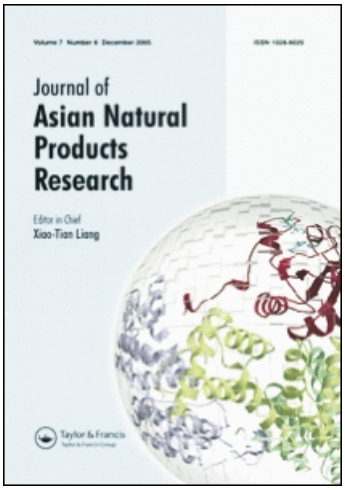

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EFFECTS OF ALPINETIN ON RAT VASCULAR SMOOTH MUSCLE CELLS

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The object of this work was to study the effects of alpinetin on cultured rat aortic smooth muscle cells. It was observed that H_2O_2 ($100\,\mu mol\,L^{-1}$) induced increase of LDH (lactate dehydrogenase) leakage in the medium of VSMC by 7.4% ($p < 0.01$) and $10^{-7}\,mol\,L^{-1}$ alpinetin significantly decreased LDH leakage induced by H_2O_2 ($p < 0.01$). Alpinetin had the effects of inhibiting VSMC proliferation in a dose-dependent manner under the condition of serum stimulation for 24 and 48 h, but with serum stimulation for 72 h adverse effects on VSMC proliferation appeared. 10^{-7} and $10^{-8}\,mol\,L^{-1}$ alpinetin had significantly inhibitory effects on VSMC migration by 67.9% ($p < 0.001$) and 34.1% ($p < 0.01$) respectively. It was also found that alpinetin (10^{-7}–$10^{-9}\,mol\,L^{-1}$) could significantly inhibit the production of NO in cultured VSMC induced by TNFα ($200\,U\,ml^{-1}$). At 10^{-7}, 10^{-8} and $10^{-9}\,mol\,L^{-1}$ the modulation of NO was by 22.6% ($p < 0.001$), 20.6% ($p < 0.01$) and 13.9% ($p < 0.05$), respectively. In summary, the data show that alpinetin has, to some extent, protective effects on VSMC.

Keywords: Alpinetin; Vascular smooth muscle cells (VSMC); Vascular injury

INTRODUCTION

The proliferation and migration of vascular smooth muscle cells (VSMC) are early pathological events leading to neointima formation following acute or chronic arterial injury [1]. Nowadays, more abundant vascular damages are evidenced by the oxygenation of endothelial cells and smooth muscle cells, and oxygen free-radical attack seems to greatly contribute dominant pathogenesis of vascular disease. In addition, oxidative agents modulate the function of vascular cells, especially VSMC, which migrate expansively into intima and proliferate noticeably, resulting in the pathology of occluding arterial lesions during atherogenesis [2]. In the neointima, the intimal SMC has a high capacity to produce NO on stimulation such as *via* tumor necrosis factor (TNF), interleukin-1(IL-1) and lipopolysaccharide (LPS), whether iNOS-derived NO actually protects or damages the neointima may depend on the amount of NO produced [3].

Alpinetin (**1**), a kind of flavonoid, is widely distributed in many medicinal plants and has been isolated from *Alpinia katsumadai*, *Amomum subulatum*, *Scutellaria rivularis* and *Scutellaria indica* [4]. It has been substantiated that **1** has endothelium-dependent and endothelium-independent vascular relaxation effects [5], but the effects on VSMC have not

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been reported. In experiments reported here, the protective effects of 1 on VSMC were investigated.

RESULTS AND DISCUSSION

Effect of 1 on VSMC Proliferation and Migration Induced by Serum Stimulation

The cultured VSMC proliferation was significantly increased after the serum simulation ($p < 0.001$); the cells were then cultured in serum-free medium for 12 h (serum deprivation) and then in normal medium containing 10% serum again. After serum deprivation, VSMC was incubated with **1** for 12 h and stimulated with serum for another 24 or 48 h; serum stimulation could significantly inhibit the proliferation in a dose-dependent manner. But when stimulated with serum for 72 h, **1** appeared to have a promotive effect (Fig. 1).

