Inhibitory effects of ligustilide and butylidenephthalide on bFGF-stimulated proliferation of rat smooth muscle cells

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Abstract: Aim To investigate the bio-affinities of ligustilide and butylidenephthalide to rat aortic smooth muscle cells and the inhibitory effects of them on bFGF-stimulated proliferation of rat vascular smooth muscle cell (VSMC). **Methods** VSMCs were cultured from rat aorta pectoralis and identified by an immunohistochemical method. The bio-affinities between solute (ligustilide or butylidenephthalide) and cell membrane were measured by rat aortic cell membrane chromatography (CMC). The inhibitory effects of ligustilide and butylidenephthalide on bFGF-stimulated VSMC proliferation were evaluated by MTT colorimetric method. **Results** Both ligustilide and butylidenephthalide had selective affinities to rat aortic smooth muscle cell as the same as verapamil, one of the calcium ion antagonists. They could potently inhibit the bFGF-stimulated VSMC proliferation at the concentrations of 5.5 and 11.1 μmol·L^{-1}, separately ($P < 0.05$), but had no effects on the normal VSMC growth. **Conclusion** Both ligustilide and butylidenephthalide can inhibit the abnormal proliferation of VSMC induced by bFGF.

Key words: ligustilide; butylidenephthalide; cell membrane chromatography; cell proliferation; smooth muscle cell; basic fibroblast growth factor

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藁本内酯和丁烯酞内酯对 bFGF诱导的大鼠平滑肌细胞异常增殖的抑制作用

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摘要: 目的 研究藁本内酯和丁烯酞内酯与大鼠主动脉平滑肌细胞的亲合活性,及其对主动脉平滑肌细胞增殖的抑制作用。方法 采用大鼠主动脉细胞膜色谱模型观察藁本内酯和丁烯酞内酯的保留特性;培养并分离纯化大鼠主动脉平滑肌细胞,采用 bFGF诱导平滑肌细胞增殖,以 MTT比色法检测藁本内酯和丁烯酞内酯对平滑肌细胞增殖的抑制作用。结果 藁本内酯和丁烯酞内酯与大鼠主动脉平滑肌细胞膜有亲和性,其保留行为与钙离子受体拮抗剂维拉帕米相似;藁本内酯和丁烯酞内酯不会引起正常大鼠主动脉平滑肌细胞增殖,但能明显抑制 bFGF诱导的大鼠主动脉平滑肌细胞的增殖。其有效浓度分别为 5.5和 11.1 μmol·L^{-1} ($P < 0.05$)。结论 藁本内酯和丁烯酞内酯与大鼠主动脉平滑肌细胞具有亲和力,能抑制血管平滑肌细胞的异常增殖。

关键词: 藁本内酯; 丁烯酞内酯; 细胞膜色谱法; 细胞增值; 平滑肌细胞; 碱性成纤维细胞生长因子

Arterial injury results in the elaboration of pro-inflammatory substances including cytokines and peptide growth factors which act to modify vascular smooth muscle cell (VSMC) proliferation and migration with resultant vessel stenosis. In response to vascular injury, inflammatory cytokines and growth factors are released and lead to VSMC phenotype and

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growth[1]. The growth factor (basic fibroblast growth factor, bFGF) has been identified in atherosclerotic lesions[2,3]. bFGF promotes proliferation through receptor tyrosine kinase pathways to modify gene transcription, translation, and cell cycle progression. Inhibition of VSMC proliferation could depress the rate of intimal hyperplasia and prevent serious atherogenesis lesions.

Ligustilide (3-butylidene-4, 5-dihydro-isobenzofuranone, BDI) and butylidenephthalide (3-butylidene-isobenzofuranone, BI) are isolated from *Ligusticum chuanxiong* (*Ligusticum chuanxiong* Hort., Umbelliferae), a traditional Chinese medicine, which has been widely used in prescriptions for cardiovascular diseases in clinic[4,5]. A few reports stated that ligustrazine from *Ligusticum chuanxiong* could inhibit VSMC proliferation[6,7], but did not mention about BDI and BI. In this study, the bio-affinities of BDI and BI on the VSMC were investigated by using the rat aortic smooth muscle CMC system[8,9]. The inhibitory effects of BDI and BI on bFGF-stimulated VSMC proliferation were determined by MTT colorimetric method.

