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The Hexosamine Biosynthesis Pathway

Contribution to the Pathogenesis of Diabetic Nephropathy

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INTRODUCTION

Prolonged hyperglycemia is the critical etiological factor in the development of the microvascular complications of diabetes including diabetic nephropathy (1–3). The tissues subject to these complications appear to be susceptible by virtue of abundant expression of cell surface facilitative glucose transporters resulting in the transport of glucose down its concentration gradient. The fact that increased glucose uptake and metabolism promotes the pathological changes leading to tissue damage has led to great interest in the pathways of disposition of glucose metabolites and their regulation. There are at least five pathways of glucose metabolism that, either directly or indirectly, appear to contribute to the complications of diabetes. This current concept, reviewed by Brownlee (4), is supported by a number of studies. The increased cellular entry of glucose results first in augmented glycolytic flux and glucose oxidation. A byproduct of

From: *Contemporary Diabetes: The Diabetic Kidney*
Edited by: P. Cortes and C. E. Mogensen © Humana Press Inc., Totowa, NJ

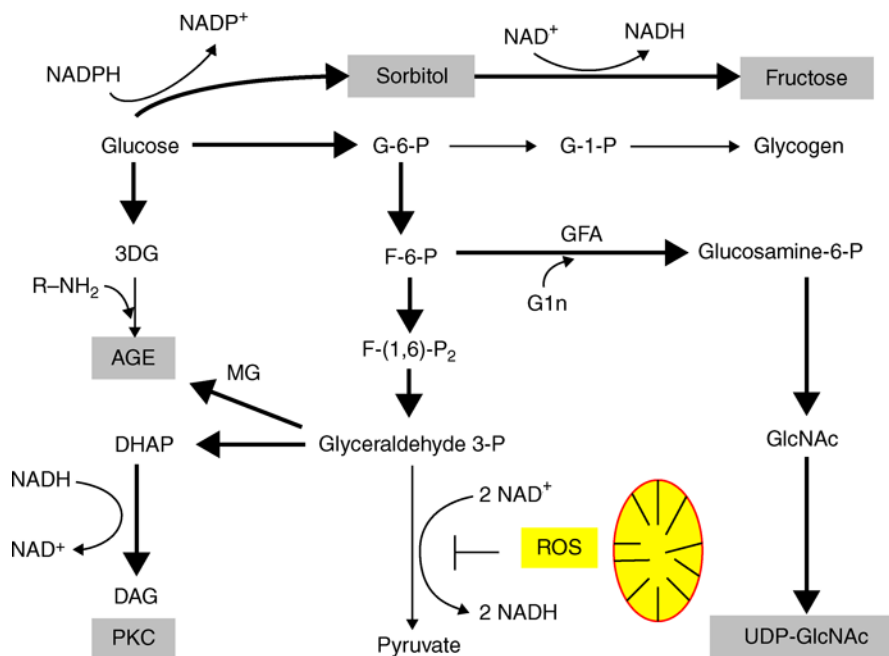


Fig. 1. Glucose metabolic pathways contributing to the microvascular complications of diabetes. Under hyperglycemic conditions, increased cellular glucose uptake and oxidative metabolism results in elevated generation of ROS and subsequent inhibition of GAPDH. Four pathways of glucose metabolism that arise at or upstream of glyceraldehyde-3-P are activated: (1) the aldose reductase/polyol pathway, (2) formation of AGEs from 3-deoxyglucosone (3DG) and methylglyoxal (MG), (3) synthesis of DAG and activation of PKC, and (4) flux through the HBP to increase UDP-GlcNAc. (Reviewed in ref. 4.)

mitochondrial substrate metabolism, i.e., electron transport and oxidative phosphorylation, is superoxide, O_2^- . The increased reactive oxygen species (ROS) production by mitochondria produces oxidative stress, a state in which the formation of ROS exceeds the capacity of cellular endogenous antioxidant removal systems. The excess ROS results in inhibition of the redox-sensitive glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), possibly via activation of poly-ADP-ribose polymerase (5). Poly-ADP-ribose polymerase activation is thought to be owing to ROS-induced DNA damage. The result of GAPDH inhibition is the increased flux of glucose metabolites through four other metabolic pathways, all of which emanate from glycolytic intermediates upstream of GAPDH. These include the following:

1. the aldose reductase or polyol pathway (6),
2. the formation of advanced glycation endproducts (AGEs) (7),
3. the formation of diacylglycerol (DAG), resulting in protein kinase C (PKC) activation (8),
4. increased flux via the hexosamine biosynthesis pathway (HBP) and generation of the end product uridine diphosphate *N*-acetyl-glucosamine (UDP-GlcNAc), a substrate used for protein glycosylation (Fig. 1).

Other chapters review oxidative stress and the polyol, AGE formation, and PKC activation pathways in detail. In this chapter, the role of the HBP is discussed.

