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Angiotensin II activation of the JAK/STAT pathway in mesangial cells is altered by high glucose

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Angiotensin II activation of the JAK/STAT pathway in mesangial cells is altered by high glucose.

Background. Both high glucose (HG) and angiotensin II (Ang II) causes glomerular mesangial cell (GMC) growth and increased synthesis of matrix proteins like collagen IV contributing to diabetic nephropathy. We have recently found that exposure of vascular smooth muscle cells to HG augments the Ang II activation of the growth promoting JAK/STAT pathway. We hypothesized that Ang II activation of the JAK/STAT pathway is altered by HG in GMC, and that this pathway might be linked to the Ang II-induced growth and overproduction of collagen IV in GMC in HG conditions.

Methods. GMC were cultured under normal glucose (NG; 5.5 mmol/L) and HG (25 mmol/L) for 48 hours and stimulated with Ang II (0.1 μmol/L) for various times. GMC lysate was then immunoprecipitated and/or immunoblotted with SHP-1, SHP-2 and phosphospecific JAK2 and STAT antibodies. The HG and Ang II induced growth and collagen IV synthesis studies were performed in GMC transfected with JAK2 antisense or JAK2 sense. GMC growth was monitored via [^{3}H]-thymidine incorporation, and collagen IV synthesis via ELISA.

Results. We found that Ang II-induced JAK2, STAT1, STAT3, STAT5A/B and SHP-2 phosphorylations were enhanced by HG, whereas that of SHP-1 was reduced. Ang II-induced growth and collagen IV synthesis also were increased under HG conditions. Transfection of GMC with JAK2 antisense oligonucleotides blocked the Ang II-induced growth and collagen IV synthesis in both NG and HG conditions.

Conclusion. These results provide evidence that activation of the JAK/STAT pathway by HG or/and Ang II may be of importance in the increased GMC cell growth and collagen IV synthesis that is seen in diabetic nephropathy.

Key words: collagen IV, mesangial cells, glomerular mesangial cells, diabetic nephropathy, glycemic control, JAK2.

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The results of the Diabetes Control and Complications Trial have shown that strict glycemic control can prevent the onset and progression of diabetic complications [1]. Several hypotheses such as hyperosmolarity, glycation end products, oxidant formation, abnormality of sorbitol and myoinositol metabolism, and protein kinase C (PKC) activation through de novo synthesis of diacylglycerol (DAG) have been proposed to explain the various pathophysiological changes induced by high glucose (HG) [2–4]. These changes, which affect many cell types such as vascular smooth muscle cells (VSMC) and glomerular mesangial cells (GMC), include increases in contractility, cellular proliferation, permeability, and extracellular matrix (ECM) and cytokine production [5]. Moreover, the beneficial therapeutical effects of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (Ang II) type 1 receptor (AT_1) antagonists in the treatment of diabetic nephropathy [6, 7] suggest that the renin-angiotensin system (RAS) plays an important role in the renal complications seen in diabetic patients with insulin-dependent (type I) diabetes mellitus (DM), such as above-normal increases in glomerular filtration rate [8, 9].

It has long been established that GMC have microfilaments that contract in response to Ang II mediated by specific Ang II receptor subtypes [10], indicating a plausible role of GMC in the regulation of glomerular size, blood flow, and filtration via contraction [11]. The effects of Ang II are exerted through high affinity membrane-bound receptors, namely AT_1 and Ang II type 2 (AT_2) receptors [12]. Most of the known effects of Ang II have been attributed to AT_1, which has a high affinity for the selective antagonist losartan. On the other hand, AT_2 has a high affinity for the selective antagonist PD 123319. Both Ang II receptor types have been localized in humans and rats but their distribution is not uniform in all somatic tissues. Some organs, such as the liver, lung and kidneys, have a nearly homogenous population of AT_1 receptors whereas others, such as the pancreas and human uterus, contain almost uniquely the AT_2 subtype [13, 14].

Upon binding to the AT_1 receptor, Ang II activates several signal transduction pathways, including the DAG-PKC pathway [15], mitogen activated protein (MAP) kinase cascade [16] and, more recently, the JAK (Janus-activated kinase)/STAT (signal transducers and activators of transcription) pathway [17]. Furthermore, it has been shown that both the DAG-PKC, MAP kinase, as well as the JAK/STAT pathways are altered under HG conditions and these alterations could be responsible for some of the vascular dysfunction observed in the diabetic state [18–21], thus suggesting that glucose and its metabolites may mediate their adverse effects by altering the various signal transduction pathways employed by certain hormones. These signaling pathways have been implicated in a variety of cells, such as GMC, that seem to play a prominent role in diabetic glomerulosclerosis [19, 22]. However, to date the effect of HG in relation to the activation of the JAK/STAT pathway by Ang II in GMC has not been elucidated.

The JAK enzymes, namely JAK1, JAK2, JAK3 and TYK2, are responsible for the activation of the STATs (STAT1, STAT2, STAT3, STAT4, STAT5A/B and STAT6), which are latent cytoplasmic transcription factors [23, 24]. STATs, when activated by tyrosine and/or serine phosphorylation, form homo- and hetero-dimers and translocate to the nucleus where they regulate the expression of various genes involved in cellular proliferation [23].

Extensive studies have clarified that both the MAP kinase and JAK/STAT pathways are activated by multistep phosphorylation cascades after ligand-cell surface receptor binding and that they transmit signals to cytosolic and nuclear targets, leading to alterations in cell growth, proliferation and other cellular functions [24]. Because Ang II has been implicated in the pathogenesis of diabetic glomerulosclerosis, it is reasonable to suspect that activation of the JAK/STAT cascade by Ang II also could be modified by HG. In this study, we have shown that not only the Ang II-activation of JAK and STAT proteins (namely JAK2, and STAT1, STAT3 and STAT5A/B) is altered by HG in cultured GMC, but that JAK2 plays a pivotal role in both the HG and the Ang II-induced GMC growth and synthesis of the ECM protein collagen IV.

METHODS

Isolation and culture of glomerular mesangial cells

Glomerular mesangial cells were obtained from isolated, collagenase-treated rat glomeruli as previously described [19]. 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 U/mL collagenase (type I) for 30 minutes at 37°C under constant, gentle shaking. GMC were plated on plastic tissue culture flasks in Dulbecco's minimal essential medium (DMEM; pH 7.4) with either normal glucose (NG; 5.5 mmol/L), or 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 (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 U/mL insulin and 10 μL/mL of 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 four days and then changed every other day until confluence. Cells were subcultured at 1:6 at seven-day intervals and the medium was changed at two-day intervals. GMC passages 1 to 6 were grown to 75 to 85% confluence, washed once with serum-free DMEM and then growth-arrested in serum-free DMEM in NG for 24 hours to synchronize the cell growth. After this time period, the media was changed to fresh serum-free media containing either NG, or HG or NG plus mannitol for 48 hours prior to Ang II stimulation.

