Heparanase Upregulation in High Glucose-Treated Endothelial Cells Is Prevented by Insulin and Heparin

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Heparan sulfate proteoglycans on the endothelial cell surface and extracellular matrix play an important role in vascular homeostasis. Previous studies have shown that the quantity of heparan sulfate is reduced in kidney and other organs in diabetes. The objectives of this study were to determine if heparanase is induced by high glucose in endothelial cells and if heparin and/or insulin or basic fibroblast growth factor (bFGF) affect this upregulation. Cultured porcine aortic endothelial cells in M199 medium were treated with high glucose (30 m*M*) and/or bFGF (1 or 10 ng/ml) or high glucose plus insulin (1 U/ml) and/or heparin (0.5 μg/ml) for 7 days. To help define the mechanism of endothelial damage, cells were also exposed to H_2O_2 (0.1 m*M*) for 1 day or mannitol (30 m*M*) for 7 days. Heparanase mRNA was detected by reverse transcription polymerase chain reaction. Heparanase activity was measured by incubating cell lysates with [^{35}S]labeled extracellular matrix of bovine corneal endothelial cells and analyzing released radioactive products by gel filtration and β-scintillation. Heparanase mRNA was found in high-glucose– and H_2O_2-treated cells; however, it was not found in control cells, mannitol- or high glucose plus insulin– and/or heparin-treated cells, or fresh porcine tissue. Heparanase activity was only found in high-glucose– and H_2O_2-treated cells. As well, bFGF did not prevent heparanase mRNA upregulation by high glucose. From these observations, we concluded that heparanase upregulation by high glucose is prevented by insulin and/or heparin but not bFGF. Reactive oxygen species, but not changes in osmolarity, may be involved in the upregulation of heparanase. Exp Biol Med 232:927–934, 2007

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Introduction

Angiopathy, a complication of diabetes mellitus, is characterized by microvascular pathology in the retina and renal glomerulus as well as changes typical of arterial disease in the macrovasculature (1–3). The endothelial cells (ECs) lining all vessels appear to be the initial target in vascular damage by hyperglycemia. EC injury is characterized by loss of antithrombotic and profibrinolytic activity (4), due to increased levels of plasminogen activator inhibitor-1 and other factors (5), and by a decrease in vasodilatory properties due to a decrease in nitric oxide (6) and prostacyclin production and an increase in endothelin activity (7). In addition, diabetes, especially type 2 diabetes, is considered an inflammatory condition characterized by upregulation of proinflammatory cytokines such as interleukin-6, tumor necrosis factor α, and C-reactive protein (8, 9). These changes may cause thrombus formation and vascular monocyte/macrophage accumulation leading to atherosclerosis. Furthermore, these changes can cause exaggerated proliferation of ECs and smooth muscle cells, as well as thickening of the extracellular matrix (ECM), including the basement membrane, leading to vascular remodeling (10). These abnormalities result in vasoconstriction, hypertension, tissue ischemia and eventually infarction, and an increase in vascular permeability (11).

Mechanisms leading to hyperglycemia-induced EC injury are multifaceted and still unclear. A complex array of mechanisms considered include binding of advanced glycosylation end products to crucial enzymes and structural proteins (8); deregulation of chemical pathways; accumulation of sorbitol (12); production of reactive oxygen species (ROS) and deficiencies in antioxidant mechanisms (12); and

modification of heparan sulfate (HS), although this has not been extensively studied.

HS is a glycosaminoglycan (GAG) associated with the cell membrane, basement membrane, and ECM (13). Depletion of HS and/or alteration in GAG metabolism may be a major mechanism of EC injury (14, 15). In previous studies, autopsies of patients with diabetic nephropathy showed decreased GAGs and HS proteoglycans (HSPGs) in the glomerulus (16, 17). Other studies have shown decreased serum HS and increased urine GAGs in patients with overt diabetic nephropathy (16). The highly negatively charged HS, due to sulfate and carboxylate residues, controls vascular permeability by acting as a "charge barrier" to the largely anionic plasma proteins (18). Although HS loss is a major cause of endothelial injury in angiopathy induced by hyperglycemia, the cause of HS degradation is still unknown.

Heparanase, an endoglucuronidase, breaks down HS. It is involved in fundamental biological and pathological processes such as inflammation, angiogenesis, autoimmunity, and cancer metastasis (19). It is normally found in cytotrophoblasts, platelets, mast cells, neutrophils, macrophages, and T and B lymphocytes (20). Heparanase activity has been found in urine, and heparanase protein is expressed in the renal glomerular cells of some diabetic patients (21). It is suggested that heparanase may be induced by hyperglycemia (22) and may contribute to EC dysfunction by degradation of HS. Indeed, our recent studies indicated that addition of heparinase I to medium results in injury to cultured endothelial cells. Although chemically distinct from mammalian heparanase, heparinase I is a bacterial lyase that breaks down HS (23). We have also previously observed that H_2O_2 causes endothelial injury (24); thus, our plan was to determine if H_2O_2 upregulates heparanase.

Previous studies have shown that insulin and/or heparin, by an unknown mechanism, prevents formation of intercellular gaps in ECs cultured in high glucose (25). These compounds may protect ECs from high-glucose injury by preventing heparanase upregulation. Basic fibroblast growth factor (bFGF) increases proliferation of capillary ECs *in vitro*, has high affinity for heparin or HSPG (26), and may inhibit HSPG degradation and protect cells from injury. Furthermore, our previous results showed that high-glucose– or heparinase I–induced EC injury is prevented by insulin and/or heparin and/or bFGF (23). Thus, our objectives were to determine if heparanase is upregulated in ECs exposed to high glucose and if heparin and/or insulin and/or bFGF suppresses heparanase expression.

