Role of the Janus kinase (JAK)/signal transducters and activators of transcription (STAT) cascade in advanced glycation end-product-induced cellular mitogenesis in NRK-49F cells

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Advanced glycation end product (AGE) is important in the pathogenesis of diabetic nephropathy, which is characterized by cellular hypertrophy/hyperplasia leading to renal fibrosis. However, the signal transduction pathways of AGE remain poorly understood. The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway has been associated with cellular proliferation in some extra-renal cells. Because interstitial fibroblast proliferation might be important in renal fibrosis, we studied the role of the JAK/STAT pathway in NRK-49F (normal rat kidney fibroblast) cells cultured in AGE/BSA and non-glycated BSA. We showed that AGE dose-dependently (10–200 μg/ml) increased cellular mitogenesis in NRK-49F cells at 5 and 7 days. However, cellular mitogenesis was unaffected by the simultaneous presence of BSA. Regarding the JAK/STAT pathway, AGE (100 μg/ml) induced tyrosine phosphorylation of JAK2 (but not JAK1, JAK3 or TYK2) at 15–60 min; it also induced the tyrosine phosphorylation of STAT1 and STAT3 at 1–2 h and 0.5–4 h respectively. Being a transcription factor, AGE also increased the DNA-binding activities of STAT1 and STAT3 AG-490 (a specific JAK2 inhibitor) (5 μM) inhibited tyrosine phosphorylation of JAK2 and the DNA-binding activities of STAT1 and STAT3. The same results were obtained by using specific 'decoy' oligodeoxynucleotides (ODNs) that prevented STAT1 and STAT3 from binding to DNA. Meanwhile, the STAT1 or STAT3 decoy ODN and AG-490 were effective in reversing AGE-induced cellular mitogenesis. We concluded that the JAK2–STAT1/STAT3 signal transduction pathway is necessary for AGE-induced cellular mitogenesis in NRK-49F cells.

Key words: decoy oligodeoxynucleotide, diabetic nephropathy, tyrosine phosphorylation.

INTRODUCTION

Diabetic nephropathy (DN) has become the leading cause of end-stage renal disease in the developed countries [1]. Unfortunately, the pathogenesis of DN is poorly defined. However, hyperglycaemia and advanced glycation end product (AGE) seem to be essential for its development [1,2]. Therefore many studies have used AGE and high glucose levels in renal cell cultures to elucidate cell biology under hyperglycaemic conditions [1,2].

DN is characterized by cellular hypertrophy/hyperplasia and extracellular matrix accumulation, which eventually lead to renal fibrosis (glomerulosclerosis and tubulointerstitial fibrosis) and end-stage renal disease [1–3]. DN is composed of glomerulopathy and tubulointerstitial changes [1,2]. In this regard, although most previous studies investigated the former, the latter might be more important in terms of renal prognosis [4]. We have therefore been studying high-glucose-cultured renal tubular cells [5,6].

Although the effects of AGE/high glucose level on renal cells have been elucidated to some extent [1,2,7], its direct relevance to renal fibrosis remains unknown. Recently, the role of fibroblast in renal fibrosis has been noted [8,9]. It is known that tubulointerstitial fibrosis is mainly composed of fibroblasts and extracellular matrices [8]. Moreover, fibroblasts have been proposed to have complex interactions with tubular cells [9]. Fibroblasts can also differentiate into myofibroblasts after various stimuli, although the effect of AGE/high glucose level on them is still unknown [10]. In this regard, the expression of myofibroblast has been shown to be related to the progression of proteinuria and renal function in DN in a recent study [11].

AGE/high glucose level can modulate cellular growth in renal cells [5–7]. However, the molecular signalling mechanisms controlling the growth of renal cells by AGE/glucose remain poorly understood. In this regard, protein kinase C, prostaglandins and oxidative stress have been shown to be important mediators in DN either *in vivo* or *in vitro* [1,7,12]. Additionally, an elevated intracellular Ca^{2+} level has also been implicated in many complications of diabetes mellitus, although its role in DN remains unproved [13].

Janus kinases (JAKs) and signal transducers and activators of transcription (STAT) are novel factors known to transduce signals initiated by several growth factors and cytokines. So far, four JAKs (JAK1, JAK2, JAK3 and TYK2) and seven STATs (STAT 1–4, 5a, 5b and 6) have been cloned. The activated JAKs tyrosine-phosphorylate the latent cytoplasmic STATs, which then homodimerize or heterodimerize and are translocated to the nucleus to become the transcription factors [14]. Moreover, the maximal activation of STATs requires both tyrosine phosphorylation by JAKs and serine/threonine phosphorylation by mitogen-activated protein kinase [15].

Previous studies have demonstrated that a synthetic double-stranded oligodeoxynucleotide (ODN) with a high affinity for a

Abbreviations used: AGE, advanced glycation end product; DMEM, Dulbecco's modified Eagle's medium; DN, diabetic nephropathy; EMSA, electrophoretic mobility-shift assay; FCS, fetal calf serum; JAK, Janus kinase; ODN, oligodeoxynucleotide; RAGE, receptor for AGE; STAT, signal transducers and activators of transcription; TYK, tyrosine kinase.

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target transcription factor can be introduced into target cells as 'decoy' to bind the transcription factor, thereby altering gene transcription [16]. Therefore the decoy approach enables us to study the molecular mechanisms of AGE-mediated transcriptional regulation.

We hypothesized that the JAK/STAT pathway might be involved in AGE-induced effects for several reasons. First, this pathway is activated by several AGE-induced cytokines or growth factors, such as interleukin 6 [17], insulin-like growth factor I [18], epidermal growth factor [19] and platelet-derived growth factor [20]. Secondly, JAK/STAT is also activated by growth hormone and angiotensin II, which have been implicated in the pathogenesis of DN [21,22]. Thirdly, JAK/STAT activates Fos and Jun, which are activated in DN [23]. Fourthly, JAK/STAT is activated by insulin [24] and might therefore be associated with glucose metabolism, whereas glucose hypermetabolism has been implicated in DN [25].

