Inhibition of the JAK/STAT Signaling Pathway Prevents the High Glucose-Induced Increase in TGF-β and Fibronectin Synthesis in Mesangial Cells

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High glucose (HG) causes glomerular mesangial cell (GMC) growth, production of transforming growth factor (TGF)-β, and increased synthesis of matrix proteins such as fibronectin, contributing to diabetic nephropathy. We recently found that exposure of cells to HG also activates the growth-promoting enzyme janus kinase 2 (JAK2) and its latent signal transducers and activators of transcription (STAT) transcription factors (STAT1, STAT3, and STAT5). Our purpose was to determine the effect that inhibition of JAK2 and these STAT transcription factors has on the HG-induced increase in TGF-β and fibronectin synthesis in GMC. Exposure of GMC to 25 mmol/l glucose caused the activation of JAK2, STAT1, STAT3, and STAT5 plus an increase in TGF-β and fibronectin synthesis, as compared with 5.5 mmol/l glucose. This HG-induced increase in synthesis of TGF-β and fibronectin was prevented by concomitant incubation with AG-490, a specific JAK2 inhibitor. The HG-induced JAK2, STAT1, and STAT3 tyrosine phosphorylations in GMC were also abolished by AG-490. Preincubation of GMC cultured in 25 mmol/l glucose with a specific JAK2 or STAT1 antisense oligonucleotide also prevented both TGF-β and fibronectin synthesis. These results provide direct evidence for linkages between JAK2, STAT1, and the glucose-induced overproduction of TGF-β and fibronectin in GMC. *Diabetes* 51:3505–3509, 2002

One of the basic underlying mechanisms of diabetic nephropathy seems to involve high glucose (HG)-induced production of transforming growth factor-β (TGF-β) and extracellular matrix molecules such as fibronectin (1,2). Glomerular mesangial cells (GMCs) cultured under HG conditions produce TGF-β and extracellular matrix molecules at a significantly faster rate than those cultured under normal glucose (NG) conditions (1,2). In addition, HG increases the de novo synthesis of the protein kinase C activator diacylglycerol (3). Thus, a mechanism by which HG induces GMC production of TGF-β and extracellular matrix molecules may be through the chronic activation of one or more isoforms of protein kinase C (4). However, other mechanisms have been suggested by which HG can stimulate GMC production of TGF-β and extracellular matrix molecules. These mechanisms include the nonenzymatic modification of macromolecules to form advanced glycation end products, changes in sorbitol and myoinositol metabolism, increased oxidant formation, and activation of mitogen-activated protein (MAP) kinase pathways (5,6).

We have recently shown that in vascular smooth muscle cells (VSMCs), exposure to HG results in the activation of the janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. That is, exposure of VSMC to HG induces the tyrosine phosphorylation of JAK2 and complex formation of JAK2 with the angiotensin II (Ang II) AT_1 receptor (7). We also demonstrated that the HG-induced tyrosine phosphorylation of JAK2 was accompanied by the tyrosine and/or serine phosphorylation of STAT1 and STAT3 and increased VSMC proliferation (7). In addition, we have shown that the activation of JAK2 was essential for both the Ang II and platelet-derived growth factor–induced MAP kinase pathway activation and VSMC growth (8). Finally, another group has recently demonstrated that the activation of JAK2 and STAT proteins was a requirement for the advanced glycation end products–induced production of extracellular matrix molecules in NRK-49F cells (9). Therefore, it seems that the activation of JAK2 and STAT proteins by HG might play an important role in both promoting cell proliferation and synthesis of extracellular matrix molecules. However, it is not known whether HG can influence JAK2 and STAT signaling in GMC. Therefore, in the present study, we examined the effects of HG on the activation of JAK2 and STAT proteins and the role that this JAK2 and STAT proteins activation might play in the HG-induced production of TGF-β and the extracellular matrix molecule fibronectin in GMC.

