O-linked β-N-acetylglucosamine supports p38 MAPK activation by high glucose in glomerular mesangial cells

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Submitted 4 March 2011; accepted in final form 27 June 2011

Goldberg H, Whiteside C, Fantus IG. O-linked β-N-acetylglucosamine supports p38 MAPK activation by high glucose in glomerular mesangial cells. Am J Physiol Endocrinol Metab 301: E713–E726, 2011. —Hyperglycemia augments flux through the hexosamine biosynthetic pathway and subsequent O-linkage of single β-N-acetyl-D-glucosamine moieties to serine and threonine residues on cytoplasmic and nuclear proteins (O-GlcNAcylation). Perturbations in this posttranslational modification have been proposed to promote glomerular matrix accumulation in diabetic nephropathy, but clear evidence and mechanism are lacking. We tested the hypothesis that O-GlcNAcylation enhances profibrotic signaling in rat mesangial cells. An adenovirus expressing shRNA directed against O-GlcNAc transferase (OGT) markedly reduced basal and high-glucose-stimulated O-GlcNAcylation. Interestingly, O-GlcNAc depletion prevented high-glucose-induced p38 mitogen-activated protein kinase (MAPK) and c-Jun NH2-terminal kinase phosphorylation. Downstream of p38, O-GlcNAc controlled the expression of plasminogen activator inhibitor-1, fibronectin, and transforming growth factor-β, important factors in matrix accumulation in diabetic nephropathy. Treating mesangial cells with thiamet-G, a highly selective inhibitor of O-GlcNAc-specific hexosaminidase (O-GlcNAcase), increased O-GlcNAcylation and p38 phosphorylation. The high-glucose-stimulated kinase activity of apoptosis signal-regulating kinase 1 (ASK1), an upstream MAPK kinase for p38 that is negatively regulated by Akt, was inhibited by OGT shRNA. Akt Thr308 and Ser473 phosphorylation were enhanced following OGT shRNA expression in high-glucose-exposed mesangial cells, but high-glucose-induced p38 phosphorylation was not attenuated by OGT shRNA in cells pretreated with the phosphatidylinositol 3-kinase inhibitor LY-294002. OGT shRNA also reduced high-glucose-stimulated reactive oxygen species (ROS) formation. In contrast, diminished O-GlcNAcylation caused elevated ERK phosphorylation and PKCδ membrane translocation. Thus, O-GlcNAcylation is coupled to profibrotic p38 MAPK signaling by high glucose in part through Akt and possibly through ROS.

mitogen-activated protein kinase; diabetic nephropathy; hexosamine biosynthesis pathway; reactive oxygen species

PROGRESSIVE DIABETIC NEPHROPATHY is characterized by pathological changes in multiple cellular compartments, including mesangial matrix expansion, culminating in glomerulosclerosis, podocyte injury and apoptosis, and tubulo-interstitial fibrosis (4, 18). A key determinant of these abnormalities is prolonged exposure to high glucose concentrations (high glucose) (14). The growing worldwide prevalence of type 2 diabetes and diabetic nephropathy and the lack of definitive treatment for diabetic nephropathy have spurred efforts to better understand the biochemical consequences of high glucose in renal cells (4, 35). A myriad of mediators of the adverse effects of high glucose in diabetic nephropathy has been identified, using cell-based and rodent models of diabetic nephropathy. These include reactive oxygen species (ROS), protein kinase C (PKC), mitogen-activated protein kinases (MAPKs) such as p38 and extracellular signal-regulated kinase (ERK), transforming growth factor-β (TGFβ), the renin-angiotensin system, elevated glomerular capillary pressure, inflammation, the small GTP-binding protein RhoA, methylglyoxal, advanced “glycation” end products (AGEs)/AGE receptors, the polyol pathway, and others (4, 22, 32, 35, 56, 82).

p38 is intimately linked to the development of diabetic nephropathy. Two MAPK kinases (MAP2Ks), MKK3 and MKK6, phosphorylate dual residues in the p38 Thr-Gly-Tyr activation loop sequence (10, 11). In turn, these MAP2Ks are phosphorylated by a range of different MAP2K kinases (MAP3Ks), including apoptosis signal-regulating kinase 1 (ASK1), TGFβ-activated kinase 1 (TAK1), MAPK/ERK kinase kinase (MEKK) 3 and 4, and others (10, 11). p38 activity is upregulated in cultured mesangial cells exposed to high glucose and in the mesangial cells and podocytes from rodents and humans with diabetic nephropathy (1, 21, 33, 45, 60, 63, 75, 80). Although the mechanisms are incompletely characterized, ROS, thioredoxin-interacting protein (TxNIP), and PKCδ have been implicated in p38 activation by high glucose (21, 60, 80). Corresponding to its general role in inflammation, fibrosis, and oxidative stress (10, 11, 33, 45, 60, 74), inhibition of p38 has been implicated in p38 activation by high glucose (21, 60, 80).

Another biochemical effect of high glucose is increased flux through the hexosamine biosynthetic pathway (HBP) (41, 52, 70). In the initial HBP reaction, the glycolytic intermediate fructose 6-phosphate is converted to glucose 6-phosphate by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Ultimately, the HBP yields uridine diphospho β-N-acetylglucosamine (UDP-GlcNAc), which is incorporated into glycolipids and complex N- or O-linked glycans on transmembrane and secreted proteins. Of interest, UDP-GlcNAc is also dynamically added to as a single monosaccharide to serine and threonine residues on intracellular proteins in a reversible, cell-wide process referred to as O-GlcNAcylation, which resembles phosphorylation (41, 52, 70). O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT), encoded by a highly conserved single gene,
OGT, is responsible for attaching single O-GlcNAc moieties to proteins, and another conserved enzyme, β-d-N-acetylglucosaminidase (O-GlcNACase), uniquely cleaves this sugar from proteins (41, 52, 70). O-GlcNAcylation controls protein function by interfering with protein-protein interactions, decreasing phosphorylation, enhancing protein stability, and altering protein function and localization (41, 52, 70). These features allow O-GlcNAc to participate in a wide variety of processes, including cardiac ischemia, cancer, development, vascular reactivity, Alzheimer’s disease, transcription, protein phosphorylation, hepatic glucose production, and possibly insulin resistance (6, 13, 20, 41, 46, 52, 68, 70, 87, 89).

O-GlcNAc may contribute to the detrimental effects of high glucose in mesangial cells, but investigations into O-GlcNAcylation have been hampered by the lack of a suitable animal model or specific OGT inhibitors and difficulties encountered in characterizing O-GlcNAc-modified proteins (23, 41, 52, 70). OGT is able to respond to a wide range of UDP-GlcNAc concentrations, enabling it to increase O-GlcNAcylation in vitro under high glucose conditions and in the kidney, aorta, heart, and atherosclerotic plaques from rodents and humans with diabetes (12, 16, 17, 19, 26, 41, 52, 70, 79, 84). As well, GFAT expression is elevated in kidneys from patients with diabetes (51). Some studies have suggested that the HBP or O-GlcNAc may upregulate profibrogenic gene expression. Glucosamine, which stimulates the HBP by bypassing GFAT, and GFAT overexpression increased mesangial cell TGFβ, plasminogen activator inhibitor-1 (PAI-1), and fibronectin expression, whereas asaraine or 6-diaz-5-oxonorleucine (DON; chemical GFAT inhibitors), antisense GFAT oligonucleotides, or OGT siRNA reversed the effects of high glucose on these genes (5, 24, 39, 69).