In view of the probable involvement of interstitial fibroblast proliferation in diabetic renal fibrosis, we studied normal rat kidney interstitial fibroblast (NRK-49F) cells cultured in AGE/BSA. We had three purposes: first, to determine whether AGE induces cellular proliferation in these cells; secondly, to elucidate whether AGE activates the JAK/STAT cascade; thirdly, to define the role of the JAK/STAT cascade in AGE-induced cellular mitogenesis.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), antibiotics, lipofectin, molecular mass standards, trypsin/EDTA, Trypan Blue stain and all medium additives were obtained from Life Technologies. Anti-phosphotyrosine (PY20), anti-JAK1, anti-JAK2, anti-JAK3, anti-TYK2, anti-STAT1, and anti-STAT3 antibodies were obtained from Santa Cruz Biotechnology. Protein A/G-coupled agarose beads and AG-490 were purchased from Calbiochem. Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody, [γ-^{32}P]ATP and the enhanced chemiluminescence kit were obtained from Amersham Corp. *N*,*N*′-methylenebisacrylamide, acrylamide, SDS, ammonium persulphate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine ('TEMED') and Tween 20 were purchased from Bio-Rad Laboratories. BSA, DMSO and all other chemicals were obtained from Sigma.

Culture conditions

NRK-49F cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). In brief, cells were grown in culture flasks and maintained in DMEM (5.5 mM D-glucose) supplemented with 100 i.u./ml penicillin, 100 mg/ml streptomycin and 5% (v/v) FCS in a humidified incubator under air/CO_2 (19:1) at 37 °C. In some experiments, cells were exposed to serum-free (0.1% FCS) DMEM supplemented with the specific JAK2 inhibitor, AG-490, for 16 h before timed exposure to FCS and AGE. AG-490 was dissolved in DMSO. Cell viability was assessed by the Trypan Blue exclusion test and was routinely more than 92%.

Preparation of AGEs

BSA fraction V (1 mM) was glycated by incubation with 0.5 M glucose in 50 mM potassium phosphate (pH 7.3)/1 mM EDTA under sterile conditions at 37 °C for 8 weeks, with the use of a modified protocol of Makita et al. [26]. The reaction mixtures were dialysed against PBS to remove the free glucose, then passed through a specific column (Pierce) to remove endotoxin. Residual glucose measured less than 0.1 mM after this procedure. Fluorescence of the AGE was determined with a Perkin-Elmer fluorescence spectrometer; the average fluorescence for AGE was 56 arbitrary units compared with 1 arbitrary unit/mg for BSA. Then AGE was stored at −20 °C until used. Control (non-glycated) BSA consisted of the same initial preparations of BSA incubated at 37 °C in the same manner, except that no glucose was present. Note that for the following experiments, BSA or AGE was added together with DMEM containing 5% (v/v) FCS.

Cellular mitogenesis

This was performed as in our previous studies [5,6]. In brief, 8.0×10^3 cells were transferred to 24-well microplates (Nunclon) and maintained in 5% FCS medium for 1 day. After a fast (0.1% FCS) for 48 h, fresh DMEM (5% FCS) containing different concentrations of AGE or BSA was added. Cells were grown for an additional 3, 5 or 7 days; 4 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL, U.S.A.) was included in all media during the last 5 h. Cells were washed; this was followed by protein precipitation with 10% (w/v) trichloroacetic acid for an additional 15 min. After aspiration of trichloroacetic acid, cells were dehydrated with 95% (v/v) alcohol for 10 min and solubilized with 0.1% SDS at 37 °C for 30 min. The lysate was harvested by scraping followed by the addition of 0.45 ml of cell mixture to 2 ml of scintillation fluid. The samples were counted on a β-counter (Beckman, Palo Alto, CA, U.S.A.). Results were plotted as c.p.m. per well. For cell number analysis, cells were manipulated as above except that six-well culture plates were used (Nunclon). Cells were harvested and counted with a haemocytometer. Each experimental data point represents the mean of duplicate wells from three independent experiments.

Preparation of nuclear extracts

Nuclear extracts were prepared by using a modification of a procedure described previously [27]. In brief, 1.5×10^7 cells were scraped in ice-cold PBS, harvested by centrifugation at 500 ***g*** for 10 min and resuspended in 400 μl of buffer A [10 mM Hepes (pH 7.9)/1.5 mM $MgCl_2$/10 mM KCl/5 mM dithiothreitol/0.1 mM EDTA/0.1 mM EGTA/5 mM PMSF/2 μg/ml aprotinin/10 μg/ml leupeptin]. After incubation on ice for 20 min, Nonidet P40 was added to a final concentration of 0.6% (v/v) and vigorously vortex-mixed for 10 s. The lysates were then centrifuged at 12000 ***g*** for 30 s at 4 °C. The nuclear pellets were resuspended in 50 μl of extraction buffer B [10 mM Tris/HCl (pH 7.4)/0.3 M KCl/3 mM $MgCl_2$/1 mM dithiothreitol/0.5 mM PMSF/2 μg/ml leupeptin/20% (v/v) glycerol] and were vortex-mixed for 15 min at 4 °C. The lysates were centrifuged at 12000 ***g*** for 5 min at 4 °C and the supernatants containing the nuclear proteins were transferred to fresh vials. Protein concentration was measured with a Bio-Rad protein assay kit. The extract was stored at −70 °C until further use.

