

Ligustilide inhibits vascular smooth muscle cells proliferation

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Abstract

Proliferation and migration of vascular smooth muscle cells (VSMCs) are believed to develop atherosclerosis and venous bypass graft disease. Ligustilide is widely used to treat some pathological settings such as atherosclerosis and hypertension. The aim of this study was to examine the effect of ligustilide on VSMCs proliferation. The results show that ligustilide significantly inhibited VSMCs proliferation and cell cycle progression. Further analysis shows that ligustilide suppressed reactive oxygen species production and extracellular signal-related kinases (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAP kinase. Cells were treated with antioxidant, superoxide dismutase, catalase, and DPI, respectively, leading to repress ERK, JNK, and p38 activation. The inhibitors of mitogen activated protein kinase (MAPK), PD98059, SB203580, and Sp600125, inhibited cell proliferation. These findings suggest the antiproliferative effect of ligustilide was associated with the decrement of reactive oxygen species resulting in the suppression of MAPK pathway. Thus, ligustilide contribute to be the effective agent in preventing cardiovascular diseases.

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1. Introduction

Angelica sinensis (Oliv.) Diels, a kind of traditional Chinese herb, is widely distributed in southwestern China, which is often used to treat atherosclerosis and hypertension with a long history for tonic effects (Sun and Wang, 2002; Fan et al., 2002). Ligustilide is one of the main active component of *A. sinensis*, which exhibits significant effect to improve blood fluidity and exhibits strong antioxidant activity (Huang and Song, 2001). In addition, ligustilide can inhibit contractile function of vascular and uterine smooth muscle (Yan and Qiao, 2000; Hu and Ding, 2003). Previous report shows that the proliferation of vascular smooth muscle cells (VSMCs) with thickening of the intima and narrowing of the vessel lumen is a hallmark of vascular stenotic disease (Zhang and Tenne, 1999; Wang et al., 2002). Signaling pathway activation in response to external stimuli plays an important role on the proliferation of VSMCs (Sundaresan et al.,

1995; Su and Karin, 1996). Mitogen activated protein kinase (MAPK) is a family of serine/threonine kinases involved in the regulation of a wide range of cellular responses, including cell proliferation, differentiation, and survival (Su and Karin, 1996). Extracellular growth factors can activate MAPK pathway, subsequently promoting proliferation of VSMCs (Dimmeler and Zeiher, 2000). On the other hand, the decrement of reactive oxygen species contributes to inhibit proliferation of VSMCs through down-regulation of MAPK pathway (Griendling and Harrison, 1999). In normal mammalian cells, the decision to proliferate is made during the G1 phase of the cell cycle (Hunter and Pines, 1994; Peters, 1994), which is regulated by cell cycle regulatory proteins (Zieske et al., 2004). And some investigations show that the expression or activation of cell cycle proteins is mediated by MAPK signaling pathway (Lavoie et al., 1996; Lee et al., 2001).

In this paper, there is considerable interest in defining the effect of ligustilide on reactive oxygen species, MAPK and the expression of p21, cyclin D1, and pRb phosphorylation activity in cultured VSMCs. The results would contribute to the search for selective methods or drugs to treat cardiovascular diseases.

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2. Material and methods

2.1. Reagents

Fetal bovine serum (FBS) was purchased from Hyclone (Perbio Science Company). Ligustilide was purchased from the Drug Research Institute of China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Dulbecco’s Modified Eagle’s Medium (DMEM), trypsin, penicillin, streptomycin, 2’,7’-Dichlorofluorescein diacetate (DCFH-DA), DPI, superoxide dismutase (SOD), catalase (CAT), PD 98059, SB 202190, Sp600125 were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies for Tyr²⁰⁴-phosphorylated ERK1/2, Thr¹⁸⁰/Tyr¹⁸²-dually phosphorylated p38, phosphorylated JNK, ERK2, p38, JNK, pRb, p21, cyclin D1, myosin, and a-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

VSMCs were prepared by the explant method from thoracic aorta of male Wistar rats (100–150 g). 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 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. VSMCs exhibited a typical “hill and valley” growth pattern and the identity was confirmed by morphological examination and

