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Ferulic acid inhibits vascular smooth muscle cell proliferation induced by angiotensin II

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Abstract

The aim of this study was to determine the effects of ferulic acid on the proliferation and molecular mechanism in cultured vascular smooth muscle cell (VSMC) induced by angiotensin II. It was shown that ferulic acid significantly inhibited angiotensin II-induced VSMC proliferation in a dose-dependent manner. Western blotting analyses suggest that the antiproliferative effect of ferulic acid was involved in the mitogen-activated protein kinases (MAPKs) pathway. While no effect on p38, ferulic acid markedly inactivated the extracellular signal-regulated kinases (ERK1/2) and c-Jun N-terminal kinases (JNK), indicating that the inhibition of ferulic acid on VSMC proliferation was associated with ERK1/2 and JNK rather than p38 pathway. On the expression of cell cycle regulatory proteins, ferulic acid elevated the protein content of $p21^{waf1/cip1}$, decreased expression of cyclin D1 and inhibited phosphorylation of retinoblastoma protein, suggesting that ferulic acid inhibited VSMC proliferation by regulating the cell progression from G_1 to S phase. The inactivation of MAPKs and modulation of cell cycle proteins of ferulic acid may be of importance in preventing cardiovascular disease.

Keywords: Ferulic acid; Vascular smooth muscle cell; Proliferation; Mitogen-activated protein kinase; Cell cycle regulatory protein; Angiotensin II

1. Introduction

The major bioactive peptide of the renin-angiotensin system, angiotensin II, plays a fundamental role, not only in controlling cardiovascular and renal homeostasis but also contributing to various cardiovascular diseases such as hypertension, atherosclerosis and heart failure. However, angiotensin II converting enzyme inhibitors appear to exert tissue protective effect against these diseases. Thus, the inhibition of growth stimulating activity likely contributes to treat the cardiovascular diseases (Dzau, 1998; Dostal and Baker, 1999).

The proliferation of vascular smooth muscle cell (VSMC) induced by angiotensin II is involved in the mitogen-activated protein kinases (MAPKs) pathway (Eguchi et al., 2001). MAPKs pathway has been described in mammalian cells, including p42/p44 extracellular signal-related kinases (ERK1/2), c-Jun N-terminal protein kinase (JNK) and p38 MAP kinase (Boulton et al., 1991; Kyriakis and Woodgett, 1994; Han et al., 1994). These stress-activated MAPKs phosphorylate specific subsets of transcriptional factors, thereby regulating cellular processes of inflammation, proliferation, differentiation, apoptosis and survival (Ip and Davis, 1998; Widmann et al., 1999). In normal mammalian cells, the decision to proliferate is made during the G_1 phase of the cell cycle (Hunter and Pines, 1994; Peters, 1994), which is regulated by cell cycle proteins (Zieske et al., 2004). Although some investigations show that the expression or activation of cell cycle proteins is mediated by MAPKs pathway (Lavoie et al., 1996; Lee et al., 2001), their interrelation on VSMC proliferation induced by angiotensin II remains unclear.

Ferulic acid is one of the active ingredients of a Chinese herbal medicine (*Ligusticum chuanxiong* hort)

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(Liu and Fang, 2001; Hou et al., 2004). As an efficient pharmaceutical, ferulic acid is clinically used to treat angina pectoris and hypertensive diseases in China (Wei, 2002). Previous investigations suggest that it is significant effects to improve blood fluidity, inhibit platelet aggregation and exhibit strong antioxidant activity (Wang et al., 1992; Zhang et al., 2001; Cheng et al., 2003). In this paper, there is considerable interest in defining the effects of ferulic acid on MAPKs pathway and cellular events from the G_1 to S phases, including the phosphorylation of retinoblastoma protein (pRb), cyclin D1 activity and the expression of $p21^{waf1/cip1}$ in cultured VSMC induced by angiotensin II.