Immunoprecipitation studies of SHP-1 and SHP-2

To determine SHP-1 and SHP-2 phosphorylation, serum-starved GMC grown in either NG or HG were stimulated with 0.1 μmol/L Ang II for various times ranging from 0 to 30 minutes. At the end of stimulation, cells were washed twice with ice-cold PBS-V (phosphate-buffered saline with 1 mmol/L Na_3VO_4). Each dish was then treated for 60 minutes with ice-cold lysis buffer [20 mmol/L Tris-HCl, pH 7.4, 2.5 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 mmol/L $Na_4P_2O_7$, 50 mmol/L NaF, 1 mmol/L Na_3VO_4 and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)], and the supernatant fraction was obtained as cell lysate by centrifugation at 58,000 × *g* for 20 minutes at 4°C. The cell lysate was incubated with 10 μg/mL of either anti-SHP-1 or anti-SHP-2 monoclonal antibodies at 4°C for two hours and precipitated by addition of 50 μL of protein A/G agarose at 4°C overnight. The immunoprecipitates were then recovered by centrifugation and washed three times with ice-cold wash buffer (TBS, 0.1% Triton X-100, 1 mmol/L PMSF, and 1 mmol/L Na_3VO_4). Immunoprecipitated proteins were dissolved in 100 mL of Laemmli sample buffer and 80 mL of each sample was resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Subsequently, samples were transferred to a nitrocellulose membrane and blocked by a 60-minute 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 10 μg/mL of affinity-purified anti-phosphotyrosine antibodies and the bound

antibodies were visualized using a Pierce Supersignal chemiluminescence detection kit (Pierce Chemical Co., Rockford, IL, USA).

Western blotting studies of JAK and STAT proteins

To ascertain the phosphorylation of the various JAK and STAT proteins, serum-starved GMC were stimulated with 0.1 μmol/L Ang II for various times ranging from 0 to 90 minutes. At the end of stimulation, cells were washed twice with ice-cold PBS-V (PBS with 1 mmol/L Na_3VO_4). Each dish was then treated for 60 minutes 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 PMSF), and the supernatant fraction was obtained as cell lysate by centrifugation at 58,000 × *g* for 25 minutes at 4°C. Protein concentration for each sample was assessed by a modification of Bradford's method [25].

Subsequently, samples were resolved by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane and blocked by a 60-minute 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 affinity-purified antiphospecific JAK and STAT antibodies (namely JAK2, and STAT1, STAT3, STAT5A/B, respectively). Subsequently, the nitrocellulose membranes were washed twice for 10 minutes each with TTBS and incubated for various times with goat anti-rabbit IgG horseradish peroxidase conjugate. After extensive washing, bound antibody was visualized on Kodak Biomax film (Eastman Kodak, Rochester, NY, USA) using the Pierce Supersignal substrate chemiluminescence detection kit. Molecular weight markers assessed specificity of the bands.

Nuclei isolation of STAT proteins

To determine the nuclear translocation of the different STAT proteins, serum-deprived cells were stimulated as described above. GMC nuclei were isolated as described previously [17] by the method of Digman, Lebovitz and Roeder [26]. Briefly, GMC were washed three times with PBS-V and scraped in PBS-V using a cell scraper. The cell pellet was resuspended in buffer A (10 mmol/L Tris-HCl, pH 7.4, 3 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 1 mmol/L Na_3VO_4), gently homogenized and incubated for 25 minutes at 4°C. The lysate was centrifuged for 5 minutes, 500 × *g* at 4°C. The pellet was resuspended in the buffer B (buffer A + 0.5% Triton X-100), incubated for 25 minutes at 4°C and centrifuged again (5 min, 500 × *g* at 4°C). Cells were then homogenized with a Dounce homogenizer, sonicated lightly, poured atop of a 350 mmol/L sucrose gradient and centrifuged (500 × *g* at 4°C, 5 min). The final pellet was then resuspended in ice-cold lysis buffer and incubated for 30 minutes at 4°C. The mixture was then centrifuged (24,000 × *g*, 4°C, 30 min) and the supernatant was collected as the nuclear extract.

JAK2 antisense oligonucleotide treatment

JAK2 antisense oligonucleotide synthesis and treatments were carried out as previously described [27]. After 24 hours, the medium was removed, 0.1% calf serum/DMEM medium in NG was added, and the cells were allowed to recover for 30 minutes. Afterwards, the GMC were washed once with serum-free DMEM and growth-arrested in serum-free DMEM for 24 hours in NG (5.5 mmol/L).

Assessment of glomerular mesangial cell growth

[^{3}H]-thymidine incorporation and [^{14}C]-leucine incorporation were used as indices of GMC growth (hyperplasia and hypertrophy, respectively) and carried out as described [28, 29]. Briefly, quiescent GMC were plated into 96-well plates and maintained in serum-free NG or HG medium as described in the experimental protocol. After 44 hours of 0.1 μmol/L Ang II exposure, the cells were pulsed with [^{3}H]-thymidine (100 μCi/mL) or [^{14}C]-leucine (50 μCi/mL) and then harvested four hours later into TCA precipitable material. Cells were then washed with PBS, incubated in 10% TCA, and dried on filter paper. The paper was then washed three times with PBS and the samples placed in scintillation liquid and counted on a scintillation counter. We assessed the increase in cell number with a Coulter counter (model ZM; Coulter, Hialeah, FL, USA).

Collagen IV ELISA

Type IV collagen was measured by competitive enzyme-linked immunosorbent assay (ELISA) [30] using type IV collagen from an Engelbreth-Holm-Swarm (EHS) tumor as the standard, rabbit anti-mouse type IV collagen as the primary antibody (both from Collaborative Research, Medford, MA, USA), and horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) as the secondary antibody. The assay was performed on media collected at the end of the experimental incubation period.

Chemicals

Molecular weight standards, acrylamide, sodium dodecyl sulfate (SDS), N-N′-methylene-bisacrylamide, N,N,-N′,N′-tetramethylenediamine, protein assay reagents and nitrocellulose membranes were purchased from Bio-Rad Laboratories, and collagenase type I was from Worthington Biochemical (Freehold, NJ, USA). Protein A/G-agarose was obtained from Santa-Cruz Biotechnology (Santa Cruz, CA, USA) whereas Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and all medium additives were obtained from Mediatech

Inc. (Herndon, VA, USA). [^{3}H]-thymidine and [^{14}C]-leucine were purchased from ICN (Costa Mesa, CA, USA). Monoclonal antibody to phosphotyrosine (PY20), SHP-1, SHP-2, JAK2, STAT1 and STAT3 were procured from Transduction Laboratories (Lexington, KY, USA). Anti-phosphotyrosine antibodies for STAT1 and STAT3 were purchased from New England Biolabs (Beverly, MA, USA), whereas anti-phosphoserine STAT1 and STAT3 antibodies were acquired from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Anti-STAT5A/B and both anti-phosphotyrosine and anti-phosphoserine STAT5A/B antibodies also were acquired from Upstate Biotechnology Inc. Anti-phosphotyrosine JAK2 antibody was obtained from Biosource International (Camarillo, CA, USA). The Pierce Supersignal substrate chemiluminescence detection kit was obtained from Pierce (Rockford, IL, USA). Goat anti-mouse IgG and anti-rabbit IgG were acquired from Amersham (Princeton, NJ, USA), and tween-20, Ang II, and all other chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO, USA).

