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Ferulic Acid Inhibits Endothelial Cell Proliferation Through NO Down-Regulating ERK1/2 Pathway

YongZhong Hou, Jie Yang, GuangRong Zhao, and YingJin Yuan*

Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, Peoples Republic of China

Abstract The aim of this study was to determine the antiproliferative mechanism of ferulic acid (FA) on serum induced ECV304 cell, a human umbilical vein endothelial line. The results suggest that FA significantly suppressed ECV304 cells proliferation and blocked the cell cycle in G_0/G_1 phase. Treatment of the cells with FA increased nitric oxide (NO) production and inactivated the extracellular signal-regulated kinase (EERK1/2), and the NO donor, sodium nitroprusside, inhibited both ECV304 cells proliferation and phosphorylation of ERK1/2. However, the NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester, caused ECV304 cells proliferation. PD 98059, the inhibitor of ERK1/2, had no effect on the NO production. These results indicate that NO suppressed ECV304 cells proliferation through down-regulating ERK1/2 pathway. Moreover, the inhibition of cell cycle progression was associated with the decrement of cyclin D1 expression and phosphorylation of retinoblastoma protein (pRb) by increment of p21 level. The findings not only present the first evidence that FA is a potent inhibitor on ECV304 cells proliferation, but also reveal the potential signaling molecules involved in its action. J. Cell. Biochem. 93: 1203–1209, 2004.

Key words: human umbilical vein endothelial cells; nitric oxide; extracellular signal-regulated kinase; cell cycle regulatory proteins

The excess proliferation of vascular endothelial cell may be development of atherosclerosis, hemangioma, and diabetes [Folkman, 1995; Pepper, 1997]. Endothelial cell proliferation and neovascularization may not only promote atherogenesis, but also accelerate plaque progression [McCarthy et al., 1999]. Consequently, treatment with inhibitors of angiogenin seems to be a powerful strategy to prevent the development of these pathologies. Mitogen-activated protein kinase (MAPK), including extracellular signal-related kinases (ERK1/2), c-Jun N-terminal protein kinase (JNK), and p38 MAP kinase [Boulton et al., 1991; Han et al., 1994; Kyriakis and Woodgett, 1994], are involved in the regulation of a wide range of cell proliferation, differentiation, and survival [Su and Karin, 1996]. ERK1/2 mediates the nitric oxide (NO) production, subsequently NO down-regulates the vascular endothelial growth factor (VEGF) stimulated endothelial cell proliferation [Cha et al., 2001], and in contrast, NO down-regulates the activation of ERK1/2 [Ingram et al., 2000]. The present investigations show that the interaction of ERK1/2 and NO remains unclear.

The induction of the D-type cyclins constitutes an essential step in the coupling of mitogenic signal [Prietzsch et al., 2002], and accompanied by phosphorylation of the retinoblastoma protein (pRb), resulting in cell proliferation [Hiebert et al., 1992]. In addition, p21, the cyclin dependent kinase inhibitors, may cause cell cycle arrest [Gartel and Tyner, 2002].

Ferulic acid (FA), as phenolic compound, is a good antioxidant against lipid peroxide damage [Bourne and Rice-Evans, 1997]. Clinically, it is widely used in the prevention of cardiovascular diseases due to water solubility of sodium ferulate [Wei, 2002]. FA significantly improves

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*Correspondence to: YingJin Yuan, Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, P.O. Box 6888, Tianjin 300072, Peoples Republic of China.
E-mail: yjyuan@public.tpt.tj.cn

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blood fluidity and inhibits platelet aggregation [Wang et al., 1992; Zhang et al., 2001]. In addition to inhibiting vascular smooth muscle cells proliferation [Yu and Wu, 2002], FA increases the level of NO production and eNOS expression on TNFα induced endothelial cell damage [Wang and Ou-Yang, 2003].

To better understand the antiproliferative mechanism of FA on serum induced ECV304 cells, we examined the effect of FA on NO production and ERK1/2 pathway, and further investigated the interaction of NO and ERK1/2.

