

S100B is required for high glucose-induced pro-fibrotic gene expression and hypertrophy in mesangial cells

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Abstract. The advanced glycation end-product (AGE)-receptor for AGE (RAGE) axis induces transforming growth factor- β (TGF- β) expression, cell hypertrophy and increases extracellular matrices that are indicated in the pathogenesis of diabetic nephropathy (DN). RAGE binds to numerous ligands besides AGE, including S100B. In the present study, the roles of S100B in high glucose-induced p21^{WAF1}, extracellular matrices, TGF- β 1 and cell hypertrophy in mouse mesangial (MES13) cells were investigated. High glucose (30 mM) time-dependently (24-72 h) induced S100B expression. High glucose and exogenous S100B (1 μ M) time-dependently increased p21^{WAF1} gene transcription and protein expression, increased type IV collagen and fibronectin protein expression, and TGF- β gene transcription and bioactivity. Exogenous S100B also time-dependently activated the extracellular regulated kinases (ERK1/2), p38 kinase and c-Jun N-terminal kinase (JNK) signaling pathways. Exogenous S100B-induced TGF- β gene transcription and bioactivity were attenuated by SB203580 (p38 kinase inhibitor) and PD98059 (ERK1/2 inhibitor). Finally, the knockdown of *S100B* by small interfering RNA (siRNA) attenuated high glucose-induced TGF- β gene transcription and bioactivity, type IV collagen and fibronectin protein expression and p21^{WAF1} protein expression. Thus, S100B induced TGF- β via the ERK1/2 and p38 kinase pathways in mesangial cells. Additionally, high glucose-induced pro-fibrotic genes (TGF- β , type IV collagen and fibronectin) and cell hypertrophy-related p21^{WAF1} are dependent on S100B.

Introduction

Chronic hyperglycemia increases advanced glycation end-products (AGE) that bind to the receptor for AGE (RAGE). The AGE-RAGE axis induces transforming growth factor- β (TGF- β), cell hypertrophy and increases extracellular matrices that are implicated in the pathogenesis of diabetic nephropathy (DN) (1-4). RAGE engagement by the specific ligands triggers DN-related signaling pathways, including extracellular regulated kinases (ERK1/2), p38 kinase and c-Jun N-terminal kinase (JNK) (5).

High glucose increases RAGE expression in mesangial cells (6) and the pivotal role of RAGE in DN can be illustrated by the finding that glomerular RAGE expression is increased in diabetic mice (7). Additionally, RAGE overexpression exacerbates, whereas RAGE deletion attenuates experimental DN (3,8). TGF- β is another central player in DN as it is pivotal in cell hypertrophy and increased extracellular matrices in DN (1,4). Diabetic renal hypertrophy is associated with cell cycle arrest and increased glomerular p21^{WAF1} (cyclin-dependent kinase inhibitor) expression (9,10), whereas p21^{WAF1}-knockout mice are protected from glomerular hypertrophy despite increased TGF- β levels (11).

RAGE binds to a number of ligands besides AGE, including S100B (a member of the S100/calgranulin family) (12). S100B is mainly expressed and secreted by the neural cells (12), whereas cerebral levels of S100B are increased in the diabetic rats (13). Notably, in two previous studies, it was found that S100B increases TGF- β 1 and fibronectin expression, while activating ERK1/2 and p38 kinase pathways in mesangial cells (14,15). However, the roles of endogenous S100B in high glucose-induced effects are not known.

Therefore, the roles of S100B in high glucose-induced p21^{WAF1}, extracellular matrices, TGF- β 1 and cell hypertrophy in mouse mesangial (MES13) cells were investigated in the present study. Additionally, the molecular mechanisms of S100B-induced TGF- β activity were assessed in terms of the ERK1/2, p38 kinase and JNK pathways.

Materials and methods

Materials. Dimethyl sulfoxide, PD98059 (ERK1/2 inhibitor), SB203580 (p38 kinase inhibitor), SP600125 (JNK inhibitor)

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and SB431542 (type I TGF- β receptor inhibitor) were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). S100B was purchased from the Abcam Co. (Cambridge, MA, USA).

Cells. The mouse mesangial cell line (MES13 cells, BCRC-60366) was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan), which obtained the cells (CRL-1927) from the American Type Culture Collection (Manassas, VA, USA). Cells were used within 30 passages of the original passage and were cultured in a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (final glucose 6.67 mM), 14 mM HEPES, 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) and 5% fetal bovine serum (FBS; Gibco) in 5% CO₂ at 37°C. MES13 cells were serum-starved for 16 h before treatment with S100B (1 μ M) or the signaling pathway inhibitors.