Immunoprecipitation and immunoblotting

For JAK activation assays, 1.5×10^7 serum-deprived cells were treated with BSA or AGE as described above. Cells were harvested by centrifugation, washed in PBS and suspended in 1 ml of TNET buffer [20 mM Tris/ HCl (pH 7.4)/40 mM NaCl/5 mM EDTA/30 mM $Na_4P_2O_7$/50 mM NaF], containing 1% (v/v) Triton X-100, 0.1% BSA, 1 mM Na_3VO_4, 20 mM *p*-nitrophenyl phosphate, 1 mM PMSF, 10 μg/ml aprotinin and

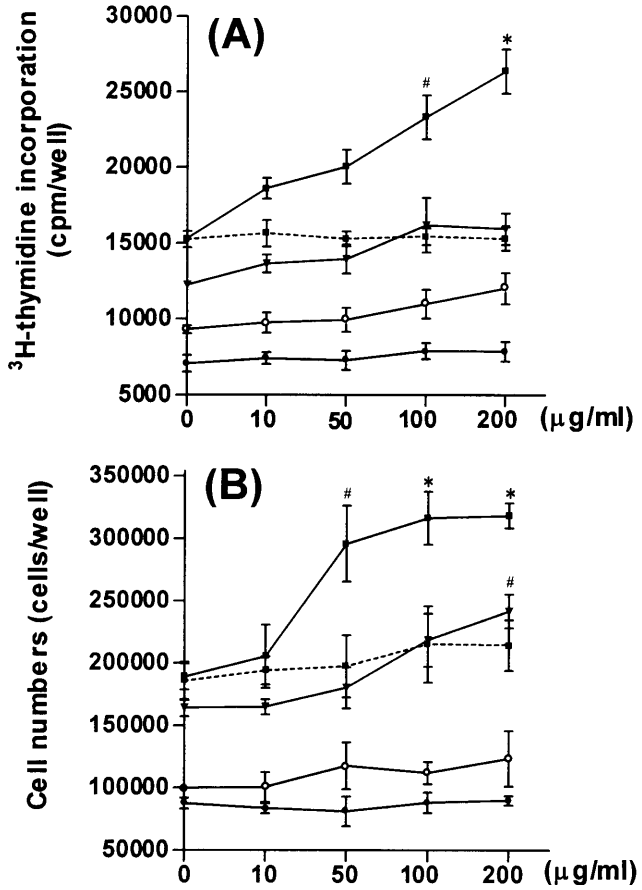

Figure 1 Time course and dose-dependent effects of AGE on cellular mitogenesis in NRK-49F cells

Serum-deprived NRK-49F cells were treated with 0, 10, 50, 100 or 200 μg/ml AGE (solid lines) in the presence of 5% (v/v) FCS for 1 (●), 3 (○), 5 (▼) and 7 days (■). AGE dose-dependently increased [^{3}H]thymidine incorporation (**A**) and cell numbers (**B**) at 5 and 7 days. The effects of BSA on cellular mitogenesis were also analysed by the above methods at 7 days (dotted lines). Results are means ± S.E.M. for three independent experiments. #$P < 0.05$; *$P < 0.01$ compared with control without AGE.

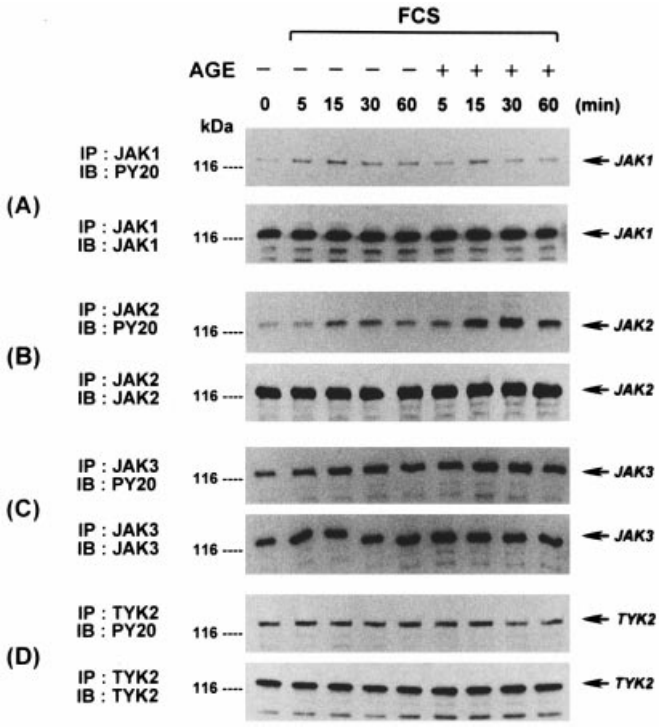

Figure 2 Time course of the effect of AGE on tyrosine phosphorylation of JAK kinases in NRK-49F cells

Total cell lysates from NRK-49F cells treated without or with AGE (100 μg/ml) for the indicated durations were immunoprecipitated (IP) with anti-JAK1 (**A**), anti-JAK2 (**B**), anti-JAK3 (**C**) or anti-TYK2 (**D**). Immune complexes were separated by SDS/PAGE [10% (w/v) gel] and analysed by immunoblotting (IB) with either antiphosphotyrosine (PY20) (upper panels) or antibodies corresponding to the above antibodies (lower panels). These are representative experiments, each performed at least three times.

10 μg/ml leupeptin. After 20 min on ice, lysates were cleared of insoluble material by centrifugation for 10 min at 12000 ***g***. Cleared extracts were immunoprecipitated for 1 h with the indicated antisera and Protein A/G-coupled agarose beads. The immune complexes were washed twice with TNET buffer and twice with kinase buffer [10 mM Hepes (pH 7.4)/50 mM NaCl/5 mM $MgCl_2$/0.1 mM Na_3VO_4 plus protease inhibitors].

The precipitated proteins were eluted by the addition of 60 μl of SDS sample buffer, resolved by SDS/PAGE [10% (w/v) gel] and then transferred to Protran membranes (0.45 μm thick; Schleicher & Schuell, Keene, NH, U.S.A.) with a Hoefer transfer apparatus (75 V for 2 h). The membranes were blocked in blocking solution [5% (v/v) non-fat milk in Tris-buffered saline/Tween (TBS-T; 20 mM Tris base/137 mM NaCl adjusted to pH 7.6 with HCl, plus 0.05% Tween 20)] for 1 h at room temperature. The membranes were subsequently probed with the following antisera, diluted in TBS-T as indicated in parentheses: anti-PY20 (0.75 μg/ml), anti-JAK1 (1:1000), anti-JAK2 (1:1000), anti-JAK3 (1:1000) or anti-TYK2 (1:1000). After 2 h, the membrane was rinsed with washing buffer (TBS-T) at room temperature. The membrane was incubated in horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody diluted 1:4000) for 1 h at room temperature, followed by the same washing steps as above. The protein bands were detected with the enhanced chemiluminescence (ECL®) system, and the percentage of phosphorylated form of protein was determined with a scanning densitometer.

