Glycocalyx, cardiometabolic disease and inflammation
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Sulfated glycosaminoglycans restore the glycocalyx barrier of endothelial cells under hyperglycemic conditions

Submitted for publication

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Abstract

High glucose levels compromise the endothelial glycocalyx barrier. Conversely, increased endothelial permeability for albumin is an early feature in patients with diabetes. Therefore, we hypothesized that exogenous glycosaminoglycan (GAG) supplementation may attenuate high glucose-induced endothelial hyperpermeability for albumin by restoring the glycocalyx-barrier properties through its association with the endothelial glycocalyx.

Methods and results: Human umbilical vein endothelial cells (HUVECs, passage P0) were cultured on semi-permeable inserts in absence and presence of unfractionated heparin (UFH) and exposed to high glucose conditions (25mM) for 5 days, of which the last 24h in presence of an exogenous GAG mixture (Sulodexide®). FITC-labeled albumin transfer determined endothelial permeability over the endothelial monolayer. UFH in the culture medium was associated with a lower basal albumin permeability compared to the absence of UFH (11.1±1.6 versus 27.5±5.6µg/ml FITC-albumin respectively P<0.01). Hyperglycemic conditions increased endothelial albumin permeability in both the presence (12.5±1.8µg/ml; P<0.05) and absence (32.4±7.0µg/ml P<0.05) of UFH in the medium. Addition of 60µg/ml Sulodexide® to HUVEC cells normalized the increased albumin permeability under high glucose conditions only in the absence of UFH (11.8±6.3% versus -1.4±5.2%, P<0.05). In line, fluorescent-labeled Sulodexide® showed a higher cell association in absence of UFH.

Conclusion: GAG supplementation by means of Sulodexide® reversed the increased trans-endothelial albumin transfer under high glucose condition in absence of UFH. This implies that GAG supplementation may attenuate vascular hyperpermeability in hyperglycaemic conditions.
Introduction

Patients with diabetes mellitus are characterized by microvascular complications, comprising neuropathy, nephropathy and retinopathy, as well as macrovascular complications like myocardial infarction and cerebrovascular events \(^1,2\). While traditional risk factors for atherosclerosis contribute to the increased propensity towards vascular damage, correlation studies have suggested that the vasculature is more vulnerable towards these risk factors in patients with diabetes mellitus (DM) \(^3\). Whereas the exact cause for this increased vulnerability has been a matter of debate, it is clear that the effect of hyperglycemia on the vessel wall lining may be involved in this process \(^4\).

The endothelial glycocalyx, a layer of proteoglycans covering the endothelium, forms a barrier against atherosclerotic stimuli and regulates vascular permeability \(^5-7\). We previously revealed that high levels of circulating glucose readily compromise this barrier \(^8\). In line, progressive glycocalyx perturbation is also associated with microalbuminuria in patients with diabetes mellitus type 1 \(^9\). Interestingly, glycosaminoglycans (GAGs) are abundantly present in the renal glomeruli where they have an important function in maintaining the negatively charged filtration barrier \(^10\). Therapeutic interventions using Sulodexide\(^\text{®}\), a mixture of GAGs containing 80% low molecular weight (LMW) heparin and 20% dermatan sulphate, have been reported to decrease microalbuminuria in both type I and type II diabetic patients \(11\). However, the beneficial effect of Sulodexide\(^\text{®}\) could not be confirmed by large clinical trials \(14\).

Since GAGs are essential constituents of the glycocalyx, and diabetic microalbuminuria is characterized by severe perturbation of the glycocalyx in diabetic patients, it has been suggested that glycocalyx restoration might explain the mechanism by which administration of Sulodexide\(^\text{®}\) may reduce microalbuminuria. In the present study, we hypothesized that exogenously administered GAGs may contribute to the restoration of the damaged endothelial glycocalyx, thereby attenuating high glucose-induced increases in endothelial permeability.