Immunoblotting. Total cell lysates were harvested, resolved by 10% SDS-polyacrylamide gel electrophoresis, and were subsequently transferred to Protran membranes (0.45 mm; Schieicher & Schuell, Keene, NH, USA). The membranes were blocked in blocking solution and subsequently probed with the following primary antibodies: S100B (ab41548, rabbit anti-rat) and col4 α 1 (ab6586, rabbit anti-human; Abcam Co.), p21^{WAF1} (sc-397, goat anti-human) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc25778, rabbit anti-human; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), fibronectin (AB1954, rabbit anti-rat; Chemicon International, Inc., Temecula, CA, USA), α -tubulin (MS-581-P0, mouse anti-chicken monoclonal antibody; Thermo Fisher Scientific, Inc., Fremont, CA, USA), ERK1/2 (9102, rabbit anti-rat), p-ERK1/2 (4370, rabbit anti-human; Thr202/Tyr204), p38 kinase (9212, rabbit anti-human), p-p38 kinase (4631, rabbit anti-human; Thr180/Tyr182), JNK (9258, rabbit anti-human) and p-JNK (9251, rabbit anti-human; Thr183/Tyr185) (Cell Signaling Technology, Inc., Beverly, MA, USA). The membrane was subsequently incubated in 4,000x diluted horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody. The protein bands were detected using the enhanced chemiluminescence system. The intensity of the immunoblot bands was quantified by densitometric analysis. Results are expressed as the ratio of intensity of the protein of interest to that of α -tubulin or the indicated protein from the same sample.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using the REzol reagent (PT-KP200CT) according to the manufacturer's instructions (Protech Technologies, Inc., Taipei, Taiwan). Briefly, cDNA was synthesized from 1 μ g RNA with the reverse transcriptase system (Promega Corp., Madison, WI, USA). RT-qPCR of the obtained cDNA was performed in triplicate on an ABI 7900 Fast RT-PCR System (Applied Biosystems, Warrington, Cheshire, UK) using SYBR Green for qPCR (Protech Corp.). The primers used were: mouse *S100B* forward, 5'-TGGTTGCCCTCATTGATGTCT-3' and reverse, 5'-CTCGTTGTTGATAAGCTCCTTCAG-3'; and mouse *18S rRNA* forward, 5'-CCAGTAAGTGC GGTCATAAGC-3' and reverse, 5'-CCTCACTAAACCATCCAATCGG-3'.

Enzyme-linked immunosorbent assay (ELISA). Medium S100B was measured using the S100B ELISA kit (Millipore Corp., Billerica, MA, USA) according to the manufacturer's instructions. The absorbance at 450 nm was recorded by the ELISA reader. The level of secreted S100B was calculated for one million cells.

Luciferase reporter plasmid transfection. The p21 promoter reporter construct p21P was a gift from Dr X.F. Wang (Department of Pharmacology, Duke University Medical Center, Durham, NC, USA) (16). The human TGF- β 1 promoter-luciferase construct pH2G5-luc was a gift from Dr J.L. Virelizier (Unité d'Immunologie Virale, Institut Pasteur, Paris, France) (17). The TGF- β bioactivity reporter p3TP-lux was a gift from Dr J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) (18). Plasmids were transiently transfected into the MES13 cells in 6-well plates (1x10⁴ cells/well) using the TurboFect reagent (Fermentas Inc., Israel). Medium containing 5% FBS was added 24 h later and cells were treated with high glucose or 1 μ M S100B for the indicated times. Cells were lysed and luciferase activity was measured by the Dynatech ML1000 luminometer (Dynatech Laboratories, Inc., Chantilly, VA, USA).

Cell hypertrophy. Cells were grown in 6-well plates until approximately 50% confluent and were subsequently made quiescent for 16 h in the medium containing 0.1% FBS. The cultures were treated with HG (30 mM) and S100B (1 μ M) for the indicated times, following which the cells were trypsinized, washed twice with phosphate-buffered saline (PBS) and counted using a hemocytometer with trypan blue staining. Equal numbers of cells were lysed in buffer [0.1% (wt/vol) SDS, 0.5% (wt/vol) sodium deoxycholate and 1.0% (wt/vol) Nonidet P-40, in PBS]. The total protein content was measured by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Cell hypertrophy was assessed by steady-state total protein (μ g)/10⁴ cells.

