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Andrographolide Suppresses High Glucose-Induced Fibronectin Expression in Mesangial Cells Via Inhibiting the AP-1 Pathway

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ABSTRACT

Mesangial cells (MCs) proliferation and accumulation of glomerular matrix proteins such as fibronectin (FN) are the early features of diabetic nephropathy, with MCs known to upregulate matrix protein synthesis in response to high glucose. Recently, it has been found that andrographolide has renoprotective effects on diabetic nephropathy. However, the molecular mechanism underlying these effects remains unclear. Cell viability and proliferation was evaluated by MTT. FN expression was examined by immunofluorescence and immunoblotting. Activator protein-1 (AP-1) activation was assessed by immunoblotting, luciferase reporter and electrophoretic mobility shift assays. Andrographolide significantly decreased high glucose-induced cell proliferation and FN expression in MCs. Exposure of MCs to high glucose markedly stimulated the expression of phosphorylated c-jun, whereas the stimulation was inhibited by andrographolide. Plasmid pAP-1-Luc luciferase reporter assay showed that andrographolide blocked high glucose-induced AP-1 transcriptional activity. EMSA assay demonstrated that increased AP-1 binding to an AP-1 binding site at −1,029 in the FN gene promoter upon high glucose stimulation, and the binding were disrupted by andrographolide treatment. These data indicate that andrographolide suppresses high glucose-induced FN expression by inhibiting AP-1-mediated pathway. J. Cell. Biochem. 114: 2562–2568, 2013.

KEY WORDS: ANDROGRAPHOLIDE; DIABETIC NEPHROPATHY; HIGH GLUCOSE; MESANGIAL CELLS; AP-1

Diabetes nephropathy is one of the most common and severe microvascular complications of diabetes mellitus and become the leading cause of end-stage renal disease [Tramonti and Kanwar, 2012]. It is characterized by an expansion of the glomerular mesangium, which is caused by mesangial cell proliferation and an excessive accumulation of extracellular matrix (ECM) proteins synthesized by mesangial cells (MCs) [Zhang et al., 2012]. Fibronectin (FN), as one of the major ingredients of ECM, is often used as an index to evaluate the levels of matrix accumulation. Therefore, inhibiting FN production is regarded as an effective strategy to ameliorate diabetic nephropathy. Hyperglycemia plays a central role in the pathogenesis of diabetic nephropathy, as shown by its prevention or retardation by strict metabolic control [Menini et al., 2006]. High glucose induces MCs proliferation and FN expression in vitro [Yano et al., 2009; Lan et al., 2011]. Activator protein-1 (AP-1) transcription factor is a heterodimeric protein, which composed of proteins

Abbreviations: AP-1, activator protein 1; EMSA, electrophoretic mobility shift assay; MCs, mesangial cells; FN, fibronectin; AP-1, activator protein-1; DMEM, dulbecco′s modified Eagle′s medium.

Tian Lan and Teng Wu authors contributed equally to this work.

Conflict of interests: None.

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belonging to the c-Fos, c-Jun, ATF, and JDP families [Ozanne et al., 2007]. Transcription of AP-1 component proteins is increased by high glucose in MCs, as is AP-1 activity [Wilmer and Cosio, 1998]. AP-1 could play a critical role in mediating severe diabetic microvascular complications [Tong et al., 2008]. Numerous genes important in the fibrotic response in diabetic nephropathy are regulated by AP-1, and the synthesis of FN is mediated by AP-1 [Chen et al., 2003b, 2012].

