Role of the hexosamine biosynthetic pathway in diabetic nephropathy

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The role of the hexosamine biosynthetic pathway in diabetic nephropathy. The hexosamine biosynthetic pathway has been hypothesized to be involved in the development of insulin resistance and diabetic vascular complications. In particular, it was demonstrated that hyperglycemia-induced production of transforming growth factor-β (TGF-β1), a proscerotic cytokine causally involved in the development of diabetic nephropathy. Several lines of evidence indicate that TGF-β1 induction is mediated by the hexosamine pathway. In cultured mesangial cells, high glucose levels induce TGF-β1 production. This effect is eliminated by inhibition of glutamine : fructose-6-phosphate-amidotransferase (GFAT), the rate-limiting enzyme of this pathway. Furthermore, stable overexpression of GFAT increased levels of TGF-β1 protein, mRNA, and promoter activity. Inasmuch as stimulation or inhibition of GFAT increased or decreased high glucose-stimulated activity of protein kinase C (PKC), respectively, the observed effects appear to be transduced by PKC. In similar experiments, involvement of the hexosamine pathway in hyperglycemia-induced production of cytokines (TGF-α and basic fibroblast growth factor [bFGF]) was demonstrated in vascular smooth muscle cells. These studies also revealed a rapid increase in GFAT activity by treatment with agents that elevated levels of cyclic adenosine 3',5' monophosphate (cAMP), thus indicating that GFAT activity is tightly regulated by cAMP-dependent phosphorylation. Using immunohistochemistry and in situ hybridization, high expression of GFAT was found in human adipocytes, skeletal muscle, vascular smooth muscle cells, and renal tubular epithelial cells, whereas glomerular cells remained essentially unstained. However, significant staining occurred in glomerular cells of patients with diabetic nephropathy. Current data indicate that the flux through the hexosamine pathway, regulated by GFAT, may be causally involved in the development of diabetic vascular disease, particularly diabetic nephropathy.

During development of diabetic nephropathy, the renal arterioles, tubules, interstitium, and particularly the glomeruli undergo structural changes that include hyalinosis of arteriolar wall, thickening of the glomerular and tubular basement membranes, and extension of the mesangial matrix [1, 2]. Quantitative morphometric studies have shown that the changes most closely related to the decline in renal function in type 1 and type 2 diabetes derived from an increase in mesangial matrix [2–5]. Immunohistochemical and biochemical studies indicated an increased deposition of various collagen types, laminin, and fibronectin, particularly in glomeruli of patients with diabetes and in experimental animal models [6–11]. Numerous studies have shown that elevated glucose concentrations can cause an increased expression of these components of the extracellular matrix in cultured mesangial cells, which suggests that these cells are an useful model in studying the pathogenesis of diabetic nephropathy [10, 12, 13]. These in vitro studies provided evidence that the high glucose-induced stimulation of mesangial and tubular matrix production is mediated by transforming growth factor-β1 (TGF-β1) [14–17]. Early glomerular and tubular increase of TGF-β1 expression has been shown in experimental and human diabetic nephropathy [18–20]. A causal involvement of TGF-β was also demonstrated given that application of TGF-β-antibodies attenuated the effects in cultured mesangial cells [15] and in experimental animals [21]. These studies indicate that elevated glucose levels induce cytokine production, particularly that of TGF-β1, which in turn induces renal extracellular matrix synthesis and finally leads to renal scarring. Different pathobiochemical pathways are thought to be involved in these damaging events. These include: the sorbitol pathway, oxidative stress, the formation and action of AGE-products and activation of protein kinase C (PKC) [22] and other kinases. Investigation of the specificity of the high glucose effect revealed that D-glucosamine but not other related hexoses efficiently mimicked the effect in stimulating TGF-β1 production in mesangial cells [23] and production of TGF-α and basic fibroblast growth factor (bFGF) in vascular smooth muscle cells [24]. In combination, these studies have indicated that the hexosamine biosynthetic pathway may be involved in mediating the effects of high levels of glucose.

