Thrombospondin-1 mediates distal tubule hypertrophy induced by glycated albumin

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Diabetic nephropathy is characterized by early hypertrophy in both glomerular and tubuloepithelial elements. However, no studies to date have established a direct causal link between hyperglycaemia and renal hypertrophy. Our previous studies have found that high glucose does not induce cellular hypertrophy or expression of TGF-β1 (transforming growth factor-β1) in distal renal tubule cells [Yang, Guh, Yang, Lai, Tsai, Hung, Chang and Chuang (1998) J. Am. Soc. Nephrol. **9**, 182–193]. In the present study, we used AGEs (advanced glycation end-products) to mimic long-term hyperglycaemia. Similar to glucose, AGEs did not induce TGF-β1 mRNA in distal renal tubule cells [MDCK (Madin–Darby canine kidney) cells]; however, TGF-β1 bioactivity was increased significantly. This result indicated post-translational regulation. Since TSP-1 (thrombospondin-1) has been demonstrated to activate latent TGF-β1 in a variety of systems, the following experiments were performed. We found that AGEs dose-dependently increased both intracellular and extracellular levels of TSP-1. Purified TSP-1, like AGEs, increased the cellular protein content. Furthermore, anti-TSP-1 neutralizing antibodies attenuated the AGE-induced increase in TGF-β1 bioactivity and hypertrophy. Thus TSP-1 might mediate AGE-induced distal renal tubule hypertrophy. In addition, we observed several putative transcription factor binding sites in the TSP-1 promoter, including those for AP-1 (activator protein-1), CREB (cAMP response element binding protein), NF-κB (nuclear factor-κB), SRF (serum response factor) and HSF (heat-shock factor), by sequence mapping. We used an enhancer assay to screen possible transcription factors involved. We showed that AP-1 and CREB were specifically induced by AGEs; furthermore, TFD (transcription factor decoy) for AP-1 could attenuate the AGE-induced increases in TSP-1 levels and cellular hypertrophy. Thus regulation of TSP-1 might be critical for hyperglycaemic distal tubule hypertrophy. Furthermore, TSP-1 TFD might be a potential approach to ameliorate diabetic renal hypertrophy.

Key words: advanced glycation, diabetic nephropathy, growth factor, transforming growth factor-β.

INTRODUCTION

Diabetic nephropathy, a common complication in patients with either Type I or Type II diabetes mellitus, has long been recognized to cause severe morbidity and mortality. Renal hypertrophy, which is characterized by cellular hypertrophy and/or hyperplasia in glomerular mesangial cells and tubulointerstitial epithelial cells, usually occurs at the first stage in the development of diabetic nephropathy.

The involvement of TGF-β (transforming growth factor-β) in the pathogenesis of diabetic renal hypertrophy has been extensively discussed [1]. This cytokine acts in an autocrine or paracrine fashion to affect cell growth and extracellular matrix accumulation. Our previous studies [2,3] and others [4] have demonstrated that most renal cells develop cellular hypertrophy under TGF-β treatment, including glomerular mesangial cells, proximal renal tubule and distal renal tubule epithelial cells. In addition, induction of TGF-β is typically found in proximal tubular epithelial cells, glomerular mesangial cells and interstitial fibroblasts [5–8] when cultured in high ambient glucose concentrations. Thus TGF-β might be a pivotal mediator of diabetic renal hypertrophy. However, this is not the case for distal renal tubule cells, since we found that high glucose *per se* did not induce TGF-β in these cells [3]; furthermore, hyperplasia rather than hypertrophy was induced. This result is compatible with the observations of Wolf and Ziyadeh [9], who found that hyperplasia of distal renal tubule segments was induced after administration of angiotensin II. So far, none of the cited studies has established a direct causal link between hyperglycaemia and distal tubular hypertrophy.

Exposure of proteins to reducing sugars such as glucose results in non-enzymic glycation, which forms reversible Schiff bases and Amadori compounds [10]. A series of further complex molecular rearrangements then yields irreversible AGEs (advanced glycation end-products). Many studies have suggested a potential role for AGEs in the pathogenesis of diabetic nephropathy [11,12]. Dramatic alterations in mesangial cells and proximal renal tubule cells have also been found after AGE treatment [13]. However, the effects of such treatment remain to be elucidated in distal renal tubule cells.

TGF-β is synthesized by virtually all cell types in a latent form that has to be activated in order to bind to cell surface receptors [14]. Therefore, most animal studies to date have used neutralizing antibodies to attenuate TGF-β activity in a variety of disease states [15–17]. Although this approach may have diagnostic or clinical use for the short-term inhibition of TGF-β activity, it would not

Abbreviations used: AGE, advanced glycation end-product; AP-1, activator protein-1; CRE, cAMP response element; CREB, CRE binding protein; FCS, fetal-calf serum; HSE, heat-shock element; HSF, heat-shock factor; MDCK, Madin–Darby canine kidney; NF-κB, nuclear factor-κB; RT-PCR, reverse transcription–PCR; SEAP, secreted form of human placental alkaline phosphatase; SRE, serum response element; SRF, serum response factor; SV40, simian virus 40; TFD, transcription factor decoy; TGF-β1, transforming growth factor-β1; TSP-1, thrombospondin-1.

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be applicable to chronic diseases such as diabetic nephropathy. Thus it is important to explore alternative ways to suppress TGF-β activity in the diabetic condition.

Physiological mechanisms for TGF-β activation remained unknown until TSP-1 (thrombospondin-1) was discovered [18–20]. TSP-1 is a homotrimeric, disulphide-bonded, multifunctional platelet and matrix glycoprotein of 450 kDa that is synthesized and secreted by a variety of cells, including glomerular mesangial cells [21] as well as renal tubule cells [22]. It is a well established finding that interactions of latent TGF-β with TSP-1 result in the activation of TGF-β [23,24]. Since TSP-1 mimics the effects of glucose in terms of fibronectin production via activation of TGF-β [25], it is essential to elucidate the regulation of TSP-1 under AGE treatment.

Tubulointerstitial changes are as important as glomerulopathy in diabetic nephropathy [26,27]. Many studies have been performed on the proximal renal tubules, but the distal nephron has also been found to be relevant for several reasons. First, significant changes (e.g. Armanni–Ebstein lesion, hyperplasia and hypertrophy) in the distal renal tubules are present in diabetes [26–29]. Secondly, Na^+,K^+-ATPase is increased in both proximal and distal renal tubule cells [30]. Thirdly, urinary excretion of distal nephron markers (e.g. Tamm–Horsfall protein, epidermal growth factor and kinin/kallikrein) is increased in diabetes [31–33]. In this regard, MDCK (Madin–Darby canine kidney) cells have been used extensively to represent distal nephron cells.

