shows that 2-NBDG uptake is much higher in the High compared with the High plus Heparin already at 2 h. Strikingly, there was nearly no 2-NBDG uptake in the cultures in normal glucose regardless of the presence or absence of heparin, providing evidence that dividing cells activate a mechanism to prevent glucose uptake as they enter G1 of the cell cycle. However, 2-NBDG uptake in high glucose alone increased continuously during the first 3 hours, but did not increase during the following 3-4 hour (Fig. 3B, High). In contrast, while uptake of glucose occurred in the first hour in high glucose plus heparin, it was subsequently blocked, with no additional uptake after 1 h (Fig. 3B, High + Heparin). These results provide strong evidence that: 1) dividing RMCs, and likely most dividing cells, rapidly stop glucose uptake as they enter G1 phase of cell division; 2) they cannot stop glucose uptake if the glucose concentration is hyperglycemic (> ~2.5 x normal (9)) for at least 3 hours, which re-programs the cells to synthesize hyaluronan in intracellular compartments in S phase; and 3) the presence of heparin re-programs the cells to stop glucose uptake by the end of the first hour into G1, which subsequently blocks the intracellular hyaluronan synthesis (23).

Glucose transporter 4 (glut4) is likely involved – Glut4 is a glucose transporter that is expressed by RMCs and is on quiescent mesangial cells of glomeruli and on quiescent smooth muscle cells of microvasculature in normal rat kidneys (24). In order to determine the role of glut4 in mediating the early glucose uptake by RMCs, non-permeabilized RMC cultures were stained for glut4 at different times up to 4 hours in G1 in normal and high glucose with and without heparin (Fig. 4). The growth arrested (G0/G1) culture (time 0) shows glut4 distributed over the RMC surface, which is internalized within 30 minutes in RMC cultures in normal glucose and nearly so in cultures in normal glucose plus heparin. In the cultures in high glucose, glut4 remains on the surface at 1 and 2 hours from G0 and is no longer on the surface by 4 hours. This indicates that it has been internalized within the next 2 hours, likely around 3 hours given the results for the uptake of 2-NBDG (Fig. 3) that showed continuous uptake for only 3 hours. The presence of heparin in the high glucose culture showed nearly complete internalization at 1 hour, which is also consistent with the 2-NBDG uptake that was blocked by 1 hour from G0. These data indicate that the intracellular mechanism required to block the initial hyperglycemic influx of glucose by heparin likely involves interaction with a cell surface receptor that re-programs the RMCs to remove glut4 from the cell surface by the end of the first hour after the cells enter G1 phase.

Cytosolic UDP-glucose increases in hyperglycemic dividing RMCs – The results in figures 3 and 4 provide evidence that continuous early uptake of glucose for 3 hours in G1 from G0
likely increases cytosolic UDP-sugars in hyperglycemic dividing cells, which are necessary for providing the substrates for the subsequent intracellular HA synthesis initiated early in S phase. Therefore, we determined to what extent early robust entry of glucose impacted the intracellular UDP-glucose, UDP-glcNAc and UDP-galNAc, the metabolites of intermediary glucose metabolism. Rat G0/G1 RMCs were stimulated to divide in normal and hyperglycemic medium for 6 hours in the presence or absence of heparin. UDP-sugars were extracted from the cell cultures and purified by ion exchange column chromatography. The sugars were then released by acid hydrolysis for analysis on FACE gels (25,26). The results show that UDP-glucose is greatly elevated within 6 h by hyperglycemic medium alone (Fig. 5). These data are consistent with the continuous 3 h increase of 2-NBDG uptake by RMCs in response to high glucose (Fig. 3) and show that high glucose did stimulate the glucose uptake and subsequent cytosolic intermediary glucose metabolism in RMCs at an early G1 cell cycle stage. Importantly, the heparin treatment prevented the high glucose induced UDP-glucose production, consistent with the finding that heparin blocked the early 2-NBDG entry into RMCs induced by high glucose. Interestingly, the intracellular UDP-glcNAc and UDP-galNAc were maintained at the basal levels regardless of the presence or absence of heparin at the 6 h time point. Further, there were no differences in terms of these intracellular UDP-sugars when comparing the 6 h cultures with the G0/G1 cells. The ratios of the UDP-glucose content in the high glucose culture to the ones in the low glucose and heparin treated high glucose cultures are ~8 and ~4 respectively. In addition, high glucose induced intracellular UDP-glucose production was observed as early as 3 h after the cells entered G1 phase (image not shown). The ratios of the UDP-glucose content in the high glucose culture to the ones in the low glucose and heparin treated high glucose cultures are ~3 and ~2 respectively. Thus, these data indicate: 1) that RMCs at the early stage of cell cycle in normal glucose or high glucose plus heparin maintain levels of cytosolic glucose concentrations well into G1 phase; and 2) that the 3 hour influx of glucose in high glucose (Fig. 3) increases cytosolic UDP-glucose concentration greatly during the 6 hours into G1.

