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Full Length Research Paper

Inhibitory effect of resveratrol on advanced glycation end products-induced mesangial cell proliferation

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Diabetic nephropathy (DN) is the major cause of end-stage renal disease. An important histological hallmark of DN is proliferation of mesangial cells, and as a result, the expansion of extracellular matrix in the mesangium. Resveratrol has been shown to ameliorate hyperglycemia in diabetic rats. However, the effects of resveratrol on DN remain unknown. The aim of the present study is to investigate the effects of resveratrol on mesangial cell proliferation induced by advanced glycation end products (AGEs). Cultured rat mesangial cells were exposed to AGEs in the absence and presence of indicated concentrations of resveratrol (2.5, 5.0 and 10.0 µmol/L). The proliferation of mesangial cells was assayed by Methylthiazoletetrazolium (MTT) assay. Cell cycle and apoptosis were analyzed using flow cytometry. Expressions of glutathione S-transferases Mu (GSTM) and nuclear factor-erythroid 2-related factor 2 (Nrf2) were detected by Western blot. Resveratrol inhibited proliferation of mesangial cells caused by AGEs, and down-regulated GSTM and Nrf2 expressions in a dose-dependent manner. S phase cell number significantly increased in the resveratrol treated groups compared with those in the AGEs group. Resveratrol inhibited mesangial cell proliferation induced by AGEs. The inhibitory effects of resveratrol were mediated in part through suppressing cell growth by arresting cells at the S phase of the cell cycle and down-regulating GSTM and Nrf2 expression. These findings suggested that resveratrol had potential preventive effects on the process of DN.

Key words: Resveratrol, diabetic nephropathy, mesangial cell, cell cycle, glutathione S-transferases Mu (GSTM), nuclear factor-erythroid 2-related factor 2 (Nrf2).

INTRODUCTION

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease (ESRD) in the developed and developing countries, which can be characterized by both glomerular and tubulointerstitial injury (Fioretto et al., 1998). An important histological hallmark of DN is proliferation of mesangial cells and increased extracellular matrix (ECM) protein synthesis, and as a result, the expansion of ECM in the mesangium (Osterby et al., 1983; Abrass, 1995; Chen et al., 2003; Yano et al., 2009). These pathological changes arise in part from the adverse effects of hyperglycaemia, leading to the formation of advanced glycation end products (AGEs) (Miyata, 1996; Simonson, 2007). Current treatments for DN mainly include strict control of hyperglycemia, hypertension, and blockade of the renin-angiotensin system. In spite of excellent glucose and blood pressure control, including administration of angiotensin-converting enzyme inhibitors and/or angiotensin II receptor blockers, DN still develops and progresses. The development of additional protective therapeutic interventions is, therefore, a major priority (Levi et al., 2011).

Resveratrol is a naturally occurring polyphenol found in more than 70 species of plants including some components of human diet, such as peanuts, grapes, and red wines (Szkudelski et al., 2007; Sulaiman et al., 2010).

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Resveratrol possesses anticancer, antioxidant, anti-inflammatory, and cardioprotective effects in various experimental models (Atten et al., 2001; El-Mowafy and Alkhalaf, 2003; Miller and Rice-Evans, 1995; Das et al., 2005; Hung et al., 2000). Resveratrol recently was shown to exert numerous beneficial effects in animal models of diabetes mellitus including improving insulin sensitivity and lowering plasma glucose (Su et al., 2006; Thirunavukkarasu et al., 2007; Zang et al., 2006). Resveratrol also was reported to alleviate diabetic vasculopathy through attenuation of AGE-receptor for AGE (AGE-RAGE) signalling pathway (Jing et al., 2010). However, the effects of resveratrol on DN remain unknown.

In the present study, we confirmed resveratrol can suppress proliferation of rat mesangial cells induced by AGEs. Moreover we explored the possible underlying mechanisms.

MATERIALS AND METHODS

Resveratrol (more than 98% pure, lot no. 0810018-22) was provided by JF-NATURAL (Tianjin, China) and solvent by dimethyl sulfoxide (DMSO). Bovine serum albumin (BSA), D-glucose, DMSO, and MTT were purchased from Sigma (USA). Rat mesangial cell line was obtained from Institute of Biochemistry and Cell Biology (Shanghai, China). Fetal bovine serum and 1640 medium were obtained from Gibco (USA). The antibodies of glutathione S-transferases Mu (GSTM), nuclear factor-erythroid 2-related factor 2 (Nrf2), and β-actin were purchased from Abcam (USA).

