Posttranscriptional Effects of Glucose on Proteoglycan Expression in Mesangial Cells

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Hyperglycemic conditions are known to increase mRNA and protein levels of several extracellular matrix molecules in cultured mesangial cells, but accompanying increases in proteoglycan mRNA have not been found, and there are discrepant reports of normal or decreased proteoglycan synthesis with or without undersulfation in diabetic kidneys and hyperglycemic cultures. We examined the effects in proliferating cells of glucose on [^{35}S]sulfate incorporation into heparan and dermatan sulfates and on mRNA levels of decorin, biglycan, and basement membrane perlecan. In both mesangial cells and vascular smooth muscle cells, 30 mmol/L glucose caused a decrease of 15% to 25% in the amount of sulfate incorporated into each proteoglycan in cultures confluent for 1 to 4 days, compared with 10 mmol/L glucose. The effect showed no specificity for the class of proteoglycan and was not a consequence of changes in total protein synthesis, which increased, or cell proliferation, which was unaffected. No decrease in charge density of any of the proteoglycan fractions was observed by ion-exchange chromatography. Therefore, the decrease in labeling was due to a decrease in synthesis and not undersulfation. mRNA levels for biglycan and perlecan increased slightly and transiently, and these changes cannot account for the decreased synthesis. Decorin mRNA was detected only in smooth muscle cells, where it and biglycan were differentially affected by glucose, apparently at the transcriptional level; stabilities of the two messages were unaffected by glucose. Although transforming growth factor-beta 1 (TGF-β_1) mRNA levels increased in response to glucose, the cytokine did not appear to regulate proteoglycan synthesis, because structural changes in proteoglycans elicited by addition of TGF-β_1 to the culture medium did not occur in the hyperglycemic cultures. On the other hand, inhibition and downregulation of protein kinase C (PKC), while decreasing net sulfate incorporation into mesangial cell proteoglycans, prevented the effect of high glucose. We conclude that a high glucose concentration causes a general decrease in the synthesis of all classes of proteoglycans at a posttranscriptional level, and can do so without affecting the charge density of individual proteoglycan molecules.

THE DIABETES CONTROL and Complications Trial has provided compelling evidence for a direct toxic role of glucose in the etiology of diabetic microvascular disease, including renal glomerulosclerosis.[1] Numerous glucose-dependent mechanisms may be involved in triggering glomerular changes in diabetes, including the accumulation of advanced glycation end products[2,3] and glycated albumin,[4] glycation of matrix molecules,[5-8] activation of mesangial cell protein kinase C (PKC),[9] multiple growth factor imbalances,[10] and glucose-dependent accumulation of transforming growth factor-beta (TGF-β).[11,12] However, the response of extracellular matrix synthesis to hyperglycemia has received considerable attention as a functional endpoint. It has been noted that the consequences of hyperglycemia are generally manifest in cells that, like the glomerular mesangial cell, are not dependent on insulin for glucose transport and therefore have an internal glucose concentration that approximates that of their milieu.[9] This, together with the central role of mesangial expansion in diabetic nephropathy,[13] has focused attention on understanding the role of glucose in modulating extracellular matrix synthesis in these cells.

High glucose concentrations (30 mmol/L) increase collagen type IV and fibronectin at the protein and mRNA levels and cause an increase in laminin mRNA in cultured cells (reviewed in Kreisberg[14]), and glucose has recently been shown to activate transcription of a collagen IV–luciferase reporter construct in mesangial cells.[15] The effect is somewhat specific for these matrix molecules—no changes are observed, for example, in collagen type I or c-*myc* expression[16]—and accompanies increased expression of tissue plasminogen activator and plasminogen activator inhibitor-1 that further influences matrix accumulation.[17] Danne et al[18] observed increased collagen type IV production in each glomerular cell type (endothelial, epithelial, and mesangial) when cells were cultured in 30 mmol/L glucose under conditions in which cell proliferation was not affected. Similar conditions produced an approximately twofold increase in mRNA for laminin, fibronectin, and collagen type IV in cultured rat mesangial cells (RMC) after 4 days.[19] Similarly, 20 mmol/L glucose increased fibronectin and its mRNA in cultured human mesangial cells.[20] Increased rates of synthesis of matrix proteins without changes in degradation rates led to their accumulation over several weeks; total protein synthesis was unaffected.[19,21] The conclusion to be drawn from such experiments is that metabolism of glucose affects extracellular matrix accumulation at the transcriptional level without affecting cell proliferation.[14]

Proteoglycans are sulfated anionic glycoproteins that subserve numerous functions in extracellular matrices and on the cell surface.[22-24] The presence of the heparan sulfate proteoglycan (HSPG), perlecan, in the glomerular basement membrane (GBM) is thought to contribute to the charge selectivity of glomerular ultrafiltration; decreases in HSPG staining in the GBM of diabetic animals[25,26] suggest that decreased synthesis, sulfation, or incorporation of

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perlecan contribute to the associated nephropathy. However, the amount of HSPG per glomerulus was not decreased in nephrotic streptozotocin-diabetic rats,[26] although newly ^{35}S-labeled HSPG was released more readily from glomeruli isolated from diabetic animals.[27] Nor was GBM HSPG reduced in renal biopsies of proteinuric human diabetic subjects.[28] Vernier et al[29] found that HSPG staining in human diabetic glomeruli was decreased in advanced nephropathy, but these decreases did not accompany microalbuminuria in the early stages of disease. Furthermore, steady-state mRNA levels perlecan were unchanged in renal cortices of diabetic mice with GBM thickening at a time when those for collagen type IV were significantly increased.[30] Earlier studies of anionic site staining in the laminae rarae interna and externa of the GBM are problematic: recent evidence strongly suggests that the laminae themselves are artifacts of fixation.[31,32]

The nature of changes in proteoglycans in the diabetic matrix remains uncertain, but several investigators have examined the effect of hyperglycemic conditions on their synthesis in cultured renal cells. Rat visceral epithelial cells in 30 mmol/L glucose (or mannitol, as an osmotic control) showed a net increase in total incorporation of sulfate into proteoglycan without a change in overall size or charge.[33] However, the amount of GBM HSPG appeared to decrease in an osmotically independent response to high glucose while other proteoglycan(s) increased in compensation. Porcine mesangial cells cultured in 40 mmol/L glucose for 48 hours in the log phase of growth accumulated approximately 50% less perlecan core protein on immunoassay compared with control cells in 5 mmol/L glucose.[34] On the other hand, Silbiger et al[35] cultured RMC in the presence of 500 mg/dL (27.8 mmol/L) glucose for 8 to 10 days and did not find any changes in the total amount of glycosaminoglycan synthesized, the size of the major proteoglycans, or the level of biglycan mRNA compared with controls in 200 mg/dL (11.1 mmol/L) glucose. However, matrix deposition of proteoglycan was increased and net charge was decreased. Similar results were obtained when RMC were cultured on filters in low serum for up to 8 weeks without passage; glucose did not affect total glycosaminoglycan synthesis or biglycan mRNA levels in this chronicity model, but decreased sulfation (ie, charge) was reported.[36] In contrast to the shorter-term studies discussed earlier, elevated glucose decreased collagen synthesis and did not affect collagen type I and IV mRNAs in these latter experiments.