Materials and Methods

EC Cultures. Primary porcine aortic ECs (PAECs) were cultured by the method of Gotlieb and Spector (27). Cells were obtained from 15 different porcine aortic segments. For the culture process, aortic segments were trimmed and washed three times in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (CMF-DPBS). After the aorta was clamped with a hemostat, the lumen was rinsed three times with CMF-DPBS and then filled with collagenase (type IV; 1 mg/ml in CMF-DPBS; Sigma-Aldrich, St. Louis, MO) for 6 mins. After the collagenase was removed, the lumen was gently rinsed with medium (M199 containing 5.5 m*M* D-glucose but no heparin or insulin; Invitrogen Corp., Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Invitrogen), 50 μg/ml penicillin (Sigma-Aldrich), and 10 μg/ml streptomycin (Sigma-Aldrich). The medium was plated onto 60-mm culture dishes that were incubated at 37°C with 5% CO_2/95% air in a humidified environment. PAECs were identified by their morphologic appearance of cobblestone-like flattened cells and the presence of von Willebrand factor in initial cultures. Before the first passage, nonendothelial-like cells, such as smooth muscle cells and fibroblasts, were destroyed cell by cell using a Pasteur pipette and mechanical suction. To pass cells, confluent cultures were washed twice with CMF-DPBS and cells were detached by trypsin (0.025% with EDTA in CMF-DPBS) for 2 or 3 mins at room temperature. The cells were resuspended in medium and transferred to 60-mm dishes for further passage. For experiments, the cells were grown in 35-mm dishes and used at confluence at passage four except when control initial confluent and nonconfluent PAECs were studied.

Reagents. Glucose (D-glucose; BDH Inc., Toronto, Canada), heparin (bovine lung; 151 USP U/mg; Upjohn Pharmaceuticals, Kalamazoo, MI), insulin (Humulin N), H_2O_2 (Sigma-Aldrich), and mannitol (Sigma-Aldrich) were prepared as stock solutions in CMF-DPBS. Stock solution concentrations were 3 *M* for glucose, 0.1 mg/ml for heparin, 100 U/ml for insulin, 10 m*M* for H_2O_2, and 600 m*M* for mannitol. Stock solutions of bFGF (Sigma-Aldrich) were prepared in M199 without serum at 1 and 0.1 ng/μl.

Treatment of Cultured Cells. Cultured PAECs in 35-mm dishes with 1 ml of medium were treated with glucose, glucose plus heparin, glucose plus insulin, glucose plus heparin plus insulin, or glucose plus bFGF for 7 days. Final concentrations in medium were 30 m*M* for glucose, 0.5 μg/ml for heparin, 1 U/ml for insulin, and 1 or 10 ng/ml for bFGF. Medium was changed and reagents were added fresh every other day for 7 days. In some experiments, mannitol was added for 7 days at a final concentration of 30 m*M*. As well, H_2O_2 at a final concentration of 0.1 m*M* was added for 24 hrs.

Control Porcine Tissue. Samples of liver, aortic smooth muscle, and kidney were collected from newly slaughtered healthy, untreated pigs. The 50-mg samples were stored in RNA stabilization reagent (RN Later; QIAGEN, Mississauga, Canada) for 3 or 4 days at room temperature.

Detection of Heparanase mRNA. To isolate RNA, porcine tissue stored in RN Later and fresh cultures were washed three times in ice-cold CMF-DPBS. Then total RNA was isolated according to the manufacturer's procedures

using an RNeasy Mini Kit (QIAGEN). The RNA concentrations were determined by ultraviolet absorption using a spectrophotometer (Cary 100 Bio UV-Visible; Varian Canada, Inc., Mississauga, Canada). A spectrophotometric ratio of absorbance between 1.7 and 2.0 at wavelengths 260/280 nm indicates pure RNA isolates. The RNA was visualized on a 1% formaldehyde agarose gel. Then 1 μg of total RNA was used to perform reverse transcription polymerase chain reaction (RT-PCR) using a SuperScript One-Step RT-PCR Kit with Platinum Taq (Invitrogen) and primers specific for the rat heparanase gene. Specific primers, Hep 458 5′-CAAGAACAGCACCTACTCA-3′ and Hep 1055 5′-CACATAAAGCCAGCTGCA-3′, were designed using Gene Fisher software to amplify a 597-bp DNA fragment. The primers were designed to span intron regions to prevent amplification of contaminating genomic DNA. As well, the internal standard house keeping gene β-actin, amplified with sense primer 5′-GGACTTCGAG-ACGGAGATGG-3′ and antisense primer 5′-GCACC-GTGTTGGCGTAGAGG-3′ for each RNA sample, was expressed as a 233-bp cDNA. The RT-PCR profile initially consisted of a cDNA synthesis step of 30 mins at 50°C followed by 5 mins at 94°C to inactivate the reverse transcriptase and activate the Taq polymerase. The RT-PCR conditions were: initial denaturation at 94°C for 2 mins and 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 mins. The RT-PCR products (10 μl) were electrophoresed on a 1.5% agarose gel containing 0.5–1 μg/ml ethidium bromide (Sigma-Aldrich) to distinguish the 597-bp cDNA fragment of the heparanase gene and 233-bp cDNA for β-actin.