AGEs synthesis

AGEs-BSA was prepared by incubating BSA (5 mg/ml) at 37°C for 12 weeks with D-glucose (500 mM). Control preparations were treated identically except that glucose was omitted. Finally, preparations were extensively dialyzed against phosphate buffer to remove free glucose. The extent of advanced glycation was assessed by characteristic fluorescence (excitation 370, emission 440 nm).

Cell culture

Rat mesangial cells (RMCs) were cultured in 1640 medium containing 10% fetal bovine serum at 37°C in 95% air/5% CO_2. RMCs were performed on 5 to 7 passages. RMCs were grown to 80 to 90%_confluence, and made quiescent by serum deprivation for 24 h (0.2% BSA), then treatment with AGEs-BSA and BSA. Before adding AGEs-BSA or control BSA to the medium, cells were preincubated with indicated concentration of resveratrol for 1 h.

Assessment of cell viability by MTT assay

RMCs (1×10^6 cells/ml) were incubated in 96-well plastic culture plates, and cultured in 1640 medium at 37°C in 95% air/5% CO_2 overnight, and preincubated with indicated concentration of resveratrol (2.5, 5, 10 μmol/L) for 1 h. Then AGEs-BSA or control BSA was added to the medium, and the concentration of AGEs-BSA was 100 μg/ml. After cultured for 48 h, 1 mg/ml MTT was added to each well and incubated for 4 h at 37°C. After the experimental periods, 10% SDS was added to the wells, which were further incubated overnight at 37°C. Optical density (OD) was measured at a wavelength of 570 nm. The OD of the control cells was assigned a relative value of 100. The experiments were performed in triplicate.

Cell cycle analysis by flow cytometric analysis

After cultured for 48 h, cells were harvested, washed with cold phosphate-buffered saline (PBS), centrifuged (600 r.p.m. for 5 min) and resuspended. After two further washing steps with cold PBS, propidium iodide (PI) solution was added and incubated at 4°C for 15 min. Then the samples were analyzed on flow cytometer (BD Biosciences, USA), and the cell cycle distribution was calculated.

Apoptosis analysis with annexin V-PI dual staining

Cells were collected and the annexin V-PI dual-staining assay was performed according to the manufacturer's instructions. Collected cells were briefly washed with cold PBS twice and resuspended in 500 μl 1 × binding buffer containing 5 μl Annexin V and 5 μl PI for 10 min at room temperature in the dark. After incubation, the cells were analyzed using a flow cytometer.

Western blot

The samples were treated with buffer, heated at 100°C for 5 min, and electrophoresed in a 12% SDS-polyacrylamide gel. After being transferred to a NC membrane (Pall), the membrane was incubated in blocking buffer (0.05% Tween 20, and 5% nonfat milk) for 1 h at room temperature, followed by 1 h incubation at room temperature in a dilution of polyclonal antibodies against GSTM, Nrf2, or β-actin, and then incubated with horseradish peroxidase-linked secondary antibody for 1 h. The membrane was developed with a chemiluminescent agent (Denville), and the band densities were measured using Image J software.

Statistical analysis

All values are expressed as means ± SE. Statistical analysis was performed using the statistical package SPSS10.0. Results were analyzed using the nonparametric test for multiple comparisons. P values < 0.05 were considered statistically significant.

RESULTS

Inhibitory effect of resveratrol on the proliferation of RMCs induced by AGEs

We examined whether resveratrol affects AGEs-induced proliferation of mesangial cells. The cells were treated with resveratrol (2.5, 5, and 10 μmol/L) for 48 h and the viability was evaluated by MTT. AGEs stimulated the proliferation of mesangial cells. However, resveratrol treated at concentrations up to 10 μmol/L exhibited dose-dependent percent decline in RMCs ranging from 102.4 to 92.6% compared with BSA control group. It indicated