In view of these differences in the apparent effects of glucose on proteoglycan synthesis among the various in vitro and in vivo studies, the present experiments were undertaken to examine the effects of hyperglycemia on the synthesis of HSPG and dermatan sulfate proteoglycan (DSPG) in RMC proliferating under defined culture conditions. A second smooth muscle cell line was assessed to examine the generality of changes potentially relevant to diabetic microvascular disease. In both cases, short-term exposure to high glucose caused a comparable decrease in both HSPG and DSPG synthesis that was not reflected by the mRNA levels of several specific proteoglycan core proteins. In contrast to the increased transcription that has been documented for several extracellular matrix molecules, the effects of glucose on proteoglycans are at the translational or posttranslational level. Despite a difference in proteoglycan mRNA profiles of the two cell types, the response of proteoglycan synthesis to glucose appears to be a general property of smooth muscle–like cells.

MATERIALS AND METHODS

Cell Culture

RMC cultures were established and characterized as described previously,[37] following collagenase treatment of freshly isolated glomeruli from 125-g male Sprague-Dawley rats according to the method of Simonson and Dunn.[38] In all experiments, RMC were used between the fifth and fifteenth passages. The A10 smooth muscle cell line originating from embryonic rat aorta[39] was obtained from the American Type Culture Collection (ATCC # CRL 1476; Rockville, MD). Both cell types were maintained in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES, penicillin G (100 U/mL), streptomycin (100 μg/mL), and 17% heat-inactivated bovine calf serum (Hyclone, Logan, UT). Cells were passaged by trypsinization, and the basal concentration of glucose in RPMI 1640 medium (10 mmol/L) was supplemented to 30 mmol/L for periods described in individual experiments. To control for possible differences due to effects of serum components on proteoglycan synthesis, the medium was premixed with serum and portions were supplemented with equal additional volumes of RPMI 1640 medium with or without added glucose to the desired final composition. In some experiments, 20 mmol/L mannitol was added instead of glucose, as an osmotic control. Protein and DNA synthesis were measured with the addition of 0.5 μCi/mL [^{3}H]leucine (60 Ci/mmol; Amersham, Arlington Heights, IL) or [^{3}H]thymidine (6.7 Ci/mmol; ICN, Irvine, CA), respectively, for 18 hours. Cell layers were then washed with ice-cold phosphate-buffered saline, fixed with three washes of ice-cold trichloroacetic acid, and solubilized in 0.1 mol/L NaOH containing 0.1% sodium dodecyl sulfate (SDS) for liquid scintillation counting.

Proteoglycan Labeling and Characterization

Proteoglycans were labeled for 18 hours with carrier-free [^{35}S]sulfate (ICN) at 0.2 mCi/mL in serum-free medium. Medium was centrifuged and desalted on Sephadex G-25, and the cell layer was extracted with 4 mol/L guanidine · HCl/50 mmol/L sodium acetate/1% Triton X-100 (pH 5.8) containing the protease inhibitors, disodium EDTA (10 mmol/L), benzamidine · HCl (5 mmol/L), 1,10-phenanthroline (3 mmol/L), tryptamine · HCl (5 mmol/L), and phenylmethylsulfonyl fluoride (1 mmol/L), as described previously.[37] Both samples were transferred to 7 mol/L urea/50 mmol/L sodium acetate/0.15 mol/L NaCl, pH 5.9, as previously described,[37] and eluted from DEAE-Sephacel with a 50-mL NaCl gradient (0.15 to 1.15 mol/L) in the same buffer. The total proteoglycan fraction eluting between 0.3 and 0.7 mol/L NaCl was pooled and desalted for enzymatic and chemical degradation.[37] To assess charge density, 0.5-mL fractions were collected, ^{35}S activity was counted by liquid scintillation on a portion of each, and NaCl concentrations in the peak fractions were measured by determining the conductivity compared with a standard curve prepared with known concentrations of NaCl in the same urea buffer. To determine the proportion of newly incorporated sulfate in each type of glycosaminoglycan, degradation with nitrous acid (selective for heparan sulfate) and chondroitinases AC and ABC (degrading chondroitin sulfates and chondroitin plus dermatan sulfates, respec-

tively; Sigma, St Louis, MO) were performed as described in detail elsewhere.[37,40]

Electrophoresis of labeled proteoglycans from the culture medium was performed on precast 4% to 12% denaturing polyacrylamide gradient gels (Novex, San Diego, CA). Samples of medium were mixed with equal volumes of 2X Laemmli sample buffer and electrophoresed at 40 mA in Tris · glycine-SDS buffer prepared from a 10X concentrate (National Diagnostics, Manville, NJ). Gels were fixed in 10% methanol/10% acetic acid, soaked for 30 minutes in Enlightening autoradiographic enhancer (Dupont/NEN, Boston, MA), dried in a commercial gel dryer (BioRad, Richmond, CA), and visualized by fluorography. Bands of radioactivity were quantified by laser densitometry.

Miscellaneous Labeling Protocols

Several experiments were performed to assess the effects of lower concentrations of glucose, PKC inhibition, and reversibility of the effects of high glucose. In each of these experiments, a simplified extraction procedure was performed to assess the effects on total cell layer proteoglycan sulfation. In each case, triplicate cultures for each condition were grown just to confluence and then transferred to the desired concentration of glucose as described earlier. At the end of the experimental period, the cell layers were washed and extracted with guanidine · HCl. The extracts were transferred to 7 mol/L urea buffer by passing through Sephadex G-50 in disposable 10-mL plastic pipettes after preconditioning with albumin, heparin, and chondroitin sulfate type C, as described previously.[37] Void volume fractions were applied to DEAE-Sephacel urea columns (0.5 mL in 3-mL plastic syringes), washed, and eluted stepwise with 0.3 and 0.7 mol/L NaCl. The fraction eluting between these limits was counted for total ^{35}S. The following protocols were used.