We also observed that serum stimulation led to VSMC migration by 75.8% ($p < 0.001$). Concentrations of 10^{-7}, 10^{-8} mol L^{-1} of **1** decreased the migration distance by 67.9% ($p < 0.001$) and 34.1% ($p < 0.01$), respectively, when VSMC was treated during serum deprivation for 12 h and serum stimulation for 12 h (Fig. 2).

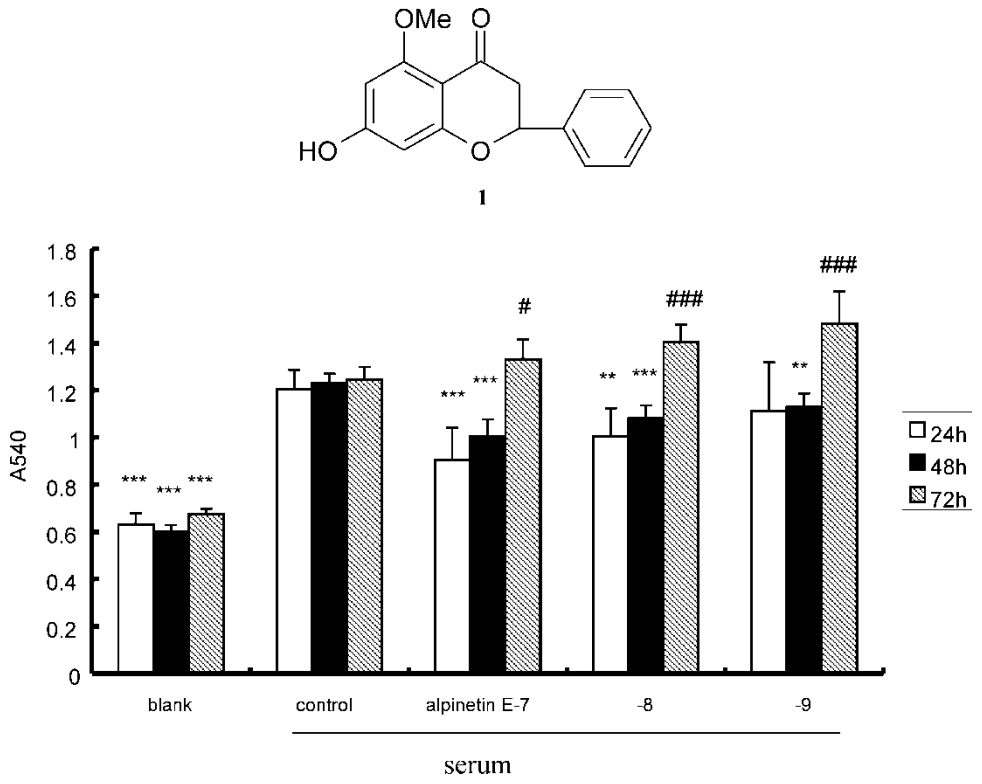

FIGURE 1 Effects of **1** on the proliferation of VSMC induced by serum stimulation. The confluenced VSMC was cultured with **1** for 12 h in serum-free medium and then stimulated with serum (10%) for another 24 h, 48 h and 72 h. The subsequent proliferation of VSMC was assessed by MTT assay. Data are expressed as mean ± SD, $n = 6$ wells, the inhibitory effects presented as $^{**}p < 0.01$, $^{***}p < 0.001$ and the promotive effects are marked as $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs.* control group.