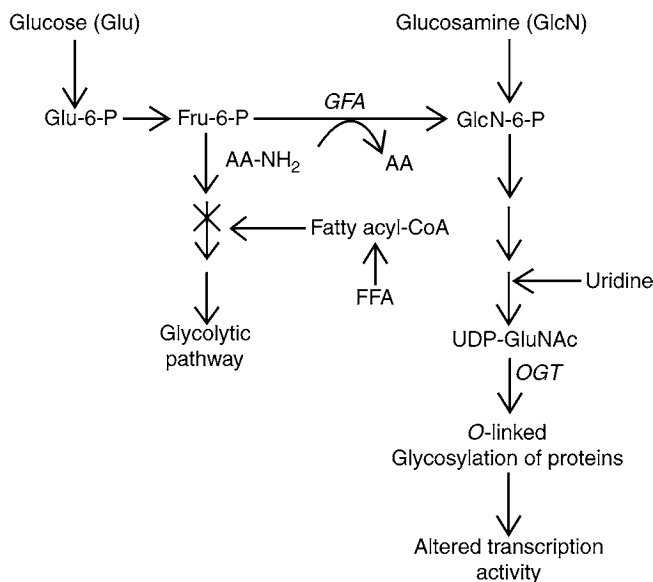


Fig. 2. The HBP. Fructose-6-P and the amino group donor, glutamine, are converted to Glc-6-P by GFA, subsequently *N*-acetylated and linked to UDP to generate UDP-GlcNAc utilized for protein glycosylation. Free fatty acid (FFA) metabolism will increase HBP flux by inhibition of glucose metabolism. Uridine availability will also enhance flux. Most commonly used in experimental systems is Glc, which bypasses the rate-limiting enzyme, GFA, and is a potent stimulator of HBP flux.

THE HEXOSAMINE BIOSYNTHETIC PATHWAY

The HBP is a glucose metabolic pathway, usually accounting for only 2–5% of total glucose metabolism that has been associated with posttranslational protein modification by glycosylation and the synthesis of glycolipids, proteoglycans, and glycosylphosphatidylinositol anchors (9–11). Classical *N*- and *O*-protein glycosylation targets secreted proteins and the extracellular domains of transmembrane proteins and takes place largely in the Golgi apparatus. More recently appreciated is the process of *O*-glycosylation of intracellular proteins on Ser/Thr residues, which is a reversible, dynamic, covalent modification analogous to phosphorylation (9–11). Because this intracellular *O*-glycosylation appears to be driven to a significant extent by the available concentration of UDP-GlcNAc (see “Protein *O*-Glycosylation and Gene Expression” section), which in turn is regulated by HBP flux, current research in the area of diabetes complications is focused largely on this phenomenon.

Interest in the relationship among glucose metabolism, diabetes, and the HBP was initiated by the discovery of its potential role in the pathogenesis of insulin resistance (12,13). Although high glucose, free fatty acids, and glucosamine (Glc) can all induce insulin resistance, several studies have suggested that the HBP contributes in each case (12–15). Furthermore, it has recently been proposed that the HBP functions in adipocytes, muscle cells, and pancreatic β -cells, as a “nutrient-sensing” pathway mediating responses to nutrient availability. For example, the adipocyte-derived hormone leptin is synthesized and secreted in response to increased HBP flux (16). Because it is beyond the scope of this chapter to review this aspect of HBP function, the reader is referred to refs. 12–16.

GLUTAMINE FRUCTOSE 6-PHOSPHATE AMIDOTRANSFERASE: THE RATE-LIMITING ENZYME AND DIABETES COMPLICATIONS

The HBP begins with the conversion of fructose-6-phosphate (F-6-P) and glutamine to Glc-6-P by the rate-limiting enzyme of this pathway glutamine:fructose-6-P amidotransferase (GFA; also abbreviated as GFAT or GFPT) (Fig. 2). A number of subsequent enzymatic steps result in the formation of UDP-GlcNAc. There are two isoforms of GFA encoded by separate genes (17). GFA1 is ubiquitously expressed, whereas GFA2 is highly expressed in the central nervous system (CNS) as well as detected in heart, skeletal muscle, lung, and placenta. The regulation of GFA is not completely understood. Much of its activity appears to be determined by its rate of expression because it has a short half-life of 1 h. The acute effects of cAMP via protein kinase A (PKA) phosphorylation are opposite on GFA1 and GFA2. Thus, phosphorylation of Ser205 of GFA1 results in inhibition (18), whereas that of Ser202 of GFA2 results in activation (19). This differential regulation would allow for tissue-specific responses of HBP pathway flux depending on the relative expression of the two isoforms in each tissue. There is also allosteric feedback inhibition by the endproduct, UDP-GlcNAc (19,20).

The importance of GFA as a rate-limiting step of the HBP and the data supporting its role in insulin resistance and diabetes complications have led to several studies of genetic associations. One report suggests an association of a polymorphism in the 5' flanking region in humans (-913 G/A) with body mass index, percent body fat, and intramyocellular lipid content (-913 G associated with higher values) (21). In another study, flanking sequence variations of GFA (GFPT1) showed a marginal association with diabetes in Caucasians (22). In the case of diabetic nephropathy, one small study showed an association with 2/7 flanking sequence variations with diabetic nephropathy in African Americans in GFPT1 and a 60% increase in GFA1 mRNA in Caucasians with nephropathy compared with diabetic subjects without nephropathy (22). In one biopsy study, immunohistochemistry revealed GFA upregulation in diabetic subjects with nephropathy (23). In contrast, a larger study of type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) subjects with and without nephropathy revealed no association with different single-nucleotide polymorphisms (SNPs) in the 60-kb GFPT1 locus (24). Recently, an amino acid substitution in GFA2, I471V, was reported to be associated with T2DM in Caucasians and a trend for an association with nephropathy in African Americans. In the same study, other variants in the 3' UTR also showed similar associations, namely, T2DM in Caucasian and diabetic nephropathy in African Americans (25). Interestingly, African Americans appeared to have a higher GFA2 mRNA in general than Caucasians. It should be noted that all these genetic association studies are small and the significance of the amino acid substitutions and the flanking region variations remain to be firmly established.

EVIDENCE FOR A ROLE OF THE HBP IN THE PATHOGENESIS OF NEPHROPATHY

Diabetic nephropathy is characterized pathologically by glomerular changes including basement membrane thickening, mesangial expansion consisting of extracellular matrix (ECM) protein accumulation, occasionally the classical Kimmelstiel-Wilson nodules, and, in advanced disease, glomerular sclerosis with obliteration of capillaries (26–28). It has been proposed that the critical event leading to progressive renal failure is the unrelenting accumulation of ECM proteins such as fibronectin, laminin, and collagen.

