FEBS Letters 582 (2008) 3484–3488

Suppressor of cytokine signaling-1 reduces high glucose-induced TGF-β1 and fibronectin synthesis in human mesangial cells

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Received 10 July 2008; revised 1 September 2008; accepted 9 September 2008

Available online 16 September 2008

Edited by Gianni Cesareni

Abstract Janus kinase (JAK) signal transducers, and activators of transcription (STAT), contribute to diabetic nephropathy. Here we show that one of the suppressors of cytokine signaling (SOCS) proteins, SOCS-1, was upregulated in human mesangial cells (HMCs) under high glucose conditions, along with the activation of JAK2, STAT1, and STAT3. Overexpression of SOCS-1 in HMCs inhibited HG-induced JAK2/STAT activation, c-Fos/c-Jun expression, and increased synthesis of TGF-β1 and fibronectin. These data suggest that SOCS-1 inhibits HG-induced overexpression of TGF-β1 and synthesis of fibronectin in HMC, which may be via JAK/STAT pathway.

Keywords: High glucose; SOCS-1; JAK2/STAT; Glomerular mesangial cell

1. Introduction

Diabetic nephropathy (DN) is one of the common causes of end-stage renal disease, which encompasses a complex of pathological changes, including renal hypertrophy, accumulation of extracellular matrical components, glomerulosclerosis, tubular atrophy and interstitial fibrosis. A basic mechanism underlying diabetic nephropathy appears to be the high glucose (HG)-induced overexpression of transforming growth factor-β (TGF-β) and the accumulation of extracellular matrix (ECM) molecules, such as collagen IV and fibronectin [1,2].

Recent studies suggest that Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling cascades may contribute to diabetic nephropathy [3]. This pathway is mainly related to renal cell growth, production of the cytokine, TGF-β, as well as the ECM proteins collagen IV and fibronectin [4,5]. Wang et al. [4] reported that the activation of JAK2 and STAT1 proteins was a requirement for the hyperglycemia-induced production of TGF-β and fibronectin in rat glomerular mesangial cells.

JAK/STAT pathway is an essential intracellular mechanism of cytokine actions and constitutes a link between activation of cell surface receptors and nuclear transcriptional event [6,7]. Control of the magnitude and duration of cytokine signaling is essential to prevent tissue damage. In this sense, recent studies have shown that JAK/STAT signaling can be regulated at many steps through different mechanisms [7]. The suppressors of cytokine signaling (SOCS) proteins have defined an important additional mechanism for the negative regulation of the JAK/STAT pathway [8,9]. However, the role of SOCS in HG-induced activation of JAK/STAT in human mesangial cell (HMC) is unknown.

Here we hypothesize that SOCS could down-regulate HG-induced TGF-β1 expression and inhibit synthesis of fibronectin via regulating the JAK/STAT pathway. To test this hypothesis, we created HMC stable cell lines overexpressing SOCS-1 to investigate the effects of SOCS-1 on the expression of TGF-β1 and synthesis of extracellular matrix protein fibronectin under high glucose condition, and further explored the activation of JAK/STAT pathway.

2. Materials and methods

2.1. Cell line and reagents

Primary HMC was obtained from Clonetics Corporation (Clonetics, San Diego, CA). RPMI medium1640 was from Gibco Invitrogen Corporation (Grand Island, NY). D-Glucose was obtained from Sigma (St. Louis, MO). The antibodies against SOCS-1, JAK2, STAT1, STAT3, c-Fos, c-Jun and fibronectin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphotyrosine antibodies for JAK2, STAT1 and STAT3 were purchased from Cell Signaling Technology (Beverly, MA). Lipofectamine 2000, pCR3.1 Vector and TRIzol reagent were obtained from Invitrogen Life Technologies (Carlsbad, CA). The RT-PCR system was obtained from Promega (Madison, WI). TGF-β1 ELISA kit was obtained from R&D Systems (Minneapolis, MN). Fibronectin ELISA system was purchased from Chemicon International (Temecula, CA).