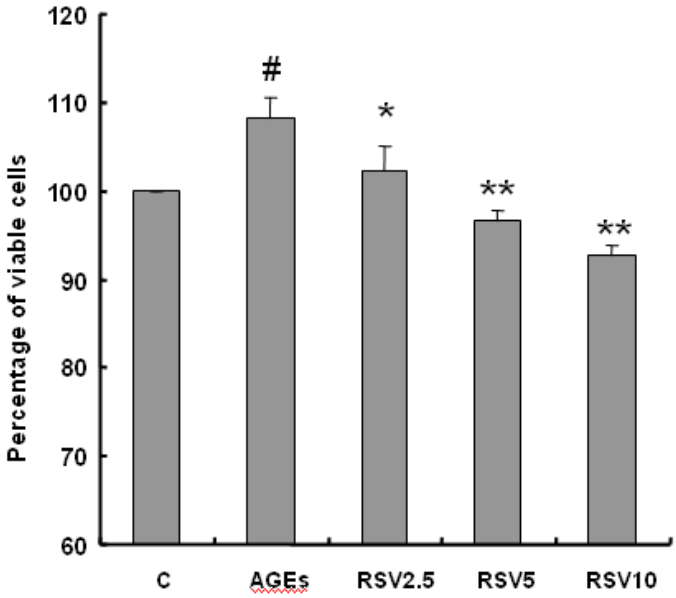


Figure 1. Effects of resveratrol (2.5, 5.0 and 10.0 µmol/L) on viability in RMCs by MTT. Results were expressed as percent of untreated cells (100%) and were given as mean± SD from three independent experiments. #P < 0.01, compared with C; *P < 0.05, **P < 0.01 compared with cells stimulated with AGEs. Photographs in C: control BAS group; AGEs: AGEs-BAS group.

that increasing resveratrol concentration resulted in decreased RMCs proliferation (Figure 1).

Effect of resveratrol on cell cycle in AGEs-induced RMCs

To explore the underlying mechanism of inhibitory effect on RMCs growth, cell cycle analysis was performed on the cells that had been treated with the indicated concentration of resveratrol (2.5, 5, and 10 µmol/L). After exposed to increasing concentrations of resveratrol for 48 h, S phase cell number significantly increased as compared with those in the AGEs-BSA group (Figure 2A). In contrast, a decreased G1 cell number was observed in the resveratrol treated groups compared with the AGEs-BSA group (Figure 2B). The data suggested that the inhibitory effect of resveratrol on cell proliferation was partly due to a dose-dependent cell cycle block in S phase.

Effect of resveratrol on apoptosis in AGEs-induced RMCs

Fluorophores annexin V and PI were used to analyze the effects of resveratrol on apoptosis in AGEs-induced RMCs. Control, AGEs-BSA cells showed rare presence of annexin V, indicating the presence of apoptosis in the

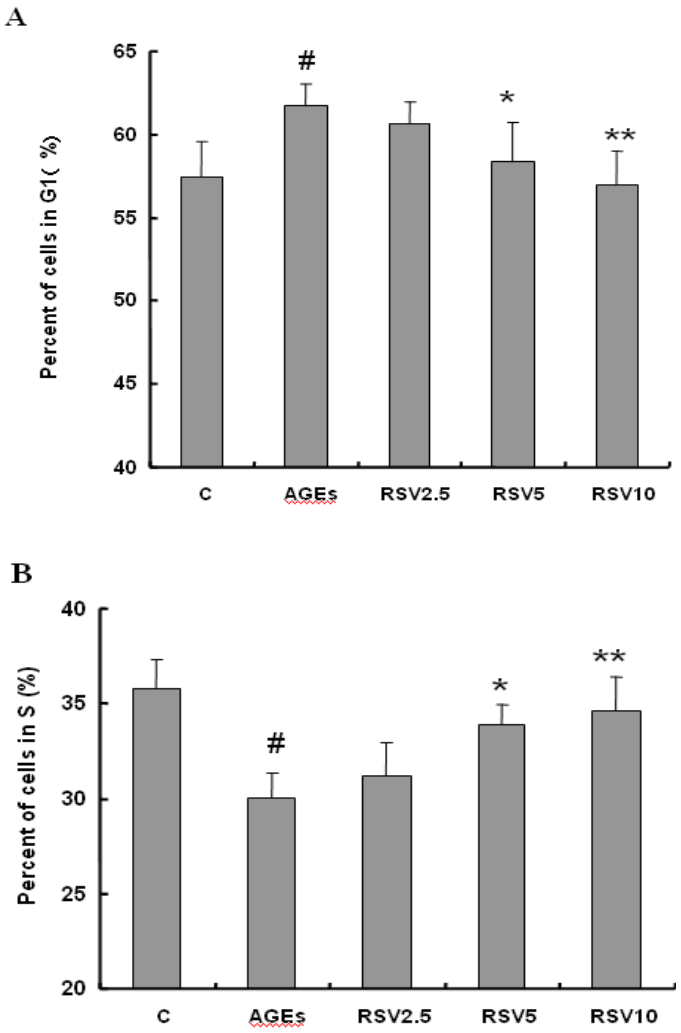


Figure 2. Effects of resveratrol (2.5, 5.0 and 10.0 µmol/L) on cell cycle of RMCs. A: The percent of cells in G1 phase. B: The percent of cells in S phase. Data were given as mean ± SD from three independent experiments. #P < 0.05, compared with C; *P < 0.05, **P < 0.01 compared with cells stimulated with AGEs. Photographs in C: control BAS group; AGEs: AGEs-BAS group.

cells at steady state. However, after resveratrol treatment, apoptosis rate of RMCs slightly increased in a dose-dependent manner compared with those of AGEs-BSA RMCs. Significantly increased apoptosis of 5 µmol/L resveratrol was noticed at the beginning, indicating the onset of the apoptotic phenomena (Figure 3).