For STAT activation assays, 2×10^7 serum-deprived cells were stimulated with BSA or AGE and harvested by centrifugation. Cell protein extracts were prepared as described above. Extracts were immunoprecipitated for 4 h with STAT monoclonal antibody and Protein A/G–agarose beads and then washed three times with isotonic wash buffer [10 mM Hepes (pH 8.0)/20 mM NaF/150 mM NaCl/0.1 mM EDTA/1 mM dithiothreitol/40 mM *p*-nitrophenyl phosphate/1 mM Na_3VO_4/1% (v/v) Triton X-100/0.5% (v/v) Nonidet P40]. Immunoprecipitated proteins were eluted with SDS sample buffer, resolved by SDS/PAGE and transferred to Protran membranes as described above. The membranes were probed with anti-PY20 (0.5 μg/ml), anti-STAT1 (1:1000) and anti-STAT3 (1:1000) antibodies; immunoreactive proteins were detected with the enhanced chemiluminescence system as described above.

Synthesis of ODNs

The sequences of phosphorothioate double-stranded ODNs used in this study were synthesized with a DNA/RNA synthesizer (Applied Biosystems Division, Perkin Elmer). STAT1 and

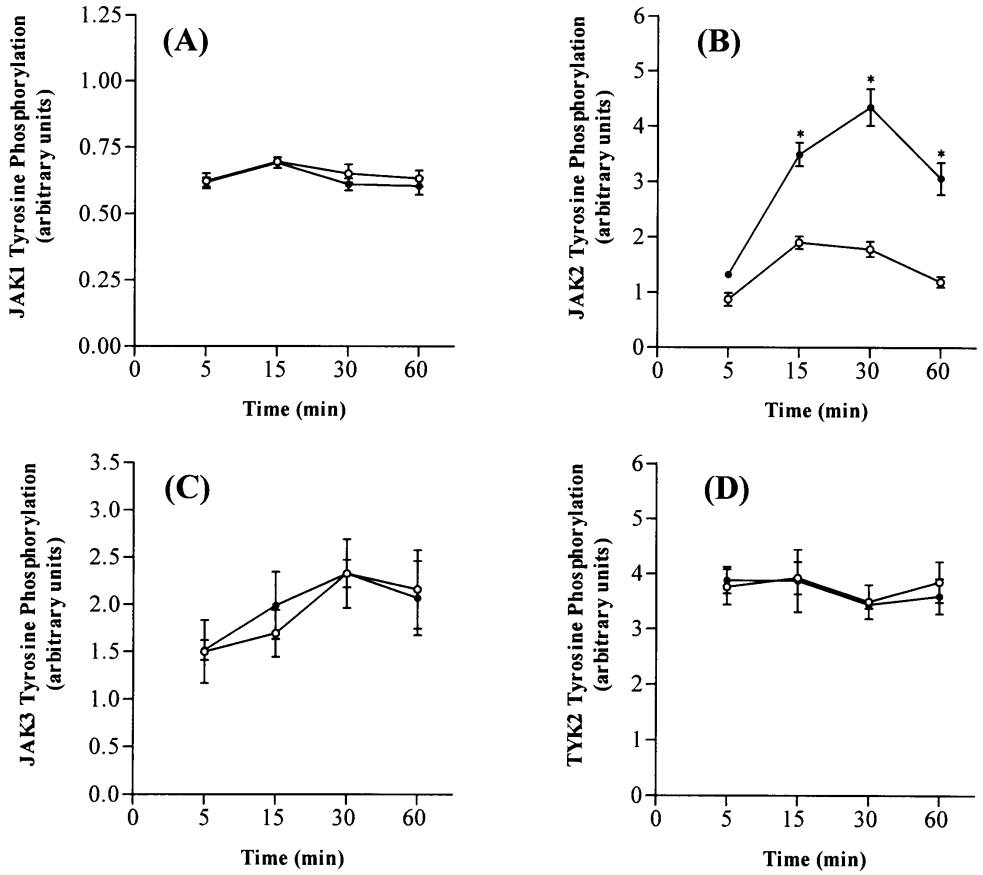

Figure 3 Laser densitometry of the gels shown in Figure 2 and two additional tyrosine phosphorylation experiments

Results are shown for JAK1 (**A**), JAK2 (**B**), JAK3 (**C**) and TYK2 (**D**) respectively. It is evident that AGE significantly increased the level of tyrosine phosphorylation of JAK2 at the indicated times. Symbols: ○, FCS; ●, FCS + AGE. Results are expressed as arbitrary units plotted against time (means ± S.E.M.; $n = 3$). $^*P < 0.01$ compared with controls without AGE for the durations indicated.

STAT3 ODNs corresponded to the high-affinity Ly-6E interferon-γ-activated site ('GAS') [28] and the acute-phase response element ('APRE') in the rat α_2-macroglobulin gene [29], respectively: STAT1 decoy ODN (consensus sequences are underlined), 5′-ATATTCCTGTAAGTG-3′, 3′-TATAAGGACATTCAC-5′; mismatched decoy ODN1, 5′-ATATTGGAGTAAGTG-3′, 3′-TATAACCTCATTCAC-5′; STAT3 decoy ODN, 5′-GATCCTTCTGGGAATTCCTAG-ATC-3′, 3′-CTAGGAAGACCCTTAAGGATCTAG-5′; mismatched decoy ODN3, 5′-GATCCTTCTGGGCCGTCCTAG-ATC-3′, 3′-CTAGGAAGACCCGGCAGGATCTAG-5′. The single-stranded ODNs were annealed for 2 h while the temperature was decreased from 80 to 25 °C.