To assess lower glucose concentrations, cells were cultured in Dulbecco's modified Eagle's medium instead of RPMI 1640 medium, to achieve 5 mmol/L glucose, and some cultures were supplemented to 10 mmol/L glucose at confluence. Cells were harvested 96 hours later after labeling for the last 18 hours with [^{35}S]sulfate.

To assess reversibility of the effects of glucose on proteoglycan sulfation, confluent cultures in RPMI 1640 medium at either 10 or 30 mmol/L glucose were grown for 96 hours and then transferred to fresh medium at 10 mmol/L glucose for an additional 48 hours, with labeling for the final 18 hours.

To study the involvement of PKC, cultures were grown in either 10 or 30 mmol/L glucose for 96 hours and labeled for the last 18 hours. In some cultures, the PKC inhibitor, staurosporine (50 ng/mL), was added together with [^{35}S]sulfate. In others, PKC was downregulated by addition of 12-*O*-tetradecanoyl phorbol-13-acetate ([TPA] 0.2 μg/mL). TPA was added 24 hours before commencing labeling, and was included in the labeling medium. We have shown elsewhere[41] that these maneuvers have the expected effects on PKC activity.

RNA Analysis

Cell layers in 75-cm^2 culture flasks were washed with phosphate-buffered saline and lysed in 2 mL 4-mol/L guanidine isothiocyanate containing 1% 2-mercaptoethanol and 0.5% sodium lauryl sarcosinate. Total RNA was prepared by ultracentrifugation on a cesium chloride cushion.[42] Twenty-microgram quantities of RNA were electrophoresed on 1% agarose gels containing 0.023 mol/L morpholinepropanesulfonic acid and 0.07 mol/L formaldehyde and transferred by overnight capillary blotting to Biotrans nylon membrane (ICN). Membranes were prehybridized for 4 hours at 42°C in 5X SSC (1X SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate) containing 5X Denhardt solution, 50 mmol/L sodium phosphate (pH 6.5), 0.1% SDS, 50% (vol/vol) formamide, and 250 μg/mL heat-denatured salmon sperm DNA (Sigma). Hybridizations were performed at 42°C for 18 hours in fresh prehybridization solution containing cDNA probes labeled with [α-^{32}P]CTP by the random primer method using a kit from Boehringer Mannheim (Indianapolis, IN). After hybridization, blots were washed three times for 5 minutes at room temperature in 2X SSC/0.1% SDS and twice for 15 minutes in 0.1X SSC/0.1% SDS before autoradiography and quantitation by laser densitometry.

The cDNA probe for mouse TGF-β_1 was a 280-bp fragment subcloned as previously described[11] by P. Kondaiah and M.B. Sporn (National Institutes of Health), from a cDNA prepared by Derynck et al.[43] Rat glyceraldehyde-3-phosphate cDNA[44] was also from M.B. Sporn. A human biglycan cDNA[45] was a gift from L.W. Fisher (National Institutes of Health). BPG7, a 1,986-bp cDNA encoding a region in domain IV of perlecan[46] cloned into the *Eco*RI site of pBR 322, was kindly supplied by J.R. Hassell (University of Pittsburgh). Rat decorin cDNA[47] was a gift from S. Kaname and E. Ruoslahti (La Jolla Cancer Research Foundation).

mRNA stability was determined by DNA-excess filter hybridization as described by Saini et al.[48] Ten micrograms of plasmid DNA was used for each filter, and control filters were prepared with 10 μg pBluescript DNA lacking inserts. Biglycan and decorin cDNAs—both in pBluescript—were immobilized on nitrocellulose filters (BA 85, 45 μm, 13 mm; Schleicher and Schuell, Keene, NH) as follows. Plasmid DNA (100 μg) was dissolved in 1 mL 20-mmol/L Tris · HCl/1-mmol/L EDTA, pH 7.6, boiled for 10 minutes, and kept at room temperature for 20 minutes after addition of an equal volume of 1-mol/L NaOH. Eight milliliters of 1.5-mol/L NaCl/0.15-mol/L sodium citrate/0.25-mol/L Tris · HCl (pH 8.0)/0.25-mol/L HCl was added, and equal amounts of the DNA were immediately transferred to 10 filters by slow suction. Each filter was washed twice with 10 mL 0.9-mol/L NaCl/0.09-mol/L citrate and baked at 80°C for 2 hours in a vacuum oven. A10 cells grown in 25-cm^2 flasks with either 10 or 30 mmol/L glucose as described earlier were incubated for 3 hours with 50 μCi [5,6-^{3}H]UTP (50 Ci/mmol; Dupont/NEN) and then washed twice with medium containing 20 mmol/L glucosamine, 5 mmol/L uridine, and 5 mmol/L cytidine.[49] Medium containing 10 or 30 mmol/L glucose was supplemented with 5 mmol/L each of uridine and cytidine and used to chase the label. At intervals, cells were lysed with guanidine isothiocyanate as described earlier, and total RNA was isolated by phenol-chloroform extraction.[50] Filters containing pBluescript DNA alone or with the biglycan or decorin inserts were prehybridized as above. Fifty-microgram quantities of labeled RNA were then hybridized to each filter under the same conditions as for Northern analysis, except that the hybridization time was extended to 24 hours. After washing the filters as described for the Northern blots, bound RNA was eluted with 200 μL 0.05-mol/L NaOH (30 minutes at 65°C), neutralized with HCl, and counted by liquid scintillation. Counts bound to filters carrying the plasmid alone were subtracted from those bound to plasmid with insert.

Statistical Analyses

Differences between parameters measured in cells cultured in 10 and 30 mmol/L glucose were assessed for significance by Student's *t* test for unpaired samples.

RESULTS

Sulfate Incorporation Into Proteoglycans

RMC were labeled with [^{35}S]sulfate for 18 hours after 7 days of culture in the presence of 10 or 30 mmol/L glucose.

Recovery of label incorporated into the proteoglycan fraction was in all cases more than 85% after extraction and DEAE-Sephacel chromatography. Labeling of both heparan and chondroitin/dermatan sulfates from the cell layer and culture medium was consistent with our previous results.[37] In both compartments, the amount of [^{35}S]sulfate incorporated into proteoglycan was decreased in 30 mmol/L glucose (Table 1). This was not an osmotic effect; supplementation of cells in 10 mmol/L glucose with 20 mmol/L mannitol did not change the rate or pattern of proteoglycan synthesis (not shown).

Under these same conditions, 30 mmol/L glucose did not significantly affect the rate of incorporation of [^{3}H]thymidine into DNA over the final 18 hours (Table 1), consistent with previous reports that glucose does not stimulate cell proliferation under these conditions.[18] There was a significant increase in the incorporation of [^{3}H]leucine into protein in the high-glucose cultures over the same period (Table 1), indicating that the decrease in sulfated proteoglycan is not part of a general decrease in protein synthesis.