In the present study, we discuss the interactions between AGEs and TSP-1 in distal renal tubule cells. We provide a novel mechanism underlying distal renal tubule hypertrophy. We also address a potential approach [i.e. TFD (transcription factor decoy)] to attenuate TGF-β activity and ameliorate diabetic renal tubule hypertrophy.

EXPERIMENTAL

Cell culture and reagents

MDCK cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in culture flasks and maintained in MEM (minimal essential medium) containing 5.5 mM D-glucose (GIBCO, New York, NY, U.S.A.) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % (v/v) FCS (fetal calf serum; PAA, Linz, Austria) in a humidified 5 % CO_2 incubator at 37 °C. Cell viability was estimated by a Trypan Blue exclusion test and was routinely > 92 %. Purified TSP-1 was purchased from Sigma (St. Louis, MO, U.S.A.), and was a lyophilized form from human platelets. Glycated albumin (AGEs) was prepared by incubating BSA with glucose (0.5 M) as described previously [34]. To ensure that they were free of endotoxin, AGEs were quantified using an endotoxin detection kit (Associates of Capecod Inc., Woods Hole, MA, U.S.A.). Unglycated BSA was used as a negative control for AGEs.

Proliferation assay and protein assay

Proliferation assays were performed using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, U.S.A.). In brief, MTS tetrazolium compound is bioreduced by viable cells into a coloured formazan product, the absorbance of which is recorded at 490 nm in a 96-well plate reader.

Protein assays were performed using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.), which is a colorimetric assay (655 nm) for protein concentration following detergent solubilization.

RT-PCR (reverse transcription–PCR) assay

RT-PCR was performed as described in our previous study [2]. Briefly, total RNA was extracted from MDCK cells. First-strand cDNA was synthesized from 4 μg of total RNA by priming with oligo(dT) using a First-strand cDNA synthesis kit (Clontech Laboratories). The primer pairs for TSP-1 were designed (Sigma, Genosys) according to the mRNA sequence of human TSP-1 (accession number XM-007606). The upstream primer (5′-AATGGACTGTTGATAGCTGCACTGA-3′) corresponds to base pairs from 1064 to 1088, while the downstream primer (5′-TGGACAGGCATCCATCAATTGGACA-3′) represents the reverse complement of nucleotides 1711–1735. This primer pair predicts a 672 bp product. The primer pair for β-actin is based on the mouse sequence [35] and predicts a 450 bp fragment. The amplification profile for TSP-1 was 94 °C for 1 min, 57 °C for 45 s and 72 °C for 45 s (35 cycles). Sample RNA that had not been subjected to reverse transcription was used as a negative control in each experiment. This sample routinely did not yield a PCR product. Quality control for RT-PCR was performed as in our previous study [36]. Thus the relative density of the PCR product increased linearly with an increasing amount of loaded RNA when the amount of RNA was less than 5 μg. Meanwhile, densitometry analysis showed a good correlation between cycle number and the intensity of the PCR product for less than 37 cycles.

Northern hybridization

The protocol was modified from our previous study [2]. In short, RNA was isolated, and 40 μg of this RNA was subjected to gel electrophoresis followed by Northern blotting. A cDNA probe was prepared as follows. RT-PCR from unconditioned MDCK cells was performed as in our previous study [2]. RT-PCR products for TGF-β1 (123 bp) and β-actin (450 bp) were eluted and purified using an Ultrafree DA kit (Millipore, Bedford, MA, U.S.A.) and were used as probes for Northern hybridization. Enzyme-labelling of the probes was performed using the Direct HRP Labeling System (Pierce, Rockford, IL, U.S.A.). Hybridization and detection were performed using the North2South™ Detection System (Pierce).

TGF-β bioassay

TGF-β bioactivity was assayed as described in our previous study [3]. In brief, cells cultured in 24-well plates under different conditions were washed twice with serum-free medium, followed by the addition of 1 ml of serum-free medium and incubation for another 24 h. A 0.45 ml sample of medium was collected and heated at 80 °C for 15 min ('total fraction'); another 0.45 ml of medium without heating was considered as the 'active fraction'. Serum-deprived mink lung epithelial cells (CCL-64; A.T.C.C.) were treated with the collected culture medium for 24 h, followed by a CellTiter 96 Aqueous One solution Cell Proliferation Assay (Promega). The ability of the respective conditioned media to inhibit the cell proliferation of mink lung cells was proportional to the bioactivity of TGF-β in each sample of culture medium.

Western blot for supernatant proteins

Cell culture supernatant (15 ml) from conditioned cells was collected and added to Amicon™ Ultra centrifugal filter devices (Ultra-15) for centrifugal concentration at 4000 ***g*** for approx.

30 min. To recover the concentrated solute, a pipette was inserted into the bottom of the filter unit and the sample was withdrawn using a side-to-side sweeping motion to ensure total recovery. A 15 μg portion of concentrated supernatant protein was subjected to SDS/7.5 %-PAGE as described in our previous study [3].

Flow cytometry (for intrastain and cell size analysis)

The protocol was modified from that of Chow et al. [37]. For the analysis of intracellular TSP-1, 1×10^6 MDCK cells were fixed with 2 % (v/v) formaldehyde for 10 min at 37 °C and permeabilized with ice-cold 100 % (v/v) methanol for 30 min at 4 °C. Cells were then washed twice with PBS [containing 4 % (v/v) FCS] and treated with anti-TSP-1 goat polyclonal antibodies (Santa Cruz Biotechnology, Inc.) at a concentration of 1 μg/100 μl in PBS plus 4 % FCS for 15 min at room temperature. Cells were then washed twice with PBS and incubated with fluorescein-conjugated anti-goat antibodies (Calbiochem, Darmstadt, Germany) at 1:200 dilution (0.5 μg/100 μl) in PBS plus 4 % FCS for 15 min at room temperature. Non-immune IgG antibody was used as a negative control. For cell size analysis, cells were detached and triturated to be a single cell suspension at a density of 5×10^5 cells/ml. Flow cytometry analysis was performed, and 10^4 cells were gated and analysed using winMDI software (version 2.8).