Natural products and their active constituents have been used for the treatment of diabetes and diabetic complications [Park et al., 2010]. Herbaceous plant andrographis paniculata is a traditional prescribed medicine in Asia for the treatment of inflammatory disorders [Xia et al., 2004; Bao et al., 2009]. Andrographolide, one of the diterpenoids, is the primary active ingredient in andrographis paniculata [Sheeja and Kuttan, 2007]. Recent studies indicated that the hypoglycemic effects of andrographolide for the treatment of diabetes [Yu et al., 2003, 2008]. Andrographolide can increase glucose utilization to lower plasma glucose in diabetic rats [Yu et al., 2003]. More recently, it has been reported that andrographolide extracted from Andrographis paniculata attenuated high glucose-induced fibrosis and apoptosis in murine renal mesangeal cell lines [Lee et al., 2010]. Synergy of andrographis paniculata polysaccharide with andrographolide improved the metabolic abnormalities in diabetic mice and prevented against the progression of diabetic nephropathy [Xu et al., 2012]. However, it is still unclear the precise mechanism by which andrographolide protects against diabetic nephropathy.

Therefore, in the present study, we investigated whether andrographolide exerts renoprotective roles in diabetic nephropathy through downregulation of FN in MCs cultured by high glucose and the potential involvement of AP-1 pathway. Our data indicate that andrographolide attenuates high glucose-induced FN expression by inhibiting the activation of AP-1 pathway, suggesting that andrographolide might ameliorate the progression of diabetic nephropathy through inhibition of ECM accumulation.

MATERIAL AND METHODS

CHEMICALS

Andrographolide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and curcumin were purchased from Sigma (St. Louis).

CELL CULTURE

Sprague–Dawley rat MCs were obtained from young SD rat kidneys as described previously [Gennero et al., 2002; Geoffroy et al., 2004]. MCs were cultured in dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum (Gibco) at 37°C in a 5% CO_2 incubator. Cells were grown to confluence and synchronized in serum-free DMEM for 24 h. Medium was then changed to (1) DMEM containing 5.5 mM glucose (normal glucose [NG]); (2) NG medium containing 16.5 mM mannitol (Mannitol [Mtol]); (3) NG medium supplemented with additional 16.5 mM glucose to a final concentration of 22 mM (high glucose [HG]) or (4) HG medium in the presence of 5 μM andrographolide (HG + Andro) for 24 h.

CELL PROLIFERATION ASSAY

The MTT assay was used to measure cell proliferation. Briefly, cells were seeded at 10^4 cells/well in 96-well plates. When cells reached confluence, cells were serum starvation for 24 h. Then cells were incubated with NG or NG with 5, 10, 20, 25, 50, and 100 μM andrographolide, or cells were treated with NG, Mtol or HG in the presence or absence of 2.5, 5, and 10 μM andrographolide for 24 h. Then 20 μl of MTT (5 mg/ml) was added to each well and incubation continued at 37°C for additional 4 h. The medium was then carefully removed and DMSO was added into each well and the absorbance of solubilized blue formazan was read at a wavelength of 570 nm using a microplate reader.

IMMUNOFLUORESCENCE

MCs were grown on glass coverslips treated with HG in the presence or absence of 5 μM andrographolide for 24 h. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and permeabilized with triton X-100 (1% in PBS) for 10 min at room temperature. After washing, cells were blocked with 10% goat serum for 1 hr at room temperature and incubated with a mouse monoclonal antibody against FN (1:1,00, Santa Cruz Biotechnology) overnight at 4°C. The signal was visualized with Alexa Fluor 488-conjugated secondary antibody (1:1,000, Invitrogen). Nuclei were co-labeled with DAPI for 10 min. The coverslips were mounted on glass slide with anti-fade mounting media (Beyotime, China), and images were collected using a confocal fluorescence microscope (Leica, Germany).