To determine if glucose affected the incorporation of sulfate into glycosaminoglycans in a type-specific manner, RMC grown in 10 or 30 mmol/L glucose were labeled with [^{35}S]sulfate for 18 hours as above. Consistent with the data in Table 1, 30 mmol/L glucose decreased total labeling of molecules in the cell layer by 17% and in the medium by 34%. The resistance of most labeled molecules to degradation by chondroitinase AC indicates that dermatan sulfate–like material predominates. However, there were no accompanying changes in the types of glycosaminoglycans being synthesized, apart from a possible increase in the proportion of label associated with heparan sulfate (Table 2). A general effect on proteoglycan synthesis is indicated, rather than a specific effect on one or more subclasses.

To identify specific proteoglycans affected by high glucose, cells were grown to confluence in 10 mmol/L glucose and then given fresh complete medium with either 10 or 30 mmol/L glucose. Media were harvested after 4 days following 18 hours' labeling with $^{35}SO_4^{2-}$, and subjected to SDS-PAGE. Three major bands are observed on autoradiography (Fig 1). Bands at approximately 245 and 115 kd (designated DSPG1 and DSPG2) are degraded by chondroitinase ABC, and have been reported to be the dermatan sulfates, biglycan and decorin, respectively, based on immunoprecipitation.[51] An additional band (HSPG1) at approximately 310 kd is identified as a heparan sulfate by its resistance to chondroitinase ABC (Fig 1) and sensitivity to heparin lyase III (not shown). Compared with 10 mmol/L glucose, 30 mmol/L glucose significantly decreased the total amount of ^{35}S incorporated at day 4, and caused a

Table 1. Effect of Glucose on Proteoglycan, Protein, and DNA Synthesis in RMC

Parameter	Glucose	
	10 mmol/L	30 mmol/L
^{35}S (cpm × 10^{-5})		
Cell layer	4.66 ± 0.26	3.35 ± 0.31
Medium	1.56 ± 0.20	1.20 ± 0.04
Total	6.22 ± 0.46	4.56 ± 0.35
Protein (^{3}H, cpm × 10^{-4})	8.48 ± 1.01	10.83 ± 0.04
DNA (^{3}H, cpm × 10^{-4})	3.30 ± 0.27	3.44 ± 0.17

NOTE. Cells were plated at a 1:10 split ratio and grown for 7 days in either complete RPMI 1640 medium (10 mmol/L glucose) or complete medium supplemented to 30 mmol/L glucose. For the final 18 hours, $^{35}SO_4^{2-}$, [^{3}H]leucine, or [^{3}H]thymidine were added and the samples processed for proteoglycan, protein, or DNA synthesis, respectively. ^{35}S results are the mean ± SE of quadruplicate wells from a single experiment, and are typical of 4 experiments in which the decrease in total ^{35}S incorporated in 30 mmol/L glucose was always between 20% and 30%. Protein synthesis (mean ± SE, n = 4) was confirmed to be increased ($P < .001$, Student's t test) and DNA synthesis unaffected in 2 independent experiments.

Table 2. Effect of Glucose on Composition of Newly Sulfated Proteoglycans in Cultured RMC

Treatment	Cell Layer (%)	Medium (%)
10 mmol/L glucose		
Nitrous acid	26.1	14.4
Chondroitinase ABC	74.3	77.7
Chondroitinase AC	11.9	10.9
30 mmol/L glucose		
Nitrous acid	33.6	23.5
Chondroitinase ABC	74.0	73.4
Chondroitinase AC	9.8	7.9

NOTE. Cells were grown on plastic for 7 days in 10 or 30 mmol/L glucose and labeled with [^{35}S]sulfate for the last 18 hours. The proportion of ^{35}S incorporated into proteoglycan and sensitive to glycosaminoglycan-specific treatments was determined. Results are from single analyses of material pooled from 2 to 4 experiments in each case.

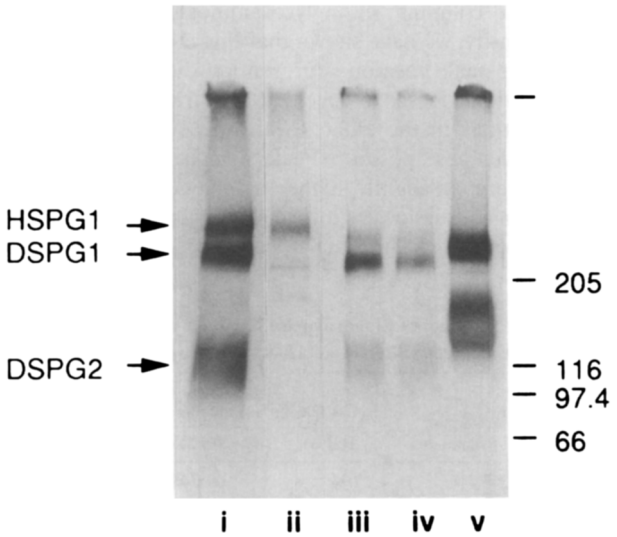

Fig 1. Electrophoresis of proteoglycans from RMC-conditioned medium. Proteoglycans were labeled with [^{35}S]sulfate for 18 hours, and the medium was electrophoresed on 4% to 12% denaturing gradient gels for fluorographic visualization. Lane i, confluent culture growing in complete medium with 10 mmol/L glucose; lane ii, conditions as in lane i after treatment with chondroitinase ABC; lane iii, culture grown 4 days past confluence in 10 mmol/L glucose; lane iv, culture grown 4 days past confluence in 30 mmol/L glucose; lane v, confluent cultures in complete medium treated for the preceding 72 hours with porcine TGF-β_1 (5 ng/mL). Positions of molecular weight markers (kd) are shown at right.

similar decrease of approximately 20% to 30% in ^{35}S incorporation by each of HSPG1, DSPG1, and DSPG2 (Fig 1 and Table 3), consistent with the above results obtained by enzyme-specific degradation of total cell layer and medium proteoglycans. Similar results were also obtained with medium conditioned by A10 cells; three bands were again observed on SDS-PAGE and decreases of 10% to 15% in $^{35}SO_4^{2-}$ content became significant with exposure to 30 mmol/L glucose for 4 days (Table 3).

Because 10 mmol/L glucose already represents a concentration that is twofold normoglycemia, we compared the effects of 5 and 10 mmol/L glucose on total cell layer ^{35}S-proteoglycan. Cells incorporated (1.76 ± 0.16) × 10^5 and (1.62 ± 0.21) × 10^5 cpm per culture in 5 and 10 mmol/L glucose, respectively (difference not significant; n = 3). Because it is generally believed that RMC require 10 mmol/L glucose to proliferate in culture,[52] and therefore integrity of the cells in the lower concentration may be suspect, subsequent experiments were performed at 10 and 30 mmol/L glucose.

