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Emodin ameliorates high-glucose induced mesangial p38 over-activation and hypocontractility via activation of PPARγ

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Abbreviation: PPARγ, peroxisome proliferator-activated receptor gamma

Abstract

Early stage diabetic nephropathy is characterized by elevated glomerular filtration. Recent studies have identified high-glucose induced p38 MAPK (p38) over-activation in mesangial cells. Mesangial hypocontractility is the major underlying mechanism, however, no ameliorating agents are currently available. We investigated the protective effects of emodin on high-glucose induced mesangial cell hypocontractility. Mesangial cells were cultured under normal (5.6 mM) and high glucose (30 mM) conditions. Emodin was administrated at doses of 50 mg/l and 100 mg/l. Angiotension II stimulated cell surface reductions were measured to evaluate cell contractility. p38 activity was detected using Western blotting. To further explore the possible mechanism of emodin, expression of the peroxisome proliferator- activated receptor γ (PPARγ) was measured and its specific inhibitor, gw9662, was administrated. Our results showed: (1) high-glucose resulted in a 280% increase in p38 activity associated with significant impairment of mesangial contractility; (2) emodin treatment dose-dependently inhibited high-glucose induced p38 over-activation (a 40% decrease for 50 mg/l emodin and a 73% decrease for 100 mg/l emodin), and mesangial hypocontractility was ameriolated by emodin; (3) both the PPARγ mRNA and protein levels were elevated after emodin treatment; (4) inhibition of PPARγ using gw9662 effectively blocked the ameliorating effects of emodin on high-glucose induced p38 over-activation and mesangial hypocontractility. Emodin effectively ameliorated p38 over-activation and hypocontractility in high-glucose induced mesangial cells, possibly via activation of PPARγ.

Keywords: diabetic nephropathies; emodin; hyperglycemia; mesangial cells; p38 mitogen-activated protein kinases

Introduction

Glomerular hyperfiltration and renal enlargement are the key indicators of early stage diabetic nephropathy (Mogensen, 1971). Many pathological processes, such as diabetes-induced oxidative stress (Santilli *et al*., 2004), altered hemodynamics (O'Bryan and Hostetter, 1997), and abnormal tubular-glomerular feedback (Thomson *et al*., 2004) have been proposed as possible mechanisms. Among these proposals, mesangial cells hypocontractility has gained interest in recent years (Stockand and Sansom, 1997; Chen *et al*., 2004). To date, several lines of research using streptotozin-induced diabetic animals have demonstrated that, in early stage diabetes, mesangial cells exhibit significantly impaired responses to several vasocontracting agents, including angiotension II and endothelin-1. These impairments are correlated with an enhanced glomerular filtration rate (Kreisberg, 1982; Osterby *et al*., 1984; Kikkawa *et al*., 1986; Dunlop and Larkins, 1990; Kanamori *et al*., 1994; Hadad *et al*., 1997). Similar results were also obtained from *in-vitro* studies. In cultured mesangial cells, high-glucose levels resulted in almost no contractile response to endothelin-1 (Hurst *et al*., 1995; Derylo *et al*., 1998). Mesangial contractile dysfunction has been widely accepted as one of the central events underlying the pathogenesis of glomerular hyperfiltration in early stage diabetic nephropathy (Stockand and Sansom, 1998).

The precise mechanism of diabetes-induced mesangial hypocontractility is not known. Recent

studies have suggested that p38 MAPK (p38) is important. The p38-mediated signal pathway involves several vasoactive agents that induce contraction of mesangial cells, including angiotension II, KCl, endothelin-1, and cadmium (Müller *et al*., 1999; Dunlop and Muggli., 2000; Tsiani *et al*., 2002; Hirano *et al*., 2005). In early stage diabetic nephropathy, many indigenous renal cells, including mesangial cells, have exhibited significantly enhanced p38 activity (Wilmer *et al*., 2001; Sakai *et al*., 2005). Inhibition of p38 has been effective in amelioration of diabetes-induced mesangial hypocontractility both *in vitro* (Dunlop and Muggli, 2000) and *in vivo* (Komers *et al*., 2007). Based on these findings, it has been proposed that inactivation of p38 can be a novel intervention for early stage diabetic nephropathy. However, no agents capable of inhibiting p38 in mesangial cells are now available.

