Angiotensin II activates the GFAT promoter in mesangial cells

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Angiotensin II activates the GFAT promoter in mesangial cells. Am J Physiol Renal Physiol 281: F151–F162, 2001.—Expression of glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme for glucose entry into the hexosamine pathway, is transcriptionally regulated. Immunohistochemical studies of human kidney biopsies demonstrate increased GFAT expression in diabetic glomeruli, but the mechanism responsible for this overexpression is unknown. Given the role of ANG II in diabetic kidney disease, we chose to study the effect of ANG II on GFAT promoter activity in mesangial cells (MC). Exposure of MC to ANG II (10−7 M) increased GFAT promoter activity (2.5-fold), mRNA (3-fold), and protein (1.6-fold). ANG II-mediated GFAT promoter activation was inhibited by the ANG II type I receptor antagonist candesartan (10−8 M) but was unaffected by the ANG II type II receptor antagonist PD-123319 (10−8 M). The intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (10−6 M), protein kinase C (PKC) inhibitors bisindoylmaleimide-4 (10−6 M) and calphostin C (10−7 M), protein tyrosine kinase (PTK) inhibitor genistein (10−4 M), Src family kinase inhibitor PP2 (2.5 × 10−7 M), p42/44 mitogen-activated protein kinase (MAPK) inhibitor PD-98059 (10−7 M), and the epidermal growth factor (EGF) inhibitor AG-1478 all attenuated GFAT promoter activation by ANG II. We conclude that the GFAT promoter is activated by ANG II via the AT1 receptor. Promoter activation is calcium dependent and PKC dependent but also involves PTK signaling pathways including Src, the EGF receptor, and p42/44 MAPK.

angiotensin II; signaling; glomerulus; mesangial cells; glutamine:fructose-6-phosphate amidotransferase

THE HEXOSAMINE PATHWAY has been implicated in some of the adverse effects of glucose (6, 8, 20, 40), and glucose flux through the hexosamine pathway may contribute to the development of diabetic kidney disease. Under physiological conditions, a small percentage (1–3%) of glucose entering cells is shunted through the hexosamine pathway (40). 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) (40). Glucose flux through the hexosamine pathway plays an important role in the development of insulin resistance in adipocytes (8, 40). Cultured cells that overexpress GFAT develop insulin resistance in the absence of hyperglycemia (6, 8), and transgenic mice that overexpress GFAT in skeletal muscle and adipose tissue are insulin resistant (20). In mesangial cells (MC), flux through the hexosamine pathway has been implicated in glucose-induced increases in transforming growth factor-β1 (TGF-β1) expression (31).

Despite these experimental observations, the regulation of expression of GFAT has not been studied extensively. In vitro, GFAT mRNA levels are transcriptionally regulated. Epidermal growth factor-α (EGF-α) activates the GFAT promoter in cells that overexpress the EGF receptor, but glucose does not activate the promoter in these cells (49). Immunohistochemical studies of human kidney biopsies have revealed increased GFAT expression in diabetic glomeruli (47), but the mechanism responsible for this effect is unknown. ANG II plays an important role in the pathogenesis of diabetic kidney injury (18, 29, 41). Blockade of the renin-angiotensin system activity by angiotensin-converting enzyme (ACE) inhibition or ANG II type I (AT1) receptor antagonism slows progression of clinical and experimental diabetic nephropathy (18, 29, 36, 37, 62). We hypothesized that ANG II increases GFAT mRNA levels in glomerular MC and sought to determine whether ANG II would activate the GFAT promoter in cultured MC.

EXPERIMENTAL PROCEDURES

Materials. d-Glucose, ANG II, phorbol 12-myristate 13-acetate (PMA), bisindoylmaleimide-4 (BIM4), calphostin C, o-diazoacetyl-1-serine (zasaserine), 6-diazo-5-oxonorleucine (DON), o-nitrophenyl-β-D-galactopyranoside (ONGP), and benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP) were obtained from Sigma-Aldrich (Mississauga, Ontario, Canada). A23187, PD-98059, PP2, and AG-1478 were purchased from Calbiochem (La Jolla, CA). Genistein and daidzein were purchased from Alexis (San Diego, CA), whereas candesartan was generously provided by AstraZeneca (Moln-
MC were incubated for 15 min at 4°C, then transferred to microcentrifuge tubes using a rubber policeman. Cell debris was pelleted by centrifugation (12,000 g, 4°C, 1 min), and the supernatant was used to assay for luciferase (0.02 ml) and β-galactosidase (0.05 ml) activities using commercially available reagents. Luciferase was measured in a luminometer (EGL&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 using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories).