Sulfate Content of Proteoglycans

Because glucose has been reported to decrease the sulfate to carbohydrate ratio of some glycosaminoglycan chains[53] and may inhibit heparan *N*-deacetylase activity in the glomerulus,[54] we investigated whether the decrease in total proteoglycan sulfate incorporated in 30 mmol/L glucose was due to production of glycosaminoglycan chains with a lower sulfate content. [^{35}S]-labeled proteoglycans from RMC grown in 10 or 30 mmol/L glucose were subjected to DEAE-Sephacel chromatography. By calibrating similar columns with glycosaminoglycans of known charge density, we have shown that this method can detect changes in peak position representing differences of one sulfate residue in six to seven disaccharide units.[55] NaCl concentrations in the eluates were measured by conductance at each peak position. Peaks from cells grown in high glucose were at slightly higher salt concentrations than peaks from cells in lower glucose (Fig 2), indicating normal sulfation and suggesting that the decrease in labeled cell layer proteoglycans is due to lower total synthesis of sulfated glycosaminoglycans. Results with the conditioned medium also show that if changes in the sulfate content of the glycosaminoglycan chains occur, they are too subtle to account for the decrease in total sulfate incorporation in the high-glucose culture (Fig 2). These experiments reproduce our previous observations[56] under the conditions of the present study, allowing a direct comparison of sulfate incorporation and charge.

Table 3. Effect of Glucose on Sulfate Incorporation Into Proteoglycans Released Into Conditioned Medium

Cell Type	Days of Treatment	Relative Band Intensity ([30 mmol/L/10 mmol/L] × 100%) HSPG1	DSPG1	DSPG2
RMC	1	104 ± 6	92 ± 8	88 ± 3
	4	74 ± 6	78 ± 9	65 ± 7
A10	1	106 ± 2	91 ± 3	110 ± 10
	4	84 ± 5	89 ± 4	87 ± 3

NOTE. RMC or A10 cells were grown to confluence and treated with fresh medium containing 10 or 30 mmol/L glucose. After 1 or 4 days, equal volumes of medium from each culture were analyzed by electrophoresis (Fig 1) after labeling with [^{35}S]sulfate for the final 18 hours of culture. After fluorography, bands were quantified by laser densitometry. The ratio of intensity of a band from the 30-mmol/L glucose culture to that from 10 mmol/L glucose is expressed as a percentage. HSPG1, DSPG1, and DSPG2 are as identified in Fig 1. The experiment was repeated 3 times. Values are the mean ± SE.

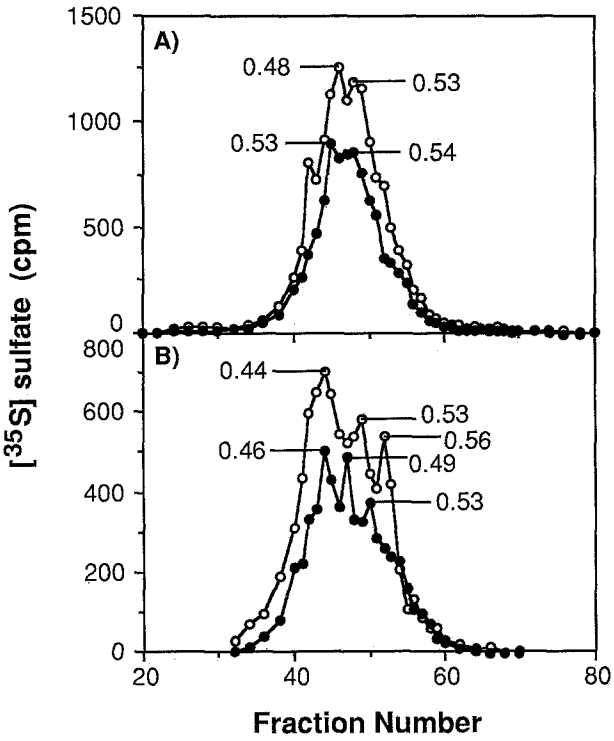

Fig 2. DEAE-Sephacel chromatography of RMC proteoglycans. Cells were grown for 7 days in 10 mmol/L (○) or 30 mmol/L (●) glucose and labeled for the last 18 hours with [^{35}S]sulfate. Labeled macromolecules from (A) the cell layer and (B) the medium were eluted in urea buffer from DEAE-Sephacel with a NaCl gradient. Numbers are the NaCl concentration (molar) in collected peak fractions, as measured by conductivity.

Reversibility of Suppressed Sulfate Incorporation

To determine whether decreased ^{35}S incorporation was an effect of high glucose present during the time of labeling, cultures treated with 10 or 30 mmol/L glucose for 4 days were then placed in fresh medium at 10 mmol/L glucose for a further 48 hours. At the end of this period, cells previously exposed to 30 mmol/L glucose incorporated 1.29 ± 0.41 × 10^5 cpm ^{35}S per culture into total cell layer proteoglycan, as compared with 2.11 ± 0.25 × 10^5 cpm per culture in cells kept at 10 mmol/L glucose throughout ($P < .05$, n = 3), suggesting that suppression is not dependent on the immediate presence of elevated glucose.

Proteoglycan mRNA

Total RNA was harvested from RMC grown for 2, 4, or 7 days in high or low glucose. Steady-state levels of biglycan

mRNA in the high-glucose group were approximately double those in the low-glucose cells throughout (Fig 3 and Table 4), when normalized to the "housekeeping" message of glyceraldehyde phosphate dehydrogenase (GAPDH). The perlecan probe hybridized weakly to a message at approximately 12 kb; this mRNA also approximately doubled in the high-glucose cells, but the effect was transient. Like biglycan mRNA, perlecan mRNA was at the highest level at day 4. Biglycan mRNA in A10 cells showed a similar response to glucose, being transiently elevated 2.5-fold at day 4 (Fig 4 and Table 4). Perlecan mRNA was not detected in A10 cells. In contrast to biglycan, decorin mRNA was not detected in RMC, was expressed only transiently in A10 cells, and was decreased in the latter by 30 mmol/L glucose. Thus, despite comparable reductions in sulfate incorporation in both proteoglycans, biglycan and decorin may be differentially regulated by glucose in A10 cells, as observed in other vascular smooth muscle, endothelial, and fibroblast cells.[57,58]

To determine if the changes in biglycan and decorin mRNA concentrations in A10 cells were a consequence of changes in message stability, RNA was pulse-labeled in 4-day postconfluent cells and the effect of glucose on message turnover rates was measured by DNA-excess filter hybridization. No effect of glucose was observed (Fig 5), indicating that changes in steady-state levels of these messages are transcriptional in origin.