In particular, there is evidence linking the HBP to MAPK signaling, a critical mediator of high glucose-induced extracellular matrix (ECM) elaboration by mesangial cells (4, 32, 33, 35, 45, 60, 66). Although O-GlcNAcylation can interfere with protein phosphorylation via steric hindrance, it is also capable of increasing phosphorylation, presumably through indirect mechanisms (41, 77). This was illustrated in an experiment in which O-GlcNAcylation was increased by inhibiting O-GlcNAcase in fibroblasts; phosphorylation was diminished in 39% of proteins, whereas it was greater in 21% of proteins (77). Glucosamine or PUGNAc activated p38 MAPK or c-Jun NH2-terminal kinase (JNK) in cultured cells, and asaraine blocked JNK stimulation by high glucose (17, 38, 47). In an ex vivo model of cardiac ischemia/reperfusion, glucosamine increased p38 phosphorylation after reperfusion, when O-GlcNAc levels were the highest (20). Adenovirus-mediated overexpression of GFAT stimulated p38 phosphorylation in mesangial cells (5). However, interpretation of the above results is limited by the considerable off-target effects associated with these reagents. For instance, glucosamine and GFAT overexpression provoke the unfolded protein response (62, 79), which is attributable to UDP-GlcNac interfening with N-linked glycosylation but not to O-GlcNAcylation (62, 79). Glucosamine can deplete NADPH, which is used to reduce oxidized glutathione, by generating excessive amounts of glucosamine 6-phosphate, an inhibitor of glucose-6-phosphate dehydrogenase (81). GFAT overexpression would theoretically have the same effect. Both glucosamine and GFAT overexpression increase the supply of UDP-GlcNac, a precursor for O-GlcNAcylation but also for N- and O-glycosylation of transmembrane proteins and glycolipids (41, 52, 70). DON and azaserine are general inhibitors of glutamine-utilizing enzymes, and asaraine has been demonstrated to exert O-GlcNAc-independent antioxidant effects (58). PUGNAc inhibits lysosomal β-hexosaminidases in addition to O-GlcNAcase, resulting in cellular ganglioside accumulation (48). As a result, PUGNAc, but not more specific O-GlcNAcase inhibitors, blocks insulin-stimulated Akt phosphorylation in adipocytes and in rodents (48, 49).

Here, we employed adenovirus-expressed OGT short-hairpin RNA (shRNA) to effectively reduce basal and high glucose-stimulated O-GlcNAcylation in mesangial cells and determine the impact of O-GlcNAcylation on signal transduction stimulated by high glucose. We found that O-GlcNAcylation promotes activation of profibrogenic p38 MAPK in response to high glucose by restraining the inhibitory actions of Akt. We also detected negative regulation of ERK and PKC isoform membrane translocation by basal O-GlcNAcylation. These results provide new insights into the specific function of O-GlcNAcylation in mesangial cells.

METHODS

Cell culture. Rat mesangial cells, passages 8–12, were characterized and as described previously (15). They were propagated in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Fisher Scientific, Ottawa, ON, Canada) and 1% penicillin-streptomycin (Invitrogen, Burlington, ON, Canada). For high-glucose experiments, the cells were grown in minimal essential medium (MEM) with 4% FBS to ~70% confluence for 2 days, washed with phosphate-buffered saline (PBS), and then washed in MEM with 0.3% FBS containing either 5.6 (normal glucose) or 25 mM glucose (high glucose) for an additional 48 h. Thiamet-G (Cayman Chemical, Ann Arbor, MI), dissolved in ethanol, was added to the mesangial cells for 24 h at final concentrations of 12.5 or 25 nM. SB-203580 (EMD Biosciences, San Diego, CA), dissolved in DMSO, was added to the mesangial cells for 48 h at a final concentration of 5 μM. LY-294002 (EMD Biosciences) was dissolved in ethanol and added to the cells for 5 h at 20 μM. The control cells were treated with an equivalent amount of ethanol or DMSO.

Adenovirus shRNA. Replication-incompetent adenoviruses encoding shRNA directed against rat OGT or a negative control sequence directed against bacterial β-galactosidase were generated with a BLOCK-iT Adenoviral RNAi Expression System kit (Invitrogen). The 19-bp target RNAi sequence specific for rat OGT (Genbank accession no. NM_017107, cds 121–3,231) was 5'-GCATCGATCTCAAAGCATT-3’ (nt 2,303–2,321). This was selected on the basis of preliminary experiments involving transient transfections with plasmids containing six different sequences into fibroblasts and a Basic Local Alignment Search Tool (BLAST) search. The control target sequence 5’-CTACAAATACTAGGATT-3’ was derived from the E. coli β-galactosidase (lacZ) gene (Genbank accession no. CP001509, nt 335,165–335,185). A BLAST search was performed to ensure lack of homology with other rat sequences. Oligonucleotides containing these sequences for OGT (5’-CAGCCGATCTC TAAAAGCATTGGTCTCAAGCATTCTTCTGGAAAATGTCTTGATCTG-3’ and 5’-AAAAGACATGTAGTCTCAAGCATTCTTCTGGAAAATGTCTTGATCTG-3’) and for lacZ (5’-CAGCCGATCTC TAAAAGCATTGGTCTCAAGCATTCTTCTGGAAAATGTCTTGATCTG-3’) were annealed, introduced into pENTR/U6 down-stream of the U6 RNA polymerase III promoter, and verified by sequencing. The vectors were then recombined in vitro into pAdBlow-iT-DEST using LR Clonase II according to the manufactur-
er’s instructions. The products pAdOGT and pAdLac were linearized with PacI and transected into 293A cells using lipofectamine 2000 (Invitrogen). The crude viral lysate derived from this transfection was amplified in 293A cells and purified using a Vivapure Adenopack 100 kit (Sartorius Mechatronics, Mississauga, ON, Canada), which is based on an adsorption to a membrane. Viral titer was estimated with an Adeno-X Rapid Titer kit (Clontech, Mountain View, CA), which detects virus hexon protein by immunohistochemistry. Quantitative real-time PCR using the oligonucleotides 5'-CACTCATATTCTCATGCCCATTATT-3' and 5'-AGAACACCATATTACCGCGTCT-3', SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA), and an Applied Biosystems 7900 HT Sequence Detection System was used to more precisely quantify adenoviral DNA and equalize the OGT shRNA and lacZ shRNA viral genomes prior to mesangial cell infection (76). The mesangial cells were grown in MEM-4% FBS for 24 h in 10-cm dishes until ~70% confluence. Adenoviruses were diluted in serum-free MEM containing 2 μg/ml poly-l-lysine and incubated for 1 h to increase transduction efficiency (2). The cells were washed with PBS and transduced with a 1:1 ratio of the adenovirus mix, 3.4 × 10^6 pfu/dish, with serum-free MEM at 37°C. After 3 h the cells were washed again with PBS, and the medium was replaced with MEM-4% FBS. The mesangial cells were then treated with or without high glucose for 48 h and harvested for Western blotting.