**RNA isolation and semiquantitative RT-PCR.** Total RNA from MC was isolated by the single-step method of Chomczynski and Sacchi (7), as we have published (27, 28, 60). Isolated RNA was stored in diethyl pyrocarbonate-treated water at −80°C. The purity and concentration were determined by measuring the optical density at 260 nm and 280 nm prior to use. The absorbance ratio, A260/A280, ranged from 1.75–1.95.

Semiquantitative RT-PCR was performed as previously reported (27, 28, 60). For β-actin the sense primer corresponded to base pairs (bp) 331–354 and the antisense to bp 550–57. GFAT sense primer corresponded to bp 1034–1053, whereas the antisense primer was bp 1515–1534 of human GFAT gene (GenBank accession no. M90516). The TGF-β1 sense primer corresponded to bp 1143–1169 and the antisense to bp 1521–1547; VCAM-1 sense primer corresponded to bp 189–211 and the antisense primer to bp 607–630. The specific primer sequences were:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense/Type</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>β-Actin</td>
<td>Sense</td>
<td>5'- AAC CCT AAG GCC AAC GGT GAA AAG 3'</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Sense</td>
<td>5'- CGA GGT GAC CTG GGC ATC CAT GAC 3'</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Sense</td>
<td>5'- CGA GAC ACT GTC ATT ATC TCC TG 3'</td>
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For amplification, 2.5 μl of the RT product were mixed with 7.5 μl of PCR mix containing 0.1 μM of each of the primer pairs and 2 U of Taq polymerase. The sample was placed onto Perkin-Elmer DNA thermal cycler (model 480) and heated to 94°C for 4 min prior to the application of temperature cycles. β-Actin was amplified to standardize the amount of RNA subjected to reverse transcription. The temperature cycle for amplification was 1) denature at 94°C for 30 s, 2) cool-anneal at 60°C for 30 s, and 3) heat-extend at 72°C for 30 s. For the β-actin, GFAT, and TGF-β1 primer pairs, the PCR product plateaued at 28 cycles, and therefore 25 cycles were chosen for the final amplification. For VCAM-1 primer pairs, the PCR product plateaued at 40 cycles, and therefore 35 cycles were chosen for final amplification as the PCR product. PCR products were separated on 1% agarose gel containing ethidium bromide, photographed, and quantitated with a transmittance/reflectance scanning densitometer (model GS 300, Hoeffer Scientific Instrument) utilizing a Macintosh class II computer (system 7.0) and Dynamax HPLC Method Management software (version 1.2).

**Western blotting of GFAT.** Nontransfected MC were exposed to ANG II (10−7 M) for 16 h, media were removed, and the cells were washed once with ice-cold PBS. Total cellular protein was obtained and used for Western blotting as we have described (27). GFAT antibody (47) was generously
ANG II activates GFAT promoter in rat MC. GFAT expression is transcriptionally regulated (49), and immunohistochemical studies of human diabetic kidney biopsies have revealed increased GFAT expression (47). However, the mechanism(s) responsible for the increased in GFAT observed in diabetic glomeruli remains unclear. Since ANG II is known to play an important role in the progression of diabetic kidney disease (18, 36, 41), we sought to determine the effect of ANG II on GFAT mRNA and protein levels in nontransfected MC. Nontransfected MC were growth arrested in DMEM/0.5% FBS, then exposed to ANG II (10^{-7} M). At the end of the incubation period, total RNA was extracted and used for RT-PCR analysis (7, 15, 16). RT-PCR analysis (Fig. 2) indicated that after 8 h of exposure to ANG II (10^{-7} M), there was a threefold increase in mRNA for GFAT (P < 0.02, n = 4).

To determine whether ANG II had any influence on the level of GFAT protein, total cellular protein was obtained after MC were exposed to ANG II (10^{-7} M) for 16 h, then used for Western blot analysis. As shown in Fig. 2B, in MC exposed to ANG II, there was a 1.6-fold increase in GFAT protein compared with cells that were not exposed to ANG II (P < 0.05, n = 3).

