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Biochimica et Biophysica Acta 1672 (2004) 36–45
BIOCHIMICA ET BIOPHYSICA ACTA
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High-glucose-induced structural changes in the heparan sulfate proteoglycan, perlecan, of cultured human aortic endothelial cells

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Received 6 November 2003; received in revised form 6 February 2004; accepted 13 February 2004

Abstract

Hyperglycemia is an independent risk factor for diabetes-associated cardiovascular disease. One potential mechanism involves hyperglycemia-induced changes in arterial wall extracellular matrix components leading to increased atherosclerosis susceptibility. A decrease in heparan sulfate (HS) glycosaminoglycans (GAG) has been reported in diabetic arteries. The present studies examined the effects of high glucose on in vitro production of proteoglycans (PG) by aortic endothelial cells. Exposure of cells to high glucose (30 vs. 5 mM glucose) resulted in decreased [^{35}S] sodium sulfate incorporation specifically into secreted HSPG. Differences were not due to hyperosmolar effects and no changes were observed in CS/DSPG. Enzymatic procedures, immunoprecipitation and Western analyses demonstrated that high glucose induced changes specifically in the HSPG, perlecan. In double-label experiments, lower sulfate incorporation in high-glucose-treated cells was accompanied by lower [^{3}H] glucosamine incorporation into GAG but not lower [^{3}H] serine incorporation into PG core proteins. Size exclusion chromatography demonstrated that GAG size was unchanged and GAG sulfation was not reduced. These results indicate that the level of regulation of perlecan by high glucose is posttranslational, involving a modification in molecular structure, possibly a decrease in the number of HS GAG chains on the core protein.

Keywords: Diabetes; Atherosclerosis; Hyperglycemia; Proteoglycan; Perlecan; Endothelial cell

1. Introduction

The major pathologic process that leads to mortality in diabetic individuals is cardiovascular disease with 80% of diabetic mortality shown to result from macrovascular disease rather than microvascular complications commonly associated with diabetes [1]. The MRFIT study showed that type II diabetic subjects have a two- to fourfold increase in cardiovascular disease risk vs. non-diabetic individuals [2]. This increased risk is independent of the classic risk factors for atherosclerosis including age, smoking, hypertension, and cholesterol levels. A major biochemical abnormality in diabetes is hyperglycemia and the Framingham Study is one of several that have demonstrated a strong association between hyperglycemia and heart disease in men and women with diabetes [3].

Little is known about mechanisms of accelerated atherosclerosis development that result from diabetes-associated hyperglycemia and very few studies have examined processes operative at the level of the artery wall that may provide a link between the two. Potentially important hyperglycemia-sensitive targets in artery are heparan sulfate proteoglycans (HSPG) that have been proposed to be anti-atherogenic by several mechanisms [4–6]. In a seminal study, it was shown that HS glycosaminoglycans (GAG), the carbohydrate moieties of HSPG, which are decreased in atherosclerotic lesions, are further decreased in arteries from diabetic patients and in diabetic arteries exhibiting atherosclerotic lesions [7]. This suggests a potential modification of HSPG as a result of hyperglycemic effects on HSPG-producing arterial cells.

The idea that HSPG may be modified by hyperglycemia is not new. In kidney, alterations in HSPG present in the basement membrane of the glomerulus have been shown to be associated with diabetic nephropathy [8,9]. Moreover, a decrease in kidney HSPG resulted in a

Abbreviations: PG, proteoglycan; GAG, glycosaminoglycan; HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; GBM, glomerular basement membrane; HAEC, human aortic endothelial cell; BAEC, bovine aortic endothelial cell; SMC, smooth muscle cell; CsABC, chondroitin ABC lyase

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doi:10.1016/j.bbagen.2004.02.005

reduction of anionic charge density and increased permeability of the glomerular basement membrane (GBM) [10]. Immunostaining studies using core protein-specific and GAG-specific antibodies showed that the reduction in HS GAG in the GBM under diabetic conditions was not accompanied by a reduction in HSPG core protein [11]. The link between reduced HS GAG in the diabetic kidney appears to be high glucose levels, as confirmed by studies demonstrating that in lines of kidney cells high glucose resulted in decreased production of HS GAG [12].

The HSPG species that is decreased in diabetic arteries has not yet been identified. A likely candidate is perlecan, previously identified in artery as a prominent component of the endothelial basement membrane as well as of atherosclerotic lesions [13]. Perlecan is the primary HSPG produced by cultured bovine aortic endothelial cells (BAEC) [14]. The present studies were undertaken to determine whether exposure of bovine and human aortic endothelial cells (HAEC) to high glucose resulted in a reduction in perlecan or modification in perlecan structure that may account for the decreased HS GAG observed in diabetic arteries.

2. Materials and methods

2.1. Cell culture

HAEC were purchased from Clonetics (San Diego, CA) and grown in Clonetics Endothelial Basal Medium supplemented with 1% hydrocortisone, 1% gentamycin, 1% amphotericin B, 0.25% bovine brain extract, and 2% fetal bovine serum (FBS) (all from Clonetics). BAEC were isolated as described [15] and grown in Dulbecco's Modified Eagle's Medium containing 5 mM glucose supplemented with 5% FBS. Human aortic smooth muscle cells (HSMC) were kindly provided by Dr. W.T. Woods, Prairie Education and Research Cooperative (Springfield, IL) and grown in Medium 199 with Earle's salts, supplemented with 10% FBS. Culture was carried out at 37 °C in a humidified atmosphere with 5% CO_2. In experiments, cells were incubated for 2, 5, or 10 days in media containing 5 mM glucose, 30 mM glucose, or 5 mM glucose + 25 mM mannitol. Experiments with HAEC, BAEC and HSMC were performed between passages 4–8, 4–15 and 4–7, respectively.

