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Effects of Ro 31-8220 on smooth muscle cell proliferation induced by fibrinogen degradation products[1]

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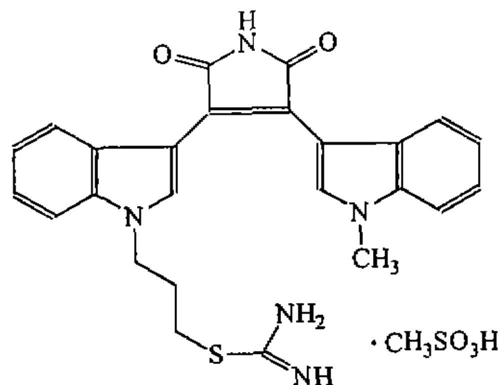
KEY WORDS Ro 31-8220; fibrin fibrinogen degradation products; protein kinase C; vascular smooth muscle; cultured cells; thoracic aorta

AIM: To study the mitogenic activity of fibrin fibrinogen degradation products (FFDP) and the effect of a new selectively potent protein kinase C (PKC) inhibitor Ro 31-8220 (Ro). **METHODS**: Rat aortic smooth muscle cells (SMC) proliferation in culture was measured by crystal violet staining assay. **RESULTS**: FFDP stimulated the proliferation of SMC during the experimental period of 72 h, Ro 0.01 – 1 $\mu mol \cdot L^{-1}$ inhibited FFDP-induced cell proliferation in a concentration-dependent manner. **CONCLUSION**: Ro exerted inhibitory effect on cell proliferation induced by FFDP.

Smooth muscle cell (SMC) proliferation is widely perceived as a key event in the formation of stenosing atherosclerotic lesions, but the stimuli primarily responsible for initiating this remain uncertain[1]. Fibrinogen is a major, primary cardiovascular risk factor[2]. Fibrin fibrinojn-derived protein was characterized and quantitated in atherosclerotic lesions[3]. Protein kinase C (PKC) is a pivotal enzyme in the intracellular signaling pathway that mediates the SMC proliferation[4]. 3-[1-[3-(Amidinothio) propyl]-3-indolyl]-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2, 5-dione methanesulfonate (Ro 31-8220[5], Ro) is a new selectively potent PKC inhibitor with an IC_{50} of 10 $nmol \cdot L^{-1}$ which had been used in the therapy of some autoimmune diseases, such as rheumatoid arthritis. This paper was to examine whether fibrin fibrinogen degradation products (FFDP) could stimulate the SMC proliferation, and the effect of Ro.



Ro 31-8220 ($C_{25}H_{23}N_5O_2S \cdot CH_3SO_3H$, M_r 553.65)

MATERIALS AND METHODS

Reagents Human fibrinogen, fibrin, plasmin, and aprotinin were purchased from Sigma (USA); Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco BRL; Ro was a generous gift from Dr Davis BRADSHAW (Roche Research Center).

Rats Wistar rats, aging 8 ± *s* 1 wk, weighing 250 ± *s* 20 g, were obtained from the Animal Center of Second Military Medical University.

Cell culture Rats were killed by cervical dislocation. The thoracic aorta was excised and placed in a Petri dish containing phosphate-buffered saline (PBS) (Ca^{2+}, Mg^{2+}-free). After the adventitia and the intima were removed with fine forceps, the cleaned aorta was cut into pieces of 1 – 2 mm^3 and put in a culture flask with the inside sticking to the bottom for incubation. Around 10 d the free smooth muscle cells derived from the explants teemed in most of the culture flask bottom. Then the cells were subcultured with 0.25 % trypsin. The cells exhibited typical hill and valley growth morphology of SMC. The typical growth experiment was performed with SMC at passage levels of 4 – 6 times.