Materials and Methods

Chemicals

M199 media, L-glutamine, antibiotic-antimycotic and trypsin were obtained from Gibco-BRL [Breda, the Netherlands], PBS pH: 7.4 from Fresenius Kabi [Bad Homburg, Germany] and Fetal Bovine Serum (FBS) from Biowhittaker [Basel, Switzerland]. The following chemicals were obtained from Sigma-Aldrich [Zwijndrecht, the Netherlands]; unfractionated heparin (UFH), endothelial cell growth supplement (ECGS) and 2-(N-morpholino)ethanesulfonic acid (MES). D(+) glucose was obtained from Merck [Schiphol-Rijk, the Netherlands]. Alexafluor 488 (goat anti-rabbit) was obtained from Invitrogen
(Breda, the Netherlands), 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide Hydrochloride [EDC] from Pierce [Etten-Leur, the Netherlands]. Fibronectin was a kind gift from the Central Laboratory for Blood transfusion [CLB, Amsterdam, The Netherlands]. Semi-permeable transwell plates with 3µm pores were purchased from Greiner [Alphen a/d Rijn, the Netherlands]. Sulodexide® was kindly provided by Alfa Wassermann [Bologna, Italy]. The study was carried out in accordance with the principles of the Declaration of Helsinki and approval was obtained from the internal review board of the Academic Medical Center.

Cell culture
HUVEC cells were isolated from human umbilical cords from the department of Obstetrics of the AMC in Amsterdam. Briefly, umbilical veins were canulated and rinsed with PBS before applying trypsin solution. The trypsin solution was incubated at 37°C to detach the endothelial cells from the vessel wall. Thereafter, the trypsin solution was collected and the vein was rinsed with PBS. The cell solution was centrifuged for 10 minutes at 1100 rpm, and the supernatant was removed after which the cell pellet was resuspended in 5ml M199 medium. Next, the cell suspension was cultured in a 10mg/ml fibronectin-coated cell culture flask of 25cm² in a M199 medium, supplemented with 20% heat-inactivated FBS, 50mg/ml ECGS, 0.2 mmol/l L-glutamine, 100U/ml Penicillin-G, 100U/ml Streptomycin sulfate and 25mg/ml Amphotericin-B at 37°C in 5% CO2. In order to determine the effect of UFH in cell culture medium, cells were grown in a second medium without UFH supplementation.

Albumin permeability assay
HUVECs were cultured on semi-permeable inserts and exposed to normo- (5mM) or hyperglycemic (25mM) conditions for 5 days, the last 24 hours in the presence 60mg/ml of the GAG mix Sulodexide®. Determining FITC-labelled bovine serum albumin transfer over the monolayer assessed endothelial permeability. Top compartment with HUVEC cells was incubated with 400mg/ml FITC-labelled albumin in 1%BSA/RPMI1640 media without phenol red. After 3 hours, the media from the bottom well was removed and FITC-Albumin content was measured by fluorescent spectrometry with a Fluostar Galaxy fluorescent spectrophotometer (BMG lab technologies, Germany).

Fluorescent labeling of Sulodexide®
The GAG mixture Sulodexide® was fluorescently labeled using the modified method by Osmond et al 15. Briefly, 1% Sulodexide® solution in 0.1M 4-morpholinoethanesulfonic acid [MES] pH:4.5 was mixed with 10% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride [EDC] in MES and 1% Alexafluor 488 [A488] in MES buffer. The mixture was protected from light and incubated overnight at 4°C while rotating. The AlexaFluor 488 labeled Sulodexide® [A488-Sulodexide] product was purified using a GE Healthcare PD10 desalting column, eluted with 10% EtOH/H₂O and vacuum dried to a powder. The quantitative GAG concentration in the fractions was determined using a color reagent dimethylmethylene blue assay 16, based on color reaction with sulfated GAG residues. To determine
the efficiency of A488 fluorescent label incorporation of the GAG mixture Sulodexide® in the collected fractions, a High Performance Gel Permeation Chromatography system was used, containing a PU-980 ternary pump with an LG-980-02 linear degasser and a FP-920 fluorescence detector (Jasco, Tokyo, Japan). Aliquots of PD10 fractions were applied to a Superdex200 (Pharmacia biotech, Diegem, Belgium) column and eluted with 1x PBS and the ratio of A488-Sulodexide and free label A488 was determined using the Borwin chromatographic software, version 1.23 (JMBS Developments, Le Fontanil, France). The Sulodexide® fraction with the highest A488 label incorporation was used for HUVEC cell association studies.