Detection of Heparanase Activity. Heparanase activity was determined by a previously published method (28) based on the ability of heparanase to break down HS in the ECM of bovine corneal endothelial cells (BCECs). [^{35}S]labeled BCEC ECM was incubated with PAEC lysates for 24 hrs at 37°C. Supernatants from the incubation were analyzed for degradation of [^{35}S]labeled HS.

To prepare ECM from primary BCECs, fresh cow eyes were sterilized by soaking in 75% ethanol. The cornea was separated using small scissors inserted into a small hole first made by puncturing the cornea with a sterilized needle. The corneal pieces were washed thoroughly with CMF-DPBS and placed in a culture dish with the endothelial side up. The BCECs were gently scraped from the cornea using a metal spatula and then transferred to a 60-mm culture dish containing 5 ml of BCEC medium (Dulbecco's modified Eagle's medium supplemented with 10% FBS, 5% calf serum [Invitrogen], 50 μg/ml gentamicin [Invitrogen], and 0.25 μg/ml fungizone [Invitrogen]). Cultures were incubated at 37°C in a humidified incubator with 5% CO_2/95% air for 5 days, the media was changed, and bFGF (1 ng/ml) was added every other day. At Day 10 cells were passed using 0.025% trypsin into 60-mm gelatinized dishes; after 24 hrs medium was changed and bFGF (1 ng/ml) was added every other day. For gelatinizing dishes, 0.2% w/v gelatin (type B; bovine skin; Sigma-Aldrich) in CMF-DPBS was autoclaved and filtered at 0.22 μm after cooling. The gelatin solution (3 ml) was added to culture dishes stored at 4°C for at least 3 hrs and was removed prior to addition of the cell suspension.

To prepare [^{35}S]labeled ECM, BCECs (passage two to four) were seeded on 35-mm culture dishes (2×10^5 cells/dish) with BCEC medium supplemented with 5% dextran (Sigma-Aldrich). Then 40 μCi $Na_2{}^{35}SO_4$ (540 to 590 mCi/mmol; Amersham Biosciences, Baie d'Urfé, Québec, Canada) was added to cultures on Days 2 and 5 without media change, although fresh media was added on Day 5 (0.5 ml/dish). At confluence 10 to 12 days after seeding, medium was removed, cells were washed three times with CMF-DPBS, and then exposed to the lysis solution (0.5% Triton X-100 and 0.025 *N* NH_4OH in CMF-DPBS) for 3–4 mins with gentle shaking at room temperature followed by washing three times with CMF-DPBS. The dishes were examined under an inverted-phase microscope for ECM.

Heparanase activity was measured in PAEC lysates. To prepare lysates, cells were washed twice with 0.5 ml of CMF-DPBS to remove medium. Cells were removed from the culture dish by addition of 0.025% trypsin in 0.01% EDTA for 3 mins, followed by addition of 0.5 ml of medium. Cells were flushed from the dish using a pipette, and the cells were added to a test tube. The final volume in the test tube was adjusted to 1.5 ml with medium. Dishes were checked under the microscope to ensure that all cells were removed. Cells were then counted using a hemocytometer. The cell suspension was centrifuged, and the cell pellet resuspended in phosphate buffer (pH 6.5) with 1 m*M* Mg^{2+} at $5–7 \times 10^5$ cells/ml. Cells were lysed by three cycles of freezing (–80°C) and thawing (37°C). Then 1 ml of the cell suspension was added to the newly made [^{35}S]labeled ECMs for 24 hrs at 37°C.

To evaluate HS degradation, a 0.8-ml aliquot of the supernatant was filtered through Sepharose 6B columns (0.9×8 cm; Amersham Biosciences). Fractions of 200 μl were eluted with CMF-DPBS at a flow rate of 6 ml/hr and counted for radioactivity by a β-scintillation counter (LS6000IC; Beckman Coulter Canada, Inc., Mississauga, Canada). In previous studies, high-Mr [^{35}S]labeled material or intact HS was eluted at peak I after V_0 (Kav < 0.2), fraction 5–10; the low-Mr [^{35}S]labeled products or degraded HS were eluted at peak II ($0.5 <$ Kav < 0.8), fraction 5–10 (28).

Results

Heparanase mRNA Expression. To determine if high glucose induced heparanase mRNA, total RNA was isolated from PAECs, in initial or passage 4 cultures, in which 30 m*M* glucose was added to medium for 7 days. RT-PCR products run on agarose gel electrophoresis showed a positive band at 597 bp (Fig. 1A, Lanes ICG and G; Fig. 1B