MATERIALS AND METHODS

Materials

FA was purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Sodium nitroprusside (SNP), trypsin, PD 98059, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-dephenyl tetrazolium bromide (MTT), N^{ω}-nitro-L-arginine methyl ester (L-NAME), were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies for phosphorylated ERK1/2, phosphorylated p38, phosphorylated JNK, ERK2, pRb, p21, cyclin D1, α-actin, and enhanced chemiluminescence (ECL) reagent kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Human umbilical vein endothelial cell line (ECV304) was purchased from China Center for Type Culture Collection. ECV304 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cell Proliferation Assay

ECV304 cells were counted and seeded into 96-well culture plates at a density of 2×10^4 cells/well. After incubation with FA for a certain period of time, each well was washed twice with phosphate-buffered saline (PBS) to remove the unabsorbed FA. Cell proliferation was assayed by MTT method. A tetrazolium salt, MTT reduction has been widely used for the quantitative assessment of cellular viability and proliferation [Mosmann, 1983]. 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 ELISA reader (BioRad 3550, Bio-Rad Laboratories, Hercules, CA).

Cell Cycle Analysis

Cell cycle was assessed by flow cytometry. Cells were harvested and adjusted to a concentration of 10^6 cells/ml, and then were fixed with ice-cold 70% ethanol. Cells were washed with PBS and incubated with 0.1 mg/ml RNase at 37°C for 10 min, stained with 50 μg/ml of propidium iodide (PI). Samples were analyzed with a flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Assay of Nitrite Production

Nitrite was measured in the culture supernatants. After indicated time periods of incubation, cell-free supernatant of 100 μl was incubated with 100 μl Griess reagent. Samples were incubated at 25°C for 10 min and absorbance was measured at 540 nm. Concentrations were determined versus a sodium nitrite standard.

Western Blot Analysis

ECV304 cells 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 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 DTT). The supernatants were obtained by centrifugation. Protein content was assayed by Bradford method. Thirty micrograms of protein was loaded 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 ECL and exposed to CL-X films (Kodak, Tokyo, Japan). The band intensities were quantified using scanning densitometry.

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 mean ± SEM. The values of $P < 0.05$ were statistically significant.

RESULTS

Effect of FA on ECV304 Cells Proliferation and Cell Cycle Progression

ECV304 cells were stimulated with 5% serum in the absence or presence FA (10, 20, 40, 80 μM)

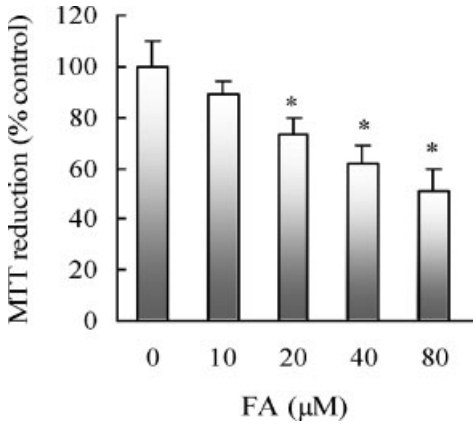

Fig. 1. Dose effect of ferulic acid (FA) on ECV304 cells proliferation. Arrested ECV304 cells (serum starved for 24 h) were stimulated with 5% serum in the absence or presence of FA (10, 20, 40, 80 μM) during 24 h. ECV304 cells proliferation was assayed by MTT method. The data points are presented as mean ± SEM of ten separate experiments. $*P < 0.05$, significant difference from control (the cells without FA).

for 24 h. The data show that FA dose dependently inhibited the serum induced ECV304 cells proliferation (Fig. 1), and the inhibitive rate was about 50% when cells were treated with 80 μM FA.

ECV304 cells were induced to enter S phase by stimulation for 24 h with 5% serum. The population of G_0/G_1 cells decreased (39.3 ± 7%), with a concomitant rise significantly in S phase cells (42.7 ± 8.6%) (Fig. 2A). Treatment with 80 μM FA inhibited serum induced G_1 to S progression, as shown by the increase in G_0/G_1 cells (63 ± 9.2%), accompanied by a concurrent decrease in S phase cells (26.2 ± 6.2%) (Fig. 2B). Therefore, FA not only inhibited ECV304 cells proliferation but also significantly arrested in G_0/G_1 phase.

Effect of FA on ECV304 Cells Nitrite Production

Nitrite, the stable end product of NO, was measured in the culture supernatants, and adjusted to the total protein content of the cell in a well. The results show that nitrate production in supernatants of ECV304 cells treated with 80 μM FA was significantly increased and peaked at 4 h (Fig. 3), suggesting that FA enhanced intracellular NO production.

