Flux Through the Hexosamine Pathway Is a Determinant of Nuclear Factor κB–Dependent Promoter Activation

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The hexosamine pathway may mediate some of the toxic effects of glucose. We hypothesized that flux through this pathway might regulate the activity of nuclear factor κB (NF-κB)-dependent genes in mesangial cells (MCs). In MCs, RT-PCR revealed that high glucose (30 mmol/l) and glucosamine (1 mmol/l) increased mRNA levels for vascular cell adhesion molecule 1 (VCAM-1) and increased the activity of an NF-κB enhancer by 1.5- and 2-fold, respectively. Overexpression of glutamine: fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme for flux through the hexosamine pathway, led to a 2.2-fold increase in NF-κB enhancer activity; the combination of GFAT overexpression and high glucose increased activity 2.8-fold, and these increases were prevented by 40 μmol/l O-diazoacetyl-L-serine (azaserine) or 6-diazo-5-oxonorleucine. High glucose, glucosamine, and GFAT overexpression increased binding of MC nuclear proteins to NF-κB consensus sequences. Immunoblotting revealed that the p65 subunit of NF-κB was O-glycosylated in MC cultured in physiologic glucose and that significant enhancement occurred with high glucose and glucosamine. Both glucose and glucosamine dose-dependently increased human VCAM-1 promoter activity. In addition, GFAT overexpression activated the VCAM-1 promoter (2.25-fold), with further augmentation by high glucose and abrogation by inhibitors of GFAT, NF-κB, and O-glycosylation. Inactivation of the two NF-κB sites in the VCAM-1 promoter abolished its response to high glucose, glucosamine, and GFAT overexpression. These results suggest that increased flux through the hexosamine pathway leads to NF-κB–dependent promoter activation in MCs. *Diabetes* 51:1146–1156, 2002

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BADGP, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; DMEM, Dulbecco's modified Eagle's medium; DON, 6-diazo-5-oxonorleucine; DTT, dithiothreitol; EMSA, electromobility shift assay; FBS, fetal bovine serum; GFAT, glutamine:fructose-6-phosphate amidotransferase; ICAM, intracellular adhesion molecule; IκB, inhibitory κB; MC, mesangial cell; IL, interleukin; NF-κB, nuclear factor κB; PAI, plasminogen activator inhibitor; PDTC, pyrolidine dithiocarbamate; PMSF, phenylmethylsulfonyl fluoride; STZ, streptozotocin; TGF, transforming growth factor; TNF, tumor necrosis factor; TTBS, Tween and Tris-buffered saline; VCAM, vascular cell adhesion molecule.

Flux through the hexosamine pathway has been implicated in some of the adverse consequences of high glucose and may also play a role in the development of diabetic kidney disease (1–3). Under physiologic conditions, a small percentage (1–3%) of glucose that enters cells is shunted through the hexosamine pathway (4). In the first step of the pathway, fructose-6-phosphate is converted to glucosamine-6-phosphate by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT; enzyme commission 2.6.1.16) (4). The end product of the hexosamine pathway, UDP *N*-acetylglucosamine is the substrate for O- and N-glycosylation of protein (5). In mesangial cells (MCs), flux through the hexosamine pathway has been implicated in glucose-induced increases in transforming growth factor β1 (TGF-β1) and plasminogen activator inhibitor type 1 (PAI-1) expression (1,2,6), cytokines that are important in tissue injury and sclerosis (7–9). O-glycosylation of protein may mediate hexosamine pathway effects (10).

Recent studies have described increased expression of inflammatory cytokines in tissues of diabetic animals. For instance, in streptozotocin (STZ)-induced diabetic apoE null mice, increased vascular cell adhesion molecule (VCAM) was identified in kidney. However, the source of the increased VCAM-1 expression in kidney was not reported (11). Macrophage infiltration has been associated with increases in glomerular expression of VCAM-1 and other inflammatory cytokines, such as intracellular adhesion molecule 1 (ICAM-1) and monocyte chemoattractant protein 1 in STZ-induced diabetic rats (12). These studies suggest that inflammation may be a critical component of the pathophysiologic mechanisms that are important in diabetic kidney disease and that the increased cytokine expression may promote the inflammatory infiltration into the glomeruli in diabetes.

The expression of many of the inflammatory cytokines is regulated by the transcription factor nuclear factor κB (NF-κB), and NF-κB activation has been associated with experimental and clinical diabetes (13–17). NF-κB is normally present in the cytoplasm in an inactive form, a condition that is determined by its interaction with its polypeptide inhibitor, inhibitory κB (IκB). After appropriate stimuli, IκB is phosphorylated by IκB kinase and ubiquitinated by a ubiquitin ligase, and both of these modifications target IκB for degradation in proteasome.

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This process releases the inhibition of NF-κB, which is transported to the nucleus where NF-κB–dependent genes are activated (18–22).

Given the reported role of the hexosamine pathway in the regulation of genes related to injury and fibrosis, we hypothesized that the hexosamine pathway is important in inflammation and that it may regulate the expression of NF-κB–dependent genes such as VCAM-1, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in MCs. Consequently, we investigated the effects of high glucose, glucosamine, and overexpression of the rate-limiting enzyme GFAT on the activities of an NF-κB enhancer element–luciferase reporter construct and NF-κB–dependent promoter-luciferase reporters for VCAM-1 in MC in the presence and absence of inhibitors of GFAT and NF-κB activation. We sought to determine whether flux through the hexosamine pathway had an effect on NF-κB nuclear binding and whether any observed effects might be associated with O-glycosylation of NF-κB components.

RESEARCH DESIGN AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and Trizol reagent were purchased from Gibco BRL (Life Technologies, Grand Island, NY). Reporter cell lysis buffer and NF-κB consensus oligonucleotides (CGGACTTTC) were obtained from Promega (Madison, WI), and Effectene transfection reagent was from QIAGEN (Mississauga, Ontario, Canada). T4 polynucleotide kinase and T4 polynucleotide kinase 10× buffer were obtained from New England Biolabs (Beverly, MA); rabbit polyclonal NF-κB p65/Rel A antibody (sc-109X) and NF-κB p50 antibody (sc-114X) were from Santa Cruz Biotechnology (Santa Cruz, CA); and [γ-^{32}P]ATP (3,000 Ci/ml) was procured from DuPont (Boston, MA). Monoclonal (mouse) anti–O-linked N-acetylglucosamine (Clone RL2) was purchased from Affinity Bioreagents (Golden, CO). Poly deoxyinosine triphosphate-deoxycytosine triphosphate (poly dI-dC) and protein A Sepharose CL-4B beads (cat # 17-0780-01) were obtained from Amersham-Pharmacia Biotech (Québec, Canada). All other reagents were of the highest grade available from Sigma-Aldrich (Mississauga, Ontario, Canada).

Preparation and culture of MCs. MCs were obtained from male Sprague-Dawley rats as described (1,23,24). The cells were cultured (37°C, 5% CO_2) in DMEM supplemented with FBS (20%), penicillin (100 units/ml), streptomycin (100 μg/ml), and glutamine (2 mmol/l). Cells were used between passages 14 and 20.

Plasmids. The plasmid pCIS (empty vector), pCIS-GFP (expresses green fluorescent protein), and pCIS-GFAT (expresses the human GFAT gene) were supplied by Dr. M.J. Quon (Cardiology Branch; National Heart, Lung and Blood Institute, National Institutes of Health; Bethesda, MD) and have been previously described (25). The plasmid pNF-κB-Luc, consisting of an NF-κB enhancer element that contains five NF-κB binding sequences in tandem upstream of a luciferase reporter gene was obtained from Stratagene (La Jolla, CA). The other promoter-luciferase reporters used in this study have been previously described. VCAM-1 promoter luciferase construct (26) was provided by Dr. J.M. Redondo (Centro de Biologia Molecular, Universidad Autonoma, Madrid, Spain); TNF-α promoter-luciferase reporter (27) was from Dr. J. Ye (National Institute for Occupational Safety and Health, Morgantown, WV); and the IL-6 promoter luciferase reporter (28) was provided by Dr. A. Nakamura (Teikyo University, Tokyo). pCMV-βgal (Promega) was used to control for variation in transfection efficiency.