RESEARCH DESIGN AND METHODS

Materials. Molecular weight standards, acrylamide, SDS, N-N′-methylenebisacrylamide, 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); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, and all medium additives were obtained from Mediatech (Herndon, VA). Monoclonal antibodies to phosphotyrosine (PY20), JAK2, STAT1, and STAT3 were procured from Transduction Laboratories (Lexing-

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Ang II, angiotensin II; ELISA, enzyme-linked immunosorbent assay; GMC, glomerular mesangial cell; HG, high glucose; JAK2, janus kinase 2; MAP, mitogen-activated protein; NG, normal glucose; STAT, signal transducers and activators of transcription; TGF, transforming growth factor; VSMC, vascular smooth muscle cell.

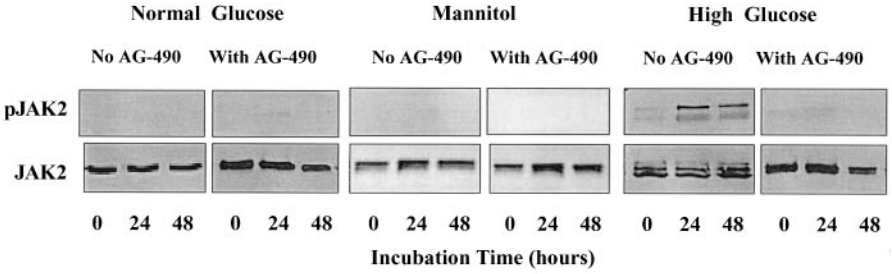

FIG. 1. Effects of the JAK2 inhibitor AG-490 on the HG-induced tyrosine phosphorylation of JAK2. Quiescent GMCs were incubated, with or without 10 μmol/l AG-490, for 24 and 48 h in serum-free medium containing either NG (5.5 mmol/l) or HG (25 mmol/l). Cells were lysed, and lysates were immunoblotted with either phosphotyrosine-specific or non–phospho-specific anti-JAK2 antibodies. Shown are representative immunoblots of three immunoblots probed with either the JAK2 phosphotyrosine-specific antibody (pJAK2) or JAK2 antibody (JAK2).

ton, KY). Antiphosphotyrosine antibodies for STAT1 and STAT3 were purchased from New England Biolabs (Beverly, MA); anti-STAT5 and antiphosphotyrosine STAT5 antibodies were acquired from Upstate Biotechnology (Lake Placid, NY).

Cell isolation and culture. GMCs were obtained from isolated, collagenase-treated rat glomeruli as previously described (10). In brief, glomeruli were harvested from male 275- to 300-g Sprague-Dawley rats by filtration with ice-cold 0.9% NaCl solution through a 200-, 150-, 120-, and 50-μm nylon mesh. Those retained on the sieve were collected, washed by centrifugation (4°C, 2000*g*), and incubated with 250 units/ml collagenase (type I) for 30 min at 37°C under constant, gentle shaking. GMCs were plated on plastic tissue culture flasks in DMEM (pH 7.4) either with NG (5.5 mmol/l) or with HG (25 mmol/l) or NG plus mannitol (19.5 mmol/l) concentrations. The culture medium was supplemented with 17% (vol/vol) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.5 units/ml insulin, and 10 μl/ml Fungizone, an antimycotic agent. The cells were incubated at 37°C in humidified 5% CO_2/95% air. The cell medium was left untouched for 4 days and then changed every other day until confluence. Cells were subcultured at 1:6 at 7-day intervals, and the medium was changed at 2-day intervals. GMC passages 1–6 were grown to 75–85% confluence, washed once with serum-free DMEM, and growth-arrested in serum-free DMEM in NG for 24 h to synchronize the cell growth. After this time, the medium was changed to fresh serum-free medium containing either NG or HG or NG plus mannitol for 24–48 h.

JAK2, STAT1, and STAT3 antisense oligonucleotide treatment. JAK2, STAT1, and STAT3 antisense oligonucleotide synthesis and treatments were conducted as previously described (11,12). After 24 h, the medium was removed, 0.1% calf serum/DMEM in NG was added, and the cells were allowed to recover for 30 min. Afterward, the GMCs were washed once with serum-free DMEM and growth-arrested in serum-free DMEM for 24 and 48 h in either NG (5.5 mmol/l) or HG (25 mmol/l).

