Overexpression of GFAT activates PAI-1 promoter in mesangial cells

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Overexpression of GFAT activates PAI-1 promoter in mesangial cells. Am J Physiol Renal Physiol 279: F718–F727, 2000.—Effects of hyperglycemia on glomerular cells may be mediated by glucose entry into the hexosamine pathway, and mesangial cell (MC) expression of the hexosamine pathway rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) is increased in diabetic glomerulosclerosis. We hypothesized that GFAT activity would be an important determinant of gene expression in glomerular MC. When overexpressed in primary MC, GFAT produced a two- to threefold increase in the activity of plasminogen activator inhibitor-1 (PAI-1) promoter. There was a 1.4-fold increase in PAI-1 promoter activity in cells exposed to high glucose (20 mM), whereas in MC overexpressing GFAT, exposure to high glucose caused a 3.5- to 4-fold increase in promoter activity. PAI-1 promoter activation was dependent on GFAT enzyme activity because o-diazo-acyetyl-L-serine and 6-diazo-5-oxonorleucine, inhibitors of GFAT enzyme activity, abrogated the activation of PAI-1 promoter in MC overexpressing GFAT. Glucosamine, which is downstream of GFAT in the hexosamine pathway, produced a 2.5-fold increase in the PAI-1 promoter activity. In addition to increasing the mRNA levels for transforming growth factor-β1 (TGF-β1), GFAT overexpression also increased mRNA levels for the TGF-β type I and type II receptors. TGF-β-neutralizing antibody did not normalize PAI-1 promoter activity in MC exposed to glucosamine or those overexpressing GFAT. We conclude that GFAT expression and activity are important determinants of gene expression in MC and that flux through the hexosamine pathway activates expression of genes implicated in vascular injury pathways.

The mechanisms responsible for diabetic glomerulosclerosis have not been fully elucidated, but clinical trials, like the Diabetes Control and Complications Trial (DCCT), have shown that glycemic control is a key determinant of diabetic microvascular injury (1). Diabetic nephropathy is characterized pathologically by expansion of the glomerular mesangium, and at the cellular level, de novo generation of diacylglycerol and activation of protein kinase C (PKC) by high glucose has been linked to expression of extracellular matrix protein genes in glomerular mesangial cells (MC) (14). However, more recently, Kolm-Litty and co-workers (28) have suggested that the glucose-induced increases in transforming growth factor-β1 (TGF-β1) expression is dependent, at least in part, on glucose flux through the hexosamine pathway.

Under physiological conditions, a small percentage (1–3%) of glucose entering cells is shunted through the hexosamine pathway (21, 33). In the first step of the pathway (Fig. 1), fructose-6-phosphate is converted to glucosamine-6-phosphate by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) (20, 32). Glucose flux through the hexosamine pathway plays an important role in the development of insulin resistance (33). Cultured cells that overexpress GFAT develop insulin resistance in the absence of hyperglycemia (6, 12), and transgenic mice that overexpress GFAT in skeletal muscle and adipose tissue are insulin resistant (23, 41). We hypothesized that enhancing flux of metabolites through the hexosamine pathway in MC by increasing GFAT expression might also activate genes implicated in vascular injury.

To test this hypothesis we first studied the effects of GFAT overexpression on activation of the plasminogen activator inhibitor-1 (PAI-1) promoter in primary cultured MC. PAI-1 is the major physiological inhibitor of tissue plasminogen activator and urokinase (31), and increased PAI-1 plasma levels have been associated with coronary artery disease and vasculopathy in patients with diabetes mellitus (5, 44). To further link GFAT overexpression to vascular injury, we studied the effect of GFAT overexpression on mRNA levels for TGF-β type I and type II receptors because TGF-β1 has been implicated in the pathogenesis of diabetic nephropathy (42, 43).