Transient transfection of MC. MC (1.5×10^5 cells/well) were plated onto six-well plastic plates (SARSTEDT), and transfection was carried out 24 h later using Effectene (QIAGEN) according to the manufacturer's specifications as we have reported (1). Briefly, MC (70–80% confluent) were co-transfected with 0.1 μg of promoter-luciferase construct or NF-κB enhancer-luciferase reporter, 0.25 μg of pGFAT, and 0.05 mg of pCMVβgal and then cultured for 18 h in DMEM containing FBS (20%) and 5.6 mmol/l D-glucose. With the use of green fluorescent protein, a transfection efficiency of 35–40% was obtained. Subsequently, the media was changed to DMEM with 0.5% FBS and glucose (5.6–30 mmol/l), glucosamine (0.25–5.0 mmol/l) with and without inhibitors azaserine (40 μmol/l), 6-diazo-5-oxonorleucine (DON; 40 μmol/l), benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP; 0.5 mmol/l), or pyrolidine dithiocarbamate (PDTC) (25 μmol/l). At various times, the cells were harvested and used for analyses as described below.

Assay of luciferase and β-galactosidase activity. Medium was aspirated, wells were washed twice with PBS, and lysis was performed using 0.2 ml/well Reporter Lysis Buffer. MC were incubated for 15 min at 4°C, then transferred to microcentrifuge tubes using a rubber policeman. Cell debris was pelleted by centrifugation (12,000*g*, 4°C, 1.0 min), and the supernatant was used to assay for luciferase (0.02 ml) and β-galactosidase (0.05 ml) activities using commercially available reagents. Luciferase activity was assessed as per the manufacturer's protocol (Promega), and β-galactosidase activity was determined using ONPG reagent (Sigma). Luciferase was measured in a luminometer (EG&G Berthold, Oak Ridge, TN), and β-galactosidase activity was based on the absorbance at 405 nm. Luciferase activity was normalized to the β-galactosidase activity and cell protein. Protein was determined on an aliquot of the supernatant obtained from cell lysis using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA).

RNA isolation and semiquantitative RT-PCR. Total RNA from MCs was isolated by the single-step method of Chomczynski and Sacchi (29), as we have published (1,30). Isolated RNA was stored in diethyl pyrocarbonate–treated water at −80°C The purity and concentration were determined by measuring the optical density at 260 nm and 280 nm before use. The absorbance ratio, A_{260}:A_{280}, ranged from 1.75 to 1.95. Semiquantitative RT-PCR was performed as previously reported (1,30–32). The specific primer sequences were as follows: β-Actin, 5′ AAC CCT AAG GCC AAC CGT GAA AAG 3′, 3′ TCA TGA GGT AGT CTG TCA GGT C 5′; VCAM-1, 5′ GGA GAC ACT GTC ATT ATC TCC TG 3′, 3′ TCC TTT CAT GTT GGC TTT TCT TGC 5′.

For amplification, 2.5 μl of the RT product was mixed with 7.5 μl of PCR mix containing 0.1 μmol/l of each of the primer pairs and 2 units of *Taq* polymerase. The sample was placed onto a Perkin-Elmer DNA thermal cycler (model 480) and heated to 94°C for 4 min before the application of temperature cycles. β-Actin was co-amplified to standardize the amount of RNA subjected to reverse transcription. The temperature cycle for amplification was as follows: *1*) denature at 94°C for 30 s, *2*) cool-anneal at 60°C for 30 s, and *3*) heat-extend at 72°C for 30 s. For the β-actin, GFAT, and TGF-β1 primer pairs, the PCR product plateaued at 28 cycles, and therefore 25 cycles were chosen for the final amplification. For VCAM-1 primer pairs, the PCR product plateaued at 40 cycles, and therefore 35 cycles were chosen for final amplification as the PCR product. PCR products were separated on 1% agarose gel containing ethidium bromide, photographed, and quantified with a transmittance/reflectance scanning densitometer (model GS 300, Hoeffer Scientific Instrument) using a Macintosh class II computer (system 7.0) and Dynamax HPLC Method Management software (version 1.2).

Nuclear protein binding to NF-κB oligonucleotide consensus sequence. The binding of nuclear protein to NF-κB consensus sequence was determined by electromobility shift assay (EMSA) (23). After washing in cold PBS, nuclear extracts of MCs were prepared by lysis in hypotonic buffer (20 mmol/l HEPES [pH 7.9], 1 mmol/l EDTA, 1 mmol/l EGTA, 20 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l $Na_4P_2O_7$, 1 mmol/l dithiothreitol [DTT], 0.5 mmol/l phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.6% Nonidet P-40), homogenized, and sedimented at 16,000*g* for 20 min at 4°C. Pelleted nuclei were resuspended in hypotonic buffer with 0.42 mmol/l NaCl and 20% glycerol and rotated for 30 min at 4°C. After centrifugation (12,000*g*, 20 min), the supernatant was collected and protein concentration was measured with the Bio-Rad assay kit.

NF-κB consensus oligonucleotides were prepared by incubating 2 μl of consensus oligonucleotide (1.75 pmol/μl), 1 μl of T4 polynucleotide kinase 10× buffer, 1 μl of [γ-^{32}P]ATP (3,000 Ci/ml; DuPont, Boston, MA), and 5 μl of nuclease-free water for 10 min at 37°C. The reaction was terminated by addition of 1 μl of 0.5 mol/l EDTA. Unlabeled ^{32}P-ATP was removed from the oligonucleotide mixture with D-25 Sephadex columns (Pharmacia, Uppsala, Sweden).

Supernatants were used as nuclear proteins for the binding assay. Nuclear proteins (3 μg) were incubated (30 min, room temperature) with 2 μg of poly(dI-dC) (Pharmacia) in binding buffer (20 mmol/l HEPES [pH 7.9], 1.8 mmol/l $MgCl_2$, 2 mmol/l DTT, 0.5 EDTA, and 0.5 mg/ml BSA) and then reacted with radiolabeled consensus oligonucleotides at room temperature for 20 min (50,000–100,000 cpm). For supershift experiments, 1.0 μg of rabbit polyclonal NF-κB p65/RelA antibody or rabbit polyclonal NF-κB p50 antibody was added to the reaction mixture and incubated for 2 h at room temperature before addition of the radiolabeled consensus oligonucleotide. Reaction mixtures were electrophoresed in a 6% polyacrylamide gel and autoradiographed. Competition experiments were performed with 100× excess unlabeled consensus oligonucleotides.

Mutagenesis of NF-κB of VCAM-1 promoter. Site-directed mutagenesis of the VCAM-1 promoter was carried out by a pair of 5′-phosphorylated primers using *pfu* polymerase (33). The intact plasmid was amplified except for the deletion region that was excluded by the two primers. After PCR amplification, parental DNA template was destroyed by addition of *Dpn*I (10–20 units)

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directly to the PCR reaction and incubated at 37°C for 1 h. *Dpn*I only cuts methylated DNA. Because parental plasmid was purified from *Escherichia coli*, it was methylated, whereas PCR-amplified DNA was not methylated. After *Dpn*I, the PCR-amplified DNA (linear) was gel-purified and circularized by ligation and transformed into *E. coli*. Colonies were then screened for the deletion. The two primers used were as follows: vcamlow1, 5′ GAGCCAGG-GAAAAAGTTTAACTGA 3′; and vcamup3, 5′ TCCGCCTCTCTAGCAAGACCCT 3′. The PCR conditions were as follows: 94°C for 3 min, followed by 35 cycles at 94°C for 45 s, 56°C for 45 s, and 72°C for 17 min.