IMMUNOBLOTTING

Cells in 60 mm-diameter dishes were harvested and lyzed by ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 2 mM EGTA, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM pepstatin A, 1 mM phenylmethyl sulphonyl fluoride). An aliquot of protein (30 μg) for each lane were subjected to 10% SDS–PAGE by electrophoresis under reducing conditions and transferred to PVDF membrane (Millipore Corporation). The blotted membrane was then blocked with 5% nonfat dry milk for 1 h at room temperature and incubated with anti-FN (1: 1,000, Santa cruz), c-jun, and p-c-jun (1:1000, Cell Signaling Technology), α-tubulin(1:5,000, Sigma) overnight at 4°C. The blotted membranes were further incubated with peroxidase conjugated anti-mouse/rabbit secondary antibodies (1:10,000, Promega), and detected by enhanced chemiluminescent (ECL) method and captured on X-ray film. The densitometry assay was performed using Quantity-one gelworks analysis software and expressed as a value relative to the density of the internal control (α-tubulin).

TRANSFECTION AND LUCIFERASE REPORTER ASSAYS

Cells were transfected using Lipofectamine 2000 (Invitrogen) in serum-free NG media with AP-1-Luc. Six hours after transfection, the cells were culture in DMEM containing 10% FBS overnight, and then exposed to NG or HG media in the presence or absence of 5 μM andrographolide for 12 h. The cells were lysed and luciferase activity was determined using the dual luciferase assay system according to the manufacturer's instructions (Promega Corp.). For controlling for differences in transfection efficiency, a plasmid that contained

Renilla luciferase was included in each transfection and used for normalization.

EMSA

Nuclear proteins for electrophoretic mobility shift assay (EMSA) were prepared using the Nuclear Extract Kit (Active Motif) according to manufacturer's instruction. The nuclear proteins (2.5 μg) were incubated with 1× binding buffer (LightShift Chemiluminescent EMSA Kit, Pierce) in the presence of 50 ng/μl poly(dI-dC), 0.05% Nonidet P-40, 5 mM $MgCl_2$ and 2.5% glycerol for 10 min and then incubated at room temperature for additional 20 min with 1 pmol of biotin-labeled AP-1 consensus oligonucleotide (Sangon Biotech Co., Ltd.). The reaction mixture was subjected to a 6% non-denaturing SDS–PAGE at 100 v for 60 min, transferred to nylon hybridization transfer membrane (Amersham) and DNA cross-linked for 10 min, and probed with horseradish peroxidase-conjugated streptavidin antibodies (1:300 dilution), then visualized with enhanced chemiluminescence and captured by X-ray film. The AP-1 probe was 5′-ATTCTCTTGCCTCAGCTTCTAC-3′ (underlined is core AP-1 binding site).

STATISTICAL ANALYSIS

All experiments were preformed in at least triplicate with similar results and the data were expressed as mean ± SD. Statistical differences between two groups were analyzed by the unpaired student's *t*-test and differences between multiple groups of data were analyzed by one-way ANOVA with Bonferroni correction (Graphpad Prism 5.0. $P < 0.05$ was considered statistically significant Figure 1.

RESULTS

ANDROGRAPHOLIDE INHIBITED HIGH GLUCOSE-INDUCED MCS PROLIFERATION

To determine the appropriate concentrations of andrographolide for the treatment of MCs, a range of the concentrations of andrographolide (0–100 μM) was added into the MCs cultured in NG medium for 24 h. The cell viability of MCs was not altered by andrographolide at the lower concentration no more than 10 μM. However, the cytotoxicity of andrographolide was exhibited at the concentration of over 20 μM (Fig. 2A). Thus, in the following experiments, MCs were treated with 5 μM andrographolide. The data showed that cell proliferation was significantly increased by HG compared with NG. In contrast, various concentrations of andrographolide (2.5, 5, and 10 μM) significantly decreased MCs proliferation cultured under HG condition. Replacement of glucose with mannitol (as an osmotic control) did not alter MCs proliferation (Fig. 2B).

