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Inhibitory effects of praeruptorin C on cattle aortic smooth muscle cell proliferation[1]

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KEY WORDS praeruptorin C; cattle; aorta; smooth muscle; cell division

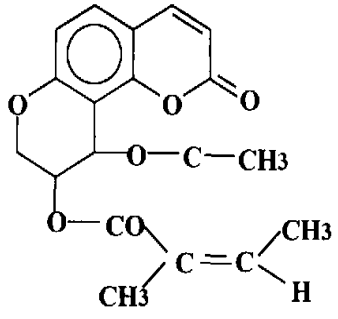
ABSTRACT

AIM: To study the effects of praeruptorin C (pra-C) on proliferation of cattle aortic smooth muscle cells (SMC). **METHODS**: The DNA synthesis of SMC was measured using the incorporation of [^{3}H] thymidine ([^{3}H] TdR). Cell cycle phase was evaluated by flow cytometry and cytotoxicity was evaluated by measuring lactic dehydrogenase (LDH) activity. **RESULTS**: Whether or not treated with angiotensin Ⅱ (Ang Ⅱ), SMC proliferation was suppressed by pra-C in a concentration-dependent manner at range from 0.001 μmol/L to 10 μmol/L. The inhibitory effects appeared to be related to G1 → S block in cell cycle traverse while the LDH activities did not change dramatically. **CONCLUSION**: Pra-C can completely inhibit SMC proliferation induced by Ang Ⅱ and partly inhibit the growth of SMC induced by bovine serum, which is important in prevention and treatment of vascular hyperplastic disease.

INTRODUCTION

Atherosclerosis (As) and hypertension are vascular hyperplastic diseases and may account for a majority of deaths attributed to coronary artery disease (CAD) in the developed as well as many developing countries. Previous studies have indicated that the change in smooth muscle cells (SMC) from a differentiated contractile type to a dedifferentiated synthetic type is the critical phenotypic response for atherogenesis and hypertension. As the change of phenotype, SMC migrates into the intima and proliferate, which leads to the narrowing of the arterial lumen physically and the production of cytokines and proteinases, causing chemical modification of the lesions and the development of atherosclerosis[1-3].

Angiotensin Ⅱ (Ang Ⅱ) can be biosynthesized and secreted in kidney and local blood vessels. Besides its vasoconstriction activity, Ang Ⅱ can stimulate hypertrophy of vascular SMC[3]. The type 1 of Ang Ⅱ (AT1) receptor is responsible for all known physiologic action of Ang Ⅱ that leads to elevated phosphoinositide hydrolysis, mobilization of intracellular Ca^{2+} and diacylglycerol, activation of Ca^{2+}/calmodulin and Ca^{2+}/phospholipid-dependent Ser/Thr kinase, as well as Ca^{2+}-regulated tyrosine kinases[4]. The reported biological activities of Ang Ⅱ might relate to flux of calcium ion tightly.



Structure of praeruptorine C

Calcium entry blockers are effective antihypertensive agents and may reverse hypertrophy in cardiac and vascular diseases. Praeruptorin C (pra-C) [2-methyl-10-(acetyloxy)-9, 10-dihydro-8, 8-dimethyl-2-oxo-2H, 8H-benzo[1,2-b;3,4-b] dipyran-9-ol ester of 2-butenolc acid] isolated from Qian-Hu, the roots of *Peucedanum praeruptorium* Dunn, was found as a kind of calcium entry blocker and to reduce the intracellular free calcium

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in rat ventricular myocytes[5,6]. Recently, pra-C was shown to reduce the responsiveness of AT1 receptor in aortic artery and inhibit the vascular contraction in renal hypertensive rats[7]. However, little is known about the effect of pra-C on the process of vascular proliferation. The aim of the present study was to investigate whether pra-C may suppress the proliferation of SMC induced by Ang Ⅱ or fetal bovine serum (FBS).

MATERIALS AND METHODS

Materials Pra-C, supplied by the Department of Phytochemistry, Jiangsu Institute of Botany (Nanjing, China), was white-colored and dissolved in polyethylenglycol 400 (PEG400).

Vascular SMC isolation and culture SMC were obtained from newborn calf thoracic aorta by nonenzymatic dissociation as described[8]. Cells between passages 5 – 10 were used and displayed the characteristic multi-layed, spindle-shaped "hills and valleys" pattern.