Our previous studies indicate that the hyperglycemic cells initiate intracellular hyaluronan synthesis (~13 h from G0) shortly after entering S phase (9,10,21,23). Thus, the increased cytosolic glucose and UDP-glucose at times later than 6 hours need to initiate the hexosamine biosynthesis pathway to synthesize UDP-glcNAc and convert UDP-glucose to UDP-glucuronic acid (UDP-glcUA), the cytosolic substrates for synthesis of hyaluronan into intracellular compartments (10,23).

Discussion

Daily intraperitoneal injection of heparin (55 mg/kg) in the diabetic rat model prevented the proteinuria and nephropathy, and sustained kidney function for 8–10 weeks (11,12). However, the cellular and molecular mechanism(s) underlying this therapeutic role of heparin are still unclear. Our previous studies have shown that glomerular mesangial cells that divide in medium with glucose concentrations three times higher than normal or greater activate hyaluronan synthesis in intracellular compartments (10,23). This initiates an autophagy and subsequent extrusion of a monocyte-adhesive hyaluronan matrix after completing cell division.

This mechanism also occurs in vivo in the streptozotocin type 1 diabetic rat model with extensive accumulation of hyaluronan in glomeruli and influx of macrophages, resulting in nephropathy, proteinuria, and kidney failure by 6 weeks. Our studies also demonstrated that heparin (2.0 µg/ml) prevented the intracellular hyaluronan synthesis and autophagy in vitro. However, after completing division, the cells synthesized an even larger extracellular monocyte-adhesive hyaluronan matrix. In vivo, glomerular hyaluronan in heparin treated diabetic rats increased greatly in weeks 1–2 and declined to near control, i.e. near absence of hyaluronan, by 6 weeks, at which time the glomeruli had extensive numbers of macrophages. Therefore, these previous results provide evidence that dividing cells have a receptor that binds heparin and initiates the intracellular responses that inhibit activation of hyaluronan synthesis in intracellular compartments during division and that reprogram the cells to address the sustained...
glucose stress after division by initiating synthesis of a large monocyte-adhesive extracellular hyaluronan matrix. Our present study provides evidence that the mechanisms involved are initiated during the very early stage of RMC division entering G1 phase.

Exposure of RMCs to high glucose for 8 h at the early G1 phase of cell cycle was sufficient to induce formation of a monocyte adhesive hyaluronan matrix after the cell division was completed. This result indicated that high glucose induced the intracellular mechanism that governs the RMC responses during subsequent cell cycle progression and after cell division is completed. Thus, this short treatment of RMCs entering G1 with high glucose was enough to induce the subsequent RMC phenotypic activation responses, including intracellular hyaluronan synthesis early in S phase and the formation of monocyte adhesive hyaluronan matrix after division (9,10,21,23). This is consistent with previous studies in which high glucose induces activation of PKC in mesangial cells within 2-3 h after serum stimulation (27-30). Thus, the early 3-4 h cellular response is important in glucose-induced formation of the extracellular monocyte adhesive hyaluronan matrix after division.