ANG II activates the GFAT promoter in a time- and concentration-dependent manner. Subsequently, we determined whether ANG II would activate a transiently transfected GFAT promoter-reporter construct in rat MC. Six hours after transfection with pGFAT and pCMV-\( \beta \)-gal, MC maintained in DMEM/FBS (0.5%)/5.6 mM glucose were exposed to ANG II (10^{-7} M) for an additional 2–48 h. Concurrent control MC, maintained in DMEM/FBS (0.5%)/5.6 mM glucose, were not exposed to ANG II. GFAT promoter activity was assessed by measuring luciferase activity, normalized to \( \beta \)-galactosidase activity and total cellular protein. GFAT promoter activity increased 1.5-fold after 4 h of exposure to ANG II (P < 0.01, n = 5) and by 2.5-fold after 24 h of ANG II exposure (Fig. 3A).

To determine whether the observed ANG II induction of GFAT was dose dependent, 6 h after transfection, MC cultured in DMEM/0.5% FBS/glucose (5.6 mM) were exposed to ANG II (10^{-10} to 10^{-7} M) for 24 h...
ANG II activates GFAT promoter in MC

Fig. 3. GFAT promoter activation by ANG II is time and dose dependent. MC (70–80% confluent, 1.5 × 10⁶ cells/well) cultured in DMEM/FBS (0.5%)/5.6 mM glucose were cotransfected with plasmids pGFAT (0.35 μg) and pCMV-βgal (0.05 μg). Six hours later, media were changed to DMEM/0.5% FBS. A: cells were incubated for an additional 2–48 h in physiological glucose concentration (5.6 mM) with or without ANG II (10⁻⁸ M). B: cells were incubated for an additional 24 h in physiological glucose concentration (5.6 mM) with or without ANG II (10⁻⁸ to 10⁻⁷ M). Cells were washed with PBS, lysis buffer (0.2 ml) was added, and cells were incubated for 15 min at 4°C. Disrupted cells were transferred to microcentrifuge tubes (1.5 ml), and cell debris was removed by centrifugation (12,000 g, 1.0 min, 4°C). Luciferase and β-galactosidase activities were determined using 0.02 ml and 0.05 ml of MC lysate, respectively. Luciferase activity was normalized to protein and β-galactosidase activity. All experiments were done in triplicate, and values are mean ± SD. For A, five separate experiments were performed, whereas for B, six separate experiments were performed. *P < 0.001 vs. control. **P < 0.01 vs. control. §P < 0.01 vs. 4, 8, and 48 h. *$P < 0.05 vs. control.

Fig. 4. Activation of the GFAT promoter by ANG II is mediated by the angiotensin type 1 (AT₁) receptor. MC (70–80% confluent, 1.5 × 10⁶ cells/well) cultured in DMEM/FBS (0.5%)/5.6 mM glucose were cotransfected with plasmids pGFAT (0.35 μg) and pCMV-βgal (0.05 μg) as before. Six hours later, media were changed to DMEM/0.5% FBS containing 5.6 mM glucose, and cells were incubated for additional 24 h in the presence (+) and absence (−) of ANG II (10⁻⁸ M), candesartan (10⁻⁸ M), or PD-123319 (10⁻⁶ M). Luciferase and β-galactosidase activities were determined and normalized to protein and β-galactosidase activity as described in EXPERIMENTAL PROCEDURES. All experiments were done in triplicate. Values are mean ± SD. *P < 0.01 vs. control (n = 3).
ANG II activation of the GFAT promoter is protein kinase C dependent. Many observed intracellular effects of ANG II are responsive to protein kinase C (PKC) inhibition, implicating PKC in ANG II signaling (3, 23, 25, 26, 43). To study the role of PKC, we examined the effect of PMA on GFAT promoter activity in transiently transfected MC. Acute exposure to PMA leads to PKC activation in many cells, whereas protracted exposure (greater than 24–48 h) leads to PKC depletion (42, 66). Consequently, MC transiently transfected with pGFAT as above were exposed to PMA (5 × 10^{-7} M) or vehicle [dimethyl sulfoxide (DMSO)] for 30 min. PMA or vehicle was removed, and cells were washed with PBS, then exposed to PKC inhibitors BIM4 (10^{-6} M) and calphostin C (10^{-7} M), or vehicle (DMSO) for 1 h. Media were removed, and cells were washed with PBS, then cultured for an additional 24 h in DMEM/0.5% FBS/5.6 mM glucose.

MC exposed to PMA exhibited an 1.75-fold increase in GFAT promoter activity (P < 0.01, n = 4). Furthermore, the addition of PKC inhibitors BIM4 (10^{-6} M) or calphostin C (10^{-7} M) to transfected MC previously exposed to PMA abrogated the PMA-induced activation of the GFAT promoter (Fig. 5B), confirming that PKC activates the GFAT promoter.