Enhancer assay

We used the Mercury™ Pathway Profiling System (Clontech Laboratories, Inc.) to assay the transactivating activity of transcription factors. Each vector contained a specific *cis*-acting DNA binding sequence [SRE (sterol response element), HSE (heat-shock element), AP-1 (activator protein-1), CRE (cAMP response element) or NF-κB (nuclear factor-κB)] located upstream from the TATA-like promoter (P_{TAL}) region of the herpes simplex virus thymidine kinase promoter, followed by a sensitive reporter gene, *SEAP* (a secreted form of human placental alkaline phosphatase). The negative control (pTAL-SEAP), lacking the enhancer element but containing the promoter and reporter gene, was used to determine uninduced background levels of reporter gene activity. The value obtained with the control vector was subtracted from the experimental value. pCTL-SEAP, which contains the SV40 (simian virus 40) early promoter inserted upstream of the *SEAP* gene and the SV40 enhancer inserted downstream, was used as a positive control. To normalize the transfection efficiency, we co-transfected a plasmid constitutively expressing β-galactosidase enzyme in target cells. The level of expression from this gene can then be used to normalize the levels of SEAP activity among different treatment groups. A 5 μg sample of plasmid DNA was used for each transfection. Transfection was performed using a Calphos™ Mammalian Transfection Kit (Clontech). SEAP activity was determined using a Great EscAPe™ SEAP kit (Clontech).

Preparation of decoy oligomers

Upper-strand and reverse-complement phosphorothioated oligonucleotides were synthesized (Unisys Biomedical Co.) and HPLC-purified. Sequences for the NF-κB TFD were designed as described by Goldring et al. [38]. The double-stranded NF-κB TFD contained three copies of a consensus NF-κB binding site found in the κ-light chain enhancer in B cells [39]. A scrambled NF-κB TFD sequence had the same base composition as the NF-κB TFD but contained no sequence predicted to bind a transcription factor, according to the TRANSFAC database (http://transfac.gbf.de/TRANSFAC/), and was used as a negative control. The 37 bp (37-mer) NF-κB TFD sequence was 5′-GGGGACTTTCCGCTGGGACTTTCCAGGGGGACTTTCC-3′ (consensus sequences underlined). The scrambled sequence (upper strand) was 5′-TGCTCTGGTGCAAGCTAGCGTTCGGTCTGTCCAGGGC-3′. The 24 bp (24-mer) AP-1 TFD sequence was 5′-TCATTTGCGTGAGTAAAGCCTGCC-3′, which was synthesized according to Adderley and Fitzgerald [40]; the scrambled sequence (upper strand) was 5′-CAGGAGAGTCTCCTGCGATGCATCTGCT-3′ [40]. The 20 bp (20-mer) CREB (CRE binding protein) TFD sequence was 5′-GGCCTGTGACTTCAAGGGCA-3′, which was modified from that of Lee et al. [41]; the scrambled sequence (upper strand) was 5′-AGGTCTGGGCCCAGTGCTAA-3′ [41].

The procedure for the preparation of double-stranded oligodeoxynucleotides was modified from that of Schmedtje et al. [42]. First, the complementary strands were annealed *in vitro* in $1 \times$ annealing buffer (20 mM Tris/HCl, 20 mM $MgCl_2$, 50 mM NaCl, pH 7.5). The mixture was heated to 80 °C and allowed to cool to room temperature slowly over 3 h. Transfection (50 μg of double-stranded oligodeoxynucleotide) was performed according to the instructions of the Calphos™ Mammalian Transfection Kit (Clontech).

Statistics

Results are expressed as means $\pm$ S.E.M. Unpaired Student's *t* tests were used for comparisons between two groups. A *P* value of < 0.05 was considered to be statistically significant.

RESULTS

AGEs did not induce expression of TGF-β1 mRNA in MDCK cells

We used AGEs rather than glucose to mimic long-term glycaemia. As with glucose, a 24 h incubation with AGEs (or BSA) did not induce a statistically significant change in the level of TGF-β1 mRNA (Figure 1). Similarly, AGEs did not affect the mRNA level of TGF-β1 in time course experiments (from 1 h to 4 days of treatment; results not shown). These results, together with our previous findings [3], implied that a completely different mechanism for distal renal tubule hypertrophy might exist, since TGF-β1 mRNA was dramatically induced in proximal renal tubule or mesangial cells under glucose or AGE treatment (reviewed in [13]).

AGEs significantly enhanced active TGF-β levels in MDCK cells

Despite the results shown in Figure 1, TGF-β might still mediate the effects of AGEs, since post-translational (bioactivity) regulation of TGF-β might occur. We used mink lung cells to monitor the presence of bioactive TGF-β in the culture media from MDCK cells exposed to a variety of conditions. As demonstrated in Figures 2(B) and 2(C), AGEs induced a significant increase in the 'active fraction' of TGF-β. This is the first demonstration that AGEs regulate TGF-β at the protein level rather than the gene level.

AGEs induced increases in the levels of TSP-1 mRNA and protein

One of the primary points of regulation of TGF-β activity is control of its conversion from the latent precursor into the biologically active form. Therefore we examined the possible

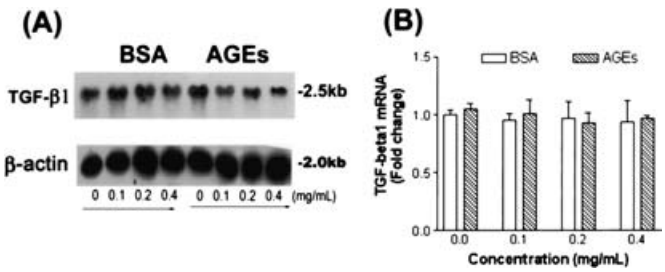

Figure 1 Northern analysis of TGF-β1 in AGEs-treated MDCK cells

Serum-starved MDCK cells were cultured in MEM with 0, 0.1, 0.2, 0.4 mg/ml BSA or AGEs in the presence of 10 % (v/v) FCS for 24 h. Total RNA was extracted and Northern blotting was performed as described in the Experimental section. Results representative of three independent experiments are shown in (**A**). The intensity of TGF-β1 mRNA was normalized to that of β-actin (**B**). It is evident that neither BSA nor AGEs induced a statistically significant change in TGF-β1 mRNA expression. Results are expressed as means $\pm$ S.E.M. of three independent experiments performed in triplicate.

involvement of TSP-1 in the effects of AGE treatment, since TSP-1 activates TGF-β in a variety of systems. It is evident that AGEs dose-dependently increased TSP-1 protein levels both intracellularly (Figure 3) and extracellularly (Figure 4). Similarly, the mRNA level of TSP-1 was also increased in response to AGE treatment (Figure 5). Treatment with BSA induced no statistically significant changes. In addition, actinomycin D pretreatment almost completely blocked the AGE-induced increase in TSP-1 mRNA expression, indicating AGEs stimulated new TSP-1 transcription (Figure 5). Therefore TSP-1 expression might be closely associated with the effects of AGE treatment in distal renal tubule cells.