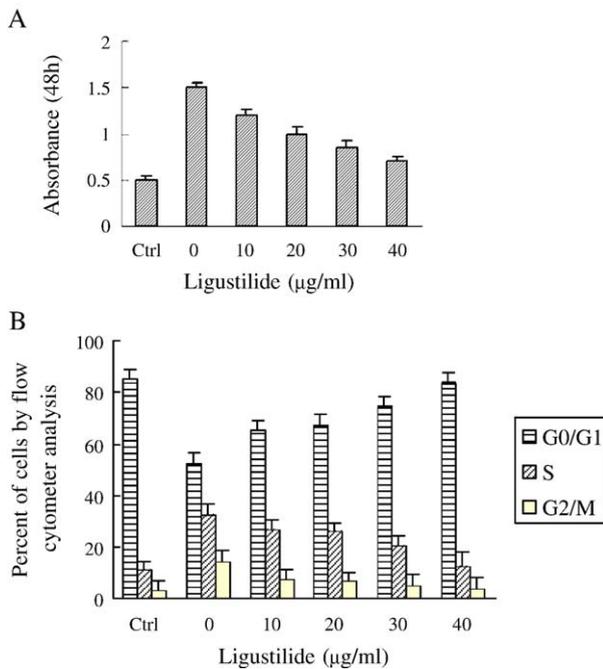


Fig. 1. Effect of ligustilide on VSMCs proliferation and cell cycle. (A) Arrested VSMCs (serum starved for 48 h) were stimulated with 10% serum in the absence or presence of ligustilide (10, 20, 30, 40 µg/ml) during 48 h. VSMCs proliferation was assayed by MTT. (B). Arrested VSMCs (serum starved for 48 h) were stimulated with 10% serum in the absence or presence of ligustilide (10, 20, 30, 40 µg/ml) during 48 h. VSMCs cell cycle was assayed by flow cytometry. The data points are presented as means±S.E.M. of four separate experiments. Ctrl means cells were treated with DMEM only.

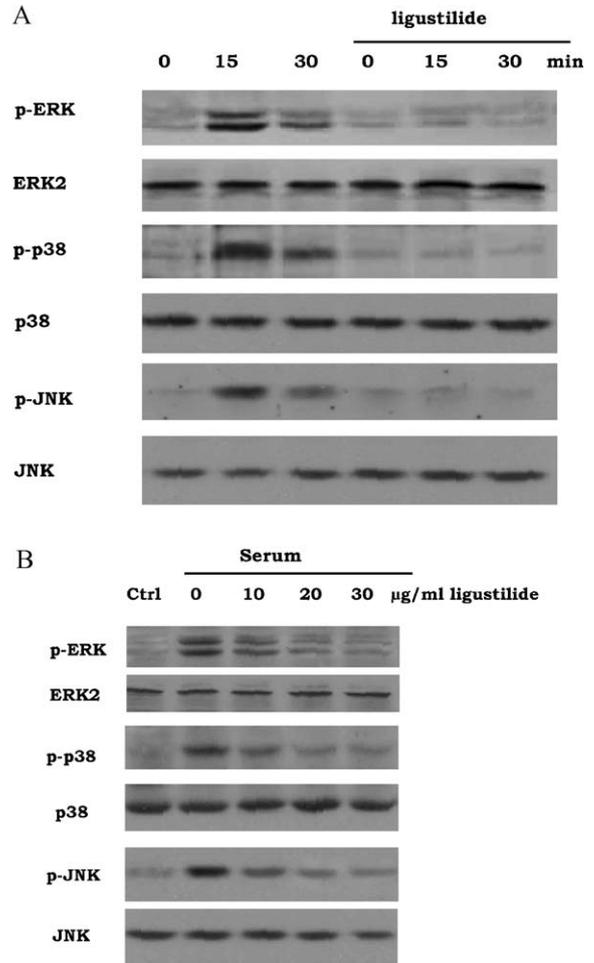


Fig. 2. Effect of ligustilide on MAPK activation. (A) Arrested VSMCs were pretreated without or with 30 µg/ml ligustilide for 1 h, and then stimulated with 10% serum for indicated times. Cell lysates were analyzed by Western blot with antibodies as indicated. (B) Arrested VSMCs were pretreated without or with various concentrations of ligustilide for 1 h, and stimulated with 10% serum for 15 min. Cell lysates were analyzed by Western blot with antibodies as indicated. Ctrl means cells were treated with DMEM only. Results are repeated three times.

staining for anti- α -smooth muscle actin and myosin antibody. Medium was replaced twice a week. The cells became confluent, followed by subculture using trypsinization. Confluent cells at passage numbers 3–6 were used for the experiments.