2. Materials and methods

2.1. Materials

Ferulic acid was purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Angiotensin II, trypsin, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT), phenyl methyl sulfonyl fluoride (PMSF), penicillin and streptomycin were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Gibco Industries). Antibodies for Tyr^{204}-phosphorylated ERK1/2, ERK2, Thr^{180}/Tyr^{182}-dually phosphorylated p38, phosphorylated JNK, $p21^{waf1/cip1}$, retinoblastoma protein (pRb), cyclin D1, α-actin and enhanced chemiluminescence (ECL) reagent kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals used were of the highest grade available commercially.

2.2. Cell culture

Healthy male Wistar rats (100–150 g) were obtained from the Laboratory Animal Center, Tianjin Institute of Pharmaceutical Research. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. VSMC was prepared by the explant method from thoracic aorta of male Wistar rats. Briefly, the aortae were freed of connective tissue and adherent fat, the endothelial cell layer of the intima was removed, and the aorta artery was cut into about 3-mm cubes. They were placed in DMEM supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. VSMC exhibited a typical "hill and valley" growth pattern and the identity was confirmed by morphological examination and by staining for α-actin. Medium was replaced twice a week. The cell became confluent, followed by subculture using trypsinization. Confluent cell at passage numbers 3–6 was used for the experiments.

2.3. Cell proliferation assay

VSMC was counted and seeded into 96-well culture plates at a density of 2×10^4 cells/well. After 24 h, the medium was changed for serum-free DMEM to make them quiescent for 48 h. VSMC was stimulated with 1 μM angiotensin II in the absence or presence of ferulic acid (20, 40, 80, 160 μM) during 48 h. Cell proliferation was assayed by MTT method as previously described (Takahashi and Abe, 2002). Briefly, a volume of 200 μl of 0.5 mg/ml MTT in DMEM medium was added to each well and incubated for 4 h. Formazan crystals were dissolved in 150 μl of dimethyl sulfoxide (DMSO) and the absorbance was measured at a wavelength of 570 nm with an enzyme-linked immunosorbent assay reader (BioRad 3550, Bio-Rad Laboratories).

2.4. Western blotting analysis

After various treatments, VSMC was harvested and lysed for 20 min in 200 μl lysis buffer (50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% NP-40, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM NaF, 0.1 mM Na_3VO_4 and 1 mM dithiothreitol). After 20 min incubation, cellular debris was removed by centrifugation at $14{,}000\times g$ for 10 min and supernatant was saved. Equal protein loading in each lane, resolved by SDS-polyacrylamide gel electrophoresis, blotted on nitrocellulose membrane. Membranes were blocked in 5% nonfat milk powder in Tris-buffered saline (TBS)/0.1% Tween-20 for 1 h at room temperature, and then incubated with specific antibodies in 5% bovine serum albumin in TBS for another 1 h. Membranes were incubated with peroxidase conjugated second antibody in blocking buffer for 1 h. The labeled proteins were detected with ECL kit.

2.5. Statistical analysis

Statistical comparison was carried out with three or more groups using one-way analysis of variance (ANOVA) and Dunnett's test. The data represent means±S.E.M. The values of $P<0.05$ were statistically significant.

3. Results

3.1. Effect of ferulic acid on VSMC proliferation

Compared with control cell treated with medium only, angiotensin II significantly stimulated VSMC proliferation (Fig. 1). Ferulic acid dose-dependently inhibited angiotensin II-induced VSMC proliferation. The inhibitive rate was about 50% when VSMC was treated with ferulic acid concentration of 80 μM. However, at higher drug doses (80 and 160 μM), the inhibitive rates of ferulic acid on VSMC were not significant change. The data suggest that ferulic