Cell growth assay The subcultured cells in DMEM medium containing 10 % FBS were seeded in 96-well culture plates at a density of 1×10^4 cells/well. After 24 h, cells were washed twice with DMEM and incubated with FFDP in serum-free DMEM at defined intervals. To test the effect of PKC

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inhibitor Ro on FFDP action, cells were incubated with FFDP (6-h digestion, 1 $g \cdot L^{-1}$) in the presence of Ro for 24 h. After incubation, cell number was measured by using a modification of Flick's procedure[6].

Preparation of FFDP FFDP was obtained by digestion of fibrin with plasmin [10 casein unit (cu)/g fibrin]. Plasmin digestion was conducted in the presence of $CaCl_2$ 1 $mmol \cdot L^{-1}$. The reaction was terminated at defined intervals by addition of aprotinin 200 kIU/cu plasmin[7].

Statistics Comparisons were carried out using t test.

RESULTS

Addition of fibrinogen did not affect the SMC proliferation (0.180 ± 0.018; $P > 0.05$ *vs* control 0.153 ± 0.011). In contrast, FFDP stimulated the growth of SMC. The mitogenic activity of FFDP was increased along with the prolongation of digestion time. The 6-h digest had the maximal activity (Tab 1).

Tab 1. Effect of digestion time on mitogenic activity of FFDP. Cells were cultured with FFDP (1 $g \cdot L^{-1}$) for 48 h. $n = 4$ homogenates (each was pooled from 4 rats), $\bar{x} \pm s$. ${}^aP>0.05$, ${}^bP<0.05$, ${}^cP<0.01$ *vs* control.

Group	Digestion time/h	$A_{595\ nm}$
Control	0	0.153 ± 0.011
FFDP	0	0.280 ± 0.030^c
	1	0.353 ± 0.026^c
	3	0.363 ± 0.011^c
	6	0.433 ± 0.019^c
	12	0.398 ± 0.022^c
	24	0.378 ± 0.022^c

Marked proliferation was noted at 24 h after incubating the cells with FFDP. The effect of FFDP was concentration- and time-dependent (Tab 2).

Tab 2. Effect of FFDP (6-h digestion) on proliferation of SMC. $n = 4$ homogenates (each was pooled from 4 rats). Percent increase over control in parentheses. $\bar{x} \pm s$. ${}^aP>0.05$, ${}^bP<0.05$, ${}^cP<0.01$ *vs* control.

FFDP $g \cdot L^{-1}$	10^3 × Absorbance at 595 nm (%) 24 h	48 h	72 h
0	168 ± 10	160 ± 14	143 ± 13
0.1	195 ± 21^b (16)	213 ± 22^c (33)	240 ± 14^c (68)
0.5	270 ± 22^c (61)	305 ± 21^c (90)	340 ± 16^c (138)
1	375 ± 21^c (123)	438 ± 29^c (174)	490 ± 26^c (243)
5	408 ± 28^c (142)	463 ± 19^c (189)	500 ± 24^c (250)

The SMC proliferation induced by FFDP was inhibited by Ro 10 – 1000 $nmol \cdot L^{-1}$ (Tab 3).

Tab 3. Effect of Ro on SMC proliferation induced by FFDP (6-h digestion, 1 $g \cdot L^{-1}$) for 24 h. $n = 4$ homogenates (each was pooled from 4 rats). Inhibitory rate over control in parentheses. $\bar{x} \pm s$. ${}^aP>0.05$, ${}^bP<0.05$, ${}^cP<0.01$ *vs* control.

Ro 31-8220 $nmol \cdot L^{-1}$	$A_{595\ nm}$ (%) Medium	FFDP
0	0.175 ± 0.026	0.360 ± 0.028
10	0.160 ± 0.014^a (8.6)	0.33 ± 0.03^a (8.3)
100	0.160 ± 0.018^a (8.6)	0.30 ± 0.03^b (16.7)
1000	0.180 ± 0.022^a (−2.8)	0.23 ± 0.03^c (36.1)

DISCUSSION

Previous study found fibrin fibrinogen deposition only in the intima of early lesions, suggesting that fibrinogen deposits might precede the deposition of LDL in the development of atherosclerosis[8]. The present study indicated that FFDP stimulated SMC proliferation and the mitogenic activity correlated with the extent of digestion, which is in accordance with the form changing of fibrinogen in the growth of atherosclerotic plaques. This observation, supported the hypothesis that fibrinogen was involved in the pathogenesis of atherosclerosis[2] and FFDP provided a pathological growth factor initiating cell proliferation.