To further examine the role of PKC in ANG II activation of the GFAT promoter, experiments were performed as above, except that MC were not exposed to PMA but were instead exposed to PKC inhibitors BIM4 (10^{-6} M) or calphostin C (10^{-7} M), for 1 h. After the medium was changed, MC were maintained in medium (DMEM/0.5% FBS/5.6 mM glucose) containing ANG II (10^{-8} M) for 24 h. Figure 5C demonstrates that the activation of the GFAT promoter by ANG II was inhibited by both BIM4 and calphostin C, implicating PKC in this signaling pathway.

ANG II activation of the GFAT promoter depends on protein tyrosine kinase. Total phosphotyrosine increase markedly in cells exposed to ANG II (25, 39, 54, 59), and many ANG II effects are sensitive to protein tyrosine kinase (PTK) inhibition (25, 39, 59). Accordingly, we tested whether inhibition of these signaling components affected the GFAT induction observed in MC exposed to ANG II. Following transfection of MC, all experiments were conducted in DMEM/0.5% FBS containing physiological (5.6 mM) glucose concentrations.

to determine the role of tyrosine phosphorylation in the intracellular signaling in response to ANG II that ultimately results in GFAT induction, transiently transfected MC were cultured in DMEM/0.5% FBS/5.6 mM glucose.

Fig. 5. Activation of the GFAT promoter by ANG II is calcium dependent and protein kinase C (PKC) dependent. Rat MC (70–80% confluent, 1.5 × 10^5 cells/well) cultured in 20% FBS/DMEM were cotransfected with plasmids pGFAT (0.35 μg) and pCMV-βgal (0.05 μg). A: 6 h later, media were changed to 0.5% FBS/DMEM containing 5.6 mM glucose. Cells were incubated for an additional 24 h in the presence and absence of ANG II (10^{-8} M) or 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (10^{-6} M) or the calcium ionophore A23187 (25 × 10^{-7} M). B: MC transiently transfected with pGFAT were exposed to phorbol 12-myristate 13-acetate (PMA, 5 × 10^{-7} M) or vehicle [dimethyl sulfoxide (DMSO)] for 30 min. PMA or vehicle was removed, and cells were washed with PBS, then exposed to PKC inhibitors bisindolylmaleimide-4 (BIM4, 10^{-6} M) or calphostin C (10^{-7} M), or to vehicle (DMSO) for 1 h. Media were removed, and cells were washed with PBS, then cultured for an additional 24 h in DMEM/0.5% FBS/5.6 mM glucose. C: MC were exposed to PKC inhibitors BIM4 (10^{-6} M) or calphostin C (10^{-7} M) for 1 h. After the medium was changed, MC were maintained in medium (DMEM/0.5% FBS/5.6 mM glucose) containing ANG II (10^{-8} M) for 24 h. For all studies, luciferase and β-galactosidase activities were determined using 0.02 ml and 0.05 ml of MC lysate, respectively. Luciferase activity was normalized to protein and β-galactosidase activity. All experiments were done in triplicate. Values are means ± SD. *P < 0.01 vs. all other conditions (n = 3). #P < 0.05 vs. all other conditions (n = 3).
mM glucose with and with ANG II (10⁻⁸ M) and the tyrosine kinase inhibitor genistein (10⁻⁴ M) or its inactive analog daidzein (10⁻⁴ M) or vehicle (DMSO). Luciferase activity was determined as before. As illustrated in Fig. 6A, genistein prevented activation of the GFAT promoter by ANG II, suggesting that tyrosine kinase activity also plays a role in the signaling that links ANG II and GFAT induction. Specificity of effect was ensured by demonstration that an inactive analog, daidzein (10⁻⁴ M), did not affect the ANG II activation of the GFAT promoter (Fig. 6A).

Src kinases have been implicated in ANG II signaling (50, 55). In addition, ANG II signaling may proceed via transactivation of the EGF receptor in a calcium-dependent manner and may lead to activation of gene transcription mediated by mitogen-activated protein kinases (MAPK) (4, 11, 45). To further define the downstream pathway(s) that may participate in ANG II-mediated activation of the GFAT promoter, we examined the effect of AG-1478 (an EGF receptor inhibitor), PP2 (an inhibitor of the Src family tyrosine kinases), and PD-98059 (an inhibitor of p42/44 MAPK) on activation of the GFAT promoter by ANG II.

