Angiotensin II blockade prevents hyperglycemia-induced activation of JAK and STAT proteins in diabetic rat kidney glomeruli

Amy K. Banes, Séan Shaw, John Jenkins, Heather Redd, Farhad Amiri, David M. Pollock, and Mario B. Marrero. Angiotensin II blockade prevents hyperglycemia-induced activation of JAK and STAT proteins in diabetic rat kidney glomeruli. Am J Physiol Renal Physiol 286: F653–F659, 2004. First published December 16, 2003; 10.1152/ajprenal.00163.2003.—Clinical and animal studies show that treatment with angiotensin-converting enzyme (ACE) inhibitors or ANG II-receptor antagonists slows progression of nephropathy in diabetes, indicating ANG II plays an important role in its development. We previously reported that hyperglycemia augments both ANG II-induced growth and activation of Janus kinase (JAK)2 and signal transducers and activators of transcription (STAT) proteins in cultured rat mesangial cells. Furthermore, we demonstrated that the tyrosine kinase enzyme JAK2 plays a key role in both ANG II- and hyperglycemia-induced growth in these cells. We hypothesized that the ACE inhibitor captopril and the ANG II-receptor antagonist candesartan would hinder hyperglycemic-induced activation of JAK and STAT proteins in rat glomeruli, demonstrating that ANG II plays an important role in the activation of these proteins in vivo. Adult male Sprague-Dawley rats were given either streptozotocin (STZ; 60 mg/kg iv) or vehicle, and glomeruli were isolated 2 wk later. Activation of JAK and STAT proteins was evaluated by Western blot analysis for specific tyrosine phosphorylation. Groups of rats were given captopril (75–85 mg·kg⁻¹·day⁻¹), candesartan (10 mg·kg⁻¹·day⁻¹), or the JAK2 inhibitor AG-490 (5 mg·kg⁻¹·day⁻¹) for the study’s duration. STZ-stimulated glomerular phosphorylation of JAK2, STAT1, STAT3, and STAT5. Phosphorylation was reduced in rats treated with captopril, candesartan, and AG-490. Furthermore, both candesartan and AG-490 inhibited STZ-induced increases in urinary protein excretion. In conclusion, our studies demonstrate that hyperglycemia induces activation of JAK2 and the STATs in vivo via an ANG II-dependent mechanism and that these proteins may be involved in the early kidney damage associated with diabetes.

glomerular mesangial cells; Janus kinase/signal transducers and activators of transcription pathway

SEVERAL LINES OF EVIDENCE indicate that the renin angiotensin system (RAS) plays an important role in the renal complications seen in patients with type 1 (insulin dependent) diabetes mellitus as well as in animals with experimental diabetes (2,6). Because ANG II is an important modulator of glomerular filtration, it is believed that its inhibition can effectively prevent glomerular structural injury. Administration of angiotensin-converting enzyme (ACE) inhibitors can maintain glomerular pressure and flow within normal limits despite the presence of pronounced hyperglycemia (5), suggesting a possible role of ANG II in the development of glomerular injury. Moreover, ANG II mediates its effects through high-affinity membrane-bound receptors, namely, the ANG II type 1 receptor (AT1) and the ANG II type 2 receptors (AT2), which have been classified recently with the aid of specific nonpeptide antagonists (9). All the vasoconstrictor and mitogenic effects of ANG II have been attributed to AT1, which has a high affinity for selective nonpeptide antagonists such as losartan and candesartan (9). On the other hand, the AT2 receptor, which has a high affinity for the selective nonpeptide antagonist PD-123319, is thought to act as a biological antagonist of the AT1 receptor because it has been shown to exert antiproliferative effects as well as mediating programmed cell death (9). In addition to this first level of classification, AT1 receptors have been further subdivided into AT1a and AT1b receptors, both of which are expressed in the rat kidney but cannot be distinguished pharmacologically (9).