Immunoprecipitation and immunoblotting of p65/RelA. MCs were incubated with glucosamine (5.0 mmol/l) for 8–12 h, medium was removed, and the cells were washed (3×) with ice-cold PBS. Cells were harvested under nondenaturing conditions on ice by incubation for 5 min with ice-cold lysis buffer (20 mmol/l Tris [pH 7.5], 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 1 mmol/l β-glycerophosphate, 1 mmol/l sodium orthovanadate, 1.0 μg/ml aprotinin, and 1 mmol/l PMSF). Cells were then scraped into microcentrifuge tubes on ice and centrifuged (12,000*g*, 4°C, 15 min). The supernatant was transferred to a fresh microcentrifuge tube, and the protein concentration was measured with Bio-Rad protein assay reagent.

Protein (500 μg) was mixed with 1 μg rabbit polyclonal NF-κB p65/Rel A antibody and incubated at 4°C overnight. Subsequently, 25 μl of protein A Sepharose CL-4B slurry (50%) was added and incubated for 90 min (4°C, gentle rocking). The mixture was centrifuged, and the pellet was washed with 2× buffer A (10 mmol/l Tris-HCl [pH 7.5]), 100 mmol/l NaCl, 2 mmol/l EDTA, 0.2% Nonidet P-40, and 0.25 mmol/l PMSF) then with 1× buffer B (10 mmol/l Tris-HCl [pH 7.5], 500 mmol/l NaCl, 2 mmol/l EDTA, 0.2% Nonidet P-40, and 0.25 mmol/l PMSF) and 1× buffer C (10 mmol/l Tris-HCl [pH 7.5] and 0.25 mmol/l PMSF). The beads were suspended in 20 μl of 3× SDS sample buffer (125 mmol/l Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue), boiled for 2 min, then centrifuged for 1 min (12,000*g*, 4°C).

The supernatant containing immunoprecipitated protein was electrophoresed in SDS-PAGE gel (8%), and protein was transferred to nitrocellulose membranes (Protran; Schleicher and Schuell, New Haven, CT). The membrane was incubated in 25 ml of blocking buffer (5% skim milk in Tween and Tris-buffered saline (TTBS) for 30 min at room temperature. Subsequently, immunoblotting was performed with RL2 antibody (1:1,000 in blocking buffer, 3 h, room temperature). The membrane was washed with TTBS (three times, 5 min/wash, room temperature) and subsequently incubated with 1:5,000 diluted horseradish peroxidase–conjugated anti-rabbit antibody (Bio-Rad, catalogue no. 170-6515) for 30 min at room temperature. The membrane was washed three times with TTBS (5 min/wash), and protein was detected with Luminol reagent (catalogue no. NN-NEL602001 KT, New England Nuclear) after exposure to x-ray film (catalogue no. NEF596; Kodak, Toronto, Ontario, Canada).

Statistical analysis. Statistical analyses were performed with the INSTAT statistical package (GraphPad Software, San Diego, CA). The difference between means was analyzed using the Bonferroni multiple comparison test. Significance was defined as $P < 0.05$.

RESULTS

High glucose and glucosamine increases VCAM-1 mRNA. To ascertain whether exposure to glucose or glucosamine led to any changes in cytokine expression in MCs, we used RT-PCR to determine VCAM-1 mRNA levels. In MCs that were exposed to glucose (30 mmol/l) for 24 h, mRNA levels for VCAM-1 increased by 2.3-fold. Similarly, in MCs that were cultured in medium with physiologic glucose concentration (5.6 mmol/l) supplemented with the hexosamine pathway product glucosamine (1 mmol/l) for 12 and 24 h, we observed a comparable increase in VCAM-1 mRNA levels at 24 h (Fig. 1).

High glucose and glucosamine activate NF-κB enhancer element activity in MC. The activity of the genes for several cytokines, including that for VCAM-1, is dependent on the activation of the transcription factor NF-κB (14,16). To determine whether high glucose or glucosamine might activate NF-κB, we studied the activity of an NF-κB–responsive luciferase reporter, pNF-κB-Luc, under various conditions.

Incubation of MCs in medium containing 30 mmol/l glucose for 48 h led to a 1.5-fold increase in luciferase

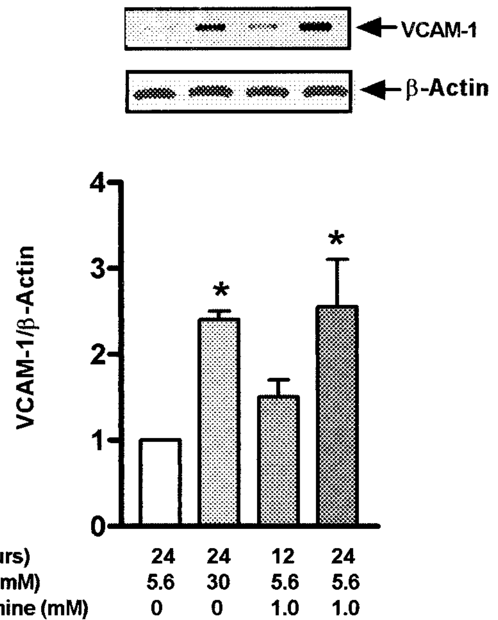

FIG. 1. High glucose and glucosamine increase VCAM-1 mRNA. MCs (70–80% confluent, 1.5 × 10^5 cells/well) were growth-arrested in DMEM/0.5% FBS/5.6 mmol/l glucose. MCs were incubated with high glucose (30 mmol/l) or physiologic glucose (5.6 mmol/l) plus glucosamine (1.0 mmol/l) for the indicated times. Total RNA was isolated, and RT-PCR was performed as per RESEARCH DESIGN AND METHODS. β-Actin was co-amplified to standardize for the amount of RNA subjected to reverse transcription. Amplification was allowed to proceed for 25 cycles. PCR product were separated on 1% agarose gel (top panel), and densitometry was performed (bottom panel). Each condition was done in duplicate in three separate experiments. Values represent the mean ± SD. *$P < 0.02$, $n = 3$.

activity ($P < 0.05$, $n = 4$) (Fig. 2*A*). This effect was not due to osmolarity because exposure of MCs to medium containing 5.6 mmol/l glucose plus 24.4 mmol/l mannitol did not reproduce the results seen with high glucose. Importantly, glucosamine (5 mmol/l) also activated the NF-κB enhancer element (Fig. 2*B*), suggesting that, like high glucose, flux through the hexosamine pathway activates NF-κB.

GFAT overexpression recapitulates the effect of high glucose and glucosamine on the NF-κB–responsive element. We have previously shown that GFAT overexpression mimics the action of high glucose and glucosamine in MCs (1,6). Hence, given the above findings that high glucose and glucosamine activated the NF-κB enhancer element, we wished to determine whether GFAT overexpression would recapitulate these observations.

MCs cotransfected with pGFAT and pNF-κB-Luc showed a 2.1-fold increase in luciferase activity compared with those transfected with the empty vector (pCIS) (Fig. 2*A*), thus complementing our observations with glucosamine (Fig. 2*B*). The effect seen with GFAT overexpression was enhanced in MCs transfected with pGFAT and cultured in medium with high glucose (30 mmol/l). This maneuver led to a 2.8-fold increase in luciferase activity. Furthermore, activation of the NF-κB enhancer element produced by overexpressing GFAT was prevented by the GFAT inhibitors azaserine and DON or by exposing MCs to PDTC (25 μmol/l), an inhibitor of NF-κB (Fig. 2*A*). These findings further suggest that hexosamine flux may play a role in NF-κB activation in MCs.