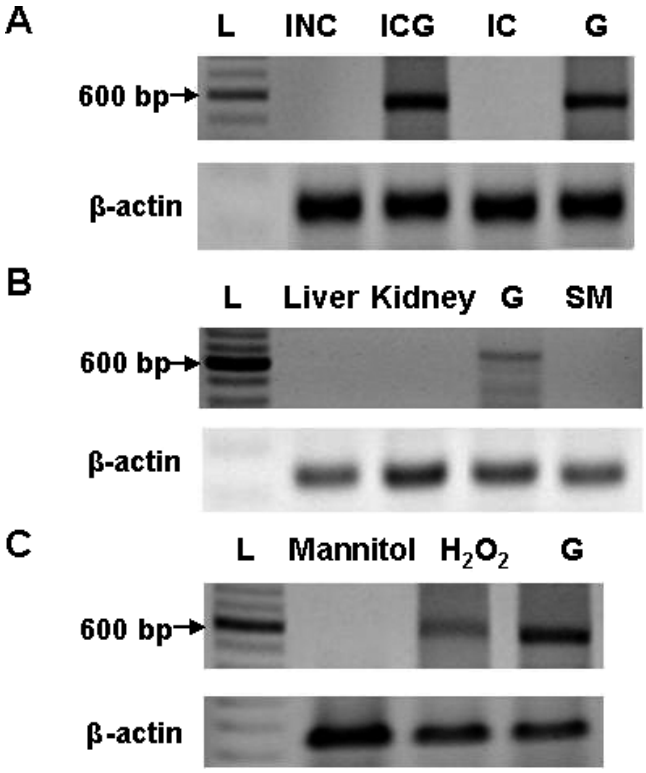

Figure 1. Heparanase mRNA in treated and untreated primary cultured PAECs and porcine tissue. Total RNA from porcine tissues and PAEC cultures was extracted, transcribed to cDNA, and subjected to PCR for heparanase. Agarose gel electrophoresis slides of RT-PCR products are shown. (A) Lane L, 100-bp DNA ladder. Lane INC, control initial nonconfluent PAECs. Lane ING, initial confluent PAECs treated with glucose (30 m*M*) for 7 days. Lane IC, control initial confluent PAECs. Lane G, PAECs treated with glucose (30 m*M*) for 7 days, passage 4. (B) Lane L, 100-bp DNA ladder. Lanes Liver and Kidney, both from untreated pigs. Lane G, PAECs treated with glucose (30 m*M*) for 7 days. Lane SM, aortic smooth muscle from untreated pigs. (C) Lane L, 100-bp DNA ladder; Lane Mannitol, PAECs treated with mannitol (30 m*M*) for 7 days. Lane H_2O_2, PAECs treated with H_2O_2 (0.1 mM) for 24 hrs. Lane G, PAECs treated with glucose (30 m*M*) for 7 days. β-actin cDNA was an internal control. These experiments were done on two or three separate occasions with similar results.

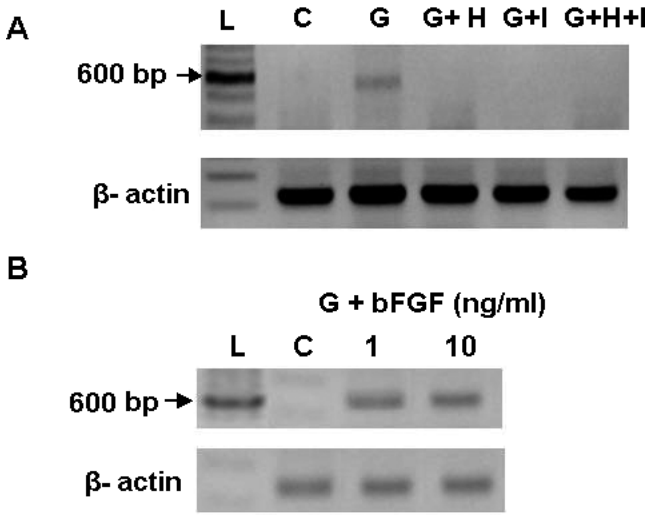

Figure 2. High-glucose–induced heparanase mRNA in PAECs is inhibited by insulin and/or heparin but not bFGF. Total RNA from passage 4 PAECs treated with glucose (30 m*M*), glucose plus heparin (0.5 μg/ml), glucose plus insulin (1 U/ml), glucose plus heparin plus insulin, and glucose plus bFGF (1 and 10 ng/ml) for 7 days was extracted, transcribed to cDNA, and subjected to PCR for heparanase. Agarose gel electrophoresis slides of RT-PCR products are shown. (A) Lanes L, 100-bp DNA ladder. Lane C, PAECs exposed to control medium. Lane G, PAECs treated with glucose (30 m*M*). Lane G+H, glucose plus heparin. Lane G+I, glucose plus insulin. Lane G+H+I, glucose plus heparin plus insulin. (B) Lane L, 100-bp DNA ladder. Lane C, PAECs exposed to control medium. Lane 1, glucose plus bFGF (1 ng/ml). Lane 10, glucose plus bFGF (10 ng/ml). β-actin cDNA was an internal control. This experiment was done on three separate occasions with similar results.

and C, Lane G). Heparanase mRNA was not detected in control untreated PAEC cultures, whether RNA was isolated from confluent or nonconfluent PAECs following initial plating (Fig. 1A, Lanes INC and IC). Further, to determine if other porcine cells produced heparanase mRNA, total RNA was isolated from untreated porcine liver, kidney, and aortic smooth muscle. RT-PCR products run on agarose gel electrophoresis were negative for heparanase mRNA, as no bands appeared at 597 bp (Fig. 1B, Lanes Liver, Kidney, and SM). To determine if ROS or changes in osmolarity also induced heparanase mRNA, total RNA was isolated from PAECs treated with H_2O_2 (0.1 m*M*) for 24 hrs or mannitol (30 m*M*) for 7 days. RT-PCR products run on agarose gel electrophoresis showed a positive band at 597 bp for H_2O_2 (Fig. 1C, Lane H_2O_2) but not mannitol (Fig. 1C, Lane Mannitol).

To determine if insulin (1 U/ml) and/or heparin (0.5 μg/ml) or bFGF (1 or 10 ng/ml) inhibit heparanase production induced by high glucose (30 m*M*), total RNA was isolated from passage 4 control PAECs and PAECs treated with high glucose and/or insulin and/or heparin or bFGF for 7 days. RT-PCR products showed a positive band at 597 bp in high-glucose–treated PAECs (Fig. 2A, Lane G) and in high glucose plus bFGF (1 and 10 ng/ml)–treated PAECs (Fig. 2B, Lanes G + bFGF [ng/ml], 1, and 10), which was not detected in the control (Fig. 2A and B, Lane C) or high glucose plus heparin– and/or insulin-treated cells (Fig. 2A, Lanes G+H, G+I, and G+H+I).