As with other G protein-coupled receptors, the signaling pathways coupled to AT1 receptors are diverse. For example, we have recently shown that in rat kidney glomerular mesangial cells (GMC), exposure to hyperglycemia results in the activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. That is, exposure of GMC to hyperglycemia induces the tyrosine phosphorylation of JAK2, which was accompanied by the tyrosine and/or serine phosphorylation of STAT1, STAT3, and STAT5 (3). In addition, we have shown that the activation of JAK2 was essential for both ANG II- and hyperglycemia-induced collagen IV production and GMC growth (3). Furthermore, we have also recently demonstrated that the activation of JAK2 and STAT1 proteins was a requirement for the hyperglycemia-induced production of transforming growth factor-β (TGF-β) and fibronectin in GMC (16). Therefore, it appears that the activation of JAK2 and STAT proteins by hyperglycemia might play an important role in both promoting cell proliferation and synthesis of extracellular matrix molecules. However, it is not known whether high levels of glucose like those found in diabetes can influence JAK2 and STAT signaling in vivo and whether blocking the action of ANG II would have any effect on the activation of the JAK/STAT pathway. Therefore, in the present study we examined the effects of hyperglycemia on the activation of JAK2 and STAT proteins in the rat kidney glomeruli and the role that ANG II may play in the hyperglycemia-induced activation of the JAK/STAT pathway in vivo.

MATERIALS AND METHODS

STZ-induced diabetes. All studies were conducted with the approval of the Medical College of Georgia animal care committee in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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accompanying with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (225–250 g) from Harlan Laboratories (Indianapolis, IN) were rendered diabetic by a single intravenous injection of streptozotocin (STZ; 60 mg/kg) made up in fresh 0.1 M citrate buffer, pH 4.5. Rats were briefly anesthetized with isoflurane (Abbott Laboratories, N. Chicago, IL), and the injection was given into the jugular vein. Age-matched control rats received buffer only. The diabetic state was confirmed 48 h later by measurement of tail blood glucose level, using an Accu-check glucometer. All rats given STZ had a blood glucose concentration exceeding 15 mM and thus were considered diabetic.

In the first series of experiments, two groups of STZ-treated rats were studied: a control diabetic group and a group treated with captopril (75–85 mg·kg⁻¹·day⁻¹) added to their drinking water (n = 12 each). A second series of experiments examined four groups: an untreated nondiabetic control group, a control group given the AT₁-receptor antagonist candesartan in their drinking water. A third series of experiments were treated with 2 U·min⁻¹·kg⁻¹ insulin (Humulin, sc, Eli Lilly, Indianapolis, IN) daily to prevent ketoadiposis (2). Animals receiving high-dose insulin (HI) to normalize their blood glucose levels were given a sustained release insulin implant (Linplant, LinShin Canada, Toronto, ON). All animals were fed standard Purina rat chow (Ralston Purina, Richmond, IN), had free access to tap water ad libitum, and were kept on a 12:12-h light-dark cycle. In the second and third series of experiments, rats were placed in metabolic cages for measurement of renal excretory function and systolic blood pressure via the tail-cuff method (14) 2 wk after STZ injection.

Isolation of glomeruli. Two weeks after STZ or control buffer injection, rats were anesthetized with pentobarbital sodium, and the glomeruli were isolated as described above except that the isolation buffers used were provided with the TransAm kit. The glomerular isolations were then analyzed by following the directions of the TransAm kit, and the absorbance was read at 450 nm on a spectrophotometer (BioTek, Winooski, VT).

Urinalysis. Urinary concentrations of sodium and potassium were determined by ion-selective electrodes (Beckman EL-ISE, Brea, CA). The protein concentration for each sample was assessed by a modification of Bradford’s method (17). Urinary creatinine concentrations were measured using a 96-well plate adaptation of the picric acid method (1).

Chemicals. Molecular mass standards, acrylamide, SDS, N,N'-methylene-bisacrylamide, N,N,N',N'-tetramethylenediamine, protein assay reagents, and nitrocellulose membranes were purchased from Bio-Rad Laboratories (Hercules, CA), and collagenase type I was from Worthington Biochemical (Freehold, NJ). Protein A/G-agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Candesartan was a kind gift from Dr. Peter Morsing at AstraZeneca Pharmaceuticals. Monoclonal antibody to phosphorysorine (PY20), SHP-1, SHP-2, JAK1, JAK2, and TYK2 were purchased from Transduction Laboratories (Lexington, KY). Anti-phosphorysorine antibodies for STAT1 and STAT3 were purchased from New England Biolabs (Beverly, MA), whereas anti-phosphorysorine STAT6 was acquired from Upstate Biotechnology (Lake Placid, NY). Anti-STAT5A/B and anti-phosphorysorine STAT5A/B antibodies were also acquired from Upstate Biotechnology. The anti-phosphorysorine antibodies for JAK1, JAK2, and TYK2 were obtained from Biosource International (Camarillo, CA). The Pierce Supersignal chemiluminescence detection kit was obtained from Pierce (Rockford, IL). The TransAm STAT family transcription factor assay kit was acquired from Active Motif North America (Carlsbad, CA). AG-490 (tyrphostin AG-490) was obtained from LC Laboratories (Woburn, MA). Goat anti-mouse IgG and anti-rabbit IgG were obtained from Amersham (Princeton, NJ), and Tween 20 and all other chemicals were purchased from Sigma (St. Louis, MO).