Emodin, an anthraquinone derivative isolated from the root and rhizome of *Rheum Palmatum*, is a Chinese herb used for immunosuppression, anti-inflammation, and anti-proliferation (Kawai *et al*., 1984; Huang *et al*., 1992; Chang *et al*., 1996). Some studies have demonstrated that most of the biological effects of emodin are mediated by suppressing p38 (Kwak *et al*., 2006; Wang *et al*., 2007). Our previous work also demonstrated that emodin can inactivate p38 in glomerular mesangial cells, and accordingly protect IL-1β induced mesangial cell proliferation (Wang *et al*., 2007). Based on these findings, we hypothesized that emodin also exerts a p38 inhibiting effect in high-glucose treated mesangial cells and, therefore, ameliorates high-glucose induced mesangial hypocontractility.

Although the inhibitory effects of emodin on p38 activation have been well documented, the precise mechanism is unclear. Recently, studies from two groups have demonstrated that emodin has peroxisome proliferator-activated receptor γ (PPARγ) activating effects (Yang *et al*., 2007; Zhou *et al*., 2008). Emodin administration both *in vivo* and *in vitro* induced PPARγ expression. PPARγ is a known regulator of the p38 signal pathway, and PPARγ activation blocks p38 activation (Boileau *et al*., 2007; Xing *et al*., 2008). Whether PPARγ activation is also involved in the emodin p38 inhibitory effect is unknown. We investigated the role of PPARγ in the protective effect of emodin in high-glucose treated mesangial cells.

Results

Emodin ameliorated high-glucose induced mesangial cell hypocontractility

Mesangial cells cultured using 5.6 mM glucose (normal group, NG) demonstrated a 39% decrease in the planar surface area after angiotension II stimulation. Compared with the NG group, cells cultured using 30 mM glucose (high gucose group, HG) only exhibited a 12% decrease in the planar surface area ($P < 0.05$), indicating impaired mesangial cell contractility. Emodin treatment ameliorated high-glucose induced mesangial hypocontractility in a dose-dependent manner, demonstrated by a 22% decrease in the cell planar surface area in the low dose emodin group (LE) (50 mg/l of emodin, $P < 0.05$) and a 30% decrease in the high dose emodin group (HE) (100 mg/l, $P < 0.05$) (Figure 1).

Emodin ameliorated high-glucose induced p38 over-activation in mesangial cells

p38 activities were evaluated by measuring the protein levels of p-p38 cells and total p38 using Western blotting. Data are presented in Figure 2. Compared with the NG group, high glucose treatment resulted in a 280% increase in the p-p38 levels ($P < 0.01$) while it did not affect the total p38 levels, suggesting elevated p38 activities induced by high glucose. Compared with the HG group, administration of 50 mg/l and 100 mg/l of emodin reduced p-p38 levels by 40% ($P < 0.05$) and 73% ($P < 0.01$), respectively, suggesting that emodin inhibits p38.