Heparanase Activity Assay. In addition to measuring heparanase mRNA, heparanase activity was measured in PAECs following various treatments to ensure that an active form of heparanase was present. Degraded HS indicates heparanase activity following incubation of [^{35}S]labeled ECM with PAEC lysates. Incubation with high-glucose– or H_2O_2-treated PAECs resulted in release of low-Mr [^{35}S]labeled degradation products into the incubation medium shown at peak II ($0.5 < Kav < 0.8$, fraction 15–25) (Fig. 3A and B). Results were similar when the ECM was incubated with 0.1 U/ml heparinase I in phosphate buffer (positive control) (Fig. 3A). Addition of lysates from control cells and cells treated with high glucose plus insulin and/or heparin or mannitol showed

release of only high-Mr [^{35}S]labeled products at peak I (Kav $<$ 0.2, fraction 5–10) with no degradation products found at peak II (Fig. 3A and B).

Discussion

The monolayer of ECs lining all blood and lymphatic vessels plays an important role in the integrity and normal function of the circulatory system. HSPGs are synthesized by ECs and are incorporated into their plasma membranes, ECM, and basement membrane. HSPGs consist of a protein core to which the GAG chains, the majority being HS, are covalently attached (29). The negatively charged HS (30) helps to control vascular permeability and prevents the transvascular movement of the large anionic plasma proteins (18). In addition, HS specifically interacts with various adhesive macromolecules in the ECM such as fibronectin, laminin, and collagen. Hence, degradation of HSPGs may disassemble the ECM and basement membrane, resulting in EC detachment and vascular damage (31). Further, HS binds to and may alter the activity of many important cationic molecules, including lipoprotein lipase, antithrombin, and Apo B and E (32), and can act as a coreceptor for many endothelial ligands (33). Thus, modifications in HS can interfere with many normal endothelial functions.

Heparanase is an endo-β-D-glucuronidase that degrades HS at specific sites. Heparanase protein is translated into an inactive form, preproheparanase, that is cleaved to become the active form, heparanase (19). Therefore, we looked at heparanase activity as well as mRNA to ensure that the active form of heparanase was produced. Although human and animal heparanase genes were recently cloned and heparanase proteins were encoded (34, 35), the porcine heparanase gene and protein has not been previously studied. Alignment of the human, mouse, and rat heparanase amino acid sequences corresponding to the 50-kDa human mature enzyme revealed 80%–93% identity (34, 35). Therefore, the rat heparanase gene sequence was used to design the primers to amplify the heparanase cDNA for porcine tissue and PAECs in our studies. Similar to observations in human studies (36), no detectable heparanase mRNA was observed in fresh untreated porcine tissue and initial primary confluent and nonconfluent PAEC cultures. Thus, PAECs can be used as an *in vitro* model to study heparanase expression in ECs for human disease.

Heparanase is rarely found in human or bovine aortic ECs following exposure to physiological activators such as bFGF, thrombin, endotoxin, interleukin-1, tumor necrosis factor, calcium ionophore, or radiation (36, 37). This suggests that EC heparanase is only released following injury that may then degrade HSPG. HSPG degradation involves vessel wall disruption and new vessel formation in both normal and pathological situations such as wound healing, tissue repair, and cancer progression (38). Maxhimer *et al*. (22) showed that the heparanase-1 gene was expressed in high-glucose–treated renal epithelial cells. Our present study is the first to show that

