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Effects of pentoxifylline and protein kinase C inhibitor on phorbol ester-induced intercellular adhesion molecule-1 expression in brain microvascular endothelial cells[1]

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KEY WORDS intercellular adhesion molecule-1; vascular endothelium; protein kinase C; tetradecanoylphorbol acetate; pentoxifylline; cultured cells

ABSTRACT

AIM: To study the potential roles of protein kinase C (PKC) on expression of intercellular adhesion molecule-1 (ICAM-1) in rat brain microvascular endothelial cells (RBMEC). **METHODS**: ICAM-1 expression in RBMEC was measured by ELISA analyses. **RESULTS**: Phorbol ester (PMA) enhanced the expression of ICAM-1 in a concentration (10 - 100 nmol·L^{-1}) and time (4 - 16 h)-dependent manner in RBMEC. Pentoxifylline (PTX) 1 - 100 μmol·L^{-1} and the PKC inhibitor H7 5 - 50 μmol·L^{-1} prevented PMA-induced stimulation of ICAM-1 expression. At PTX 100 μmol·L^{-1} and H7 50 μmol·L^{-1}, they reached maximal inhibitory effects [ICAM-1 expression (A) from (0.410 ± 0.014) to (0.175 ± 0.022) and (0.182 ± 0.013), respectively; $P < 0.01$]. **CONCLUSION**: Activation of PKC in RBMEC is associated with increased expression of ICAM-1 in RBMEC. PTX and H7 preincubation may inhibit PKC-induced up-regulation of ICAM-1.

INTRODUCTION

Chronic inflammatory diseases such as multiple sclerosis and experimental allergic encephalomyelitis (EAE) are characterized by intense leukocytic infiltration into the central nervous system (CNS)[1,2]. Brain microvessels are the most relevant part of the vascular system with regard to CNS inflammation. Cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) play an important role in leukocyte's adhesion to and transmigration through endothelial cells[3]. Cytokines such as TNF_α and IL-1 induce ICAM-1 expression in brain microvascular endothelial cells (BMEC) and stimulate their adhesion (no published article). However, the signaling mechanisms responsible for the induction of adhesion molecules in BMEC are poorly understood.

Pentoxifylline (PTX), a derivative of the methylxanthine, has been used for many years in the treatment of peripheral vascular diseases. Increased red blood cell flexibility, reduction of blood viscosity, and decreased potential of platelet aggregation are the basic actions of PTX, resulting in therapeutic benefits due to improved microcirculation and tissue oxygenation[4]. Phorbol ester (PMA) is a protein kinase C (PKC) activator.

In the present study, cultured rat brain microvascular endothelial cells (RBMEC) were used to study the role of PKC in expression of ICAM-1 and protective effects of PTX and PKC inhibitor-H7.

MATERIALS AND METHODS

Rats Wistar rats of either sex, 2 - 3 wk (n = 36, Grade Ⅱ, Certificate № D02-25-4), were obtained from the Animal Center of Second Military Medical University.

Chemicals PMA, H7, and *o*-phenylenediamine (OPD) were purchased from Sigma Co; Anti-ICAM-1-MAb (1A29, mouse IgG) was purchased

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from Seikagaku Co, Japan; PTX was purchased from Shijiazhuang № 1 Pharmaceutical Co.

Cell culture Primary cultures of RBMEC were isolated by a modification of the method described by duan *et al*[15]. Briefly, rat cerebral cortex was obtained from rats. Larger blood vessels were carefully removed. Brain specimens were cut into small pieces and homogenized in Medium 199 containing 2 % fetal bovine serum (FBS). The homogenate was filtered through 149 μm nylon net and 74 μm nylon net. The crude microvessels on 74 μm nylon net were collected and digested in a solution containing collagenase Ⅱ 1 g·L^{-1} in Medium 199 at 37 ℃ for 40 min. Then the solution was centrifuged at 800 × *g* for 10 min. The rat brain microvessels were plated on plastic plate and grown in Medium 199 with 1 % *L*-glutamine, 20 % FBS, heparin 100 kU·L^{-1}, benzylpenicillin 100 kU·L^{-1}, streptomycin 100 mg·L^{-1} and bovine brain extract[16] 200 mg·L^{-1} for 5 – 7 d. Then the medium including microvessels was changed with new medium. Cells were grown at 37 ℃ in a humidified atmosphere with 5 % CO_2 and 95 % air. RBMEC were identified with immunofluorescent staining with anti-Factor Ⅷ. 2 nd – 4 th passage confluent cultures of RBMEC were used.

Treatment of RBMEC with PMA Confluent cultures of RBMEC in 96-well plates were stimulated for 0 – 16 h with serum-free Medium 199 containing various concentration of PMA. ICAM-1 expression in RBMEC was measured with ELISA.

ELISA ICAM-1 expression in RBMEC was quantitated by measuring the binding of rat monoclonal antibody to RBMEC on quadruplicate wells of confluent monolayers of RBMEC in 96-well flat-bottomed plates[7]. Briefly, RBMEC were incubated with PMA and/or drugs, then the tissue medium was removed. The cells were washed twice with warm Medium 199 before fixed with 1 % paraformaldehyde at 25 ℃ for 15 min. After washing the fixed cells three times with Medium 199, unbound sites were blocked by adding a 2 % solution of Bovine Serum Albumin (BSA) diluted in Medium 199 and incubated at 37 ℃ for 1 h. After removing the blocking solution, a total of 100 μL of anti-ICAM-1-MAb was added and plates were incubated at 37 ℃ for 1 h. The plates were then washed three times with Medium 199, and 100 μL of a 1/1000 dilution of the developing antibody (anti-mouse IgG, HRP conjugate) in 1 % BSA-Medium 199 was added. The plates were then incubated at 37 ℃ for 1 h. The enzyme conjugated was removed and the cells were washed four times with Medium 199. Next 100 μL of OPD substrate was added to each well, and the plates were incubated at 37 ℃. Controls were included in each assay. The plates were read at 492 nm with type-511 micro-elisa reader (Shanghai № 3 Analytical Instrument Factory) between 5 and 30 min after incubation.