Western blotting for JAK2 and STAT tyrosine phosphorylations. For ascertaining the effects of HG on the tyrosine phosphorylation of JAK2, STAT1, STAT3, and STAT5, serum-starved GMCs were placed in either NG or HG medium for 24 and 48 h. At the end of desired periods, cells were washed twice with ice-cold PBS with 1 mmol/l Na_3VO_4. Each dish was then treated for 60 min with ice-cold lysis buffer (20 mmol/l Tris-HCl [pH 7.4], 2.5 mmol/l EDTA, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1% SDS, 10 mmol/l $Na_4P_2O_7$, 50 mmol/l NaF, 1 mmol/l Na_3VO_4, and 1 mmol/l phenylmethylsulfonyl fluoride), and the supernatant fraction was obtained as cell lysate by centrifugation at 58,000*g* for 25 min at 4°C. Protein concentration for each sample was assessed by a modification of Bradford's method (13).

Subsequently, samples were resolved by 10% SDS-PAGE. The gel was then transferred to a nitrocellulose membrane and blocked by 60-min incubation at room temperature (22°C) in TTBS (TBS with 0.05% Tween-20, pH 7.4) plus 5% skimmed milk powder. The nitrocellulose membrane was incubated overnight at 4°C with antiphosphotyrosine-specific JAK2 and STAT antibodies. Subsequently, the nitrocellulose membranes were washed twice for 10 min each with TTBS and incubated for various times with goat anti-rabbit IgG horseradish peroxidase conjugate. After extensive washing, the bound antibody was visualized on Kodak Biomax film, Pierce Supersignal substrate chemiluminescence detection kit. Molecular weight markers assessed specificity of the bands.

Fibronectin Western blots. Fibronectin secreted from cultured GMCs at 48 h in either NG or HG in the presence or absence of the JAK2 inhibitor or JAK2, STAT1, or STAT3 antisense oligonucleotides was analyzed by Western blot analysis. The respective supernatant containing an appropriate amount of protein was subjected to SDS-PAGE in a 10% gel. After completion of electrophoresis, proteins were transferred onto nitrocellulose membrane in a transfer buffer (50 mmol/l Tris-HCl [pH 7.0], 380 mmol/l glycine, and 20% methanol). The blots were blocked in 5% nonfat dry milk dissolved in PBS with 0.1% Tween 20. The membranes were incubated for 1 h with a polyclonal rabbit anti-rat fibronectin antibody (Chemicon International, Temecula, CA). The secondary antibody was peroxidase-conjugated goat anti-rabbit IgG antibody (Cappel, Durham, NC). The luminescence detection of peroxidase was performed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

TGF-β and fibronectin enzyme-linked immunosorbent assay. For quantifying the level of TGF-β or fibronectin proteins produced under the different experimental conditions, total TGF-β or fibronectin proteins were measured in the GMC culture supernatant using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit for TGF-β (R&D Systems, Minneapolis, MN) or competitive inhibition ELISA for fibronectin (Chemicon International) according to the manufacturer's descriptions.

Data analysis. All statistical comparisons were made using Student's t test for paired data and ANOVA. Data are reported as means ± SE for the number of separate plates of GMCs tested. Significance was $P < 0.05$.

RESULTS

Effects of HG and AG-490 on the tyrosine phosphorylation of JAK2, STAT1, STAT3, and STAT5. For determining whether HG induces the tyrosine phosphorylation of JAK2, GMCs were incubated for 24 and 48 h in serum-free medium containing either NG (5.5 mmol/l glucose) or HG (25 mmol/l glucose) with or without 10 μmol/l AG-490. Cells were then lysed, and equal amounts of lysate from each condition (30 μg of protein) were immunoblotted with a JAK2 phosphotyrosine-specific antibody that recognizes the tyrosine-phosphorylated but not the nonphosphorylated form of JAK2. As shown in Fig. 1, under NG conditions, there is no tyrosine phosphorylation of JAK2 (130-kDa band) detected. However, in HG conditions, JAK2 was tyrosine-phosphorylated at a significant level above NG conditions, at both 24 and 48 h, suggesting that HG promotes a constitutive activation of JAK2 even in the absence of growth factors. There was no significant difference, however, in the HG-induced JAK2 tyrosine phosphorylation between the 24- and 48-h HG exposure. In addition, AG-490 completely inhibited the HG-induced JAK2 tyrosine phosphorylation at both 24 and 48 h (Fig. 1). Lysates were also immunoblotted with an anti-JAK2 antibody that recognizes both phosphorylated and nonphosphorylated forms of JAK2. Equal amounts of JAK2 were detected for all conditions by the non–phospho-specific antibody, indicating that the differences detected with the phosphotyrosine-specific antibody were not due to differences in the amount of total JAK2 protein loaded in each lane (Fig. 1).