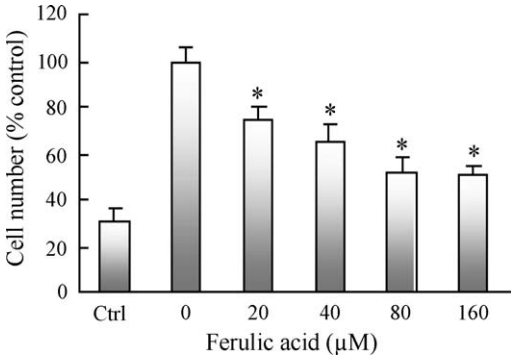

Fig. 1. Dose effect of ferulic acid on VSMC proliferation. Arrested VSMC (serum starved for 48 h) were stimulated with 1 μM angiotensin II in the absence or presence of ferulic acid (20, 40, 80, 160 μM) during 48 h. Control group was not treated with angiotensin II and ferulic acid. VSMC proliferation was assayed by MTT. The data points are presented as means±S.E.M. of six separate experiments. *P<0.05, significant difference from the cell treated with angiotensin II only.

acid markedly inhibited VSMC proliferation induced by angiotensin II.

3.2. Effect of ferulic acid on the MAPKs activation

Arrested VSMC was exposed to 1 μM angiotensin II for 0, 15 and 30 min. The activations of ERK1/2, JNK and p38 were examined by Western blotting with the phospho-specific antibody. The data show that ERK1/2, JNK and p38 were activated by angiotensin II and peaked at 15 min (Fig. 2). In contrast, when arrested VSMC was pretreated with 80 μM ferulic acid for 1 h, the activations of ERK1/2 and JNK

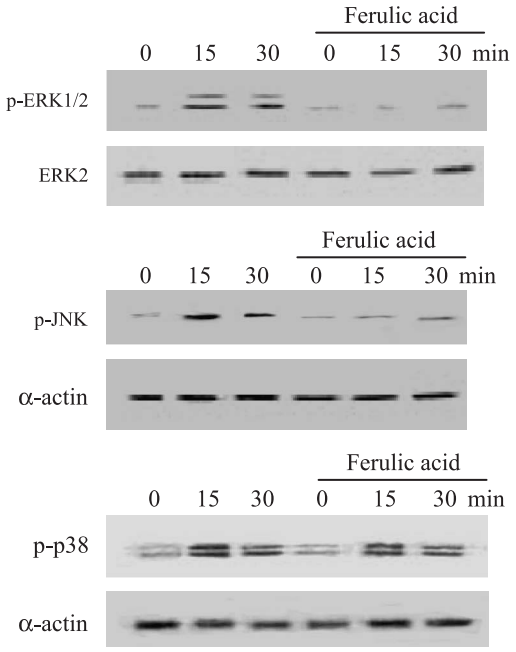

Fig. 2. Time effect of ferulic acid on MAPKs activation. Arrested VSMC was pretreated without and with 80 μM ferulic acid for 1 h, and stimulated with 1 μM angiotensin II for 0, 15, 30 min. Cell lysates were analyzed by Western blotting with antibodies as indicated.

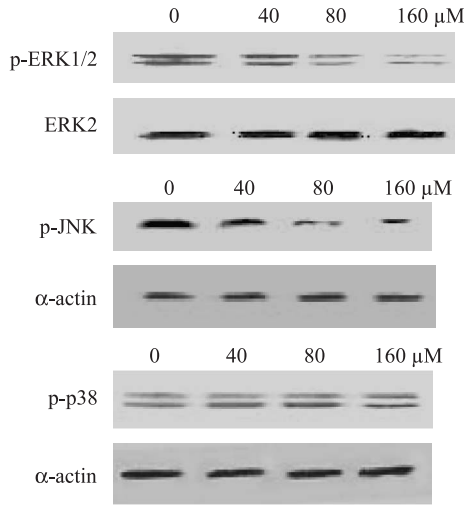

Fig. 3. Dose effect of ferulic acid on MAPKs activation. Arrested VSMC was pretreated without and with various concentration ferulic acid for 1 h, and stimulated with 1 μM angiotensin II for 15 min. Cell lysates were analyzed by Western blotting with antibodies as indicated.

were significantly inhibited. p38 was also activated by angiotensin II, but it was not suppressed by ferulic acid.