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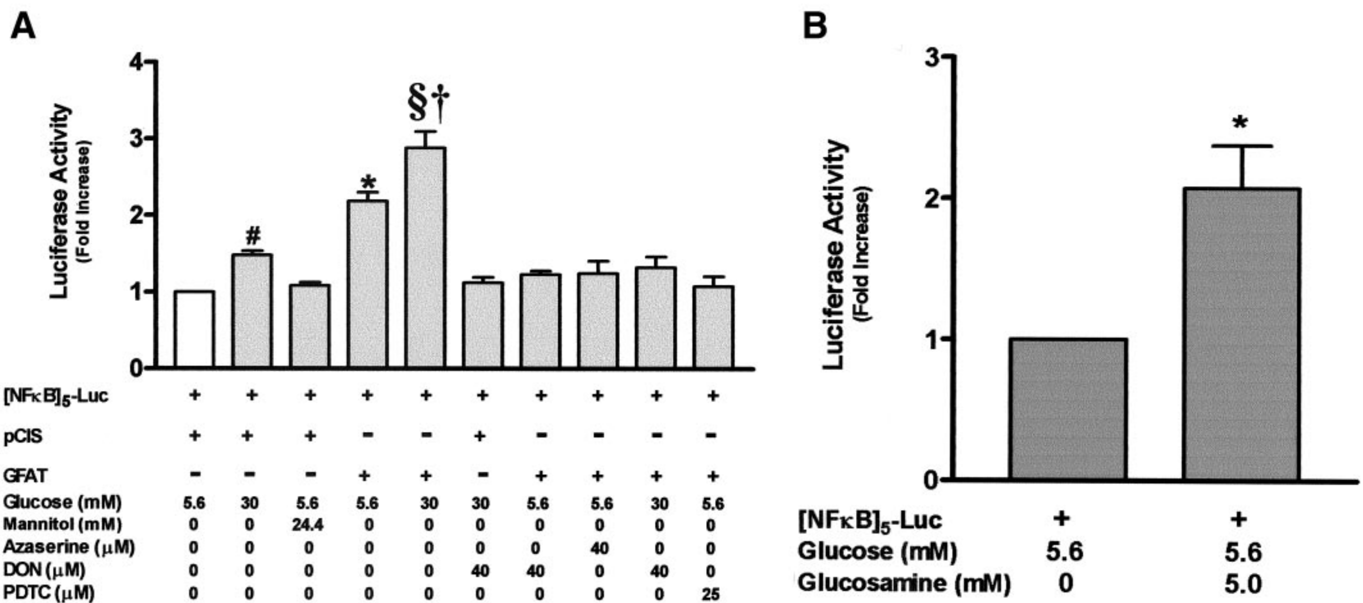

FIG. 2. high glucose, glucosamine, and GFAT overexpression activates NF-κB enhancer element. *A*: MCs cultured in 20% FBS/DMEM/5.6 mmol/l glucose were cotransfected with pNF-κB (0.15 μg/35-mm well), pCIS, or pCIS-GFAT (0.25 μg/35-mm well) and pCMVβgal (0.05 μg/35-mm well). Eighteen hours after transfection, medium was changed to 0.5% FBS/DMEM/5.6 or 30 mmol/l glucose, and cells were incubated for an additional 48 h with and without azaserine or DON (40 μmol/l) or PDTC (25 μmol/l). Mannitol (24.4 mmol/l) was used as an osmotic control. *B*: MCs were plated as above and then cotransfected with pNF-κB and pCMVβgal. Eighteen hours after transfection, medium was changed to 0.5% FBS/DMEM/5.6 mmol/l glucose with and without glucosamine (5.0 mmol/l). Luciferase activity was normalized to protein and β-galactosidase activity. All experiments were done in triplicate, and values represent the mean ± SD. *$P < 0.02$ versus control, $n = 4$; #$P < 0.05$ versus control, $n = 4$; §$P < 0.01$ versus control, $n = 4$; †$P < 0.05$ versus GFAT in 5.6 mmol/l glucose, $n = 4$.

High glucose, glucosamine, and GFAT overexpression increases binding of nuclear proteins to NF-κB consensus sequence. After activation, NF-κB is translocated to the nucleus, where it participates in the activation of NF-κB–dependent gene transcription. The increase of NF-κB levels in the nucleus is reflected by augmented NF-κB binding to consensus sequences (18,20,22). To determine whether high glucose, glucosamine, or GFAT overexpression increases binding of nuclear proteins to NF-κB consensus sequence (GGGATTTCCC), we performed EMSA (23).

The binding of nuclear protein to radiolabeled NF-κB consensus sequence was increased by high glucose (Fig. 3*A*, *B*, and *E*) and glucosamine (Fig. 3*E* and *F*). Like high glucose and glucosamine, GFAT overexpression increased the binding of nuclear protein to NF-κB consensus sequence (Fig. 3*C* and *D*). This was attenuated in cells that were transfected with pCIS-GFAT and exposed to the GFAT inhibitors azaserine and DON (Fig. 3*C* and *D*), suggesting that the increased nuclear binding was related to GFAT activity. Supershift experiments performed with antibodies to the p50 and p65 subunits of NF-κB demonstrated retardation by p65 antibody (Fig. 3*E*, band 3).

High glucose and glucosamine increase O-glycosylation of p65/Rel A NF-κB subunit. Several proteins, including transcription factors Sp1 and c-myc and RNA polymerase II, are O-glycosylated, and this posttranslational modification is believed to be important in protein stability and function (34–39). Our finding that flux through the hexosamine pathway may increase NF-κB nuclear activity by increasing p65 protein led us to examine whether p65 is O-glycosylated and how this may be affected by high glucose and glucosamine.

NF-κB p65/RelA subunit was immunoprecipitated with anti–NF-κB p65 antibody and subsequently immunoblotted with RL2 antibody to detect O-glycosylated products. Figure 4 demonstrates that in MCs cultured in physiologic glucose, there is minor O-glycosylation of the p65 subunit of NF-κB. Incubation of MCs in either high glucose or glucosamine significantly increases O-glycosylation of the p65 subunit of NF-κB (Fig. 4).

GFAT overexpression activates promoters for VCAM-1, TNF-α, and IL-6 in MCs. The transcriptional activities of several inflammatory genes and adhesion molecules are induced by NF-κB. Three of the most studied are TNF-α, IL-6, and VCAM-1 (40–44). In view of our observation that GFAT overexpression activated the NF-κB enhancer element, we sought to determine whether the same would hold for NF-κB–dependent promoter activity.

In MCs that overexpressed GFAT, we observed a 2.25-, 1.70-, and 1.5-fold induction of the VCAM-1, TNF-α, and IL-6 promoter, respectively (Fig. 5). Because the response of the VCAM-1 promoter was most marked, we chose to characterize further the activation of this promoter in MCs.

VCAM-1 promoter activation is dependent on flux through the hexosamine pathway. In these experiments, MCs were transiently transfected with the VCAM-1 promoter-luciferase reporter alone, or the former construct was cotransfected with pCIS-GFAT or pCIS. We found that both glucose and glucosamine caused dose-dependent increases in VCAM-1 promoter activity (Fig. 6*A* and *B*). TNF-α, a known activator of VCAM-1 gene expression in several cells, including MCs (16,45,46), also activated the VCAM-1 promoter in MCs (Fig. 7). In MCs overexpressing GFAT, the activity of the VCAM-1 promoter was also increased, and incubation in high glucose for 48 h led to additional enhancement of VCAM-1 pro-

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moter activity, above that seen in physiologic (5.6 mmol/l) glucose ($P < 0.05$, $n = 4$) (Fig. 7). The effect of high glucose on VCAM-1 promoter activity was not due to increased osmolarity because in MCs cultured in 5.6 mmol/l glucose plus 24.4 mol/l D-mannitol, the VCAM-1 promoter was not activated (Figs. 6*A* and 7).