Materials and methods

Chemicals and Materials Verapamil, trypsin, and bFGF were obtained from Sigma-Aldrich (St Louis, MO, USA). Antibiotic drugs (penicillin G, 8×10^5 u and streptomycin sulfate, 1×10^6 u) were obtained from Shandong Lukang Medicine Co. (Shandong, China). Dulbecco's modified Eagle's media (DMEM) was obtained from GIBCO (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biotechnology Co. (Hangzhou, China). Other reagents used in the experiment were analytical grade from commercial sources. Hanks balanced salt solution (HBSS) and phosphate buffered saline (PBS), autoclaved sterilization (121 ℃, 20 min), were prepared in the Lab. CO_2 incubator was from Rheoyne (Rheoyne, USA). BDI and BI (purity >98% by HPLC) were separated from *Ligusticum chuanxiong* in the Lab. The initial solution of BDI and BI were dissolved with DMSO, sterilized for 30 min in steam, and stored in refrigerator for further use. Male or female SD rats, weighing 180 - 220 g, were supplied from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China).

CMC analysis SD rats were anaesthetized with ether and sacrificed by decapitation. The aorta pectoralis was immediately removed and immersed in cold (4 ℃) PBS (pH 7.4). Then it was dissected from adherent tissue and homogenized. The aorta cell membrane stationary phase was prepared and its enzymatic bioactivity was determined as reported previously[9,10]. The chromatographic column was filled with this stationary phase in high pressure. The chromatographic conditions were as following: rat aortic cell membrane column (50 mm × 2.0 mm ID) was used. Mobile phase was 50 mmol·L^{-1} sodium phosphate buffer (pH 7.4) with a flow rate of 0.5 mL·min^{-1}, and the detection wavelength was at 236 nm under the column temperature of 37 ℃. BDI and BI were screened by the CMC system under the conditions above. An affinity between solute (compound) and stationary phase (rat aortic cell membrane) is commonly expressed in the logarithm of capacity factor ($\log k'$) of the solute as following equation:

$$\log k' = \log [(t_R - t_0) / t_0]$$

Where t_R is the retention time of the solute and t_0 is the void time of the solvent which will not act on rat artery cell membrane in CMC system.

Cell culture and isolation[11] Rat VSMCs were isolated from segment of SD rat thoracic aorta. Briefly, the segment of aorta was removed out of the thoracic cavity into cold (4 ℃) HBSS and subsequently rinsed, trimmed, and cut open longitudinally in DMEM supplemented with antibiotics (100 u·mL^{-1}). Endothelial and adventitial surfaces were scraped off from the aorta pectoralis by using scalpel and the remaining medial layer was rinsed in HBSS and chopped into pieces (about 1 mm × 1 mm in size). The rat aortic pieces were suspended with a complete media in a tissue culture flask coated with 1% gelatin and placed in a 37 ℃, 5% CO_2 incubator. After cultured for seven days, the VSMCs grew out of the aortic pieces. Their morphology was observed as typical "hill and valley" by phase contrast microscopy (Chongqing, China). The isolation of VSMCs was performed by adherence firstly compared with other kinds of cells and this procedure was performed in triplicate. The purity of VSMCs was identified by an immunohistochemical method.

Assay of cell proliferation VSMC proliferation was assayed by using MTT colorimetric method. Rat VSMCs were detached by trypsin-EDTA solution (0.25%), and inoculated at 4 000 cells per well in a 96-well plate (Costar, Cambridge, MA) in 200 μL of

DMEM medium containing 10% FBS and incubated at 37 ℃, 5% CO_2 for 24 hours. The medium was then changed to 5% FBS DMEM and incubated for 48 hours (24th - 72nd hour from passage). Then various concentrations of BDI (5.5 - 43.8 μmol· L^{-1}) and BI (5.6 - 44.3 μmol· L^{-1}) were added, separately, to the wells and incubated for further 48 hours (72nd - 120th hour from passage), respectively. The stimulant, bFGF was added simultaneously into the 96-well plate. The blank was cultured in normal condition, and only the same amount of bFGF was added into the VSMC control. At the last 4 hours of incubation, 10 μL of the MTT reagent (12 mmol· L^{-1}) was added. After incubation, the medium was taken off and 200 μL of the solubilization solution (DMSO) was added into each well. After 30 min, the dissolved purple formazan was then recorded at 490 nm with a microtiter plate reader (Bio-rad, Hercules, CA, USA). All experiments were performed at least three times in more than quadruplex in one 96-well plate. The intensity of cell proliferation was subsequently expressed as absorbance (A). Data were presented as mean values ± standard error and analysis of variance was used to analyze the differences between experimental groups. Statistical significance was accepted within 95% confidence limits.

Results

1 Retention features in CMC system

The solutes had affinity with rat aortic cell membrane and the log k' values of the solutes in CMC system were calculated with the equation above-mentioned. In this rat aortic CMC system, verapamil as control had a stronger affinity with a log k' value of 1.35 (Figure 1A). Under the same conditions, there were broad chromatographic peaks with a log k' value of 1.07 for BDI (Figure 1B) and 1.01 for BI, separately (Figure 1C). It demonstrated that there was affinity between solute (BDI or BI) and stationary phase (protein and receptor on rat aortic cell membrane). Vitamin C, which has no interaction with rat arteria aorta, was chosen as a negative control and did not show any retention in CMC system (Figure 1D).