Data analysis. Data from in vivo studies were evaluated using ANOVA analysis’s protected least significant difference post hoc test to compare significant differences between individual means. Densitometry was performed using a digital imaging system (Alpha Innotech, Staffordshire, UK) and analyzed with ANOVA and a
Physiological effects of the AT$_1$-receptor antagonist candesartan. In agreement with previous findings (6), treatment of rats with STZ resulted in a significant increase in urinary volume compared with the nondiabetic control rats (Table 1). Creatinine excretion significantly increased in STZ-treated rats, consistent with hyperfiltration at this stage of diabetes. Treatment with the AT$_1$-receptor antagonist candesartan had no effect on the elevated levels of sodium, potassium, and creatinine excretion in the STZ-induced diabetic rats (Table 1).

In addition, treatment with candesartan lowered the systolic blood pressure in nondiabetic rats but not in the STZ-treated rats (Table 1). In the STZ-treated diabetic rats, concurrent treatment with candesartan significantly reduced protein excretion (Fig. 1).

Physiological effects of the JAK2 inhibitor AG-490. As seen in the previous experimental groups, rats treated with STZ showed a significant increase in urinary volume compared with the nondiabetic control rats (Table 2). Interestingly, STZ-induced diabetic rats treated with AG-490 showed a significant reduction in urine output (Table 2) as well as a significant reduction in fluid intake (data not shown). Creatinine excretion was again significantly increased in STZ-treated rats. Treatment with the JAK2 inhibitor AG-490 had no effect on the elevated levels of sodium, potassium, and creatinine excretion in the STZ-induced diabetic rats (Table 2). However, in STZ-treated diabetic rats, treatment with AG-490 significantly reduced protein excretion (Fig. 2).

Treatment with AG-490 had no effect on systolic blood pressure of either the control or STZ-treated rats. Treatment of rats with STZ that also received insulin to normalize their blood glucose levels (88 ± 3.1 mg/dl) had no effect on any of the parameters measured (data not shown).

ANG II AT$_1$ receptor blockade effects on hyperglycemia-induced activation of JAK and STAT proteins in vivo. While we have previously documented that both hyperglycemia and ANG II have the ability to activate JAK and STAT proteins through the AT$_1$ receptor in GMC in vitro (3), this activation by both hyperglycemia and ANG II has not been studied under in vivo conditions in rat kidney glomeruli. Therefore, we investigated the ability of hyperglycemia to stimulate the tyrosine phosphorylation of the different JAK and STAT proteins found within rat kidney glomeruli. We found that hyperglycemia induced the tyrosine phosphorylation of JAK2, and tyrosine and serine phosphorylation of STAT1, STAT3, and STAT5, whereas JAK1, TYK2, and STAT6 tyrosine phosphorylation and the serine phosphorylation of STAT6 were not affected compared with controls (Figs. 3–5). In rats treated with STZ and insulin to normalize their blood glucose levels, there were no differences in the levels of phosphorylated JAK2 between control and STZ-treated rats with normalized blood glucose levels (data not shown).

We next examined the tyrosine and serine phosphorylation of STAT1, STAT3, and STAT5A/B in diabetic rats with the AT$_1$-receptor antagonist candesartan. Candesartan treatment obstructed the hyperglycemia-induced activation of JAK2 and the STAT transcription factor proteins (Figs. 3 and 4). We also found that treatment of normal rats with candesartan alone had no effect on the activation of JAK2 and the STAT proteins (Figs. 2 and 3). Furthermore, we have also found that treatment of diabetic rats that were administered the ACE inhibitor captopril prevented the hyperglycemia-induced tyrosine and serine phosphorylation of STAT1, STAT3, and STAT5, which strongly suggests that ANG II plays a key role in the hyperglycemic-induced activation of the STAT transcription factors (Fig. 6).