Possible Role of TGF-β

Because TGF-β increases biglycan levels in RMC,[51] decreases decorin expression in some cells,[59] and mediates collagen synthesis in RMC cultures in response to glucose,[12] it was of interest to determine whether the changes in decorin and biglycan mRNA could be mediated by this cytokine. In both cell types, glucose treatment causes an increase in the transcript at 2.5 kb detected by a cDNA encoding mouse TGF-β_1, although the increase is more pronounced and sustained in A10 cells (Figs 3 and 4 and Table 4). The response of TGF-β_1 mRNA to 30 mmol/L

Table 4. Effects of Glucose on Proteoglycan and TGF-β mRNA Concentrations in RMC and A10 Cells

mRNA	Fold Increase		
	Day 2	Day 4	Day 7
Biglycan			
RMC	1.8	2.5	1.3
A10	0.7	2.5	1.0
Decorin			
RMC	—	—	—
A10	0.40	0.25	0.43
Perlecan			
RMC	0.9	1.8	0.6
A10	—	—	—
TGF-β_1			
RMC	0.6	1.6	1.3
A10	1.1	2.6	4.6

NOTE. RNA was isolated from cells grown past confluence for 2 to 7 days in 10 or 30 mmol/L glucose. Values are the ratio of the intensity of the Northern hybridization band from 30-mmol/L samples to that from 10-mmol/L samples. All signals are corrected for intensity of the GAPDH signal in the same lane. Northern blots are shown in Figs. 3 and 4. Proteoglycan results are typical of those obtained in 3 separate experiments. TGF-β blots were repeated once with similar results.

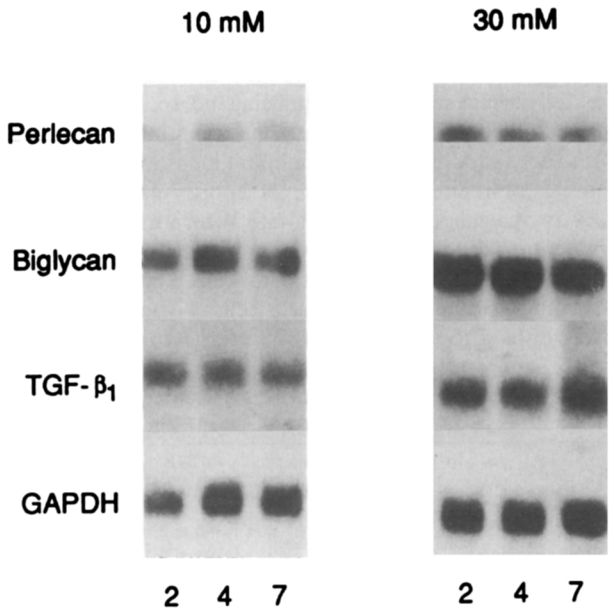

Fig 3. Northern blot analysis of proteoglycan mRNA in RMC. Confluent cultures were transferred to 10 or 30 mmol/L glucose and lysed for RNA extraction 2, 4, or 7 days later. The same blot was hybridized with cDNAs encoding domain IV of perlecan, biglycan, TGF-β_1, and GAPDH, which detected the indicated bands at 12, 2.6, 2.5, and 1.3 kb, respectively. Results are typical of 2 or 3 experiments (see Table 4 NOTE).

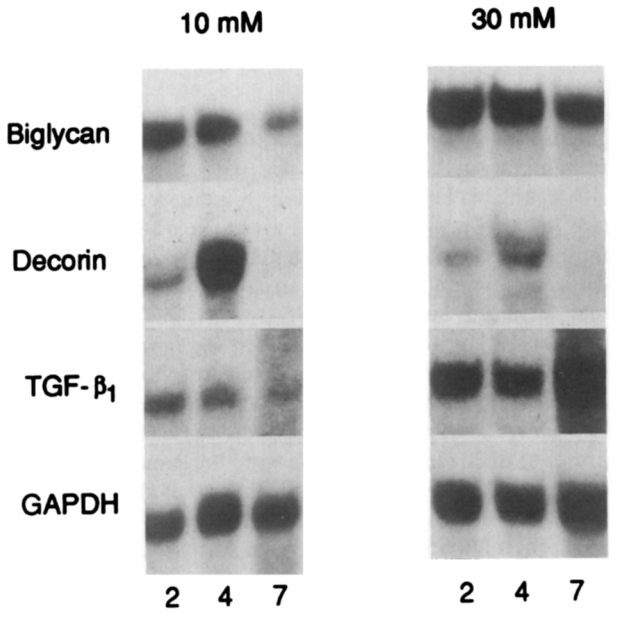

Fig 4. Northern blot analysis of proteoglycan mRNA in A10 cells. Confluent cultures were transferred to 10 or 30 mmol/L glucose and lysed for RNA extraction 2, 4, or 7 days later. The same blot was hybridized with cDNAs encoding biglycan, decorin, TGF-β_1, and GAPDH, which detected the indicated bands at 2.6, 2.2, 2.5, and 1.3 kb, respectively. Results are typical of 2 or 3 experiments (see Table 4 NOTE).