We also investigated the ability of HG to stimulate the tyrosine phosphorylation of the different STAT proteins found within GMC. We found that HG induced the tyrosine phosphorylation of STAT1, STAT3, and STAT5 (Fig. 2). That is, in HG conditions, STAT1, STAT3, and STAT5 all were tyrosine-phosphorylated at a significant level above NG conditions, at both 24 and 48 h, suggesting again that HG promotes a constitutive activation of these STATs even in the absence of growth factors. Furthermore, we found that whereas the JAK2 specific inhibitor AG-490 completely inhibited the HG-induced STAT1 and STAT3

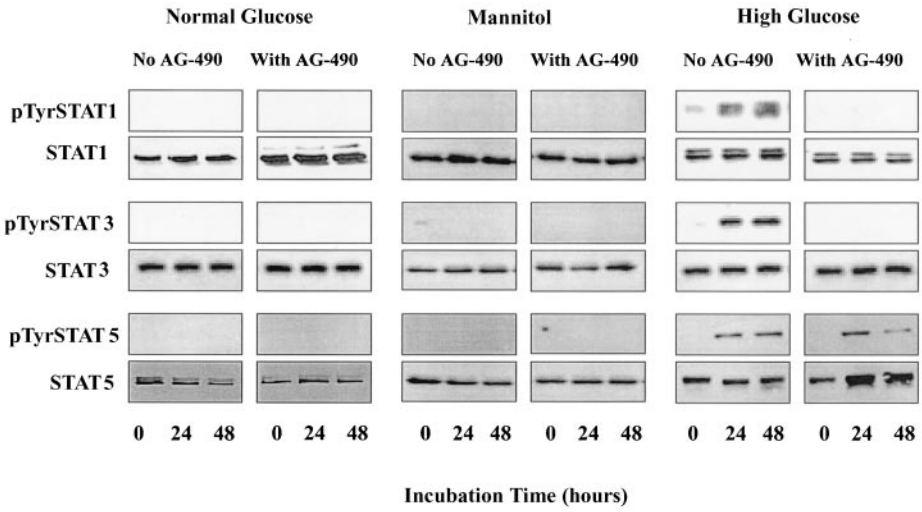

FIG. 2. Effects of the JAK2 inhibitor AG-490 on the HG-induced tyrosine phosphorylation of STAT1, STAT3, and STAT5. Quiescent GMCs were incubated, with or without 10 μmol/l AG-490, for 24 and 48 h in serum-free medium containing either NG (5.5 mmol/l) or HG (25 mmol/l). Cells were lysed, and lysates were immunoblotted with phosphotyrosine-specific or non–phospho-specific anti-STAT1, STAT3, and STAT5 antibodies. Shown are representative immunoblots of three immunoblots probed with either the STAT1, STAT3, and STAT5 phosphotyrosine-specific antibodies (pSTAT) or STAT antibody (STAT).

tyrosine phosphorylations at both 24 and 48 h (Fig. 2), STAT5 tyrosine phosphorylation was not affected (Fig. 2). These results suggest that JAK2 plays an important role in the HG activation of STAT1 and STAT3 but not STAT5.

Effects of mannitol and AG-490 on the tyrosine phosphorylation of JAK2, STAT1, STAT3, and STAT5. In the experiments shown in Figs. 1 and 2, as well as all others described in the present study, controls were included to determine whether the effects of HG might be due to hyperosmolarity. Thus, in addition to the NG control (5.5 mmol/l glucose), we included a control of 5.5 mmol/l glucose plus 19.5 mmol/l mannitol. As shown in Figs. 1 and 2, 5.5 mmol/l glucose plus 19.5 mmol/l mannitol had no effect on either JAK2 (Fig. 1) or STAT (STAT1, STAT3, and STAT5; Fig. 2) tyrosine phosphorylation. Furthermore, in all other experiments described thereafter, no effects of hyperosmolarity (glucose plus mannitol) were seen on any of the other signaling events examined (TGF-β or fibronectin synthesis; data not shown).