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The hexosamine pathway. Glucose enters the mesangial cell via a specific facilitative glucose transporter (GLUT-1). Under normal conditions, 1–3% of glucose entering cells is shunted through the hexosamine pathway. Fructose-6-phosphate (Fruc-6-P) is converted to glucosamine-6-phosphate (GlcN-6-P) by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Subsequently, GlcN-6-P is converted to uridine diphosphate N-acetylgluco- somine (UDP-GlcNAc), which serves as a substrate for the O-glycosylation of intracellular proteins. O-glycosylation of transcription factors has been implicated in the regulation of gene transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**
- d-Glucose, d-glucosamine, o-diazoacetyl-L-serine (azaserine), 6-diaz-o-5-oxonorleucine (DON), N-nitrophenyl-β-D-galactopyranoside, and ATP bioluminescence somatic cell assay kit were obtained from Sigma-Aldrich (Mississauga, ONT). DMEM, fetal bovine serum (FBS), and Trizol reagent were purchased from Gibco-BRL (Life Technologies, Grand Island, NY). Reporter cell lysis buffer was obtained from Promega (Madison, WI), and effectene reagent was obtained from Qiagen (Mississauga, ONT). Rabbit anti-TGF-β antibody was supplied by R&D Systems (Minneapolis, MN).

**Preparation and Culture of MC**

Primary MC were obtained from male Sprague-Dawley rats as described (25). The cells were cultured (37°C, 95% air-5% CO₂) in DMEM supplemented with FBS (20%), penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM). The purity and concentration were determined by measuring the optical density (OD) at 260 and 280 nm before use. The OD260/280 ratio ranged from 1.75–1.95.

**Plasmids**

Plasmid PL12, containing the human PAI-1 promoter (∼740 to +44) attached upstream to the luciferase reporter gene, was prepared as described (35). Plasmids pCIS, pCIS-GFAT, and pCIS-GFP kindly provided by Dr. M. J. Quon, have been previously described (6). To control for variation in transfection efficiency, a β-galactosidase expression plasmid (pCMV-βgal) was used.

**Transient Transfection of MC**

Twenty-four hours before transfection, MC (1.5 × 10⁵ cells/well) were plated onto six-well plastic plates (Sarstedt). The following day, transfection was carried out by using effectene (Qiagen) as described by the manufacturer. Cells (70–80% confluent) were transfected with 0.05 μg pCMV-βgal, 0.1 μg PL12, and 0.25 μg pCIS or pCIS-GFAT. Transfected MC were cultured (37°C, 95% air-5% CO₂) for 18 h in DMEM containing FBS (20%) and 5.6 mM d-glucose. The media was changed to DMEM/0.5% FBS, glucose (5.6 or 30 mM), or 1, 5, or 10 mM d-glucosamine. At this point, inhibitors of GFAT, azaserine (40 μM) or DON (40 μM) were added to some wells as required. Forty-eight hours later, the cells were harvested and used for analyses as described below.

**Assay of Luciferase and β-Galactosidase Activity**

Media was aspirated, wells were washed twice with PBS, and lysis was performed by using 0.2 ml of reporter lysis buffer. Cells were incubated for 15 min at 4°C, then transferred to microcentrifuge tubes by using a rubber policeman. Cell debris was pelleted by centrifugation (12,000 g, 4°C, 10 min), and the supernatant was used to assay for luciferase (0.02 ml) and β-galactosidase (0.05 ml) activities by using commercially available reagents. Luciferase was measured in a luminometer (EG&G, Berthold, TN), and β-galactosidase activity was based on the absorbance at 405 nm. Luciferase activity was normalized to the β-galactosidase activity and cell protein. Protein was determined on an aliquot of the supernatant obtained from cell lysis by using a Bio-Rad protein assay dye reagent (Bio-Rad).

**ATP Assay**

Cellular ATP content was determined by using a bioluminescent kit (Sigma-Aldrich). Media was aspirated, wells were washed twice with PBS, and assay for cellular ATP content was performed according to the manufacturer’s specification. Cellular ATP was expressed as micromoles ATP per milligram cell protein.

**RNA Isolation and Semiquantitative RT-PCR**

Total RNA from MC was isolated by the single-step method of Chomczynski and Sacchi (8) as published previously (25, 42, 43). Isolated RNA was stored in diethyl pyrocarbonate-treated water at −80°C. The purity and concentration were determined by measuring the optical density (OD) at 260 and 280 nm before use. The OD260/280 ratio ranged from 1.75–1.95.

Semiquantitative RT-PCR was performed as previously reported (25, 42, 43). For β-actin the sense primer corresponded to bp 331–354 and the antisense to bp 550–57. The TGF-β1 sense primer corresponded to bp 1143–1169 and the antisense to bp 651–671 (GenBank accession no. L26110), whereas the TGF-β2 type I receptor sense primer corresponded to bp 331–354 and the antisense to bp 550–57. The TGF-β2 type II receptor sense primer corresponded to bp 818–837 and the antisense to bp 1129–1146. GFAT sense primer corresponded to 1034–1053, whereas the antisense primer was 1515–1534 of human GFAT gene (GenBank accession no. M90516). The specific primer sequences were the following.