Thus, mechanisms that increase the synthesis and/or decrease degradation of the ECM have been proposed to play important pathogenic roles. One such key effector is transforming growth factor (TGF)- β , an autocrine/paracrine growth factor that is upregulated by high glucose and stimulates ECM protein gene expression (29,30). One of the first studies implicating the HBP in the pathogenesis of diabetic nephropathy showed that in cultured porcine glomerular mesangial cells, Glc could mimic high glucose to increase expression of fibronectin and TGF- β (31). Glc is taken up by cells via glucose transporters and converted directly to Glc-6-P, thereby bypassing the rate-limiting enzyme GFA (Fig. 2). In this way, the HBP is very strongly activated and therefore, Glc has often been used as a probe of the effects of the HBP. Importantly, in that study the effect of high glucose on TGF- β and fibronectin was inhibited by azaserine, a glutamine analog and inhibitor of GFA, as well as by markedly decreasing GFA protein content using antisense oligonucleotides. Although Glc mimics high glucose, it likely has other effects, so that these additional approaches to modulate HBP flux in the presence of high glucose provided critical supportive data. Taken together, the evidence suggested that enhanced HBP flux increased TGF- β_1 gene expression and consequently ECM protein expression. It is noted that earlier studies had already implicated HBP activation in the stimulation of gene expression, in that case, of TGF- α and basic fibroblast growth factor in vascular smooth muscle cells (32).

Another protein upregulated in diabetic nephropathy, which inhibits ECM protein degradation, is plasminogen activator inhibitor (PAI)-1. PAI-1 mRNA is also induced by high glucose via the HBP, but is independent of TGF- β_1 (33). Similarly, inhibition of GFA by 6-diazo-5-oxo-1-norleucine (DON) blocked the effect of high glucose to increase PAI-1 (33). Apart from using Glc, another method for driving the HBP is to overexpress GFA. This experimental maneuver can increase HBP flux even in the presence of normal glucose. Experiments in cells and transgenic mice overexpressing GFA confirm the increased expression of various proteins, for example, TGF- β_1 (34,35), TGF- β type 1 and type 2 receptors (35), PAI-1 (35), and fibronectin (36) in mesangial cells, angiotensinogen in renal proximal tubular cells (37), and in adipose tissue, leptin (38).

POTENTIAL MECHANISMS OF ALTERED GENE EXPRESSION BY HBP FLUX

Although flux through the HBP has now been well documented to influence gene expression, a number of questions have been raised about the mechanism. One is whether these effects on gene expression are dependent on the formation of the endproduct, or on some other HBP intermediate.

Oxidative Stress

In this context, it has been noted that Glc-6-P, the immediate product of GFA, can inhibit glucose-6-P-dehydrogenase, the enzyme that promotes glucose metabolism via the pentose phosphate pathway (PPP) (39). One important function of the PPP is the generation of NADPH, necessary for the regeneration of reduced glutathione (GSH), a critical and major cellular antioxidant. Thus, in the presence of elevated Glc-6-P and consequent PPP inhibition caused by exposure to Glc, there occurs an associated oxidative stress. Indeed, oxidative stress has been implicated in the effects of Glc to induce pancreatic β -cell dysfunction (40) as well as teratogenesis (41). In β -cells, both high glucose and Glc increased expression of *c-myc*, whereas in the day 7.5 mouse embryo, both glucose and Glc depleted GSH and inhibited expression of Pax-3, resulting in neural

tube defects. The protection against these changes by administration of GSH ethyl ester or *N*-acetylcysteine (NAC), to maintain endogenous GSH concentrations, supports the notion that oxidative stress may be, in some cases, a key trigger of Glc action. This is important for several reasons. First, many studies that support the HBP as a mediator of hyperglycemia-induced cellular perturbations have utilized Glc. The conclusion that any of these effects are owing to increased *O*-glycosylation resulting from increased HBP flux may be erroneous. Second, both glucose and Glc may cause oxidative stress. However, they appear to act differently. Thus, Glc will inhibit the PPP, whereas glucose enhances mitochondrial oxidative metabolism and ROS generation (*see* Introduction and Fig. 1). Marshall (42) recently demonstrated that, at least in adipocytes, high glucose does not increase Glc-6-P concentrations, whereas exposure to 2 mM Glc (commonly used in in vitro experiments) results in a marked elevation. Similarly, Glc results in much higher levels of UDP-GlcNAc (about three- to fourfold) than high glucose.

In order to determine the contribution of the HBP to the pathophysiology of diabetic complications, a number of different approaches will be necessary to dissociate those consequences of increased HBP flux that are not relevant to hyperglycemia.

Protein O-Glycosylation and Gene Expression

Because the major function of the HBP appears to be the generation of UDP-GlcNAc used for glycosylation, increasing numbers of studies have focused on this process. Whereas more complex glycosylation of secreted and extracellular domains of proteins appears to be regulated by enzymes localized to the endoplasmic reticulum and Golgi apparatus, the addition of a single *O*-GlcNAc moiety to protein Ser/Thr residues is catalyzed by uridine diphospho-*N*-acetyl-Glc:polypeptide β -*N*-acetylglucosaminyltransferase (OGT) (EC 2.4.1.94). OGT is encoded by a single gene and resides largely in the nucleus. An alternatively spliced variant is localized to the mitochondria (43,44). Although the regulation of OGT is not completely understood, Hart has proposed that the intracellular levels of UDP-GlcNAc are limiting for intracellular protein *O*-glycosylation (9,11). For this reason, glucose concentration and activity of GFA are important determinants of overall levels of protein *O*-glycosylation. OGT targeting via protein-protein interactions likely imposes some substrate specificity under certain conditions (45). There is also one enzyme, *O*-GlcNAcase (*O*- β -*N*-acetylglucosaminidase EC 3.2.1.52 [hexosaminidase C]) which removes the *O*-GlcNAc moiety (46,47). This results in reversible alterations of protein function.