Effects of HG on TGF-β production and the role of JAK2 and STAT1. With the TGF-β ELISA, using acid-activated conditioned media, we found a significant increase in TGF-β protein under HG at 48 h (Fig. 3). To test the effects of JAK2, STAT1, and STAT3 on the TGF-β production, we used two approaches. Our first approach was to use the JAK2 specific inhibitor AG-490 (14), and the second approach was the antisense approach using JAK2, STAT1, and STAT3 antisense oligonucleotides. We have already demonstrated, in a previous study (11), that the JAK2 antisense directed against the JAK2 translation initiation site inhibited the JAK2 synthesis in GMC. In this study, we now show the both STAT1 and STAT3 antisense oligonucleotides directed against their translation initiation sites (12) also prevents the synthesis of both STAT1 and STAT3 (Fig. 4).

We found that by either depleting the GMC of JAK2 by preincubating the cells with the JAK2 antisense or inhibiting JAK2 with the specific inhibitor AG-490, the HG-induced TGF-β protein synthesis in GMC was significantly inhibited (Fig. 3). In addition, we found that depleting the GMC of STAT1 by preincubating the cells with the STAT1 antisense also significantly inhibited the HG-induced TGF-β protein synthesis (Fig. 3). However, depleting the GMC of STAT3 by preincubating the cells with the STAT3 antisense had no effect on the HG-induced TGF-β protein synthesis (Fig. 3). These results suggest that JAK2 activation of STAT1 plays a key role in the HG-induced TGF-β synthesis.

Effects of HG on fibronectin production and the role of JAK2 and STAT1. The effect of HG on the synthesis of

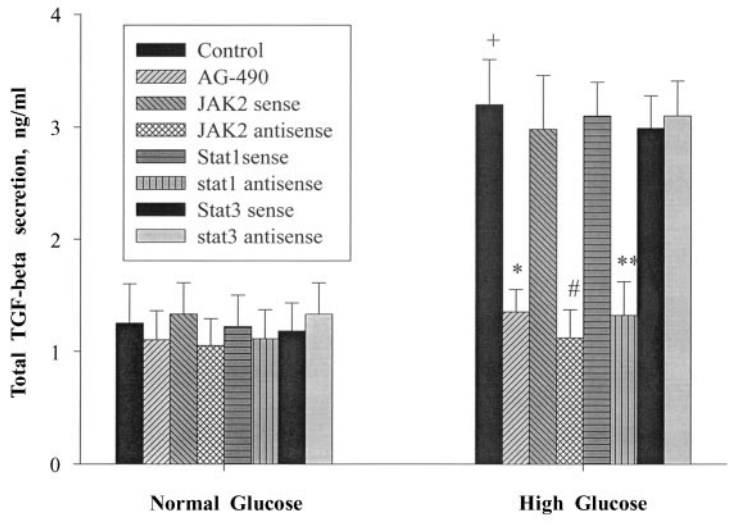

FIG. 3. Effect of glucose and JAK2 or STAT inhibition on TGF-β production in GMCs. Quiescent GMCs were incubated, with or without 10 μmol/l AG-490, for 48 h in serum-free medium containing either NG (5.5 mmol/l) or HG (25 mmol/l) or pretreated for 24 h with either JAK2, STAT1, or STAT3 sense or JAK2, STAT1, or STAT3 antisense oligonucleotides. GMCs were cultured and the TGF-β production was assessed via ELISA as described in RESEARCH DESIGN AND METHODS. Results represent the mean ± SE of six independent cultures and are expressed as nanograms of TGF-β protein produced per milliliter of cultured medium. HG significantly induced (+P < 0.01) TGF-β synthesis, which was significantly inhibited by preincubation with the JAK2 inhibitor AG-490 (*P < 0.01). HG-induced TGF-β synthesis was also significantly inhibited by preincubation with the JAK2 antisense oligonucleotide (#P < 0.01) or the STAT1 antisense oligonucleotide (P < 0.01). STAT3 antisense oligonucleotide pretreatment had no effect.**