Fluorescent labeled Sulodexide® association with HUVEC monolayer
HUVECs were cultured in absence and presence of UFH in culture media under normo- and high glucose conditions for 5 days on permanox slide chambers (NUNC, Sigma-Aldrich, Zwijndrecht, the Netherlands). The cells were rinsed with normal conditioned media and incubated with 23.2µg/ml A488-Sulodexide® in conditioned media for 30 minutes at room temperature. The fluorescent-labeled GAG mixture used for cell incubation contained 96% A488-Sulodexide®. Cells were washed with conditioned medium to remove unbound labeled A488 Sulodexide® and cells were imaged using Nikon Eclipse TE 2000-U fluorescence microscope (with a DXM1 200F camera). For data analysis, NIS elements AR2.3 software from Laboratory imaging and ImageJ software from NIH (National Institutes of Health) were used.

Statistical analysis
For statistical analysis, two-way paired and unpaired t-tests were used. A value of p < 0.05 was considered statistically significant. Statistical analyses were performed using Graphpad prism 5. Values are means ± SE.

Results

Effect of unfractionated heparin on albumin permeability
The HUVECs were cultured in normoglycemic (NG) (5mM) and hyperglycemic (HG) (25mM) conditions for 5 days in the absence and presence of UFH. Figure 1 shows that the HUVEC monolayer cultured without UFH was more permeable for albumin compared to the monolayer cultured with UFH in the medium (27.5±5.6 without UFH vs. 11.1±1.6µg/ml with UFH, p<0.01). Hyperglycemic conditions significantly increased albumin leakage in HUVECs cultured in both the absence and the presence of UFH (32.4±7.0 vs. 12.6±1.8µg/ml, p<0.01).
Figure 1. Albumin permeability through the HUVEC monolayer is increased without UFH in the medium: 27.5 ± 5.6µg/ml compared to cells cultured under the addition of 50µg/ml UFH: 11.1 ± 1.6µg/ml (p<0.01, n=15). High glucose conditions increased albumin permeability both in the absence and in the presence of UFH (32.4 ± 7µg/ml vs. 12.6 ± 1.8µg/ml; p<0.05, n=15). White column = normoglycemic, black column = hyperglycemic, *= p<0.05, **= p<0.01.

Albumin permeability under normoglycemic conditions after 24 hours Sulodexide

No effect was observed on albumin permeability in cells cultured under NG conditions in the absence of UFH, exposed to 60mg/ml Sulodexide® for the last 24 hours (36.8±10.6µg/ml Sulodexide® vs. 27.5±5.6µg/ml no Sulodexide® FITC-Albumin, p=ns) (figure 2). In the presence of UFH, Sulodexide® supplementation increased albumin permeability under NG conditions (17.3 ± 3.0µg/ml Sulodexide® vs. 11.1 ± 1.6µg/ml no Sulodexide® FITC-albumin, p<0.05).