Small interfering RNA (siRNA) transfection. S100B siRNA (sense, 5'-GCC CUC AUU GAU GUC UUC CAC CAG U-3' and antisense, 5'-ACU GGU GGA AGA CAU CAA UGA GGG C-3') were purchased from the Invitrogen Corp. (Grand Island, NY, USA). Negative control siRNA (Trilencer-27 siRNA) was from the OriGene Technologies Inc. (Rockville, MD, USA). We transfected 50 nM siRNA into cells in 6-well plates (10x10⁴ cells/well) by using the TurboFect reagent (Fermentas Inc.). Cells were exchanged with fresh 5% FBS-containing medium 24 h after transfection.

Statistics. Data are expressed as the means \pm standard error of the mean. *In vitro* experimental data were collected from ≥ 3 repeated experiments. One-way analysis of variance followed by post-hoc Dunnett tests was used for the comparison between the treated and control groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

High glucose increases S100B protein and mRNA expression in mes13 cells. S100B mRNA was measured by RT-qPCR, while

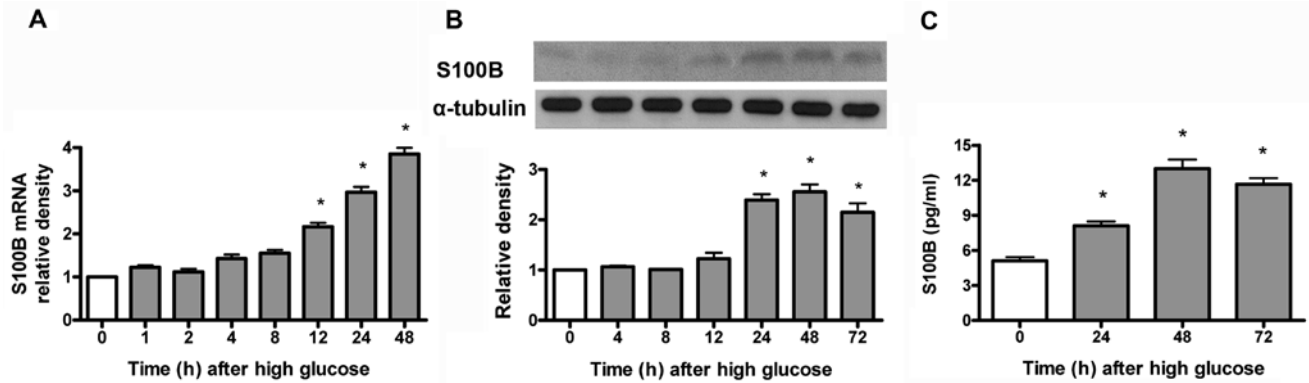


Figure 1. High glucose increases S100B protein and mRNA expression in MES13 cells. Serum-fasted cells were exposed to normal glucose (5.5 mM, open bars) or high glucose (30 mM, closed bars). (A) *S100B* mRNA was measured by RT-qPCR. Time-dependent (1-48 h) effects of high glucose on *S100B* mRNA expression are shown. (B) S100B protein was measured by immunoblotting and normalized to α -tubulin. Time-dependent (4-72 h) effects of high glucose on S100B protein expression are shown. (C) Medium S100B was measured using the S100B ELISA kit. Time-dependent (24-72 h) effects of high glucose on medium S100B concentration are shown. Data are expressed as the means \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. 0 h.