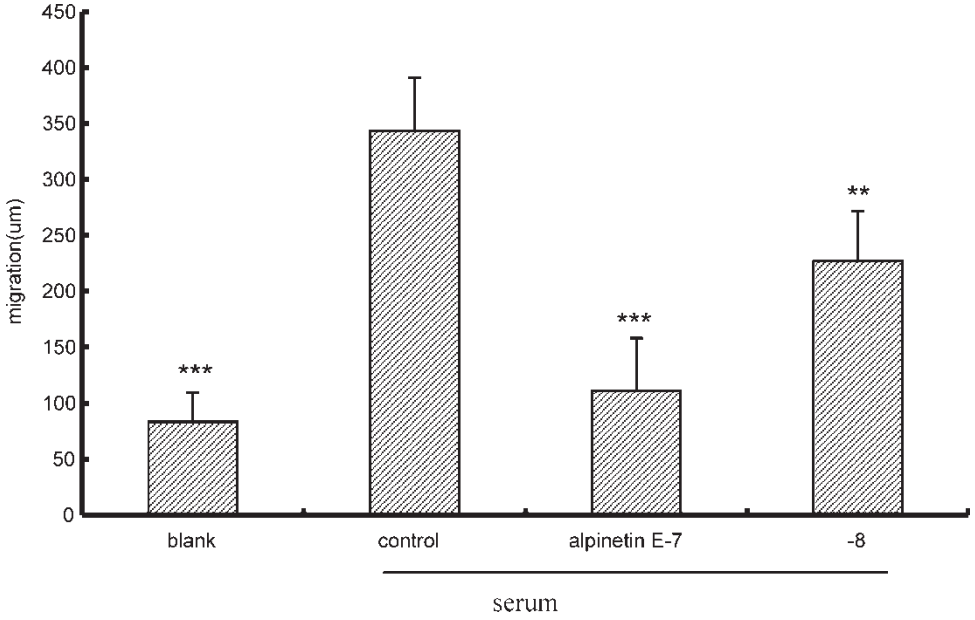

FIGURE 2 Inhibitory effects of **1** on the migration of VSMC induced by serum stimulation. The confluenced VSMC was incubated with **1** in serum-free medium for 12 h and stimulated with serum (10%) for a further 12 h; the subsequent migration of VSMC was then assessed by the methods of Sarker. Data are expressed as mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control group.

Inhibitory Effects of 1 on Production of NO in VSMC Induced by TNFα

The production of NO in VSMC was increased by 24.5% ($p < 0.01$) when VSMC were treated with TNFα (200 U ml^{-1}) for 72 h. Concentrations of 10^{-7}, 10^{-8} and 10^{-9} mol L^{-1} of **1** inhibited the production of NO by 22.6% ($p < 0.001$), 20.7% ($p < 0.01$) and 13.9% ($p < 0.05$), respectively, when VSMC were incubated with **1** 30 min before TNFα was added (Fig. 3).

Inhibitory Effect of 1 on Leakage of LDH from VSMC Injured by H_2O_2

H_2O_2 (100 μmol L^{-1}) could cause the LDH leakage from VSMC that occurs when they were incubated together for 1 h. Compound **1** could inhibit the leakage of LDH from VSMC by 7.1% ($p < 0.01$) (Fig. 4).

VSMC proliferation is a pathogenic hallmark of many vascular diseases [1]. During restenosis following arterial injury, VSMC form a neointimal layer in arteries by changing from a differentiated, contractile phenotype to a dedifferentiated, migratory, and proliferative phenotype. Several growth factors, cytokines, have been implicated in these phenotypic changes [6]. Both VSMC proliferation and migration contribute to lesion formation. In present experiments, we found that **1** could inhibit the proliferation and migration of VSMC stimulated by serum. These results suggest that **1** may play a therapeutic role in the formation of vascular diseases, especially in the atherosclerosis.

The concept of an endothelium-derived relaxing factor (EDRF) put forward in 1980 by Furchgott and Zawadzki [7] implies that nitric oxide (NO) produced by NO synthase (NOS) in the endothelium diffuses to the underlying vascular smooth muscle, where it modulates vascular tone as well as VSMC proliferation. According to this concept, VSMC do not express NOS by