Data analysis

For Figure 7, a two-factor analysis of variance model was used to determine if differences in mean collagen IV synthesis existed between Ang II concentrations (0, 0.01, 0.1, 1.0 μmol/L) within glucose levels (normal and high) and between glucose levels within concentrations. For Figures 8, and 10 to 13, a two-factor analysis of variance (ANOVA) model was used for each figure to determine if differences in mean growth parameter (collagen IV synthesis, cell number, thymidine and leucine incorporation) existed between groups (Control, Ang II, AG-490, AG-490 + Ang II for Figure 8; Control, Ang II, antisense, Ang II + antisense, sense, Ang II + sense for Figures 10 to 13) within glucose level (normal and high) and between glucose levels within group. For all analyses, an alpha level of 0.05 was used to determine statistical significance. Tukey multiple comparison tests were used to determine differences regardless of whether the two-factor interaction in each model was statistically significant.

RESULTS

Effect of high glucose on the activation of JAK2 and SHP proteins by Ang II

We have shown previously that Ang II binding to the AT_1 receptor in VSMC induces a rapid tyrosine phosphorylation and activation of JAK2 under NG conditions [17]. However, it is not currently known whether the activation of JAK2 by Ang II is different in GMC and altered by HG. Therefore, we set forth to determine the effects of HG on the activation of JAK2 by Ang II in GMC. Under NG condition JAK2 was tyrosine phosphorylated in response to Ang II within 5 to 10 minutes, and this activation returned to basal levels after longer exposure (30 min) to Ang II (Fig. 1). Conversely, under HG conditions both basal and Ang II activated JAK2 tyrosine phosphorylation were enhanced when compared to NG conditions (Fig. 1). To ensure that the observed increase in JAK2 phosphorylation was due to elevated glucose concentration and not to an osmolarity effect, GMC also were grown in NG plus high (19.5 mmol/L) mannitol concentrations. As expected, no differences in JAK2 phosphorylation were found between normal and elevated mannitol concentrations (data not shown).

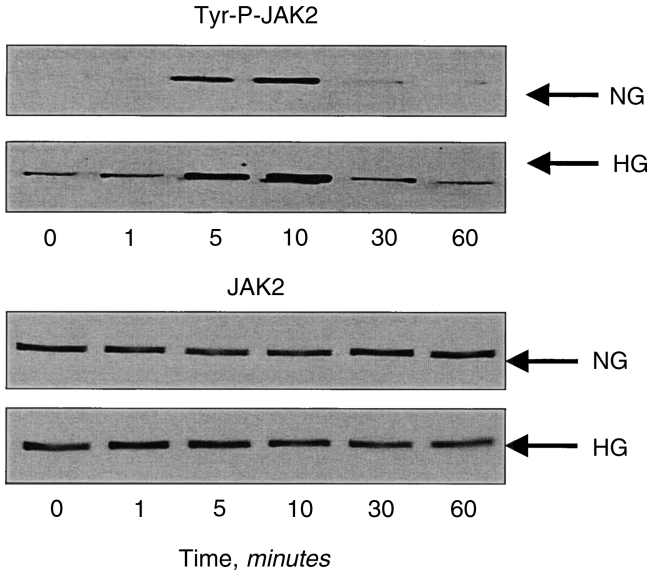

Fig. 1. Effects of high glucose on basal and angiotensin II (Ang II)-induced tyrosine phosphorylation of JAK2. Glomerular mesangial cells, incubated in normal glucose (NG; 5.5 mmol/L) or high glucose (HG; 25 mmol/L), were stimulated with Ang II (0.1 μmol/L) for various times (0, 1, 5, 10, 30, 60 min). Cells were lysed and immunoblotted with phosphotyrosine-specific and nonphosphospecific anti-JAK2 antibodies. Results shown are representative of three experiments.

Our previous study showed that the phosphorylation state of JAK2 is tightly regulated by the two cytoplasmic phosphotyrosine phosphatases (PTPase) SHP-1 and SHP-2 [31]. Therefore, we investigated the activation of these two cytosolic PTPases under NG and HG conditions in GMC by examining their tyrosine phosphorylation states. SHP-1 phosphorylation was completely abolished under HG conditions (Fig. 2). SHP-2 tyrosine phosphorylation, on the other hand, was increased under both basal and Ang II stimulation (Fig. 2). These results suggest that the JAK2 sustained tyrosine phosphorylation under HG concentration in mesangial cells might be due partly to changes on SHP-1 and SHP-2 activation. As with JAK2 phosphorylation, there were no differences between SHP-1 and SHP-2 phosphorylation

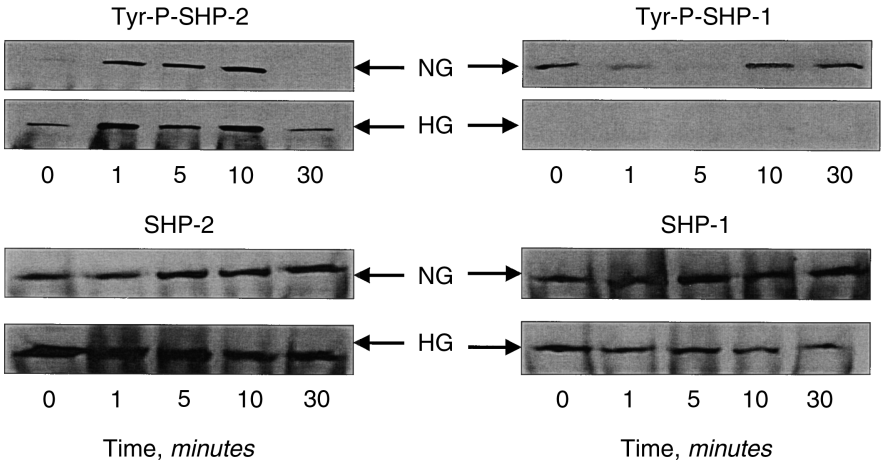

Fig. 2. Effects of high glucose on the Ang II-induced tyrosine phosphorylation of SHP-1 and SHP-2. Glomerular mesangial cells, incubated in normal glucose (5.5 mmol/L) or high glucose (25 mmol/L), were stimulated with Ang II (0.1 μmol/L) for various times (0, 1, 5, 10, 30 min). Cells were lysed, and SHP-1 or SHP-2 was immunoprecipitated from lysates (1 mg of protein for each condition) with either an anti-SHP-1 or anti-SHP-2 antibody. Immunoprecipitates were then immunoblotted with anti-phosphotyrosine antibody or with either an anti-SHP-1 or anti-SHP-2 antibody. Similar results were obtained in three experiments.