Figure 2. Sulodexide® had no effect on baseline permeability of the HUVEC monolayer cultured under normoglycemic conditions without UFH; 27.5 ± 5.6 versus 36.8 ± 10.6µg/ml; (p=ns), n=8. Normoglycemic albumin permeability is increased in HUVECs cultured in the presence of both 50µg/ml UFH and 60µg/ml Sulodexide® compared to UFH without Sulodexide®: 17.3 ± 3.0µg/ml vs. 11.1 ± 1.6µg/ml FITC-albumin; (p<0.05) n=7. White column = normoglycemic, without Sulodexide, striped column = normoglycemic, with Sulodexide, *= p<0.05.
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Effect of Sulodexide on albumin permeability under high glucose conditions
First, the GAG mixture Sulodexide® was added to a HUVEC monolayer with hyperglycemia induced albumin permeability in order to determine the optimal glycocalyx-restoring dose. Then, cultured HUVECs were incubated for the last 24 hours with Sulodexide® at 60mg/ml. Supplementation of 60µg/ml Sulodexide® had a beneficial effect on high glucose-induced permeability of the monolayer in the absence of UFH. Figure 3 shows that Sulodexide® supplementation normalized albumin permeability to control levels without UFH in the medium [-1.4±5.2% Sulodexide® vs. 11.8±6.3% no Sulodexide®, p<0.05]. In contrast, the experiments conducted with UFH in the culture medium showed no significant effect of Sulodexide® on permeability of the monolayer under HG conditions [10.8±3.0% Sulodexide® vs. 13.7±5.6% no Sulodexide®, p=ns].

Figure 3. Sulodexide® at a concentration of 60µg/ml attenuated the high glucose increase in permeability of the HUVEC monolayer cultured in the absence of UFH [11.8±6.3% vs. -1.36±5.2%; (p<0.05), n=8]. HUVECs cultured in the presence of 50µg/ml UFH showed no reduction of high glucose induced albumin permeability by Sulodexide® [16.5 ± 9.3% vs. 14.7±5.7%; (p=ns), n=7]. Black column = hyperglycemic, without Sulodexide, striped column = hyperglycemic, with Sulodexide, * = p<0.05.

The association of fluorescent-labeled Sulodexide® with HUVEC monolayer
Figure 4 shows that the A488 Sulodexide® staining of the monolayer is concentrated at specific cells in the culture instead of having a uniform association. HUVECs cultured without UFH in the culture medium showed more fluorescent A488-Sulodexide® association with the cells compared to cultures grown with UFH [25.7 ± 2.4 vs. 6.3 ± 0.5 arbitrary units (a.u.) fluorescence, p<0.05]. HG conditions did not influence A488-fluorescent Sulodexide® staining, independent of UFH addition [25.7±2.4 NG vs. 22.4±3.1 HG (p=ns)] with UFH and [6.3±0.5 NG vs. 9.6±2.0 HG a.u. fluorescence (p=ns)] without UFH.
Figure 4. The imaging results show more cell association of fluorescent-labeled Sulodexide® with cells cultured without UFH (25.7±2.4 a.u. fluorescence) compared to cells cultured with UFH in the medium (6.3±0.5 a.u. fluorescence, \( p<0.05 \)). Hyperglycemic conditions did not influence fluorescent Sulodexide® staining in the absence nor presence of UFH; 25.7±2.4 versus 22.4±3.1 (\( p=ns \)) and 6.3±0.5 versus 9.6±2.0 a.u. fluorescence (\( p=ns \)). White column = normoglycemic, black = hyperglycemic, * = \( p<0.05 \).

Discussion

The present study shows that Sulodexide®, a mixture of 80% LMW heparin and 20% dermatan sulfate, restores hyperglycemia-induced albumin permeability through a cultured monolayer of HUVECs, only in the absence of unfractionated heparin (UFH) in the medium. Under normoglycemic conditions however, addition of Sulodexide on top of UFH increased albumin permeability. These data imply that supplementation of exogenous GAGs may restore hyperglycaemia-associated hyperpermeability by restoring the glycocalyx barrier in diabetes but the mechanisms underlying this effect are unclear.