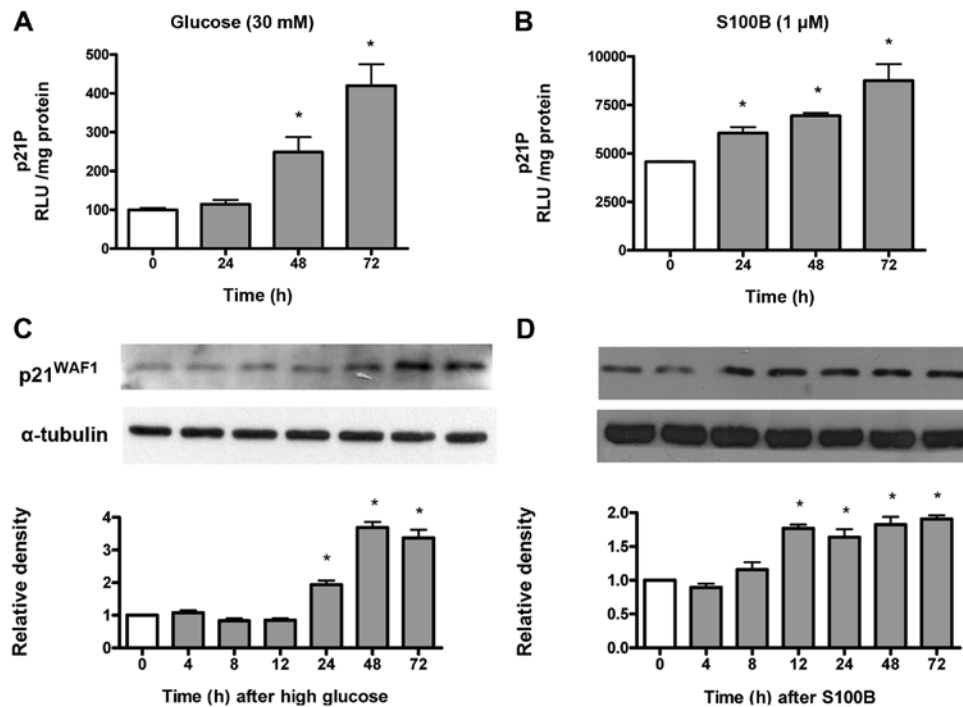


Figure 2. High glucose and S100B time-dependently increase *p21^{WAF1}* gene transcription and protein expression. Transcriptional activity of *p21^{WAF1}* was measured by transient transfection of the *p21^{WAF1}* promoter reporter construct p21P. Protein expression of *p21^{WAF1}* was measured by immunoblotting and normalized to α -tubulin. Time-dependent (24-72 h) effects of (A) high glucose (30 mM, closed bars) and (B) S100B (1 μ M, closed bars) on *p21^{WAF1}* gene transcription was expressed as the relative light units (RLU). Time-dependent (4-72 h) effects of (C) high glucose (30 mM, closed bars) and (D) S100B (1 μ M, closed bars) on *p21^{WAF1}* protein expression. Data are expressed as the means \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. 0 h.

S100B protein was measured by immunoblotting and ELISA. As shown in Fig. 1, high glucose (30 mM) time-dependently (12-48 h) increased *S100B* mRNA (Fig. 1A) and time-dependently (24-72 h) increased S100B protein (Fig. 1B) expression and medium S100B protein levels (Fig. 1C).

High glucose and S100B time-dependently increase *p21^{WAF1}* gene transcription and protein expression. High glucose-induced cell hypertrophy is associated with increased *p21^{WAF1}* expression in mesangial cells (10). Gene transcriptional activity of the *p21^{WAF1}* gene was measured by p21P,

while protein expression was measured by immunoblotting. As shown in Fig. 2A, high glucose time-dependently (48-72 h) increased the *p21^{WAF1}* gene transcriptional activity. Similarly, S100B (1 μ M) time-dependently (24-72 h) increased the *p21^{WAF1}* gene transcriptional activity (Fig. 2B). As shown in Fig. 2C, high glucose time-dependently (48-72 h) increased *p21^{WAF1}* protein expression. Similarly, S100B (1 μ M) time-dependently (12-72 h) increased *p21^{WAF1}* protein expression (Fig. 2D).

High glucose and S100B time-dependently increase type IV collagen and fibronectin protein expression. Type IV collagen