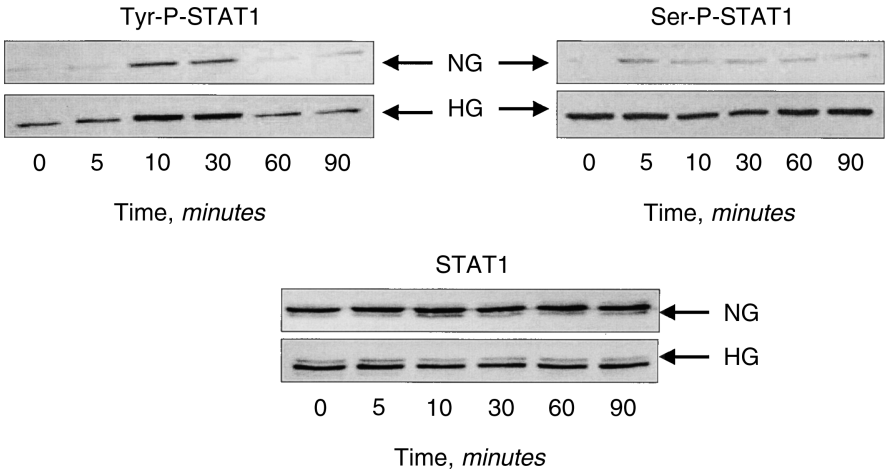

Fig. 3. Effects of high glucose on Ang II-induced tyrosine and serine phosphorylation of STAT1. Glomerular mesangial cells, under normal glucose (5.5 mmol/L) and high glucose (25 mmol/L), were stimulated with Ang II (0.1 μmol/L) for various times (0, 5, 10, 30, 60, 90 min). Cells were lysed and immunoblotted with phosphotyrosine- and phosphoserine-specific, and nonphosphospecific anti-STAT1 antibodies. Results shown are representative of four experiments.

in GMC cultured in NG and high mannitol (data not shown).

Effect of high glucose on the activation of STAT proteins by Ang II

Others and we have previously documented that Ang II has the ability to activate STAT proteins through the AT_1 receptor [17, 28, 32]. Nevertheless, this activation by Ang II has not been studied under HG conditions in GMC. Therefore, we investigated the ability of Ang II to stimulate tyrosine and/or serine phosphorylation of the different STAT proteins found within GMCs under NG and HG conditions. In the NG concentration, Ang II-induced STAT1 tyrosine phosphorylation occurred at 10 minutes and this STAT1 tyrosine phosphorylation returned to basal levels at 60 minutes, whereas STAT1 serine phosphorylation occurred around 5 minutes and remained stimulated even after 90 minutes of Ang II stimulation (Fig. 3). Under HG conditions, STAT1 was both tyrosine and serine phosphorylated under basal conditions. Furthermore, both the tyrosine and serine phosphorylation of STAT1 remained elevated at HG conditions when compared to NG conditions (Fig. 3).

When examining the phosphorylation of STAT3 and STAT5A/B under basal conditions, STAT3 serine, but not tyrosine, phosphorylation was greatly enhanced under HG in comparison to incubation of the GMC under the NG concentration (Fig. 4). In addition, similar to STAT1, HG augmented both the tyrosine and serine phosphorylation of STAT3 induced by Ang II (Fig. 4). Finally, HG enhanced both the basal and the Ang II-induced STAT5A/B tyrosine and serine phosphorylation in GMC (Fig. 5).

As previously established, once STAT proteins are activated by tyrosine and serine phosphorylation, they translocate to the nucleus [23]. Therefore, we investigated the nuclear translocation of the different STAT proteins in response to Ang II. Under NG concentrations, the STAT1 protein translocated to the nucleus when GMC were stimulated by Ang II (Fig. 6A). Also, there was further stimulation of STAT1 nuclear translocation under HG conditions. With respect to STAT3

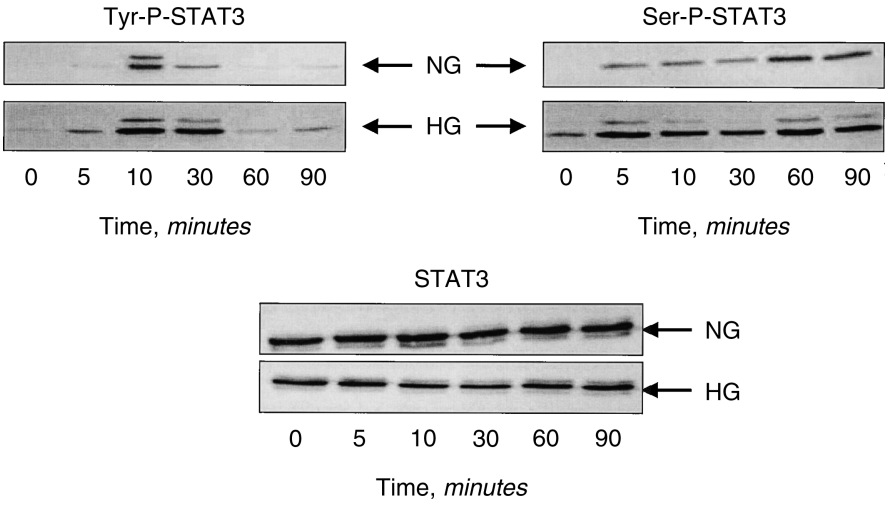

Fig. 4. Effects of high glucose on Ang II-induced tyrosine and serine phosphorylation of STAT3. Glomerular mesangial cells, under normal glucose (5.5 mmol/L) and high glucose (25 mmol/L), were stimulated with Ang II (0.1 μmol/L) for various times (0, 5, 10, 30, 60, 90 min). Cells were lysed and immunoblotted with phosphotyrosine- and phosphoserine-specific, and nonphosphospecific anti-STAT3 antibodies. Similar results were obtained in four experiments.

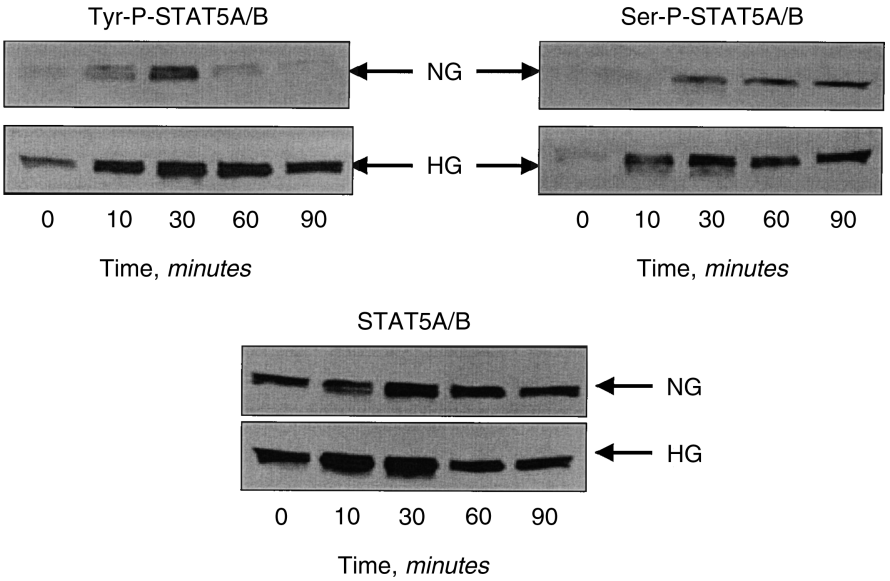

Fig. 5. Effects of high glucose on Ang II-induced tyrosine and serine phosphorylation of STAT5A/B. Glomerular mesangial cells, under normal glucose (5.5 mmol/L) and high glucose (25 mmol/L), were stimulated with Ang II (0.1 μmol/L) for various times (0, 5, 10, 30, 60 min). Cells were lysed and immunoblotted with phosphotyrosine- and phosphoserine specific, and nonphosphospecific anti-STAT5A/B antibodies. Results shown are representative of four experiments.

nuclear translocation, HG also augmented the Ang II-induced nuclear translocation of STAT3 (Fig. 6B). Similar to STAT1 and STAT3, STAT5A/B was translocated to the nucleus upon Ang II stimulation (Fig. 6C). Again, the Ang II-induced STAT5A/B translocation to the nucleus was increased under HG conditions (Fig. 6C). In addition, the differences observed in both the phosphorylations and the nuclear translocations of the various STAT proteins were due to HG and not hyperosmolarity, since no differences were observed in GMC cultured under NG conditions or NG plus elevated mannitol concentration (data not shown).