Statistics Data were expressed as $x \pm s$ and compared by a paired *t* test.

RESULTS

Time and dose course of ICAM-1 expression in RBMEC induced by PMA RBMEC can express basal ICAM-1 at rest. Time kinetics studies showed that the increasing effect of PMA on ICAM-1 expression could be detected as early as 4 h after treatment (Tab 1), and reached maximal levels at 8 h. ICAM-1 expression increased after stimulation of the RBMEC with PMA (10 – 100 nmol·L^{-1}) (Tab 2). The effects of PMA showed a concentration-dependent tendency. ICAM-1 expression reached maximum at

Tab 1. PMA-induced ICAM-1 expression in RBMEC. $n = 4$ wells and repeated for 3 independent experiments. $x \pm s$. $^cP < 0.01$ *vs* control (0 h).

Time/h	PMA/nmol·L^{-1}	ICAM-1 expression (*A*)
0	0	0.134 ± 0.013
4	100	0.248 ± 0.016^c
8	100	0.444 ± 0.009^c
12	100	0.327 ± 0.017^c
16	100	0.282 ± 0.017^c

Tab 2. Concentration-dependence of PMA-induced ICAM-1 expression in RBMEC for 8 h. $n = 4$ wells and repeated for 3 independent experiments. $x \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

PMA/nmol·L^{-1}	ICAM-1 expression (*A*)
0	0.132 ± 0.010
10	0.199 ± 0.027^b
50	0.354 ± 0.003^c
100	0.454 ± 0.041^c

100 nmol·L^{-1}, so PMA 100 nmol·L^{-1}, 8 h was selected in our experiments.

Effects of PTX and H7 on induction of ICAM-1 expression by PMA When RBMEC were preincubated with a range of concentrations of PTX (1 – 100 μmol·L^{-1}) and H7 (5 – 50 μmol·L^{-1}) for 15 min followed by 8 h with PMA (100 nmol·L^{-1}) in the continued presence of the drugs, ICAM-1 expression was concentration-dependently inhibited (Tab 3).

Tab 3. Effect of drugs on ICAM-1 expression induced by PMA. $n = 4$ **wells and repeated for 3 independent experiments.** $\bar{x} \pm s$. $^cP < 0.01$ *vs* **PMA group.**

Drug/μmol·L^{-1}		PMA/nmol·L^{-1}	ICAM-1 expression (A)
		0	0.136 ± 0.009
		100	0.410 ± 0.014
H7	5	100	0.320 ± 0.006^c
	10	100	0.280 ± 0.012^c
	50	100	0.182 ± 0.013^c
PTX	1	100	0.341 ± 0.008^c
	10	100	0.287 ± 0.010^c
	100	100	0.175 ± 0.022^c

DISCUSSION

Adhesion of leukocytes to endothelial cells is dependent upon the expression of adhesion molecules in both endothelial cells (EC) and leukocytes[8,9]. The present study found that PMA stimulated ICAM-1 up-regulation in RBMEC and H7 could inhibit this effect. PKC had been proven to play an important role in ICAM-1 expression in EC. So activating PKC might be involved in the mechanism of cell adhesion molecule expression in EC and adhesion between EC and leukocytes.

Our study also found that PTX inhibited PMA-induced ICAM-I expression in brain EC. This might explain that PTX might delay infiltration of inflammatory cells in the CNS of mice with EAE[10].

In conclusion, the present study showed the inhibitory effects of PTX and H7 on the PMA-induced ICAM-1 up-regulation in RBMEC, and provided a theoretical basis in the prevention and treatment of cardiovascular and cerebrovascular diseases with PTX and PKC inhibitors.

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己酮可可碱与蛋白激酶 C 抑制剂对佛波醇酯诱导脑微血管内皮细胞表达细胞间粘附分子-1 的影响[1]

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关键词 细胞间粘附分子-1；血管内皮；蛋白激酶 C；14-烷基佛波醇乙酸酯；己酮可可碱；培养的细胞

目的：研究佛波醇酯（PMA）诱导大鼠脑微血管内皮细胞（RBMEC）表达细胞间粘附分子-1（ICAM-1）及 PKC 抑制剂 H7 与己酮可可碱（PTX）的抑制作用．**方法**：采用 ELISA 方法测定培养 RBMEC 表达 ICAM-1．**结果**：PMA 在 10－100 nmol·L^{-1}范围内剂量依赖性地诱导 RBMEC 表达 ICAM-1；在 4－16 h 范围内时间依赖性诱导 RBMEC 表达 ICAM-1．H7 和 PTX 分别在 5－50 μmol·L^{-1}和 1－100 μmol·L^{-1}范围内剂量依赖性抑制 PMA 诱导的 RBMEC 表达 ICAM-1．PTX 100 μmol·L^{-1}，H7 50 μmol·L^{-1}时，抑制作用达最大[吸光度分别从（0.410 ± 0.014）降至（0.175 ± 0.022）和（0.182 ± 0.013），$P<0.01$]．**结论**：PKC 抑制剂及己酮可可碱能抑制 PMA 诱导 RBMEC 表达 ICAM-1，表明 PKC 参与 RBMEC ICAM-1 表达调控．（责任编辑 刘俊娥）