Transfection of ODNs into cells

Commercially available cationic liposomes (Lipofectin Reagent) were used to facilitate the transfection of ODNs into the NRK-49F cells, as described previously [16]. In brief, ODNs dissolved in DMEM were mixed with Lipofectin, which was also dissolved in DMEM at a ratio of 1 nmol/μg, and incubated for 20 min at room temperature. The ODN–liposome complexes were added to flasks or 24-well plates. We had determined the specific conditions under which cationic liposomes could be used to transfect the decoy ODN into NRK-49F cells successfully without causing cellular toxicity.

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed by using previously published protocols [30] with minor modifications. In brief, ^{32}P-labelling of STAT1 and STAT3 decoy ODNs was performed with T4-polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.) and [γ-^{32}P]ATP (3000 Ci/mmol). Labelled DNA was separated from the unincorporated radioactivity by using an ion-exchange column (Schleicher and Schuell, Keene, NH, U.S.A.). Binding reactions were performed by adding 5 μg of nuclear protein to 20 μl of binding buffer [10 mM Tris/HCl (pH 7.5)/1 mM $MgCl_2$/0.5 mM EDTA/0.5 mM dithiothreitol/50 mM NaCl/10 % (v/v) glycerol/0.2 % (v/v) Nonidet P40/2 μg of poly(dI-dC) (Boehringer Mannheim)] containing [γ-^{32}P]ATP labelling STAT oligonucleotide probes (2.5×10^4 c.p.m., or approx. 2.5 fmol per reaction). Where indicated, unlabelled competitive oligonucleotides or antibodies were included during the pre-incubation periods. Samples were incubated at room temperature for 25 min and fractionated by electrophoresis in a 6 % (w/v) non-denaturing polyacrylamide gel in TGE buffer [20 mM Tris (pH 7.5)/150 mM glycine/1 mM EDTA] that had been pre-electrophoresed for 1 h at 80 V. Gels were run at 100 V for 1.5 h. After electrophoresis, gels were transferred to 3 MM paper, dried in a gel dryer under vacuum at 75 °C and exposed to X-ray Hyperfilm-MP at −70 °C with an intensifying screen. The results were quantified with a scanning densitometer.

Statistics

The results are expressed as means ± S.E.M. Unpaired Student's *t* tests were used for the comparison between two groups. One-way analysis of variance followed by unpaired *t* test was used for comparison between more than three groups. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Proliferation of NRK-49F cells in response to AGE

Cellular mitogenesis, determined by the [^{3}H]thymidine incorporation assay, was measured in NRK-49F cells after timed exposures to AGE or BSA. As shown in Figure 1(A), AGE dose-dependently (10–200 μg/ml) increased [^{3}H]thymidine incorporation in NRK-49F cells at 5 and 7 days. AGE also significantly increased cell numbers in these cells (Figure 1B). However, NRK-49F cells exposed to non-glycated BSA for 7 days exhibited no change in cellular mitogenesis. The experiments lasting 1, 3 and 5 days also yielded similar findings (results not shown).

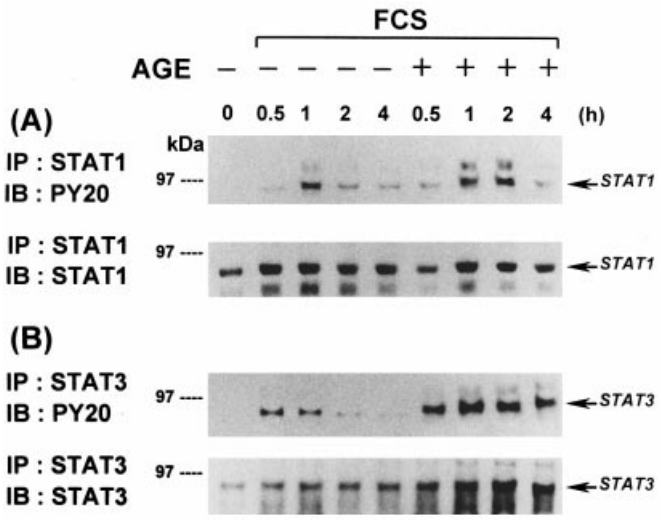

Figure 4 Time course of the effect of AGE on tyrosine phosphorylation of STAT1 and STAT3 in NRK-49F cells

Total cell lysates from NRK-49F cells treated without or with AGE (100 μg/ml) for the indicated durations were immunoprecipitated (IP) with anti-STAT1 (**A**) or anti-STAT3 (**B**). Immune complexes were separated by SDS/PAGE [10% (w/v) gel] and immunoblotted (IB) with either antiphosphotyrosine (PY20) antibodies (upper panels) or antibodies corresponding to the above antibodies (lower panels). These are representative experiments, each performed at least three times.

Effects of AGE on tyrosine phosphorylation of JAK kinases

For an initial exploration of the role of the JAKs in AGE-mediated signalling events, the ability of AGE to phosphorylate various JAKs in NRK-49F cells was examined. Tyrosine phosphorylation of JAK kinases by FCS and AGE (100 μg/ml) treatments was studied by immunoprecipitation (anti-JAK antibodies) followed by immunoblotting (anti-PY20 antibody). As shown in Figures 2 and 3, AGE induced the time-dependent tyrosine phosphorylation of JAK2 (but not JAK1, JAK3 or TYK2) at 5–60 min. Although FCS alone might induce tyrosine phosphorylation of JAK2, there seemed to be a large increase in JAK2 activation in the presence of AGE. These results implied that AGE-induced NRK-49F cell proliferation might be through the JAK cascade and be dependent on the activation of JAK2.

Effects of AGE on tyrosine phosphorylation of STAT1 and STAT3

Because STATs are downstream effectors of JAKs, the tyrosine phosphorylation of STAT1 and STAT3 was also studied in these cells. As shown in Figures 4(A) and 5(A), FCS induced the tyrosine phosphorylation of STAT1 at 0.5–4 h. The addition of AGE (100 μg/ml) resulted in a greatly prolonged and increased tyrosine phosphorylation of STAT1. Similarly, AGE (100 μg/ml) also enhanced the tyrosine phosphorylation of STAT3 at 0.5–4 h (Figures 4B and 5B).