Effect of FA on MAPK Pathway

Although serum activated ERK1/2 and peaked at 10 min, did not activate p38 and JNK (Fig. 4), suggesting that ERK1/2 pathway may regulate ECV304 cells proliferation. On the other hand, 80 μM FA inhibited the activation of ERK1/2 and peaked at 10 min (Fig. 5). The results indicate that the antiproliferative effect of FA involves the inhibition of the ERK1/2 pathway.

Effect of FA on the NO and ERK1/2

NO and MAPK pathway have shown to play a role in the regulation of endothelial cell proliferation [RayChaudhury et al., 1996; Sylvie et al., 2000]. In the presence of 5% serum, 3 μM PD 98059 (ERK1/2 inhibitor) inhibited ECV304 cells proliferation (Fig. 6A) rather than affecting the NO production (Fig. 6B), suggesting that ERK1/2 may not regulate NO production. On the other hand, 10 μM SNP (NO donor) not only inactivated ERK1/2 (Fig. 7) but also inhibited the serum induced cells proliferation (Fig. 6A), indicating that NO may down-regulate the activation of ERK1/2. Moreover, 150 μM L-NAME, the nonselective NO synthase inhibitor, led to ECV304 cells proliferation (Fig. 6A) and inhibited the NO production (Fig. 6B). This findings suggest that NO

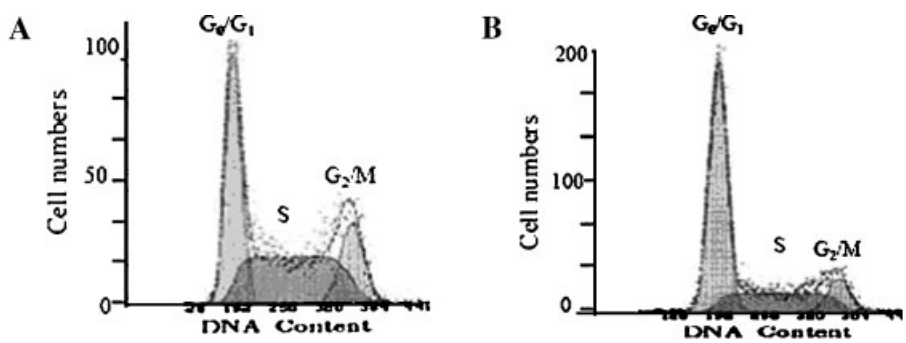

Fig. 2. Effect of FA on the cell cycle. Arrested ECV304 cells (serum starved for 24 h) were stimulated with 5% serum in the absence or presence of 80 μM FA during 24 h. Cellular DNA content was determined by flow cytometry. **A**: Serum; (**B**) serum + 80 μM FA.

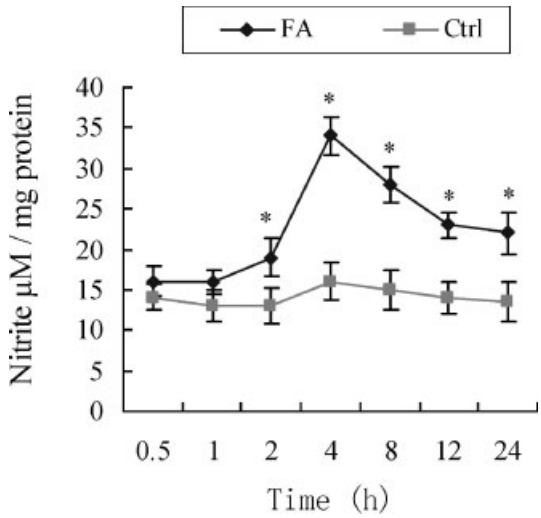

Fig. 3. Time effect of FA on nitrite production. Arrested ECV304 cells (serum starved for 24 h) were stimulated with 5% serum in the absence or presence of 80 μM FA for indicated time periods. Nitric oxide (NO) production was measured by the Greiss reagent after treatment with FA and the results were normalized to protein content of the cultures. The data points are presented as mean ± SEM of four separate experiments. $*P < 0.05$, significant difference from control (the cells without FA).