MC transiently transfected with the GFAT promoter-luciferase were exposed to either AG-1478 (250 nM), PD-98059 (10⁻⁴ M), PP2 (10⁻⁵ M), or vehicle (DMSO) for 1 h. The media were removed, and cells were washed with PBS, then subsequently MC were cultured for 24 h in medium (DMEM/0.5% FBS/5.6 mM glucose) in the presence and absence of ANG II (10⁻⁸ M). As illustrated in Fig. 6B, both PP2 and AG-1478 abrogated the effect on ANG II on the GFAT promoter. The MAPK inhibitor PD-98059 significantly attenuated activation of the GFAT promoter by ANG II (P < 0.02 vs. ANG II, n = 4; P < 0.05 vs. PD-98059, n = 4). These findings suggest that activation of the GFAT promoter by ANG II depends on src kinases and EGF receptor transactivation and on downstream activation of p42/44 MAPK.

The hexosamine pathway participates in ANG II-induced increases in mRNA for TGF-β1 and VCAM-1. ANG II activates the genes for TGF-β1 and VCAM-1, and both TGF-β1 and VCAM-1 have been implicated in glomerular injury (46, 61, 63). Therefore, to determine whether activation of GFAT is important in the downstream effects of ANG II on genes that have been implicated in glomerular injury, we examined whether the effect of ANG II on TGF-β1 and VCAM-1 mRNA levels in MC was dependent on flux through the hexosamine pathway.

MC transiently transfected with the GFAT promoter-luciferase construct were exposed to the GFAT inhibitors azaserine (2 × 10⁻⁵ M) and DON (2 × 10⁻⁵ M) for 24 h. As shown in Fig. 7A, these inhibitors had no effect on basal activity of the GFAT promoter or on the activation of the GFAT promoter by ANG II.

To further determine whether ANG II-mediated increase in gene expression was related to hexosamine pathway flux, we studied the effect of BADGP, a specific inhibitor of O-glycosylation (21, 33), on activation of the VCAM-1 promoter by ANG II. In MC transiently transfected with the VCAM-1 promoter-luciferase reporter and subsequently exposed to ANG II (10⁻⁷ M), there was a 2.6-fold increase in luciferase activity that was attenuated when BADGP (1 mM) was present in the medium (Fig. 7B).

We next studied the effect of DON and azaserine on MC mRNA levels for VCAM-1 and TGF-β1 in the absence and presence of ANG II. Neither DON nor azaserine affected basal expression of VCAM-1 and TGF-β1 (Fig. 7, C and D). When MC were exposed to ANG II for 16 h, there was a significant increase in mRNA levels for both VCAM-1 and TGF-β1, which was attenuated by DON and azaserine (Fig. 7, E and F).
Fig. 7. The hexosamine pathway participates in ANG II-induced increases in mRNA for transforming growth factor-β1 (TGF-β1) and vascular cell adhesion molecule-1 (VCAM-1). A: ANG II activation of the GFAT promoter is unaffected by azaserine and 6-diazo-5-oxonorleucine (DON). MC transiently transfected with the GFAT promoter-luciferase construct as before were exposed to ANG II (10^{-8} M) and DON (2 × 10^{-5} M) were added to the medium, and cells were cultured for 24 h. Control MC were not exposed to ANG II, azaserine, or DON. B: benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP) prevents activation of the VCAM-1 promoter by ANG II. MC were transiently transfected with pVCAM (0.35 μg) and pCMV-βgal (0.05 μg) and subsequently cultured (DMEM/0.5% FBS/5.6 mM glucose) for 24 h with and without ANG II (10^{-8} M) or BADGP (1 mM), an inhibitor of O-glycosylation. Control MC were not exposed to ANG II or BADGP. Luciferase activity was determined as described earlier. C–F: DON and azaserine do not affect basal expression of TGF-β1 (C) or VCAM-1 (D) but attenuate ANG II-induced increase in mRNA for TGF-β1 (E) and VCAM-1 (F). Nontransfected MC were cultured (DMEM/0.5% FBS/5.6 mM glucose) without or with ANG II, azaserine (2 × 10^{-5} M), or DON (2 × 10^{-5} M) for 16 h. Control MC were not exposed to ANG II, azaserine or DON. Total RNA was isolated and RT-PCR was performed per main text. β-Actin was coamplified to standardize for the amount of RNA subjected to reverse transcription. Amplification was allowed to proceed for 25 cycles. PCR product were separated on 1% agarose gel (top), and densitometry was performed (bottom). Experiments were performed in triplicate, and a representative autograph is shown. Values are mean ± SD, and all conditions were done in triplicate. *P < 0.01 vs. control, DON, or azaserine only (n = 3). †P < 0.01 vs. control, DON, or azaserine only (n = 3). **P < 0.02 vs. control (n = 3). ‡P < 0.01 vs. ANG II (n = 3). §P < 0.05 vs. ANG II (n = 3).
High glucose increases GFAT mRNA and activates the GFAT promoter in MC. A final series of experiments were designed to determine whether glucose had any effect on the mRNA levels for GFAT and on GFAT promoter activity in MC. Six hours after transfection with pGFAT and pCMV-βgal, MC were exposed to ANG II (10^{-8} M) for 24 h in medium (DMEM/0.5% FBS) containing either physiological (5.6 mM) or high (30 mM) glucose concentrations, and promoter activity was assessed by measuring luciferase activity normalized to β-galactosidase activity. Control experiments with MC cultured in DMEM/0.5% FBS containing physiological glucose concentration (5.6 mM) were performed concurrently.