- **β-Actin**
  - 5’ AAC CCT AAG GCC AAC CGT GAA AAG 3’
  - 3’ TCA TGA GGT AGT CTG TCA GGT C 5’
  - TGF-β1
  - 5’ CGA GGT GAC CTG GGC ATC CAT GAC 3’
  - 3’ CTG CTC CAC CCTT GGG CTT CCG ACC CAC 5’
  - TGF-β2 type I receptor
  - 5’ TGG TCC AGT CTG CTT CGT CT 3’
Overexpression of GFAT in MC Activates the PAI-1 Promoter

To determine whether GFAT overexpression would activate the PAI-1 promoter, MC were cotransfected with the PAI-1 promoter-luciferase construct (PL12) and plasmid pCIS (empty vector) or pCIS containing the human GFAT gene (pCIS-GFAT), and growth arrested in 0.5% FBS for 48 h in 5.6 mM glucose. We observed that transient transfection of pCIS-GFAT led to a fourfold increase in the steady-state mRNA levels for GFAT compared with cells transfected with the empty vector, pCIS (Fig. 2). In addition, cotransfection of GFAT in the presence of the GFAT inhibitor azaserine did not influence the level of expression in the MC (Fig. 2).

Transient transfection of pCIS-GFAT led to a 2.5- to 3-fold increase in luciferase activity in MC maintained in 5.6 mM glucose, compared with cells transfected with pCIS (Fig. 3; \( P < 0.001, n = 4 \)). To determine whether the increase in PAI-1 promoter activity in MC transfected with pCIS-GFAT was dependent on an increase in GFAT activity, luciferase activity was measured in cell lysates from MC transfected with pCIS-GFAT in the presence of two GFAT inhibitors, azaserine and DON. Inhibition of GFAT activity abrogated the effect of pCIS-GFAT transfection on PAI-1 promoter activity, as illustrated in Fig. 3.

High Glucose Further Activates the PAI-1 Promoter in MC Transfected With GFAT

Previous studies have shown that increased GFAT sensitizes cells to the effects of glucose, specifically their response to insulin (12). We hypothesized that high glucose might lead to a further increase in PAI-1 promoter activity in cells transfected with pCIS-GFAT. To test this hypothesis, we compared luciferase activity in MC transiently transfected with either pCIS or pCIS-GFAT in 5.6 and 30 mM glucose.

In MC cotransfected with pCIS and PL12, then exposed to varying glucose concentrations, luciferase activity increased by ~35% in 20 and 30 mM glucose compared with physiological glucose concentration (Fig. 4A; \( P < 0.05, n = 4 \)). Cotransfection with pCIS-
GFAT and PL12 led to a 2.8-fold increase in luciferase activity in 5.6 mM glucose that was further augmented by 30 mM glucose so that MC transfected with pCIS-GFAT and exposed to 30 mM glucose exhibited a 3.5- to 4-fold increase in the luciferase activity (*P < 0.001, n = 4). High-glucose-induced PAI-1 promoter activity in pCIS-GFAT-transfected cells was not an osmotic effect because the addition of mannitol (24.4 mM) to the media containing glucose (5.6 mM) did not further increase PAI-1 promoter activity in pCIS-GFAT-transfected cells (Fig. 4B).