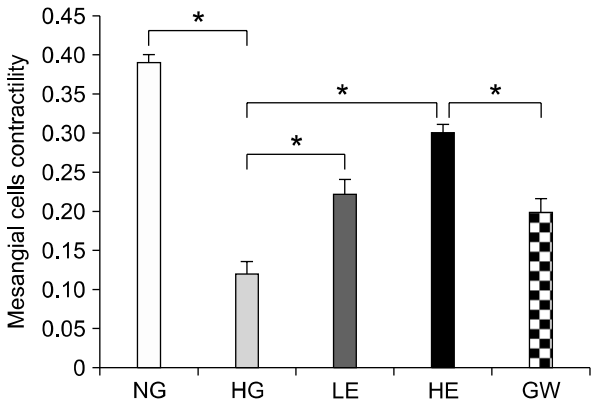

Figure 1. Emodin ameliorates high-glucose induced mesangial cell hypocontractility. Mesangial cell contractility was evaluated by measuring the decrease in the planar surface area after angiotension II stimulation. In the NG group, angiotension II stimulation resulted in a 39% decrease in the planar surface area. In the HG group, angiotension II induced a 12% decrease in the planar surface area, suggesting impaired contractility due to a high glucose level ($P < 0.05$). Compared with the HG group, emodin significantly elevated mesangial contractility in a dose-dependent manner (elevated angiotension II induced a planar surface area decrease of 22% in the LE group and a 30% decrease in the HE group, $P < 0.05$). Administration of GW9662 effectively blocked the ameliorating effects of emodin on mesangial hypocontractiliy with only a 20% decrease in the planar surface area ($P < 0.05$). Values are mean $\pm$ SD. NG: 5.6 mM glucose. HG: 30 mM glucose. LE: 30 mM glucose with 50 mg/l of emodin. HE: 30 mM glucose with 100 mg/l of emodin. GW: 30 mM glucose with 100 mg/l of emodin and 10 μM gw9662. *$P < 0.05$.

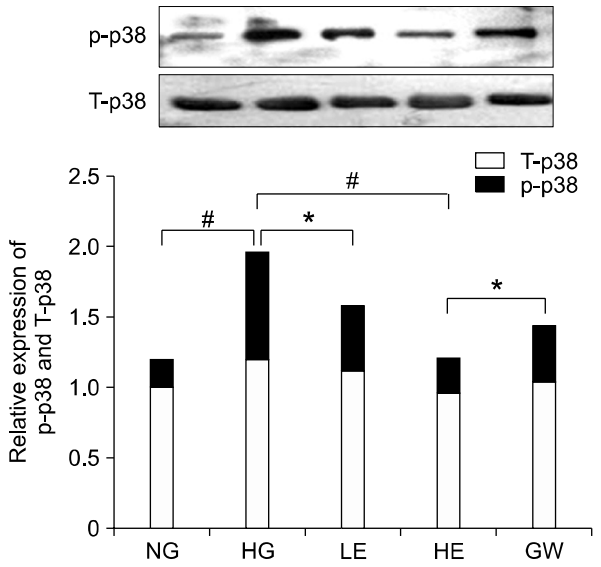

Figure 2. Emodin ameliorates high-glucose induced p38 over-activation in mesangial cells. Compared with the NG group, high glucose treatment resulted in a 280% elevation in p-p38 protein levels ($P < 0.01$). Compared with the HG group, p-p38 protein levels decreased by 40% ($P < 0.05$) in the LE group and 73% in the HE group ($P < 0.01$). Compared with the HE group, administration of gw9662 elevated the p-p38 protein level by 96% ($P < 0.05$). No changes in total p38 levels were detected among the five groups. The value of T-p38 in the NG group was designated as 1.0. Values are mean ± SD, $P < 0.05$ is considered statistically significant. NG: 5.6 mM glucose. HG: 30 mM glucose. LE: 30 mM glucose with 50 mg/l of emodin. HE: 30 mM glucose with 100 mg/l of emodin. GW: 30 mM glucose with 100 mg/l of emodin and 10 μM gw9662. *$P < 0.05$; #$P < 0.01$.

Emodin treatment did not affect p38 expression as no changes in the total p38 protein levels were observed.

Emodin elevated PPARγ expression in mesangial cells

Expression of PPARγ was evaluated by measuring mRNA and protein levels using real-time PCR and Western blotting. Data are presented in Figures 3 and 4. Compared with the HG group, administration of 50 mg/l and 100mg/l of emodin resulted in a 151% ($P < 0.05$) and 177% ($P < 0.01$) increase in the PPARγ mRNA levels, respectively. Consistent with these results, the protein content of PPARγ was also elevated by emodin treatment (196% elevation in the LE group and 421% elevation in the HE group, $P < 0.05$). These results suggest that emodin has PPARγ-activating effects.

GW9662 administration blocked the protective effects of emodin on high-glucose induced mesangial hypocontractility

To further investigate whether the ameliorating effects of emodin on high-glucose induced mesangial cell p38 over-activation and hypocontractility are mediated by PPARγ, the specific PPARγ inhibitor GW9662 was administrated to the HE group. Results showed that, compared with the HE group, GW9662 administration resulted in a 96% elevation of p-p38 protein levels ($P < 0.05$). Consistent with changes in p-p38, angiotension II induced mesangial cell contractility also decreased after GW9662 treatment ($P < 0.05$), (Figure 1 and 2). These findings suggest that the ameliorating effects of emodin on high-glucose induced mesangial cell hypocontractility are mediated partially or totally by activation of PPARγ.