Measurement of DNA synthesis[9] Cells were adjusted to a density of 2×10^8 cells per liter with Dulbecco's modified Eagle medium (DMEM) (Gibco/BRL) containing 10 % FBS and added into 96-well tissue culture plates (Nunclon Co) at 5×10^4 cells per well. Quiescence was induced in confluent cells by switching cells to DMEM with 0.1 % FBS for 48 h. The quiescent cells were divided into control group (10 % FBS-supplemented DMEM); solvent group (10 % FBS-supplemented DMEM + PEG400); pra-C group (10 % FBS-supplemented DMEM + pra-C 0.001 – 10 μmol/L). After incubated for 0.5 h at 37 ℃, partial cells were treated with Ang Ⅱ 0.1 μmol/L (Sigma) to induce the growth of SMC and the others' condition was not changed. After 24 h, 37 mBq [^{3}H]TdR (Chinese Atomic Nucleus Research Institute, Beijing, China) was added to each well for 6 h, unincorporated [^{3}H]TdR was removed by washing three times with Hank's medium. Cells were harvested using a cell harvester and the incorporation of radioactive thymidine into cells was determined by liquid scintillation spectrometry.

Analysis of cell cycle phases by flow cytometry Cells (2×10^5) were seeded on 96-well plates. When reaching confluence, cell growth was arrested by serum deprivation for 48 h. Mitogenesis was induced by 10 % FBS-supplemented DMEM in the presence or absence of Ang Ⅱ. After incubated at 37 ℃ for 24 h, control and treated cells were fixed by adding 70 % ethanol. They were then stained with 10 mg/L of propidium iodide (Molecular Probe, Inc Eugene, OR) in the presence of 100 mg/L of RNase A as described before[9]. Cell cycle phase distribution was analyzed using flow cytometry (FACS Calibur, BD Co, USA).

Measurement of lactic dehydrogenase (LDH) activity Cells were plated into 96-well tissue culture plates as described in the section of measurement of DNA synthesis. Confluent cells were cultivated for 24 h at 37 ℃. Supernatant was collected and the LDH activity was measured with LDH assay kit provided by Nanjing Jiancheng Biotechnological Institute.

Statistical analysis Data were expressed as $\bar{x} \pm s$, and compared with paired t-test.

RESULTS

Effects of pra-C on incorporation of [^{3}H]TdR into DNA in cultured SMC In 10 % FBS-containing DMEM, Ang Ⅱ (0.1 μmol/L) increased the [^{3}H]TdR incorporation into DNA of SMC from 270.0 Bq (control) to 361.8 Bq (Ang Ⅱ). But pra-C inhibited [^{3}H]TdR incorporation into DNA of SMC with or without Ang Ⅱ. In the presence of Ang Ⅱ, pra-C 0.001 μmol/L, 0.01 μmol/L, 0.1 μmol/L, 1 μmol/L, and 10 μmol/L suppressed the incorporation of radioactive thymidine into DNA by 34 %, 45 %, 52 % ($P<0.05$), 55 % ($P<0.05$), and 59 % ($P<0.05$) respectively (Fig 1A). While in the absence of Ang Ⅱ, the inhibitory rates of pra-C (0.001 μmol/L – 10 μmol/L) were separately 11 %, 17 %, 33 %, 62 % ($P<0.05$), and 63 % ($P<0.05$) (Fig 1B).

Change of cell cycle phase by pra-C To explore the underlying basis for the anti-mitogenic properties of pra-C, cell cycle phase traverse in SMC was measured. Ang Ⅱ 0.1 μmol/L increased the percentage of cells in S phase from 35 % to 45 %, while pra-C (0.1 μmol/L) reduced the S phase of cells to 33 % in the absence of Ang Ⅱ and 36 % in the presence of Ang Ⅱ. Simultaneously, the amount of cells in G_0G_1 phase was increased. Compared with control group, the solvent PEG400 could not affect the cell cycle phase and the percentage of cells in S phase was not altered (37 %). In addition, no apoptotic wave of cells treated with pra-C was found by FACS.

Evaluation of cell toxicity of pra-C To investigate if the inhibitory effects of pra-C on SMC proliferation resulted from its cell toxicity, the LDH activity of cell culture medium was measured. The LDH

activity of control group was 697.67 U/L and no statistical difference of LDH activity was found between control and pra-C-treated groups.