2.2. Cell culture and preparation of stable HMC cell lines overexpressing SOCS-1

HMCs were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum, 12.5 mmol/l HEPES, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 atmosphere. Stable transfections of HMC with pCR3.1 vector and pCR3.1/SOCS-1 (kindly provided by Dr. C.J. Auernhammer, Maximilians-Universität, Germany) were performed with Lipofectamine 2000 according to the manufacturer's instructions. Subsequently, cells were cultured in selection medium containing 0.5 mg/ml geneticin for 4

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Abbreviations: SOCS, suppressor of cytokine signaling; HG, high glucose; HMC, human mesangial cell; DN, diabetic nephropathy; ECM, extracellular matrix; JAK, Janus kinase; STAT, signal transducers and activators of transcription

0014-5793/$34.00
doi:10.1016/j.febslet.2008.09.014

weeks before single clones were isolated. The clones were further expanded in selection medium containing geneticin (0.5 mg/ml). HMCs were grown to 75–85% confluence, washed once with serum-free RPMI medium 1640, and then growth-arrested in serum-free RPMI medium 1640 in normal glucose (NG, 5.5 mmol/l) for 24 h to synchronize the cell growth. After this time period, the media were changed to fresh serum-free media containing NG, HG, or NG plus mannitol stimulation at indicated time points.

2.3. RT-PCR analysis

Total RNA and then cDNA were prepared from cultured cells using TRIzol reagent and RT-PCR kits. The primers used were: SOCS-1, forward 5′-CGCGACTACCTGAGCTCCTTC-3′, reverse 5′-AGTTCAGGTCCTGGCTCCAGA-3′, giving 301 bp PCR product; TGF-β1, forward 5′-CCAACTATTGCTTCAGCTCCA-3′, reverse 5′-TTATGCTGGTTGTACAGGG-3′, giving 196 bp PCR product; GADPH, forward 5′-ATCCCATCACCATCTTCCAG-3′, reverse 5′-CCATCACGCCACAGTTTCC-3′, giving 382 bp PCR product. The PCR products were subjected to 2% agarose gel electrophoresis and analyzed with a GDS-8000 Bioimaging system (UVP, upland, CA) and LabWorks 4.5 software (UVP). RNA expression was quantified by comparison with internal-control GAPDH.

2.4. Western blot analysis

Whole cell extracts or the culture medium (50 μg of protein/lane) was loaded, separated by SDS–PAGE, transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with anti-SOCS-1, c-Fos, c-Jun, fibronectin and β-actin antibodies. Subsequently, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse IgG horseradish peroxidase conjugate, and then exposed to X-ray film using enhanced chemiluminescence system. To determine the activation of JAK/STAT pathway, the membranes were firstly blotted with phospho-JAK2, STAT1 and STAT3 antibodies and then the phospho-antibodies were stripped off and blotted with JAK2, STAT1 and STAT3 antibodies. The JAK2, STAT1 and STAT3 phosphorylation were quantified by comparing the phospho and total protein levels from the same blot. The intensity of the bands was measured using LabWorks 4.5.

2.5. Enzyme-linked immunosorbent assay

After the cells were cultured in 6-well plates under the different experimental conditions for 48 h, the supernatants were collected. The TGF-β1 or fibronectin protein was quantified using a commercial quantikine enzyme-linked immunosorbent assay (ELISA) kit for TGF-β1 or competitive sandwich ELISA for fibronectin according to the manufacturer's descriptions.