The protein targets of *O*-glycosylation are those primarily involved in transcriptional regulation, for example, RNA polymerase-associated proteins, transcription factors, and coactivators and corepressors (9–11). Several functional consequences of *O*-glycosylation have been documented such as increased DNA-binding (48), altered protein-protein binding (49,50), and decreased protein degradation (51). Depending on the cellular context, activation or repression of gene expression has been documented (52–55).

In the context of diabetic nephropathy, as outlined above, the expression of several genes; TGF- β_1 , laminin, fibronectin, and PAI-1 were stimulated by high glucose, Glc, and overexpression of GFA. Furthermore, inhibition of GFA blocked the effect of high glucose but not Glc (Fig. 3). These data support increased *O*-glycosylation secondary to HBP flux as playing a role.

Transcription Factor Regulation by the HBP

In the case of PAI-1, our laboratory has examined the role of the transcription factor Spl. Although the PAI-1 promoter can be stimulated by TGF- β_1 via a Smad-binding

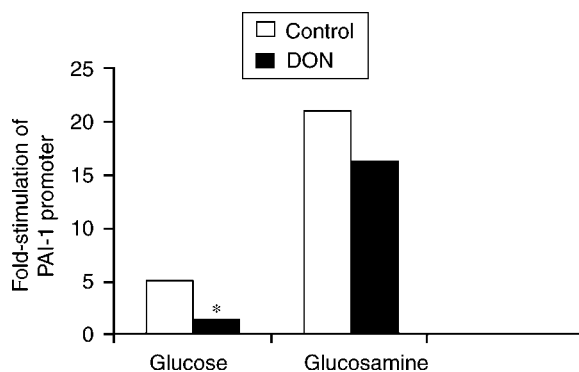


Fig. 3. Inhibition of GFA by DON blocks high glucose but not Glc stimulation of the PAI-1 promoter. Rat mesangial cells were transfected with a PAI-1 promoter (nucleotides from -740 to $+44$) fused to the luciferase reporter gene and then exposed to 20 mM, high glucose (glucose) or 2 mM Glc, in the presence and absence of DON to inhibit GFA. Cells were harvested and luciferase activity determined. Values are expressed relative to basal glucose defined as 1.0 . (Adapted from ref. 33.)

site, we found that HBP flux could increase gene expression independent of $TGF-\beta_1$ (33). Other PAI-1 promoter sequences had been identified, which bound the transcription factor specificity protein 1 (Sp1), a known target of *O*-glycosylation (56). We were able to demonstrate that a PAI-1 promoter luciferase reporter gene was activated by Glc and mutation of the Sp1-binding sites abolished this stimulation (Fig. 4). In keeping with at least one function of *O*-glycosylation, *O*-glycosylated Sp1 showed enhanced DNA binding (33). It was also shown that under hyperglycemic conditions, there is increased glycosylation and reciprocally decreased overall phosphorylation of Sp1 (34). It has been proposed that one mechanism by which protein *O*-glycosylation alters function is by competition with phosphorylation which may occur on the same or adjacent sites (9) (Fig. 5). It is important to note, however, that in the case of Sp1, with at least nine potential *O*-glycosylation sites, it is possible that specific sites may regulate different or even opposing functions.

Although we noted that the enhanced DNA binding was relatively modest ($\sim 30\%$), there was a marked stimulation of the promoter (33). Thus, the transactivation function of Sp1 was examined by fusing the entire transcription factor (holo Sp1) or only the transactivation domain (TAD) with the yeast GAL4 DNA-binding domain. This chimeric, fused Sp1 GAL4 was cotransfected into mesangial cells with an expression vector encoding a luciferase reporter gene driven by a GAL4-binding promoter (GAL4-Luc). Both Glc and high glucose strongly stimulated luciferase gene expression and the glucose effect was blocked by DON, a GFA enzyme inhibitor (57) (Fig. 6). Thus, it appeared that increased flux through the HBP could alter gene expression by modulating transcription factor function in at least two ways, namely, DNA binding and transactivation. The mechanism of altered transactivation could be direct, i.e., mediated by Sp1 *O*-glycosylation, or indirect, for example, glycosylation-independent and mediated by an upstream HBP product such as Glc-6-P, and/or *O*-glycosylation of other targets that regulate Sp1 function such as signaling proteins or transcriptional coactivators/corepressors. To test whether “upstream” HBP metabolites were involved, we took advantage of cells derived from embryos of EMeg32 knockout mice (58). Glc-6-P *N*-acetyltransferase (EMeg32) catalyzes the acetylation of Glc-6-P. The targeted deletion of EMeg32 is embryonic lethal but embryonic fibroblasts could be generated. These cells have