2 Rat VSMC identification

Primary rat VSMCs were isolated from the adult rat aorta pectoralis as described previously. The rat VSMCs were observed under phase contrast microscopy (Figure 2A) and demonstrated immunohistochemically

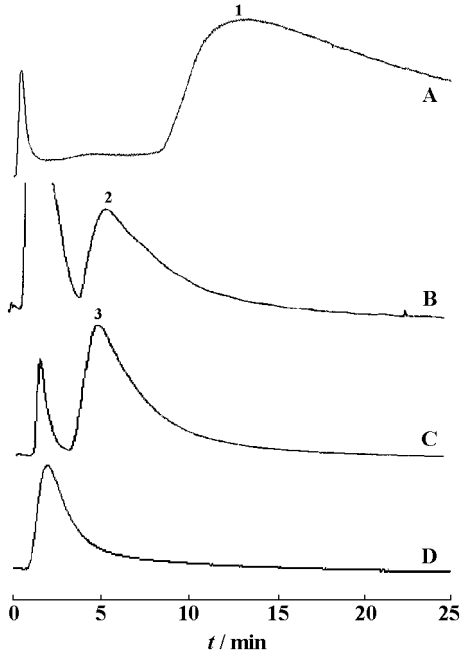

Figure 1 Chromatograms of verapamil (0.20 mg· mL^{-1}, A), BDI (0.22 mg· mL^{-1}, B), BI (0.25 mg· mL^{-1}, C) and vitamin C (D) on the model of rat aorta CMC. 1: Verapamil; 2: BDI; 3: BI

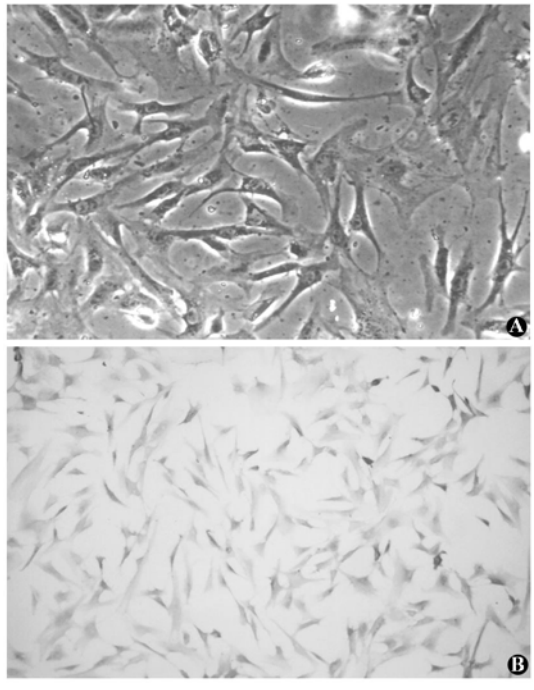

Figure 2 Graph of rat VSMC (10 × 20) observed under phase contrast microscopy (A) and immunohistochemical result stained with α-smooth muscle actin (B)

with α-smooth muscle actin staining, as well as lack of staining for the endothelial cell surface antigen (Figure 2B). The results showed that the cells were typical in morphology with a high purity observed by immunohis-

tochemical method. The rat VSMCs, between 4th passage and 8th passage, were used for the following experiments.

3 Rat VSMC growth curve

The rat VSMC growth curve was observed before proliferation experiments. The rat VSMCs grew logarithmically between 3 and 6 days. The solutes were added at the third day after passage to verify their bioactivities to cells growth in following experiments.

4 bFGF-induced VSMC proliferation

As shown in Figure 3, bFGF could induce rat VSMC proliferation. The percentage of growth rate was 24.0%, 26.0%, 38.7%, 47.3%, 36.7% and 30.0% at 1.7, 3.4, 6.8, 13.6, 27.0 and 54.0 AU· L^{-1}, respectively, which was highly significant when compared with the control. Maximal bFGF stimulation was observed at 13.6 AU· L^{-1} (0.221 ± 0.028, $P < 0.01$ *vs* control). Based on these results, bFGF concentration of 13.6 AU· L^{-1} was chosen to induce VSMC proliferation in subsequent experiments.