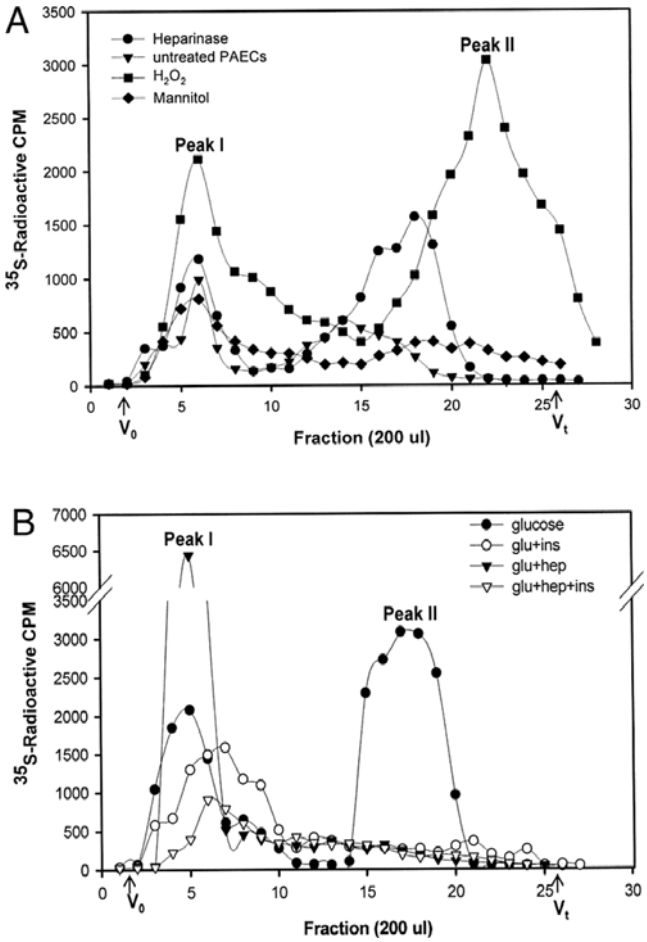

Figure 3. Heparanase activity in PAECs. PAECs treated with mannitol (30 m*M*) or glucose (30 m*M*) and/or heparin (0.5 μg/ml) and/or insulin (1 U/ml) for 7 days or H_2O_2 (0.1 m*M*) for 24 hrs. [^{35}S]labeled ECM from BCECs was incubated with PAEC lysates (1 ml) at 37°C for 24 hrs. The supernatant (0.8 ml) was filtered in Sepharose 6B columns. Eluted fractions (200 μl) were counted by β-scintillation. Shorter-chain fragments eluted at peak II indicates HS degradation and thus heparanase activity, whereas nondegraded HS eluted at peak I showed no heparanase activity. (A) Heparinase I (0.1 U/ml) (positive control) (●), untreated PAECs (negative control) (▼), H_2O_2 (■), mannitol (◆). (B) glucose (●), glucose plus heparin (▼), glucose plus insulin (○), glucose plus heparin plus insulin (▽). V_0, void volume; V_t, total volume. This experiment was done on three separate occasions with similar results with variation in elution position (Kav values) <10%. These results are from a representative experiment.

heparanase mRNA and activity were detected in PAECs treated with high glucose and H_2O_2, which cause EC injury. Indeed, PAEC injury was demonstrated by a decrease in the number of live cells per culture and increase in lactate dehydrogenase (LDH) release into medium in high-glucose–, heparinase I–, or H_2O_2-treated compared with control cells (23, 24, 39). An increase in osmolarity is likely not the cause of heparanase upregulation because heparanase mRNA and activity was not found in mannitol-treated cells. ROS may be involved in the pathway by which hyperglycemia induces heparanase upregulation since heparanase was expressed in H_2O_2-treated cells.

Previous studies found decreased serum HS and increased urine GAGs in patients with overt diabetic nephropathy (40, 41) and decreased GAGs in the glomerular basement membranes of patients with diabetic nephropathy compared with nondiabetic subjects (17). In patients with diabetic nephropathy, the decreased quantity of HSPGs in the glomerular basement membrane correlated with the degree of proteinuria (42), as did changes in HSPG content and structure (43). Increased glomerular permeability to largely anionic plasma proteins may result from loss of HSPGs, the negative "charge barrier" (14), due to HS degradation by heparanase. Heparanase activity has been shown in renal glomerular cells and in the urine of diabetic patients (21). Kidney biopsies from patients with diabetic nephropathy show a marked decrease in glomerular and tubular HS, which is associated with increased heparanase expression. An upregulation of heparanase, associated with a disappearance in glomerular HS, has also been seen in rats and mice with streptozotocin-induced diabetes (44). In addition, a decrease in HS in the aortic intima of diabetic patients was observed (45), suggesting that abnormalities in HS metabolism may occur in the whole cardiovascular system, linking cardiovascular complications and nephropathy, although the site of heparanase production is unclear.

The ECs in macrovessels and microvessels have different properties, but both are characterized by the same pathological features in diabetes mellitus, such as exaggerated proliferation of ECs and thickening of the basement membrane resulting in narrowing of the vessel lumen, which contributes to premature thrombosis, and ischemia (11, 46). Heparanase upregulation could also be a common pathology in injured ECs from both large and small vessels.

This is the first study to provide evidence that heparanase upregulation caused by high glucose can be prevented by heparin and/or insulin. Heparin is found endogenously in mast cells and is used as an antithrombotic drug. Heparin ameliorates increased vascular permeability caused by various polycationic substances (47, 48). The ability of heparin to protect endothelium has been shown in several studies. Heparin accumulates in endothelium against a concentration gradient (49) and protects cultured ECs from injury by ROS (24). Heparin treatment results in increased formation of HS in cultured ECs (50). Insulin regulates glucose metabolism and promotes glucose uptake and utilization to reduce plasma glucose concentrations. Insulin and/or heparin prevent the intercellular gaps in cultured ECs exposed to high glucose (25). Our previous experiments showed that heparin and insulin reduce the increase in live cell number and decrease in LDH release in PAECs treated with high glucose (39) and reduce ultrastructural changes in PAECs treated with high glucose for 6 days (51). We have also shown that insulin and/or heparin and/or bFGF protect ECs treated with high glucose or heparinase I alone (23). Our present results, showing that insulin and/or heparin inhibit heparanase mRNA and activity in high-glucose–treated cells, agree with the protective effects demonstrated in these previous studies and also support the idea that GAGs and heparin-like polyanionic molecules inhibit heparanase upregulation (52). This study further demonstrated, at the gene level, inhibition of EC heparanase expression in high-glucose–treated cells by insulin and/or heparin but not bFGF. Although bFGF activity is dependent on HS integrity, bFGF has the ability to enhance cell proliferation, growth, and differentiation, and bFGF loses its stimulating functions on cells when HS is degraded (53), it likely does not regulate heparanase expression.

In summary, diabetic vascular complications are primarily caused by EC injury. Degradation of HSPGs contributes to EC injury, resulting in loss of endothelial anionic charges and increasing vascular permeability as well as other pathological effects. Our studies confirmed that heparanase expression is associated with high glucose levels, which can degrade HS and could contribute to diabetic vascular complications. Furthermore, insulin and heparin, alone or in combination, but not bFGF downregulate heparanase expression and may protect ECs from high-glucose injury. These results provide an innovative background for further studies in pharmacological intervention of diabetic vascular disease.