2.2. Metabolic radiolabeling

When uniformly confluent, monolayers were washed with balanced salt solution and labeling was performed for 48 h with media containing the indicated glucose concentrations and 50 μCi/ml [^{35}S] sulfate, 1 μCi/ml [^{3}H] serine, 10 μCi/ml [^{3}H] glucosamine, or 30 μCi/ml [^{35}S] Met/Cys, all from NEN, Boston, MA. This long radiolabeling period was chosen to achieve steady state conditions for all PG pools.

2.3. PG isolation

2.3.1. Secreted PG

Culture media were removed and cleared of cell debris by centrifugation at $300 \times g$ for 10 min. Media were then dialyzed extensively against 0.03 M Na_2SO_4 and 0.02 M NaCl at 4 °C to remove free radiolabel. To confirm that PG were not lost by aggregation and precipitation during low salt dialysis, yields were compared with an alternative method of isolation in which media samples were adjusted to 8 M urea and isolated by chromatography on DEAE Sephacel mini columns [16]. This method serves to remove free radiolabel and concentrate samples without dialysis. Total yield was similar ($P = 0.6$) by the dialysis and ion exchange methods, $3.12 \pm 0.22 \times 10^5$ dpm/mg cell protein and $3.37 \pm 0.37 \times 10^5$ dpm/mg cell protein, respectively. Percent of total [^{35}S] sulfate in HSPG, as measured by resistance to chondroitin ABC lyase (CsABC), was similar (62.1 ± 6 and 60.8 ± 9 in samples from dialysis and ion exchange methods, respectively).

2.3.2. Cell associated PG

Cell sheets were washed with balanced salt solution (137 mM NaCl, 2.7 mM KCl, 1.45 mM KH_2PO_4, 20.3 mM Na_2HPO_4) and incubated in 0.05% trypsin and 0.02% EDTA in PBS for 5 min at 37 °C. Serum-containing medium was then added to inhibit the trypsin action. The trypsin fluids contained the cell-associated PG and were separated from the intracellular fraction by centrifugation at $300 \times g$ for 10 min. The cell associated fraction was then dialyzed extensively against 0.03 M Na_2SO_4 and 0.02 M NaCl at 4 °C to remove free radiolabel [17]. The pelleted cells were washed with BSS and used for measurement of cell number by hemocytometry and for analysis of protein levels using a BCA protein assay kit (Pierce, Rockford, IL). In some samples the cells were separated from the subendothelial matrix by addition of 20 mM NH_4OH + 0.1% Triton X-100 for 5 min at room temperature, followed by three washes with BSS, and three washes with media to remove detached cells.

2.3.3. Sephacel purification for PG characterization

Samples were dialyzed into 7 M urea in 0.05 M Tris pH = 7.6 and applied to a 15-ml DEAE Sephacel Column equilibrated in the same buffer. After washing with two bed volumes of loading buffer, a 200-ml continuous gradient of 0 to 1 M NaCl in urea/Tris buffer was used to elute PG from the column. Fractions (2.5 ml) were collected and radioactivity was measured by scintillation counting.

2.3.4. CPC precipitation

PG were precipitated in 1% 1-hexadecyl pyridinium chloride (CPC) for 24 h at 25 °C in the presence of 100

μg CS carrier. The PG–CPC complexes were then spun at 800 × *g* for 1 h to pellet and then resolubilized in 1-ml 2 M NaCl/ethanol. Three milliliters of absolute ethanol was added to dissociate the PG from the CPC. After 24 h at 25 °C the samples were pelleted by centrifugation at 800 × *g* for 1 h [18]. Samples were analyzed by scintillation counting and used for further characterization.

2.3.5. GAG identification by enzymatic digestion

Aliquots of PG were dialyzed against ABC buffer (0.1 M Tris, 0.03 M sodium acetate, 0.01 M EDTA, pH = 8.0). Identification of HSPG and CS/DSPG was performed as follows. Aliquots of dialyzed PG samples were incubated with 0.05 U of CsABC (Seikagaku Inc., Ijamsville, MD) in ABC buffer containing protease inhibitors (0.001 M phenylmethylsulfonyl fluoride (PMSF), 0.07 mM pepstatin, 0.01 M *N*-ethylmalemide) and 1 mg/ml BSA for 16 h at 37 °C. Following this, ethanol was used to precipitate the intact HSPG, and CS/DSPG was calculated by subtracting HSPG from the total PG. To confirm the identity of HS GAG, aliquots of purified PG were subjected to nitrous acid degradation by incubation with an equal volume of 20% butyl nitrite: 1 M HCl for 2 h at 25 °C with shaking followed by neutralization with 1 M sodium hydroxide. In addition, in other experiments, digestion of HS GAG was accomplished by incubation of PG with 1.5 U Heparinase I and 3.0 U of Heparinase III (Sigma, St. Louis, MO) in 0.1 M Tris, 0.03 M sodium acetate and 4 mM calcium chloride, pH 8.0 for 16 h at 37 °C.

2.3.6. Size exclusion chromatography for GAG chain size

In order to separate HS GAG from the core proteins, aliquots of CsABC-treated samples were incubated in 1 N NaOH at 25 °C overnight with continuous shaking. Samples were neutralized with 1N HCl and loaded onto a Sepharose CL-6B column (60 × 0.9 cm), eluted with 0.2 M NaCl, at a flow rate of approximately 3 ml/h. Fractions (1 ml) were collected and analyzed for radioactivity. The excluded (V_o) and total (V_t) volumes of the column were determined using blue dextran and tryptophan, respectively.

2.3.7. Immunoprecipitation

Purified PG were adjusted to 2.5 mg/ml BSA and were incubated with 50 μg/ml of the perlecan antibody for BAEC (Upstate Biotech, NY) or HAEC (Zymed, CA) for 4 h at 4 °C followed by the addition of 50 μg of a 50% slurry of protein-A beads (Amersham Pharmacia Biotech, NJ). Samples were incubated at 4 °C overnight with constant movement. Beads were separated from unbound antibody and PG by centrifugation at 10,000 × *g* for 15 s and washed extensively with lysis buffer (150 μM NaCl, 25 mM Tris–HCl 1% Triton, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). Beads were resuspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 2 min to release bound antigen before separation by SDS-PAGE or scintillation counting.