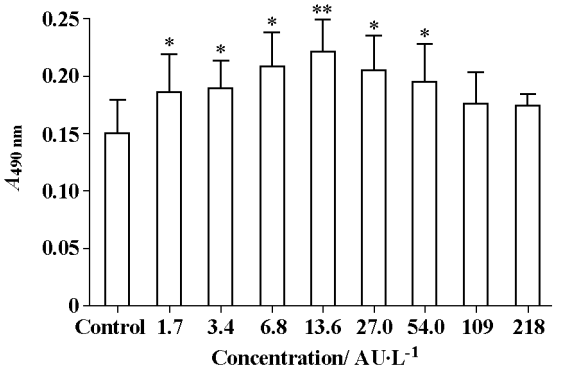

Figure 3 VSMC proliferation induced by bFGF (n = 8). The VSMCs were stimulated with bFGF for 48 hours (72nd - 120th hour from passage). Then bFGF-induced VSMC proliferation was detected by MTT colorimetric method. * $P < 0.05$, ** $P < 0.01$ *vs* control

5 Inhibition of BDI and BI on VSMC proliferation

The results showed that there was no effect on VSMC proliferation ($P > 0.6$ *vs* control at all doses), and also no toxicity to VSMC even at the doses of 50 μmol· L^{-1}. BDI- and BI-treated cells remained viable with Trypan blue staining. BDI could inhibit bFGF-induced VSMC proliferation in a dose-dependent manner (Figure 4A), its IC_{50} value was 17.18 μmol· L^{-1}. The percentage of inhibition was 23.08%, 40.66%, 57.14% and 89.01% at 5.5, 11.0, 21.9 and 43.8 μmol· L^{-1}, respectively, which was highly significant when compared with the control. BI also could inhibit bFGF-induced VSMC proliferation in a dose-dependent manner (Figure 4B), its IC_{50} value was 25.38 μmol· L^{-1}. The percentage of inhibition was 29.67%, 47.14% and 67.03% at 11.1, 22.2 and 44.3 μmol· L^{-1}, respectively, which was highly significant when compared with the control.

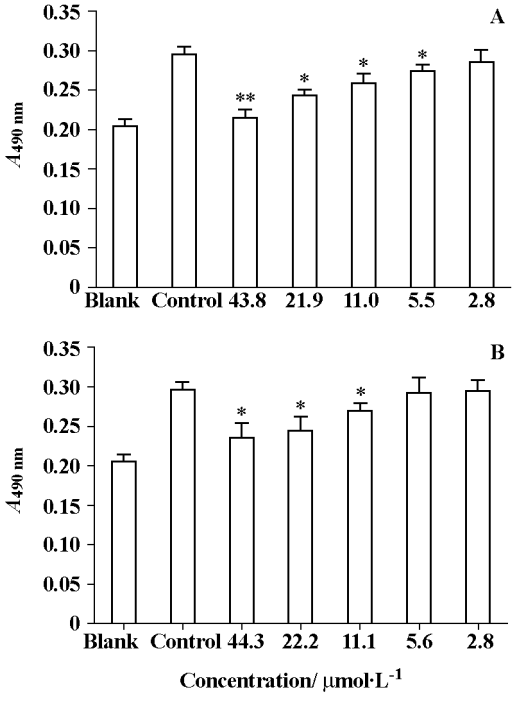

Figure 4 The inhibitory effects of BDI (A) and BI (B) on bFGF-induced VSMC proliferation (n = 8). * $P < 0.05$, ** $P < 0.01$ *vs* control

Discussion

Determination of the bio-affinity of components to the target cell is a key step for the screen analysis of traditional Chinese medicine. In the previous studies, CMC models had been successfully used to screen effective components from *Angelica sinensis*, *Herba epimedii*, *Leontice robustum*[12-15]. In this paper, the rat aortic CMC model has been established to measure the affinities of BDI and BI to the VSMCs, separately. It is found from the results that both BDI and BI have the similar retention features as verapamil as the control, in the model. Based on this, BDI and BI can act on the aortic smooth muscle cell. The log k' values of BDI and BI will reflect their bio-affinities. However, it needs further work to investigate how they exert their effect on the target cell.

As it is well known, the human atherosclerosis is associated with vascular smooth muscle proliferation. Decreased bFGF availability contributed to the vascular impairment and depressed severity of atherosclerotic lesions[16]. bFGF promotes proliferation through

receptor tyrosine kinase pathways to modify gene transcription, translation, and cell cycle progression. In order to depress the rate of intimal hyperplasia and then prevent serious atherogenesis lesions, it will be important to inhibit bFGF-induced VSMC proliferation. According to some reports[17-19], calcium channel blockers such as verapamil could depress VSMC proliferation and retard the progression of atherosclerosis. The inhibitory effects of BDI and BI on bFGF-induced VSMC proliferation were tested by primary culture of rat aorta pectoralis and MTT colorimetric method. These results showed that both BDI and BI could potently inhibit this kind of proliferation in a dose-dependent manner at the range of 5.5 - 43.8 μmol• L^{-1} and 11.1 - 44.3 μmol• L^{-1}, respectively.

In conclusion, this study showed that the screening results by the model of rat aorta CMC are closely correlative to pharmacological effects *in vitro*. In addition, both BDI and BI can inhibit bFGF-induced proliferation and depress migration in vascular injury, and as a result decrease the risk of intimal hyperplasia and atherogenesis.

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