Subsequently we examined the effects of treatment with non-glycated BSA by monitoring the tyrosine phosphorylation of JAK2, STAT1 and STAT3. Incubation with 100 μg/ml BSA for 0.5 h did not affect the tyrosine phosphorylation of JAK2 in comparison with control cells incubated with FCS alone (Figure 6A). The experiments on STAT1 and STAT3 also yielded similar findings when cells were treated with BSA for 2 h (Figures 6B and 6C).

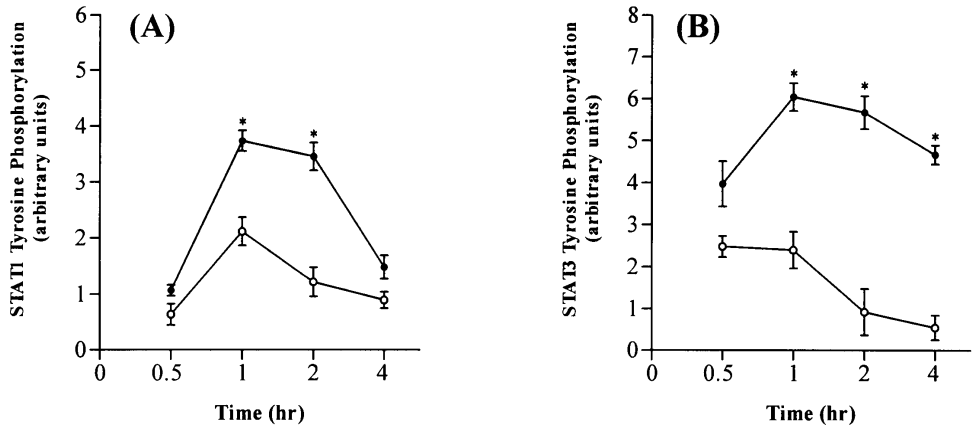

Figure 5 Laser densitometry of the gels shown in Figure 4 and two additional tyrosine phosphorylation experiments

The effects of AGE on tyrosine phosphorylation of STAT1 and STAT3 were analysed. It is evident that AGE significantly increased the levels of tyrosine phosphorylation of STAT1 (**A**) and STAT3 (**B**) at 1–2 h and 0.5–4 h respectively. Symbols: ○, FCS; ●, FCS + AGE. Results are expressed as arbitrary units plotted against time (means ± S.E.M.; $n = 3$). *$P < 0.01$ compared with controls without AGE for the durations indicated.

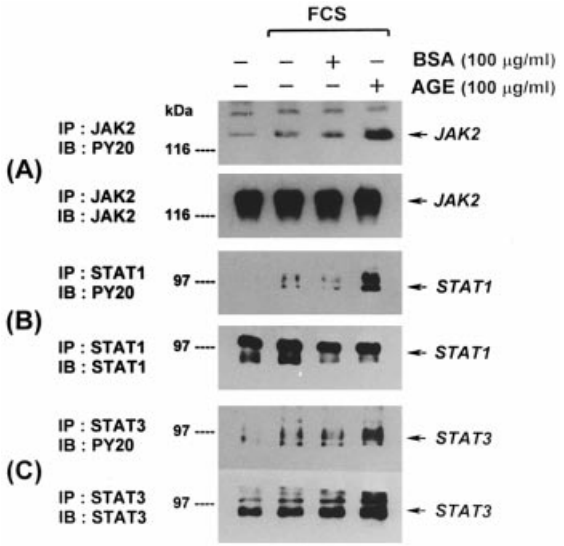

Figure 6 Effects of BSA and AGE on the tyrosine phosphorylation of JAK2, STAT1 and STAT3

Total cell lysates from NRK-49F cells treated with 100 μg/ml AGE or BSA were immunoprecipitated (IP) with anti-JAK2 (**A**), anti-STAT1 (**B**) or anti-STAT3 (**C**). Immune complexes were separated by SDS/PAGE [10% (w/v) gel] and immunoblotted (IB) with either antiphosphotyrosine (PY20) antibodies (upper panels) or antibodies corresponding to the above antibodies (lower panels). These are representative experiments, each performed at least three times.

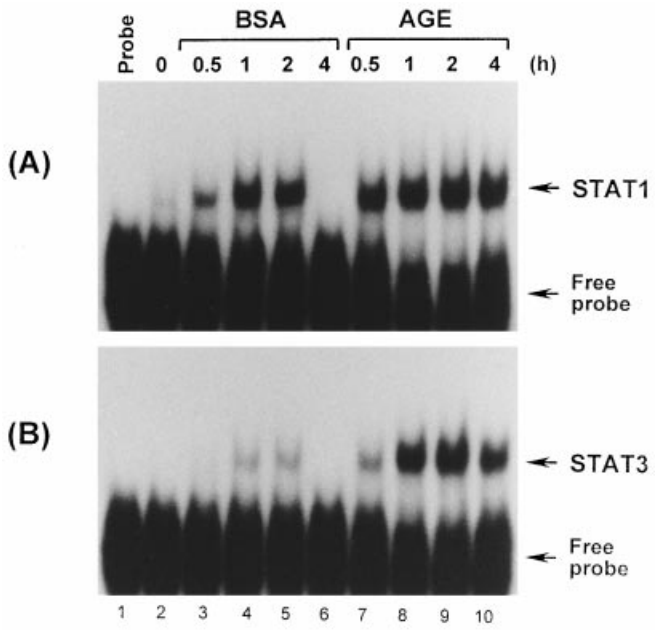

Figure 7 Time course of the effects of BSA and AGE on the DNA-binding activities of STAT1 and STAT3 proteins in NRK-49F cells

Serum-deprived cells were treated with 100 μg/ml BSA or AGE for the indicated times. Nuclear extracts were prepared and assayed for STAT1 and STAT3 activities, as described in the Materials and methods section. In lane 1 (probe), only labelled specific STAT1 or STAT3 ODN probe was added to the binding reaction. This is a representative experiment performed independently three times.