The above results suggested that activation was dependent on flux through the hexosamine pathway and that GFAT overexpression sensitizes MCs to the effect of high glucose. This is further supported by the observation that inhibition of GFAT enzymatic activity by incubating cells with DON or azaserine prevented the increase in VCAM-1 promoter activity (Fig. 7). We observed similar inhibition of activation of the VCAM-1 promoter by GFAT overexpression when the experiments were performed in the presence of PDTC (25 μmol/l), a compound that is known to interfere with NF-κB activation.

BADGP has been shown to limit O-glycosylation by preventing addition of N-acetylglucosamine to proteins such as mucin (47,48). Recently, we also demonstrated that BADGP may attenuate the activity of the hexosamine pathway (30). Therefore, to characterize further the activation of the VCAM-1 promoter by GFAT, we assessed promoter activity in the presence and absence of this inhibitor. As shown in Fig. 7, BADGP (0.5 mmol/l) attenuated the activation of the VCAM-1 promoter that occurred with GFAT overexpression.

Mutagenesis of NF-κB sites in VCAM-1 promoter moderates its responsiveness. Two NF-κB interacting sites located at −73 and −57 have been identified in the VCAM-1 promoter (49). We used site-directed mutagenesis to modify these sites (Fig. 8*A*), then we studied the impact of this modification on the activation of the promoter by high glucose, glucosamine, or GFAT overexpression. The results, shown in Fig. 8*B*, indicate that the mutated promoter was not activated by high glucose, glucosamine, and GFAT overexpression. This further suggested that activation of the VCAM-1 promoter by increasing hexosamine pathway flux was dependent on NF-κB.

DISCUSSION

Recent studies have suggested that flux through the hexosamine pathway, which produces the substrates for O- and N-glycosylation of proteins, may allow cells to sense the level of glucose in the extracellular environment (50) and may also be an important determinant of hyperglycemic tissue injury (1,50–54). O-glycosylation of serine and threonine residues is important for posttranslational modification affecting the behavior of many nuclear and cytosolic proteins, including some transcription factors (5,34,35). O-glycosylation of transcription factors is believed to be important in the regulation of gene expression (34–36,38,39). Indeed, protein O-glycosylation has been likened to protein phosphorylation, and for some signaling molecules, these two processes may be competitive (5,34,36,55). However, the exact role of the hexosamine pathway and O-glycosylation in gene expression remains unclear.

In humans with type 1 diabetes, kidney sections show increased immunostainable GFAT (3), and GFAT activity is elevated in skeletal muscle from patients with type 2 diabetes (56). Our recent studies indicate that GFAT activity is an important determinant of expression of genes implicated in diabetic glomerular disease. As such, we demonstrated that GFAT overexpression or glucosamine activated the promoter for PAI-1 in an Sp1-dependent manner and increased mRNA levels for TGF-β1 and its receptors (1,6).

Inflammatory cytokines such as VCAM-1 are expressed by MCs in culture (57), and VCAM-1 expression is increased in glomeruli from STZ-induced diabetic rats and mice (11,12). Because expression of the genes for these cytokines is NF-κB–dependent (58) and NF-κB is activated by high glucose (13,14,17), we speculated that flux through the hexosamine pathway might also play a role in the activation of NF-κB–dependent genes. Indeed, our first major observation was that either high glucose or glucosamine increased VCAM-1 mRNA levels (Fig. 1) and activated an NF-κB enhancer element (Fig. 2). The magnitude of the activation was comparable to that reported for high glucose–mediated activation of NF-κB–dependent genes by several other investigators (11,14,16,59).

GFAT overexpression also activated the NF-κB enhancer element, and synergistic activation was observed with the combination of high glucose and GFAT overexpression. The observed increase in luciferase activity was dependent on GFAT activity because it was prevented when MCs were incubated with the GFAT inhibitors azaserine and DON. Because activation occurred when MCs were cultured in medium that contained physiologic glucose concentrations (5.6 mmol/l), these results indicate GFAT overexpression alone is sufficient to activate the NF-κB enhancer element and are consistent with our findings for the PAI-1 promoter (1). A hyperosmolar stimulus with mannitol did not reproduce the effect of 30 mmol/l glucose (Fig. 2*B*). Our observation that either glucosamine or GFAT overexpression increases the activity of the NF-κB enhancer element (Fig. 2*B*) bolsters the argument that NF-κB transcriptional elements may be activated by flux through the hexosamine pathway.

The transcription factor NF-κB serves as a critical regulator of the inducible expression of many genes. NF-κB is activated by multiple stimuli and is a critical component of the signal transduction machinery required for tissue remodeling and development (44,58,60–65). Accordingly, we sought to characterize the effects of hexosamine flux on NF-κB–dependent gene transcription.

Our second major observation was that high glucose and glucosamine increased binding of nuclear proteins to

