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Verapamil, cyproheptadine, and anisodamine antagonized $[Ca^{2+}]_i$ elevation induced by TNFα in a single endothelial cell[1]

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KEY WORDS tumor necrosis factor; calcium; endothelium; verapamil; cyproheptadine; anisodamine; cultured cells; confocal microscopy

ABSTRACT

AIM: To study the effect of tumor necrosis factor alpha (TNFα) on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and the effects of verapamil (Ver), cyproheptadine (Cyp), and anisodamine (Ani) on TNFα-induced $[Ca^{2+}]_i$ changes in single endothlial cell, and to explore the mechanisms of TNFα-mediated shock and antishock actions of Cyp and Ani. **METHODS:** Human umbilical vein endothlial cell strains (ECV304) were seeded in 35-mm tissue culture dish with 2 mL DMEM culture medium. The cultured cells were loaded by Fluo-3/AM. The spatial distribution and the dynamic changes of $[Ca^{2+}]_i$ in single endothelial cell were determined by laser scanning confocal microscopy. **RESULTS:** After stimulation with TNFα, $[Ca^{2+}]_i$ in single endothelial cell rapidly increased in a concentration-dependent manner and arrived at the peak value within 60 s, afterwards, decreased and kept above the basal level. The confocal scanning image showed that $[Ca^{2+}]_i$ elevation was more obvious in nuclear than in cytoplasma and decreased slowly. Ver (1, 2 μmol/L), Cyp (30, 60 μmol/L), and Ani (20, 40 μmol/L) markedly inhibited TNFα 1.2 nmol/L-induced $[Ca^{2+}]_i$ elevation. **CONCLUSION:** TNFα markedly induces elevation of $[Ca^{2+}]_i$ in a single endothelial cell, it may be an important mechanism of TNFα-induced shock and tissue injury. That Cyp and Ani obviously suppress TNFα-induced $[Ca^{2+}]_i$ elevation probably is one of the mechanisms of their antishock effects.

INTRODUCTION

Tumor necrosis factor alpha (TNFα) is a polypeptide cytokine that has been found to occupy a pivotal role in the development of shock and tissue injury during septicemia[1,2]. Infusion of rat with TNFα results in a syndrome of shock that was pathologically similar to septic shock[1]. Clinical studies have demonstrated that serum TNF levels predict morbidity and mortality in human meningococcemia and in clinical septic shock of other etiologies[2]. Anti-TNF monoclonal antibodies prevent septic shock during lethal bacteremia[3]. Our studies had found that plasma TNFα levels in rats markedly increased after lipopolysaccharides (LPS) challenge, and inhibiting TNFα production had a obvious anti-endotoxic shock effect[4]. In this study, using laser scanning confocal microscopy (LSCM), we detected the spatial distribution and the dynamic changes of $[Ca^{2+}]_i$ in single endothelial cell after TNFα stimulation and investigated the effects of Ver, Cyp, and Ani on the changes of $[Ca^{2+}]_i$ induced by TNFα, so as to elucidate the mechanisms of TNFα-mediated shock and antishock actions of Cyp and Ani.

MATERIALS AND METHODS

Drugs and reagents TNFα (provided by Biotinge Biomedicine Co); Ver (Jiangsu Lianyungang Pharmaceutical Factory); Cyp (Jinan Yongning Pharmaceutical Co); Ain (Wuhu Changjiang Pharmaceutical Co); Fluo-3/AM (Molecular probes, Eugene, Oregon, USA); Dulbecco's modified Engle's medium (DMEM) and fetal bovine serum (Gibco BRL, USA); HEPES (Boehringer Mannbeim, Germany); Other chemicals were of AR grade.

Cell culture Human umbilical vein endothelial

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cell strains (ECV304, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) was seeded onto 35-mm tissue culture dish with 2 mL of DMEM culture medium (DMEM 10 g/L, HEPES 35.76 g/L, $NaHCO_3$ 37 g/L, fetal bovine serum 100 mL, penicillin 100 kU/L, streptomycin 100 kU/L, pH 7.2-7.4) and incubated at 37 ℃ in 5 % CO_2 atmosphere (CO_2 humidified incubator, Heraeus, Germany). The culture medium was renewed every 2 d.

Fluo-3/AM-loaded cells Fluo-3/AM in Me_2SO was added into Hanks' solution (final concentration 5 μmol/L). Freshly prepared fluorescent solution 2 mL replaced the medium in the dish. The cells were incubated at 37 ℃ for 45 min for analysis[5].