TSP-1 mediated the AGE-induced increases in TGF-β bioactivity and cell size

To further elucidate the role of TSP-1 in the effects of AGEs, anti-TSP-1 antibodies were used in the presence of AGE treatment. Anti-TSP-1 antibody (from Santa Cruz Biotechnology) is an affinity-purified goat polyclonal antibody. Mouse non-specific antibody MOPC-104E (M2521) was used as a non-immune control (Sigma, St. Louis, MO, U.S.A.). As Table 1 demonstrates, AGEs at 0.4 mg/ml induced significant increases in both TGF-β bioactivity and cell size. Interestingly, 15 μg/ml anti-TSP-1 antibodies could partly reverse both of these AGE-induced increases. These observations strongly suggested that TSP-1 might play a pivotal role linking the activation of TGF-β and the effects of AGE treatment.

AGEs and TSP-1 exhibited similar effects on cell proliferation and protein content

If TSP-1 mediates AGE-induced growth effects, TSP-1 might exhibit similar effects as AGEs. Indeed, administration of either AGEs or purified TSP-1 significantly induced cellular hypertrophy (as shown by protein content) and growth inhibition (as shown by proliferation assay) (Figure 6). Taken together with the results in Figures 3–5, this indicates that TSP-1 might be a significant mediator of the effects of AGE treatment in distal renal tubule cells.

AGEs enhanced the transactivating activity of AP-1, CREB and NF-κB

According to the TRANSFAC® database (http://www.cbrc.jp/research/db/TFSEARCH.html), we observed putative binding

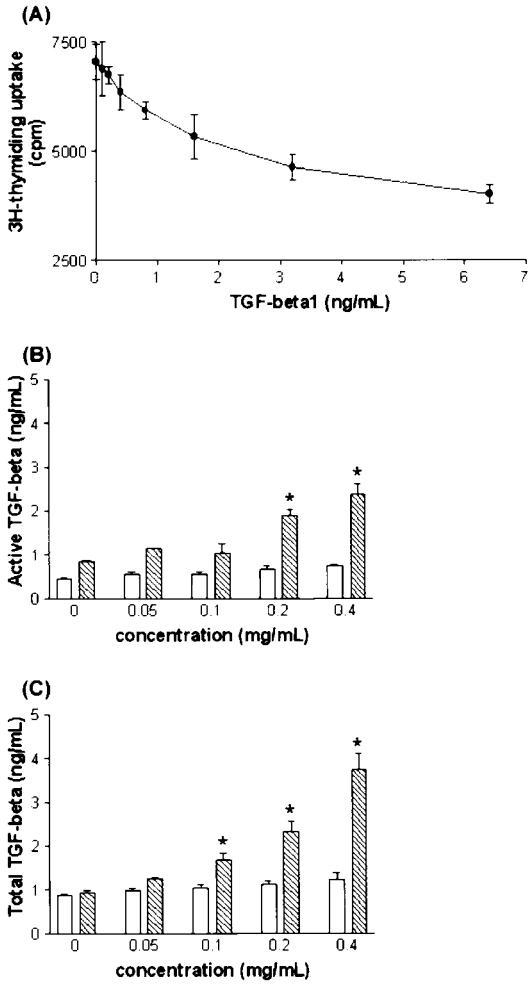

Figure 2 Dose-dependent effects of AGEs/BSA on TGF-β bioactivity

(**A**) Standard growth inhibition curve for mink lung cells treated with TGF-β1 (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng/ml). The percentage growth inhibition is proportional to the amount of active TGF-β in the culture medium. (**B**, **C**) Serum-starved MDCK cells were cultured in MEM with 10 % (v/v) FCS and 0, 0.1, 0.2, 0.3 or 0.4 mg/ml BSA (empty bars) or AGEs (hatched bars) for 24 h. Culture media were collected and the cell growth inhibition assay was performed as described in the Experimental section. AGEs, but not BSA, dose-dependently increased active (**B**), and total (**C**), TGF-β. Results are expressed as means $\pm$ S.E.M. of three independent experiments performed in triplicate; $^*P < 0.05$ compared with absence of AGEs (or BSA)

sites for CREB (-1595 to -1588), AP-1 (-459 to -453), NF-κB (-1315 to -1306; -1758 to -1749), SRF (serum response factor; -1218 to -1207) and HSF (heat-shock factor; more than ten sites) in the TSP-1 promoter (accession number M62449). To elucidate the effects of AGE treatment on gene regulation, these transcription factors were monitored in a time-course experiment. As Figure 7(D) demonstrates, the transactivating abilities of AP-1, CREB and NF-κB were significantly induced within 2 h of AGE treatment; however, in control experiments (Figure 7E), NF-κB was activated after BSA treatment. Thus AP-1 and CREB might play roles in the biological effects induced by AGEs.