Discussion

In addition to structural support for glomerular capillary tufts, mesangial cells also regulate the capillary filtration surface area and, therefore, modulate the glomerular filtration rate (Stockand and Sansom, 1998). Meseangial cell regulating effects

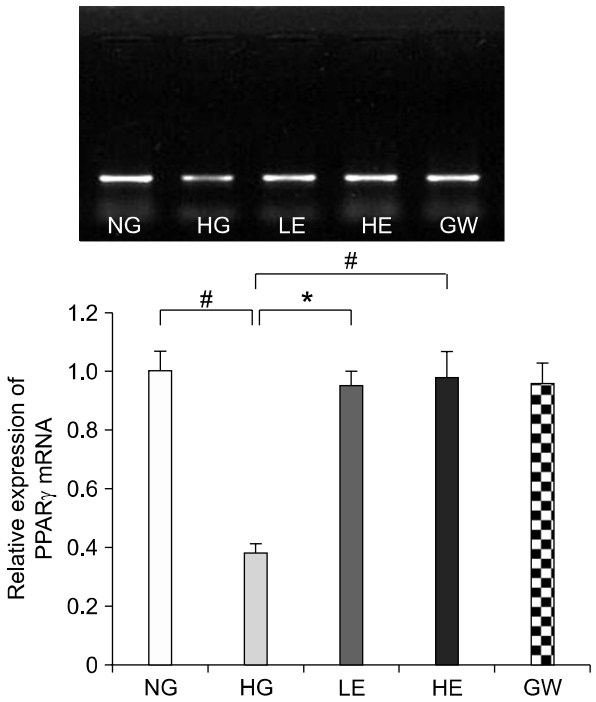

Figure 3. PPARγ mRNA levels. Compared with the NG group, high glucose reduced PPARγ mRNA expression by 62% ($P < 0.01$). Compared with the HG group, 50 mg/l and 100 mg/l of emodin elevated PPARγ mRNA levels by 151% ($P < 0.05$) and 177%, respectively, ($P < 0.01$). Values are mean ± SD, $P < 0.05$ is considered statistically significant. NG: 5.6 mM glucose. HG: 30 mM glucose. LE: 30 mM glucose with 50 mg/l of emodin. HE: 30 mM glucose with 100 mg/l of emodin. GW: 30 mM glucose with 100 mg/l of emodin and 10 μM gw9662. *$P < 0.05$; #$P < 0.01$.

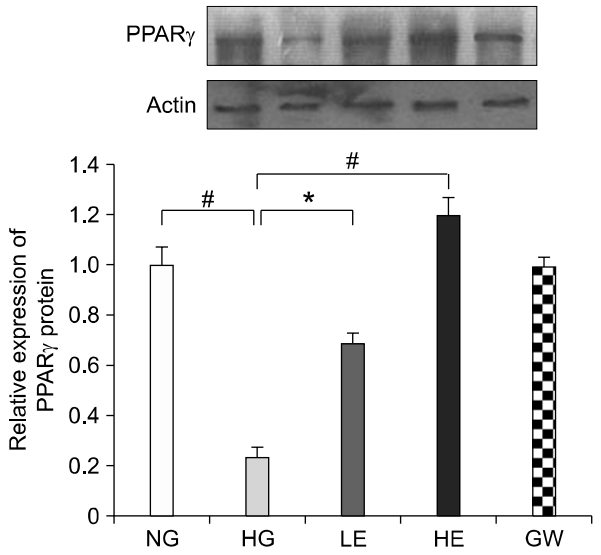

Figure 4. PPARγ protein levels. Compared with the NG group, high glucose resulted in a 77% decrease in the PPARγ protein level ($P < 0.01$). Compared with the HG group, 50 mg/l and 100 mg/l of emodin elevated PPARγ protein levels by 196% ($P < 0.05$) and 421% ($P < 0.01$), respectively. Values are mean ± SD, $P < 0.05$ is considered statistically significant. NG: 5.6 mM glucose. HG: 30 mM glucose. LE: 30 mM glucose with 50 mg/l of emodin. HE: 30 mM glucose with 100 mg/l of emodin. GW: 30 mM glucose with 100 mg/l of emodin and 10 μM gw9662. $^{*}P < 0.05$; $^{\#}P < 0.01$.