Enhancement of DNA-binding activities of STAT1 and STAT3 by AGE

The tyrosine phosphorylation of STAT in mammalian cells is associated with the acquisition of the ability to bind DNA. Therefore the induction of DNA binding activities in the nuclei of AGE-stimulated NRK-49F cells was determined by using EMSAs with an oligonucleotide probe containing a high-affinity target sequence for STAT factors. Figure 7(A) shows that AGE enhanced the DNA-binding activity of STAT1 protein to the interferon-γ-activated site oligonucleotide at 0.5–4 h. The effect of FCS alone on the DNA-binding activity of STAT1 protein was similar to that observed after the administration of BSA to cells (results not shown). Interestingly, AGE (100 μg/ml) also increased the DNA-binding activity of STAT3 at 0.5–4 h (Figure 7B). These findings indicate that both STAT1 and STAT3 are responsible for the AGE-mediated signalling events.

AG-490 and the STAT decoy ODN inhibit the AGE-enhanced DNA-binding activities of STAT protein and cellular mitogenesis in NRK-49F cells

To examine further the possibility that AGE-dependent proliferative responses were mediated through the JAK2–STAT1/STAT3 cascade, we investigated the effect of AG-490, a specific JAK2 inhibitor. AG-490 belongs to the tyrphostin family of tyrosine kinase inhibitors, which block protein tyrosine kinases by binding to the substrate-binding site [31]. For EMSA, as shown in Figure 8(A) (lane 3), pretreatment with AG-490 (5 μM) for 16 h inhibited the AGE-enhanced DNA-binding activity of STAT1. A similar result was found for STAT3 (Figure 8B, lane 3). To provide evidence that the STAT decoy ODN prevented the binding of STAT to its target sites, we also performed EMSAs in the presence of the STAT1 and STAT3 decoy ODNs and the mismatched control ODNs (Figure 8). We found that the AGE-increased binding activities of STAT1 and STAT3 were abolished by treatment with STAT1 (2 μM) and STAT3 (2 μM)

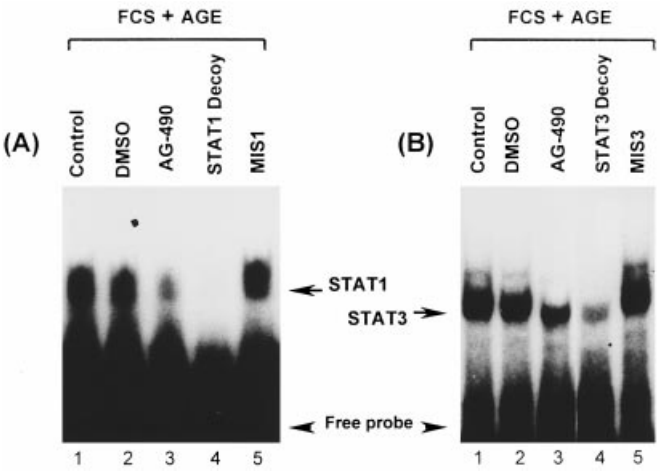

Figure 8 EMSA for the DNA-binding activities of STAT1 (A) and STAT3 (B) proteins in NRK-49F cells

Lanes 1, nuclear extracts from FCS- and AGE-treated NRK-49F cells used as controls; lanes 2 and 3, cells pretreated with DMSO or AG-490 (5 μM) for 16 h before exposure to FCS and AGE; lanes 4, FCS and AGE-stimulated NRK-49F cells transfected with STAT1 or STAT3 decoy ODN; lanes 5, FCS and AGE-stimulated NRK-49F cells transfected with mismatched ODN1 (MIS1) or mismatched ODN3 (MIS3). This is a representative experiment performed independently three times.

Table 1 Effects of AG-490 (JAK2 inhibitor), STAT1 and STAT3 decoy ODNs on AGE-induced cellular mitogenesis in NRK-49F cells

NRK-49F cells were exposed to AGE (100 μg/ml) or BSA (100 μg/ml) for 7 days. The [^{3}H]thymidine incorporation and cell number analysis were determined as described in the Materials and methods section. Values are expressed as percentages of the control value. Results are means $\pm$ S.E.M. for four cultures pooled from three experiments. *Significant effect of the agonist compared with control: $P < 0.05$.

Treatment	[^{3}H]Thymidine incorporation (% of control)	Cell number analysis (% of control)
AGE (100 μg/ml) (control)	100	100
AGE + DMSO	91 $\pm$ 8.6	92 $\pm$ 1.5
AGE + AG-490 (5 μM)	49 $\pm$ 2.7*	55 $\pm$ 4.7*
AGE + STAT1 decoy ODN (2 μM)	52 $\pm$ 3.2*	51 $\pm$ 4.1*
AGE + mismatched ODN1 (2 μM)	102 $\pm$ 8.3	96 $\pm$ 3.8
AGE + STAT3 decoy ODN (2 μM)	48 $\pm$ 3.0*	49 $\pm$ 4.2*
AGE + mismatched ODN3 (2 μM)	96 $\pm$ 2.2	98 $\pm$ 1.7
Medium alone	58 $\pm$ 1.8*	56 $\pm$ 2.1*
BSA (100 μg/ml)	51 $\pm$ 3.6*	59 $\pm$ 4.4*

decoy ODNs respectively. However, the addition of the mismatched ODNs did not inhibit STAT binding. Finally, we examined the effects of AG-490 and the STAT decoy ODN on cellular mitogenesis 7 days after treatment with AGE (Table 1). Pretreatment with AG-490 (5 μM) for 16 h inhibited AGE-induced NRK-49F cell proliferation. In addition, cellular mitogenesis was blunted by transfection of STAT1 and STAT3 decoy ODNs, whereas the mismatched ODNs did not have this effect. These results demonstrate that AGE couples to the induction of the JAK2–STAT1/STAT3 signalling pathway in NRK-49F cells, which suggests that this pathway might contribute to AGE-regulated cell growth.

DISCUSSION

Previous studies have shown that AGE/high glucose level modulates multiple functions of mesangial cells and renal tubular cells, including mitogenesis, hypertrophy and the production of extracellular matrix and cytokine/growth factors [5–7]. However, the present study is the first demonstration that AGE can also induce cellular mitogenesis in a renal interstitial fibroblast cell line NRK-49F. Given that interstitial fibroblast proliferation might be involved in renal fibrosis [8], our finding could have important implications in DN.