**Immunoblot analysis.** Mesangial cells were washed three times with ice-cold PBS and scraped in RIPA buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 50 mM β-glycerolphosphate, 30 mM sodium fluoride, 10 mM sodium pyrophosphate, 100 mM okadaic acid, protease inhibitors (Complete, Roche Applied Science, Laval, QC, Canada), which is boiled in a lysis buffer consisting of 50 mM Tris, pH 7.5, 135 mM NaCl, 25 mM β-glycerolphosphate, 5 mM EGTA, 5 mM EDTA, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 2 mM sodium pyrophosphate, 25 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 0.5 mM DTT, 100 mM okadaic acid, and protease inhibitors (Complete). After centrifugation at 13,000 g for 10 min at 4°C to remove cell supernatant, the supernatant was mixed with 4X Laemmli sample buffer and boiled for 5 min. Lysates containing equivalent amounts of protein (5–10 μg) (Bio-Rad protein assay kit) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Pall, Ville St. Laurent, QC, Canada). The blots were incubated overnight with the indicated primary antibodies, washed, and exposed to horseradish peroxidase (HRP)-conjugated anti-IgG secondary antibodies for 1 h and immunoreactive bands visualized by enhanced chemiluminescence (Lumiglo; KPL, Gaithersburg, MD). The resulting X-ray films were analyzed by scanning and ImageJ software (National Institutes of Health, Bethesda, MD). To normalize to protein loading, blots were stripped in a solution consistent of 62.5 mM Tris, pH 6.8, and 2% SDS at 50°C for 30 min, washed, and then reprobed with antibodies directed against β-actin or various kinases. Polyclonal rabbit anti-ERK, JNK, p38, Akt, ASK1, PKCβ1, and PKCδ, goat anti-ASK1, and monoclonal anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK, phospho-JNK, phospho-p38, phospho-Akt, and phospho-stress-activated protein kinase/ERK kinase (SEK1) antibodies were from Cell Signaling Technology (Danvers, MA). Monoclonal anti-O-GlcNAc (RL-2), rabbit anti-PAI-1, rabbit anti-ASK1, and monoclonal TxNIP antibodies were from Abcam (Cambridge, MA), American Diagnostica (Stamford, CT), Novus Biologicals (Littleton, CO), and MBL International (Woburn, MA), respectively. HRP-conjugated anti-IgG secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

**Mesangial cell viability.** Mesangial cells were seeded in 60-mm dishes, transduced with OGT shRNA or control adenoviruses, and incubated with 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) for 4 h in MEM containing 0.3% FBS. The medium was removed, the formazan crystals resulting from mitochondrial metabolism of MTT were solubilized with 2 ml isopropanol-40 mM HCl, and the absorbance was measured at 570 nm in a spectrophotometer.

**PAI-1-luciferase reporter assay.** A firefly luciferase reporter gene harboring nucleotides –740 to +44 from the human PAI-1 promoter pPAI-Luc was described previously (26). PRL-CMV (Promega, Madison, WI) expresses Renilla luciferase under control of a CMV promoter. Mesangial cells (50% confluent) in six-well plates were transiently transfected with 0.6 μg DNA/well, consisting of pAdOGT or pAdLac, pPAI-Luc, and pRL-CMV in a 75:13:1 ratio, using 1.2 μl of FuGENE-6 (Roche Applied Science) in MEM-4% FBS. The cells were washed with PBS after 4 h, maintained in MEM-4% FBS overnight, and then exposed to 25 mM glucose or 5.6 mM glucose/19.4 mM mannitol for 48 h in MEM-0.3% FBS. Luciferase assays were performed with a Dual Luciferase Reporter Assay kit (Promega) and a GloMax20/20 luminometer (Promega) as described by the manufacturer. PAI-1 promoter firefly luciferase values were normalized to Renilla luciferase activity to control for transfection efficiency.

**ELISA for fibronectin and TGFβ.** Mesangial cells were cultured in 60-mm dishes, transduced with adenoviruses, and then incubated for 48 h in MEM containing 10 μg/ml bovine insulin (Sigma). Conditioned medium was collected and centrifuged at 13,000 g to remove cellular debris. Latent TGFβ was activated by adding 20 μl of 1 N HCl to 100 μl of cell culture supernatant for 10 min and neutralizing with 13 μl of 1.2 N NaOH-0.5 M HEPES. Sandwich enzyme-linked immunosorbent assay (ELISA) kits were used to detect total TGFβ (R & D Systems, Minneapolis, MN) and fibronectin (Kamiya Biomedical, Seattle, WA) according to the manufacturers’ instructions. The results were calculated based on a standard curve constructed with recombinant proteins and normalized to cellular protein content.

**ASK1 immune complex kinase assay.** Two 10-cm dishes of mesangial cells were washed three times with ice-cold PBS and scraped into a lysis buffer consisting of 50 mM Tris, pH 7.5, 135 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10% glycerol, 1% Igepal, 0.5% sodium deoxycholate, 10 mM sodium fluoride, 2 mM sodium pyrophosphate, 25 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 0.5 mM DTT, 100 mM okadaic acid, and protease inhibitors (Complete). After incubation for 30 min on ice and centrifugation at 13,000 g for 10 min at 4°C, equal amounts of protein were subjected to immunoprecipitation overnight with 4 μg of goat anti-ASK1 antibody using protein A-agarose. Theagarose beads were washed twice with lysis buffer and twice with kinase buffer consisting of 50 mM HEPES, pH 7.5, 20 mM MgCl2, 1 mM EDTA, 5 mM EGTA, 2 mM sodium fluoride, 25 mM β-glycerolphosphate, 0.5 mM sodium orthovanadate, 0.5 mM DTT, 100 mM okadaic acid, and protease inhibitors (Complete). For the kinase assay, the immunoprecipitates were resuspended in kinase buffer containing 100 μM ATP and 0.7 μg of GST-SEK (Abnova Antibodies, Walnut, CA) and incubated for 60 min at 30°C, similar to the assay described previously for MEKK1 (72). After centrifuging at 10,000 g for 30 s at 4°C, the supernatant and pellet were boiled in sample buffer and immunoblotted for phospho-SEK (Ser261) and ASK1 (rabbit antibody), respectively. Blotting for phospho-SEK (Ser261) avoided the detection of SEK autophosphorylation. The phospho-SEK blots were stripped and reprobed for SEK.