HEXOSAMINE BIOSYNTHETIC PATHWAY

Amino sugars are physiologically synthesized through the hexosamine biosynthetic pathway (Fig. 1). In this path-
The hexosamine biosynthetic pathway merges from glycolysis using fructose-6-phosphate (Fruc-6-P) to form glucosamine-6-P (GlucN-6-P). Glutamine (GluN) serves as donor of the aminogroup. The reaction is catalyzed by the rate-limiting enzyme glutamine:fructose-6-phosphate-amidotransferase (GFAT). GlucN-6-P is rapidly acetylated, isomerized to N-acetylglucosamine-6-phosphate (GlucNAc-1-P) and activated to UDP-N-acetylglucosamine (UDP-GlucNAc) that serves as common precursor for all amino sugars used for the synthesis of glycoproteins, lipids, and proteoglycans. Glucosamine (GlucN) can also enter the cell through the glucose transporter and is rapidly phosphorylated by hexokinase yielding GlucN-6-P, thereby bypassing the rate-limiting first step of the hexosamine biosynthetic pathway.

Figure 1. The hexosamine biosynthetic pathway. Glucose (Gluc) enters the cell through the glucose transporter and is metabolized. The hexosamine biosynthetic pathway merges from glycolysis using fructose-6-phosphate (Fruc-6-P) to form glucosamine-6-P (GlucN-6-P). Glutamine (GluN) serves as donor of the aminogroup. The reaction is catalyzed by the rate-limiting enzyme glutamine:fructose-6-phosphate-amidotransferase (GFAT). GlucN-6-P is rapidly acetylated, isomerized to N-acetylglucosamine-6-phosphate (GlucNAc-1-P) and activated to UDP-N-acetylglucosamine (UDP-GlucNAc) that serves as common precursor for all amino sugars used for the synthesis of glycoproteins, lipids, and proteoglycans. Glucosamine (GlucN) can also enter the cell through the glucose transporter and is rapidly phosphorylated by hexokinase yielding GlucN-6-P, thereby bypassing the rate-limiting first step of the hexosamine biosynthetic pathway.
of the flux through the hexosamine pathway. The flux through the hexosamine pathway may also be regulated by GFAT gene expression. Little is known about the half-life of GFAT protein in vivo, but studies in isolated adipocytes suggest a half-life of less than 1 hour [32]. To elucidate regulation of gene expression, the promoter of mouse GFAT has been cloned and partially characterized [33]. Detailed analysis of the GC-boxes indicated that Sp1 binds to three elements and that this transcription factor plays an important role in the regulation of GFAT gene expression. Earlier studies have shown that GFAT gene transcription can be regulated by epidermal growth factor, glucose and glucosamine in human cells [34] and may be increased by α-pheromone in yeast [35].

The GFAT gene has been localized on chromosome 2p13 [36]. Recently, the existence of a second GFAT (GFAT2) was deduced from human cDNA showing 75.6% homology to GFAT1 [37]. The properties of GFAT are summarized in Table 1.

Table 1. Properties of glutamine : fructose-6-phosphate- 
amidotransferase (GFAT)

<table>
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<tr>
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<td>Chromosomal location GFAT1: 2p13i; GFAT2: 5q</td>
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Involvement of the hexosamine pathway in high-glucose-level induced TGF-β1 production

Investigation of the specificity of the effect of high glucose on mesangial cells showed that the effect is specific for D-glucose, whereas L-glucose, sorbitol, and 3-O-methyl-D-glucose produced no effect [23]. However, 12 mmol/L D-glucosamine was much more potent than 30 mmol/L D-glucose itself in enhancing the production of TGF-β1 protein and subsequent mesangial production of matrix components. D-glucosamine also promoted conversion of latent TGF-β to the active form. These data indicated that the hexosamine biosynthetic pathway is involved in high glucose-level-induced mesangial TGF-β1 production. To prove the involvement of this pathway, GFAT activity was blocked by the substrate analog azaserine and by inhibition of GFAT protein synthesis with antisense-oligonucleotide. Both interventions prevented the high glucose-level-induced increase in cellular glucosamine metabolites, TGF-β1 mRNA, protein and bioactivity and the subsequent effects on enhanced matrix production. As expected, inhibition of GFAT activity did not abolish the glucosamine-induced effects because glucosamine bypasses the GFAT-catalyzed step. Similarly, glucosamine has been shown to be more potent than glucose in stimulating TGF-β1 transcription in cultured renal proximal tubular cells (abstract; Daniels MC, J Am Soc Nephrol 6:1040, 1995). These data indicate that the flux of glucose metabolism through the GFAT-catalyzed hexosamine biosynthetic pathway mediates the hyperglycemia-induced renal production of TGF-β leading to renal matrix expansion.