AP-1 TFD could reverse the AGE-induced increase in TSP-1 levels and hypertrophy

As shown in Table 2, 0.4 mg/ml AGEs stimulated a significant increase in cellular TSP-1 levels and in TGF-β activity and

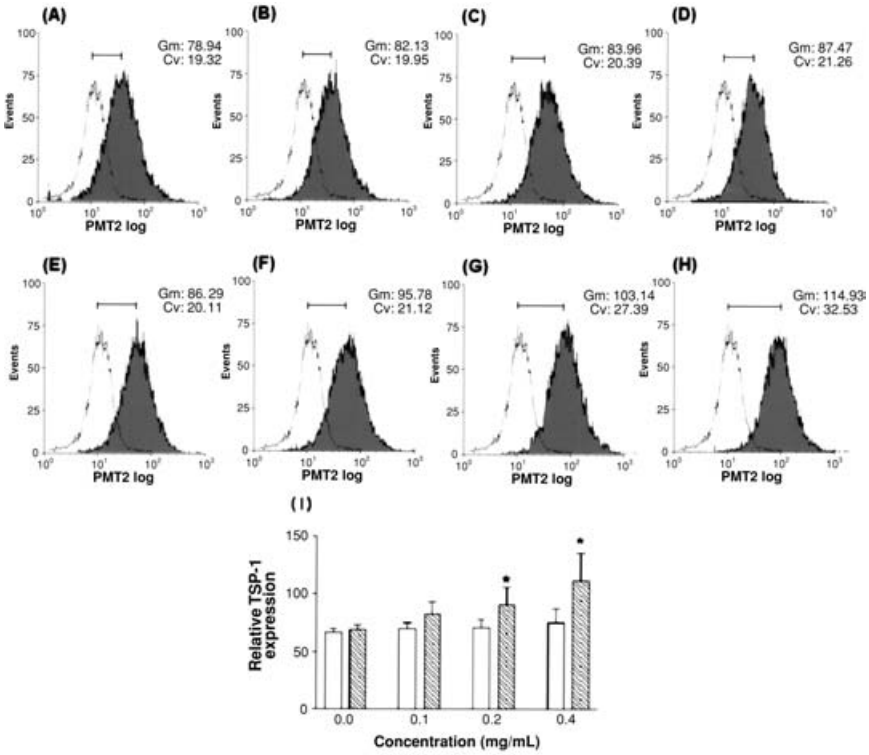

Figure 3 Evaluation of intracellular accumulation of TSP-1 in MDCK cells

Serum-starved MDCK cells were cultured in MEM with 0, 0.1, 0.2 or 0.4 mg/ml BSA (**A**, **B**, **C** and **D** respectively) or AGEs (**E**, **F**, **G** and **H** respectively) in the presence of 10 % (v/v) FCS for 24 h. Expression of TSP-1 was evaluated in 2 % (v/v) formaldehyde-fixed MDCK cells by indirect staining with anti-TSP-1 antibodies followed by treatment with FITC-conjugated anti-goat IgG (right-hand peaks). Negative controls (left-hand peaks) represent MDCK cells stained with non-immune antibodies, followed by the secondary antibodies. The horizontal axis shows relative TSP-1 protein expression detected by fluorescence intensity (logarithmic scale); the vertical axis shows relative cell number. Results are expressed as mean fluorescence (PMT2) intensity. Gm, geometric mean; Cv, coefficient of variation. (**I**) The non-immune background (mean 12.32) was subtracted for each sample. Results are expressed as means ± S.E.M. of three independent experiments; $^*P < 0.05$ compared with absence of AGEs (or BSA). It is evident that AGEs dose-dependently increased intracellular TSP-1 content.

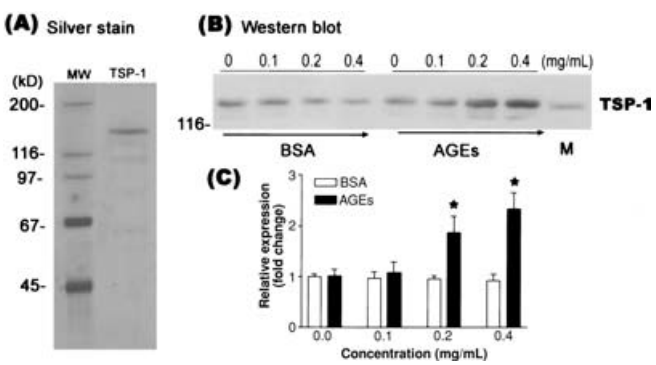

Figure 4 Effects of AGEs on extracellular accumulation of TSP-1 in MDCK cells cultured in conditioned media

(**A**) Purified TSP-1 (0.75 μg) was resolved by electrophoresis followed by silver staining. It is evident that the molecular mass of TSP-1 is between 116 and 200 kDa. Lane MW contained molecular mass markers. (**B**) Serum-starved MDCK cells were cultured in MEM with 0, 0.1, 0.2 or 0.4 mg/ml BSA or AGEs in the presence of 10 % (v/v) FCS for 24 h. Supernatant proteins were concentrated and portions of 15 μg were resolved by SDS/7.5 %-PAGE followed by immunoblotting with anti-TSP-1 antibodies. A positive control (2 μg of purified TSP-1; designated M) was performed simultaneously with the immunoblotting procedure as described in (**A**). (**C**) The intensity of each band from (**B**) was scanned, and results are expressed as means ± S.E.M. of three independent experiments performed in triplicate; $^*P < 0.05$ compared with absence of AGEs (or BSA). It is evident that AGEs dose-dependently increased the level of extracellular TSP-1.

Table 1 Regulation of cell size and TGF-β bioactivity by AGEs in MDCK cells

Serum-starved MDCK cells were cultured in 10 % (v/v) FCS with no additions (Control), 0.4 mg/ml BSA or 0.4 mg/ml AGEs for 24 h, with or without 15 μg/ml anti-TSP-1 antibodies or non-immune antibodies. Measurement of cell size and TGF-β bioactivity assays were performed as described in the Experimental section. It is evident that anti-TSP-1 antibodies could partly reverse the AGE-induced increases in both cell size and TGF-β bioactivity (active fraction). Results are expressed as means ± S.E.M. of three independent experiments; $^*P <$ 0.05 compared with control

		TGF-β bioactivity	
Condition	Cell size (forward light scatter)	Active (ng/ml)	Total (ng/ml)
Control	242 ± 10	0.7 ± 0.02	2.1 ± 0.10
+ BSA	253 ± 14	0.7 ± 0.03	2.3 ± 0.19
+ AGEs	314 ± 15*	2.7 ± 0.16*	2.9 ± 0.26
+ AGEs + anti-TSP-1 Ig	288 ± 13	1.3 ± 0.05	2.7 ± 0.17
+ AGEs + non-immune Ig	325 ± 14*	2.9 ± 0.25*	2.4 ± 0.27

protein level. Furthermore, TFDs for AP-1 and CREB could specifically reverse the AGE-induced effects; scrambled TFDs exhibited no differences compared with the group treated with 0.4 mg/ml AGEs. Thus AP-1 and CREB might mediate the AGE-induced increases in TSP-1 levels and protein content. In addition, there is likely to be a close interaction between the expression of TSP-1 and active TGF-β in culture media, since