**ROS detection.** Intracellular ROS were detected with the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Invitrogen) (7). Mesangial cells were seeded in 60-mm dishes, transduced with adenovirus, and incubated with 25 or 5.6 mM glucose in MEM-0.3% serum for 48 h. After being washed with PBS, cells were loaded with 6.4 μM CM-H2DCFDA in serum-free MEM (without phenol red) for 30 min at 37°C, washed again, and switched back to serum-free MEM (without phenol red). Green fluorescent images were acquired with an Olympus 1X71 inverted fluorescence microscope (Tokyo, Japan) equipped with a Q Imaging Retiga Exi charge-coupled device camera using the ×40 objective, processed, and colorized with Q Capture Pro software (Q Imaging, Surrey, BC, Canada). They were quantified with ImageJ. Exposure to the light source was minimized to prevent photobleaching.

**PKC membrane content.** Total cell membranes were isolated essentially as we described previously (15). In brief, mesangial cells
were washed three times with ice-cold PBS, scraped into ice-cold buffer A containing 50 mM Tris, pH 7.5, 10 mM EGTA, 2 mM EDTA, 1 mM DTT, and protease inhibitors (Complete), and homogenized. Cell lysates were kept on ice for 30 min and then centrifuged at 100,000 g at 4°C for 1 h. The pellets were washed with and solubilized in buffer A with 1% (vol/vol) Triton X-100. The extracts were centrifuged again at 100,000 g for 30 min at 4°C, and the supernatant was saved as the total membrane fraction.

Statistical analysis. Results are expressed as means ± SD. Statistical analysis was performed by one-way analysis of variance followed by the Newman-Keuls post hoc test for multiple comparisons using Prism 4 (Graph Pad Software, La Jolla, CA). The Student t-test was used for single comparisons. A probability of value of P < 0.05 was considered to be statistically significant.

RESULTS

To determine whether O-GlcNAc controls MAPK activation in cultured mesangial cells, we aimed to reduce but not eliminate OGT expression with siRNA, given that targeted deletion of the Ogt gene is lethal (52, 70). In a previous study, transfection of siRNA oligonucleotides into serum-deprived mesangial cells was associated with toxicity from the transfection reagents (26), and here we were unable to select stable mesangial cell lines expressing shRNA against OGT (not shown). Furthermore, a small molecule OGT inhibitor (67) was toxic to serum-deprived mesangial cells. Accordingly, we developed an adenovirus encoding OGT shRNA and used it to deplete mesangial cell OGT.

Transduction of rat mesangial cells with OGT shRNA adenovirus for 3 days reduced OGT protein expression to ~20% of control in cells incubated in 5.6 or 25 mM glucose (high glucose; Fig. 1A). This was accompanied by a dramatic reduction in basal and high-glucose-stimulated O-GlcNAcylation in immunoblots of whole cell extracts probed with the O-GlcNAc-specific monoclonal antibody RL-2 (Fig. 1B). Not all protein bands were affected equally. One ~60-kDa O-GlcNAcylated protein was much less sensitive to OGT shRNA, possibly because of slower turnover of its O-GlcNAc. We quantified an ~75-kDa O-GlcNAcylated protein (indicated by the arrow in Fig. 1B) that was particularly sensitive to high glucose. The intensity of this band was increased by ~40% after incubation of the mesangial cells in 25 mM glucose and markedly reduced to ~17 and ~11% of control in OGT shRNA-treated cells maintained in 5.6 or 25 mM glucose, respectively. Other bands on the O-GlcNAc immunoblot in adenovirus-transduced cells were only slightly increased or not affected by high glucose. Mesangial cell viability, determined by MTT assay, was not affected by OGT shRNA adenovirus (Fig. 1C).

Expression of the profibrotic serine protease inhibitor PAI-1 is upregulated by high glucose in mesangial cells and in diabetic nephropathy, where PAI-1 increases ECM accumulation by inhibiting ECM degradation (26, 31, 44). Previously, we reported that OGT siRNA oligonucleotides blocked PAI-1 induction by high glucose in cells incubated in medium with a baseline glucose concentration of 1 mM (26). We confirmed this result using OGT shRNA adenovirus and 5.6 mM basal glucose levels. As illustrated in Fig. 1D, high glucose upregulated PAI-1 protein levels 1.8-fold, and adenovirus-mediated OGT shRNA prevented this effect. Mannitol, a control for osmotic effects, did not augment PAI-1 levels (Fig. 2D). Cotransfection of a plasmid expressing OGT shRNA blocked activation of a transiently transfected PAI-1 promoter luciferase reporter gene by high glucose (Fig. 1G). In addition, we studied the effect of diminished O-GlcNAc on other proteins that contribute to mesangial matrix accumulation. Fibronectin and TGFβ protein levels, as determined by ELISA in mesangial cell conditioned medium, were 2.9- and twofold higher, respectively, in cells exposed to 25 mM glucose compared with cells maintained in 5.6 mM glucose (Fig. 1, E and F), and these effects were interrupted by OGT shRNA.

Of the three classical MAPK families, ERK generally promotes cellular growth, whereas p38 and JNK are involved in cellular reactions to stress, inflammation, and apoptosis (10, 11, 61). ERK and p38 activation by high glucose in mesangial cells are well established (4, 32, 33, 35, 60, 75, 80), whereas JNK stimulation by high glucose has been reported previously in endothelial cells (47). To evaluate the effect of reduced O-GlcNAcylation on MAPKs, we transduced mesangial cells with OGT shRNA adenovirus and assessed the phosphorylation status of all three MAPKs by immunoblotting. Mesangial cells incubated in medium supplemented with 25 mM glucose exhibited increased p38, JNK, and ERK phosphorylation 1.8-, 1.9-, and 1.8-fold, respectively, compared with cells incubated in 5.6 mM glucose (Fig. 2, A–C). Upregulation of p38 was not due to osmotic effects, since mannitol did not increase p38 phosphorylation (Fig. 2D). However, mannitol caused a modest 1.3-fold increase in ERK phosphorylation, suggesting that it is partially affected by glucose-induced changes in osmolarity (Fig. 2D). Interestingly, activation of the proinflammatory MAPKs p38 and JNK by high glucose was prevented by OGT shRNA (Fig. 2, A and B). Conversely, ERK phosphorylation was higher in OGT shRNA cells than in control shRNA cells in both 5.6 and 25 mM glucose (Fig. 2C).