Effect of the JAK2 inhibitor AG-490 on the in vivo activation of the JAK2. To directly implicate JAK2 activation in vivo, we administered the JAK2 inhibitor AG-490. We found that AG-

### Table 1. Urinary excretion rates and blood pressure measurements

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<th>Control</th>
<th>Candesartan</th>
<th>STZ+ Candesartan</th>
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<tr>
<td>Urinary volume, ml/day</td>
<td>14±1</td>
<td>15±1</td>
<td>180±6*</td>
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<td>Sodium excretion, mmol/day</td>
<td>1.3±0.1</td>
<td>1.5±0.1</td>
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<td>Potassium excretion, mmol/day</td>
<td>5.2±0.4</td>
<td>5.8±0.2</td>
<td>11.0±0.5*</td>
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<td>Creatinine excretion, g/day</td>
<td>1.01±0.08</td>
<td>1.01±0.11</td>
<td>1.36±0.18*</td>
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<tr>
<td>Systolic blood pressure, mmHg</td>
<td>139±3</td>
<td>103±5*</td>
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Values are means ± SE; n = 12/group. Urinary excretion rates and blood pressure in control and streptozotocin (STZ)-treated rats with or without candesartan treatment are shown. *P < 0.05 vs. control.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AG-490</th>
<th>STZ+ AG-490</th>
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<tbody>
<tr>
<td>Urinary volume, ml/day</td>
<td>17±1</td>
<td>24±2</td>
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<td>Sodium excretion, mmol/day</td>
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<td>Potassium excretion, mmol/day</td>
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<td>Creatinine excretion, g/day</td>
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<td>Systolic blood pressure, mmHg</td>
<td>131±3</td>
<td>130±6*</td>
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Values are means ± SE; n = 6/group. Urinary excretion rates and blood pressure in control and STZ-treated rats with or without AG-490 treatment are shown. *P < 0.05 vs. control.
490 (5 mg·kg⁻¹·day⁻¹ ip) significantly reduced the phosphorylation of JAK2 in rat kidney glomeruli in vivo (Fig. 7). In control rats administered AG-490, we found no difference between the control levels of JAK2 phosphorylation and those in the control rats with AG-490 (Fig. 7).

Effect of AG-490, candesartan, and hyperglycemia on transcriptional activation of STATs. Using the TransAM transcriptional assay, we investigated the effects of the JAK2 inhibitor AG-490, the AT₁-receptor antagonist candesartan, and hyperglycemia on the transcriptional ability of STAT1, STAT3, and STAT5. We found that hyperglycemia alone increased the transcriptional activation of STAT5A, STAT5B, STAT3, and STAT1 (Fig. 8). This was significantly reduced by administration of AG-490 and candesartan. In addition, we found that STZ administration without an increase in blood glucose levels had no effect (Fig. 8). These data further suggest that an
increase in blood glucose levels is a key factor in the activation of the JAK/STAT pathway in vivo.

Effect of hyperglycemia on the activation of the tyrosine phosphatases SHP-1 and SHP-2 in rat kidney glomeruli. We have previously shown that the phosphorylation state of JAK2 is tightly regulated by the two cytoplasmic phosphotyrosine phosphatases (PTPases), SHP-1 and SHP-2, in GMC (3). Therefore, we investigated the activation of these two cytosolic PTPases under hyperglycemic conditions in rat kidney glomeruli by examining their tyrosine phosphorylation states. We found that SHP-1 phosphorylation was completely abolished under hyperglycemic conditions (Fig. 9). SHP-2 tyrosine phosphorylation, on the other hand, was increased by hyperglycemia (Fig. 9). Furthermore, as with JAK2 phosphorylation, we also observed that the AT1-receptor antagonist candesartan significantly reduced hyperglycemia-induced tyrosine phosphorylation of SHP-2. These results suggest that sustained JAK2 activation under hyperglycemic conditions might be partly due to decreased SHP-1 and increased SHP-2 phosphorylation. These results are in accordance with our previous findings that clearly demonstr-

DISCUSSION

In the present study, we have shown that hyperglycemia altered the activation of the JAK/STAT pathway in vivo through ANG II in rat kidney glomeruli by inducing the phosphorylation of JAK2 kinase and different STAT proteins, namely, STAT1, STAT3, and STAT5A/B. Activation of STAT proteins by many different extracellular proteins including plasma-derived growth factor (11), epidermal growth factor (15), vascular endothelial growth factor (7), and ANG II via the AT1 receptor (11, 12) has provided a mechanism of action for the exaggerated growth seen in many pathophysiological situations such as diabetic glomerulosclerosis (5, 6). Moreover, use of ACE inhibitors and AT1-receptor antagonists in diabetic nephropathy has suggested that ANG II may be an important modulator of GMC growth (5, 6). As reported previously (3, 4), ANG II has the ability to activate STAT proteins by phosphorylating JAK kinases. However, our studies provide new information that the activation process, which we measured as phosphorylation levels, occurs in an in vivo model of diabetic nephropathy that exhibits abnormal glomerular function determined by protein excretion.