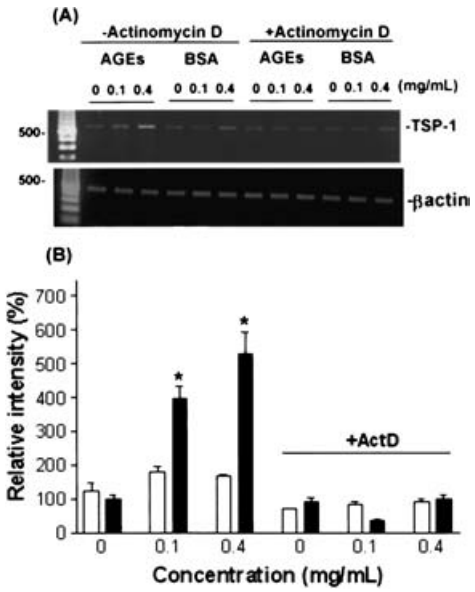

Figure 5 TSP-1 mRNA expression was significantly induced in MDCK cells after AGEs treatment

Serum-starved MDCK cells were cultured in 10 % (v/v) FCS with AGEs (0, 0.1 or 0.4 mg/ml) or BSA (0, 0.1 or 0.4 mg/ml). Actinomycin D (ActD; 10 μg/ml) was added 2 h prior to treatment. Total RNA was extracted and RT-PCR was performed as described in the Experimental section (**A**). Relative intensity was determined by a densitometer (**B**). It is evident that TSP-1 mRNA was dose-dependently induced by AGE treatment (■). Furthermore, actinomycin D pretreatment completely blocked the AGE-induced increase in TSP-1 mRNA expression, indicating that AGEs stimulated new TSP-1 transcription. Results are expressed as means ± S.E.M. of three independent experiments performed in triplicate; $^*P < 0.05$ compared with no AGEs/BSA in the absence of actinomycin D.

parallel results were observed. Furthermore, AGEs induced a significant increase in cell size, which could be specifically reversed by AP-1 TFD (Figure 8), but not by CREB TFD (results not shown). Therefore AP-1 TFD might be a potential approach to ameliorating AGE-induced renal hypertrophy.

DISCUSSION

We have demonstrated previously that distal renal tubule cells exhibit a completely different growth pattern under hyperglycaemic stimulus compared with mesangial cells or proximal renal tubule cells [3]. In this ongoing study, we provide some novel viewpoints with regard to diabetic distal renal tubule hypertrophy. First, we demonstrated that AGEs increased cellular hypertrophy, whereas high glucose increased cellular hyperplasia, in distal renal tubule cells [3]. Secondly, up-regulation of TSP-1 rather than TGF-β1 might be responsible for distal renal tubule hypertrophy, since either AGEs (Figure 1) or high glucose (Y.-L. Yang, L.-Y. Chuang, J.-Y. Guh, S.-F. Liu, M.-Y. Hung, T.-N. Liao, Y.-L. Huang and T.-A. Chiang, unpublished work) induced significant increases in the mRNA level of TSP-1, but not TGF-β1. Thirdly, manipulation of TSP-1 expression might be a potential approach for treating diabetic renal distal tubule hypertrophy (Table 2 and Figure 8).

In the present study, we investigated the effects of AGEs on distal renal tubule cells for three reasons. First, as our previous study demonstrated [3], short-term high glucose culture did not affect TGF-β mRNA or its bioactivity in distal renal tubules; therefore AGEs were used instead, since they mimic long-term hyperglycaemia. Secondly, AGEs are actively reabsorbed and cleared by the proximal convoluted renal tubule in normal rats; however, in patients with a damaged proximal renal tubule (such as patients with end-stage renal disease or diabetes), AGE-induced biological alternations in distal renal tubule cells could still occur [43]. This notion is compatible with that of Nouwen et al. [44], showing induction of distal renal tubule hypertrophy followed by acute proximal tubular injury *in vivo*. Thirdly, albumin is one of the major glycated proteins in the serum and urine of diabetic patients with nephropathy. Glycated albumin is a more reliable marker of lack of glycaemic control than HbAlc (glycated haemoglobin) or fructosamine [45]; therefore we use glycated albumin to mimic the diabetic condition.

We believe that culture in medium containing glycated albumin in the soluble phase, in amounts representative of those found in diabetic serum and urine, represents more closely the *in vivo* milieu than dose culture of cells in plates on to which AGE adducts have been immobilized. Thus we believe our results are more relevant to the actual diabetic condition and thus valuable for clinical reference. Although the detailed signal transduction mechanisms by which glycated albumin leads to growth arrest, hypertrophy (Figure 6A) and an increase in active TGF-β (Figure 2B) are currently unknown, it is possible that binding of this glycated protein to a plasma membrane site triggers receptor-induced events, analogous to many other models of ligand–receptor interactions.

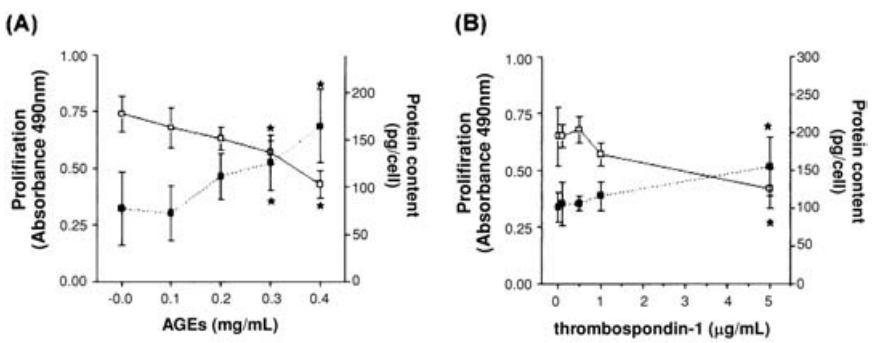

Figure 6 Effects of AGEs and TSP-1 on cell proliferation and protein content

Serum-starved MDCK cells were cultured in MEM containing AGEs (**A**) or purified TSP-1 (**B**) in the presence of 10 % (v/v) FCS for 24 h. Assays for cell proliferation (left vertical axis; □) and protein content (right vertical axis; ■) were performed as described in the Experimental section. It is evident that either AGEs (0, 0.1, 0.2, 0.3 or 0.4 mg/ml) and purified TSP-1 (0, 0.1, 0.5, 1 or 5 μg/ml) exhibited similar dose-dependent effects, namely inducing an increase in protein content and a decrease in cell proliferation. Data are expressed as means ± S.E.M. of three independent experiments performed in triplicate; $^*P < 0.05$ compared with absence of AGEs or TSP-1.