2.3.8. SDS-PAGE

Purified PG were either left intact, digested with Heparinase I and III, CsABC or all three enzymes as described above and then ethanol-precipitated overnight. Samples were resolubilized in sample buffer containing 62.5 mM Tris–HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 1% 2-mercaptoethanol, and 0.03 mM bromophenol blue was added to each sample before boiling for 2 min. The samples were resolved by 4–12% gradient SDS-PAGE for autoradiography and Western blotting.

2.3.9. Western blotting

The samples were transferred to nitrocellulose and blocked overnight with PBS containing 0.1% Tween and 5% nonfat dry milk and then washed extensively with wash buffer containing 0.1% Tween in PBS. The samples were incubated in PBS containing 0.1% Tween and 2.5% nonfat dry milk and an anti-perlecan antibody (Zymed), which reacts with the core protein of perlecan.

2.3.10. Data analysis

Experiments were done in triplicate and repeated with consistent results. Data were analyzed by Student *t* test and ANOVA. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. High glucose decreases sulfate incorporation into secreted HSPG of HAEC and BAEC

The effect of high glucose on PG synthesis was first explored by incubating both HAEC and BAEC in media containing low (5 mM) or high (30 mM) glucose in the presence of [^{35}S] sodium sulfate. PG synthesis was measured by sulfate incorporation into high molecular weight macromolecules isolated from the culture media and cell layer (cell associated PG). In BAEC, [^{35}S] sulfate incorporation into total secreted PG was decreased in the presence of 30 mM glucose compared to 5 mM glucose (115.7 ± 8.4 vs. 175.6 ± 7.2 dpm/10^3 cells, $n = 3$, P=.003). To determine whether a specific PG type was reduced by high-glucose conditions, radioactive PGs were identified by susceptibility or resistance to CsABC or nitrous acid. As shown in Fig. 1A, the lower sulfate incorporation was accounted for by a 37% decrease in ^{35}S incorporation specifically into HSPGs. There was no difference in [^{35}S] sulfate incorporation into CS/DS PG produced under high or low glucose conditions. In cell-associated PG, ^{35}S incorporation into total PG was not different between low- and high-glucose treatments

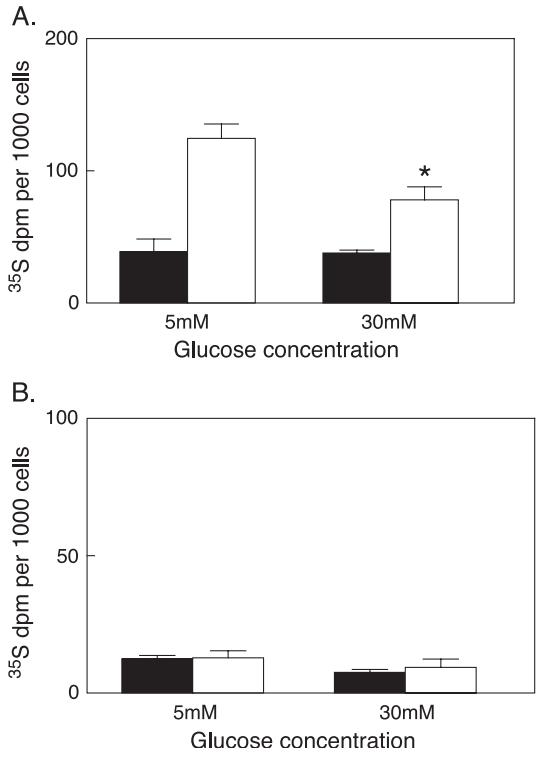

Fig. 1. BAEC-secreted HSPG, but not CS/DSPG, is reduced in high-glucose conditions. The effect of low (5 mM) and high (30 mM) glucose on secreted PG (A) and cell-surface PG (B) was determined in BAEC cultured with 5 or 30 mM glucose for 10 days as described and radiolabeled with [^{35}S] sodium sulfate for the last 48 h. The media and cell-surface fractions were dialyzed against 0.02 M NaCl and 0.03 M Na_2SO_4. Radioactivity associated with CSPG/DSPG (solid bars) and HSPG (open bars) in each fraction was measured based on susceptibility or resistance to enzymatic digestion. Values are mean ± S.E. of experiment performed in triplicate. *$P < 0.05$ vs. 5 mM glucose.

(18 ± 2.6 vs. 25 ± 2.5 dpm/10^3 cells) nor were differences measured in sulfate incorporation specifically into HSPG or CS/DSPG (Fig. 1B).

Similar experiments were conducted with HAEC. As shown in Fig. 2, high-glucose treatment resulted in a 33% reduction in sulfate incorporation into total secreted PG. This reduction was observed following only 2 days of exposure to the elevated glucose and was retained for 10 days of treatment (Fig. 2A). As had been observed in BAEC (data not shown), treatment with mannitol for 10 days did not result in a significant decrease in [^{35}S] sulfate incorporation into PG, indicating that effects of glucose on PG were not simply due to hyperosmolar conditions. As in BAEC, the reduction in sulfate incorporation in high-glucose-treated cells was the result of effects on HSPG (Fig. 2B). Although this decrease represented only a 25% reduction in sulfate incorporation from the osmotic control, this difference was consistent and statistically significant in all experiments, whereas the slight reduction (16%) in sulfate incorporation into CS/DSPGs consistently did not reach significance.

3.2. High glucose has no effect on sulfate incorporation into arterial SMC PG

Because SMC are also a source of HSPG in artery, we examined the effect of elevated glucose on PG synthesis by human aortic SMC. In these cells, glucose did not cause decreased sulfate incorporation into HSPG in the secreted or cell-associated fraction (Table 1).