Effects of high glucose and Ang II on collagen IV protein production

First, the in vitro effect of Ang II on the production of collagen IV in GMC incubated in either NG or HG media was examined. When GMC were incubated with the medium alone for 48 hours, the baseline concentrations of collagen IV were 395 ng/mg cell protein for GMC grown in NG media and 825 ng/mg cell protein for GMC grown in HG media. These results indicate that collagen IV was constitutively produced by GMC and that HG induced a significant increase in its synthesis (Fig. 7). Incubation of GMC with various concentrations of Ang II for 48 hours significantly increased the production of collagen IV in a dose-dependent manner, and the peak concentration was observed at 0.1 μmol/L for GMC grown in both NG and HG media (Fig. 7). In summary, Ang II induced a significant increase in collagen IV protein production in the media under NG conditions, which was significantly augmented under HG conditions. In addition, NG plus high mannitol did not significantly augment the Ang II-induced collagen IV

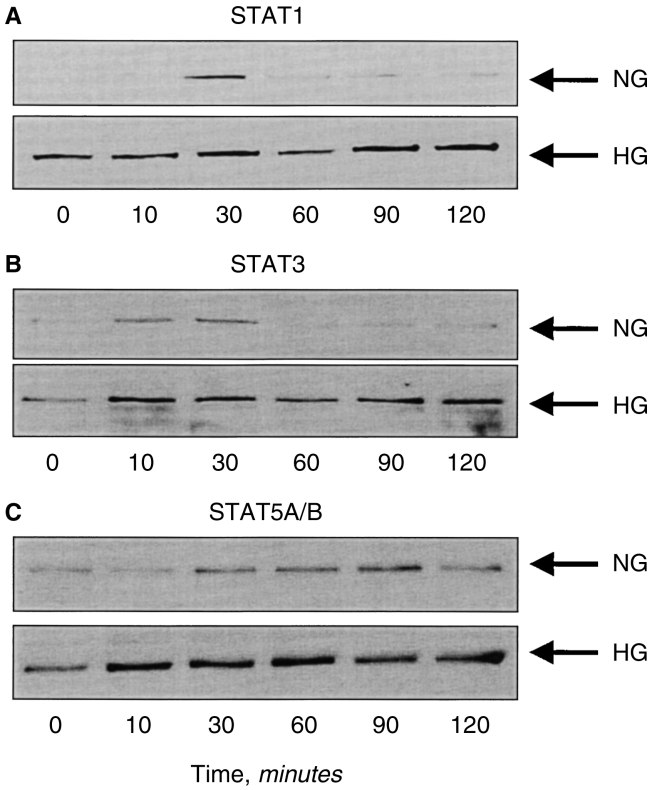

Fig. 6. Effects of high glucose on Ang II-induced nuclear translocation of STAT1, STAT3 and STAT5A/B. Glomerular mesangial cells, under normal glucose (5.5 mmol/L) and high glucose (25 mmol/L), were stimulated with Ang II (0.1 μmol/L) for the times shown. Cells nuclei were isolated and immunoblotted with anti-STAT1, STAT3 and STAT5A/B antibodies. Similar results were obtained in four experiments.

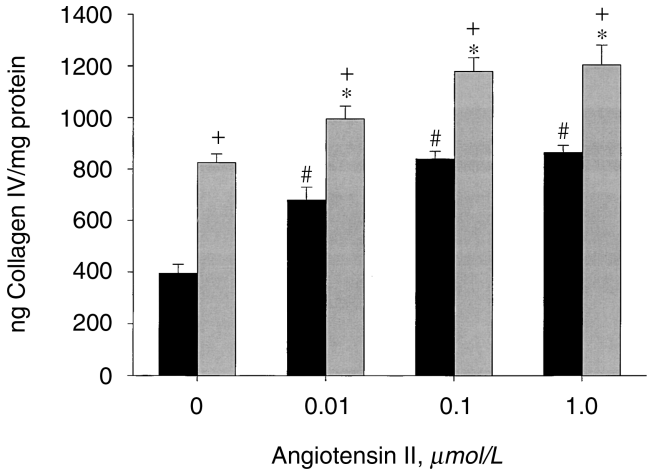

Fig. 7. Ang II-induced collagen IV production in normal (■) and high (□) glucose concentrations. Glomerular mesangial cells were cultured for 48 hours in DMEM containing either normal glucose (5.5 mmol/L) or high glucose (25 mmol/L) with or without Ang II at the indicated concentrations. From each condition, media were collected for immunoassay of collagen IV as described in the text. Results represent the mean ± SEM of four independent cultures and are expressed as nanograms of collagen IV per cell protein content. Glomerular mesangial cells exposure to different concentrations of Ang II caused significant increases in collagen IV synthesis in normal glucose ($\#P < 0.05$) and high glucose ($*P < 0.05$) conditions reaching a maximum at 0.1 μmol/L Ang II for both normal and high glucose. Significant differences were also found between glucose levels. In each instance, high glucose had significantly higher ($+P < 0.05$) mean collagen IV levels than normal glucose.

protein synthesis (data not shown), suggesting that hyperosmolality per se could not explain the effect of HG.

Role of JAK2 on the high glucose- and Ang II-induced production of collagen IV

Our prior study showed that Ang II induced the tyrosine phosphorylation of JAK2, and that this phosphorylation was enhanced under HG condition. In addition, the JAK/STAT pathways have been implicated in cell growth, proliferation and other cellular functions [24]. Therefore, in order to test the role of JAK2 on both the HG and the Ang II-induced collagen IV production, we used the JAK2-specific inhibitor AG-490. We have already tested the effects of this JAK2 inhibitor on the JAK/STAT pathway [27]. In the present study, pre-incubating the GMC for one hour with 10 μmol/L of AG-490 significantly inhibited both the Ang II- and the HG-induced collagen IV protein synthesis in GMC (Fig. 8). Thus, JAK2 appears to play a role in both the Ang II- and the HG-induced synthesis of collagen IV.