2.3. Cell proliferation and cell cycle assay

VSMCs was counted and seeded into 96-well culture plates at a density of 2×10^4 cells/well. After incubation with drugs for a certain period of time, each well was washed twice with phosphate-buffered saline (PBS) to remove the unabsorbed drugs. Cell proliferation was assayed by MTT method. 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 and the absorbance was measured at a wavelength of 570 nm with an enzyme linked immunosorbent assay (ELISA) reader (BioRad 3550, Bio-Rad Laboratories). Cell cycle was assessed by flow cytometer. Cells were harvested and adjusted to a concentration of 10^6 cells/ml, and then were fixed with ice-cold 70% ethanol. Cells were

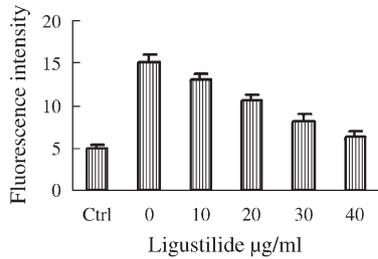


Fig. 3. Effect of ligustilide on serum induced VSMCs reactive oxygen species production. VSMCs were stimulated with 10% serum in the absence or presence of ligustilide (10, 20, 30, 40 $\mu\text{g/ml}$) during 2 h. Results are expressed as mean \pm S.D. ($n=4$). Ctrl means cells were treated with DMEM only.

washed with phosphate-buffered saline and incubated with 0.1 mg/ml RNase at 37 $^{\circ}\text{C}$ for 10 min, stained with 50 $\mu\text{g/ml}$ of propidium iodide (PI). Samples were analyzed by flow cytometry (FACSCalibur cytometer BD Biosciences).

2.4. Reactive oxygen species assay

Reactive oxygen species were determined by 2',7'-Dichlorofluorescein diacetate (DCFH-DA) fluorescence assay. VSMCs were treated with various conditions, the cells were washed twice with PBS and incubated in Krebs Ringer HEPES solution containing 10 μM DCFH-DA at 37 $^{\circ}\text{C}$ for 30 min. The fluorescence intensity was determined with excitation wavelength of 485 nm and emission wavelength of 524 nm (Hatachi, Model F-2000).

2.5. Western blot analysis

VSMCs were harvested and lysed for 20 min in 200 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.1% NP-40, 0.5 mM Phenylmethylsulfonyl fluoride (PMSF), 10 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin, 1 mM NaF, 0.1 mM Na_3VO_4 , and 1 mM dichlorodiphenyltrichloroethane (DTT)). The supernatants were obtained by centrifugation. Protein content was assayed by Bradford method. 30 μg of protein was loaded in each lane and resolved by SDS-PAGE gel electrophoresis, blotted on nitrocellulose membrane. Blots were probed with specific antibodies, and then membranes were incubated with peroxidase-conjugated second antibody. Blots were treated with enhanced chemiluminescence and exposed to CL-X films (Kodak).

2.6. 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 \pm S.D. and $P < 0.05$ was statistically significant.

3. Results

3.1. Effect of ligustilide on VSMCs proliferation

The VSMCs were treated with various concentration of ligustilide (0, 10, 20, 30 and 40 $\mu\text{g/ml}$) for 48 h, and the cell

proliferation was analyzed by MMT method. Our results show that ligustilide significantly inhibited the proliferation of VSMCs induced by 10% serum, in a dose dependent manner (Fig. 1A). The inhibitive rate was about 50% when VSMCs were treated with 40 $\mu\text{g/ml}$ ligustilide. To better understand that ligustilide suppresses cell proliferation, the cell cycle was assayed by flow cytometry on VSMCs treated with various concentrations of ligustilide. The results show that ligustilide significantly suppressed cells from G1 to S phase progress in a dose dependent manner (Fig. 1B). These findings suggest that ligustilide not only inhibited cell proliferation but also cell cycle progression.