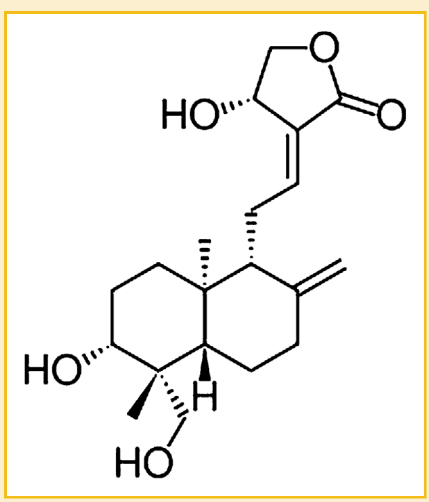

Fig. 1. Chemical structure of andrographolide.

ANDROGRAPHOLIDE INHIBITED HIGH GLUCOSE-INDUCED FN EXPRESSION IN MCS

Exposure of MCs to high glucose for 24 h resulted in significant increase in the FN expression as evaluated by confocal microscopic analysis. Interestingly, high glucose-induced FN expression was abolished in the presence of andrographolide (Fig. 3A). Likewise, Western blotting analysis also indicated that high glucose-induced

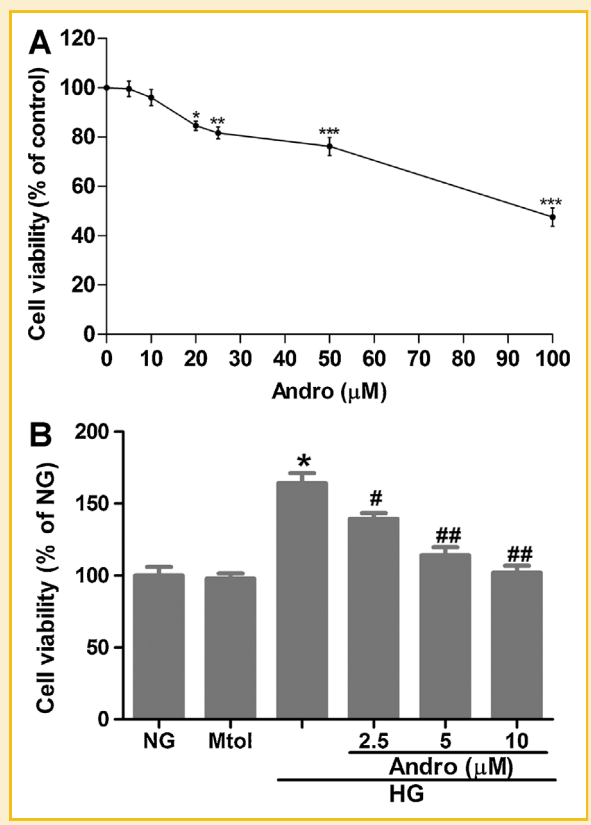

Fig. 2. Andrographolide inhibited high-glucose induced MCs proliferation. A: MCs were treated with normal glucose (NG) DMEM cultured with/without different concentrations of andrographolide and cell viability was examined by MTT. B: MCs were cultured with normal glucose (NG) or high glucose (HG) or high glucose with indicated concentration of andrographolide (2.5, 5, and 10 μM), respectively. MCs were cultured with mannitol as an osmotic control. MCs proliferation was assessed by MTT. $^{*}P < 0.01$ versus NG; $^{\#}P < 0.01$ versus HG.