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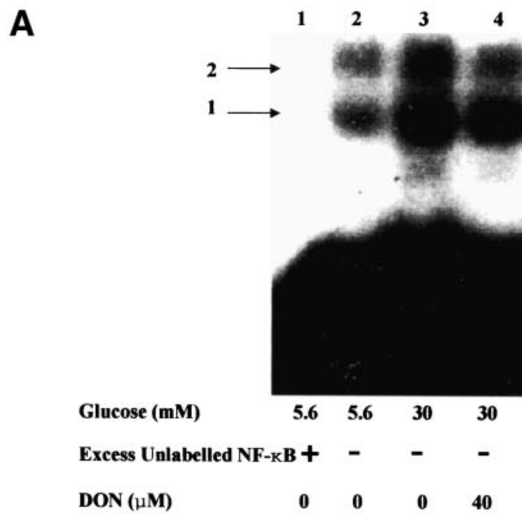
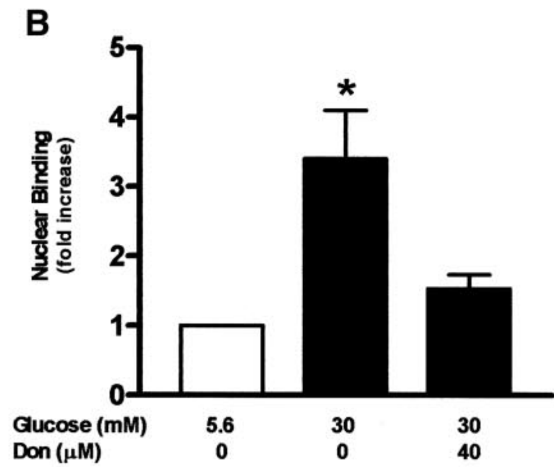
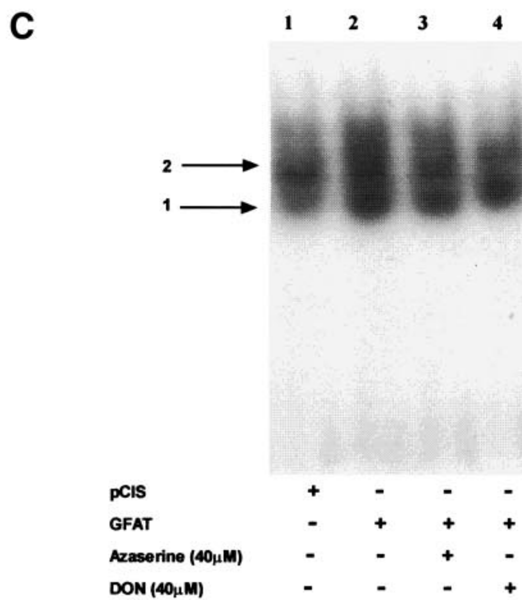
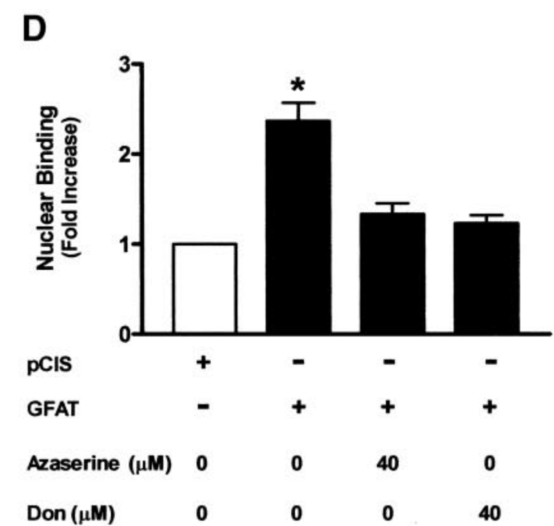
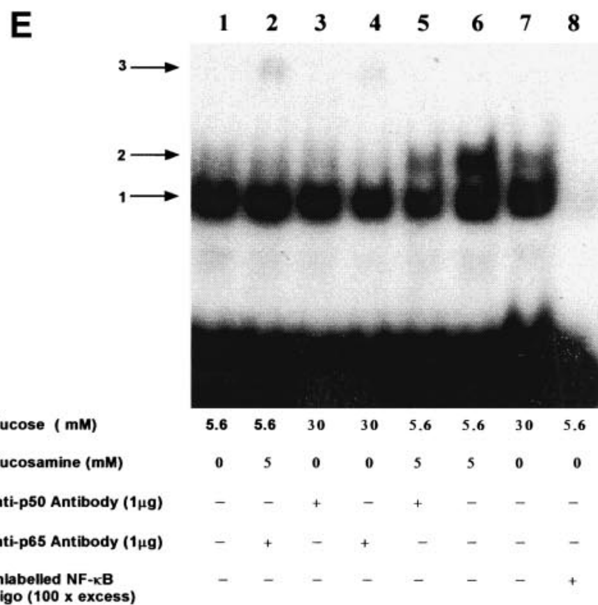
FIG. 3. High glucose, glucosamine, and GFAT overexpression increases binding of nuclear protein to NF-κB consensus sequence. Untransfected MCs were plated overnight in 20% FBS/DMEM/5.6 mmol/l glucose. Subsequently, medium was changed to 0.5% FBS/DMEM/5.6 or 30 mmol/l glucose (*A* and *E*) without or with glucosamine (5.0 mmol/l) (*E*), and cells were incubated for 24 h. *C*: MCs were transfected with pCIS or pCIS-GFAT (0.40 μg/35-mm well), and 18 h after transfection, medium was changed to 0.5% FBS/DMEM/5.6 mmol/l glucose. Cells were incubated for 24 h with and without azaserine or DON (40 μmol/l). Nuclear extracts of MCs were prepared, and EMSA was performed. For supershift experiments, 1 μg of rabbit polyclonal NF-κB p65/Rel A antibody or rabbit polyclonal NF-κB p50 antibody was added to the reaction mixture and incubated for 2 h at room temperature before addition of the radiolabeled consensus oligonucleotide. Reaction mixtures were electrophoresed in a 6% polyacrylamide gel and autoradiographed. Competition experiments were performed with 100× excess unlabeled consensus oligonucleotides. Three separate experiments were performed in duplicate for each condition. Graphs (*B*, *D*, and *F*) show densitometry of the gels. Numbers 1 and 2 indicate the NF-κB–retarded bands, and 3 denotes the supershifted band. *$P < 0.05$ versus control, $n = 3$; #$P < 0.02$ versus control, $n = 3$.

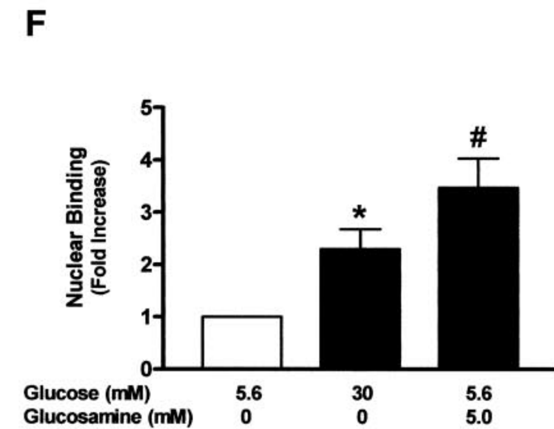












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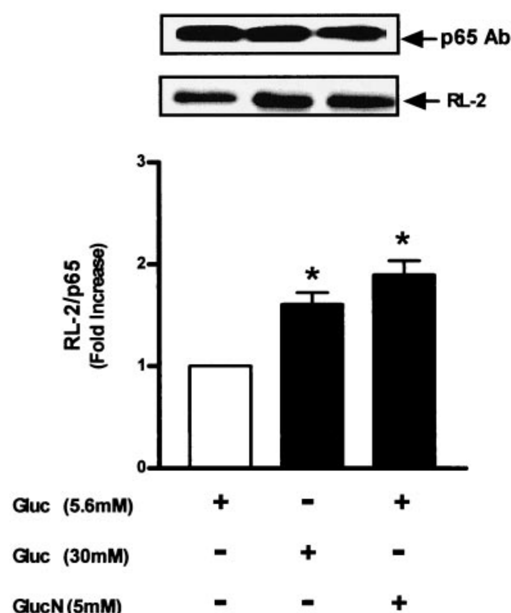

FIG. 4. High glucose and glucosamine increase O-glycosylation of p65/Rel A NF-κB subunit. MCs were plated overnight in 20% FBS/DMEM/5.6 mmol/l glucose. Subsequently, medium was changed to 0.5% FBS/DMEM/5.6 or 30 mmol/l glucose, with or without glucosamine (5.0 mmol/l), and cells were incubated for an additional 8–12 h. Protein (500 μg) was used for immunoprecipitation with 1 μg of rabbit polyclonal NF-κB p65/Rel A antibody. Immunoprecipitated protein was electrophoresed in SDS-PAGE gel (8%), and protein was transferred to nitrocellulose membranes. Immunoblotting was performed with RL2 antibody (1:1,000 in blocking buffer, 3 h, room temperature). Protein was detected with Luminol reagent after exposure to x-ray film. The experiment was done in triplicate. The top panel shows representative autoradiograph images for immunoprecipitated protein (p65) and immunoblotted protein (RL2). The bottom panel is a graphic representation of the densitometric ratios of RL2/p65. Values are the mean ± SD. *P < 0.05 versus control (time 0, glucose 5.6 mmol/l, glucosamine 0 mmol/l), n = 3.

NF-κB consensus sequences (Fig. 3*A*, *B*, *E*, and *F*). Similarly, GFAT overexpression increased the binding of nuclear proteins to an NF-κB consensus sequence, which was prevented by GFAT inhibition with azaserine and DON (Fig. 3*C* and *D*). The high glucose finding is supported by recent studies in which high glucose was found to induce similar magnitudes of NF-κB activation in vascular smooth muscle cells (16), peripheral blood mononuclear cells from patients with diabetes (17), and cultured endothelial cells (66). Our observation that the hexosamine pathway is a determinant of NF-κB activation is novel and may provide one possible mechanism whereby high glucose leads to activation of NF-κB–dependent genes.