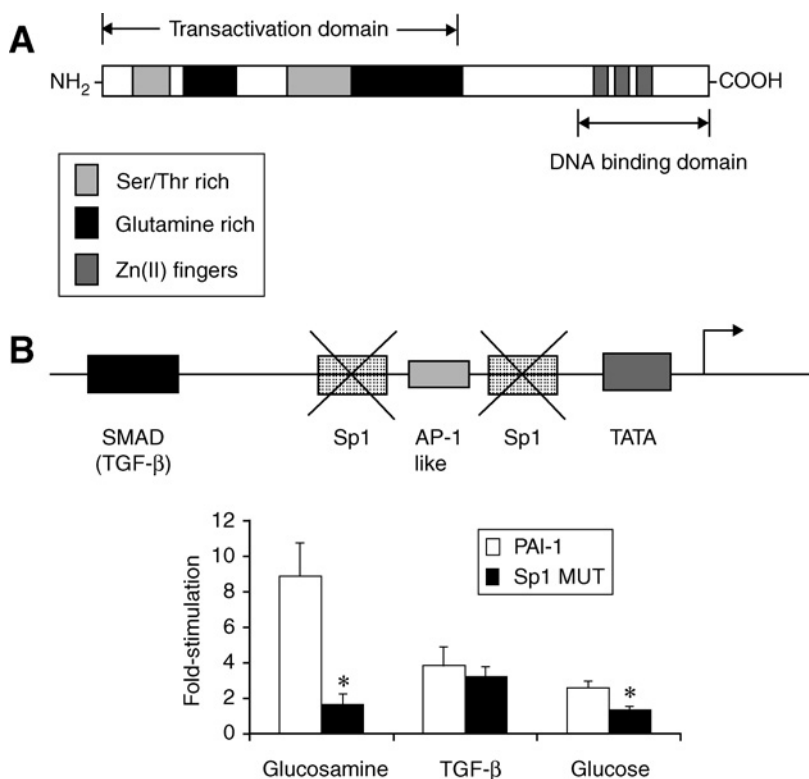


Fig. 4. Activation of the PAI-1 promoter by high glucose and Glc is dependent on Sp1 binding sites. **(A)** Schematic representation of the transcription factor Sp1, which contains nine potential sites of *O*-glycosylation on Ser/Thr residues. Note the Ser/Thr rich regions in the TAD. **(B)** A portion of the PAI-1 promoter (nucleotides from -740 to +44) fused to a luciferase reporter (PAI-1, open bars) or the PAI-1 promoter -Luc with the Sp1 sites mutated (Sp1 mut, filled bars) was transfected into rat mesangial cells which were subsequently exposed to 2 mM Glc, 20 ng/mL TGF-β, or 20 mM glucose. Cells were harvested and luciferase activity measured. Although Glc and high glucose stimulated the wild-type PAI-1 promoter, mutation of the Sp1 sites abolished this effect. However, TGF-β₁ was able to stimulate both wild type and mutant promoters, presumably via the intact smad, transcription factor, binding site. **p* < 0.05 vs stimulation of wild-type promoter. (Adapted from ref. 33.)

extremely low levels of UDP-GlcNAc that do not rise on exposure to elevated glucose. When transfected with the PAI-1 promoter luciferase-expressing cDNA, basal levels of expression were low and did not increase in response to glucose or Glc (Fig. 7). However, addition of serum did lead to an increase demonstrating the specificity of a lack of the HBP end product to high glucose-induced gene expression (not shown).

Altered Cell Signaling

Another effect of increased HBP flux that we considered was altered cell signaling. There were a number of suggestions that Glc could activate PKC enzymes (59,60). It had been well documented that high glucose results in PKC activation, which appears to be a key event in the pathogenesis of the complications of diabetes (8). Furthermore, PKC signaling had been noted to activate Sp1-mediated gene expression

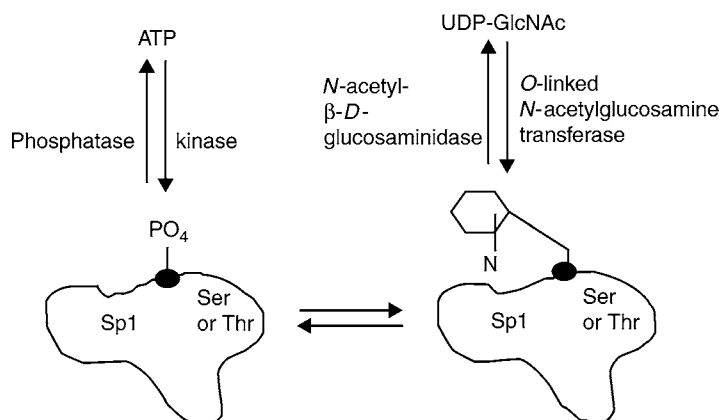


Fig. 5. Reciprocal phosphorylation and *O*-glycosylation. A number of transcription factors, including Sp1, are subject to both phosphorylation and *O*-glycosylation which may occur at the same site and modulate protein function. Such alternating covalent modification may serve as an additional level of control to that exerted by activation and inhibition of kinases, phosphatases, OGT, and *O*-GlcNAcase. (Reviewed in ref. 9.)

(61). We found that both high glucose and Glc-activated PKC- β and - δ in cultured mesangial cells, and that specifically inhibiting each isoform by transfection of dominant negative (DN) PKCs was able to block PAI-1 promoter-driven luciferase expression. Moreover, the DN PKC- β_1 (but not DN PKC- δ) and a PKC- β specific pharmacological inhibitor, LY379196, were able to block the transactivation function of the Sp1-GAL4 chimeric transcription factor (Fig. 8) (57). These data implicated PKC activation, and specifically PKC- β , as part of the mechanism. Although high glucose is known to activate cPKCs (conventional PKCs) such as PKC- β by stimulating *de novo* DAG synthesis, blocking the HBP flux with DON, surprisingly, also decreased PKC activation (Fig. 8). The precise contribution of the HBP to PKC activation remains unclear; however, *O*-glycosylation of PKCs was not detected (not shown).