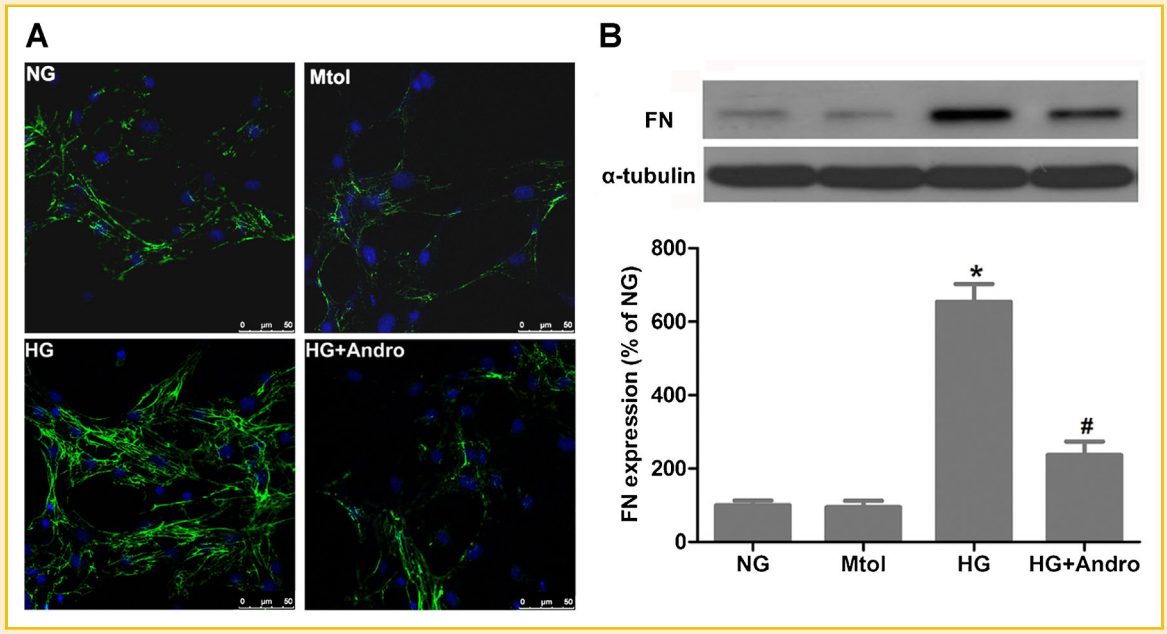

Fig. 3. Andrographolide suppressed high glucose-induced FN expression. MCs were cultured by high glucose in the presence or absence of andrographolide (5 μM) for 24 h. A: Immunofluorescence staining for FN by the laser scanning confocal microscopy (magnifacation 630 ×), and green fluorescence indicates localization of FN. B: Immunoblotting for FN expression in MCs. $^{*}P < 0.01$ versus NG; $\#P < 0.01$ versus HG.

upregulation of FN were significantly reduced by andrographolide treatment (Fig. 3B). In contrast, Mtol, served as hyperosmotic control, has no effect on the expression of FN. Together, these results demonstrated that andrographolide significantly attenuated high glucose-induced FN expression in MCs, suggesting that andrographolide plays renoprotective roles in the diabetic nephropathy.

ANDROGRAPHOLIDE INHIBITED HIGH GLUCOSE-INDUCED PHOSPHORYLATION OF C-JUN IN MCS

Transcription factor AP-1 is a menagerie of dimeric basic region-leucine zipper proteins, consisting of homodimers of jun or heterodimers of fos and jun [Shaulian and Karin, 2001, 2002]. To explore the potential involvement of AP-1 in the inhibition of FN expression by andrographolide, we measured the phosphorylation of c-jun in MCs cultured in HG medium. Figure 4 showed that high glucose markedly increased phosphorylated c-jun (p-c-jun) in MCs, whereas andrographolide significantly decreased the protein levels of p-c-jun induced by high glucose. These data indicated that andrographolide has inhibitory effects on the AP-1 activation induced by high glucose might result from the suppression of phosphorylation of AP-1.

ANDROGRAPHOLIDE REDUCED HIGH GLUCOSE-INDUCED TRANSCRIPTIONAL ACTIVITY OF AP-1 IN MCS

We therefore examined andrographolide suppresses high glucose-induced FN depends on the inhibition of AP-1 activity. We first evaluated endogenous AP-1 transcriptional activity in cultured cells using a luciferase reporter assay. As shown in Figure 5, when MCs were transfected with pAP-1-Luc reporter plasmid, the induction of luciferase activity by high glucose was blocked by andrographolide as well as the AP-1-specific inhibitor curcumin. These data suggest a key role of AP-1 in high glucose-induced FN expression and predict a cis-AP-1 element in the FN gene promoter that mediates the high glucose-induced FN expression. Indeed, the rat FN promoter identified a putative AP-1 binding site 5′-TGCCTCA-3′ at −1029, which shares a high degree of homology to the canonical AP-1 site 5′-TGAGTCA-3′.