The NF-κB/Rel family of transcription factors consists of five members (p50, p52, p65 [RelA], RelB, and c-Rel) that form homo- and heterodimers that are capable of binding to DNA and subsequently activating NF-κB targeted gene (58,67). p50/p65 heterodimers have been the most extensively studied, and specific DNA binding sites for this dimer have been identified in the promoter region of genes for a diverse array of cytokines, adhesion molecules, enzymes, and receptors (58,67). In the present study, we used antibodies to p50 and p65 to ascertain whether these subunits were responsible for the increased binding of nuclear protein to NF-κB consensus sequences upon exposure of untransfected MCs to high glucose (30 mmol/l) or glucosamine (5 mmol/l). Under our experimental conditions, only nuclear protein extracts that were incubated with the p65 antisera produced a supershift (Fig. 3*E*, band 3). This suggests that in MCs that were exposed to high glucose or glucosamine, the increase in nuclear protein binding to NF-κB oligonucleotide consensus sequences was due primarily to p65/RelA. This is consistent with the recent observations of increased nuclear p65 translocation in MCs exposed to high glucose (68). Furthermore, several reports support a role for selective activation of Rel/NF-κB family members, and some NF-κB target genes contain binding sites that preferentially bind p65 homodimers (16,69–72). In addition, adenovirus-mediated expression of a dominant-negative p65 was found to inhibit induction of several proinflammatory genes, including ICAM-1, VCAM-1, and IL-8 in porcine aortic endothelial cells (62).

Flux through the hexosamine pathway is believed to exert an effect on gene expression by increasing the intracellular concentration of UDP N-acetylglucosamine, a substrate for the O-glycosylation of proteins (10,34,35,53,73). Intracellular levels of O-linked glycosylated proteins correlate with GFAT activity, and blockade of GFAT activity or inhibition of GFAT expression with antisense oligonucleotides lowers the intracellular levels of O-GlcNAc–modified proteins (10,73). The posttranslational modification of serine residues in transcription factors by O-glycosylation can affect the activity of the transcription factors. For instance, O-glycosylation of Sp1 stabilizes the protein and prevents proteasomal degradation (74) and may also play a role in the stability of the tumor suppressor p53 in relation to its ability to regulate transcription (75). In light of this, our third major observation was that p65/RelA is O-glycosylated in untransfected MCs that are exposed to high glucose or glucosamine (Fig. 4).

Our finding that activation of an NF-κB enhancer was

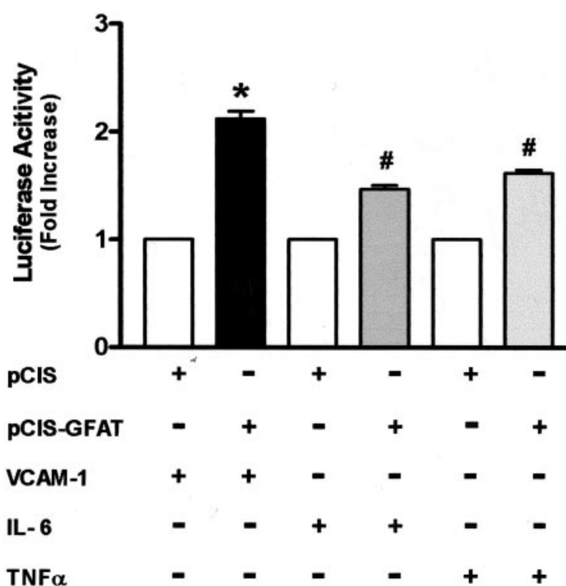

FIG. 5. GFAT overexpression activates promoters for VCAM-1, TNF-α, and IL-6 in MCs. MCs were transiently cotransfected with pCIS-GFAT or pCIS (0.2 μg/well), pCMVβgal (0.05 μg/well), and the indicated promoter-luciferase reporter construct (0.1 μg/well). Eighteen hours after transfection, medium was changed to 0.5% FBS/DMEM/5.6 mmol/l glucose, and cells were incubated for 48 h. Promoter activity was determined by measuring luciferase activity. Values are the mean ± SD. *P < 0.01, n = 4; #P < 0.02, n = 4.

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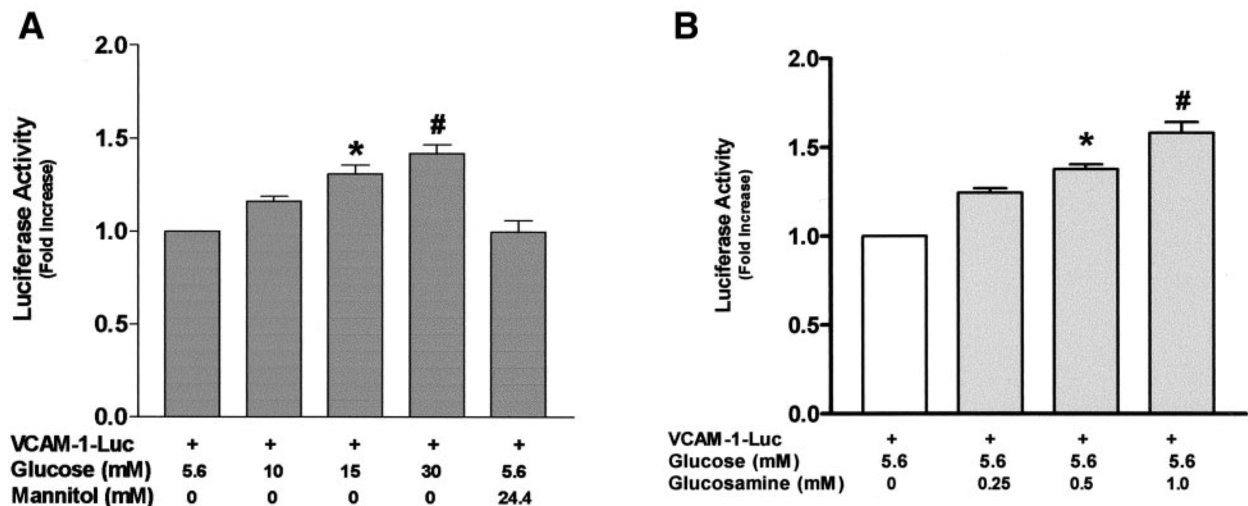

FIG. 6. Glucose and glucosamine increase VCAM-1 promoter activity. MCs were cotransfected with VCAM-1 promoter luciferase and pCMVβgal. Eighteen hours after transfection, medium was changed to 0.5% FBS/DMEM containing 5.6–30 mmol/l glucose (*A*) or 5.6 mmol/l glucose (*B*) with and without glucosamine (0.25–1.0 mmol/l). Cells were incubated for 48 h. Luciferase activity was determined as above. All experiments were done in triplicate, and values represent the mean ± SD. *$P < 0.05$ versus control, $n = 3$; #$P < 0.02$ versus control, $n = 3$.

dependent on flux through the hexosamine pathway led us to hypothesize that the increased flux through the hexosamine pathway might also activate NF-κB–dependent inflammatory genes. To test this hypothesis, we transiently co-transfected MCs with pCIS or pCIS-GFAT and promoter-luciferase reporter constructs for VCAM-1, TNF-α, and IL-6, respectively. Our fourth major observation was that overexpression of GFAT in MCs activated promoter constructs for VCAM-1, TNF-α, and IL-6 (Fig. 5). To relate these effects of GFAT overexpression to NF-κB–dependent promoter activation, we further characterized the effect of glucose and glucosamine on VCAM-1 promoter activity. We observed that both glucose and glucosamine dose-dependently increased the activity of the VCAM-1 promoter (Fig. 6*A* and *B*), whereas mannitol was without effect (Fig. 6*A*). We further found that activation of the VCAM-1 promoter by overexpression of GFAT could be inhibited by azaserine and DON (Fig. 7), strengthening a role for flux through the hexosamine pathway in activation of VCAM-1 promoter. BADGP and PDTC prevented activation of VCAM-1 promoter in GFAT-overexpressing MCs