To elucidate further the involvement of the hexosamine pathway in TGF-β1 induction, human GFAT was stably overexpressed in NIH-3T3 fibroblasts, yielding a 2.5-fold increase in GFAT protein as determined by Western blot (Weigert and Schleicher, unpublished results). To verify that the hexosamine pathway was activated by GFAT overexpression, cellular concentrations of UDP-GlucNAC were determined by capillary electrophoresis as previously described [38]. A twofold increase in UDP-GlucNAC concentration indicated increased flux through the hexosamine pathway after 24 hours (Fig. 2A), whereas no difference could be found after 48 hours. Determination of TGF-β1 expression in these cells revealed that TGF-β1 mRNA increased approximately twofold after 24 hours (Fig. 2B). Although production of mesangial TGF-β1 protein was low in the first 24 hours, GFAT overexpressing cells showed a twofold production after 24 and 48 hours (Fig. 2C). The effect of GFAT overexpression on TGF-β1 gene expression was also studied by using the human TGF-β1 promoter [39] ligated to the reporter gene luciferase. With this sensitive assay, we were able to demonstrate that GFAT overexpressing cells exhibit approximately a 50% increase in TGF-β1 promoter activity (Weigert and Schleicher, unpublished results). These results further support the involvement of the hexosamine pathway in the gene regulation of TGF-β1.

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Glucosamine similarly induced the translocation of PKC isoenzymes PKCα, β, and ε whereas PKCξ was unaffected. Glucosamine similarly induced the translocation of these PKC isoforms as rapidly and effectively as the widely used PKC stimulator phorbol myristate acetate. Treatment of mesangial cells with high glucose caused a translocation of PKC β whereas other isoforms were less affected. The presence of azaserine, an inhibitor of GFAT activity, ameliorated the effect of high glucose levels on PKC β translocation. These data also indicate that the high glucose level-induced activation that has been described by various researchers [12, 13, 40] may be mediated by the hexosamine pathway. In a report by Filippis et al [42], the effect of high glucose and glucosamine on PKC activity was studied in adipocytes. Both high glucose and glucosamine caused a threefold increase in PKC activity; the effect of high glucose, but not that of glucos-
Fig. 2. Overexpression of GFAT in NIH-3T3 fibroblasts increases cellular UDP-GlucNAc concentration (A), TGF-β1 mRNA (B), and TGF-β1 protein levels (C). Fibroblasts were stably transfected with the expression vector pcDNA3 containing the human GFAT cDNA under control of the cytomegalovirus promoter or pcDNA3 alone as control. (A) Stably transfected fibroblasts were cultured for 24 or 48 hours in Dulbecco’s modified Eagle’s medium 2% Ultroser (Gibco, Eggenstein, Germany). Preparation of cell extracts and determination of UDP-GlucNAc with capillary electrophoresis were previously described [38]. DNA content of the cells was determined by fluorometry with bisbenzimidazol and used for normalization of cell number [23]. (B) Stably transfected fibroblasts were cultured for 24 hours as described in (A) and total RNA was prepared. Northern blotting was carried out with 25 μg RNA. For hybridization, digoxigenin-labeled RNA probes for TGF-β1 or glyceraldehyde 3-phosphate dehydrogenase were used [23]. Three different control and GFAT clones are shown. (C) Supernatants of stably transfected fibroblasts cultured in Dulbecco’s modified Eagle’s medium without fetal calf serum or Ultroser were collected after 24 and 48 hours, and TGF-β1 was determined by a commercially available enzyme-linked immunosorbent assay (R & D Systems, Wiesbaden, Germany).