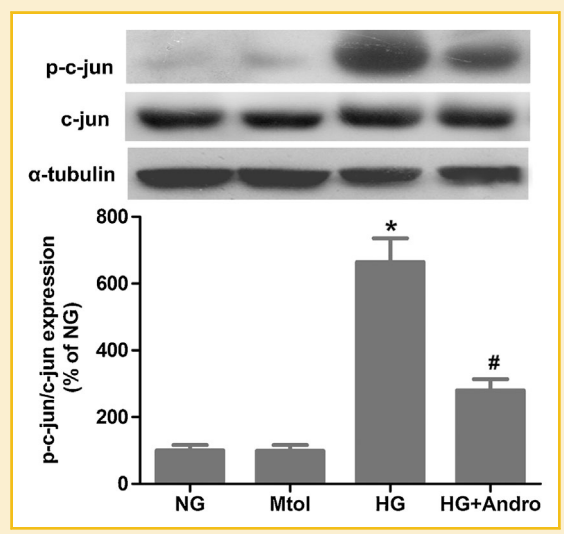

Fig. 4. Andrographolide inhibited high glucose-induced phosphorylation of c-jun. MCs were cultured by high glucose in the presence or absence of andrographolide (5 μM) for 12 h. The expression of p-c-jun and c-jun in MCs was examined by immunoblotting. $^{*}P < 0.01$ versus NG; $\#P < 0.01$ versus HG.

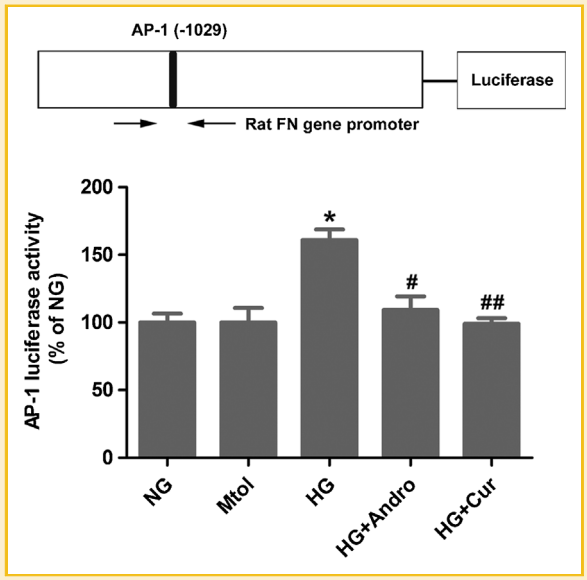

Fig. 5. Andrographolide inhibited FN promoter activity in MCs cultured by high glucose. A: Schematic map of FN promoter-luciferase reporter construct. The putative rat FN gene promoter from—2000 contains AP-1 binding sites at −1029, as indicated. B: Luciferase reporter assay. MCs were transfected with pFN-Luc reporter plasmid for 6 h, then cells were pretreated with or without andrographolide (5 μM) for 3 h followed by exposure to NG or HG media for 12 h. MCs were pretreated with curcumin for 3 h as positive control. $^{*}P < 0.01$ versus NG; $^{\#}P < 0.01$ versus HG.