To determine the activation of the JAK/STAT pathway, we initially looked at the hyperglycemia-induced JAK2 tyrosine phosphorylation. We found a significant difference in the phosphorylation levels of JAK2 under hyperglycemic conditions compared with control. Interestingly, phosphorylation levels of JAK1 and TYK2 were not increased under hyperglycemic conditions, suggesting specificity and differential activation of members of the JAK kinase family. Consequently, we investigated the possible cause and effects of increased JAK2 tyrosine phosphorylation. Among the leading causes of an increased JAK2 tyrosine phosphorylation is the possible down-regulation of tyrosine phosphatases (13). To this end, we investigated the phosphorylation of two cytosolic tyrosine phosphatases, SHP-1 and SHP-2. We found that while SHP-1 phosphorylation was completely abolished under hyperglycemic conditions, SHP-2 phosphorylation was increased. We also found that blockade of AT1 receptors significantly reduced hyperglycemia-induced tyrosine phosphorylation of SHP-2. These results suggest that sustained JAK2 activation under hyperglycemic conditions might be partly due to decreased SHP-1 and increased SHP-2 phosphorylation. These results are in accordance with our previous findings that clearly dem-

Fig. 6. A: representative Western blots of stimulation of STAT1, STAT3, and STAT5 tyrosine phosphorylation in glomeruli of control, STZ, and captopril-treated STZ rats. B: densitometric analysis of Western blots (n = 3). *P < 0.05 vs. control. †P < 0.05 vs. STZ.

Fig. 7. A: representative Western blot of stimulation of pJAK2 and the effects of the JAK2 inhibitor AG-490 on tyrosine phosphorylation in glomeruli of control nondiabetic rats, control nondiabetic rats treated with AG-490, STZ-induced diabetic rats, and STZ-induced diabetic rats treated with AG-490. B: densitometric analysis of Western blots (n = 6). *P < 0.05 vs. control. †P < 0.05 vs. STZ.
strate that SHP-1 is the phosphatase responsible for JAK2 protein dephosphorylation, whereas SHP-2 appears to play a role in the phosphorylation of JAK2 (13). We propose that SHP-2 is acting as an adaptor protein for JAK2 association with the AT1 receptor, thereby facilitating JAK2 phosphorylation and activation as we have previously demonstrated in vitro with GMC cultures (3).

We have also determined, in this study, that the STAT proteins are activated in vivo by hyperglycemia in rat kidney glomeruli. It has been postulated that tyrosine phosphorylation plays a critical role in STAT protein activation (8). Therefore, we employed anti-phosphospecific STAT antibodies, and found that, while hyperglycemia induces the phosphorylation of these STAT proteins on tyrosine residues, the ACE inhibitor, the AT1-receptor antagonist and the JAK2 inhibitor significantly blocked these phosphorylations. The tyrosine phosphorylation of the various STAT proteins observed under hyperglycemic conditions is not unique to this study (3, 4, 16). However, this is the first time that the activation of STAT proteins has been demonstrated in an in vivo setting. It has also been well established that JAK2 plays an important role in growth and proliferation of GMC in vitro (3). Again, our observations are the first to demonstrate that the activation of JAK2 occurs in an in vivo setting model of glomerular dysfunction. These findings provide further support for the hypothesis that the JAK/STAT pathway plays an important role in GMC growth and extracellular matrix deposition in diabetic nephropathy. Therefore, future studies will need to focus on the

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**Fig. 9.** A: representative Western blots of the effects of candesartan on STZ-induced tyrosine phosphorylation of tyrosine phosphatases SHP1 and SHP2 in rat kidney glomeruli. B: densitometric analysis of Western blots (n = 3). *P < 0.05 vs. control. †P < 0.05 vs. STZ.
function of JAK2 and STAT proteins and their role in growth and extracellular matrix deposition under in vivo conditions.

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GRANTS

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