Serum starved G0/G1 RMCs in normal glucose maintained glucose transporter 4 (glut4) on the cell surface and rapidly removed it when addition of serum stimulated reentry into the G1 cell cycle with subsequent blockage of 2-NBDG uptake. This provides evidence that glucose utilization is not necessary during the cell cycle and must be blocked. In contrast, reentry of G1 in high glucose sustained cell surface glut4 and 2-NBDG uptake for 3 h. This indicates that there was an early high glucose induced glucose influx into the cells in response to extracellular high glucose leading to the activation of intracellular mechanisms that mediate the subsequent pathological responses. Therefore, preventing glucose entry into cells at the early stage of cell cycle is required for the normal cell division. This supports our hypothesis that major pathological responses are generated from hyperglycemic dividing cells.

Importantly, this high 2-NBDG induced glucose influx into the cells at the entry of G1 stage of the cell cycle was blocked within 1 hour by heparin by removal of glut4 from the cell surface. This indicates that direct interaction between heparin and cells at this early G1 stage initiated intracellular pathways to prevent sustained glucose uptake into the cells, thereby preventing the intracellular glucose activation of the mechanism(s) that interfere with the normal cell cycle progression and compromise cell functions after division. This interpretation is supported by our previous findings that heparin interacts with cell surface receptors to regulate the MAP kinase dependent and independent pathways (19-21).

The serum-starved G0/G1 cells maintained glut4 on the cell surface, consistent with the finding in other serum-starved cells, which have high activities that use glucose metabolic pathways to support their survival (24). Upon the serum stimulation to re-enter cell cycle in normal glucose, the glut4 was rapidly removed from the cell surface to avoid the excessive glucose influx interfering with the subsequent normal cell cycle progression.

Since excessive glucose was inside of cells in the high glucose cultures, the question is whether this intracellular glucose activates the intermediate glucose metabolism during the early stage of the cell cycle. Our data demonstrated that UDP-glucose was greatly increased in the cells cultured in high glucose for 6 h. The contents of this UDP-glucose in the normal glucose with or without heparin were at basal levels equivalent to the serum starved cells, indicating that abnormal production of UDP-glucose by intermediate glucose metabolism is a pathological response of RMCs to sustained glucose uptake for 3 hours in high glucose medium. As expected, heparin treatment prevented this activation of intermediate glucose metabolism for formation UDP-glucose by activating a mechanism to remove glut4 from the surface within 1 hour and block further 2-NBDG uptake. This also demonstrated that one of the beneficial roles of heparin in diabetic nephropathy is to prevent the activation of high glucose induced intermediate metabolism by blocking excessive glucose uptake at the early stage of the RMC cell cycle.

In summary, this study has provided strong evidence that: 1) high glucose-induced early responses in the cell cycle progression are crucial for the later changes in the cell functions; 2) high glucose induced the excessive influx of 2-NBDG into the cells through cell surface glut4, which
activates abnormal intermediate cytosolic glucose metabolism; and 3) interaction between heparin and a putative cell surface receptor on RMCs initiates a mechanism to remove the glut4 from the cell surface shortly after entering G1 thereby preventing excessive glucose uptake and subsequent activation of intermediate glucose metabolism. One of our ongoing studies is to determine the cell surface receptor and what the signaling pathways are that remove glut4 and prevent excessive glucose influx in order to maintain normal RMC functions.

**Experimental Procedures**

**Reagents** – Streptomyces hyaluronidase, Streptococcal hyaluronidase and chondroitinase ABC were from Seikagaku America Inc. (Rockville, MD). Antibody against glucose transporter 4 was from Abcam (Cambridge, MA), and 2-NBDG was from Cayman Chemical (Ann Arbor, MI).