Glycation (nonenzymatic glycosylation) results from exposure over time of proteins, lipids, and DNA to elevated glucose. We next tested whether the decreased incorporation of [^{35}S] sulfate into the secreted HSPG of HAEC was the result of glucose per se, or the effect of glycation of either media proteins or endothelial cell components. This question was explored by incubating HAEC in low or high glucose for 10 days in the presence or absence of aminoguanidine, an inhibitor of glycation reactions. The decreased sulfate incorporation into secreted PG persisted in the presence of high glucose and aminoguanidine (Table 2). These data together with those showing that short exposure to elevated glucose was sufficient to cause a decrease in secreted HSPG (Fig. 2)

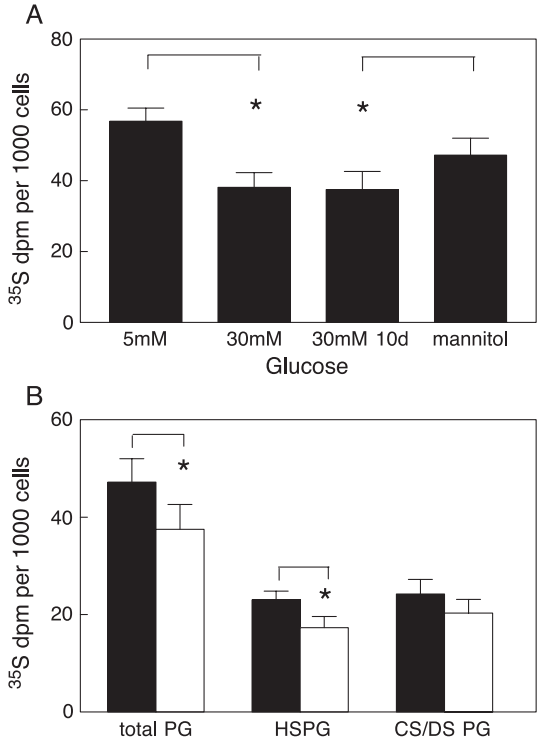

Fig. 2. HAEC-secreted HSPG, but not CS/DSPG, is reduced in high-glucose conditions. (A) Total secreted PG. HAEC were incubated with 5 or 30 mM glucose for 2 or 10 days or 5 mM glucose + 25 mM mannitol for 10 days including [^{35}S] sodium sulfate for the final 48 h. The media was harvested and dialyzed as described. Radiolabel incorporation into total PG is depicted. (B) Secreted PG (10 day) described in (A) were digested with GAG-degrading enzymes to determine effect of glucose on radiolabel incorporation into specific GAGs vs. mannitol osmotic control. Solid bars, osmotic control; open bars, 30 mM glucose. Values are mean ± S.E. of experiment performed in triplicate. *$P < 0.05$.

Table 1
HSPG production by human aortic SMC is unaffected by glucose conditions

	Secreted PG		Cell-associated PG	
	dpm [^{35}S] sodium sulfate per 1000 cells			
	HSPG	CSPG	HSPG	CSPG
5 mM glucose	153 ± 31	665 ± 33.6	50.1 ± 4.7	67.2 ± 3.7
30 mM glucose	225 ± 15	666 ± 24.4	49.3 ± 4.1	65.2 ± 3.2

HSMC were incubated with media containing 5 or 30 mM glucose for 10 days and [^{35}S] sodium sulfate for the final 48 h. Media or cell-associated fractions were dialyzed and PG were subjected to GAG-degrading enzymes in order to determine radiolabel incorporation into specific GAG species. Values are mean ± S.E. of experiment performed in triplicate.

led us to conclude that the hyperglycemia-induced alterations in secreted HSPG were not due to effects of glycation.

3.3. High glucose does not cause a decrease in HS chain length or sulfation

The decrease in [^{35}S] sulfate incorporation into HSPG secreted by endothelial cells exposed to high glucose may indicate structural modifications in HSPG (e.g. decreased sulfation, decreased GAG chain length, decreased numbers of GAG) or decreased numbers of HSPG.

To determine whether the high-glucose treatment caused decreased sulfation of HS GAG chains, BAEC and HAEC were incubated in media containing low or high glucose for 2 or 5 days and [^{35}S] sulfate and [^{3}H] glucosamine were used to radiolabel both sulfate and sugar residues in the GAG. In addition to the decrease in [^{35}S] sulfate incorporation, there was a significant decrease in the [^{3}H] glucosamine incorporation into HSPG secreted by both cell types when cultured in high glucose (Table 3). The effect of high glucose on glucosamine incorporation was in fact greater than that on sulfate incorporation and the ratio of ^{35}S/^{3}H increased in the high-glucose-treated cells. These data suggest that although high glucose may cause a decrease in numbers of GAG on the core protein or affect the length of GAG, the GAG that are present are at least if not more highly sulfated.

A confounding factor in interpreting effects of different glucose concentrations on incorporation of radiolabeled glucosamine is that an increased flow of glucose into the hexosamine pathway will alter the specific activities of the UDP-*N*-acetylhexosamine precursors. For this reason, we used an indirect method [19] to determine that the lower [^{3}H] glucosamine incorporation into HS was not simply the result of pool dilution. In this method, [^{35}S] sulfate was used as a reference isotope because it rapidly equilibrates with the small endogenous pool of sulfate. Specific activity of the hexosamines was then calculated based on incorporation of both [^{35}S] sulfate and [^{3}H] glucosamine into CS disaccharides.