**Overexpression of GFAT Increases TGF-β Receptor mRNA Levels in MC**

To determine whether GFAT overexpression would increase mRNA levels for TGF-β and type I and type II receptors, MC were transfected with the plasmid pCIS or pCIS-GFAT and growth arrested in 0.5% FBS for 48 h in 5.6 mM glucose. As illustrated in Fig. 5, transient transfection of MC with pCIS-GFAT led to a 2.5-fold increase in the ratio of mRNA levels of TGF-β1 to β-actin (*P < 0.05 vs. pCIS), a 2-fold increase in the ratio of mRNA levels of TGF-β type II receptor to β-actin (*P < 0.05 vs. pCIS), and a 1.8-fold increase in the ratio of mRNA levels of TGF-β type I receptor to β-actin (*P < 0.05 vs. pCIS). Because the efficiency of transfection was estimated at 25–30% on the basis of green fluorescent protein expression, the impact of GFAT overexpression on mRNA levels is likely to be much greater than measured because only a fraction of
Taq and 2 U of 2 h. RT product was mixed with 0.1 nM virus RT was added, and the mixture was incubated at 42°C for RNAsin and random hexamers and heated. Moloney mouse leukemia was incubated for an additional 48 h. Total RNA was isolated by the transfection, media was changed to 0.5% FBS/DMEM and cells were cultured in 20% FBS/DMEM were transfected with plasmid pCIS or pCIS2-GFAT (0.4 μg/35-mm well). Eighteen hours after transfection, media was changed to 0.5% FBS/DMEM and cells were incubated for an additional 48 h. Media was aspirated, wells were washed twice with PBS, and cellular ATP was performed by using a bioluminescent kit according to the manufacturer's specification (Sigma-Aldrich). Cellular ATP was expressed as μmol ATP/mg cell protein. Brackets indicate concentration. *P, not significant; n = 4.

Glucosamine Activates the PAI-1 Promoter in MC

To further implicate flux through the hexosamine pathway in the activation of the PAI-1 promoter by GFAT overexpression, we studied the effect of glucosamine on the PAI-1 promoter because glucosamine is downstream of the rate-limiting enzyme GFAT in

Table 1. Cellular protein and β-galactosidase activity under various experimental conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cellular Protein, μg</th>
<th>P Value</th>
<th>β-Galactosidase (OD405)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCIS</td>
<td>71.9 ± 12.8</td>
<td></td>
<td>0.118 ± 0.049</td>
<td></td>
</tr>
<tr>
<td>pCIS-HiGlc</td>
<td>84.2 ± 11.8</td>
<td>&gt;0.1</td>
<td>0.129 ± 0.077</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>GFAT</td>
<td>74.8 ± 16.2</td>
<td>&gt;0.1</td>
<td>0.154 ± 0.084</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>GFAT-HiGlc</td>
<td>73.5 ± 18.1</td>
<td>&lt;0.1</td>
<td>0.116 ± 0.067</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>GFAT-azaserine</td>
<td>77.7 ± 9.8</td>
<td>&gt;0.1</td>
<td>0.125 ± 0.095</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>GFAT-DON</td>
<td>64.8 ± 5.4</td>
<td>&gt;0.1</td>
<td>0.100 ± 0.016</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 experiments, each in triplicate. HiGlc, high glucose (30 mM); GFAT, glutamine: fructose-6-phosphate amidotransferase; azaserine, o-diazoacetyl-L-serine; DON, 6-diazo-5-oxo-lornicure. β-Galactosidase activity was estimated by using a commercially available colorimetric reagent (Sigma-Aldrich), and levels were based on the optical density at 405 nm (OD405).

GFAT Overexpression Does Not Influence Cellular ATP

A recent report (24) suggests that the impact of glucosamine on cells, including induction of insulin resistance, may be related to depletion of intracellular ATP. To determine whether overexpression had any impact on cellular ATP levels, we compared ATP levels of lysate from untransfected cells, cells transfected with pCIS, and cells transfected with pCIS-GFAT. We have found previously that exposure of MC to glucosamine did not affect cellular ATP levels (18). Similarly, there was no difference in cellular ATP levels between untransfected cells or cells transfected with pCIS or pCIS-GFAT (Fig. 6).

PAI-1 Promoter-Luciferase Reporter Activity in Cells Overexpressing GFAT is Unrelated to Cellular Protein Content or β-Galactosidase Activity

In the present study, to determine PAI-1 promoter activity under the various experimental conditions, luciferase activity was normalized to cellular protein and β-galactosidase activity. To be certain that the differences in PAI-1 promoter activity among the various conditions were not related to differences in cellular protein or β-galactosidase activity, we compared the total cellular protein and β-galactosidase activity (represented by optical density at 405 nm) for the various conditions. As shown in Table 1, there was no difference in cellular protein or β-galactosidase activity in cells transfected with pCIS or pCIS-GFAT and subsequently exposed to high glucose or inhibitors of GFAT activity.

Glucosamine Activates the PAI-1 Promoter in MC

To further implicate flux through the hexosamine pathway in the activation of the PAI-1 promoter by GFAT overexpression, we studied the effect of glucosamine on the PAI-1 promoter because glucosamine is downstream of the rate-limiting enzyme GFAT in

the total MC population in culture is transfected with the pCIS-GFAT construct.