2.6. Statistical analysis

Statistical analysis was performed by one-way ANOVA. The results were presented as means ± S.E.M. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. High glucose induces expression of SOCS-1 in HMC

The synthesis of SOCS-1 protein in HG-stimulated cells was analyzed by Western blot. The expression level of SOCS-1 protein increased within 1 h of HG stimulation, peaked at 4 h, and gradually decreased to baseline at 24 h (Fig. 1A). HG-induced time-dependant mRNA levels of SOCS-1 were evaluated by RT-PCR analysis. As shown in Fig. 1B, the increased expression of SOCS-1 was detected at 1 h and peaked at 4 h. In addition, no differences were found in HMC cultured under conditions of NG or NG plus mannitol among different time points (data not shown).

3.2. HG activates the JAK/STAT pathway in HMC

To determine the effect of HG on activation of the JAK/STAT signal pathway, we examined the phosphorylation of JAK2, STAT1, and STAT3 by Western blot analysis. As shown in Fig. 2, HG-induced time-dependent tyrosine phosphorylation of JAK2, STAT1, and STAT3; the phosphorylation levels were peaked at 12–24 h, and remained at high levels until 48 h.

3.3. SOCS-1 overexpression inhibits HG-induced tyrosine phosphorylation of JAK2, STAT1 and STAT3

To investigate the modulation of JAK/STAT signal pathway by SOCS-1 under HG condition, HMCs were transfected with expression vector for SOCS-1 (pCR3.1/SOCS-1) or control vector (pCR3.1). The expression of SOCS-1 protein was 5.8-fold greater than that of the control group (Fig. 3A). Compared with those of the NG groups, the phosphorylation levels of JAK2, STAT1, and STAT3 significantly increased in HG group; whereas, overexpression of SOCS-1 in HMCs significantly suppressed HG-induced JAK2, STAT1 and STAT3 phosphorylation (Fig. 3B).

3.4. SOCS-1 overexpression reduces the synthesis of TGF-β1 and fibronectin

TGF-β1 levels in the culture medium were measured with ELISA. A significant increase in TGF-β1 protein was seen after HMCs were cultured under HG condition at 48 h, whereas the HG-induced overexpression of TGF-β1 was

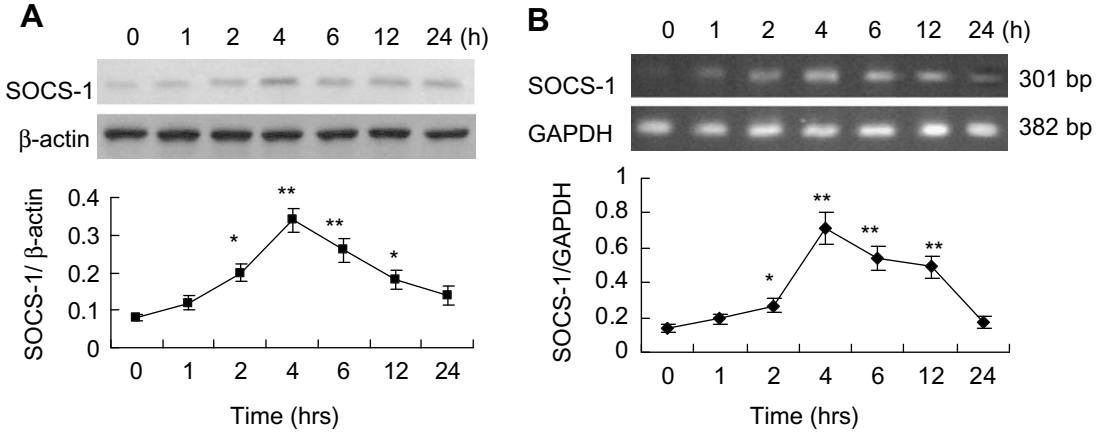

Fig. 1. Time course of the effects of HG on SOCS-1 protein and mRNA expression. HMCs were incubated with HG (30 mM) at the indicated times (0–24 h). (A) The expression of SOCS-1 was analyzed by immunoblotting ($n = 6$). (B) The expression of SOCS-1 mRNA was detected by RT-PCR analysis ($n = 6$). Values are expressed as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (0 h).