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1. Deckert T, Yokoyama H, Mathiesen E, Ronn B, Jensen T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen JS. Cohort study of predictive value of urinary albumin excretion for atherosclerotic vascular disease in patients with insulin dependent diabetes. BMJ 312:871–874, 1996.
2. Gall MA, Borch-Johnsen K, Hougaard P, Nielsen FS, Parving HH. Albuminuria and poor glycemic control predict mortality in NIDDM. Diabetes 44:1303–1309, 1995.
3. Stehouwer CD, Schaper NC. The pathogenesis of vascular complications of diabetes mellitus: one voice or many? Eur J Clin Invest 26: 535–543, 1996.
4. Stehouwer C. von Willebrand factor, dysfunction of the vascular endothelium, and the development of renal and vascular complications in diabetes. In: Mogensen CE, Ed. The Kidney and Hypertension in Diabetes Mellitus (4th ed.). Boston, Kluwer Academic Publishers, pp199–208, 1998.
5. Borsum T. Biochemical properties of vascular endothelial cells. Virchows Arch B Cell Pathol Incl Mol Pathol 60:279–286, 1991.
6. Elliott TG, Cockcroft JR, Groop PH, Viberti GC, Ritter JM. Inhibition of nitric oxide synthesis in forearm vasculature of insulin-dependent diabetic patients: blunted vasoconstriction in patients with microalbuminuria. Clin Sci (Lond) 85:687–693, 1993.
7. Verma S, Arikawa E, McNeill JH. Long-term endothelin receptor blockade improves cardiovascular function in diabetes. Am J Hypertens 14:679–687, 2001.
8. Wautier JL, Schmidt AM. Protein glycation: a firm link to endothelial cell dysfunction. Circ Res 95:233–238, 2004.
9. Dandona P, Chaudhuri A, Aljada A. Endothelial dysfunction and hypertension in diabetes mellitus. Med Clin North Am 88:911–931, 2004.
10. Kefalides NA. Basement membrane research in diabetes mellitus. Coll Relat Res 1:295–299, 1981.
11. Colwell JA, Lopes-Virella MF. A review of the development of large-vessel disease in diabetes mellitus. Am J Med 85:113–118, 1988.