We observed a 2.5-fold induction of the GFAT promoter activity by ANG II (10^{-8} M) in physiological glucose (5.6 mM) compared with control MC at 24 h (P < 0.01, n = 5) (Fig. 8A). In contrast, high glucose (30 mM) produced a 1.75-fold increase in GFAT promoter activity over the same time. When cells were exposed to ANG II (10^{-8} M) and high glucose (30 mM), there was no significant increase in the GFAT promoter activity compared with that with ANG II alone (Fig. 8A). Subsequent dose-response studies showed that GFAT promoter activity increased significantly following exposure to 20 mM glucose for 24 h, with no further increases in medium containing 30 mM glucose (data not shown).

To examine the effect of glucose on GFAT mRNA levels, nontransfected MC were growth arrested in DMEM/0.5% FBS, then exposed to ANG II (10^{-8} M) in physiological glucose (5.6 mM) or to 20 mM glucose for 8 h. At the end of the incubation period, total RNA was extracted and used for RT-PCR analysis (9, 16, 17). RT-PCR analysis (Fig. 8B) indicated that after 8 h of exposure to ANG II (10^{-8} M) or glucose (20 mM), there was a 1.8-fold increase in GFAT mRNA.

**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. ANG II plays a central role in the progression of diabetic glomerular disease (5, 18, 36, 41, 69). ACE inhibition or the use of an AT1 receptor antagonist abrogates the ANG II-mediated increase in TGF-β1 and attenuates the development of glomerular sclerosis in both diabetic and nondiabetic models (5, 18, 36, 41, 70).

Recently, a new metabolic pathway for intracellular glucose, the hexosamine pathway, has been described, and it may play a role in diabetic injury. Kolm-Litty and co-workers (31) have suggested that glucose flux through the hexosamine pathway (Fig. 1) is important in the pathogenesis of diabetic glomerulopathy. In the hexosamine pathway, fructose-6-phosphate is first converted to glucosamine-6-phosphate by the rate-limiting enzyme GFAT (40). Under normal physiological conditions, only a small percentage (1–3%) of glucose entering cells is shunted through this pathway (40). The end product of the hexosamine pathway, uridine diphosphate N-acetylgalactosamine (UDP-GlcNAc), is a substrate for O-glycosylation of intracellular proteins (19,
5.6 mM glucose (O32, 40, 52, 56).

EXPERIMENTAL PROCEDURES.

and 1

and exposed to ANG II for 16 h. Total RNA was isolated as before, cells/well) were growth arrested in DMEM/0.5% FBS/5.6 mM glucose

media were changed to DMEM/0.5% FBS/5.6 mM glucose, and cells

cultured in 20% (FBS)/DMEM were cotransfected with plasmids pGFAT (0.35

amount of RNA subjected to reverse transcription. Amplification was

allowed to proceed for 25 cycles. PCR products were separated on 1%

agarose gel (top), and densitometry was performed (bottom). All

experiments were performed in triplicate, and a representative au-

tograph is shown. In A and B, values are means ± SD. *P < 0.01 vs. 5.6 mM glucose (n = 5), **P < 0.05 vs. 5.6 mM glucose (n = 5). *P < 0.01 vs. control (n = 3).

GFAT mRNA levels in MC.