To further investigate the dose-dependent effect of ferulic acid on ERK1/2, JNK, and p38, arrested VSMC was pretreated with various concentration ferulic acid (40, 80, 160 μM), and then stimulated with 1 μM angiotensin II for 15 min. The data suggest that ferulic acid markedly inhibited ERK1/2 and JNK activations, while not significantly affected the activation of p38 with increase dose of ferulic acid (Fig. 3). Thus, ferulic acid inhibited the activations of ERK1/2 and JNK, but not p38 MAPKs pathway in a dose- and time-dependent manner.

3.3. Effects of ferulic acid on cyclin D1 and p21$^{waf1/cip1}$

Ferulic acid led to a reduction of cyclin D1 protein content in VSMC induced by angiotensin II (Fig. 4A). At higher drug concentrations (80 and 160 μM), the expression of cyclin D1 was not detectable. On the other hand, p21$^{waf1/cip1}$ is known

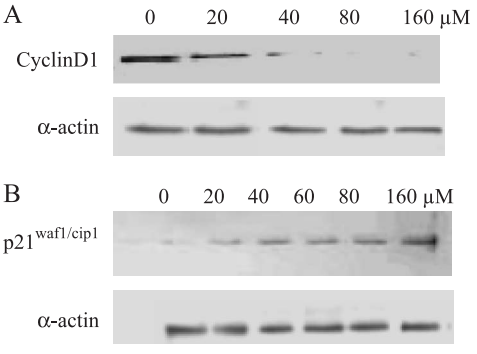

Fig. 4. Dose effect of ferulic acid on expressions of cyclin D1 and p21$^{waf1/cip1}$. Arrested VSMC was pretreated without and with various concentration ferulic acid for 1 h and stimulated with 1 μM angiotensin II for 6 h. Cell lysates were analyzed by Western blotting with antibodies as indicated.

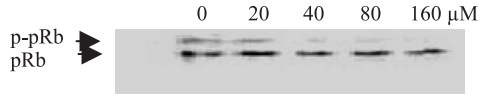

Fig. 5. Effect of ferulic acid on activation of pRb. Arrested VSMC was pretreated without and with various concentration ferulic acid for 1 h, and stimulated with 1 μM angiotensin II for 6 h. Cell lysates were analyzed by Western blotting with antibodies as indicated.

to inhibit the activities of many cyclin/CDK complexes, so we examined the effect of ferulic acid on $p21^{waf1/cip1}$ expression. Ferulic acid (20–160 μM) significantly increased $p21^{waf1/cip1}$ expression on angiotensin II-induced VSMC in a dose-dependent manner (Fig. 4B). The data suggest that the antiproliferative effect of ferulic acid on VSMC may be involved in the expression of $p21^{waf1/cip1}$, subsequently inhibited the level of cyclin D1 protein.

3.4. Effect of ferulic acid on the pRb

The G_1-to-S phase transition is accompanied by phosphorylation of the retinoblastoma protein (pRb), resulting in transcription of early genes required for mitosis (Hiebert et al., 1992). A mobility shift of pRb is indicative of increased phosphorylation (p-pRb) on angiotensin II-induced VSMC, while the pretreatment of ferulic acid significantly inhibited its phosphorylation (Fig. 5). The degrees of phosphorylation were diminution with dose increment of ferulic acid. Although the phosphorylation of pRb was not significantly suppressed at lower dose (20 μM), its almost complete inhibition was observed at 40 μM or greater dose.

4. Discussion

Vascular smooth muscle cell proliferation and migration induced by various growth factors can develop a variety of pathological processes including atherosclerosis, hypertension and restenosis after balloon angioplasty (Ross, 1986). It is well known that angiotensin II induces VSMC proliferation (Newby and George, 1993), and accelerated VSMC proliferation is a characteristic feature in arteries of hypertensive patients and animals (Cho et al., 1997). Consequently, inhibition of VSMC proliferation represents a potentially important therapeutic strategy for the treatment of diseases such as atherosclerosis and restenosis. In this paper, our data suggest that ferulic acid significantly inhibited VSMC proliferation induced by angiotensin II. This finding is consistent with previous reports showing that ferulic acid inhibited VSMC proliferation induced by oxidized lipoprotein and endothelin-1 (Yu and Wu, 2002; Wang et al., 1999). These results indicate that ferulic acid may be a potential pharmaceutical to prevent VSMC proliferation.