Fig. 1. O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT) short-hairpin RNA (shRNA) markedly lowers O-GlcNAcylation and prevents plasminogen activator inhibitor-1 (PAI-1), fibronectin, and transforming growth factor-β (TGFβ) induction by high glucose. A–F: mesangial cells were transduced with an adenovirus expressing OGT shRNA (OGT) or control shRNA against bacterial β-galactosidase (control) and then incubated with 25 (high glucose) or 5.6 mM glucose (normal glucose). Whole cell extracts were immunoblotted for OGT (A, O-GlcNAc (B), or PAI-1 (D)). Mesangial cell viability was assessed with 3-(4,5-dimethyl-2-thiazolyl)-2,5-dipheny1-2H-tetrazolium bromide (MTT) (C). Fibronectin (E) and total TGFβ (F) protein levels were determined by ELISAs in conditioned medium. G: mesangial cells were transiently cotransfected with a PAI luciferase reporter and plasmids expressing Renilla luciferase and OGT shRNA (OGT) or control shRNA (control). The cells were incubated for 48 h with 25 mM glucose or 19.4 mM mannitol-5.6 mM glucose (mannitol), and firefly luciferase activity was measured and normalized to Renilla luciferase. The graphs show the amounts of specific protein (A, B, D, E, and F), cell viability (C), or luciferase activity (G) after normalization for protein loading or transfection efficiency, respectively. The results are expressed as fold change relative to 5.6 mM glucose-control shRNA (A–D), protein in culture medium relative to cellular protein (E) and total TGFβ (F), or 5.6 mM glucose and mannitol-control shRNA (G). For B, the ~75-kDa band indicated by the arrow was quantified. Values are means ± SD; n = 3 for A–C, n = 4 for D–G. *P < 0.05, 25 mM glucose-control shRNA vs. 5.6 mM glucose-control shRNA, 5.6 mM glucose-OGT shRNA vs. 5.6 mM glucose-control shRNA, or high glucose-OGT shRNA vs. high glucose-control shRNA.
Fig. 2. OGT downregulation blocks activation of p38 MAPK and JNK, but not ERK, by high glucose. A–C: mesangial cells were transduced with OGT shRNA (OGT) or control shRNA (control) adenoviruses and then incubated with 25 or 5.6 mM glucose for 48 h. Cell extracts were immunoblotted for phospho-p38 MAPK (A), phospho-JNK (B), or phospho-ERK (C). D: mesangial cells were incubated for 48 h in medium containing 5.6 mM glucose (control) or 5.6 mM glucose-19.4 mM mannitol (mannitol) and cell extracts immunoblotted for phospho-p38, phospho-ERK, or PAI-1. E: mesangial cells were maintained in medium containing 5.6 or 25 mM glucose in the presence of 5 μM SB-203580 or vehicle for 48 h. Cell extracts were blotted for PAI-1. The blots were reprobed for p38, JNK, ERK, or β-actin as loading controls. The graphs show the amounts of specific protein after normalization for protein loading as fold change relative to 5.6 mM glucose-control shRNA (A–C), 5.6 mM glucose (D), or 5.6 mM glucose-vehicle (E). Values are means ± SD; n = 3 for B and E, n = 4 for A and D, and n = 5 for C. *P < 0.05, 25 mM glucose-control shRNA vs. 5.6 mM glucose-control shRNA, 5.6 mM glucose-OGT shRNA vs. 5.6 mM glucose-vehicle or 25 mM glucose-vehicle, 25 mM glucose-OGT shRNA vs. 25 mM glucose-control shRNA, mannitol vs. control, 25 mM glucose-vehicle vs. 5.6 mM glucose-vehicle, or 25 mM glucose-SB-203580 vs. 25 mM glucose-vehicle.
O-GlcNAC-sensitive p38 was linked to PAI-1; thus, pretreatment of mesangial cells with the p38 inhibitor SB-203580 blocked the increase in expression of PAI-1 in response to high glucose (Fig. 2E). To test the effect of increasing O-GlcNAcylation on p38 phosphorylation, O-GlcNACase was inhibited with a potent and highly selective O-GlcNACase inhibitor, thiamet-G, which unlike PUGNAc does not inhibit hexosaminidase-β (48). Following mesangial cell incubation with either 12.5 or 25 nM thiamet-G for 48 h, Western blot analysis (Fig. 3A) revealed that both 12.5 and 25 nM thiamet-G robustly increased whole cell O-GlcNAcylation and significantly enhanced p38 phosphorylation in normal glucose (5.6 mM; Fig. 3B). In view of the prosurvival/anti-inflammatory effects of pharmacologically elevating O-GlcNAcylation post-cardiac ischemia (Refs. 20, 41, 42, 52, 78, and 89; also see below), we reasoned that high levels of O-GlcNAC might suppress the activation of proinflammatory p38 by high glucose. However, as shown in Fig. 3C, thiamet-G did not inhibit high glucose-induced p38 phosphorylation (Fig. 3C).

To probe the mechanism of p38 activation, we examined some of the signaling components that regulate p38. One of the pivotal MAP3Ks for p38 is ASK1, which responds to oxidative stress and cytokines and plays a vital role in inflammation (30). In fact, depletion of ASK1 attenuates prolonged activation of p38 and JNK by the above stimuli in mice and cultured cells (30). As well, a previous study demonstrated that high glucose increased the amount and activity of ASK1 in endothelial cells, resulting in elevated PAI-1 expression (85). These data prompted us to investigate whether ASK-1 is influenced by O-GlcNAcylation. The lack of antibodies reactive with phosphorylated rat ASK1 and its relatively low expression level precluded evaluation of ASK1 phosphorylation directly by immunoblotting. Instead, we conducted in vitro ASK1 kinase assays by incubating immunoprecipitated ASK1 with recombinant MKK4, an endogenous ASK1 substrate. After incubation of mesangial cells in high glucose, ASK1 kinase activity was stimulated 3.3-fold (Fig. 4A), and this effect was abrogated when OGT was silenced by shRNA.

A number of positively and negatively acting proteins converge to regulate ASK1 via phosphorylation and protein-protein interactions (30). Among these proteins is thioredoxin (Trx), a 12-kDa thiol oxidoreductase that binds noncovalently to the NH2 terminus of ASK1 and blocks ASK1 autophosphorylation (30). Trx is directly inhibited by TxNIP (also called Vitamin D3 upregulated protein-1, and Trx-binding protein-2), which forms disulfide bonds with the Trx catalytic center (65). Upon induction by high glucose or oxidative stress, TxNIP activates ASK1 by binding to Trx2, causing it to dissociate from ASK1 (65). Relevant to the current study, TxNIP is required for p38 activation by high glucose in mesangial cells (60), and experiments using glutamine and azaserine suggest that the HBP, and possibly O-GlcNAcylation, might regulate TxNIP expression (8). To evaluate the role of O-GlcNAcylation in TxNIP induction by high glucose in mesangial cells, we transduced mesangial cells with OGT or control shRNA adenoviruses and carried out TxNIP immunoblots. Compared with cells incubated in 5.6 mM glucose, mesangial cells exposed to 25 mM glucose were observed to have modestly higher (1.3-fold) TxNIP levels (Fig. 4B). OGT shRNA reduced the levels of TxNIP by 40% in medium containing 5.6 mM glucose but did not decrease fold stimulation by high glucose or the absolute levels of TxNIP in cells incubated in 25 mM glucose (Fig. 4B). These results suggest that OGT shRNA does not suppress high-glucose-induced p38 activation through suppression of TxNIP induction by high glucose.
ROS activate ASK1 and p38 (30, 80). To explore the impact of reducing O-GlcNAcylation on ROS levels, mesangial cells were loaded with the cell-permeable fluoroprobe CM-H2DCFDA and viewed by fluorescence microscopy. CM-H2DCFDA is deesterified intracellularly and becomes fluorescent when oxidized to 2’,7’-dichlorofluorescin (DCF) by ROS such as hydrogen peroxide (7). Using this assay, we found that when oxidized to DCF by ROS, H2DCFDA was deesterified intracellularly and becomes fluorescent (Fig. 4C). Following exposure to high glucose, DCF-sensitive ROS levels were increased 5.1-fold at 48 h relative to normal glucose, as we and others have reported previously (Fig. 4C) (8, 28, 60, 82). Notably, the ability of high glucose to increase ROS levels was abolished by adenovirus-mediated OGT shRNA without any reduction of basal ROS levels (Fig. 4C).