on the capillary filtration surface area are based on the normal cell ability to respond to endogenous vasoactive agents, including both vaso-contraction and vaso-relaxation (Stockand and Sansom, 1998). To date, numerous vaso-active agents have been identified in such biological processes, including angiotension II, endothelin-1, and atrial natriuretic peptide (ANP) (Okuda *et al*., 1986; Simonson and Dunn, 1990; Hadad *et al*., 1997). In the normal state, glomerular filtation is constantly and accurately controlled by a balance between the actions of these vaso-contracting and vaso-relaxing agents (Stockand and Sansom, 1998). In a diabetic state, this balance is disrupted because the response of mesangial cells to vaso-contracting agents is significantly impaired (Kreisberg, 1982; Osterby *et al*., 1984; Kikkawa *et al*., 1986; Dunlop and Larkins, 1990; Kanamori *et al*., 1994; Hadad *et al*., 1997; Stockand and Sansom 1997; Chen *et al*., 2004). This is believed to be the major event accounting for diabetes-induced glomerular hyperfiltration and renal hypertrophy. Drugs to normalize the mesangial cell response to vaso- contracting agents have a great clinical significance for intervention in early diabetic nephropathy. However, no such drugs are currently available.

Emodin is an anthraquinone derivative isolated from the Chinese herb *Rheum Palmatum* and has been demonstrated to have several biological effects, including anti-inflammation, anti-firbosis, and immunosuppression (Kawai *et al*., 1984; Huang *et al*., 1992; Chang *et al*., 1996). Emodin is widely used in the treatment of disease, including cancer, inflammation, atherosclerosis, and uremia. We have demonstrated that emodin is also effective for high-glucose induced mesangial cells hypocontractility.

Angiotension II is an important member of the renin-angiotensin system (RAS) and is known for multiple biological effects. Angiotension II can regulate glomerular filtration via stimulation of mesangial contraction and can induce mesangial proliferation and extracellular matrix production (Kennefick and Anderson, 1997). In early stage diabetic nephropathy, the impaired response of mesangial cells to angiotension II is the major factor underlying diabetes-induced glomerular hyperfiltration. In late stage diabetic nephropathy, over-production and over-activation of angiotension II exist. Angiotension II over-activation is believed to be an important mechanism accounting for diabetes-induced progressive proteinuria and renal function decline because of its pro-proliferative and pro-fibrosis effects. However, because angiotension II is one of the most potent mesangial contractile agonists, it is widely used as a stimulator to investigate mesangial cells contractility. In cultured mesangial cells, high glucose treatment resulted in a 70% impairment of mesangial cell contractility (a 12% decrease in the cell surface area in the HG group vs. a 39% decrease in the NG group). However, such impairment is significantly ameliorated by emodin. Furthermore, the ameliorating effect of emodin is dose-dependent. Emodin at 50 mg/l elevated angiotension II induced cell contraction by 83.3% whereas at 100 mg/l cell contraction was elevated by 150%. These results provide direct evidence that emodin effectively normalizes the high-glucose induced hypo-response to vaso-contracting agents in mesangial cells.

The precise mechanism underlying vaso-contracting agents inducing mesangial contraction is not known. Recent research has suggested that the p38 mediated signal pathway plays a key role (Müller *et al*., 1999; Dunlop and Muggli, 2000; Wilmer *et al*., 2001; Tsiani *et al*., 2002; Hirano *et al*., 2005; Sakai *et al*., 2005; Komers *et al*., 2007). As demonstrated by Müller and colleagues (1999), 2 μM angiotension II stimulation resulted in a significant elevation of p38 activity in cultured rat glomerular mesangial cells, while administration of SB 203580, an inhibitor of p38, almost completely abolished angiotension II induced cell contraction. Similar results have also been demonstrated in