Fig. 5. GFAT overexpression increases mRNA levels for transforming growth factor-β1 (TGF-β1), TGF-β type II (RII), and type I receptor (RI). Mesangial cells (70–80% confluent, 1.5 × 10⁵ cells/well) cultured in 20% FBS/DMEM were transfected with plasmid pCIS2 or pCIS2-GFAT (0.4 μg/35-mm well). Eighteen hours after transfection, media was changed to 0.5% FBS/DMEM and cells were incubated for an additional 48 h. Total RNA was isolated by the single-step method (8). One microgram of total RNA was mixed with RNAsein and random hexamers and heated. Moloney mouse leukemia virus RT was added, and the mixture was incubated at 42°C for 2 h. RT product was mixed with 0.1 μM of each of the primer pairs and 2 U of Taq polymerase and amplified as per EXPERIMENTAL PROCEDURES. β-Actin was coamplified to standardize for the amount of RNA subjected to reverse transcription. Amplification was allowed to proceed for 28 cycles. PCR product were separated on 1% agarose gels (A), and densitometry was performed (B). All experiments were done in triplicate. Values are means ± SD. *P < 0.02, n = 3.
the hexosamine pathway (Fig. 1). Primary cultured MC were transiently transfected with the plasmid PL12. MC were growth arrested in 0.5% FBS and exposed to 1–10 mM glucosamine. Luciferase activity was assayed in cell lysates after 48 h and compared with MC in 5.6 mM glucose. As illustrated in Fig. 7A, there was a dose-dependent effect of glucosamine on activation of the PAI-1 promoter, and 10 mM glucosamine led to a twofold increase in luciferase activity ($P < 0.02$ vs. 5.5 mM glucose, $n = 3$). Similarly, as illustrated in Fig. 7B, when cells were exposed to glucosamine there was a 2.3-fold increase in mRNA for TGF-β1 ($P < 0.02$, $n = 3$), a 1.5-fold increase in mRNA for type I receptor ($P < 0.05$, $n = 3$), and a 2.75-fold increase in mRNA for type II receptor ($P < 0.02$, $n = 3$).

The PAI-1 promoter construct PL12 used in these studies is TGF-β1 responsive. Because Kolm-Litty and co-workers (28) have reported that flux through the hexosamine pathway increases TGF-β1 production by MC, we sought to determine whether activation of the PAI-1 promoter by GFAT overexpression and/or glucosamine was due to autocrine stimulation by TGF-β1. As expected, Fig. 8A shows that the PL12 promoter construct is TGF-β1 responsive and that coincubation with a pan-specific neutralizing antibody to TGF-β can prevent promoter activation. In these studies, 5 ng/ml of recombinant TGF-β1 led to a threefold increase in luciferase activity after 48 h, and 30 μg/ml of neutralizing antibody normalized luciferase activity. In contrast, TGF-β-neutralizing antibody did not normalize luciferase activity in MC overexpressing GFAT or in MC exposed to 10 mM glucosamine (Fig. 8, B and C).