Recent studies have demonstrated that the three major MAPKs (ERK1/2, JNK, p38) are activated on angiotensin II-induced VSMC proliferation via the Gq-coupled angiotensin II type 1 receptor (Eguchi et al., 2001). In this paper, angiotensin II significantly stimulated VSMC proliferation and activated the MAPKs pathway, our results also support above reports. Ferulic acid significantly inactivated the ERK1/2 and JNK at 15 min on angiotensin II-induced VSMC, but the activation of p38 was not suppressed and it was not dose- and time-dependent manner. Therefore, the antiproliferative effect of ferulic acid on VSMC was associated with the inactivation of ERK1/2 and JNK rather than p38 pathway.

On the other hand, the intracellular signaling target activation in response to extracellular stimuli is mediated through a network of interacting proteins that regulate a large number of cellular processes. Reactive oxygen species production of VSMC induced by angiotensin II activates the downstream MAPKs pathway (Dimmeler and Zeiher, 2000). Some investigations show that the antioxidants can inhibit MAPKs activation, subsequently inhibit proliferation of VSMC (Tsai et al., 1996; Kyaw et al., 2002). As a potent antioxidant, ferulic acid can scavenge free radical and inhibit lipid oxidation (Concepción et al., 1999; Zhao and Yu, 2001; Zhao et al., 2002). Taken together with these results, it needs to further investigate that whether the inactivation of MAPKs pathway is associated with the ability of ferulic acid scavenging reactive oxygen species.

The activation of MAPKs can stimulate the downstream transcription gene expression, resulting in protein synthesis and cell proliferation (Eguchi and Inagami, 2000). The activation of ERK1/2 caused cyclin D1 expression and phosphorylation of pRb on angiotensin II-induced VSMC (Kintscher et al., 2003). Our data suggest that angiotensin II markedly stimulated the cyclin D1 expresson and phosphorylation of pRb, which involved in MAPKs pathway, and it was consistent with previous findings (Watanabe et al., 1996; Lee et al., 2001; Kintscher et al., 2003). Ferulic acid significantly decreased the cyclin D1 level and inhibited phosphorylation of pRb, suggesting that proliferation of VSMC involved in the cell cycle regulatory proteins activation or expression.

The expression or activity of cyclin D1 affects the cell progression from G_1 to S phase (Sherr, 1994; Lavoie et al., 1996). Cyclin D1 binds and activates CDK4(6), and then phosphorylates its target protein, pRb (Zieske et al., 2004). Subsequently, the release of transcription factors E2F by phosphorylation of pRb promotes cell proliferation. The high level of $p21^{waf1/cip1}$ can inhibit the cyclin D1 expression, resulting in decline of pRb phosphorylation (Zieske et al., 2004). $p21^{waf1/cip1}$ has broad specificity and binds to various G_1 cyclin/CDK complexes, which can inhibit DNA replication, and the VSMC from G_1 to S phase was blocked (Waga et al., 1994; Chang et al., 1995). In atherosclerotic arteries, the expression of $p21^{waf1/cip1}$ level contributes to inhibit cell proliferation during arterial repair (Tanner et al., 1998). The results indicate that the inhibition of ferulic acid on the phosphorylation of pRb was associated with diminution of cyclin D1 by increase of $p21^{waf1/cip1}$ level on angiotensin II-induced VSMC.

In the present studies, ferulic acid significantly inhibited proliferation on VSMC induced by angiotensin II. The antiproliferative effect is involved in inhibition of ERK1/2 and JNK activation, which may result in down-regulation the phosphorylated pRb by reduction of cyclin D1 due to increase of $p21^{waf1/cip1}$ level. Our data provide a possible molecular mechanism mediating the inhibitive effect of ferulic acid on VSMC proliferation. The results also give evidences that ferulic acid may be an effective agent for cardiovascular disease.

Acknowledgements

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