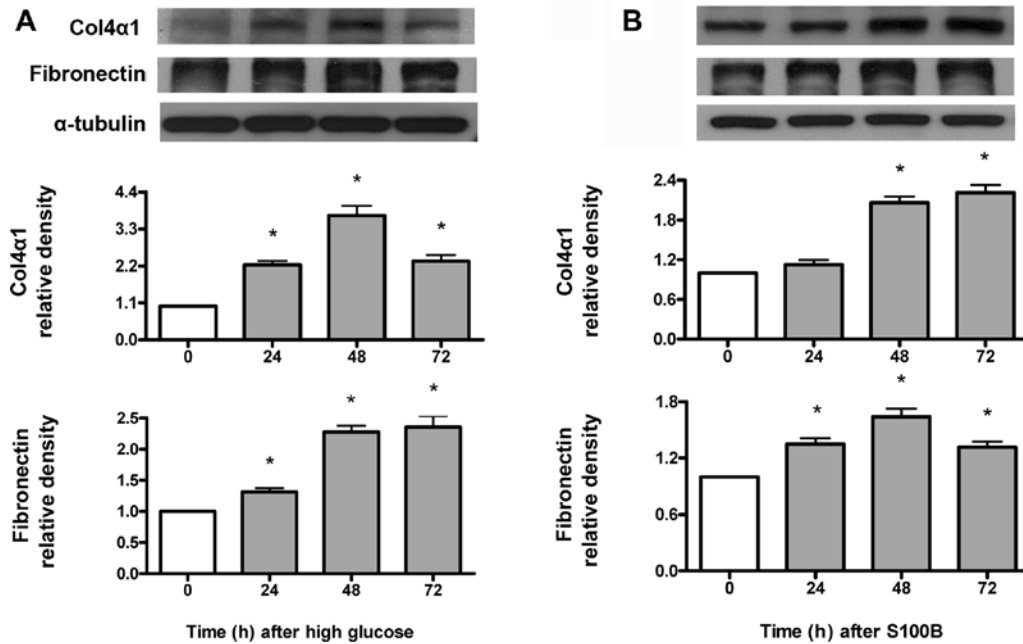


Figure 3. High glucose and S100B time-dependently increase type IV collagen and fibronectin protein expression. Expression of type IV collagen (col4α1) and fibronectin protein was measured by immunoblotting and normalized to α -tubulin. Time-dependent (24–72 h) effects of (A) high glucose (30 mM, closed bars) and (B) S100B (1 μ M, closed bars) on col4α1 and fibronectin protein expression. Data are expressed as the means \pm standard error of the mean of three independent experiments. * P <0.05 vs. 0 h.

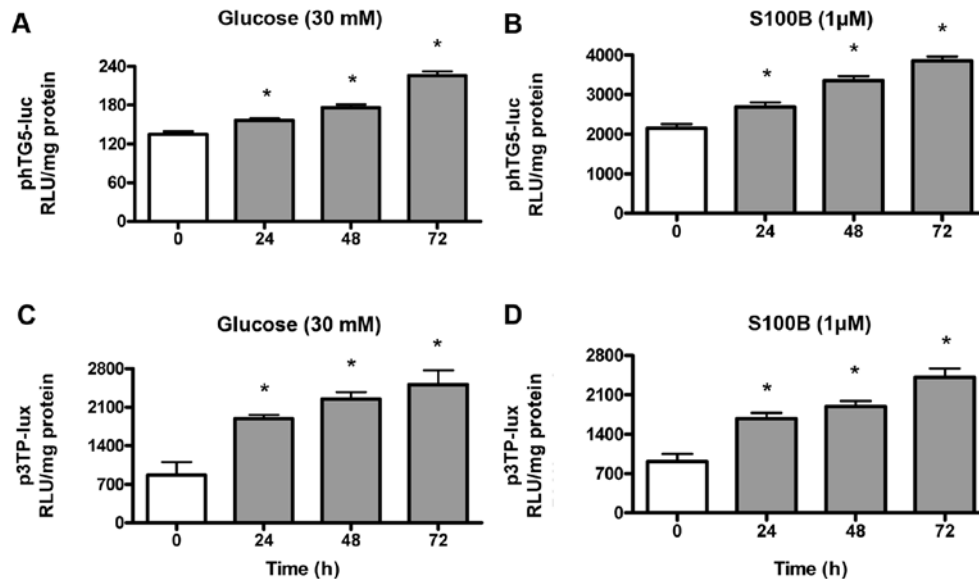


Figure 4. High glucose and S100B time-dependently induce *TGF- β* gene transcription and bioactivity. *TGF- β* transcriptional activity or bioactivity (expressed as the relative light units, RLU) were measured by transient transfection of phTG5-luc (*TGF- β* promoter plasmid) or p3TP-lux (*TGF- β* bioactivity reporter plasmid), respectively. Time-dependent (24–72 h) effects of (A) high glucose (30 mM, closed bars) or (B) S100B (1 μ M, closed bars) on *TGF- β* transcriptional activity. Effects of (C) high glucose (30 mM, closed bars) and (D) S100B (1 μ M, closed bars) on *TGF- β* bioactivity. Data are expressed as the means \pm standard error of the mean of three independent experiments. * P <0.05 vs. 0 h. *TGF- β* , transforming growth factor- β .

and fibronectin protein expression was measured by immunoblotting. As shown in Fig. 3A, high glucose (30 mM) increased type IV collagen and fibronectin protein expression at 24–72 h. Similarly, S100B (1 μ M) increased type IV collagen protein expression at 48–72 h, but increased fibronectin protein expression at 24–72 h (Fig. 3B).