A

Fig. 8. High glucose activates the GFAT promoter and increases GFAT mRNA levels in MC. A: MC (70–80% confluent, 1.5 x 10⁵ cells/well) were growth arrested in DMEM/0.5% FBS/5.6 mM glucose (5.6 or 30 mM). Cells were washed with PBS and lysed, and luciferase and β-galactosidase activities were determined. B: nontransfected MC (70–80% confluent, 1.5 x 10⁵ cells/well) were growth arrested in DMEM/0.5% FBS/5.6 mM glucose and exposed to ANG II for 16 h. Total RNA was isolated as before, and 1 μg of total RNA was used for RT-PCR as described in EXPERIMENTAL PROCEDURES. β-Actin was coamplified to standardize for the amount of RNA subjected to reverse transcription. Amplification was allowed to proceed for 25 cycles. PCR products were separated on 1% agarose gel (top), and densitometry was performed (bottom). All experiments were performed in triplicate, and a representative autorograph is shown. In A and B, values are means ± SD. *P < 0.01 vs. 5.6 mM glucose (n = 5). **P < 0.05 vs. 5.6 mM glucose (n = 5). *P < 0.01 vs. control (n = 3).

GFAT is transcriptionally regulated (50), ANG II also activated the GFAT promoter in a dose- and time-dependent fashion. Most of the actions of ANG II are mediated by binding to the AT1 receptor at the cell surface (4, 27, 39). ANG II-induced activation of the GFAT promoter was mediated by the AT1 receptor, because candesartan, the specific AT1 receptor antagonist, completely prevented GFAT promoter activation by ANG II, whereas the AT2 receptor antagonist was without effect.

The AT1 receptor is a seven-transmembrane protein that lacks inherent PTK activity, and the activity of this receptor is coupled to G proteins at the plasma membrane (2, 26, 38, 59). Following the ligation of the AT1 receptor by ANG II, intracellular signaling may proceed by three possible pathways (2, 26, 38). ANG II binding to the AT1 receptor leads to activation of phospholipase C-γ and the generation of inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ promotes calcium release from the endoplasmic reticulum and consequently increases intracellular calcium levels that may lead to activation of gene transcription (43, 44). On the other hand, DAG activates PKC, and the latter may then influence target gene expression (23, 38, 39). Finally, when ANG II binds to the AT1 receptor, intracellular tyrosine kinase signaling pathways, including MAPK, are activated, leading to changes in gene transcription (24, 39).

Our data indicate that ANG II regulates GFAT promoter activity by modulating signaling pathways that include calcium, PKC, and tyrosine kinase cascades. A direct role for PKC in ANG II-mediated GFAT promoter activation is suggested by the observations that PMA was sufficient to activate the promoter and, second, that inhibitors of PKC prevented GFAT promoter activation by ANG II. On the other hand, our observation that an intracellular calcium chelating agent, BAPTA, attenuated the activation of the GFAT promoter by ANG II suggests calcium was necessary for ANG II-mediated activation of the GFAT promoter. However, the inability of the calcium ionophore A23187 to activate the promoter suggests calcium did not have an independent effect on GFAT promoter activation. The effect of ANG II on isolated glomeruli may depend on interactions between PKC and calcium signaling (12), and calcium may enhance PKC activity (48). Therefore, it is possible that calcium-PKC interactions may play a role in GFAT promoter activation by ANG II. Similarly, calcium-dependent tyrosine
phosphorylation of intracellular protein has been described (22); therefore, it is also possible that calcium may participate in GFAT promoter activation via this latter mechanism.

Some of the actions of ANG II are dependent on activation of tyrosine kinase signaling cascades (39, 54). We first looked at the effect of a nonspecific PTK inhibitor (genistein) and its inactive analog on ANG II-induced GFAT promoter activity. The activation of the GFAT promoter by ANG II was abolished by the tyrosine kinase inhibitor genistein but not its inactive analog, implicating tyrosine kinase signaling in the promoter response to ANG II. To further characterize the effect of PTK inhibition on ANG II-induced GFAT promoter activation, we studied the Src kinase inhibitor PP2 and the EGF-specific receptor tyrophostin AG-1478, because src kinases and transactivation of the EGF have been implicated in ANG II signaling (50, 55). Finally, we studied the effect of PD-98059, an inhibitor of p42/44 MAPK, on activation of the GFAT promoter by ANG II. The src kinase inhibitor, the EGF receptor tyrophostin, and PD-98059 inhibited the response to ANG II. Taken together, these results suggest that one important pathway linking ANG II and activation of the GFAT promoter is via transactivation of the EGF receptor with the subsequent downstream activation of p42/44 MAPK. Transactivation of the EGF receptor and MAPK activation has been implicated in the signaling response to ANG II (4, 11, 17, 45, 67, 68). Furthermore, signaling pathways including src, calcium and PKC have been implicated in the response to ANG II by vascular smooth muscle cells (55) and in the response to neuromodulators by Swiss 3T3 cells (53). The regulatory elements in the GFAT promoter (58) (GenBank accession no. U39442) that are responsible for ANG II-induced activation were not identified in the current study. However, the PKC dependence, taken together with the observation that PMA is sufficient to activate the promoter, suggests that two AP1 sites, located at positions −542 and −309 of the promoter, may be responsible for the effects of ANG II (4, 34, 35).