Akt (also called protein kinase B) is a phosphatidylinositol-3-kinase (PI3K)-dependent Ser/Thr kinase that enhances glucose uptake and cellular survival (27). One of the antiapoptotic functions of Akt is to inactivate proapoptotic ASK1 by directly phosphorylating it on Ser83 (37, 86). Akt also phosphorylates and inhibits another p38 MAP3K, MEKK3 (29). Hence, an inverse relationship exists between p38 and Akt activation in streptozotocin-induced diabetes in mice and in cultured renal proximal tubule cells exposed to high glucose (59). Full Akt activation requires phosphorylation on Akt Ser473 by mammalian target of rapamycin complex 2 or other kinases and phosphorylation on Akt Thr308 by phosphoinositide-dependent kinase 1 (27). Akt Ser473 phosphorylation was unchanged in high-glucose-treated cells relative to cells incubated in normal glucose (Fig. 5A), implying that increasing O-GlcNAcylation above basal levels does not affect Akt. However, under high glucose conditions the phosphorylation of both Akt Ser473 and Thr308 was significantly higher in OGT shRNA-treated cells than in control shRNA-expressing cells (Fig. 5, A and B). We then examined the consequences of this effect for p38. On the basis of a previously reported strategy (29), mesangial cells were incubated with 20 μM LY-294002 for 5 h to inhibit PI3K and Akt. This dose was found in a preliminary experiment to be the approximate minimal dose required to inhibit Akt phosphorylation in cells transduced with either control or OGT shRNA (not shown). As expected, pretreatment with LY-294002 eliminated Akt phosphorylation and increased p38 phosphorylation (Fig. 5C). Consistent with Akt being an O-GlcNAc effector, OGT shRNA decreased p38 phosphorylation in vehicle-treated cells but not in LY-294002-exposed cells. Taken together, these results indicate that suppression of Akt phosphorylation by basal O-GlcNAcylation is a key mechanism by which O-GlcNAc regulates p38.

We also investigated PKCδ as a possible mediator of O-GlcNAc actions on p38. Hyperglycemia can activate p38 via O-GlcNAcylation on ROS levels was increased 5.1-fold at 48 h relative to normal glucose, as we and others have reported previously (Fig. 4C) (8, 28, 60, 82). Notably, the ability of high glucose to increase ROS levels was abolished by adenovirus-mediated OGT shRNA without any reduction of basal ROS levels (Fig. 4C).

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in PKCδ-deficient mice (21). As well, we found previously that PKCδ was required for PAI-1 promoter activation by high glucose (25). To address whether this PKC isoform is regulated by O-GlcNAcylation, mesangial whole cell extracts and membrane fractions were immunoblotted for PKCδ. Total cellular PKCδ levels were unaltered following expression of OGT shRNA by adenovirus (Fig. 6A). PKCδ membrane levels were not greater in high-glucose-treated mesangial cells than in cells incubated in normal glucose under the present experimental conditions (Fig. 6B). In 5.6 mM glucose, membrane PKCδ abundance was significantly higher in OGT shRNA-transduced compared with control shRNA-transduced cells, whereas there was no difference in high glucose. This result demonstrates that O-GlcNAcylation limits PKCδ membrane translocation in medium containing 5.6 mM glucose but not in high-glucose-containing media.

**DISCUSSION**

O-GlcNAcylation could potentially link metabolism to profibrotic signal transduction in mesangial cells, given the reversible and dynamic nature of this posttranslational modification and its ability to interfere with phosphorylation, but specific evidence is incomplete. We tested this idea with adenovirus-mediated OGT shRNA, which efficiently reduced OGT protein expression (Fig. 1A), and both basal and high glucose stimulated whole cell O-GlcNAcylation (Fig. 1B). Thus, the observed effects of adenovirus OGT shRNA in the present experiments can be attributed to reductions in basal and high-glucose-induced O-GlcNAcylation.

Immunoblots of overall cellular O-GlcNAcylation highlighted the prominent basal O-GlcNAcylation present in mesangial cells. We detected a 1.4-fold increase in the O-GlcNAcylation of an ~75-kDa band in control shRNA-treated cells incubated in high glucose compared with 5.6 mM glucose, but there was only a slight or no change in other bands (Fig. 1B). Although the use of adenoviruses may have raised baseline O-GlcNAcylation, as reported previously (88), this result suggests that the O-GlcNAcylation of many proteins is saturated at basal glucose levels. Consistent with this finding, a previous study of hearts from rats with type 2 diabetes identified increased O-GlcNAcylation in only three of 11 bands on a total cellular O-GlcNAc immunoblot (19). Increased total O-GlcNAc immunohistochemical staining was noted in kidney biopsies from patients with diabetic nephropathy (12), but immunoblots have not been reported.