Resveratrol down-regulated GSTM and Nrf2 expression in AGEs-induced RMCs

In mesangial cells, AGEs significantly increased mRNA expression of GSTM. A diminished GSTM expression was observed in cells treated with resveratrol for 1 h. The inhibitory effect of resveratrol on GSTM expression was in a dose-dependent manner. Next, we tested the

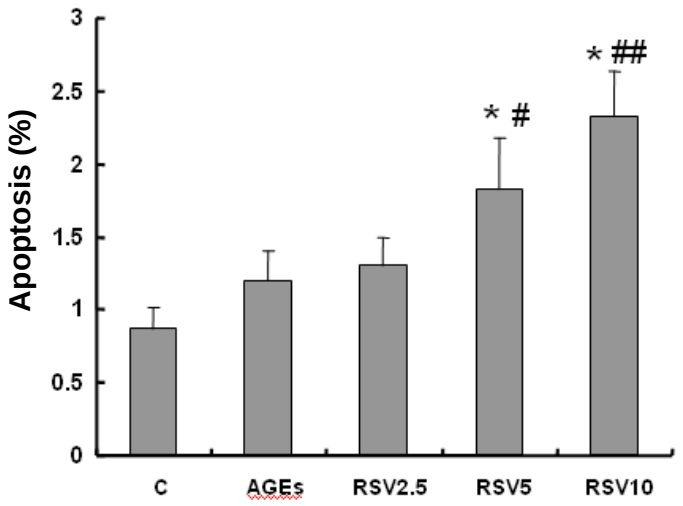
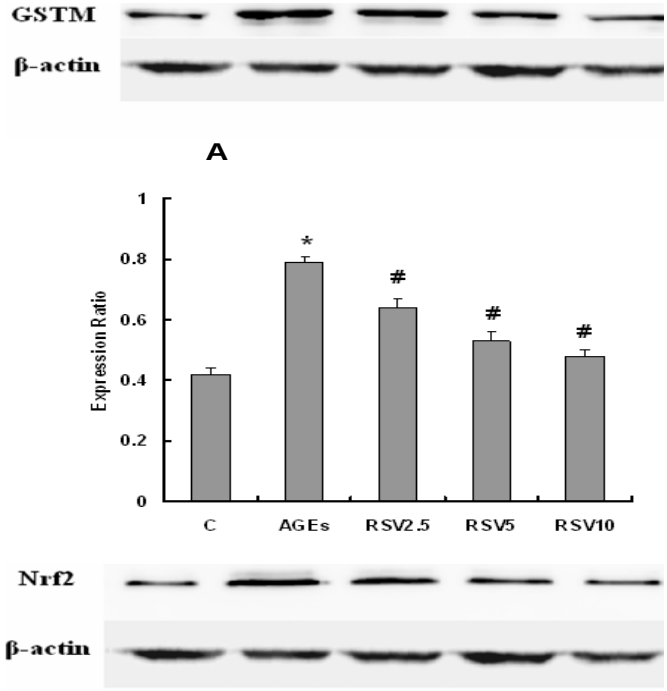


Figure 3. Effect of resveratrol (2.5, 5.0 and 10.0 µmol/L) on apoptosis of RMCs. Data were given as mean ± SD from three experiments. *P < 0.01, compared with C; #P < 0.05, ##P < 0.01 compared with AGEs. Photographs in C: control BAS group; AGEs: AGEs-BAS group.



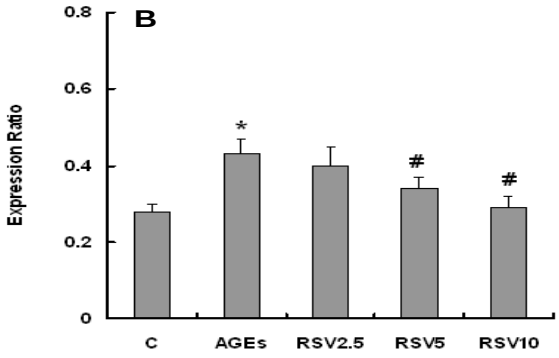


Figure 4. Effect of resveratrol (2.5, 5.0 and 10.0 µmol/L) on expressions of GSTM and Nrf2 in RMCs. A: Resveratrol down-regulated GSTM expression in RMCs. Data were expressed as GSTM/β-actin and given as mean ± SD from three experiments. *P < 0.01, compared with C; #P < 0.01, compared with AGEs. Photographs in C: control BAS group; AGEs: AGEs-BAS group. B: Resveratrol down-regulated Nrf2 expression. Data were expressed as Nrf2/β-actin and given as mean ± SD from three experiments. *P < 0.01, compared with C; #P < 0.01, compared with AGEs. Photographs in C: control BAS group; AGEs: AGEs-BAS group.