High glucose and S100B time-dependently induce TGF- β gene transcription and bioactivity. Gene transcriptional activity of the *TGF- β* gene was measured by transient transfection of phTG5-luc, while *TGF- β* bioactivity was measured by transient transfection of p3TP-lux. As shown in Fig. 4A and B, high glucose (30 mM) and S100B (1 μ M) time-dependently (24–72 h)

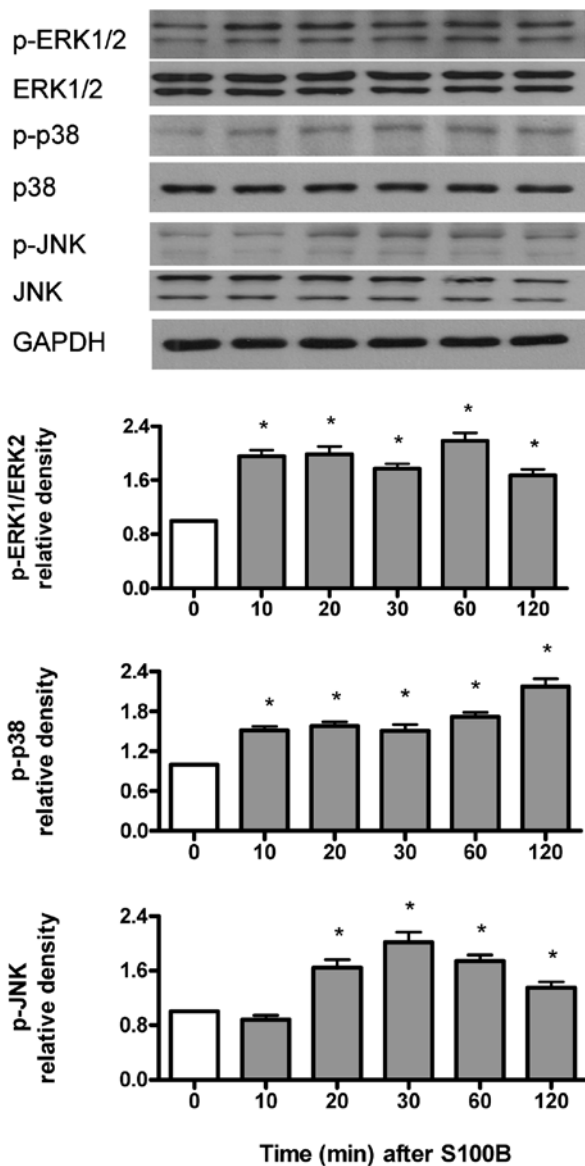


Figure 5. S100B time-dependently activates ERK1/2, p38 and JNK kinases. Phospho-ERK1/2 (p-ERK1/2), ERK1/2, p-p38 kinase, p38 kinase, p-JNK, JNK and GAPDH were measured by immunoblotting. Expression of phosphorylated proteins was normalized to that of the respective unphosphorylated proteins. Time-dependent (10-120 min) effects of S100B (1 μ M) on p-ERK1/2, p-p38 and p-JNK expression are shown. Data are expressed as the means \pm standard error of the mean of three independent experiments. *P<0.05 vs. 0 h. ERK1/2, extracellular regulated kinases; JNK, c-Jun N-terminal kinase.

increased the *TGF- β* gene transcriptional activity. As shown in Fig. 4C and D, high glucose (30 mM) and S100B (1 μ M) time-dependently (24-72 h) increased *TGF- β* bioactivity.

S100B time-dependently activates ERK1/2, p38 and JNK kinases. p-ERK1/2, p-p38 kinase and p-JNK were measured by immunoblotting and were normalized to their respective unphosphorylated forms. As shown in Fig. 5, S100B (1 μ M) increased p-ERK1/2 and p-p38 kinase at 10-120 min, but increased p-JNK at 20-120 min.

*S100B-induced *TGF- β* gene transcription and *TGF- β* bioactivity are dependent on ERK1/2, p38 and *TGF- β* receptors.* Gene transcriptional activity of the *TGF- β* gene was measured