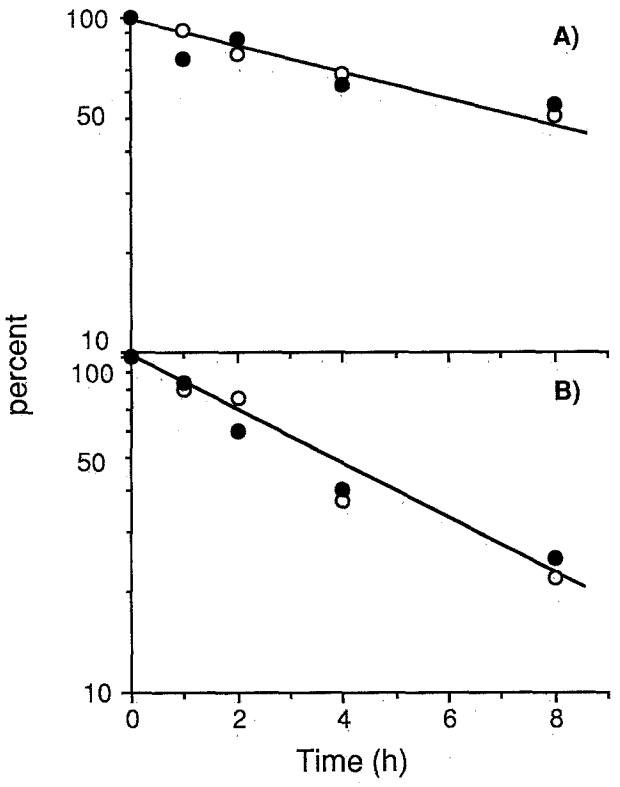

Fig 5. Biglycan and decorin mRNA turnover measured by DNA-excess filter hybridization. Confluent cultures of A10 cells were treated with 10 mmol/L (○) or 30 mmol/L (●) glucose for 4 days and pulse-labeled with [^{3}H]uridine for the last 3 hours in culture. Total RNA was harvested from 0 to 8 hours during the chase period, and ^{3}H hybridizing to filters of immobilized biglycan (A) or decorin (B) cDNA in pBluescript was counted. Values are expressed as the percentage of counts hybridizing in RNA obtained at time zero, and are corrected for counts hybridizing to filters of immobilized pBluescript alone.

glucose is modest in RMC; it continues to increase more prominently in A10 cells.

It has been observed that TGF-β_1 increases the apparent size of newly synthesized biglycan in vascular smooth muscle cells by increasing the length of the glycosaminoglycan chains,[60] and therefore, a structural change in biglycan may serve as a marker for TGF-β_1 activity. This effect was reproduced in the present set of experiments (Fig 1, DSPG1), and similar changes in electrophoretic mobility are seen in bands with lower molecular mass in Fig 1. However, the apparent sizes of proteoglycans made in the presence of 30 mmol/L glucose were the same as in control cells, indicating that regardless of glucose-mediated changes in TGF-β_1 mRNA levels, the cytokine does not exert an apparent influence on proteoglycan synthesis.

Possible Role of PKC

Because elevated glucose has been shown to increase PKC activity transiently in RMC,[9] we also assessed the role of PKC in proteoglycan synthesis by using a PKC inhibitor, staurosporine, and by downregulating PKC with a phorbol ester. Both maneuvers decreased total sulfate incorporation into cell layer proteoglycans compared with control cells, and eliminated the further suppressive effect of 30 mmol/L glucose (Table 5). In fact, 30 mmol/L glucose caused a significant increase in sulfation in both cases.

DISCUSSION

Changes in the synthesis of specific proteoglycans that may accompany pathophenotypic changes have not been well documented, although their potential role in the glomerular and vascular lesions of diabetes has been considered.[35,54,61] In general, glucose enhances matrix synthesis in cultured RMC without increasing cell proliferation.[14,19,20] However, this response can be modified by cytokines and the state of growth of the culture. For instance, in the presence of insulin-like growth factor-I, glucose increased protein synthesis while decreasing [^{3}H]thymidine incorporation.[62] In contrast, 25 mmol/L glucose increased [^{3}H]thymidine incorporation and early-response gene expression in serum-starved murine mesangial cells.[63] Silbiger et al[36] used RMC growing slowly in long-term, low-serum culture with the aim of better mimicking in vivo conditions, and found that glucose suppressed both proliferation and collagen synthesis. In the present study, we examined proteoglycan expression in postconfluent cultures proliferating slowly in serum-replete medium to isolate the effects of glucose from those attributable to abrupt changes in growth medium or substratum. The cells continue to proliferate slowly and accumulate matrix, and may be a useful model for examining factors that affect matrix composition.[64] The RMC were used between passages five and 15, a time when the proteoglycan profile is constant,[37] comparable to previous studies on the effects of glucose on these cells.[35,65,66] Under these experimental conditions, glucose did not change the rate of synthesis of DNA ([^{3}H]thymidine incorporation) and significantly increased protein synthesis ([^{3}H]leucine incorporation) at the time sulfate labeling of proteoglycans was studied.

In these experiments, 30 mmol/L glucose was found to cause a reproducible decrease of approximately 15% to 20% in the amount of [^{35}S]sulfate incorporated over 24 hours into each of the major proteoglycans, including heparan and dermatan sulfates, synthesized by both mesangial and vascular smooth muscle cells. Hyperglycemia in vivo can lead to changes in tissue sulfate content and, in

Table 5. Effects of PKC Modulation on Sulfate Incorporation Into Proteoglycans Associated With the Cell Layer

Treatment	^{35}S Incorporated (cpm/10^3 cells)		
	10 mmol/L Glucose	30 mmol/L Glucose	*P*
Control	155 ± 20	96 ± 31	<.05
Staurosporine	58 ± 4	83 ± 9	<.02
TPA	99 ± 12	114 ± 1	<.01

NOTE. Cells were grown in 10 or 30 mmol/L glucose for 4 days past confluence and labeled with [^{35}S]sulfate for the final 18 hours (control). In some cultures, PKC was inhibited with staurosporine during labeling; in others, PKC was downregulated by treatment with TPA 24 hours before and during labeling. Values are the mean ± SD from 3 separate cultures.

turn, affect the specific activity of radiolabeled sulfate.[67,68] However, in cell culture sulfate from the medium rapidly enters cells and equilibrates in the phosphoadenosyl phosphosulfate pool—the donor for proteoglycan sulfation.[69] Sulfate uptake by RMC is unaffected by glucose.[68] Therefore, specific activity of the label is determined by the sulfate concentration in the medium, and differences in specific activity are unlikely to account for the differences in incorporated label in the present studies. Undersulfation of proteoglycans—that is, less sulfate incorporated per glycosaminoglycan chain, leading to a lower charge density—has been inconsistently reported to occur in experimental diabetes. Kjellén et al[53] observed small shifts on ion-exchange chromatograms of HSPG from diabetic rat livers, which they interpreted as undersulfation. Inhibition by glucose of *N*-deacetylase, an activity required to expose additional sites for sulfation during heparan synthesis, has been advanced as an explanation.[54] Inhibition of *N*-deacetylase by glucose would affect heparan sulfate synthesis, but it is not required for chondroitin/dermatan sulfates, which remain fully *N*-acetylated, and so cannot account for the global suppression of proteoglycan synthesis observed here. Kanwar et al[70] reported a hexose-dependent decrease in sulfate incorporation into glomerular proteoglycans by the perfused kidney that was overcome by supplementing the perfusate with adenosine triphosphate. The decrease was in part due to undersulfation; proteoglycans newly labeled in the presence of higher glucose eluted from DEAE-Sephacel at lower salt concentrations. However, such a deficit is unlikely to occur as an isolated phenomenon in vivo. Rather, it is likely that if adenosine triphosphate levels were low enough to affect glycosaminoglycan sulfation, synthesis of other macromolecules would also be affected. In fact, the energy derived from the oxidative production of uronic acid balances the cost of addition to glycosaminoglycans of one sulfate per disaccharide.[71] Nor is sulfate likely to become limiting. Cultured RMC sustain the charge density of newly synthesized proteoglycans in the absence of any added sulfate by deriving sufficient amounts from oxidation of amino acids.[55] Drug-metabolizing sulfotransferase activity has been found to be diminished in diabetes,[72] but it is not clear that the Golgi enzymes involved in dermatan and heparan sulfation are similarly affected. DEAE-Sephacel chromatography failed to show any differences in sulfate content of proteoglycans isolated from kidneys of proteinuric streptozotocin- or BioBreeding-diabetic rats and appropriate controls.[56] In the present study, no change in charge density was demonstrable by ion-exchange chromatography, indicating that glucose can decrease the synthesis of glycosaminoglycans independently of the sulfation of individual chains. Whereas 4 weeks of continuous culture in high glucose diminishes the net charge of the unfractionated glycosaminoglycan/proteoglycan pool,[36] initial glucose-dependent changes in glycosaminoglycan synthesis appear to be restricted to the quantity produced.