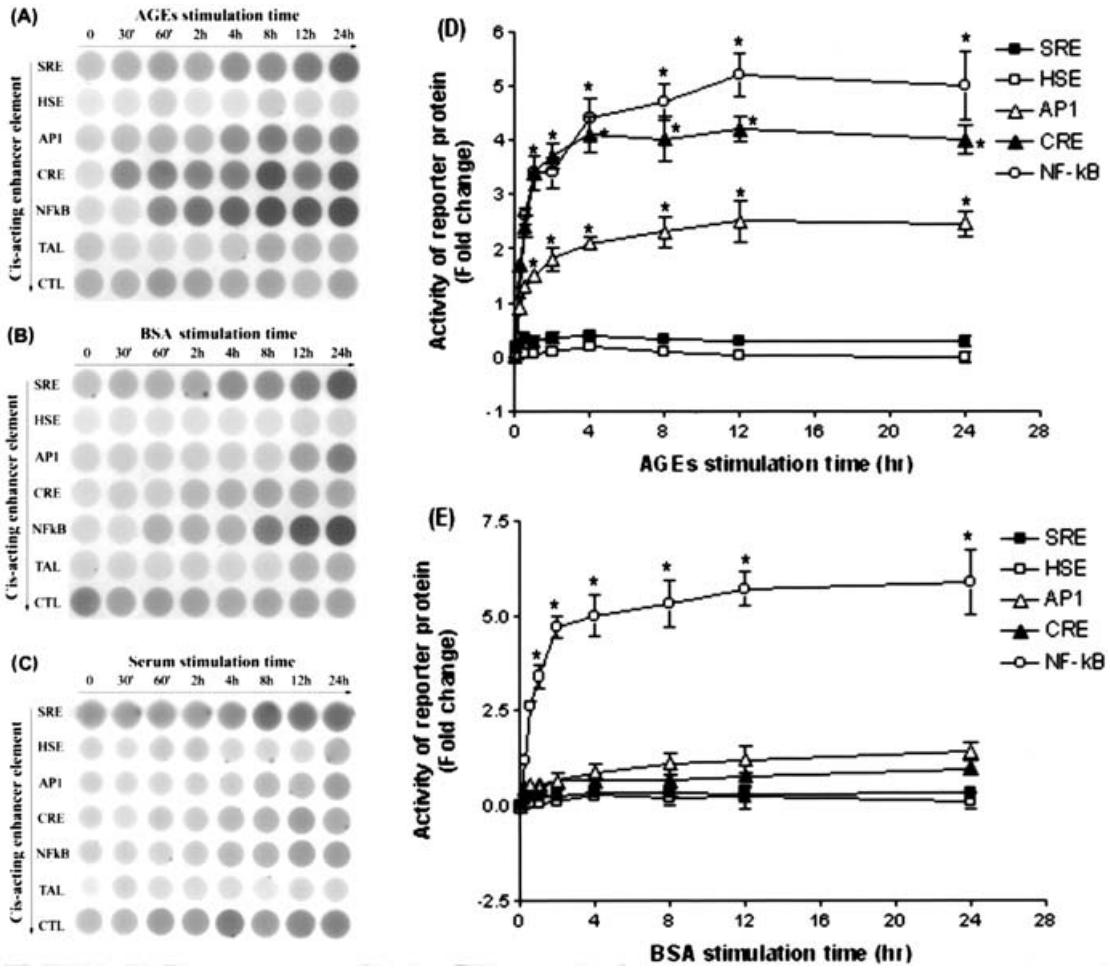

Figure 7 Transcription factors induced by AGEs treatment

MDCK cells were plated in six-well culture plates and transfected with different reporter vectors (pSRE-SEAP, pHSE-SEAP, pAP-1-SEAP, pCRE-SEAP, pNFκB-SEAP, pTAL-SEAP and pCTL-SEAP) that contained a specific *cis*-acting enhancer element and a sensitive reporter gene, *SEAP*. After transfection, cells were treated with 0.4 mg/ml AGEs (**A**), 0.4 mg/ml BSA (**B**) or vehicle (MEM with 0.5 % FCS; **C**). Aliquots (15 μl) of supernatants were collected at indicated times (0, 30 min 60 min, 2 h, 4 h, 8 h, 12 h, 24 h) from the same well. Activation of differential transcription factors was determined by the activity of the reporter protein (SEAP) secreted into the culture medium. Dot density was determined by densitometry. In (**D**) and (**E**), results are expressed following subtraction of the control density (**C**) from the values in (**A**) and (**B**) respectively. Data are expressed as means $\pm$ S.E.M. of three independent experiments performed in triplicate; $^*P < 0.05$ compared with control (time 0). It is evident that AGEs specifically activated transcription factors AP-1 and CREB in MDCK cells. TAL, negative control lacking the enhancer element but containing the promoter and reporter gene; CTL, positive control containing the SV40 early promoter inserted upstream of the *SEAP* gene and the SV40 enhancer inserted downstream.

The decoy strategy is a powerful new tool that is useful for gene therapy and in the study of transcriptional regulation. This approach is particularly attractive for several reasons. First, transcription factors are plentiful and readily identifiable. Secondly, the synthesis of sequence-specific decoys is relatively simple and can be targeted to specific tissues. Thirdly, knowledge of the exact molecular structure of the targeted transcription factor is unnecessary. Fourthly, decoy oligonucleotides might be more effective than antisense oligonucleotides in blocking constitutively expressed factors as well as multiple transcription factors that bind to the same *cis* element. Thus, in the present study, we use TFDs to monitor the role of three transcription factors induced after AGE treatment. We demonstrated that AP-1 might be one of the most important transcription factors implicated in diabetic renal hypertrophy of the distal segment. In fact, many previous studies have suggested a role for AP-1 in diabetic nephropathy [46,47].