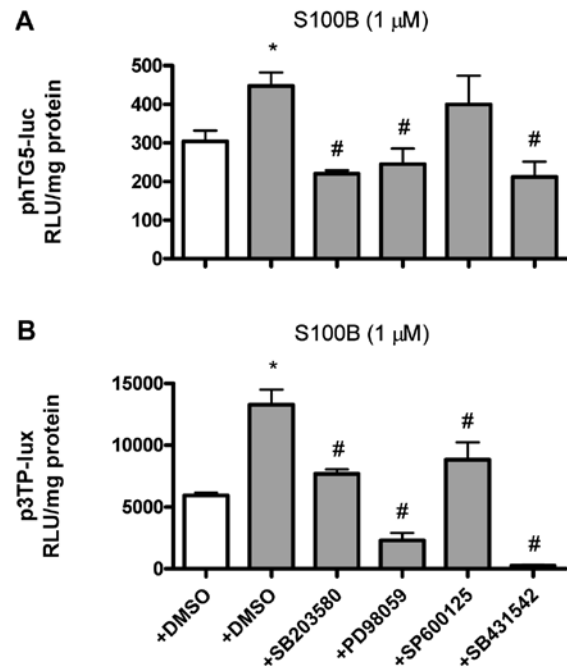


Figure 6. S100B-induced *TGF- β* gene transcription and bioactivity are dependent on ERK1/2, p38 and *TGF- β* receptor. *TGF- β* transcriptional activity or bioactivity (expressed as the relative light units, RLU) were measured by transient transfection of phTG5-luc (*TGF- β* promoter plasmid) or p3TP-lux (*TGF- β* bioactivity reporter plasmid), respectively. SB203580, PD98059, SP600125 and SB431542 were dissolved in dimethyl sulfoxide (DMSO) (final concentration 0.1%). (A) Effects of SB203580 (10 μ M, p38 inhibitor, pre-treated for 1 h), PD98059 (10 μ M, ERK1/2 inhibitor, pre-treated for 1 h), SP600125 (10 μ M, JNK inhibitor, pre-treated for 1 h) and SB431542 (10 μ M, *TGF- β* receptor type I inhibitor, pre-treated for 1 h) on S100B (1 μ M, closed bars)-induced *TGF- β* transcription at 24 h. (B) Effects of SB203580, PD98059, SP600125 and SB431542 on S100B (1 μ M, closed bars)-induced *TGF- β* bioactivity at 24 h. Data are expressed as the means \pm standard error of the mean of three independent experiments. *P<0.05 vs. lane 1. #P<0.05 vs. lane 2. *TGF- β* , transforming growth factor- β ; ERK1/2, extracellular regulated kinases; JNK, c-Jun N-terminal kinase.

by phTG5-luc, while *TGF- β* bioactivity was measured by p3TP-lux. SB203580 (p38 kinase inhibitor), PD98059 (ERK1/2 inhibitor) and SB431542 (type I *TGF- β* receptor inhibitor) attenuated S100B (1 μ M)-induced *TGF- β* gene transcriptional activity (Fig. 6A) and *TGF- β* bioactivity (Fig. 6B) at 24 h. By contrast, SP600125 (JNK inhibitor) only attenuated S100B (1 μ M)-induced *TGF- β* bioactivity (Fig. 6B), but not *TGF- β* gene transcriptional activity (Fig. 6A) at 24 h.

*High glucose-induced *TGF- β* and pro-fibrotic genes are dependent on S100B.* Gene transcriptional activity of the *TGF- β* gene was measured by transient transfection of phTG5-luc, while *TGF- β* bioactivity was measured by p3TP-lux. Type IV collagen, fibronectin, S100B and p21^{WAF1} proteins were measured by immunoblotting. The role of S100B was measured by *S100B* siRNA. *S100B* (but not the scrambled) siRNA attenuated high glucose-induced *TGF- β* gene transcriptional activity (Fig. 7A) and bioactivity (Fig. 7B) at 24 h. Additionally, *S100B* (but not the scrambled) siRNA increased high glucose-induced type IV collagen, fibronectin, S100B and p21^{WAF1} protein expression at 48 h (Fig. 7C). Finally, *S100B* (but not the scrambled) siRNA