Fig. 5. Diminished OGT expression increases Akt activation. Mesangial cells were transduced with OGT shRNA (OGT) or control shRNA (control) adenoviruses. A and B: cells were incubated with 25 (high glucose) or 5.6 mM glucose (normal glucose) for 48 h. C: cells were incubated in 25 mM glucose for 48 h and then treated with 20 μM LY-294002 or vehicle for the last 5 h. Cell extracts were immunoblotted for phospho-Akt (Ser473; A), phospho-Akt (Thr308; B), or phospho-p38 and phospho-Akt (Thr308; C). The blots were reprobed for Akt or p38 as loading controls. The graphs show the amount of phospho-Akt or phospho-p38 relative to 5.6 mM glucose-control shRNA in A and B or relative to vehicle-control shRNA in C. Values are means ± SD; n = 3. *P < 0.05, 25 mM glucose-OGT shRNA vs. 25 mM glucose-control shRNA, vehicle-OGT shRNA vs. vehicle-control shRNA, or LY-294002-control shRNA vs. vehicle-control shRNA.
O-GlcNAcylation than high glucose. We demonstrated that expression of the profibrotic proteins PAI-1, fibronectin, and TGFβ, which contribute to mesangial matrix deposition in diabetic nephropathy (33, 35, 66), was dependent on O-GlcNAc (Fig. 1, D–G). PAI-1 was found to be dependent on p38 in the current experiments (Fig. 2E), and fibronectin and TGFβ have previously been shown to be regulated by p38 (33, 60, 66), in keeping with the profibrotic effects of p38 in diabetic nephropathy (33, 45, 66). Other mediators of fibrosis and inflammation, an antecedent of fibrosis, have been reported to be O-GlcNAc dependent. For example, NF-κB activation in response to high glucose or increased glucose uptake induced by p53 deficiency has been shown to require O-GlcNAcylation of either IκB kinase-β or NF-κB p65 (36, 84). RhoA promotes fibrosis in diverse settings (64) and is essential for ECM accumulation in murine diabetic nephropathy models and in mesangial cells in vitro (56). OGT siRNA blocked RhoA activation by endothelin in vascular smooth muscle cells (46). O-GlcNAcylation of the transcriptional repressor Snail1 prevented its degradation and triggered epithelial-mesenchymal transition, a key process in renal tubulointerstitial and other types of fibrosis (55). Collectively, these data argue that O-GlcNAcylation could be a component of a profibrogenic program in diabetic nephropathy.

By contrast, other studies suggest that O-GlcNAc has extensive anti-inflammatory effects. Pretreatment with glucosamine, PUGNAc, and more specific O-GlcNAcase inhibitors, 1,2-dideoxy-2-methyl-α-D-glucopyranosyl-[2,1-d]-Δ-2′-thiazoline derivatives, improved cardiac function and decreased inflammation in cardiac ischemia models (20, 41, 42, 52, 78, 89). In the reverse experiment, inducible cardiac deletion of OGT worsened cardiac dysfunction, apoptosis, and fibrosis postcoronary artery ligation in mice (78). Evidence for anti-inflammatory effects of O-GlcNAcylation has emerged from other settings as well. For example, glucosamine and PUGNAc suppressed inflammation, including the activation of NF-κB and p38, in a number of in vivo models of inflammation (41, 43, 52, 83, 89). Increasing O-GlcNAcylation with uridine and N-acetylgalactosamine reduced JNK phosphorylation in leukemia cells (67). OGT overexpression suppressed lipopolysaccharide (LPS)-induced NF-κB activation, whereas OGT shRNA increased NF-κB activation by LPS in cultured cells (89).

The above data appear to conflict with our finding that basal O-GlcNAcylation supports activation of the proinflammatory, profibrotic kinase p38 in response to high glucose in mesangial cells. However, there are a number of differences between these previously reported experiments and the present study that could account for this apparent discrepancy. Most importantly, it is likely that distinct signaling pathways that may be differentially affected by O-GlcNAc were activated. The experiments that demonstrate the anti-inflammatory effects of O-GlcNAc involved signaling by cytokines, Toll-like receptors, and/or NF-κB, but these factors may not be relevant in the model of high-glucose-induced p38 phosphorylation in cultured mesangial cells. We evaluated the effect of decreasing O-GlcNAcylation, whereas most of the studies on cardiac ischemia have sought to increase O-GlcNAcylation supports activation of the proinflammatory, profibrotic kinase p38 in response to high glucose in mesangial cells.

The present data show that basal O-GlcNAcylation regulates high-glucose-stimulated p38 MAPK, as indicated by our finding that OGT shRNA interrupted activation of p38 by high glucose (Fig. 2A). The absence of an inhibitory effect of OGT shRNA on ERK (Fig. 2C) ruled out nonspecific effects of O-GlcNAc reduction on cell signaling or glucose uptake. Elevating O-GlcNAcylation with thiamet-G increased p38 phosphorylation (Fig. 3B), although it should be noted that thiamet-G was associated with a much larger increase in
of inflammation and O-GlcNAcylation were based on glucosamine or PUGNac, which have significant non-O-GlcNAc-related effects (see above). It is also conceivable that the relatively high levels of O-GlcNAcylation induced by glucosamine or PUGNac compared with high glucose inhibit inflammatory signaling, including p38. However, the experiments performed with thiamet-G (Fig. 3C), which achieved large increases in O-GlcNAcylation, argue against this possibility by showing that high levels of O-GlcNAcylation did not suppress p38 phosphorylation in mesangial cells. Finally, one of the major functions of O-GlcNAcylation is to confer a survival program in response to pathophysiological stress (41, 52), as documented in cultured cells in response to osmotic stress, heat shock, or ischemia, and in vivo after cardiac ischemia/reperfusion or hemorrhagic shock (20, 42, 78, 88, 89). Therefore, the anti-inflammatory effects of O-GlcNAcylation post-cardiac ischemia could be secondary to increased cell survival rather than direct effects of O-GlcNAcylation on inflammation.

Direct O-GlcNAcylation of p38 is unlikely to explain the present results. Although p38 has been shown to coimmunoprecipitate with OGT following glucose deprivation, OGT interacted primarily with previously activated p38 rather than inactive p38 (9). Both p38 and JNK phosphorylation were modified by OGT shRNA in the current experiments, suggesting that the target of O-GlcNAcylation regulates both of these MAPKs. Last, O-GlcNAcylation usually interferes with protein-protein interactions and impedes phosphorylation if the O-GlcNAc-modified residues are close to phosphorylation sites (13, 36, 41, 52, 55, 70).

Mechanistically, we propose that basal O-GlcNAcylation facilitates high-glucose-induced p38 activation by curbing the phosphorylation of negatively acting Akt. This notion is supported by increased Akt Ser473 and Thr308 phosphorylation under high glucose conditions, when OGT was depleted by shRNA (Fig. 5, A and B), and by the inability of OGT shRNA to reduce p38 phosphorylation in cells that were pretreated with the PI3K inhibitor LY-294002 (Fig. 5C). The lack of an inhibitory effect of high glucose on Akt phosphorylation suggests that basal O-GlcNAcylation maximally inhibits mesangial cell Akt. Our results agree with the reported reciprocal relationship between p38 and Akt in proximal tubule cells exposed to high glucose, diabetic nephropathy, and other settings. These effects could occur through Akt-mediated phosphorylation of the p38 MAP3K ASK1 on Ser83. Indeed, OGT shRNA attenuated high-glucose-stimulated ASK1 kinase activity (Fig. 4A) in support of this hypothesis.