**DISCUSSION**

One-third of diabetic patients will develop kidney disease characterized by progressive mesangial matrix expansion, proteinuria, and declining glomerular filtration rate, but the mechanisms responsible for diabetic glomerular injury remain poorly understood. There is a relationship between blood glucose control and microalbuminuria, and the DCCT has shown that blood glucose level is a key determinant of microalbuminuria, and the DCCT has shown that blood glucose level is a key determinant of microalbuminuria, proteinuria, and declining glomerular filtration rate, but the mechanisms responsible for diabetic glomerular injury remain poorly understood. There is a relationship between blood glucose control and microalbuminuria, and the DCCT has shown that blood glucose level is a key determinant of microalbuminuria, proteinuria, and declining glomerular filtration rate, but the mechanisms responsible for diabetic glomerular injury remain poorly understood. There is a relationship between blood glucose control and microalbuminuria, and the DCCT has shown that blood glucose level is a key determinant of microalbuminuria, proteinuria, and declining glomerular filtration rate, but the mechanisms responsible for diabetic glomerular injury remain poorly understood. There is a relationship between blood glucose control and microalbuminuria, and the DCCT has shown that blood glucose level is a key determinant of microalbuminuria, proteinuria, and declining glomerular filtration rate, but the mechanisms responsible for diabetic glomerular injury remain poorly understood. There is a relationship between blood glucose control and microalbuminuria, and the DCCT has shown that blood glucose level is a key determinant of microalbuminuria, proteinuria, and declining glomerular filtration rate, but the mechanisms responsible for diabetic glomerular injury remain poorly understood.
In the hexosamine pathway, fructose-6-phosphate is first converted to glucosamine-6-phosphate by the rate-limiting enzyme GFAT (33). Under normal physiological conditions, only a small percentage (1–3%) of glucose entering cells is shunted through the hexosamine pathway (21, 33). The end product of the hexosamine pathway, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), is a substrate for O-glycosylation of intracellular proteins (22, 30, 39, 40). Although GFAT activity is low in the kidney compared with other tissues like the liver, fat, and skeletal muscle (38), recent in vivo studies of human kidney biopsy samples have revealed that GFAT immunostaining increases in MC of glomeruli exhibiting diabetic glomerulosclerosis (36). A twofold increase in GFAT activity is sufficient to induce insulin resistance in rat-1 fibroblasts (11, 12), and GFAT overexpression sensitizes adipocytes and tissues to glucose-induced insulin resistance so that insulin responsiveness is impaired at lower concentrations of glucose in cells that overexpress GFAT (12). These studies lead us to speculate that increased GFAT activity might be an important determinant of gene expression in diabetic glomerulus. To test our hypothesis, we sought to determine whether GFAT overexpression was sufficient to activate gene expression in primary MC. We chose to study the effect of GFAT overexpression on activation of the PAI-1 promoter because PAI-1 is the major physiological inhibitor of tissue plasminogen activator and urokinase (5, 31, 44). An increase in PAI-1 is predicted to decrease extracellular matrix protein degradation by reducing plasmin activation and thus may contribute to the accumulation of extracellular matrix protein in the glomerulus. Moreover, increased PAI-1 plasma levels have been associated with vasculopathy in patients with diabetes mellitus (5, 44).

Our first major observation was that transient overexpression of GFAT with pCIS-GFAT activated the PAI-1 promoter in primary cultured MC in comparison to cells transfected with the empty vector, pCIS. The increase in PAI-1 promoter activity was dependent on GFAT activity because both of the GFAT inhibitors, azaserine and DON, prevented the increase in PAI-1 promoter activity in pCIS-GFAT-transfected cells. Neither inhibitor affected GFAT expression. All of the experiments were first performed in 5.6 mM glucose, and therefore our results are similar to the studies of Cook and co-workers (11, 12) on insulin resistance in rat-1 fibroblasts, in that GFAT overexpression is sufficient to activate the PAI-1 promoter.

High glucose activated the PAI-1 promoter (Fig. 4A), and we also observed a further increase in PAI-1 promoter activity in MC overexpressing GFAT after exposure to 30 mM glucose (Fig. 4B). Again, this response was dependent on GFAT and was not due to an unex-
pected effect of the transfection protocol because cells transfected with pCIS did not exhibit a similar increase in PAI-1 promoter activity when they were exposed to 30 mM glucose. The response was also dependent on glucose because an osmotic stimulus with mannitol did not reproduce the effect of 30 mM glucose (Fig. 4B). These findings suggest that overexpression of GFAT sensitizes MC to effects of high glucose.

PAI-1 transcription is highly induced by TGF-β1 (7, 35). The PL12 promoter construct used in this study contains the promoter region from +699 to +54, and this region has several TGF-β1-responsive elements, including an activator protein-1 site and two CAGA boxes (7, 35). Because glucosamine induces TGF-β1 expression (13, 28), it was possible that activation of the PAI-1 promoter in our experiments was due to autocrine effect of TGF-β1. Therefore, we cocultivated transfected MC with a pan-TGF-β-neutralizing antibody to inhibit autocrine stimulation by TGF-β1. The concentration of antibody was determined by studying TGF-β1-induction of our PAI-1 promoter construct (PL12). The TGF-β-neutralizing antibody completely inhibited the activation of the PAI-1 promoter by recombinant TGF-β1, whereas activation by glucosamine or by overexpression of GFAT was only partially blocked by the neutralizing antibody (Fig. 8). These results suggest that receptor-mediated TGF-β1 activity was only partially responsible for activation of the PAI-1 promoter in cells exposed to glucosamine or those in which GFAT was overexpressed. Thus in our primary MC, overexpression of GFAT or provision of the downstream substrate glucosamine activates the PAI-1 promoter independently of autocrine or paracrine TGF-β1 activity. We have made similar observations in MC cotransfected with a dominant-negative TGF-type II receptor construct (18).