both endothelin-1 and cadmium induced mesangial contraction (Dunlop and Muggli, 2000; Tsiani *et al*., 2002; Hirano *et al*., 2005). These findings suggest that p38 activation acts as a common step in mesangial contraction induced by different vasoactive agents. In a diabetic state, over-activation of p38 exists in mesangial cells (Wilmer *et al*., 2001; Sakai *et al*., 2005; Komers *et al*., 2007) and this is proposed as the major mechanism responsible for mesangial cell hypo-responsiveness to vaso-contracting agents. Wilmer *et al*. (2001) demonstrated that a 30 mM glucose treatment for seven days resulted in a 250% increase in the p38 activity in mesangial cells, and blocking p38 using SB 203580 significantly ameliorated high-glucose induced mesangial dysfunction. A recent study further revealed that *in-vivo* usage of a p38 inhibitor was also effective in ameliorating glomerular hyperfiltration in STZ treated rats (Komers *et al*. 2007). Based on these findings, it has been proposed that inhibition of p38 is an important intervention target for early diabetic nephropathy. We have demonstrated that the ameliorating effects of emodin on high-glucose induced mesangial hypocontractility occur via p38 inhibition. Emodin at 50 mg/l and 100 mg/l reduced p-p38 levels by 40% and 73%, respectively. This finding is consistent with other *in-vitro* studies using human umbilical vein endothelial cells (Kwak *et al*., 2006), human lung non-small cell carcinoma cells (Yeh *et al*., 2003), and retina ganglion cells (Lin *et al*., 2007) in which the pharmacological effect of emodin was mediated via inhibition of p38. Our previous study also demonstrated that emodin normalizes IL-1β induced mesangial cell p38 over-activation (Wang *et al*., 2007). Thus, p38 inhibition is the probable mechanism underlying the protective effects of emodin on high-glucose induced mesangial hypocontractility.

Recent studies have suggested that emodin has a PPARγ-activating effect. In high-fat diet treated ApoE-knockout mice, administration of emodin resulted in a significant elevation of PPARγ expression in aortic atherosclerotic plaques (Zhou *et al*., 2008). Using a surface plasmon resonance experiment, Yang and colleague (2007) demonstrated that emodin binds to PPARγ directly and enhances PPARγ mRNA expression. Similar results have also been demonstrated herein. Both the PPARγ mRNA and protein levels were elevated after emodin treatment. GW9662 is a specific blocker of PPARγ and a 10 μM GW9662 treatment resulted in a 96% increase in p-p38 protein levels, indicating elevated p38 activation. Along with p38 activation, mesangial cell contractility also decreased. These findings suggest that emodin inactivates p38 and ameliorates mesangial hypocontractility via, at least partially, PPARγ activation.

The regulatory effect of PPARγ activation on the p38 signal pathway is far from clear. Results from different research have yielded different conclusions. For example, in an osteoarthritis animal model (Boileau *et al*., 2007), administration of pioglitazone, a PPARγ agonist, resulted in significant p38 inhibition in cartilage specimens. The inhibitory effects of PPARγ on the activation of p38 have also been demonstrated in cultured mesencephalic neuron cells (Xing *et al*., 2008). On the contrary, PPARγ activation leads to p38 activation in renal epithelium cells (Kwon *et al*., 2008). These inconsistent findings indicate that the regulatory effect of PPARγ on the p38 signal pathway is probably tissue-specific. Current evidence is not sufficient to explain these differences. The relationship between PPARγ and p38 needs to be investigated.

In conclusion, we have demonstrated that emodin partially or totally ameliorates high-glucose induced p38 over-activation via activation of PPARγ and, therefore, ameliorates hypocontractility in mesangial cells (Figure 5).

Methods

Cell culture

Established rat glomerular mesangial cells were obtained from Wuhan Life-Science Academy (Wuhan, China). Cells were cultured in RPMI 1640 (GIBCO/Invitrogen Corp, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37^{o}C under 5% CO_2. Cells between