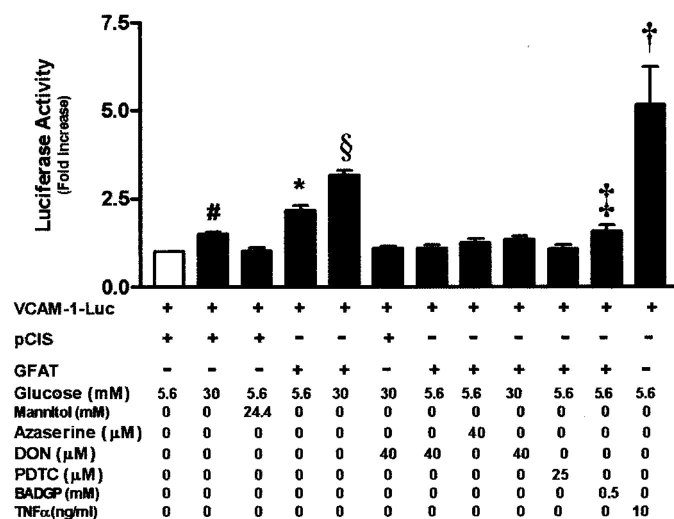

FIG. 7. VCAM-1 promoter activation is dependent on flux through the hexosamine pathway. MCs were cotransfected with VCAM-1 promoter-luciferase, pCIS or pCIS-GFAT, and pCMVβgal and subsequently maintained in 0.5% FBS/DMEM/5.6 or 30 mmol/l glucose for 48 h with and without azaserine (40 μmol/l), DON (40 μmol/l), PDTC (25 μmol/l), or BADGP (0.5 mmol/l). TNF-α (10 ng/ml) served as a positive control. Normalized luciferase activity was determined as before. All experiments were done in triplicate, and values represent the mean ± SD. *$P < 0.02$ versus control; #$P < 0.05$ versus control; §$P < 0.05$ versus GFAT in 5.6 mmol/l glucose; †$P < 0.001$ versus control; ‡$P < 0.05$ versus GFAT in 5.6 mmol/l glucose; $n = 4$ in all cases.

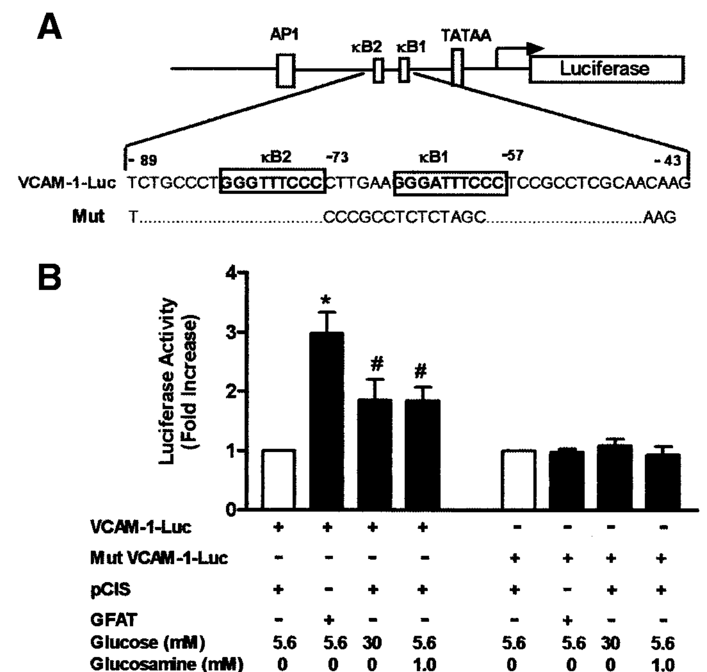

FIG. 8. Mutagenesis of the VCAM-1 promoter attenuates its response to high glucose, glucosamine, and GFAT overexpression. MCs were cotransfected with pCIS or pCIS-GFAT and pCMVβgal and wild-type or mutated (mut) VCAM-1 promoter-luciferase. *A*: Wild-type and mutated (mut) VCAM-1 promoter depicting the sequence of the mutated region that contains two NF-κB binding sites (κB1 and κB2) as outlined in the text. The promoter region between positions −43 and −88 was replaced with the sequence CCCGCCTCTCTAGC as shown. *B*: Results for four separate experiments that were performed in triplicate. *$P < 0.02$ versus control; #$P < 0.05$ versus control; $n = 4$.

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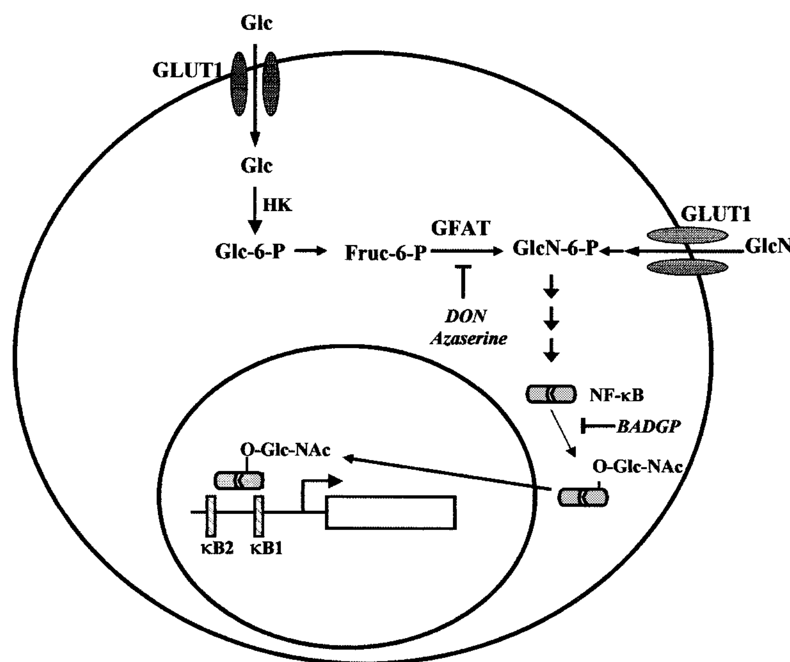

FIG. 9. Summary of the role of the hexosamine pathway in NF-κB–dependent promoter activation in MC. Glucose (Glc) enters the MC via a specific facilitative glucose transporter (GLUT 1) and is converted to fructose-6-phosphate (Fruc-6-P). Under normal conditions, most of the glucose that enters the cells is used for energy production and glycogen synthesis as necessary, and only 1–3% of glucose that enters cells is shunted through the hexosamine pathway. Fruc-6-P is converted to glucosamine-6-phosphate (GlcN-6-P) by the rate-limiting enzyme GFAT. Glucosamine (GlcN) may also enter MC directly, likely via GLUT1, and is phosphorylated by hexokinase (HK). Subsequently, GlcN-6-P is converted to UDP N-acetylglucosamine (Glc-NAc), which serves as a substrate for the O-glycosylation of intracellular proteins. O-glycosylation of transcription factors has been implicated in the regulation of gene transcription. DON and azaserine are inhibitors of GFAT enzymatic activity; BADGP inhibits O-glycosylation.

(Fig. 7), supporting a role for O-glycosylation and NF-κB activation, respectively, in VCAM-1 induction by increasing hexosamine flux.

Recent studies have demonstrated increased inflammatory cytokine expression, including VCAM-1, in the glomeruli of STZ-induced diabetic mice and rats (11,12). On the basis of these findings, it has been suggested that hyperglycemia-mediated increased expression of cytokines such as VCAM-1 may promote infiltration of inflammatory cells in diabetes and that inflammation may be an important component of the response of various tissues to chronic hyperglycemia (12). Our observations suggest that glucose flux through the hexosamine pathway may also be a determinant of VCAM-1 expression in MCs.

In summary, our findings support the general hypothesis that metabolic pathways linked to the development of insulin resistance may also influence glucose-mediated inflammation in vascular smooth muscle–like cells. More specific, our findings suggest that the rate-limiting enzyme in the hexosamine pathway, GFAT, sensitizes MCs to the effects of high glucose by regulating flux through the hexosamine pathway, and through this action, the hexosamine pathway may contribute to injury and inflammation by influencing the expression of NF-κB–dependent genes (Fig. 9).

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