To further confirm that JAK2 plays this role, an antisense strategy was utilized. A JAK2 antisense oligonucleotide, which has been shown by our group to suppress

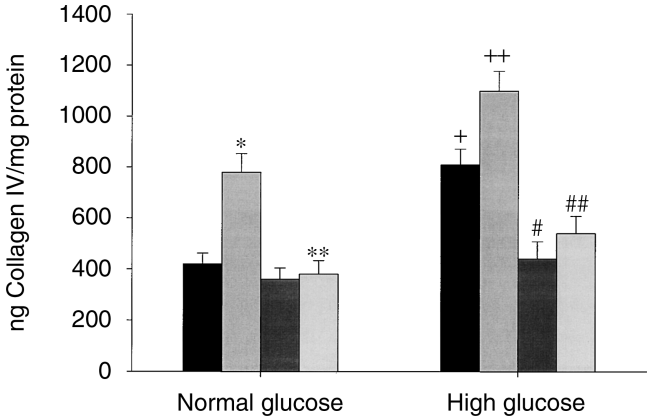

Fig. 8. Effect of high glucose and JAK2 inhibition on Ang II-induced collagen IV production. Glomerular mesangial cells were cultured for 48 hours in DMEM containing either 5.5 mmol/L or 25 mmol/L glucose with or without Ang II at 0.1 μmol/L in the presence or absence of the JAK2 inhibitor AG-490 at 10 μmol/L. Symbols are: (■) control; (□) Ang II; (■) AG490; (□) Ang II + AG490. Collagen IV production was assessed as described in Figure 7. Results represent the mean ± SEM of four independent cultures and are expressed as nanograms of collagen IV per cell protein content. Ang II induced a significant increased in collagen IV synthesis in both the normal glucose ($*P < 0.01$) and high glucose ($++P < 0.05$) conditions when compared to Control levels. This Ang II-induced production of collagen IV was significantly inhibited by AG-490 in normal glucose ($**P < 0.01$) and high glucose ($\#\#P < 0.01$). High glucose alone also induced a significant increased ($+P < 0.01$) in collagen IV synthesis that was significantly inhibited ($\#P < 0.05$) by AG-490.

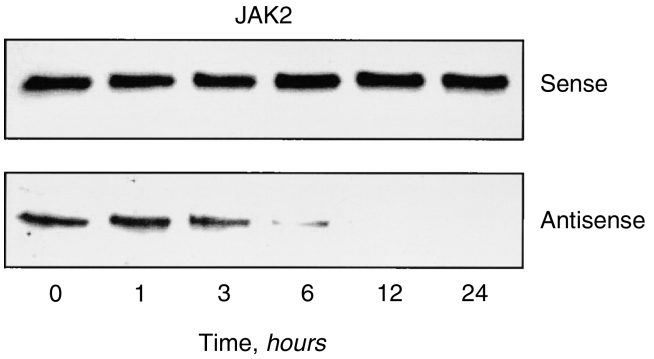

Fig. 9. Effect of JAK2 sense and antisense oligonucleotides on JAK2 expression in glomerular mesangial cells. Glomerular mesangial cells were treated with JAK2 sense and antisense oligonucleotides for the times indicated, the cells were lysed, and JAK2 was immunoprecipitated from the lysates with the anti-JAK2 antibody. Precipitated proteins were then immunoblotted with the anti-JAK2 antibody.

JAK2 expression in VSMC [27], was tested for its inhibitory effects on JAK2 expression in GMC. As a negative control, a complementary JAK2 sense oligonucleotide was tested. GMC were treated for various times with either the antisense or the sense oligonucleotide, and the levels of JAK2 protein expression were demonstrated by immunoblotting. As shown in Figure 9, the JAK2 antisense completely suppressed JAK2 expression after 12 hours of treatment. In contrast, the sense oligonucleotide had no effect. Experiments were then carried out in which GMC were treated with either the antisense or sense oligonucleotide for 12 hours and then stimulated the cells with HG alone or Ang II in either NG or HG for 48 hours as described before. We found that pre-incubating the GMC with the JAK2 antisense oligonucleotide (but not the JAK2 sense oligonucleotide) significantly inhibited the Ang II-, HG- and Ang II plus HG-induced collagen IV synthesis in GMC (Fig. 10).

Effect of Ang II and HG on glomerular mesangial cell growth and the role of JAK2

As shown in Figure 11, GMC exposed to Ang II for 48 hours under NG conditions resulted in a significant increased in [^{3}H]-thymidine incorporation when compared to control cells. Exposure of cells to HG alone also resulted in a significant increased in [^{3}H]-thymidine incorporation when compared to NG. In addition, the Ang II-induced [^{3}H]-thymidine incorporation was significantly enhanced in cells incubated in HG when compared to cells incubated in NG. Finally, just like our previous finding with the collagen IV synthesis, pre-incubating the GMC with the JAK2 antisense oligonucleotide (but not the JAK2 sense oligonucleotide) significantly inhibited the Ang II-, HG- and Ang II plus HG-induced [^{3}H]-thymidine incorporation (Fig. 11). In a similar experimental condition, the number of cells at the end of respective incubations was also counted. Parallel to the alterations in DNA synthesis, Ang II and HG individually stimulated cell number and produced a significant increase in cell replication under Ang II/HG conditions (Fig. 12). Again, pre-incubating the GMC with the JAK2 antisense oligonucleotide (but not the JAK2 sense oligonucleotide) significantly inhibited the Ang II-, HG- and Ang II plus HG-induced increased in cell proliferation. Now in order to determine whether HG response to cell proliferation was not due to increased osmolarity, the effect of 19.5 mmol/L mannitol on [^{3}H]-thymidine incorporation was also examined. Mannitol had no significant stimulatory effect on [^{3}H]-thymidine incorporation compared with the effect under NG conditions (data not shown).

Finally, to determine the effect of Ang II on [^{14}C]-leucine incorporation under NG or HG conditions as an index of total protein synthesis, cultures were exposed to Ang II in a fashion similar to the assessment of DNA synthesis except 0.5 μCi/mL [^{14}C]-leucine was added in the incubating medium. In all of the conditions tested (Ang II, HG or Ang II plus HG) there was no significant effect on [^{14}C]-leucine incorporation (Fig. 13), suggesting that hypertrophy is not a feature of increase GMC growth.

DISCUSSION

The present study showed that HG altered the activation of the JAK/STAT pathway by Ang II in GMC by inducing the phosphorylation of JAK2 kinase and different STAT proteins, namely STAT1, STAT3 and STAT5A/B. It is the activation of STAT proteins by many different extracellular proteins, including growth factors (PDGF, epidermal growth factor and VEGF) and Ang II via the AT_1 receptor, which has provided a mechanism of action for the exaggerated growth seen in many pathophysiological situations, such as diabetic glomerulosclerosis. Moreover, recent evidence has accumulated with the use of ACE inhibitors and AT_1 receptor antagonists in diabetic nephropathy have suggested that Ang II may be an important modulator of GMC growth. As reported previously [17, 20, 27], Ang II has the ability to activate STAT proteins by phosphorylating JAK kinases. However, to date this activation process has not been demonstrated in GMC, which under HG conditions exhibit abnormal growth that can serve as an in vitro model of diabetic glomerulosclerosis [33].