**Establishment of RMC cultures and assay for glucose uptake** – RMC cultures were established from isolated glomeruli and characterized as described previously (31,32). RMCs were used between passages 5 and 15 when they still contract in response to angiotensin II and endothelin, and they exhibit growth suppression in the presence of heparin (1 µg/ml), which are additional characteristics of mesangial cells (19,33,34). RMCs were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and passaged at confluence by trypsin digestion for 5 min with a solution of 0.025% trypsin, 0.5 mM EDTA. To render cells quiescent (19), cultures at 40% confluence (2x10^4 cells/cm^2) were washed with RPMI 1640 medium and placed in fresh medium containing 0.4% FBS for 48 h (yielding 70 – 80% confluent cultures).

Glucose uptake by RMCs was assayed with 2-NBDG as previously described (35). Briefly, serum starved RMCs on chamber slides were stimulated by 10 % FBS in RPMI 1640 medium with concentrations of 5.6 and 25.6 mM D-glucose containing 3.9 µmol 2-NBDG per mmol glucose in the presence or absence of 1 µg/ml heparin. At 1, 2, 3 and 4 h after stimulation, RMC images were collected on a fluorescence microscope, and the fluorescent intensities of collected images were quantified with Image J software.

**Immunohistochemistry** – Paraformaldehyde-fixed RMC cultures on chamber slides were stained for glucose transporter 4 (glut4) with antibody, and for nuclei with 4,6-diamidino-2-phenylindole (DAPI), as described previously (9,10) or according to the manufacturer’s instruction. Samples were treated with the antibody at a 1:75 dilution, washed and treated with anti-mouse IgG Cy3 antibody at 1:200 dilution. Stained samples were mounted in VectaShield containing DAPI (Vector Laboratories) for staining the nuclei of cells. Confocal images of the samples were obtained with a Leica TCS-NT laser scanning confocal microscope equipped with four lasers for excitation at 351, and 561 wavelengths. The same settings of the confocal microscope and laser scanning were used for both control and treated samples.

**Assay for monocyte adhesion** (9,22) – Serum starved RMCs in 6-well plates were treated up to 72 h with 10 % FBS and with concentrations of 5.6 and 25.6 mM D-glucose. U937 cells were cultured in suspension in RPMI 1640 medium containing 5% FBS and passaged at a 1:5 ratio (2x10^5 cells/ml) every 48 h (22). Assays for monocyte adhesion were done as described previously (9,22). After washing, the cell cultures were imaged by microscopy with a Polaroid digital camera (9), and the numbers of monocytes per culture area were counted using Image-Pro software. Each culture was equally divided into four regions, and a culture area for imaging was randomly picked in each region. Streptomyces hyaluronidase treatment (1 TRU/ml at 37° C for 15 min) of RMCs before monocyte incubation was used to determine the extent of the hyaluronan-mediated adhesion. Analysis of hyaluronan contents in mesangial cell cultures was performed as previously described (9).

**Fluorophore Assisted Carbohydrate Electrophoresis (FACE) analysis of reducing saccharides** (25,26) – Serum starved RMCs in 6-well plates were treated for 6 h with 10 % FBS and concentrations of 5.6 and 25.6 mM D-glucose with or without 1 µg/ml heparin. The cell cultures were extracted with 1 ml of 75% cold ethanol for 10 mins. The ethanol lysates were dried by centrifugal evaporation. The dried samples were dissolved in 200 µl mini-Q water, and 200 µl chloroform was used to remove proteins from the
samples. Then UDP-sugar extracts were mixed with 50 μl Q-Sepharose beads and, after washing with 100 μl mini-Q water for 5 times, the saccharides of UDP-sugars bound to the beads were released in 50 μl 20% acetic acid at 100° C for 10 mins. The acid supernatants were dried by centrifugal evaporation and re-dissolved in 20 μl of 0.1 M ammonium acetate, pH 7.0. The saccharides were dried by centrifugal evaporation in microtubes and then subjected to reductive amination with 2-aminoacridone as described previously (9). At the end of the incubation, the samples were each mixed with glycerol to 20%, and 5 μl aliquots were then subjected to electrophoresis on Glyko Mono Composition gels with Mono Running buffer from ProZyme Inc (San Leandro, CA). Running conditions were 500 V at 4° C in a cold room for 1 h. Gels were imaged on an Ultra Lum transilluminator (365 nm). Images were captured with a Quantix cooled charge-coupled device camera from Roper Scientific/Photometrics and analyzed with the Gel-Pro Analyzer program version 3.0 (Media Cybernetics).
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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.
Author contributions