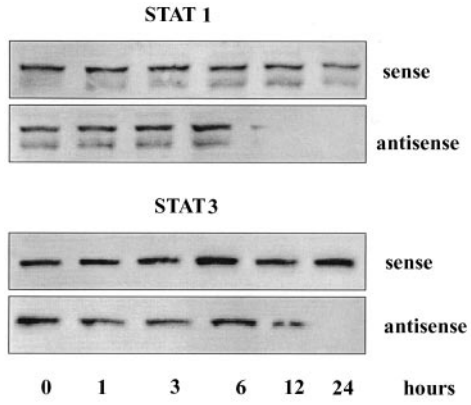

FIG. 4. Effect of STAT1 and STAT3 sense and antisense oligonucleotides on STAT1 and STAT3 expression in GMCs. GMCs were treated with STAT1 or STAT3 sense and antisense oligonucleotides for the times indicated and lysed, and STAT1 or STAT3 were immunoprecipitated from the lysates with the specific anti-STAT1 or anti-STAT3 antibody. Precipitated proteins were then immunoblotted with the specific anti-STAT1 or anti-STAT3 antibody.

fibronectin was also studied by measuring fibronectin concentration in the medium by either ELISA (Fig. 5) or Western analysis (Fig. 6). As anticipated, accumulation of fibronectin in the medium over 48 h was significantly higher in GMC cultures that contained 25 mmol/l glucose than in cultures that contained 5.5 mmol/l glucose. As demonstrated for TGF-β, preincubation with the JAK2 inhibitor AG-490 or exposure to the JAK2 and STAT1 antisense oligonucleotide significantly prevented the increase in fibronectin accumulation in GMCs grown in 25 mmol/l glucose (Figs. 5 and 6). Again, preincubation with the STAT3 antisense oligonucleotide did not significantly prevent the increase in fibronectin accumulation in GMCs grown in 25 mmol/l glucose (Figs. 5 and 6).

DISCUSSION

The results of these experiments demonstrate that HG stimulates JAK2, STAT1, STAT3, and STAT5 activity in renal GMCs and indicate that activation of both JAK2 and STAT1 participates in the HG-induced stimulation of TGF-β and production of the extracellular matrix protein fibronectin by these cells. This interpretation is based on the observations that JAK2 and STAT1 tyrosine phosphorylation increases in rat GMCs incubated in HG media versus rat GMCs incubated in NG and that both preincubation with the inhibitor of JAK2, AG-490, or the JAK2 and STAT1 antisense prevented the increases in TGF-β and fibronectin production that usually occur on exposure of rat kidney GMCs in culture to HG.

TGF-β is a key cytokine for matrix protein production, and HG has been previously shown to induce coordinated increases in TGF-β protein, the TGF type II receptor, and the extracellular matrix protein, fibronectin (1,2). HG has been shown to trigger a series of events, including protein kinase C and MAP kinase activation, elevation of reactive oxygen species, plus increased expression of *c-fos* and *c-jun* proto-oncogenes, which form a heterodimer, AP-1, that activates the TGF-β gene promoter (5). Therefore, given that JAK2 and STAT1 activation promotes *c-fos* gene expression and MAP kinase activation (8,15), our findings in the current study strongly suggest that the HG-induced activation of both JAK2 and STAT1 is linked to the HG-induced TGF-β and fibronectin overproduction.

The mechanism by which HG promotes JAK2 activation is speculative but may relate to an interaction of JAK2 with reactive oxygen species induced by HG. For example, it was shown recently that reactive oxygen species stimulate the activity of JAK2 in both fibroblasts and A-431 cells (16). Furthermore, another group of investigators have also shown that the reactive oxygen species formation inhibitor diphenylene iodonium significantly inhibited activation of JAK2 by Ang II in rat aorta smooth muscle cells, indicating that reactive oxygen species production contributes to JAK2 activation in response to Ang II (17). Therefore, all of these findings suggest that the JAK-STAT pathway responds to intracellular reactive oxygen species and that the vasoactive peptide Ang II uses reactive oxygen species as a second messenger to regulate JAK2 activation.