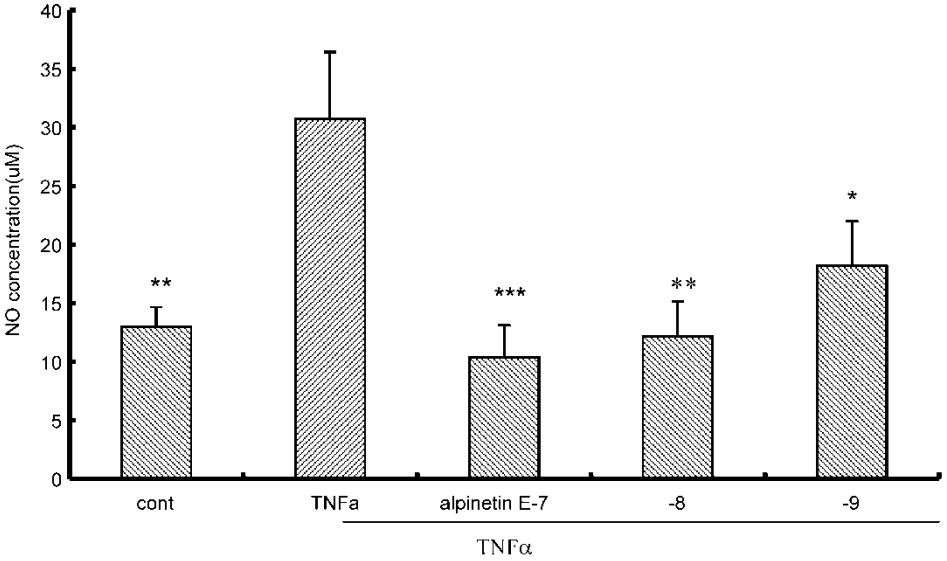

FIGURE 3 Effect of **1** on the production of NO in VSMC medium supernatant. VSMC were treated with TNFα (200 U ml^{-1}) for 72 h, then NO in medium supernatant was assessed. **1** was added 30 min prior to the addition of TNFα. Data are expressed as mean ± SD, $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* control group.

themselves. This attractive, simple scheme is now under considerable debate [8]. Buchwalow and his colleague testify that VSMC in various blood vessels express all three NOS isoforms, depending on the blood vessel type. Yan and his co-workers [3] also show that the intimal smooth muscle cells (SMC) are the main iNOS (inducible nitric oxide synthase) expressing cell type in the injured artery, and that it responds more vividly to iNOS-inducing cytokines. Our data show

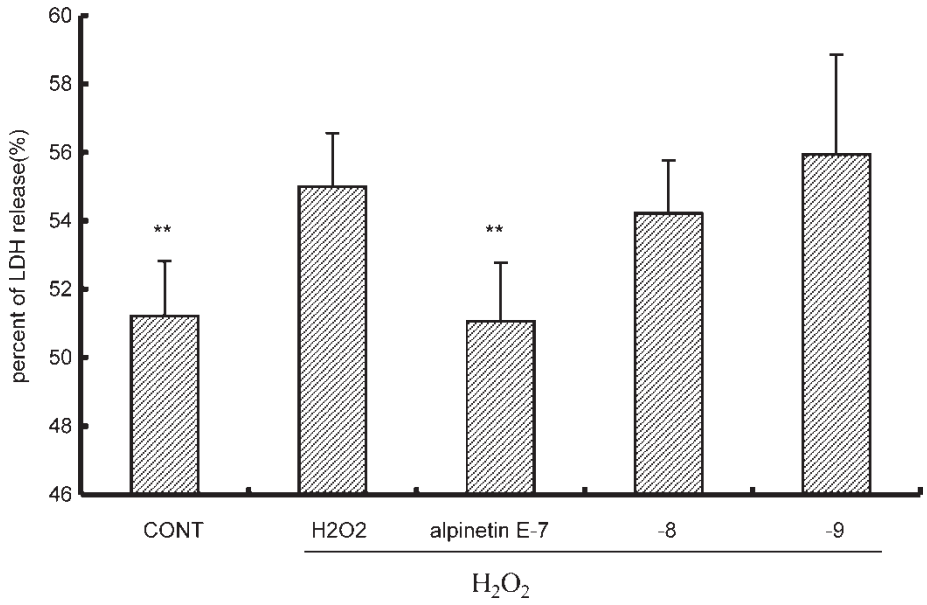

FIGURE 4 Effects of **1** on the leakage of LDH in VSMC medium supernatant. VSMC were treated with H_2O_2 (100 μmol L^{-1}) for 1 h, then LDH in the medium supernatant was assessed. **1** was added 30 min prior to the addition of H_2O_2. Data are expressed as mean ± SD, $n = 6$ wells, $^{**}p < 0.01$ *vs.* control group.