Table 2. Expression of GFAT in human and rat tissues

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<th>Tissue</th>
<th>Protein (human)</th>
<th>mRNA (human)</th>
<th>Activity (rat)</th>
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<tbody>
<tr>
<td>Kidney tubules</td>
<td>+ +</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Kidney glomeruli</td>
<td>+ +</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Muscle</td>
<td>+ + +</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>Smooth muscle cells</td>
<td>++/++</td>
<td>++/++</td>
<td>+++</td>
</tr>
<tr>
<td>Liver</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Fat</td>
<td>++ +</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Testis</td>
<td>ND</td>
<td>ND</td>
<td>+++</td>
</tr>
</tbody>
</table>

- below detection limit; + to +++, mild to strong expression; ND, not determined.

GFAT protein was determined by immunohistochemistry or in situ hybridization in normal human subjects [44].

1. Expression of rat GFAT mRNA was evaluated by Northern blotting [36].

6. GFAT activity by measuring the enzyme activity in tissue extracts [45].

Results of GFAT mRNA expression in human tissues

Localization of GFAT expression in human tissues

Investigation of the cellular expression of GFAT is important because it represents the rate-limiting enzyme of the hexosamine pathway. Expression of GFAT in
Fig. 3. Possible pathobiocchemical link between hyperglycemia and mesangial TGF-β1 production. Elevated flux through the hexosamine pathway results in activation of PKC. Activated and translocated PKC in turn activates transcription factors (S) to form a complex that induces transcription of TGF-β1. Finally, TGF-β1 protein is secreted and acts autocrine/paracrine as prosclerotic cytokine.

various tissues has been studied in normal human subjects [44]. For this immunohistochemical study, a peptide-antibody raised against the C-terminus of GFAT was used. Given that GFAT1 and GFAT2 are identical at this location, the antibody recognizes both isoenzymes. This study revealed that GFAT protein expression is very different in different tissues. Strong staining was seen in all epithelia including renal tubuli or epithelial cells whereas endothelial and glomerular cells remained essentially unstained. Noteworthy, GFAT was significantly expressed in vascular smooth muscle cells. Furthermore, GFAT was strongly expressed in adipocytes and skeletal muscle cells, which supports the hypothesis that the hexosamine biosynthetic pathway may be involved in the pathogenesis of insulin resistance. The results obtained for GFAT protein expression could be confirmed on the mRNA level using situ hybridization. Northern blot analysis essentially confirmed the findings; the use of specific probes for GFAT2 revealed that this subtype was preferentially expressed throughout the nervous system [37]. The data are summarized in Table 2. The unexpected finding that GFAT is not significantly expressed in normal glomeruli suggested that it may be regulated by diabetes. Analysis of GFAT expression in renal sections obtained from patients with diabetes showed significant expression in glomerular cells indicating that the enzyme is induced in diabetes. In line with this finding may be the report on increased GFAT activity in human skeletal muscle of patients with type 2 diabetes [46]. However, detailed analysis of GFAT regulation in diabetes or related diseases has not yet been performed.

CONCLUSIONS

Recent results provided evidence that the hexosamine pathway, previously solely regarded as biosynthetic pathway for amino sugars, exhibits regulatory functions. These include induction of gene expression (e.g., growth factors in smooth muscle cells or renal mesangial cells and of leptin gene expression in muscle and fat [47]) and the induction of insulin resistance [27]. Therefore, this pathway may be regarded as a glucose-sensing pathway [47]. Two possible molecular mechanisms have been elucidated to explain how increased flux through this pathway may transduce its effects by activating PKC [41, 42] alone or also by increasing glycosylation of transcription factors thereby increasing their activity [30]. The possible molecular mechanism of the hyperglycemia-induced production of the prosclerotic cytokine TGF-β1 through the hexosamine biosynthetic pathway is shown in Fig. 3. Although recent studies have increased our knowledge on the regulatory function of this pathway, the relevance for the patient with diabetes remains to be established.

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