Requirement of *O*-Glycosylation: OGT

The evidence thus far strongly suggested, but did not directly prove the requirement of *O*-glycosylation for the stimulation of PAI-1 gene expression and Sp1 activation by high glucose. To demonstrate this we have recently employed genetic and pharmacological approaches to specifically modulate *O*-glycosylation. Thus, transfection of a DN-OGT (lacking the catalytic domain) blocked the high glucose-induced increase in expression of the endogenous PAI-1 mRNA and the PAI-1 promoter-driven luciferase reporter gene. Similar results were obtained with transfection of an OGT siRNA that depleted OGT by 60–80%. Third, overexpression of *O*-GlcNAcase, to rapidly remove the *O*-GlcNAc from proteins, also inhibited the effect of high glucose. This was demonstrated for endogenous PAI-1 mRNA, as well as reporter gene expression driven by either the PAI-1 promoter or the GAL4 promoter (in the presence of the fused Sp1-GAL4 transcription factor), indicating that the stimulation of the transactivation function of Sp1 was *O*-glycosylation-dependent. Finally, inhibition of *O*-GlcNAcase with PUGNAc, in the presence of normal physiological glucose concentrations, increased protein *O*-glycosylation and mimicked high glucose to

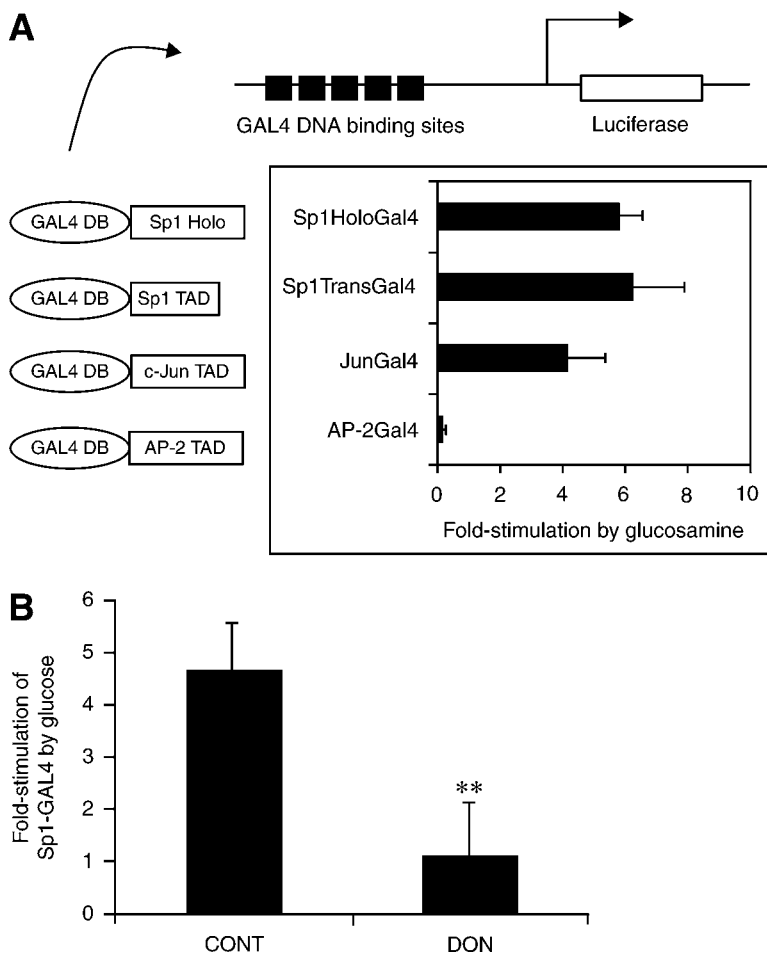


Fig. 6. High glucose and Glc stimulate the transactivation function of Sp1 via the HBP. (A) A cDNA coding for the entire transcription factor Sp1 (Sp1 Holo) or its TAD (Sp1 Trans), or the *c-Jun* TAD (Jun), or a portion of the AP-2 TAD was fused to the yeast GAL4 DNA binding domain (GAL4). These cDNAs were cotransfected into mesangial cells with an expression vector for the luciferase reporter gene driven by a promoter containing GAL4 DNA binding sites and exposed to Glc. Glc strongly stimulated the transactivation of Sp1, as well as Jun (likely via the AP-1 like site in the promoter, *see Fig. 4B*), but not AP-2. (B) Rat mesangial cells were cotransfected as in A with plasmids expressing the Sp1 GAL4 “chimeric” transcription factor and the GAL4-Luc reporter and exposed to 20 mM glucose in the presence and absence of 20 μ M DON to inhibit GFA. Inhibition of GFA completely blocked the stimulation of Sp1 transactivation function by high glucose. (Adapted from ref. 57.)

stimulate PAI-1 mRNA and luciferase expression (62). These results formally prove that the effects of HBP flux on gene expression, at least in the case of PAI-1, and likely for many other genes, is mediated by increased *O*-glycosylation. It should be emphasized that, although correlating with Sp1 glycosylation, the relevant target protein(s) of OGT, which enhance gene expression remain unknown. In the case of Sp1, for example, glycosylation of at least one amino acid, Ser484, has been associated with decreased transcriptional activity (53). In the case of enhanced TGF- β_1 expression by high glucose and the HBP, the transcription factor USF (upstream stimulatory factor) has been implicated, but increased glycosylation of USF was not detected (63). Furthermore, cAMP response element-binding protein (CREBP) phosphorylation