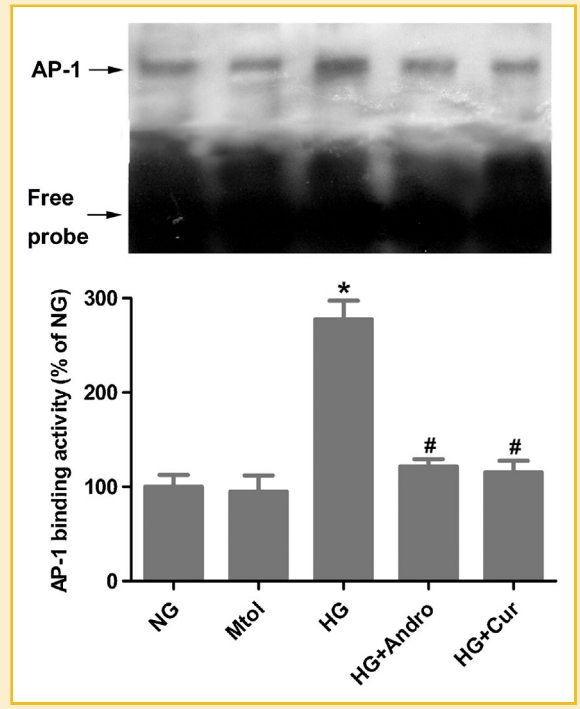

Fig. 6. Andrographolide inhibited high glucose-induced AP-1 DNA binding activity. MCs were pretreated with or without andrographolide (5 μM) in NG medium for 3 h, then medium were changed to NG, Mtol or HG for 3 h. Nuclear extracts were subjected to EMSA to examine AP-1 DNA binding activity. MCs were pretreated with curcumin for 3 h as positive control. $^{*}P < 0.01$ versus NG; $^{\#}P < 0.01$ versus HG.

ANDROGRAPHOLIDE ATTENUATED HIGH GLUCOSE-INDUCED AP-1 DNA BINDING ACTIVITY IN MCS

AP-1 activation is also involves in increased DNA binding activity. To assess whether the putative AP-1 site in the FN promoter is functional, we performed EMSA using a biotin-labeled double strand probe corresponding to this AP-1 and its flanking sequence. As shown in Figure 6, the formation of the DNA-AP-1 complex was markedly induced by high glucose, and the high glucose-induced complex was markedly reduced when the cells were treated with 5 μM andrographolide. These data strongly suggest that andrographolide blunted high glucose-induced FN expression by blocking the AP-1 binding activity.

DISCUSSION

The key findings of the present study are that andrographolide inhibited MCs from high glucose-induced cell proliferation and FN expression through inhibition of AP-1 pathway.

Andrograhis paniculata is a traditional Chinese medicine used in many Asia countries for the treatment of colds, fever, laryngitis and diarrhea. An ethanolic extract of Andrographis paniculata demonstrated anti-diabetic properties in streptozocin (STZ)-induced diabetic rats [Zhang and Tan, 2000]. Andrographolide (Fig. 1), the primary active component of andrograhis paniculata, exhibits several pharmacological activities, including anti-inflammatory, immunoregulatory, anti-cancer, and hypoglycemic actions [Jada et al., 2008]. The cytotoxicity of andrographolide was exhibited at the concentration of over 20 μM in MCs in our study. Similar concentrations of andrographolide were used in previous studies. Andrographolide (10 μM) inhibited cell viability and induced apoptotic cell death in both androgen-stimulated and castration-resistant human prostate cancer cells without causing significant toxicity to normal immortalized prostate epithelial cells [Chun et al., 2010]. The activation of NF-κB in leukocytes was significantly inhibited by treatment with 15 μM andrographolide. Thus, in the current study, a lower concentration of andrographolide (5 μM) was used in the treatment of MCs without cytotoxicity. Our data showed that andrographolide markedly decreased MCs proliferation induced by high glucose, suggesting that andrographolide might attenuate diabetic renal hypertrophy in vivo.