In the present study, we first demonstrated that post-translational regulation of TGF-β might play a more important role than transcriptional regulation in distal renal tubule hypertrophy under diabetic conditions. As shown in Figures 1 and 2, we observed that AGEs enhanced TGF-β bioactivity rather than the TGF-β1 mRNA level. In addition, AGEs induced growth arrest and enhanced cellular hypertrophy (Figure 6A). We also observed that TSP-1 protein and mRNA expression were significantly induced after AGE treatment in distal renal tubule cells (Figures 3–5). Furthermore, AGE-induced increases in cell size and protein content (Y.-L. Yang, L.-Y. Chuang, J.-Y. Guh and S.-F. Liu, unpublished work) could be reversed by the administration of TSP-1-specific antibodies and AP-1 TFD (Tables 1 and 2, and Figure 8). This is the first observation linking AGE-induced cellular hypertrophy with TSP-1.

Consistent with the use of anti-TSP-1 antibodies or AP-1 TFD for attenuating cellular hypertrophy in the present study, Yevdokimova et al. [48] have also demonstrated that inhibition of TGF-β1 activation by a synthetic TSP-1-based peptide (GGWSHW) under high-glucose conditions completely suppressed increases in fibronectin expression. Furthermore, a TSP-1

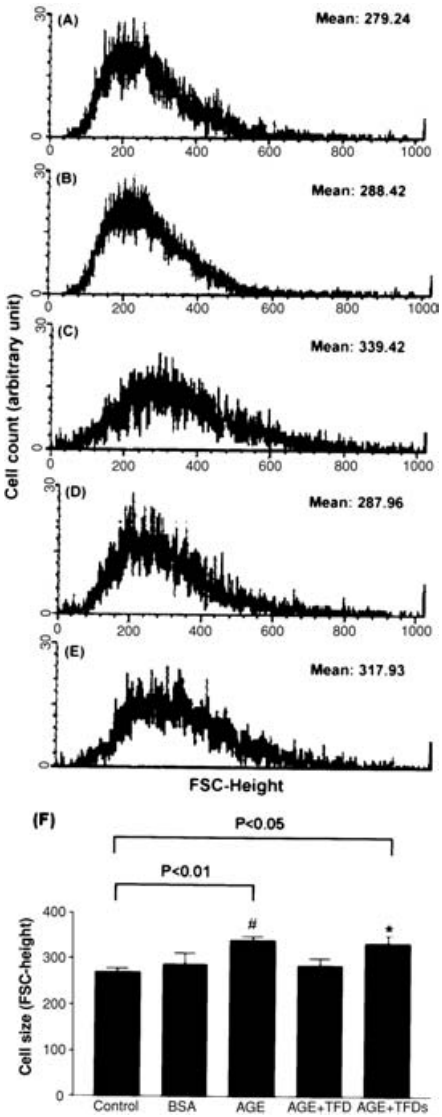

Figure 8 AP-1 TFD partly reverses the AGEs-induced increase in cell size

Serum-starved MDCK cells were transfected with AP-1 TFD (**D**) or its scrambled sequence (**E**), as described in the Experimental section. The cells were then cultured in 10% FCS in the absence of AGEs (**A**), or with 0.4 mg/ml AGEs (**C**–**E**) or BSA (**B**) for another 24 h. Approx. 10^4 cells were gated and subjected to winMDI software analysis. Cell size was expressed as mean FSC (forward scatter) height (**F**). It is evident that the AGE-induced increase in cell size could be specifically attenuated by AP-1 TFD. Data are expressed as means ± S.E.M. of three independent experiments; $^*P < 0.05$, $\#P < 0.01$ compared with control.

antisense oligonucleotide prevented TGF-β1 activation, and normalized the expression of fibronectin. This observation is consistent with our finding. Thus we suggest that regulation of TSP-1 may help to provide an alternative approach in attempts to halt the progress of diabetic nephropathy.

To sum up, we have demonstrated that: (a) hypertrophy was induced by AGEs in distal renal tubule cells; (b) TSP-1 (mRNA and protein) was induced by AGEs in MDCK cells; (c) *in vitro* treatment with purified TSP-1 induced an increase in cellular hypertrophy; and (d) blocking TSP-1 expression with anti-TSP-1 antibodies or AP-1 TFD reversed AGE-induced cellular hypertrophy. These data provide convincing evidence that TSP-1 mediates distal renal tubule hypertrophy induced by glycated albumin. In addition, we suggest that regulation of TSP-1 expression might be a potential approach to ameliorating diabetic renal hypertrophy.

Table 2 Modulation of bioactivity of secreted TGF-β, level of intracellular TSP-1 and protein content by TFDs in MDCK cells

Serum-starved MDCK cells were transfected with different TFDs or their scrambled sequences (sTFD) as described in the Experimental section. The cells were then cultured in 10% (v/v) FCS with no additions (Control), 0.4 mg/ml BSA or 0.4 μg/ml AGEs for another 24 h. To evaluate TSP-1 expression, cells were scraped, formaldehyde-fixed, and indirectly stained with anti-TSP-1 antibodies followed by addition of FITC-conjugated secondary antibodies. Meanwhile, culture media were collected as described in the Experimental section and mink lung cell growth inhibition assays were performed. Protein assays were performed using a Bio-Rad DC protein assay kit. It is evident that TFDs for both AP-1 and CRE could partly reverse AGE-induced increases in cellular TSP-1 expression, active TGF-β and protein content. Results are expressed as means ± S.E.M. of three independent experiments performed in triplicate; $^*P < 0.05$ compared with control.

Condition	TSP-1 (fluorescence intensity)	Active TGF-β (ng/ml)	Protein content (pg/cell)
Control	81.2 ± 19.1	0.6 ± 0.03	87.0 ± 19.3
BSA	86.6 ± 19.7	0.8 ± 0.04	91.8 ± 19.3
AGEs	119.2 ± 26.4*	2.4 ± 0.12*	149.8 ± 29.0*
AGEs + AP-1 TFD	91.2 ± 20.1	1.1 ± 0.09	106.3 ± 24.2
AGEs + AP-1 sTFD	116.3 ± 24.5*	2.9 ± 0.31*	140.2 ± 43.5*
AGEs + CRE TFD	95.5 ± 20.2	1.3 ± 0.11	120.8 ± 58.0*
AGEs + CRE sTFD	124.9 ± 23.8*	2.0 ± 0.20*	154.7 ± 48.3*
AGEs + NF-κB TFD	125.4 ± 25.4*	2.1 ± 0.18*	154.7 ± 14.5*
AGEs + NF-κB sTFD	123.8 ± 23.3*	2.6 ± 0.25*	145.0 ± 33.8*

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