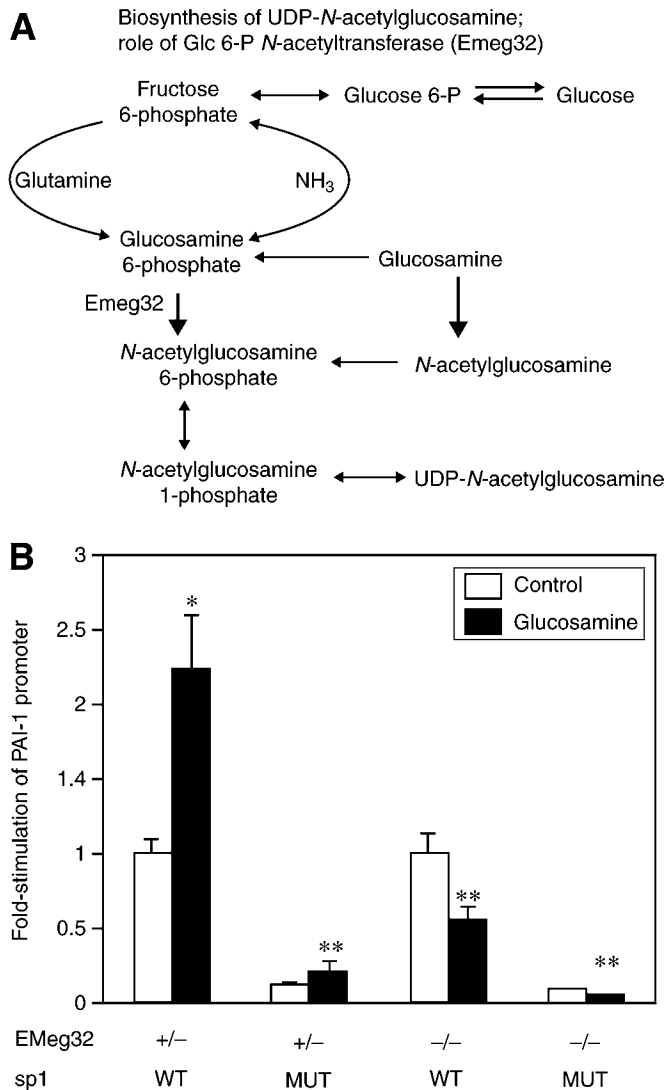


Fig. 7. EMEG32^{-/-} cells with an impaired HBP do not respond to Glc. **(A)** The HBP includes the enzyme, Glc-6-P *N*-acetyltransferase (EMeg 32), which is necessary for the generation of the end product, UDP-GlcNAc. **(B)** EMEG32^{+/-} (heterozygote) and EMEG32^{-/-} (knockout) embryonic fibroblasts were cultured and transfected with the wild-type PAI-1 promoter or the PAI-1 promoter with the Sp1 sites mutated fused to the luciferase reporter as in Fig. 4. The cells were exposed or not to 2 mM Glc. The EMEG32^{+/-} cells containing the wild-type promoter responded to Glc whereas the mutation of the Sp1 sites blocked this effect. In contrast, in the EMEG32^{-/-} cells, there was no stimulation of the wild-type PAI-1 promoter by Glc. UDP-GlcNAc concentrations were very low in the EMEG32^{-/-} cells and not altered by Glc. **p* < 0.05 compared with control. ***p* < 0.01 compared with Glc in EMEG32^{+/-} with wild-type promoter.

and activation has also been associated with enhanced HBP flux (64). Thus, in addition to transcription factors, proteins which alter cell signaling (e.g., kinases, phosphatases [65,66]), or coactivator/corepressor proteins resulting in altered protein-protein interactions with transcription factors, may be equally or more important targets of *O*-glycosylation depending on the cellular context and specific gene being studied.

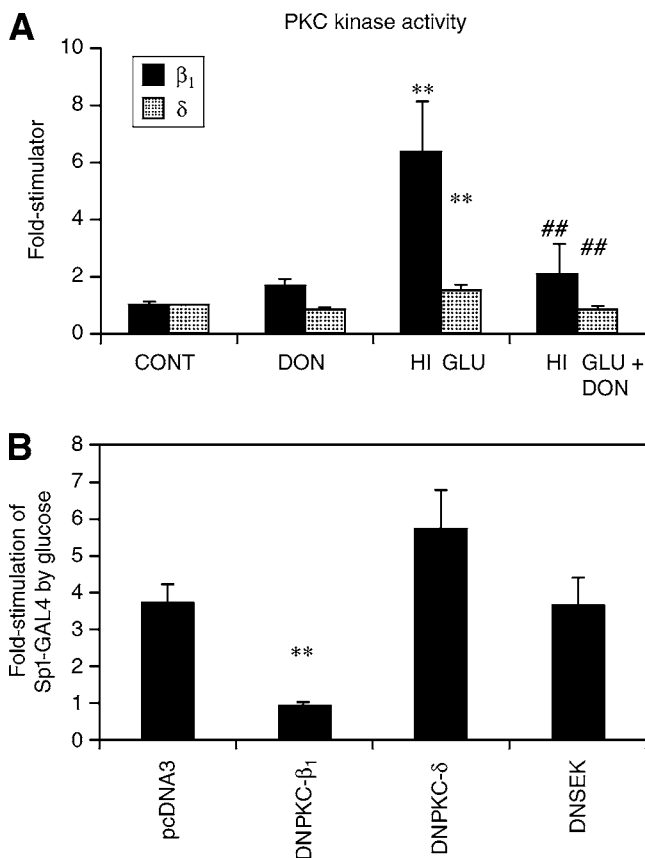


Fig. 8. High glucose activates PKC- β and - δ and PKC- β is required to stimulate Sp1 transactivation. (A) Cultured rat mesangial cells were exposed to 20 mM glucose (HI GLU) for 4 d and with or without 20 μ M DON for the final 2 d. Cells were homogenized and PKCs immunoprecipitated with isoform-specific antibodies and subjected to in vitro kinase assays. High glucose activated PKC- β_1 and PKC- δ , which were both inhibited in the presence of the GFA inhibitor, DON. ** $p < 0.01$ vs PKC activity in basal glucose. ## $p < 0.01$ vs HI GLU in the absence of DON. (B) Mesangial cells were cotransfected with the expression vectors for Sp1-GAL4 and GAL4-Luc as in Fig. 6, along with an empty vector or DN mutants of PKC- β_1 , PKC- δ , or stress-activated protein kinase/extracellular signal activated protein kinase kinase. Only DN-PKC- β_1 blocked the transactivation function of Sp1. ** $p < 0.01$ vs empty vector pcDNA3. (Adapted from ref. 57.)