3.2. Effect of ligustilide on MAPK activation

To better understand the effect of ligustilide on MAPK pathway, we measured the activation of ERK, JNK and p38 by Western blot with the phospho-specific antibodies. Arrested VSMCs were pretreated with ligustilide for 1 h, and were stimulated with 10% serum for 0, 15, 30 min. The data suggest that ligustilide markedly inhibited ERK, JNK and p38 activation, and the inhibitive effect was up to maximal within 15 min (Fig. 2A). To further investigate the dose dependent effect of ligustilide on ERK, JNK and p38, they were stimulated by 10% serum within 15 min. The results suggest that the activation of ERK, JNK and p38 were significantly inhibited

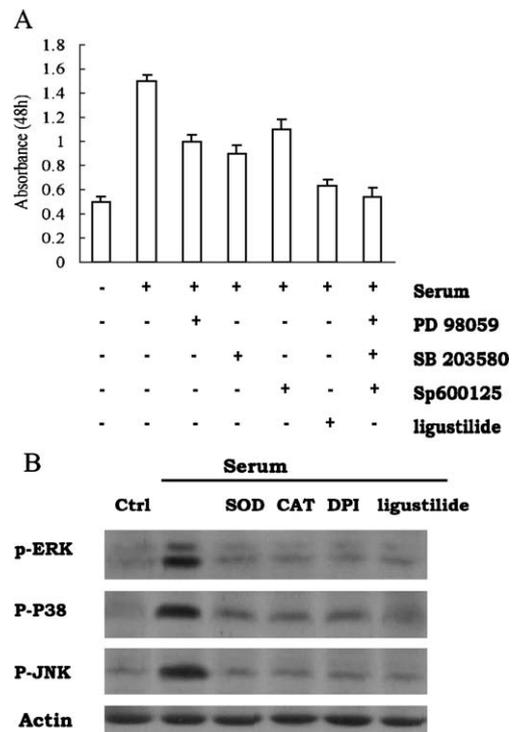


Fig. 4. Ligustilide suppresses MAPK associated with the decreased reactive oxygen species production. (A) Arrested VSMCs were stimulated with 10% serum in the absence or presence of 20 μM PD98059, 10 μM SB203580, 20 μM Sp600125, 30 $\mu\text{g/ml}$ ligustilide, respectively, for 48 h. VSMCs proliferation was assayed by MTT. (B) Arrested VSMCs were pretreated without or with the DPI (10 μM), SOD (500 units/ml), CAT (500 U/ml), 30 $\mu\text{g/ml}$ ligustilide for 2 h and stimulated with 10% serum for 20 min. Cell lysates were analyzed by Western blot with antibodies as indicated. Ctrl means cells were treated with DMEM only. Results are repeated three times.

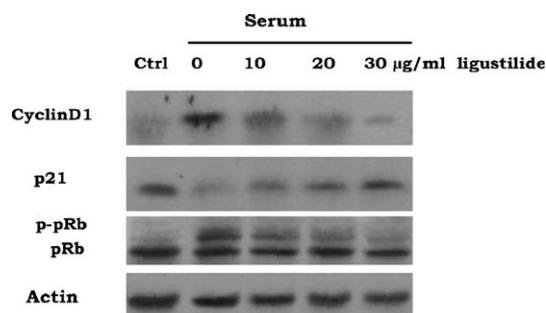


Fig. 5. Effect of ligustilide on the expressions of cyclin D1, p21, and pRb. Arrested VSMCs were pretreated without or with various concentration of ligustilide for 1 h, and stimulated with 10% serum for 6 h. Cell lysates were analyzed by Western blot with antibodies as indicated. Ctrl means cells were treated with DMEM only. Results are repeated three times.

with increasing ligustilide concentrations from 10 to 30 $\mu\text{g/ml}$ after VSMCs were pretreated with drug for 1 h (Fig. 2B). Therefore, ligustilide inhibited the activation of MAPK pathway which may result in the decreased cell proliferation.

3.3. Effect of ligustilide on the reactive oxygen species

The production of reactive oxygen species was examined on VSMCs induced by 10% serum for 2 h. The results show that stimulation of the VSMCs with serum treatment resulted in an intense DCF fluorescent signal. The increment of DCF fluorescence was prevented by treatment with various concentrations of ligustilide (10, 20, 30, 40 $\mu\text{g/ml}$) (Fig. 3). The data suggest that ligustilide significantly inhibited serum induced intracellular reactive oxygen species production.