Glycocalyx and GAG supplementation

We observed a decreased albumin permeability of the HUVEC layer in the presence of UFH compared to those cultured without UFH. The improvement in endothelial permeability following heparin exposure can be explained by a loss of intercellular gaps due to heparin-induced reorganization of the cytoskeletal structure. In comparison, studies in rabbits also reported decreased glomerular albumin leakage following UFH administration. In porcine aortic endothelial cells, it has been reported that cell-associated GAGs were decreased following exposure to high glucose conditions with a concomitant increase in GAGs in the medium. In fact, GAG synthesis was stimulated in these cells following the addition of heparin to the medium. In the present study, we observed less association of cultured cells with fluorescent-labeled Sulodexide® in the case of presence of UFH in the medium. In this setting, one can speculate that fewer binding sites are available for the GAG mixture due to
either the presence of heparin or the increased production of GAGs induced by heparin. In support of a beneficial impact of substrate supplementation, a mixture of heparan- and heparin sulfate could also prevent ox-LDL–induced deceleration of rolling leukocytes 20. Similarly, endothelial glycocalyx degradation following hyaluronidase exposure, an enzyme that catalyses the hydrolyses of hyaluronan, could be reconstituted by infusing a GAG-mixture containing hyaluronan and chondroitin sulfate. Treatment with either GAG molecule separately however, had no clear effect 21. Collectively, the present data suggest that exogenous GAGs may have the capacity to protect the vessel wall from pathologic stimuli by restoring a locally perturbed glycocalyx layer.

**Hyperglycemia and Glycocalyx Perturbation**

The increase in endothelial albumin permeability under high glucose conditions in the present in vitro model supports previous studies which show that both acute and chronic hyperglycemia induce a reduction of glycocalyx volume in humans 8, 9. During hyperglycemia in vivo, several mechanisms could be involved in the loss of glycocalyx volume. First, GAGs are formed from amino-sugars or hexosamines to form large complexes of repeated disaccharide units. It has been suggested that activation of the hexosamine pathway might be partially responsible for the adverse effects of chronic hyperglycemia by shunting of excess intracellular glucose into the hexosamine pathway 22. This may eventually reduce the production of essential components within the glycocalyx. Second, patients with DM are characterized by an increase in circulating GAGs from the glycocalyx, e.g. hyaluronan, in the presence of increased levels of glycocalyx degrading enzymes, comprising hyaluronidase 9. Apparently, high glucose levels induce glycocalyx destruction through upregulation of glycocalyx degrading enzymes. On the other hand, injury to the vasculature may cause increased shedding of GAGs, resulting in an up-regulation of GAG synthesis to compensate for the increased degradation 23, 24, which can also be responsible for the increase in circulating GAG levels. High glucose conditions have been shown to increase heparanase activity and heparin compounds are able to inhibit this activity 25. The fact that we observed no effect of Sulodexide® supplementation on high glucose treated HUVECs cultured in the presence of UFH may relate to inability of the Sulodexide® compound to reach desirable sites on endothelial cells due to the presence of excess high molecular weight UFH.

**Clinical Implications**

Glycocalyx damage has been suggested to be involved in the deterioration of vascular barrier function that can ultimately contribute to both micro- as well as macro-vascular diabetic disease 26. Efforts aiming to restore the endothelial glycocalyx by exogenous administration of GAG components may offer a new strategy to prevent complications in patients with DM. Future studies will have to explore the value of GAG supplementation in a clinical setting.
Study limitations

The present study has certain limitations. First, it should be mentioned that UFH, often used in the medium of cultured endothelial cells, influences the effect of Sulodexide® on albumin permeability and should be taken into account when studying the glycocalyx in cultured cells. Second, HUVECs were used as a representation of endothelial cell behaviour in general and as a surrogate for conditions in vivo. The dimension of the glycocalyx is influenced by several environmental factors like culture conditions, inflammation induced by preparation of cells and (absence of) shear stress. But despite these unfavourable circumstances in vitro, it has been shown by immunostaining that glycocalyx constituents are present at the surface of cultured cells. This indicates that there is production of glycocalyx constituents under in vitro cell culture conditions. However in recent work, other authors have expressed concern about the remaining functions of a glycocalyx layer produced under such conditions. Altogether this debate points out the urgent need for new in vitro models with the capacity to study the endothelial glycocalyx under circumstances more similar to the environment in vivo, including the presence of shear stress. Such models are under development now and will be essential to further elucidate glycocalyx behaviour and potential therapies restoring glycocalyx damage.
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Reference List