CONCLUSIONS

The HBP begins with conversion of the glycolytic intermediate F-6-P, in combination with glutamine, to Glc-6-P. Subsequent steps yield the endproduct, UDP-GlcNAc, utilized in glycosylation reactions. Flux through the HBP, usually comprising only 2–5% of glucose metabolism, is elevated in diabetes by hyperglycemia. This is due not only to increased cellular glucose uptake but also to an oxidative stress induced block of glycolysis, downstream of F-6-P, at the level of GAPDH. The augmented synthesis of UDP-GlcNAc drives intracellular protein *O*-glycosylation, a posttranslational, dynamic, and reversible modification of Ser/Thr residues analogous to phosphorylation. Many of the targets of *O*-glycosylation, namely, nuclear proteins, for example, transcription factors, cofactors, and signaling molecules, for example, eNOS, regulate

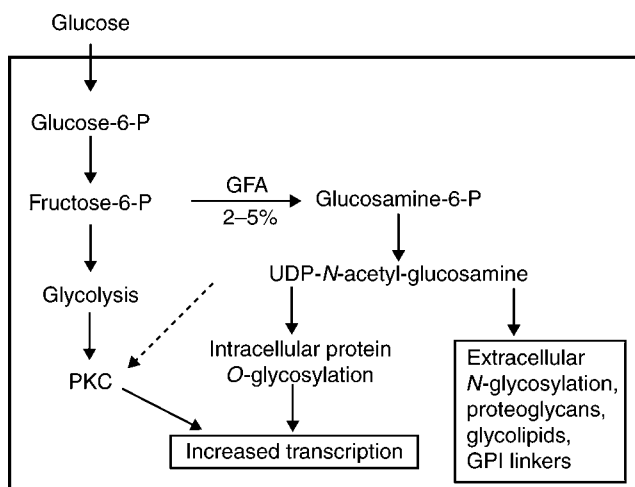


Fig. 9. Proposed mechanism of the contribution of the HBP to the pathogenesis of diabetic nephropathy. Increased flux through the HBP results in elevated UDP-GlcNAc levels that drive intracellular protein *O*-glycosylation. This posttranslational modification of nuclear proteins, for example, transcription factors, cofactors and potentially of signaling molecules, leads to altered gene expression. Increased expression of such prosclerotic proteins as TGF- β_1 , fibronectin, laminin, collagen, and PAI-1, which are documented to contribute to diabetic nephropathy are mediated, at least in part, by the HBP. The most relevant protein targets of *O*-glycosylation that play a role in the pathogenesis of nephropathy and altered gene expression remain to be identified.

gene expression. Indeed, expression of prosclerotic proteins, for example, TGF- β_1 , fibronectin, laminin, collagen, and PAI-1, has been found to be stimulated in the kidney via enhanced HBP flux. These proteins are critical components of the development and progression of diabetic nephropathy, involved in basement membrane thickening, extracellular mesangial matrix accumulation, and glomerulosclerosis. Thus the HBP, along with other glucose metabolic pathways, appears to be a significant contributor to the altered tissue structure and function observed in diabetes caused by hyperglycemia. A goal of future research is to test whether inhibition of the increased *O*-glycosylation *in vivo* is a feasible and effective approach to prevent and treat diabetic nephropathy.

In conclusion, the HBP is implicated as a major contributor to the microvascular complications of diabetes, along with the polyol pathway, PKC activation, and AGE formation. It has been associated not only with diabetic nephropathy, but also more recently with diabetic embryopathy (41), cardiomyopathy (67), and cataract formation (68). This pathway has been shown to be a contributor to insulin resistance, particularly in the context of high glucose, and to β -cell dysfunction. Taken together, the data support augmented intracellular *O*-glycosylation as one of the major mediators of “glucose toxicity.” In this chapter and in the context of diabetic nephropathy, we have focused on altered gene expression as the major consequence of increased HBP flux (Fig. 9). However, proteins other than transcriptional regulators are known to be modified by *O*-glycosylation, which results in functional consequences. Important examples are the Rpt2 ATPase subunit of the 26S proteasome, which results in inhibition of protein degradation (51), and endothelial nitric oxide synthase (eNOS) which is *O*-glycosylated on Ser1146, the amino acid target of phosphorylation by Akt/PKB (66). This results in resistance of eNOS activation by insulin and other Akt/PKB activators in endothelial

cells. Such dysregulation may contribute to atherosclerosis, a process that begins with endothelial dysfunction.

We are just beginning to discover the multiple targets and functional consequences of reversible, intracellular protein *O*-glycosylation (11). The HBP and this posttranslational modification appear to play a critical role in the physiology and pathophysiology of diseases associated with abnormal glucose metabolism. Further *in vivo* work is required to explore these relationships and determine if, and under what circumstances, inhibition of the HBP and/or protein *O*-glycosylation may be a feasible and effective therapeutic strategy to limit the vascular complications of diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institutes for Health Research and the Juvenile Diabetes Research Foundation International. The authors would like to thank Dr. G. Boehmelt and J. Dennis for the EMeg32^{-/-} cells and G. Hart for helpful discussion. D. Topic was supported by summer studentships from the Banting and Best Diabetes Centre, University of Toronto, and the Samuel Lunenfeld Research Institute, Mount Sinai Hospital.

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