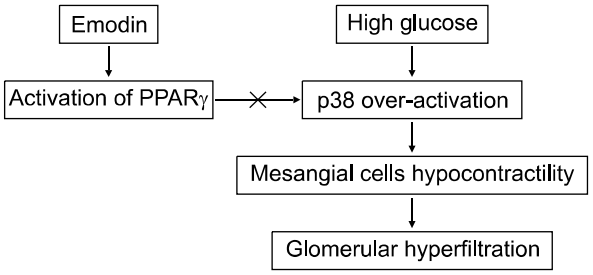

Figure 5. Possible mechanism underlying the protective effects of emodin on high-glucose induced mesangial hypocontractility. In a high-glucose state, p38 was over-activated in glomerular mesangial cells. This is the central event leading to an impaired response to vaso-contractile agents. The impaired contractility of mesangial cells unavoidably results in elevated glomerular filtration, the hallmark of early stage diabetic nephropathy. Emodin, possibly via activation of PPARγ, inhibits high-glucose induced p38 over-activation and, therefore, inhibits ameliorated mesangial cells hypocontractility.

passages 10 and 18 were used for experiments. After a 24-h preincubation period, mesangial cells were divided according to glucose concentration and different compounds added into the five groups of 1) normal glucose group (NG, 5.6 mM glucose, served as control); 2) high glucose group (HG, 30 mM glucose); 3) low-dose emodin group (LE, 30 mM glucose with 50 mg/l of emodin); 4) high-dose emodin group (HE, 30 mM glucose with 100 mg/l of emodin); and 5) PPARγ blocking group (GW, 30 mM glucose with 100 mg/l of emodin and 10 μM gw9662). Cells were incubated for another 48 h before analysis. Emodin and gw9662 were purchased from Sigma (St Louis, MO).

Mesangial cell contractility assay

Mesangial cell contractility was evaluated by measuring alternations in the cellular planar surface area. AngiotensionII, obtained from Sigma (St Louis, MO), was used as a contractile agonist at a dosage of 1 μM. Cells were visualized using an inverted fluorescence microscope (Nikon TS2000) and images were captured before and 30 min after angiotension II stimulation. Images were analyzed using Image J Software and changes in the cell planar surface area after angiotension II stimulation were evaluated.

Western blot analysis

Western blotting was performed as described by Wang *et al*. (2007) and Liu *et al*. (2006). Briefly, after treatment with different compounds, mesangial cells were harvested and lysed using a lysis buffer containing 25 mM HEPES-NaOH, 1.5 mM $MgCl_2$, 0.3 M NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 100 mM $NaVO_4$, 2 mg/ml of leupeptin, and 100 mg/ml of PMSF. Protein concentrations were determined using the Lowry method. Equal amounts of protein were loaded, then separated using SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% skim milk, the membranes were then incubated overnight at 4°C with specific antibodies for total p38, phospho-p38 (p-p38, Thr^{180}/Tyr^{182}), and PPARγ. After incubation with the respective second antibodies, the immune complexes were detected using the ECL method and immunoreactive bands were quantified using an Alphaimager 2200. Values were corrected using the absorbency of the internal control (actin). Antibodies for total p38 and p-p38 were purchased from Cell Signaling Technologies (Beverly, MA), while other antibodies were a product of Santa Cruz Biotechnology (Santa Cruz, CA).

Real-time PCR

PPARγ mRNA levels were detected using real-time PCR (Liu *et al*., 2006; Wang *et al*., 2007). Cells were harvested and total RNA was extracted using the standard Trizol RNA isolation method. Reverse transcription of 1 μg of RNA was carried out according to the instructions for the TaKaRa RT kit (Shiga, Japan). Specific primers designed against rat PPARγ and GAPDH (internal control) were verified using NCBI Blast. Primer sequences along with annealing temperatures are shown in Supplemental Data Table S1. Real-time PCR was performed using a Quantitect SYBR Green kit (Qiagen). The reaction volume was 25 μl, and 100 ng of cDNA was used as template. Fluorescence was detected using an ABI Prism 7700 Detection System. PCR products were visualized using gel electrophoresis to confirm a single product of the correct size. Ratios of the target gene to GAPDH were calculated.

Data analysis

All experiments were repeated at least three times. Values are reported as mean ± SD. Data were analyzed using SPSS 10.0 software. Statistical significance was assessed using ANOVA and an unpaired Student *t* test and *P* values of less than 0.05 were considered statistically significant.

Supplemental data

Supplemental Data include a Table and can be found with this article online at http://e-emm.or.kr/ article/article_ files/SP-41-9-05.pdf.

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