HPLC separated CS disaccharides from control HAEC had a ^{3}H/^{35}S ratio of 1.81 compared to 1.13 and 1.02 from cells treated for 2 and 5 days with high glucose. This translated into a reduction in specific activity of the [^{3}H] glucosamine pool from 2398 Ci/mmol into control cells to 1351 Ci/mmol in the high-glucose-treated cells. Based on this, the estimated incorporation of [^{3}H] glucosamine in HSPG as a result of pool changes would decrease from 18.1×10^4 dpm/mg cell protein in control cells to 10.3×10^4 dpm/mg cell protein (43% reduction) in high-glucose-treated cells. Actual dpm of ^{3}H measured in HS (Table 3) were 5.5×10^4 dpm (69% reduction) and 4.3×10^4 dpm/mg cell protein (70% reduction) for cells in high glucose for 2 and 5 days, respectively. These data indicate that the observed reduction in [^{3}H] glucosamine incorporation into HS chains in cells exposed to high glucose is due, in part, to dilution of the precursor pool, but also to structural differences in the HSPGs.

To further examine possible structural changes in HSPG resulting from high glucose, the size of the HS GAG chains was measured. BAEC were incubated for 2 or 5 days in low or high glucose and labeled with [^{35}S] sodium sulfate. CS/DS GAG chains from the culture media were degraded with CsABC. HSPG were base-treated to separate GAG from the

Table 2
Aminoguanidine does not prevent high-glucose-induced reduction of HSPG in HAEC

	− Aminoguanidine	+ Aminoguanidine
	dpm [^{35}S] sodium sulfate per 1000 cells	
5 mM glucose	27.0 ± 1.2	24.5 ± 2.4
30 mM glucose	17.3 ± 2.0*	14.5 ± 0.5*

HAEC were incubated with media containing 5 or 30 mM glucose for 10 days in the presence or absence of 5 mM aminoguanidine and [^{35}S] sodium sulfate for the final 48 h. Media were harvested and dialyzed as described. PG were subjected to CsABC digestion as described in Section 2, to isolate HSPG. Values are mean ± S.E. of experiment performed in triplicate.

* $P < 0.05$ compared to 5 mM control.

Table 3
Effect of high glucose on [^{3}H] glucosamine and [^{35}S] sulfate incorporation into HS GAG chains

	dpm ^{3}H glucosamine/mg protein	^{35}S/^{3}H
HAEC		
5 mM glucose	181,503 ± 7259	0.050 ± 0.001
30 mM glucose (2-day)	55,363 ± 6304*	0.177 ± 0.014*
30 mM glucose (5-day)	43,003 ± 2823*	0.151 ± 0.007*
BAEC		
5 mM glucose	201,902 ± 17,405	0.115 ± 0.010
30 mM glucose (2-day)	59,505 ± 2478*	0.321 ± 0.013*
30 mM glucose (5-day)	83,640 ± 6615*	0.295 ± 0.011*

HAEC and BAEC were incubated in media containing 5 or 30 mM glucose for 2 or 5 days including [^{3}H] glucosamine and [^{35}S] sodium sulfate for the final 48 h. The media was harvested and dialyzed as described and PGs were isolated by DEAE Sephacel chromatography. PGs were subjected to CsABC enzyme digestion, as described in Section 2, in order to isolate HSPG. Values are mean ± S.E. of experiment performed in triplicate.

* $P < 0.05$ compared to 5 mM control.

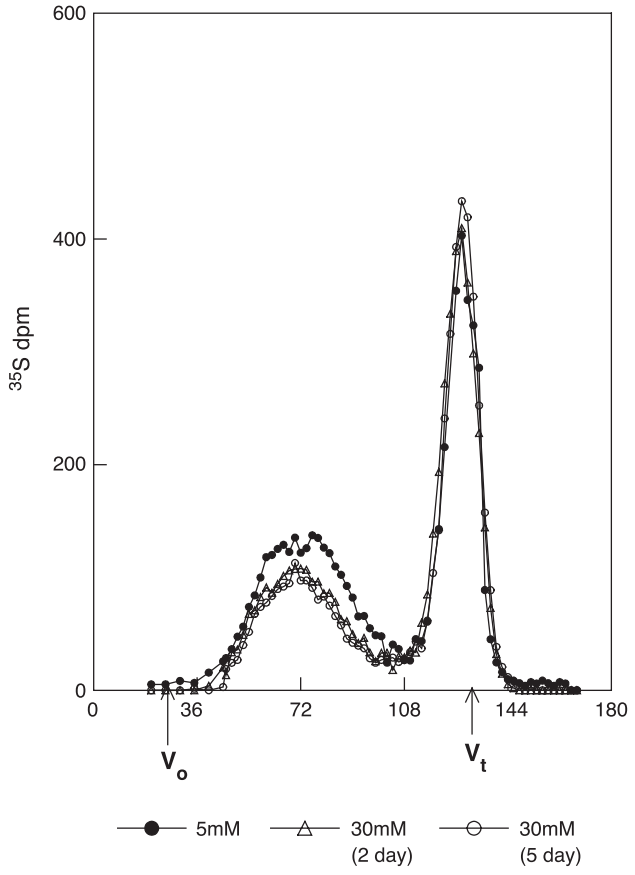

Fig. 3. Size-exclusion chromatography of PG synthesized by HAEC cultured under low or high-glucose conditions. HAEC were incubated with 5 or 30 mM glucose for 2 or 5 days including [^{35}S] sodium sulfate for 48 h. The media was harvested and dialyzed as described and PGs were isolated by DEAE Sephacel chromatography. PGs were subjected to CsABC treatment, as described in Section 2, to isolate HSPG. PG were treated with NaOH to remove the GAG chains and then applied to CL-6B column. Material at the V_t represents degraded CS, and material remaining in the column represents HS chains.

core proteins and loaded onto a CL-6B Sepharose column. The profiles (Fig. 3) revealed an HS GAG peak that eluted similarly in all treatment groups, with no difference in the calculated K_{av} of GAG produced under low- or high-glucose conditions. When compared to a published calibration curve [20], the HS GAGs produced in high or low glucose had an estimated average molecular size of 26 kDa. These data suggest that the decrease in [^{3}H] glucosamine and [^{35}S] sulfate incorporation into HSPG in high-glucose conditions is not the result of a decrease in GAG size.