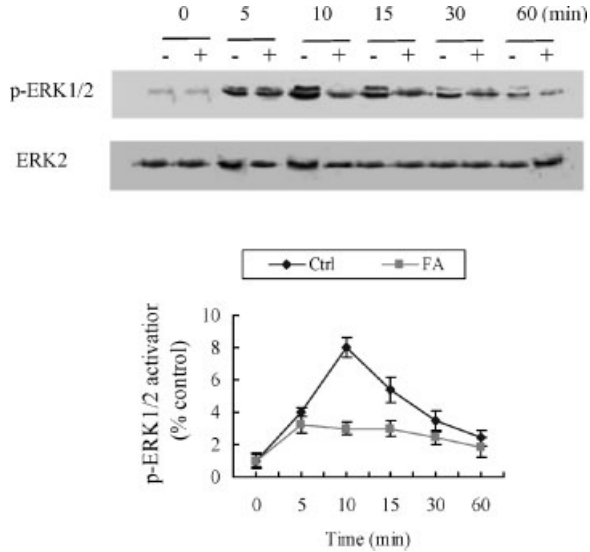

Fig. 5. Time effect of FA on ERK1/2 activation. Arrested ECV304 cells were stimulated with 5% serum in the absence or presence of 80 μM FA for indicated time periods. Cell lysates were analyzed by Western blot with antibodies as indicated. Relative activities were quantified by scanning densitometry and the figure showed the level of each activity as relative value of the maximal level. The results were repeated at least three times.

appeared the upstream molecule of ERK1/2, and down-regulated the activation of it.

Effect of FA on the Expression of Cell Cycle Regulatory Proteins

FA inhibited the cyclin D1 protein expression in ECV304 cells induced by serum (Fig. 8B), and in contrast, FA enhanced p21 protein level (Fig. 8A). The G_1-to-S phase transition is accompanied by phosphorylation of the pRb, resulting in transcription of early genes required for proliferation [Hiebert et al., 1992]. A mobility shift of pRb is indicative of increased phosphorylation (p-pRb). Our data suggest that FA markedly inhibited pRb phosphorylation on ECV304 cells induced by serum (Fig. 8C).

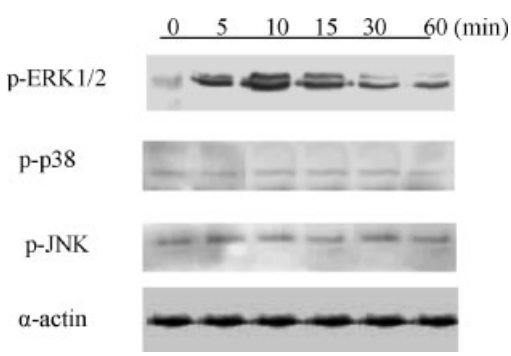

Fig. 4. Time effect of serum on ERK1/2, p38, JNK activation. Arrested ECV304 cells were stimulated with 5% serum for indicated time periods. Cell lysates were analyzed by Western blot with antibodies as indicated. The results were repeated at least three times.

DISCUSSION

In the present study, FA not only inhibited serum induced ECV304 cells proliferation, but also prevented cells from G_1 to S progression. Further investigations show that ECV304 cells treated with NO donor (SNP) resulted in inhibition of cell proliferation, and in contrast, L-NAME (NO synthase inhibitor) led to cell proliferation. The results indicate that NO may limit the endothelial cell proliferation, which was also consonant with previous investigation [Ahmed et al., 1997]. NO has been suggested to contribute to the pathophysiological mechanism of a variety of cardiovascular diseases, and one mechanism of cardiovascular protection for NO is through the induction of a G_0/G_1 cell cycle arrest, preventing cell proliferation [Sarkar et al., 1997; Ma et al., 2003]. ECV304 cells treated with FA increased NO production, suggesting that the NO production was connected to the inhibition of ECV304 cells proliferation. However, these results are in contrast to other reports that NO leads to VEGF induced