The present study did not address the mechanism responsible for the effect of flux through the hexosamine pathway on activation of the PAI-1 promoter; however, flux through the hexosamine pathway is believed to exert an effect on gene expression by increasing intracellular concentrations of GlcNAc and UDP-GlcNAc, which are substrates for the O-glycosylation of proteins (22, 39, 40, 45). The intracellular levels of O-linked glycosylated proteins correlate with GFAT activity, and blockade of GFAT activity or inhibition of GFAT expression with antisense oligonucleotides lowers the intracellular levels of O-GlcNAc-modified proteins (39, 40). The posttranslational modification of serine residues in transcription factors by O-glycosylation can affect the activity of the transcription factors. For example, O-glycosylation of Sp1 stabilizes the protein and prevents proteosomal degradation (20). There are Sp1 consensus sequences in the PAI-1 promoter, and we have found that site-directed mutagenesis of two adjacent Sp1 binding sites in the PAI-1 promoter attenuates glucosamine-induced activation of the PAI-1 promoter (18).

Recently, Hresko and others (24) reported that glucosamine-induced insulin resistance in fat cells was secondary to depletion of cellular ATP. In the present study, there was no difference in cellular ATP for untransfected MC, cells transfected with the empty vector, or those overexpressing GFAT (Fig. 6). We have also found that glucosamine did not affect cellular ATP levels (18), and our observations of a lack of effect of glucosamine on cellular ATP is supported by other studies (10).

A number of studies have implicated TGF-β1 in the pathogenesis of diabetic nephropathy (3, 48, 50), and recent studies have shown that glucosamine increases TGF-β1 in porcine MC and that flux through the hexosamine pathway may be responsible for glucose-induced increases in TGF-β1 expression in MC (28). In addition, glucosamine activates TGF-β1 promoter-luciferase reporter in various cells in culture (13). TGF-β1 influences extracellular matrix protein synthesis by interacting with specific cell surface receptors, TGF-β type II and TGF-β type I receptors (32, 34, 47), but the effect of GFAT overexpression on TGF-β1 and TGF-β receptor expression in primary cultured MC has not been defined. We hypothesized that increased flux through the hexosamine pathway in MC overexpressing GFAT might stimulate expression of TGF-β1 type I and type II receptors, in addition to TGF-β1, in MC. The rationale for these studies was further supported by the observations that expression of TGF-β type I and type II receptors is commonly increased after injury in a variety of tissues including skin (17), vascular endothelium (46), and glomerulus (37, 49).

Our third major observation was that GFAT overexpression was sufficient to increase TGF-β receptors and TGF-1 expression in MC in physiological glucose concentrations (5.6 mM glucose). Interestingly, TGF-β expression is increased in dermal wound healing (36), a setting in which TGF-β receptor expression also increases (17). Although the mechanism underlying the effect of GFAT overexpression on TGF-β receptors and TGF-1 expression was not determined, like the PAI-1 promoter, Sp1-regulatory elements are present in the promoters of all three of these genes (2, 16, 26, 27). It is also possible that other regulatory elements are responsible for the effect of the hexosamine pathway on the expression of these genes. For example, the AP-1 transcription factor(s) can be O-glycosylated, and Kolm-Litty and co-workers (29) have also recently reported that glucosamine activates PKC, which may also contribute to these changes in gene expression.

High glucose produced a modest activation of the PAI-1 promoter, but we were unable to demonstrate a consistent effect of high glucose on the mRNA levels for TGF-β1 and type I and type II receptors. This observation does not rule out an impact of glucose on mRNA for TGF-β1 and type I and type II in our primary MC. Instead, we believe that it reflects low flux through the hexosamine pathway in these primary cells. This conclusion is further supported by the finding that in cells overexpressing GFAT that there is further augmentation of PAI-1 promoter activity in high glucose.

In summary, our findings support the general hypothesis that metabolic pathways implicated in insulin resistance may also be important in the development of
diabetic complications. More specifically, our findings suggest that the rate-limiting enzyme in the hexosamine pathway, GFAT, sensitizes MC to the effects of high glucose by regulating flux through the hexosamine pathway and, through this action, the hexosamine pathway may contribute to diabetic glomerular injury by influencing expression of genes implicated in vascular injury.

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