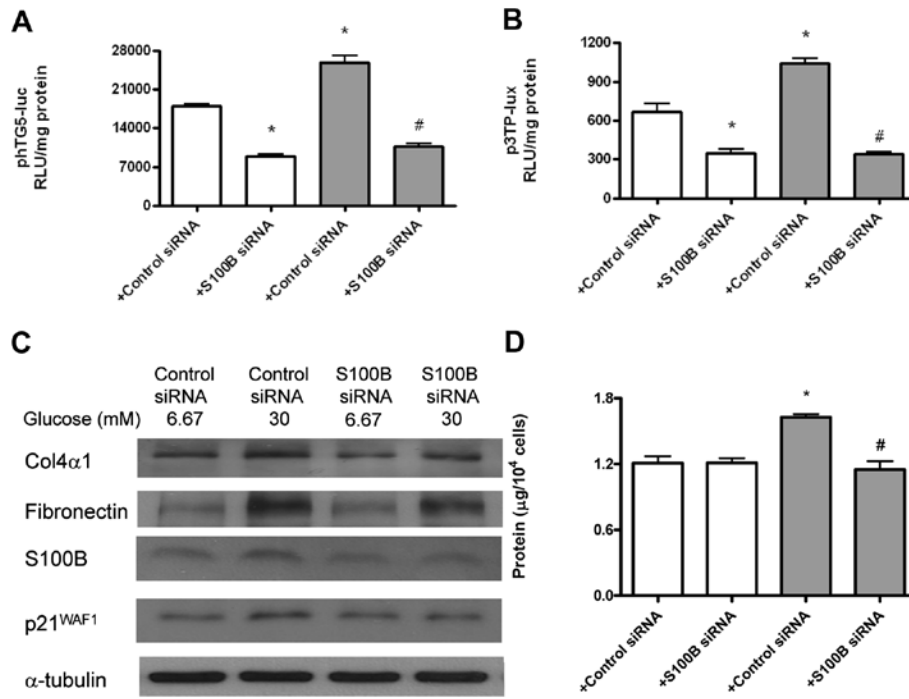


Figure 7. High glucose-induced TGF- β and pro-fibrotic genes are dependent on S100B. *S100B* siRNA (50 nM) was transiently transfected into cells. TGF- β transcriptional activity or bioactivity (expressed as the relative light units, RLU) was measured by transient transfection of pHSG5-luc (TGF- β promoter plasmid) or p3TP-luc (TGF- β bioactivity reporter plasmid), respectively. Expression of type IV collagen (col4 α 1), fibronectin, S100B and p21^{WAF1} proteins was measured by immunoblotting and normalized to α -tubulin. Cell hypertrophy was assessed by total protein (μ g)/10⁴ cells. Effects of *S100B* siRNA on high glucose (30 mM, closed bars)-induced (A) TGF- β gene transcriptional activity and (B) bioactivity at 24 h. Effects of *S100B* siRNA on high glucose-induced (C) col4 α 1, fibronectin, S100B and p21^{WAF1} proteins and (D) cell hypertrophy at 48 h. Data are expressed as the means \pm standard error of the mean of three independent experiments. *P<0.05 vs. lane 1. #P<0.05 vs. lane 3. TGF- β , transforming growth factor- β ; siRNA, small interfering RNA.

attenuated high glucose-induced cell hypertrophy (Fig. 7D) at 48 h.

Discussion

To the best of our knowledge, this is the first study to demonstrate that high glucose increased S100B in mesangial cells and that high glucose and S100B increased p21^{WAF1}, type 4 collagen, fibronectin and TGF- β activity. Additionally, S100B-induced TGF- β is dependent on ERK1/2 and p38 kinase, whereas high glucose-induced expression of p21^{WAF1}, type IV collagen, fibronectin and TGF- β and cell hypertrophy are dependent on S100B. These findings shed light on the roles of S100B in DN.

The finding that high glucose increased *S100B* expression in mesangial cells is similar to two previous studies showing increased *S100B* mRNA levels in diabetic human glomeruli (19) or diabetic rat renal cortex (20) in the Gene Expression Omnibus microarray database (21), although the original studies did not mention it. High glucose and exogenous S100B were also found to induce the p21^{WAF1} gene transcription and protein expression, type IV collagen and fibronectin expression and TGF- β gene transcription and bioactivity. These findings prompted us to further elucidate the roles of S100B in high glucose-induced effects.

High glucose-induced TGF- β gene transcription and bioactivity, p21^{WAF1} gene transcription and protein expression, and type IV collagen and fibronectin expression are

dependent on S100B. In addition, high glucose-induced cell hypertrophy is also dependent on S100B. As S100B is a specific ligand for RAGE (3), it is likely that the above high glucose-induced effects are dependent on RAGE. However, it is also likely that intracellular S100B acts without engaging RAGE (22).

ERK1/2, p38 kinase and JNK pathways have been indicated in the pathogenesis of DN (1,5). These pathways are also the downstream targets of the RAGE signaling (5). S100B activated the ERK1/2, p38 kinase and JNK pathways in the present study. Additionally, S100B-induced TGF- β transcription and bioactivity are dependent on the TGF- β receptor and the ERK1/2 and p38 kinase pathways. These findings are compatible with the notion of the auto-induction of TGF- β (23) and also corroborate two previous findings that TGF- β 1 induction is dependent on ERK1/2 and p38 kinase in mesangial cells (24,25). By contrast, S100B-induced TGF- β bioactivity (but not gene transcription) is dependent on the JNK pathway. This finding is compatible with the notion that JNK is a part of the TGF- β non-Smad signaling pathways (26).

In conclusion, S100B induced TGF- β via the ERK1/2 and p38 kinase pathways. The significance of high glucose-induced S100B is demonstrated by the findings that high glucose-induced pro-fibrotic genes (TGF- β , type IV collagen and fibronectin) and cell hypertrophy-related p21^{WAF1} are dependent on S100B. Thus, S100B may be a novel target for the treatment of DN.

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