3.4. HSPG core protein synthesis is not decreased by high glucose

To test whether core protein synthesis was reduced or whether the amount of sulfation per core protein was decreased, HAEC were double-labeled with [^{3}H] serine and [^{35}S] sulfate in the presence of low or high glucose for 2 or 5 days. Radiolabeled HSPGs were isolated from the culture media and purified by treatment with CsABC and CPC precipitation. As shown in Fig. 4A, there was no effect of the high glucose on [^{3}H] serine incorporation into the HSPG core protein at 2 or 5 days of treatment. Fig. 4B shows the ratio of [^{35}S] sulfate/[^{3}H] serine which decreased by day 2 and further by day 5 due to the decrease in [^{35}S] sulfate incorporation in the high-glucose condition. These data indicate that there is not a decreased production of intact HSPG as a result of elevated glucose but rather that high glucose causes modifications affecting GAG chains.

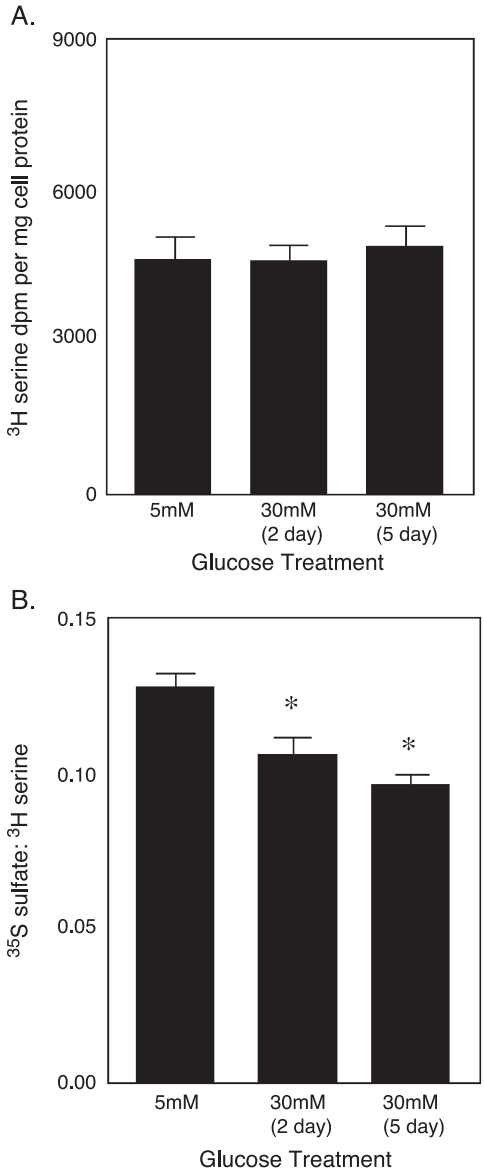

Fig. 4. Secreted HSPG core protein is unaffected by glucose conditions. HAEC were incubated for 2 or 5 days in media that contained 5 or 30 mM glucose and [^{3}H] serine and [^{35}S] sodium sulfate for the final 48 h. The media was harvested and dialyzed as described and PGs were isolated by precipitation with 1% CPC and subsequent ethanol precipitation. PGs were subjected to CsABC treatment, as described in Section 2, to isolate HSPG. (A) [^{3}H] serine in HSPG, (B) [^{35}S] sulfate/[^{3}H] serine ratio in HSPG. Values are mean ± S.E. of experiment performed in triplicate. *$P < 0.05$ vs. 5 mM control.

3.5. Identification of high-glucose-induced structural modifications in perlecan of BAEC

The major HSPG species produced by cultured BAEC has been shown to be perlecan [14], which has also been found in the cell layer [21,22]. To confirm a specific effect on perlecan and to determine why we had been unable to detect an effect of high glucose on cell layer HSPG (Fig. 1B), BAEC were exposed to 5 or 30 mM glucose for 5 days and radiolabeled with [^{3}H] serine and [^{35}S] sulfate. PG, isolated from the media and cell layer, were immunoprecipitated with an anti-perlecan core protein antibody. Fig. 5 shows radioactivity associated with perlecan. Although total HSPG in the cell fraction was similar in low- and high-glucose-treated cells (3904 ± 681 and 4720 ± 832 dpm [^{35}S] sulfate/mg cell protein for 30 and 5 mM glucose-treated cells, respectively), a significant decrease in [^{35}S] sulfate associated with perlecan was measured in both media and cell layer fractions from high-glucose-treated cells. Moreover, the ratio of [^{35}S] sulfate/[^{3}H] serine was significantly lower ($P = 0.01$) in perlecan from cells exposed to high glucose. These data confirm that in BAEC, both media and cell layer perlecan are structurally modified with a decrease in GAG/core protein ratio.

3.6. Identification of perlecan as the secreted HSPG of HAEC

SDS-PAGE and Western blotting were used to analyze the PG produced by HAEC. [^{35}S] Met/Cys-labeled PG were isolated from the HAEC media by CPC precipitation and then subjected to GAG-degrading enzymes and run on a 4–12% gradient polyacrylamide gel. The resulting autoradiogram (Fig. 6A) indicated the presence of a high molecular weight PG sensitive to enzymatic digestion with heparinase which resulted in the appearance of a protein that corresponds to the 470-kDa core protein of perlecan. In addition, the media contained two lower molecular weight core proteins, one at 250 kDa that did not appear to be sensitive to either CS/DS or HS degrading enzymes, and a band at 150 kDa. In order to confirm the presence of perlecan, [^{35}S] Met/Cys-labeled media PG were immunoprecipitated with an antibody to domain III of the perlecan core protein. The autoradiogram of the core protein-radiolabeled immunoprecipitated perlecan (Fig. 6B) established that the secreted HAEC perlecan consists of a higher molecular weight core protein at 400 kDa in addition to a lower molecular weight core protein at 200–250 kDa. The immunoprecipitate was also resolved on a 4–12% gradient gel and transferred to nitrocellulose. The resulting Western blot (Fig. 6C) confirmed that the antibody recognized both the high molecular weight band at >400 kDa and the band at 250 kDa, but not the band at 150 kDa.