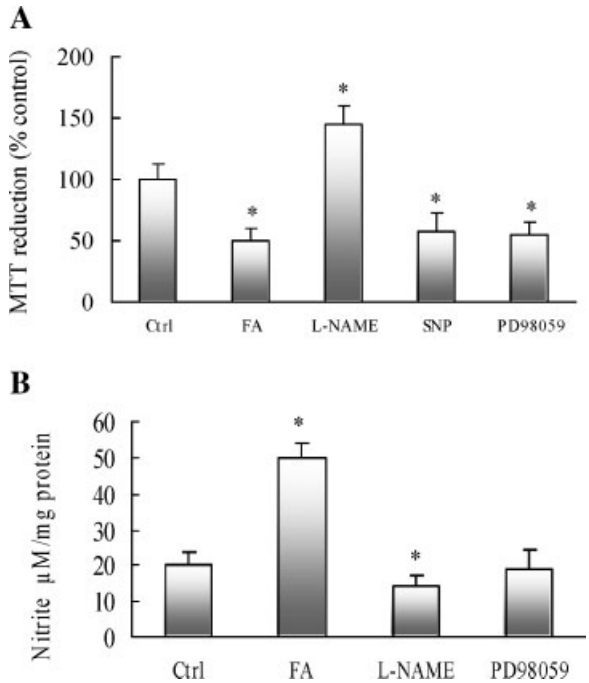

Fig. 6. Effect of L-NAME, SNP, and PD 98059 on serum induced ECV304 cells proliferation and NO production. **A**: Cells were stimulated with 5% serum in the absence or presence of 80 μM FA, 150 μM L-NAME, 3 μM PD 98059, 10 μM SNP for 24 h. Cells proliferation was assayed by MTT method. **B**: Cells were stimulated with 5% serum in the absence or presence of 80 μM FA, 150 μM L-NAME, 3 μM PD 98059, for 4 h. NO production was examined in cultured ECV304 cells. The data points are presented as mean ± SEM of five separate experiments. $^*P < 0.05$, significant difference from control (the cells without drugs).

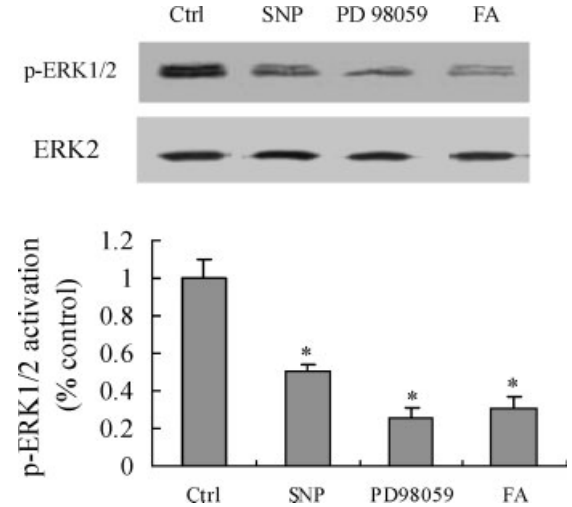

Fig. 7. Effect of FA, PD 98059, and SNP on ERK1/2 activation. Arrested ECV304 cells were stimulated with 5% serum in the absence or presence of 80 μM FA, 3 μM PD 98059, 10 mM SNP for 10 min. Cell lysates were analyzed by Western blot with antibodies as indicated. Relative activities were quantified by scanning densitometry and the figure showed the level of each activity as relative value of the maximal level. The results were repeated at least three times.

endothelial cell proliferation [Morbidelli et al., 1996; Parenti et al., 1998]. The reasons for this discrepancy may be related to the cell types.

A number of extracellular stimuli, including serum, have been reported to induce endothelial cell proliferation by activating ERK1/2 pathway [Shin et al., 1999; Cha et al., 2001; Liu et al., 2001]. We observed that serum activated ERK1/2 pathway rather than JNK and p38, and the inhibition of ERK1/2 with PD 98059 abolished the ECV304 cells proliferation, suggesting that serum induced ECV304 cells proliferation was involved in the activation of ERK1/2 pathway. FA inhibited the activation of ERK1/2, indicating that the antiproliferative effect was associated with the inhibition of ERK1/2. This study is also in line with the finding that imidazolium *trans*-imidazoledimethyl sulfoxide tetrachlororuthenate inhibits serum induced ECV304 cells proliferation by suppressing the ERK1/2 pathway [Pintus et al., 2002]. However, angiostatin and endostatin inhibit VEGF induced endothelial cell proliferation without affecting ERK1/2 pathway known to regulate endothelial cell migration and proliferation [Eriksson et al., 2003]. Moreover, ERK1/2 mediates the NO production rather than being involved in the beWo cells proliferation [Cha et al., 2001]. The results suggest that it may be different pathway for regulation of endothelial cell proliferation.