The glucose-dependent decrease in proteoglycan synthesis appears to be at the translational or posttranslational level. While core protein message levels are variably affected by glucose, synthesis of both chondroitin/dermatan and heparan sulfates is uniformly depressed. Nor need changes in mRNA stability be invoked to explain these results. We examined mRNA stability of decorin and biglycan in A10 cells because these are abundant messages with strongly hybridizing probes for use in the filter binding assay, and change in opposite directions in response to glucose. Whereas biglycan and decorin mRNA levels in A10 cells are differentially affected by glucose, both mRNA turnover rates remain constant in 10 and 30 mmol/L glucose, with half-life values of approximately 8 and 4 hours, respectively. Therefore, whereas transcriptional effects appear to be involved in determining levels of decorin and biglycan mRNA in response to glucose, later events must account for the observed decrease in sulfated product. It is interesting that Mahadevan et al[73] have recently shown a glucose-dependent increase in hyaluronic acid synthesis that appears to lead to decreased synthesis of other (sulfated) glycosaminoglycan chains by an unknown mechanism(s). The failure of synthesis to return to normal 30 to 48 hours after returning RMC to 10 mmol/L glucose from 30 mmol/L in our studies is consistent with accumulation of such an inhibitory product.

The nature of DSPG2 is not clear. Decorin mRNA was not detected in confluent RMC cultures, although the message does appear during the first passage through the cell cycle when quiescent RMC are stimulated with serum (A. Wang and D.M. Templeton, unpublished) and is readily detected by Northern blotting in A10 cells under some conditions. Furthermore, whereas incorporation of sulfate into DSPG2 is increased markedly by TGF-β, in parallel to biglycan, decorin is decreased in some cells by TGF-β[58,74] in response to a TGF-β–negative regulatory element in the decorin gene promoter.[59] Decorin binds to collagen and prevents collagen fibrillogenesis in vitro,[75] but may stabilize matrix in vivo. Both decorin and biglycan bind to and neutralize the biological activity of TGF-β.[76] The matrix- and growth factor–binding properties of DSPG2 are not known, but it may be predicted that the decreased synthesis of biglycan and DSPG2 observed in response to 30 mmol/L glucose will influence matrix stability and cell-matrix interactions in the diabetic milieu.

TGF-β has been found to be elevated in human and experimental diabetes[11] and in mesangial cells cultured in high glucose.[12] In the present study, steady-state TGF-β mRNA levels were increased by high glucose, but the increased size of biglycan produced in response to TGF-β[60] (Fig 1) was not observed in these cultures. The possibility remains that increased TGF-β is responsible for the increased level of biglycan mRNA observed in response to glucose. However, the increases in glycosaminoglycan chain size and sulfate elicited by active TGF-β added to the culture are not observed in response to glucose. Increased mRNA levels may not reflect increased active protein in these experiments, or increases may occur that are insufficient to affect proteoglycan synthesis. Alternatively, glucose may overcome the effects of TGF-β on proteoglycan struc-

ture. The role, if any, of TGF-β in determining proteoglycan expression in high-glucose cultures remains to be elucidated.

Another well-documented effect of glucose on cultured mesangial cells is an elevation of PKC activity,[9] probably secondary to elevated diacylglycerol levels.[77] The present studies show that downregulation or inhibition of PKC abrogates the effect of high glucose on proteoglycan synthesis. However, both treatments also lead to a decrease in net sulfate incorporation in the cell-layer proteoglycans in 10 mmol/L glucose. This would suggest that PKC activity is necessary for normal proteoglycan synthesis. It then seems contradictory that conditions associated with increased PKC activity (ie, 30 mmol/L glucose in the absence of PKC inhibitors) cause decreased synthesis. An explanation may be that downregulation or inhibition of PKC affects other cellular signals that may influence proteoglycan synthesis at other levels that have not been addressed in the present studies. The demonstration that staurosporine causes a greater suppression of synthesis than TPA, under the same conditions for which we have shown the latter to more effectively block phorbol ester–dependent induction of c-*fos*,[41] suggests that other factors are involved. For instance, although staurosporine has a lower K_i for PKC than for other kinases, kinase inhibitors in general lack strong specificity.[78] Nevertheless, the fact that decreased PKC activity achieved by two independent means removes the suppression and actually increases ^{35}S incorporation in 30 mmol/L glucose supports the conclusion that PKC is involved in the response.

A general response of cultured mesangial cells to high glucose is an increased accumulation of extracellular matrix[14] reminiscent of the mesangial expansion of diabetic nephropathy.[13] The role, if any, of decreased proteoglycan synthesis in this process is unclear, but may be predicted to affect matrix organization. There may also be effects on cell signaling. Heparin and heparan sulfate suppress proliferation of mesangial[37] and vascular smooth muscle[79] cells, apparently at least in part by interfering with PKC signaling.[80] Mesangial cell PKC is increased in response to hyperglycemia,[9] and it is conceivable that the effects of increased PKC signaling are exacerbated by a deficiency of heparan sulfate. Mesangial cell TGF-β is increased by high glucose[12] and may play an important role in matrix accumulation in the diabetic glomerulus.[11] Decorin and biglycan can neutralize some activities of TGF-β,[81] and, again, the effects of increased TGF-β signaling might be exacerbated by a deficiency in these DSPGs. However, in contrast to the effects of heparan sulfate on PKC signaling, the effect of decorin and biglycan on TGF-β is a property of their core proteins and not the glycosaminoglycan chains.[81] The present studies do not address whether core protein levels are affected by glucose.

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