Diabetic nephropathy is a serious complication in diabetes. MCs proliferation and hypertrophy, ECM accumulation have been recognized as major pathogenic events during the progression of renal failure in diabetic nephropathy [Schena and Gesualdo, 2005; Ichinose et al., 2007]. Major typical morphological changes are the results of changes in the ECM. Thus, basement membranes are thickened and the glomerular mesangial matrix and the tubulointerstitial space are expanded, due to increased amounts of ECM [Kolset et al., 2012]. Deposition of ECM protein such as FN cause glomerulosclerosis. For the decade, andrographolide was found to be an anti-diabetic compound in vivo and in vitro assays [Yu et al., 2003, 2008; Lee et al., 2010]. Andrographolide can lower blood glucose in STZ-diabetic rats by increasing glucose utilization [Yu

et al., 2003]. Another literature demonstrated that andrographolide increased expression of the glucose transporter subtype 4 (GLUT4) to lower plasma glucose in diabetic rats [Yu et al., 2008]. Recently, it was reported that andrographolide had anti-diabetic nephropathy effects on high glucose-cultured MCs. Andrographolide inhibited the expression of fibrosis markers such as TGF-β [Lee et al., 2010]. Our data showed that andrographolide inhibited the expression of FN in MCs induced by high glucose. These results indicated that andrographolide might prevent the pathogenesis of diabetic nephropathy not only by its hypoglycemic effect, but also by attenuating FN expression which is the direct cause of the glomerulosclerosis.

Transcription factor AP-1 is a menagerie of dimeric basic region-leucine zipper proteins, consisting of homodimers of Jun or heterodimers of Fos and Jun [Shaulian and Karin, 2001, 2002]. A binding site of AP-1 in FN promoter region has been identified in rat MCs, and AP-1 is an important transcription factor regulating FN expression [Tamura et al., 1998]. AP-1 is a mediator of glucose responses and activated by high glucose in MCs and diabetic kidneys [Wilmer and Cosio, 1998; Kim et al., 2003; Lan et al., 2011; Huang et al., 2013], and high glucose-induced FN upregulation is dependent on AP-1 activation [Peng et al., 2008]. Together, these evidences suggest that activation of AP-1 enhances FN expression in MCs under high glucose condition.

Adding to the understanding of the renoprotective activity of andrographolide in MCs, we presented evidences that andrograholide regulates FN expression via inhibiting the activity of AP-1. We showed that treatment with andrographolide blocked the unregulation of FN by high glucose through inhibition of the FN gene promoter. Accompanying the suppression of phosphorylated c-jun, andrographolide also inhibited the transcriptional activity and DNA binding activity of AP-1 demonstrated by both luciferase and EMSA assays. Since the FN promoter contains an AP-1-binding site [Chen et al., 2003a], it is highly likely that the effect of andrographolide on HG-induced FN expression is through the regulation of AP-1 promoter activity.

Curcumin, the active ingredient of the spice turmeric, exhibits extensive pharmacological activities including anti-oxidant, anti-inflammatory and hypoglycemic effects [Nishiyama et al., 2005; Strimpakos and Sharma, 2008], Recent studies showed that curcumin improved renal function in STZ-induced diabetic rats and attenuated FN expression through inhibition of AP-1 activity in MCs cultured by high glucose [Sharma et al., 2006; Tikoo et al., 2008; Huang et al., 2013]. Several studies indicated that curcumin can inhibited the activity of AP-1 and was widely used as an AP-1 inhibitor [Aggarwal and Shishodia, 2006; Anand et al., 2008; Lan et al., 2011]. Therefore, in the current study, curcumin was used as AP-1 inhibitor in the luciferase reporter and EMSA assays. The data showed that treatment with curcumin significantly inhibited AP-1 activity in MCs cultured by high glucose. The inhibitory potency of andrographolide was similar to curcumin, suggesting that inhibiting AP-1 activity is one of the therapeutic targets preventing diabetic nephropathy.

Collectively, in the current study, we identified that andrographolide not only downregulated FN in MCs under high glucose condition, but also inhibited AP-1 pathway, suggesting that AP-1 is the core of andrographolide regulating FN expression in high glucose-cultured MCs. Our findings provide new insight into the mechanism of andrographolide in the treatment of early diabetic nephropathy.

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