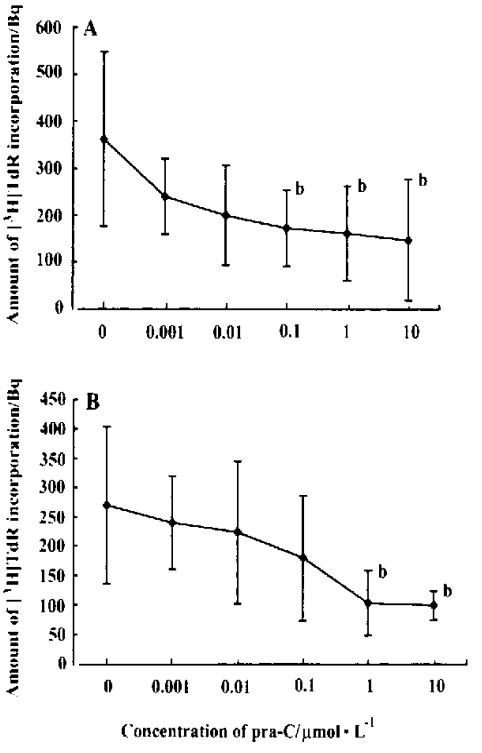

Fig 1. Effects of pra-C on proliferation of cattle aortic smooth muscle cells (SMC). Confluent cells were induced into quiescence by serum deprivation and were treated by pra-C in the presence (A) or absence (B) of Ang Ⅱ (0.1 μmol/L) for 30 h. [³H]TdR (37 mBq per well) was also present during the last 6 h of incubation. $n=8$. $\bar{x}\pm s$. $^bP<0.05$ *vs* control group.

DISCUSSION

Ang Ⅱ is a biofunctional growth factor that activated both proliferative and antiproliferative pathways to induce aortic SMC hypertrophy or hyperplasia, depending on the cellular melieu and intrinsic genetic characters[10]. Consistent with the results reported by Wang *et al*[10], in 10 % FBS-containing DMEM, Ang Ⅱ significantly promoted cell DNA synthesis and increased the ratio of cells in S phase. The results suggest that Ang Ⅱ can serve as a growth factor for SMC in the presence of higher concentration's serum.

As a kind of calcium entry blocker, pra-C was shown to reduce the responsiveness of AT1 receptor in aortic artery[7] and improve the vascular hypertrophy by decreasing the size of SMC[11]. In this study we tested the potential effect of pra-C on cell division. It was shown that pra-C effectively suppressed the Ang Ⅱ-induced proliferation of SMC. This effect was not attributed to the cellular toxicity because no changes were detected in LDH activity and only relative low percentage of programmed cell death (less than 1 %, data not shown) was detected by flow cytometry in the pra-C group. The treatment of pra-C resulted in the S phase of cell population decreased to the control level, which was paralleled by a concomitant increase of G_0G_1 phase in the presence of Ang Ⅱ. This result was consistent with that of [³H] TdR incorporation experiment. Thus, we postulate that pra-C exerts its inhibition on the proliferation of SMC through a partial blockage in G1→S traverse. The bioactivity of Ang Ⅱ is related to Ca^{2+}-mediated signal transduction pathway. Pra-C not only inhibited Ang Ⅱ-induced vascular SMC proliferation by blocking of Ca^{2+} influx, but also reduced the amount of FBS-induced DNA synthesis, suggesting that pra-C might suppress directly the FBS-induced SMC division.

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前胡丙素对牛主动脉平滑肌细胞增殖的抑制作用[1]

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关键词 前胡丙素；牛；主动脉；平滑肌；细胞分裂

目的：探讨前胡丙素（pra-C）对牛主动脉平滑肌细胞（SMC）增殖的影响。**方法**：细胞DNA的合成量由^{3}H-胸腺嘧啶核苷（^{3}H-TdR）掺入试验测定，应用流式细胞术测定细胞周期，用乳酸脱氢酶活性测定观察药物的毒性。**结果**：无论有无血管紧张素Ⅱ（Ang Ⅱ），在0.001 μmol/L到10 μmol/L浓度范围内，pra-C均可呈浓度依赖性地抑制SMC的增殖，抑制作用主要与阻止细胞进入有丝分裂期有关，而与细胞毒性无关。**结论**：pra-C能够完全阻断Ang Ⅱ诱导SMC的增殖效应，部分阻止小牛血清诱导的细胞分裂，这对于血管增生性疾病的防治有重要意义。

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