Ro inhibited the FFDP-induced SMC proliferation. PKC has been proven to play a pivotal role in SMC proliferation. So activating PKC may be involved in the mechanism of FFDP action.

In conclusion, the present study showed the inhibitory effect of Ro against the FFDP-induced SMC proliferation, and provided a theoretical basis in the prevention and treatment of cardiovascular diseases with effective PKC inhibitors.

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Ro 31-8220 对纤维蛋白原降解产物诱导的平滑肌细胞增殖的影响

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关键词 Ro 31-8220; 纤维蛋白原降解产物; 蛋白激酶 C; 血管平滑肌; 培养的细胞; 胸主动脉

目的: 研究纤维蛋白原降解产物的致有丝分裂原活性及一种新型 PKC 抑制剂 Ro 31-8220 (Ro)的作用. 方法: 大鼠主动脉平滑肌细胞增殖采用结晶紫染色法测定. 结果: 纤维蛋白原降解产物促进大鼠主动脉平滑肌细胞的增殖, Ro (0.01 - 1 μmol$\cdot$L^{-1})剂量依赖地抑制增殖. 结论: Ro 抑制纤维蛋白原降解产物诱导的平滑肌细胞的增殖.

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Uptake of $^{99m}Tc^{5+}$-complexes in ischemic myocardial slices and their dissociable ability

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KEY WORDS drug stability; myocardial ischemia; technetium compounds; diphosphates; succimer; glucoheptonate

AIM: To find how some technetium-complexes to deliver the active species, TcO_4^{3-}, to the target tissue from a dissociable polynuclear Tc^{5+} species in preserved states *in vivo*. **METHODS**: Effect of dissociation ability of the polynuclear Tc^{5+} complexes on their accumulation in ischemic myocardium was tested. Ability of dissociation as having an appropriate conformation to become biologically functional after entering the blood circulation was tested using a simple dilution method by thin layer chromatography (TLC) analysis. Various degree of ischemic myocardium slices of rat were incubated with 1/100 diluted $^{99m}Tc^{5+}$-succimer, $^{99m}Tc^{5+}$-GH and $^{99m}Tc^{5+}$-PPi. **RESULTS**: The TLC patterns of $^{99m}Tc^{5+}$-GH and $^{99m}Tc^{5+}$-PPi showed the presence of a fast increasing of free Tc-species as dilution degree increased. The relative radioactivity of peak of free pertechnetate ($R_f = 0.85 - 1.0$) with 1:500 dilution was: $^{99m}Tc^{5+}$-succimer 0 %, $^{99m}Tc^{5+}$ - GH 28.1 % ± 1.3 %, and $^{99m}Tc^{5+}$-PPi 46.0 % ± 2.9 % respectively. The uptake of the myocardium after ischemia for 3 h was $^{99m}Tc^{5+}$-succimer 420 % ± 110 % dose/g tissue, $^{99m}Tc^{5+}$-GH 710 % ± 180 % dose/g tissue, and $^{99m}Tc^{5+}$-PPi 1295 % ± 390 % dose/g tissue respectively. **CONCLUSION**: The dissociation and myocardial uptake showed: $^{99m}Tc^{5+}$-succimer < $^{99m}Tc^{5+}$-GH < $^{99m}Tc^{5+}$-PPi, the uptake by the ischemic myocardium is positively correlated to their dissociation.

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$^{99m}Tc^{5+}$-succimer as a tumor imaging agent has been developed[1]. It was perhaps a result of the similarity of the TcO_4^{3-} pentavalent core to the