VSMC cultured *in vitro* could produce NO on stimulation of TNFα, which is consistent with Buchwalow, suggesting that local NOS expression may modulate vascular functions in an endothelium-independent manner [8]. Muniyappa *et al.* [9] verify that iNOS in VSMC is upregulated in arterial injury and plays a role in regulating VSMC proliferation and restenosis. Inflammatory cytokines, *e.g.* IL-1beta, released during vascular injury induce iNOS. Our data indicate that, compared with control, the production of NO induced by TNFα is significantly increased ($p < 0.01$), and that **1** at three different concentrations could significantly inhibit NO production; it is suggested that **1** may inhibit the expression of iNOS in VSMC. We also observed that **1** could inhibit the leakage of LDH in VSMC induced by H_2O_2, suggesting that **1** could protect VSMC against peroxide damage *in vitro*.

The present experiments show that **1** could not only inhibit the proliferation and migration of VSMC, but also protect VSMC from injury induced by H_2O_2 and TNFα. In summary, alpinetin has protective effects on VSMC and may exert therapeutic effects on vascular injury diseases such as atherosclerosis and restenosis.

EXPERIMENTAL

Cell Culture

VSMC were isolated from the medial layer of the thoracic aorta of 6-week-old Wistar rats, and intima and adventitia were removed as described previously [10]. A 1 mm^3 tissue slice was inoculated in a culture flask and kept inverted at 37°C in a 5% CO_2 humidified-atmosphere incubator for 6 h; it was then turned over, changing the culture medium every 3 days, VSMC began to grow out in about a week. The VSMC so-obtained were cultivated in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal calf serum (FCS), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). Cells from passages 10–20 were used for the present studies.

Tests of VSMC Proliferation Induced by Serum Stimulation

Cells were plated at $1-2 \times 10^5$ per well in a 96-well plate and allowed to attach in DMEM/10%FCS. After attachment, they were deprived of serum for 12 h, cells were then incubated with **1** (purchased from National Institute for the Control of Pharmaceutical and Biological Products) at concentrations of 10^{-7}, 10^{-8} and 10^{-9} mol L^{-1} for a further 12 h, followed by the addition of DMEM/10%FCS for 24, 48 and 72 h. At the indicated time, the MTT method was used to measure the absorbance to represent VSMC proliferation.

Tests of VSMC Migration Induced by Serum Stimulation

The VSMC migration test was carried out using a modification of Sarkar's method [11]. Cells were plated into the cover glass in a 6-well plate and allowed to attach in DMEM/10%FCS. After attachment, they were deprived of serum, the cover glass was taken out using a razor blade to scrape off the confluent cultured cells, and the cultivation continued without serum; meanwhile 10^{-7} and 10^{-8} mol L^{-1} of **1** were added for 12 h, followed by the addition of DMEM/10%FCS for a further 12 h. Seven visual fields (10×10) were then observed for each wounding injury. The maximum distances migrated were determined in each field and averaged for each injury.

Nitrite Assay

The accumulation of NO_2^-, a stable end-product of NO formation in conditioned medium was measured as an indicator of NO production by VSMC [12].

VSMC were seeded in 96-well plates ($1-2\times10^5$ cells per well), cultured with DMEM/10%FCS until 90% confluence, and growth-arrested by replacing the medium with DMEM/0.2%FCS for 24 h. The cells were then incubated with **1** at concentrations of 10^{-7}–10^{-9} mol L^{-1} for a further 0.5 h, followed by the addition of TNFα (200 U ml^{-1}) for 72 h; NO detection was then carried out according to the method described previously [13].

LDH Detection

VSMC were seeded in 96-well plates ($1-2\times10^5$ cells per well), cultured with DMEM/10%FCS until 90% confluence, and growth-arrested by replacing the medium with DMEM/0.2%FCS for 24 h. The cells were then incubated with **1** at concentrations 10^{-9}–10^{-7} mol L^{-1} for a further 0.5 h, followed by the addition of H_2O_2 (100 μmol L^{-1}) for 1 h. The culture medium was collected to measure lactate dehydrogenase (LDH) activity according to the method described in lactate dehydrogenase kits.

Statistical Analysis

All results were expressed as mean ± SD; the data were analyzed with the Student's *t*-test.

Acknowledgements

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