12. Bayraktutan U. Free radicals, diabetes and endothelial dysfunction. Diabetes Obes Metab 4:224–238, 2002.
13. Kjellen L, Lindahl U. Proteoglycans: structures and interactions. Annu Rev Biochem 60:443–475, 1991.
14. Kanwar YS, Linker A, Farquhar MG. Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. J Cell Biol 86:688–693, 1980.
15. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Enevoldsen A. Albuminuria reflects widespread vascular damage. The Steno hypothesis. Diabetologia 32:219–226, 1989.
16. Shimomura H, Spiro RG. Studies on macromolecular components of human glomerular basement membrane and alterations in diabetes. Decreased levels of heparan sulfate proteoglycan and laminin. Diabetes 36:374–381, 1987.
17. Parthasarathy N, Spiro RG. Effect of diabetes on the glycosaminoglycan component of the human glomerular basement membrane. Diabetes 31:738–741, 1982.
18. Hardebo JE, Kahrstrom J. Endothelial negative surface charge areas and blood-brain barrier function. Acta Physiol Scand 125:495–499, 1985.
19. Vlodavsky I, Goldshmidt O, Zcharia E, Metzger S, Chajek-Shaul T, Atzmon R, Guatta-Rangini Z, Friedmann Y. Molecular properties and involvement of heparanase in cancer progression and normal development. Biochimie 83:831–839, 2001.
20. Vlodavsky I, Eldor A, Haimovitz-Friedman A, Matzner Y, Ishai-Michaeli R, Lider O, Naparstek Y, Cohen IR, Fuks Z. Expression of heparanase by platelets and circulating cells of the immune system: possible involvement in diapedesis and extravasation. Invasion Metastasis 12:112–127, 1992.
21. Katz A, Van Dijk DJ, Aingorn H, Erman A, Davies M, Darmon D, Hurvitz H, Vlodavsky I. Involvement of human heparanase in the pathogenesis of diabetic nephropathy. Isr Med Assoc J 4:996-1002, 2002.
22. Maxhimer JB, Somenek M, Rao G, Pesce CE, Baldwin D, Jr., Gattuso P, Schwartz MM, Lewis EJ, Prinz RA, Xu X. Heparanase-1 gene expression and regulation by high glucose in renal epithelial cells: a potential role in the pathogenesis of proteinuria in diabetic patients. Diabetes 54:2172–2178, 2005.
23. Han J, Mandal AK, Hiebert LM. Endothelial cell injury by high glucose and heparanase is prevented by insulin, heparin and basic fibroblast growth factor. Cardiovasc Diabetol 4:1–12, 2005.
24. Hiebert LM, Liu JM. Heparin protects cultured arterial endothelial cells from damage by toxic oxygen metabolites. Atherosclerosis 83:47–51, 1990.
25. Mandal AK, Puchalski JT, Lemley-Gillespie S, Taylor CA, Kohno M. Effect of insulin and heparin on glucose-induced vascular damage in cell culture. Kidney Int 57:2492–2501, 2000.
26. Broadley KN, Aquino AM, Woodward SC, Buckley-Sturrock A, Sato Y, Rifkin DB, Davidson JM. Monospecific antibodies implicate basic fibroblast growth factor in normal wound repair. Lab Invest 61:571–575, 1989.
27. Gotlieb AI, Spector W. Migration into an in vitro experimental wound: a comparison of porcine aortic endothelial and smooth muscle cells and the effect of culture irradiation. Am J Pathol 103:271–282, 1981.
28. Matzner Y, Bar-Ner M, Yahalom J, Ishai-Michaeli R, Fuks Z, Vlodavsky I. Degradation of heparan sulfate in the subendothelial extracellular matrix by a readily released heparanase from human neutrophils. Possible role in invasion through basement membranes. J Clin Invest 76:1306–1313, 1985.
29. Muir H. Structure and function of proteoglycans of cartilage and cell-matrix interactions. Soc Gen Physiol Ser 32:87–99, 1977.
30. Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68:729–777, 1999.
31. Kraemer PM. Heparan sulfates of cultured cells. II. Acid-soluble and -precipitable species of different cell lines. Biochemistry 10:1445–1451, 1971.
32. Kolset SO, Salmivirta M. Cell surface heparan sulfate proteoglycans and lipoprotein metabolism. Cell Mol Life Sci 56:857–870, 1999.
33. Hileman RE, Fromm JR, Weiler JM, Linhardt RJ. Glycosaminoglycan-protein interactions: definition of consensus sites in glycosaminoglycan binding proteins. Bioessays 20:156–167, 1998.
34. Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L, Pecker I. Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. Nat Med 5:793–802, 1999.
35. Hulett MD, Freeman C, Hamdorf BJ, Baker RT, Harris MJ, Parish CR. Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis. Nat Med 5:803–809, 1999.
36. Godder K, Vlodavsky I, Eldor A, Weksler BB, Haimovitz-Freidman A, Fuks Z. Heparanase activity in cultured endothelial cells. J Cell Physiol 148:274–280, 1991.
37. Nakajima M, Irimura T, Nicolson GL. A solid-phase substrate of heparanase: its application to assay of human melanoma for heparan sulfate degradative activity. Anal Biochem 157:162–171, 1986.
38. Moscatelli DA, Presta M, Mignatti P, Mullins DE, Crowe RM, Rifkin DB. Purification and biological activities of an angiogenesis factor from human placenta. Anticancer Res 6:861–863, 1986.
39. Mandal AK, Ping T, Han J, Hiebert LM. Protective effects of insulin and heparin on basic fibroblast growth factor in high glucose treated cultured endothelial cells (abstract). J Am Soc Nephrol 340A, 2002.
40. Yokoyama H, Sato K, Okudaira M, Morita C, Takahashi C, Suzuki D, Sakai H, Iwamoto Y. Serum and urinary concentrations of heparan sulfate in patients with diabetic nephropathy. Kidney Int 56:650–658, 1999.
41. Perez-Blanco FJ, Arrabal-Martin M, Villen I, Zuluaga A, Rodriguez-Cuartero A. Urinary activity of N-acetyl-beta-glucosaminidase in patients with nephrolithiasis. Nephron 86:518–519, 2000.
42. Tamsma JT, van den BJ, Bruijn JA, Assmann KJ, Weening JJ, Berden JH, Wieslander J, Schrama E, Hermans J, Veerkamp JH. Expression of glomerular extracellular matrix components in human diabetic nephropathy: decrease of heparan sulphate in the glomerular basement membrane. Diabetologia 37:313–320, 1994.
43. Rosenzweig LJ, Kanwar YS. Removal of sulfated (heparan sulfate) or nonsulfated (hyaluronic acid) glycosaminoglycans results in increased permeability of the glomerular basement membrane to 125I-bovine serum albumin. Lab Invest 47:177–184, 1982.
44. van den Hoven MJ, Rops AL, Bakker MA, Aten J, Rutjes N, Roestenberg P, Goldschmeding R, Zcharia E, Vlodavsky I, van der Vlag, Berden JH. Increased expression of heparanase in overt diabetic nephropathy. Kidney Int 70:2100–2108, 2006.
45. Wasty F, Alavi MZ, Moore S. Distribution of glycosaminoglycans in the intima of human aortas: changes in atherosclerosis and diabetes mellitus. Diabetologia 36:316–322, 1993.
46. Richardson JV, Wright CB, Hiratzka LF. The role of endothelium in the patency of small venous substitutes. J Surg Res 28:556–562, 1980.
47. Fairman RP, Sessler CN, Bierman M, Glauser FL. Protamine sulfate causes pulmonary hypertension and edema in isolated rat lungs. J Appl Physiol 62:1363–1367, 1987.
48. Peterson MW, Stone P, Shasby DM. Cationic neutrophil proteins increase transendothelial albumin movement. J Appl Physiol 62:1521–1530, 1987.
49. Hiebert LM, Wice SM, McDuffie NM, Jaques LB. The heparin target organ—the endothelium. Studies in a rat model. Q J Med 86:341–348, 1993.
50. Nader HB, Toma L, Pinhal MA, Buonassisi V, Colburn P, Dietrich CP. Effect of heparin and dextran sulfate on the synthesis and structure of heparan sulfate from cultured endothelial cells. Semin Thromb Hemost 17(Suppl 1):47–56, 1991.

51. Mandal AK, Ping T, Caldwell SJ, Bagnell R, Hiebert LM. Electron microscopic analysis of glucose-induced endothelial damage in primary culture: possible mechanism and prevention. Histol Histopathol 21: 941–950, 2006.
52. Bar-Ner M, Eldor A, Wasserman L, Matzner Y, Cohen IR, Fuks Z, Vlodavsky I. Inhibition of heparanase-mediated degradation of extracellular matrix heparan sulfate by non-anticoagulant heparin species. Blood 70:551–557, 1987.
53. Folkman J, Klagsbrun M. Angiogenic factors. Science 235:442–447, 1987.