3.4. Ligustilide suppresses MAPK in associated with decreased reactive oxygen species

We have demonstrated that ligustilide reduced reactive oxygen species production and suppressed MAPK, here we further found that cells were treated with the inhibitors of MAPK (PD98059 for ERK, SB203580 for p38, and Sp600125 for JNK, respectively) that led to the inhibition of cell proliferation. In addition, cells were treated with all the three kinds of inhibitors which significantly suppressed cell proliferation (Fig. 4A). To better understand if the antioxidant is involved in the suppression of the MAPK pathway, the cells were treated with the antioxidant, SOD, CAT, DPI, respectively, leading to suppressed ERK1/2, p38, and JNK (Fig. 4B). These findings suggest that ligustilide suppressed cell proliferation was associated with decreased reactive oxygen species production resulting in the inactivation of MAPK pathway.

3.5. Effects of ligustilide on cyclin D1, p21 and pRb

The data suggest that ligustilide markedly inhibited cyclin D1 protein expression on serum induced VSMCs (Fig. 5). In addition, p21 is known to inhibit the activities of many cyclin/CDK complexes, so we examined the effect of ligustilide on p21 expression. Ligustilide (10 to 30 $\mu\text{g/ml}$) significantly increased

p21 expression on serum induced VSMCs (Fig. 5). The data indicate that the antiproliferative effect of ligustilide on VSMCs may be involved in the over-expression of p21, which subsequently inhibited the level of cyclin D1 protein. The G1-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 VSMCs induced by serum, while the pretreatment of ligustilide significantly inhibited its phosphorylation (Fig. 5).

4. Discussion

Vascular smooth muscle cells 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 the growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and angiotensin II can induce VSMC proliferation (Newby and George, 1993). Consequently, inhibition of VSMCs proliferation represents a potentially important therapeutic strategy for the treatment of diseases such as atherosclerosis and restenosis. Our results show that ligustilide significantly inhibited the serum induced VSMCs proliferation and cell cycle progress.

MAPK pathway has been described in mammalian cells, including p42/p44 extracellular signal-related kinases (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAP kinase (Boulton et al., 1991; Kyriakis and Woodgett, 1994; Han et al., 1994). The activation of MAPK is associated with VSMCs proliferation due to stimulation of the downstream earliest transcriptional event genes c-Jun, c-Fos and Elk-1 mRNA expression, subsequently increasing DNA synthesis of VSMCs (Jin et al., 2000; Eguchi and Inagami, 2000). In this paper, the serum significantly activated MAPK pathway (Fig. 2A and B), which was abolished by the ligustilide. These results indicate that the antiproliferative effect of ligustilide was involved in the inactivation of MAPK pathway.

In addition, 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. In VSMCs, the reactive oxygen species production involve not only cell proliferation but also activation of MAPK pathway (Dimmeler and Zeiher, 2000), but the activation of MAPK comes from a variety of upstream signaling molecular regulation (Eguchi and Inagami, 2000; Bogoyevitch, 2000). As a potent antioxidant, ligustilide significantly decreased intracellular reactive oxygen species production. This finding was also in line with previous report that antioxidant inhibit MAPK pathway, resulting in inhibition of cell proliferation (Tsai et al., 1996; Kyaw et al., 2002). Here we also demonstrated that VSMCs were treated with the PD98059, SB203580, and Sp600125, respectively, led to inhibition of cell proliferation. On the other hand, SOD, CAT, or DPI suppressed the ERK, JNK, and p38 activation, suggesting that the antiproliferative effect of ligustilide was associated with the decrement of reactive oxygen

species resulting in the suppression of mitogen activated protein kinase pathway.

The expression or activity of cyclin D1 affects the cells progression from G1 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 can inhibit the cyclin D1 expression, resulting in decline of pRb phosphorylation (Chang et al., 1995). p21 has broad specificity and binds to various G1 cyclin/CDK complexes, which can inhibit DNA replication (Chang et al., 1995; Waga et al., 1994). In atherosclerotic arteries, the expression of p21 levels contributes to inhibit cell proliferation during arterial repair (Tanner et al., 1998). Our results indicate that the suppression of phosphorylation of pRb was associated with decrement of cyclin D1 and increment of p21 level on serum induced VSMCs proliferation.

In conclusion, ligustilide significantly inhibited VSMCs proliferation and cell cycle progression, which was associated with the inhibition of MAPK pathway by decrement of reactive oxygen species production. Our data provide a possible molecular mechanism mediating the inhibitive effect of ligustilide on VSMCs proliferation. The results also give new evidences that ligustilide may be an effective agent for cardiovascular diseases.

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