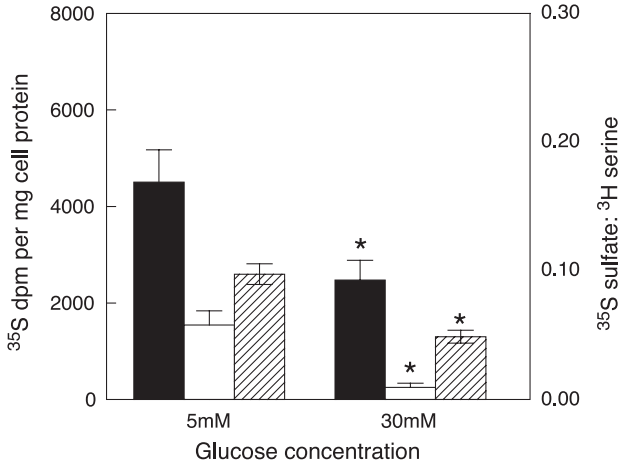

Fig. 5. High-glucose-induced reduction of [^{35}S] incorporation into perlecan of BAEC. Cells were incubated for 5 days in media that contained 5 or 30 mM glucose and included [^{3}H] serine and [^{35}S] sodium sulfate for the final 48 h. PG were isolated from the media and cell layer as described and immunoprecipitated with an antibody to the core protein of perlecan. Solid bars, secreted perlecan; open bars, cell associated perlecan; striped bars, ratio of [^{35}S] sodium sulfate/[^{3}H] serine in immunoprecipitated perlecan. Values are mean ± S.E. of experiment performed in triplicate. *$P < 0.05$ compared to 5 mM glucose.

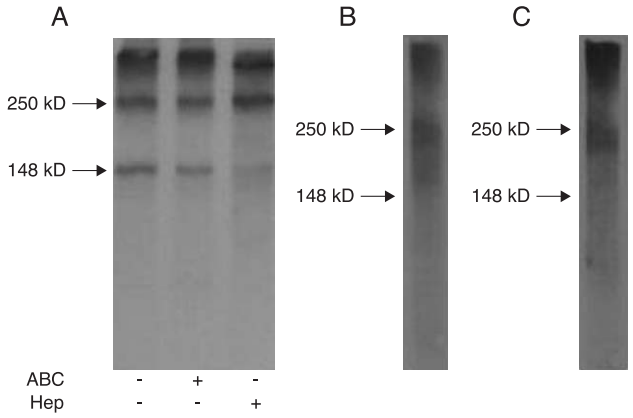

Fig. 6. Identification of secreted HSPG of HAEC. Autoradiograms and Western Blot of HAEC secreted PG. HAEC were radiolabeled with [^{35}S] methionine for 48 h. The media was removed and dialyzed as described and PGs were isolated by precipitation with 1% CPC and subsequent ethanol precipitation. Aliquots were treated with CsABC (ABC) or Heparinase I and III (Hep) and applied to a 4–12% polyacrylamide gel (1.5-mm thick) (A). The CPC-precipitated PG described in (A) was immunoprecipitated with 10 μg anti-perlecan antibody + protein-A sepharose as described in Section 2, applied to a 4–12% polyacrylamide gel and the resulting autoradiogram is shown (B). The immunoprecipitated perlecan described in (B) was resolved on a 4–12% gradient gel, transferred to nitrocellulose and probed with the anti-perlecan antibody (1:500) (C). The migration positions of known molecular weight standard markers are shown.

4. Discussion

These studies have demonstrated that in both HAEC and BAEC in culture, high-glucose exposure results in a structural modification of the HSPG, perlecan. The evidence for this includes a high-glucose-induced decrease in metabolic radiolabeling of two perlecan GAG chain components, glucosamine and sulfate, with no change in core protein labeling.

These data are consistent with and extend earlier studies by Ebara et al. [23], who showed that both BAEC and HepG2 cells respond to high-glucose conditions by reduced sulfate incorporation into HSPG. Moreover, in the HepG2 cells, as in the present studies, the use of double GAG radiolabeling precursors indicated that sulfation per disaccharide was not decreased as a result of high glucose. Present data demonstrate a lower ratio of radiolabeled GAG to core protein in the PG from high-glucose-treated cells indicating a greater than 30% reduction in the number of disaccharide units per core protein. This may be the result of smaller-sized GAG. However, a size difference of that magnitude should have been measurable on the size exclusion column that failed to detect any difference in GAG size resulting from high-glucose treatment. The sizes of the GAG-chains were similar to the 28 kDa reported for HS GAG of rat mesangial cells from either normal or diabetic rats [24].

An alternative explanation for the reduced GAG to core protein ratio is a reduction in the number of GAG chains on the perlecan core. Since perlecan may contain three or four GAG [25], the 30% lower GAG to core ratio in PG produced under high-glucose conditions could result from the loss of one HS chain. Since perlecan may contain both CS and HS chains [25–27], one explanation for these results would be that under high-glucose conditions, CS GAG may be substituted for HS. This would be consistent with studies showing that culture conditions may determine whether CS or HS GAG are added to the core protein of the cell surface HSPG, syndecan 1 [28]. It is unlikely that high glucose has caused such a substitution in perlecan however, since there was no increase in radiolabel incorporation into CS GAG to correspond with the loss of incorporation into HS GAG.