AW and VCH designed and coordinated the study, and wrote the paper. AJW designed and analyzed glucose uptake, glucose transporter, and the UDP-sugars, and contributed to the preparation of the manuscript. JR analyzed high glucose induced monocyte adhesion and hyaluronan matrix formation. AA provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.
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Footnotes

The abbreviations used are: DAPI, 4,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; FACE, fluorophore-assisted carbohydrate electrophoresis; FBS, fetal bovine serum; glut4, glucose transporter 4; glcNAc, N-acetylglucosamine; glcUA, glucuronic acid; 2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; RMCs, rat mesangial cells; and UDP, uridine diphosphate.
Figure legends:

1. U937 monocyte adhesion to RMC cultures. Panel A: The RMCs were stimulated to divide and incubated for 72 h in normal glucose (5.6 mM, left), high glucose (25.6 mM, middle), or high glucose only for first 8 h and then normal glucose (right). Micrographs of U937 monocytes bound to the cultures are shown. Panel B: The bar graph shows the number of bound U937 monocytes normalized to area as described in Methods for 3 cultures for each treatment. The unpaired Student’s t test was used to compare the means of two groups (the asterisks indicated P<0.01).

2. Hyaluronan matrices. The RMCs were stimulated to divide and incubated for 72 h in normal glucose (5.6 mM), high glucose (25.6 mM), or high glucose only for first 8 h and then normal glucose. The bar graphs show the relative hyaluronan contents in the cell layers of RMC cultures (mean +/- SD for 3 replicate cultures).

3. Fluorescent 2-NBDG uptake by RMCs. RMC cultures were stimulated to divide from G0 in normal and high glucose with or without heparin. The fluorescent labeled glucose analog, 2-((N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) was added to the cultures at time 0, and cultures were analyzed for fluorescence at 1, 2, 3 and 4 h. Panel A: The fluorescent micrographs for the 2 hour cultures are shown. Panel B: The bar graphs show the relative 2-NBDG fluorescent contents in the cell layers of RMC cultures (mean +/- SD for 3 replicate cultures).

4. Glut4 on RMCs. RMC cultures were stimulated to divide from G0 in normal and high glucose with or without heparin up to 4 h. At the indicated incubation time points, non-permeabilized RMC cultures were stained with a glut4 antibody.

5. FACE analysis for UDP-sugars from RMCs. RMC cultures were stimulated to divide from G0 in normal and high glucose with or without heparin for 6 h. The UDP-sugars were extracted from RMC cultures, hydrolyzed and then analyzed by FACE as described in the Methods.
Fig. 3

A  
2h Cultures
High  Low  High + Heparin  Low + Heparin

B  
Fluorescent 2-NBDG contents

|               | 1h     | 2h     | 3h     | 4h     |
|---------------|--------|--------|--------|--------|
| Low           |        |        |        |        |
| Low + Hep     |        |        |        |        |
| High          |        |        |        |        |
| High + Hep    |        |        |        |        |

Values shown as mean ± S.E.M.
Fig. 5

[Image of a gel electrophoresis experiment with labeled samples and annotations]

- UDP-sugars
- Serine
- GlcNAc
- Glc
- GlcNAc

| Condition           | +  | +  | +  | +  |
|---------------------|----|----|----|----|
| Glucose 5.6mM       |    |    |    |    |
| Glucose 25.6mM      |    |    |    |    |
| Heparin             |    |    |    |    |
Heparin affects cytosolic glucose responses of hyperglycemic dividing mesangial cells
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