To determine the activation of the JAK/STAT pathway, we initially looked at the Ang II-induced JAK2 phosphorylation. There was a significant difference in the phosphorylation levels of JAK2 under HG conditions when compared to NG. Consequently, the possible

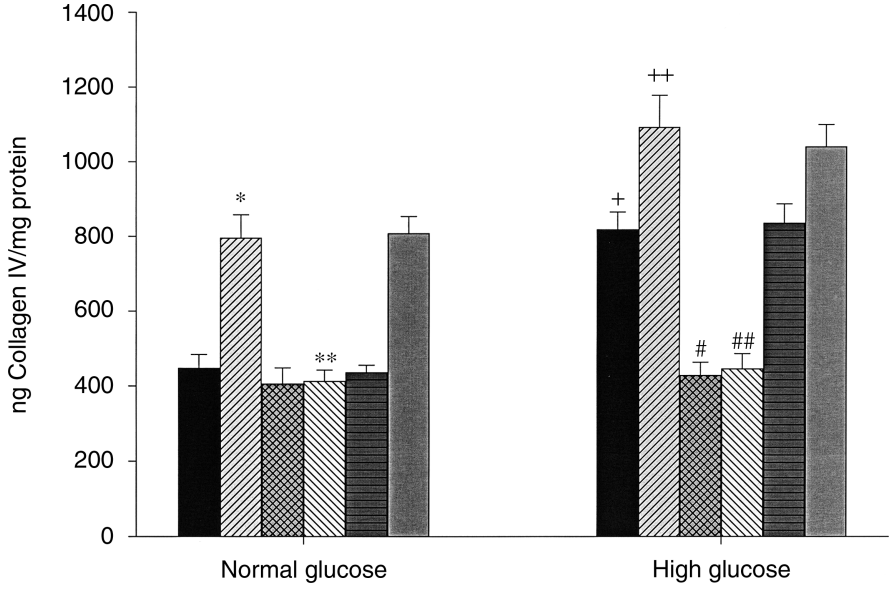

Fig. 10. Effect of JAK2 antisense on Ang II-induced collagen IV production in normal and high glucose conditions. Glomerular mesangial cells were pretreated for 12 hours with either JAK2 sense or JAK2 antisense oligonucleotides. Symbols are: (■) control; (▨) Ang II; (▩) antisense; (▧) Ang II + antisense; (▤) sense; (▦) Ang II + sense. Mesangial cells were cultured as described in Figure 8, and collagen IV production was assessed as described in Figure 7. Results represent the mean ± SEM of four independent cultures and are expressed as nanograms of collagen IV per cell protein content. The Ang II-induced increased in collagen IV synthesis in normal glucose ($*P < 0.01$ vs. Control) and high glucose ($++P < 0.01$ vs. Control) was significantly inhibited by JAK2 antisense oligonucleotide preincubation ($**P < 0.01$, normal glucose; $\#\#P < 0.01$, high glucose), while JAK2 sense oligonucleotide preincubation had no effect. High glucose alone significantly induced ($+P < 0.01$) collagen IV synthesis, which also was significantly inhibited by preincubation with the JAK2 antisense oligonucleotide ($\#P < 0.01$). JAK2 sense oligonucleotide pretreatment had no effect.

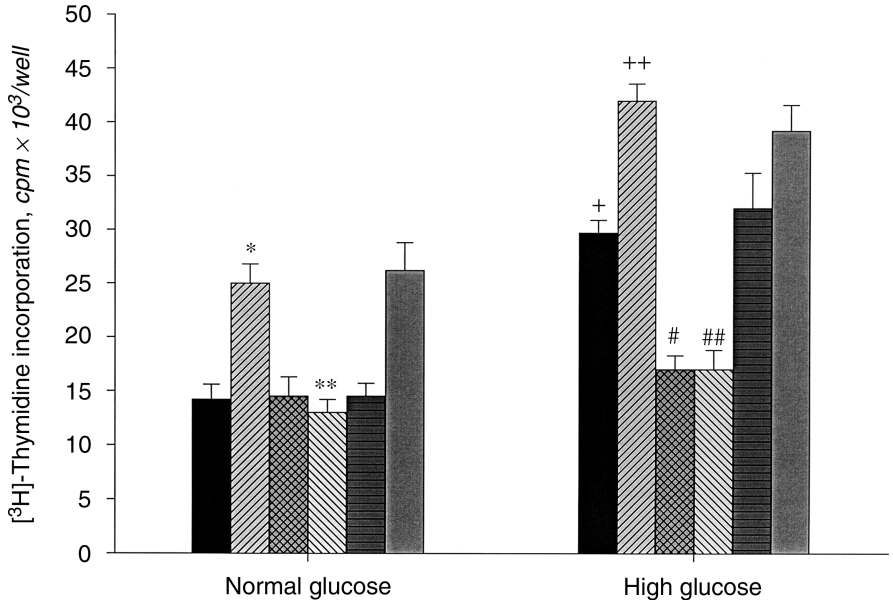

Fig. 11. Effect of JAK2 antisense on Ang II-induced [^{3}H]-thymidine incorporation in normal and high glucose conditions. JAK2 sense and JAK2 antisense twelve hours pretreated mesangial cells were cultured for 48 hours in DMEM containing either 5.5 mmol/L or 25 mmol/L glucose with or without Ang II at 0.1 μmol/L. [^{3}H]-thymidine incorporation was assessed as an index for DNA synthesis as described in the Methods section. Symbols are: (■) control; (▨) Ang II; (▩) antisense; (▧) Ang II + antisense; (▤) sense; (▦) Ang II + sense. Results represent the mean ± SEM of four independent cultures and are expressed as cpm × 10^3/well. Ang II-induced significant increases in [^{3}H]-thymidine incorporation in either normal glucose ($*P < 0.01$) or high glucose ($++P < 0.01$) conditions. The [^{3}H]-thymidine incorporation was significantly inhibited by JAK2 antisense oligonucleotide in both normal ($**P < 0.01$) and high glucose ($\#\#P < 0.01$) conditions. The JAK2 sense oligonucleotide preincubation had no effect. High glucose alone significantly induced [^{3}H]-thymidine incorporation ($+P < 0.01$), which was also significantly inhibited ($\#P < 0.01$) by the JAK2 antisense oligonucleotide.

cause and effects of increased JAK2 tyrosine phosphorylation was investigated. Among the leading causes of an increased JAK2 tyrosine phosphorylation is the possible down-regulation of tyrosine phosphatases. To this end, we investigated the phosphorylation of two cytosolic tyrosine phosphatases, SHP-1 and SHP-2. SHP-1 phosphorylation was completely abolished under HG conditions whereas SHP-2 phosphorylation was increased under basal and Ang II stimulation. These results suggest that JAK2 sustained activation under HG concentration is partly due to decreased SHP-1 and increased SHP-2 phosphorylation. These results are in accordance with our previous findings that clearly demonstrate that SHP-1 is the phosphatase responsible for JAK2 protein dephosphorylation, whereas SHP-2 appears to play a role in the phosphorylation of JAK2 perhaps by acting as an adaptor protein for JAK2 association with the AT_1 receptor, thereby facilitating JAK2 phosphorylation and