An intriguing finding of these studies was that the GAG-reduction effects were specific for perlecan, with no high-glucose-associated changes measured in CS/DSPG. This suggests a unique effect of high glucose on the perlecan core protein that affects GAG addition, rather than an effect on enzymes involved in GAG biosynthesis and modification. GAGs are synthesized in the Golgi apparatus on the linkage tetrasaccharide [GlcAβ1-3Galβ1-3 Galβ1-4Xylβ1-(Ser)] attached to serine residues of the core polypeptide [29]. The linkage regions of CS, DS, and HS chains have a common structure. GAG biosynthesis is initiated by transfer of Xyl from UDP-Xyl to a hydroxyl group of specific serine residues on the polypeptide chain of the core protein. A specific recognition signal for the xylosyltransferase determines the number of GAG chains initiated, and peptide mutation studies have shown that Glu/Asp-X-Ser-Gly (X being any amino acid) is a common amino acid sequence that is recognized by the enzyme [30]. A second signal determines the species of GAG to be synthesized and unique enzymes are required for chain elongation and modification of CS/DS and HS. HS priming is initiated through αGlcNAc transferase1 adding GlcNAc to GlcA of the linkage tetrasaccharide, and CS/DS through β GalNAc transferase1 adding GalNAc to the GlcA. Evidence suggests that CS initiation represents the default pathway [31], but the recognition signal for β GalNAc transferase1 initiating CS chain elongation is unclear. Site-directed mutations of consensus amino acid sequences adjacent to GAG attachment sites on domain 1 of perlecan have demonstrated that synthesis of HS chains requires a cluster of acidic amino acids in close proximity to the Ser–Gly acceptor sequences [32]. In addition, the presence of a SEA module, an 80-amino-acid sequence that is also found in the HSPG, agrin, was found to enhance HS priming. Three potential HS attachment sites have been identified on domain 1 of perlecan [26,32] and one on domain V [27]. Our studies demonstrate that high glucose induces a decrease in the GAG/core protein ratio that is consistent with the loss of a GAG chain and may involve the HS attachment sites on the core protein.

The high-glucose-induced decrease in HS GAG but not core protein of endothelial cell perlecan is consistent with findings in the GMB of diabetic kidneys where immunofluorescence studies on renal biopsies showed a decrease in staining of HS GAG without changes in core protein staining [11]. These findings were also supported by GAG but not core decreases in HSPG of isolated kidney cells cultured in high glucose [12]. HS GAGs are decreased in arterial tissue of diabetic patients [7], however, it is unknown whether this decrease is accompanied by, or independent of, a decrease in HSPG core protein. Our recent studies have shown that in diabetic monkeys, the content of arterial HS GAG is negatively correlated with plasma glucose concentration (Edwards, I.J., Wagner, J.D., Litwak, K.N., and Cefalu, W.T., unpublished data).

Although EC are the major producer of HSGAG in the artery wall, SMC also contribute to arterial HS content. Our studies with arterial SMC were interesting since no change in sulfate incorporation was seen in arterial SMC incubated in high-glucose conditions. The differential effects of high glucose on PG produced by EC vs. SMC may be a result of different glucose-transport activity into the two cell types. It has been established that the GLUT-1 isoform of the glucose transporter is the only transporter present in both EC and VSMC [33]. This transporter is present in most cells at low levels and has been shown to be negatively regulated by glucose in many cells types including rat skeletal muscle cell lines and 3T3L1 fibroblasts [34]. However, several studies have shown that while VSMC have an autoregulatory mechanism in hexose transport and were able to reduce protein levels of GLUT-1 in high-glucose conditions, the same is not true for vascular endothelial cells [33]. Retinal capillary endothelial cells were also shown to lack this important autoregulatory function [35]. Our studies demonstrating a differential effect of high glucose on PG produced by EC vs. SMC indicate that the ability to regulate glucose uptake may be an important factor in PG production.

Oxidant stress-associated processes have been implicated in most of the pathological alterations in the vasculature of diabetic individuals [36]. In the present studies, the short high-glucose exposure times of cells in many of our experiments do not favor a major role for glycoxidation products

in the altered GAG synthesis. Alternatively, elevated glucose may increase mitochondrial superoxide anion production which in turn leads to activation of protein kinase C and NFκB [37]. Thus, major regulatory pathways may be modified and we cannot rule out the involvement of intracellular reactive oxygen species in the glucose-associated reduction in HSGAG.

The biological significance of high-glucose-induced structural changes in perlecan secreted by arterial endothelial cells may relate to its ability to bind and regulate FGF-2. HS has been shown to be an important positive regulator of FGF activity by acting as a co-receptor for FGF at the cell surface [38,39]. Alternatively, it may serve as a negative regulator by sequestration of FGF in the extracellular matrix, thus preventing the growth factor from binding to its cell surface receptor [40,41]. Reduced BAEC perlecan expression increases FGF-mediated smooth muscle cell proliferation in culture [42]. In the diabetic condition, high-glucose-induced reduction in HS of perlecan secreted by EC may impair its ability to sequester FGF, thereby allowing increased activation of the FGF receptor on SMC by its ligand. This may lead to increased proliferation of SMC in the artery wall contributing to atherosclerosis progression.

The biological importance of our findings may also be linked to the role of HS in retention of monocytes in the subendothelial matrix, a process thought to be a key in initiation of the atherosclerotic lesion. A subendothelial matrix deficient in HS has been shown to exhibit increased adhesion of THP-1 monocytes to exposed fibronectin-binding proteins in the subendothelial matrix [6]. Studies in our laboratory indicate that HSPG in the subendothelial matrix are also modified when HAEC are exposed to high glucose [43]. In a recent study, decreased perlecan in the livers of diabetic mice was proposed to result in impaired remnant clearance from plasma [23]. This implicated diabetes-associated changes in HSPG as atherosclerosis-promoting through the production of atherogenic lipoproteins. Whether in the liver or in the arterial wall, perlecan appears to be a hyperglycemia-sensitive target and thus a potentially important link between diabetes and atherosclerosis.

Acknowledgements

The authors thank Jerry Reagan, PhD, and Richard St. Clair, PhD, for critical review of the manuscript. This work was supported by a Research Award from the American Diabetes Association (IJE) and by National Institutes of Health Training Grant HL07115-27 in Atherosclerosis Research (CAVW).

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