The findings reported herein demonstrate that HG modulates both JAK2 and STAT1, STAT3, and STAT5 activities in GMC and does so at concentrations of glucose that are typically found in patients with diabetes. Therefore, it is possible that the events observed in rat kidney GMC may occur in humans, but direct experimental confirmation is required. These observations afford new insight into the pathophysiological mechanisms that contribute to extracellular matrix accumulation in diabetic renal disease and

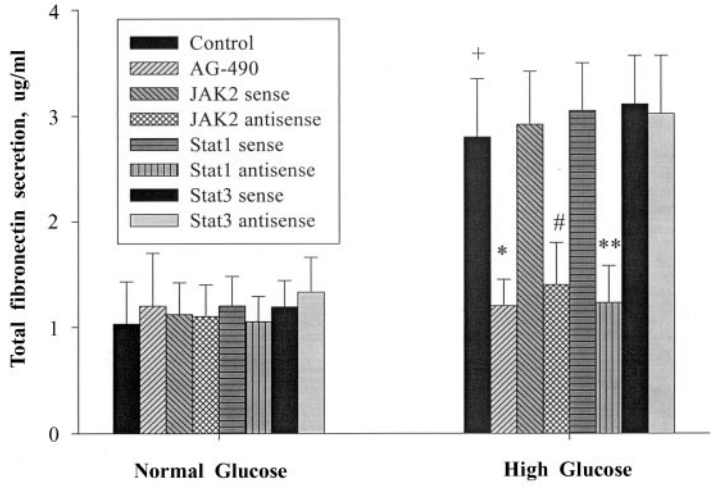

FIG. 5. Effect of glucose and JAK2 or STAT inhibition on fibronectin production in GMCs. Quiescent GMCs were incubated, with or without 10 μmol/l AG-490, for 48 h in serum-free medium containing either NG (5.5 mmol/l) or HG (25 mmol/l) or pretreated for 24 h with either JAK2, STAT1, or STAT3 sense or JAK2, STAT1, or STAT3 antisense oligonucleotides. GMCs were cultured, and fibronectin production was assessed via ELISA as described in RESEARCH DESIGN AND METHODS. Results represent the mean ± SE of six independent cultures and are expressed as micrograms of fibronectin protein produced per milliliter of cultured medium. HG significantly induced (+$P < 0.01$) fibronectin synthesis, which was significantly inhibited by preincubation with the JAK2 inhibitor AG-490 (*$P < 0.01$). HG-induced fibronectin synthesis was also significantly inhibited by preincubation with the JAK2 antisense oligonucleotide (#$P < 0.01$) or the STAT1 antisense oligonucleotide ($P < 0.01$). STAT3 antisense oligonucleotide pretreatment had no effect.**

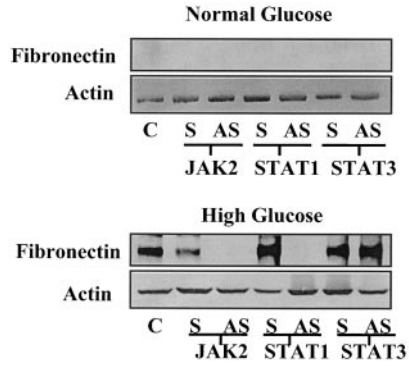

FIG. 6. Effect of glucose and JAK2 or STAT inhibition on fibronectin synthesis in cultured GMCs. Treatment of the GMCs was the same with the method described in Fig. 5, i.e., mesangial cells were treated with serum-free DMEM containing NG (5.5 mmol/l) or HG (25 mmol/l) for 48 h in the presence of JAK2, STAT1, or STAT3 sense (S) or antisense oligonucleotides (AS). Thirty micrograms of proteins in the respective supernatant was applied to a 10% SDS-PAGE. After completion of electrophoresis, fibronectin was analyzed by ECL-linked Western blot. Shown are representative immunoblots of three immunoblots probed with either a polyclonal antifibronectin or anti-actin antibody.

suggest that compounds such as AG-490 eventually could be effective treatment for prevention of diabetic nephropathy.

In conclusion, we have shown that HG stimulates TGF-β and fibronectin production in rat kidney GMC in culture in a JAK2-STAT1–dependent manner. Therefore, these results suggest that the JAK-STAT signaling cascade is an important mechanism by which elevated concentrations of glucose may contribute to nephropathy associated with diabetes.

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