Other studies have also revealed that Akt is inhibited by basal O-GlcNAcylation. Chemical inhibition of OGT by a small molecule inhibitor increased Akt Thr308 phosphorylation in leukemia cells (67). Adenovirus-mediated overexpression of O-GlcNAcase in HepG2 cells maintained in 5 mM glucose or in the livers of db/db or euglycemic wild-type mice resulted in increased Akt phosphorylation (13, 71). Knockout of the HBP pathway enzyme glucosamine-6-phosphate acetyltransferase (EMeg32), which substantially decreased O-GlcNAcylation, was associated with elevated Akt phosphorylation in murine embryo fibroblasts (3). The dominant role played by basal O-GlcNAcylation in Akt regulation was further demonstrated by the inability of highly selective O-GlcNAcase inhibitors to suppress insulin-stimulated Akt phosphorylation in adipocytes in vitro or in rodents (48, 49). Although PI3K activity was not affected by O-GlcNAcase expression in HepG2 cells (71), the precise mechanism of the inhibitory effects of basal O-GlcNAcylation on Akt has not yet been defined. Several reports provided evidence for the direct modification of Akt by O-GlcNAc on the basis of precipitation with wheat germ agglutinin, immunoblotting-immunoprecipitated Akt with O-GlcNAc-specific antibodies, and mass spectroscopy (34, 67, 71), but the exact functional role of Akt O-GlcNAcylation remains uncertain.

Apart from inhibiting Akt, basal O-GlcNAcylation may regulate other signaling systems that enhance p38 activation, such as ROS (23, 30, 80). In the present study, high glucose increased mesangial cell ROS, as monitored by DCF, and OGT shRNA antagonized this response (Fig. 4C). O-GlcNAc may influence the expression or function of a variety of proteins involved in high-glucose-induced ROS production. On the other hand, it is equally plausible that much of this effect of OGT shRNA on ROS is secondary to inactivation of p38, which can phosphorylate the NADPH oxidase subunit p47phox (57) and upregulate the expression of the NADPH oxidase Nox4 (54). Reinforcing this view, the p38 inhibitor SB-203580 has been shown to block high-glucose-induced increases in ROS in mesangial cells (60). OGT overexpression diminished and O-GlcNAcase overexpression increased ROS generated by hypoxia/reoxygenation or hydrogen peroxide treatment in neonatal cardiomyocytes due to changes in the expression of catalase (53). As discussed above for p38, the different outcomes of reducing O-GlcNAcylation on ROS formation probably reflect the involvement of disparate signal transduction pathways in high-glucose-treated cells compared with other settings.

The failure of OGT shRNA to reduce stimulation of TxNIP in the presence of high glucose (Fig. 4B) indicates that TxNIP is not a mediator of O-GlcNAc effects on p38 under high-glucose conditions. Uridine diphospho-β-N-acetylglucosamine (UDP-GlcNAc) is produced by the hexosamine biosynthesis pathway (HBP), a side branch of glycolysis. OGT adds single N-acetylglucosamine moieties to intracellular proteins (O-GlcNAc). We hypothesize that basal O-GlcNAcylation inhibits Akt phosphorylation in mesangial cells, relieving repression of the p38 MAP3K ASK1 by Akt. This results in activation of the ASK1 and p38 MAPK pathway. In the nucleus, upregulated p38 induces the expression of PAI-1, fibronectin, and TGFβ, which contribute to diabetic glomerulosclerosis. O-GlcNAc may also increase ROS, which can stimulate ASK1, but further investigations are required to establish whether this is a primary effect.

Fig. 7. Proposed model for the regulation of mesangial cell p38 MAPK signaling by O-GlcNAc. Uridine diphospho-β-N-acetylglucosamine (UDP-GlcNAc) is produced by the hexosamine biosynthesis pathway (HBP), a side branch of glycolysis. OGT adds single N-acetylglucosamine moieties to intracellular proteins (O-GlcNAc). We hypothesize that basal O-GlcNAcylation inhibits Akt phosphorylation in mesangial cells, relieving repression of the p38 MAP3K ASK1 by Akt. This results in activation of the ASK1 and p38 MAPK pathway. In the nucleus, upregulated p38 induces the expression of PAI-1, fibronectin, and TGFβ, which contribute to diabetic glomerulosclerosis. O-GlcNAc may also increase ROS, which can stimulate ASK1, but further investigations are required to establish whether this is a primary effect.
glucose conditions. However, TxNIP levels were somewhat diminished by OGT shRNA in medium containing 5.6 mM glucose, possibly reflecting decreased stability of TxNIP, but the exact basis for the heterogeneous impact of OGT shRNA on TxNIP in different glucose concentrations is unclear.

In contrast to its suppressive effects on p38, we found that OGT shRNA exaggerated signal transduction through other profibrogenic pathways, such as ERK and PKC (22, 32), consistent with the broad range of O-GlcNac effects. ERK phosphorylation was higher in medium containing 5.6 or 25 mM glucose when OGT shRNA was expressed (Fig. 2C). Opposite effects on ERK have been reported previously; for instance, increasing O-GlcNAcylation in β-cells with N-acetylglucosamine and uridine led to higher basal phospho-ERK levels (67), and treatment of hippocampal slices with a specific O-GlcNACase inhibitor also upregulated ERK phosphorylation (73). Unexpectedly, OGT knockdown led to an increase in basal membrane PKCβ content in medium containing 5.6 but not 25 mM glucose (Fig. 6B). The basis for differential effects in normal vs. high glucose is unknown, but as in the case of TxNIP, the O-GlcNAcylation of certain proteins may be decreased to a much greater extent by OGT shRNA in 5.6 vs. 25 mM glucose medium, leading to distinct effects. Many PKC isoforms appear to be modified by O-GlcNAc (50) based on immunoblots, but a definitive analysis of the impact of O-GlcNAcylation on PKC activity has not been described yet. The present data agree with the observation that PUGNAc decreased basal membrane association of PKCα and -ε (50). Earlier experiments with glucosamine and the GFAF inhibitors azaserine and DON suggested that the HBP causes PKC membrane translocation or increased intrinsic PKC kinase activity (25, 40), but given the significant non-O-GlcNAc-related effects of these reagents, the role of O-GlcNAcylation requires further study.

In summary, we demonstrate here that O-GlcNAcylation is coupled to the phosphorylation of profibrotic p38 MAPK in response to high glucose in mesangial cells, and this in turn supports increased PAI-1 expression. The mechanism partly involves the restriction of Akt phosphorylation by basal O-GlcNAcylation, alleviating Akt-dependent inhibition of p38 activation. A schematic representation is shown in Fig. 7. Reduced ROS levels may also facilitate the negative effects of OGT shRNA on p38. Intriguingly, O-GlcNAc is known to regulate other profibrotic proteins, such as RhoA, Sna1I, and NF-κB, suggesting that the positive effects of basal O-GlcNAc on p38 may be part of a larger profibrotic program orchestrated by O-GlcNAc. On the other hand, decreasing O-GlcNAcylation led to increased ERK phosphorylation and basal PKCβ membrane translocation. These diverse overall actions of O-GlcNAc could account in part for the different effects in various tissues and experimental conditions. Specific genetic and chemical probes, along with stoichiometric characterization of substrate modification, will be required to completely understand the functions of O-GlcNAcylation in vivo.

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GRANTS

This work was supported from by a grant from the Canadian Institute of Health Research (MOP 49409) to I. G. Fantus.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.


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