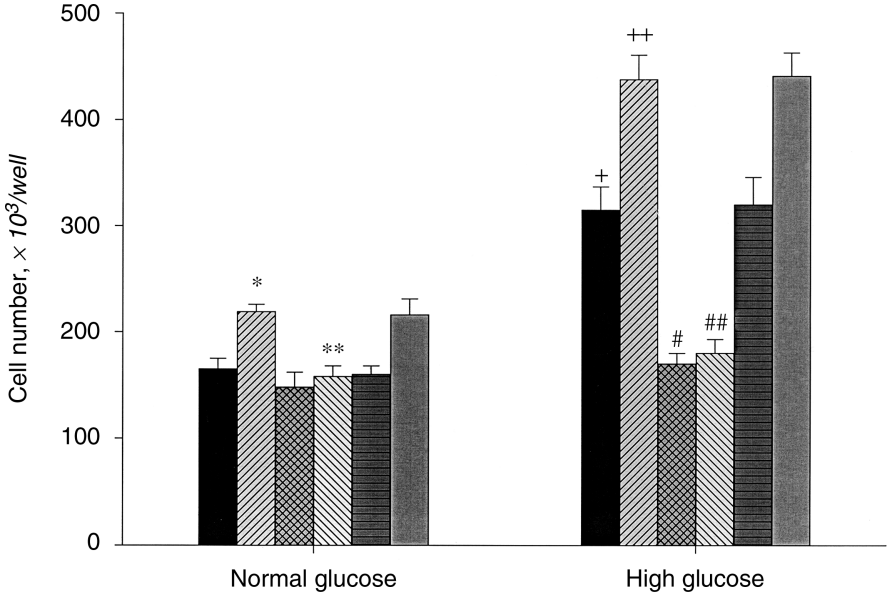

Fig. 12. Effect of JAK2 antisense on the Ang II-induced increased cell number in normal and high glucose conditions. Glomerular mesangial cells cultures were processed in a fashion similar to Figure 10, and cell number was counted at the end of respective incubations. Symbols are: (■) control; (▨) Ang II; (▩) antisense; (▧) Ang II + antisense; (▤) sense; (▦) Ang II + sense. Results represent the mean ± SEM of four independent cultures. Ang II-induced significant increases in cell number in both the normal ($*P < 0.05$) and high glucose ($++P < 0.01$) conditions that were significantly inhibited by the JAK2 antisense oligonucleotide in both the normal ($**P < 0.05$) and high glucose ($\#\#P < 0.01$) conditions. The JAK2 sense oligonucleotide, on the other hand, had no effect. High glucose without Ang II also significantly induced ($+P < 0.05$) an increase in cell number that was also significantly inhibited ($\#P < 0.05$) by the JAK2 antisense oligonucleotide. Again preincubation with the JAK2 sense oligonucleotide had no effect.

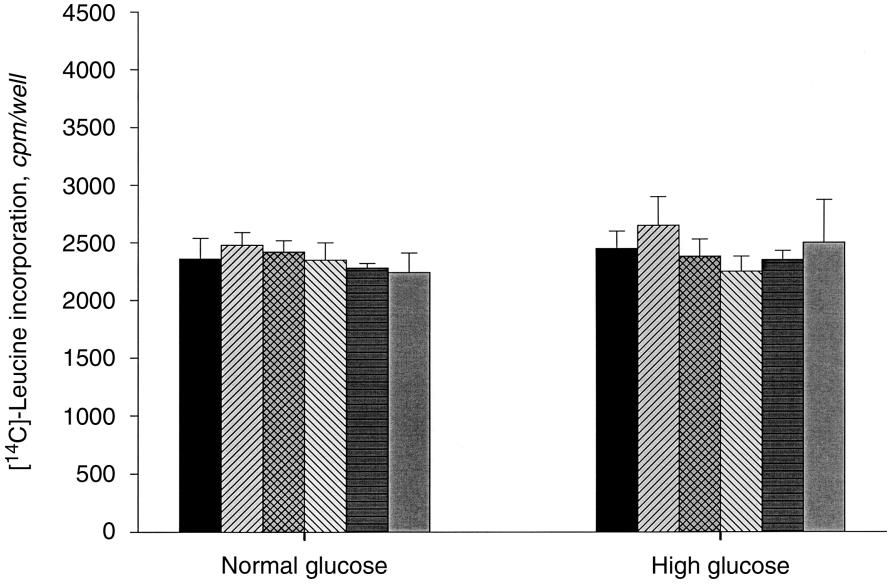

Fig. 13. Effect of JAK2 antisense on Ang II-induced [^{14}C]-leucine incorporation in normal and high glucose conditions. Glomerular mesangial cells cultures were processed in a fashion similar to Figure 10, and [^{14}C]-leucine incorporation was assessed as an index for total protein synthesis as described in Methods section. Symbols are: (■) control; (▨) Ang II; (▩) antisense; (▧) Ang II + antisense; (▤) sense; (▦) Ang II + sense. Results represent the mean ± SEM of four independent cultures. No significant differences ($P > 0.05$) were detected in all the conditions tested.

activation as we have previously demonstrated in VSMC [31]. In addition, these effects are due to HG and not to hyperosmolarity, since no alterations in the tyrosine phosphorylation of both SHP-1 and SHP-2 were observed under elevated mannitol concentration.

The next logical step was to determine the STAT proteins activated by Ang II treatment in GMC grown under NG and HG conditions. Moreover, it has been postulated that serine phosphorylation in addition to tyrosine phosphorylation plays a critical role in STAT protein activation [34, 35]. We employed anti-phosphospecific STAT antibodies, and found that, while Ang II induces the phosphorylation of these STAT proteins on both tyrosine and serine residues under NG conditions, these phosphorylations are greatly enhanced under HG conditions. Furthermore, this effect was not due to increased osmolarity because no effects on STAT protein phosphorylation were observed in GMC cultured in elevated mannitol.

The constitutive phosphorylation (at time = 0 min) of the various STAT proteins observed under HG conditions is not unique to our study [20, 36]. A plausible explanation for such a constitutive activation is the HG-induced activation of both the PKC and MAP kinase pathways [18, 19], which have been shown to crosstalk with the JAK/STAT pathway [28]. In addition, it has

been shown that MAP kinase also has the ability to serine phosphorylate various STAT proteins [37]. Furthermore, in an elegant study, Zhang et al have shown that the serine phosphorylation of STAT proteins is essential for their translocation to the nucleus [34], and since it is well accepted that once STAT proteins are activated they translocate to the nucleus [23], we examined Ang II-induced STAT nuclear translocation. Under HG conditions the Ang II-induced nuclear translocation of STAT1, STAT3 and STAT5A/B was enhanced when compared to NG conditions. Therefore, the augmentation of the JAK/STAT pathway induced by Ang II under HG conditions provides a plausible explanation for the beneficial effects of RAS blockade in diabetic nephropathy.

It has been established that increased glomerular collagen IV production in streptozotocin-diabetic rats plus stimulation of cell growth and matrix transcripts by HG levels in short-term GMC culture provide evidence that stimulation of cell growth and extracellular matrix synthesis is important in early diabetic glomerulopathy and plays an important role in the pathophysiological manifestations of diabetic nephropathy [19, 21, 38]. Furthermore, it has been proposed that GMC growth and collagen IV production under HG concentrations is an in vitro model of diabetic glomerulosclerosis [21, 38]. To date cell growth and collagen IV production in GMC have been attributed to increase activity of key-growth kinases, such as PKC and MAP kinase [19, 21, 39]. However, we report, to our knowledge for the first time, that the increased cell growth plus the production of collagen IV in GMC by both HG and Ang II also can be attributed to the activation of JAK2. It has been well established that JAK2 plays an important role in growth and proliferation of cells such as VSMC [28], which resemble GMC morphologically [40]. Moreover, the HG augmentation of the Ang II-induced nuclear translocation of the various STAT proteins suggests that these STATs may play an important role in the GMC growth and extracellular matrix deposition in diabetic nephropathy. Future studies will need to focus on the function of these STAT proteins and their role in GMC growth and extracellular matrix deposition under HG conditions.

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