possibility that resveratrol could regulate the Nrf2 expression. A similar result was observed: resveratrol prevented Nrf2 expression in a dose-dependent manner which was increased in the AGEs exposed condition (Figure 4).

DISCUSSION

Glomerulosclerosis in diabetic nephropathy is caused by accumulation of ECM proteins in the mesangial interstitial space (Alsaad and Herzenberg, 2007). One important reason of ECM protein accumulation is increased synthesis by mesangial cells. Several studies have unraveled significant mesangial cell proliferation in the early stages of diabetic nephropathy (Young et al., 1995; Awazu et al., 1999), which precedes the increases in the ECM proteins and glomerular sclerosis. Mesangial cells have been confirmed to express receptors for AGEs, such as AGE-R1 and AGE-R2. Meanwhile AGEs can increase mesangial cell proliferation and mesangial synthesis of ECM proteins (Suzuki et al., 2006; He et al., 2000). Consistent with these studies, we also observed increased proliferation in mesangial cells exposed to AGEs condition in our present study. Furthermore, we invested the effects of different concentrations of resveratrol (2.5, 5.0, 10 µmol/L) on AGEs-induced RMCs. In our previous study, polyphenol as antioxidant were observed to decrease of AGEs level *in vivo* (Li et al., 2008). *In vitro* our data demonstrated resveratrol inhibited AGEs-induced RMCs proliferation in a dose-dependent manner. The result suggested that resveratrol had the potential prevention effects on the process of DN.

In the present study, we analyzed the effects of resveratrol on cell cycle and apoptosis to explore the possible mechanisms about its inhibition on RMCs proliferation. The data showed S phase cell number significantly increased in the resveratrol treated groups as compared with those in the AGEs-BSA group. Indeed, the results indicated that resveratrol suppresses cell growth by arresting cells at the S phases of the cell cycle. Apoptosis has been documented in the course of

renal injury both in diabetic patients and rats (Choudhury et al., 2010; Kelly et al., 2009). Mesangial cell apoptosis possibly keeps in check an enhanced proliferative response and accumulated ECM (Ortiz, 2000). To evaluate the effects of resveratrol on apoptosis in AGEs-induced RMCs, we measured apoptosis rate using flow cytometer. After resveratrol treatment, apoptosis rate of RMCs slightly increased in a dose-dependent manner compared with those of RMCs without resveratrol treatment. The results showed resveratrol induced apoptosis in RMCs, which can partly explain the inhibitory effect of resveratrol on the proliferation of mesangial cells.

Moreover we analyzed GSTM expression on AGEs-induced RMCs. The results showed that expression of GSTM was increased, and Nrf2 expression was also increased on AGEs-induced RMCs. Apoptosis signal-regulating kinase 1(ASK1), a mitogen activated protein kinase (MAPK) (Ichijo et al., 1997), is an important kinase in the intracellular signal transduction system leading to cell proliferation and ECM protein synthesis. It is demonstrated that GSTM directly interacts with the N-terminal portion of ASK1 both *in vivo* and *in vitro*. This interaction results in suppression of ASK1 activity as well as ASK1-dependent apoptotic cell death (Cho et al., 2001). However, up-regulation of GSTM expression on mesangial cells resulted in suppression of ASK1-dependent apoptosis, which furthermore resulted in mesangial cells proliferation. Meanwhile we investigated the effect of different concentrations of resveratrol (2.5, 5.0, 10 µmol/L) on GSTM and Nrf2 expressions. Our data suggested resveratrol inhibited the expressions of Nrf2 and GSTM in a dose-dependent manner. The down-regulated expression of GSTM resulted in activation of ASK1-dependent apoptosis, which can explain the effect of resveratrol on the apoptosis in mesangial cells.

In conclusion, we demonstrated that resveratrol inhibited AGEs-induced RMCs proliferation. The inhibitory effects of resveratrol were mediated in part through suppressing cell growth by arresting cells at the S phases of the cell cycle and down-regulating Nrf2 and GSTM expression. The result suggested that resveratrol had the potential prevention effects on the process of DN.

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