The goal of the present study was to determine whether NO inhibited ECV304 cells proliferation by regulation of ERK1/2 pathway. In this article, it was of interest to find that FA markedly increased NO production and inhibited phosphorylated ERK1/2 on serum induced ECV304 cells, which suggest that NO production and inhibition of ERK1/2 were important for regulation of ECV304 cells proliferation. On the other hand, the addition of SNP alleviated the phosphorylated ERK1/2 and the inhibition of ERK1/2 with PD 98059 had no effect on NO production, suggesting that NO may be the up-stream of ERK1/2 pathway and down-regulated the activation of ERK1/2 pathway. Thus, NO may affect the proliferation of ECV304 cells by regulating ERK1/2 pathway. The result also supports previous finding that NO attenuates ERK1/2 activity via generation

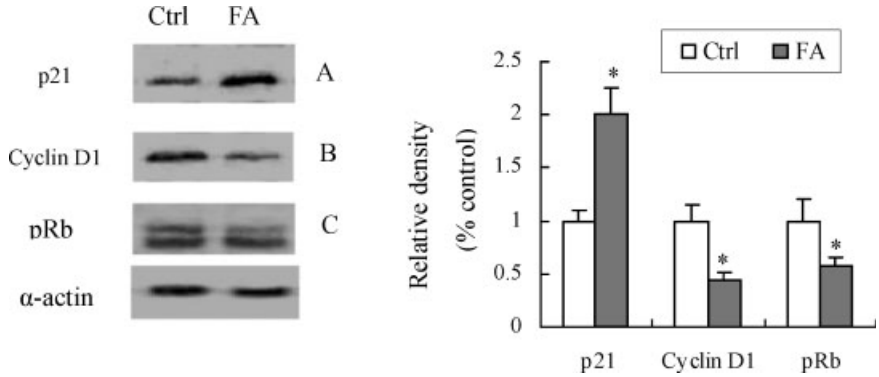

Fig. 8. Dose effect of FA on p21, cyclin D1, and pRb. Arrested ECV304 cells were stimulated with 5% serum in the absence or presence of 80 μM FA for 6 h. Cell lysates were analyzed by Western blot with antibodies as indicated. Relative activities were quantified by scanning densitometry and the figure showed the level of each activity as relative value of the maximal level. The results were repeated at least three times.

of cyclic guanosine monophosphate (cGMP) [Ingram et al., 2000], and NO can directly inhibit the activity of ERK1/2 [Raines et al., 2004]. However, other reports show that ERK1/2 mediates the NO production and PD 98059 abolishes the NO production [Zheng et al., 1999; Cha et al., 2001], and the findings are in contrast to other result that the activation of ERK1/2 had no effect on the NO production [Lorenz et al., 2004]. On the other hand, the activation of the ERK1/2 cascade by NO lead to VEGF induced endothelial cell proliferation [Parenti et al., 1998]. In addition, the pharmacological effects of various NO donors are different [Kimura et al., 2002]. Thus, it does highlight the need to examine multiple cell types or NO donors in order to understand the discrepancy. Taken together with our results, NO down-regulated the activation of ERK1/2 pathway, subsequently, inhibited the proliferation of ECV304 cells. However, whether NO may be a dependent pathway to inhibit the endothelial cell proliferation, needs further investigation.

ERK1/2 has been shown to regulate cyclin D1 expression [Lavoie et al., 1996]. The expression of cyclin D1 affects the cell progression from G_1 to S phase [Sherr, 1994; Lavoie et al., 1996]. Cyclin D1 can increase the phosphorylation of pRb [Zieske et al., 2004], resulting in cell proliferation [Weinberg, 1995]. The high level of p21 can inhibit the cyclin D1 expression, resulting in decline of pRb phosphorylation [Zieske et al., 2004]. p21 has broad specificity and binds to various G_1 cyclin/CDK complexes, which can inhibit DNA replication [Waga et al., 1994]. In atherosclerotic arteries, the expression of p21 level contributes to inhibit cell proliferation during arterial repair [Tanner et al., 1998]. Our data show that FA suppressed cyclin D1 expression and activity of pRb by increment of p21 level, resulting in inhibition of cell cycle progression.

In conclusion, FA significantly inhibited ECV304 cells proliferation, to our knowledge, this is very interesting to demonstrate that NO inhibited the activation of ERK1/2. FA mediated NO production, subsequently down-regulated the ERK1/2 pathway affecting ECV304 cells proliferation. Thus, NO inhibits ERK1/2 pathway may be important in preventing the cardiovascular diseases.

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