ANG II is known to activate transcription of TGF-β1 and other profibrotic and proinflammatory cytokines (30, 46, 61, 62, 69, 71). In addition, ANG II induces VCAM-1 expression (63), and ACE inhibition decreases the level of soluble VCAM-1 in type II diabetics (13, 14). We have also shown that GFAT overexpression leads to activation of genes that are important in fibrosis (28). Thus we sought to determine whether flux through the hexosamine pathway mediated some of the downstream effects of ANG II. Inhibition of GFAT, the rate-limiting enzyme for flux through the hexosamine pathway, with azaserine or DON, attenuated but did not abolish the effect of ANG II on VCAM-1 and TGF-β1 mRNA levels in MC. Furthermore, using BADGP, an inhibitor of O-glycosylation (21, 33), also prevented the ANG II-mediated increase in GFAT promoter activity. These results suggest that part of the MC response to ANG II is dependent on flux through the hexosamine pathway.

Both the Diabetes Control and Complications Trial Research Group and the UK Prospective Diabetes Study Group trials (10, 64) have also shown that glycemic control is a key determinant of diabetic microvascular injury. Accordingly, we further sought to determine whether ANG II and high glucose concentrations would have independent, additive, or synergistic effects on GFAT promoter activity in MC. In a previous study, Paterson and Kudlow (49) found that elevated glucose concentrations alone had no impact on GFAT promoter activity in a breast cell cancer line, but when combined with EGF, glucose (25 mM) attenuated activation of the GFAT promoter by EGF. In contrast to this report, we observed that high glucose levels (20 and 30 mM) activated the GFAT promoter in cultured MC. Thus there are cell-specific differences in the regulation of the GFAT promoter. High glucose concentrations have been shown to downregulate AT1 receptor density in MC and to increase PKC isoform expression (1). The sum of these effects on ANG II-dependent signaling responses in the MC is difficult to predict, so we looked at the activation of the GFAT promoter by ANG II in MC maintained in 30 mM glucose. Although each condition led to an increase in promoter activity, there was no additive or synergistic effect between glucose and ANG II.

We believe that our finding that ANG II activates the promoter for GFAT has important implications in view of the role of ANG II in the progression of renal disease, particularly because ANG II is known to activate transcription of TGF-β1 and other profibrotic and proinflammatory cytokines (30, 46, 61, 62, 69, 71). In addition, we have also shown that GFAT overexpression leads to activation of genes for plasminogen activator inhibitor-1 (PAI-1), TGF-β1, and TGF-β receptors in MC (28). Therefore, ANG II may also affect expression of genes that promote glomerulosclerosis by influencing glucose flux through the hexosamine pathway.

Although the regulation of GFAT promoter activity by ANG II has been studied with a mouse promoter, we believe that these results are relevant to humans. The cDNA for mouse GFAT has 91% homology with the human sequence, and the amino acid sequence of mouse GFAT shows 98.6% homology with human GFAT (57). Given that mice and rats have been extensively used as models for studying diabetes mellitus and its complications (including glomerular diseases), we believe that our model system will have relevance to understanding how GFAT is regulated in human cells and will provide insights into the role that the hexosamine pathway may play in the pathogenesis of diabetic complications such as nephropathy.

In summary, our findings support the hypothesis that ANG II, acting via the AT1 receptor, increases expression of GFAT, the rate-limiting enzyme for glucose entry into the hexosamine pathway. ANG II regulates GFAT promoter activity in cultured MC by modulating signaling pathways that include calcium, PKC, src kinases, the EGF receptor, and p42/44 MAPK. These findings suggest that a link between ANG II and the metabolic fate of glucose may contribute to the
development of diabetic complications such as vascular and glomerular injury.

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