The role of renal interstitial fibroblasts in DN has rarely been studied [11]. As with non-renal fibroblasts, skin fibroblasts have been shown to overproduce collagen in high-glucose culture [32]. Skin fibroblasts from patients with DN also have cellular hypertrophy and abnormalities in cell cycles [33]. Additionally, high glucose levels can modulate the growth of gingival fibroblasts [34]. Because we found that AGE induced the proliferation of renal interstitial fibroblasts and it is known that both tubular cells and fibroblasts produce many cytokines or growth factors and extracellular matrices [8,9], their interaction might result in tubulointerstitial fibrosis [9].

Although AGE induces some well-known biological effects in renal cells [7], its signal transduction pathway is less well known. One mechanism through which AGE modulates cellular function is through an interaction with specific cell-surface acceptor molecules. The receptor for AGE (RAGE) is such a receptor and is a newly identified member of the immunoglobulin superfamily expressed on the surface of various cells [35]. Interaction of AGEs with endothelial surface RAGE generates intracellular oxidative stress, resulting in activation of nuclear factor κB transcription factor [35]. Moreover, mitogen-activated protein kinase and its downstream protein, the AP-1 transcription factor, might also be activated by AGE in tubular cells [36]. However, the present study is the first demonstration that the JAK/STAT pathway is associated with mitogenic response to AGE in renal interstitial fibroblasts. Studies are under way to determine whether the regulation of JAK/STAT activation is mediated by a RAGE-dependent pathway in these cells.

The JAK/STAT pathway has been thought to be important in cellular proliferation and differentiation in immune and blood-borne cells [14], whereas immune activation has not traditionally been thought to be related to the pathogenesis of DN [1]. However, tubulointerstitial infiltration of inflammatory cells has been found in the diabetic rat [4]. Additionally, a recent study has shown that AGE could induce an immune response [37]. Thus AGE could activate the JAK/STAT pathway by eliciting the immune response. In contrast, JAK/STAT is part of the angiotensin II signal transduction pathway [38]. In view of the importance of non-haemodynamic factors of angiotensin II in DN [22], AGE could also activate the JAK/STAT pathway by inducing angiotensin II. Finally, it is interesting to note that leptin also activates JAK/STAT [39]. Given that leptin might be associated with obesity and insulin resistance in patients with non-insulin-dependent diabetes mellitus [40] and that leptin receptors are present in the kidney [39], it remains to be studied whether leptin could be induced by AGE.

In this study, we found that the activation of JAK2, STAT1 and STAT3 was associated with the AGE-induced proliferation of NRK-49F cells. Moreover, the specific inhibition of JAK2 by AG-490 abolished the AGE-induced proliferation of NRK-49F cells. This finding establishes that JAK2 is necessary in cellular mitogenesis in our model. This is important because the proliferation of interstitial fibroblasts might contribute to renal fibrosis. Our results therefore raise the possibility that inhibiting the JAK/STAT activity might be a useful pattern in the prevention or treatment of diabetic renal fibrosis.

AGE might induce cellular proliferation either directly or indirectly by inducing cytokines or growth factors. Evidence for the direct pathway is that the molecular signalling mechanisms controlling the growth of fibroblasts have been proposed to involve the JAK/STAT pathway [41]. Therefore it is known that JAK/STAT activates several immediate-early genes that are critical in cellular growth (e.g. Fos and Jun, which form AP-1, and Myc) [42]. In addition, STAT3 can activate the cell-cycle-regulatory protein cyclin D, which is critical for cell cycle progression [43]. Finally, STAT3 was recently shown to activate the angiotensinogen gene in a model of cardiac hypertrophy [44], whereas angiotensin II is known to be a growth factor for many

cells [22,38]. The above mechanisms might therefore interact or synergize to induce cellular proliferation in NRK-49F cells. Indeed, many of the above mechanisms have been shown to be operative in hepatic regeneration [42]. As a corollary, Na_3VO_4 induces mesangial proliferation [45] while activating the JAK/STAT pathway [46].

Evidence for the indirect pathway of AGE-induced cellular proliferation is that AGE induces several cytokines or growth factors that use the JAK/STAT signalling pathway [17–20]. Additionally, JAK/STAT has been shown to contribute to smooth-muscle cell proliferation induced by platelet-derived growth factor and angiotensin II [38]. In summary, JAK/STAT is directly or indirectly involved in AGE-induced cellular proliferation in NRK-49F cells.

In the present study, of the four JAK kinases only JAK2 was increased by AGE. This observation is corroborated by the fact that JAK2 is activated by several cytokines associated with DN, such as insulin, insulin-like growth factor I, angiotensin II, epidermal growth factor, platelet-derived growth factor and growth hormone [14,38,47]. Similarly, of the seven STATs, we chose only STAT1 and STAT3 for this study because STAT1/STAT3 was predominantly activated by the above-mentioned cytokines or growth factors.

Recently, transcription-factor-decoy technology has been developed to inhibit the expression of several genes by using synthetic double-stranded ODNs containing the consensus binding sequence of a transcription factor. Previous studies had reported that the transfection of AP-1 decoy ODN did not alter DNA synthesis and cell number in response to angiotensin II in vascular smooth-muscle cells [48]. However, the administration of E2 promoter binding factor decoy inhibited the proliferation of mesangial cells [49]. In the present study we also employed STAT transcription factor decoys to block NRK-49F cell proliferation. The results of ODN experiments (Figure 8) suggested that our transfection system was able to suppress AGE-induced binding activities of STAT1 and STAT3. In addition, STAT1- and STAT3-decoy treatments both significantly decreased AGE-enhanced NRK-49F cell mitogenesis. Taken together, these observations indicate that STAT1 and STAT3 are important in AGE-induced NRK-49F cellular proliferation. Given that renal interstitial fibroblast proliferation might be critical for renal fibrosis, we speculate that JAK/STAT might be involved in diabetic renal fibrosis.

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