Measurement of $[Ca^{2+}]_i$ The fluorescent intensity was increased as the Fluo-3 binding to intracellular free Ca^{2+}. The changes of fluorescent intensity might represent the corresponding alteration of $[Ca^{2+}]_i$. Cells were scanned with a 1 μm spot argon ion laser beam at 355 nm. Emissions at 405 and 485 nm from the illuminated spot on the cells were directed to a sensitive photomultiplier tube and acquisition interface. The ratio of the intensities of fluorescent emission at 530 and 630 nm with excitation at 488 nm was measured with the same system. The $[Ca^{2+}]_i$ in various parts of single cells was calculated from the ratio of fluorescence at each emission wavelength using standard calibration curve of Ca^{2+} concentration. Using a 5 W argon ion laser emitting at 488 nm and 514 nm, the phase-contrast microscope (× 20), and Time Series scanning program, we continually observed the spatial distribution and the dynamic changes of $[Ca^{2+}]_i$ in a single cell by laser scanning confocal microscope (Bio-Tek Meridian Instruments, USA)[6,7].

Experimental protocol The endothelial cell loaded-well with Fluo-3 in culture dish was found in the screen, the normal fluorescent image and $[Ca^{2+}]_i$ in a single resting cell were detected. Then, the samples were divided into two groups. (1) TNFα group: After the fluorescence was in the steady state, seven concentrations of TNFα were added into the dish. Final concentrations of TNFα in Hanks' solution were 0 (the saline replaced the TNFα), 0.3, 0.6, 1.2, 1.8, 2.4, and 3 nmol/L, respectively. The spatial distribution and the dynamic changes of $[Ca^{2+}]_i$ in a single cell were observed. (2) Drug treated group: pretreatment with Ver 1 or 2 μmol/L, Cyp 30 or 60 μmol/L, and Ani 20 or 40 μmol/L (final concentrations in Hanks' solution), the effects of Ver, Cyp, and Ani on the fluorescent image and curve of $[Ca^{2+}]_i$ were observed. After 1 min, TNFα (final concentration of 1.2 nmol/L) was added. The effects of Ver, Cyp, and Ani on the changes of $[Ca^{2+}]_i$ induced by TNFα in a single endothlial cell were observed.

Analysis of data The changes of $[Ca^{2+}]_i$ were indicated by the percentage of the fluorescent intensity combining Fluo-3 with Ca^{2+}. The formula is shown as follows: Percentage of increase in $[Ca^{2+}]_i$ fluorescent intensity (%) = $(F_{max} - F_0)/F_0 \times 100$ %, where F_{max} is the peak value of the fluorescent intensity of the $[Ca^{2+}]_i$ elevation induced by TNFα. F_0 is the fluorescent intensity before administrating drug.

Data were expressed as $\bar{x} \pm s$ and cmpared with t-test.

RESULTS

The effects of TNFα on $[Ca^{2+}]_i$ in single endothelial cell After stimulated with TNFα, the fluorescent value of $[Ca^{2+}]_i$ in a single cultured endothelial cell rapidly increased and arrived at the peak value within 60 s, afterwards, decreased and kept above the basal level. The confocal scanning image of spatial distribution of $[Ca^{2+}]_i$ showed that $[Ca^{2+}]_i$ elevation was more obvious in nuclear than in cytoplasma, and decreased slowly (Fig 1). TNFα-induced $[Ca^{2+}]_i$ elevation was in a concentration-dependent manner. With increase of TNFα concentration, the fluorescent intensity of $[Ca^{2+}]_i$ rose more obviously and declined more slowly (Tab 1).

Tab 1. Effect of TNFα on $[Ca^{2+}]_i$ in a single cultured endothelial cell. $n = 6$ samples. $\bar{x} \pm s$. $^cP < 0.01$ *vs* basal fluorescent values.

TNFα/nmol·L^{-1}	Percentage of increase in fluorescent intensity/%
0 (saline control)	0
0.3	97 ± 19^c
0.6	176 ± 27^c
1.2	216 ± 34^c
1.8	280 ± 37^c
2.4	323 ± 39^c
3	393 ± 41^c

Effects of Ver, Cyp, and Ani on $[Ca^{2+}]_i$ elevation induced by TNFα Pretreated with Ver 1 or

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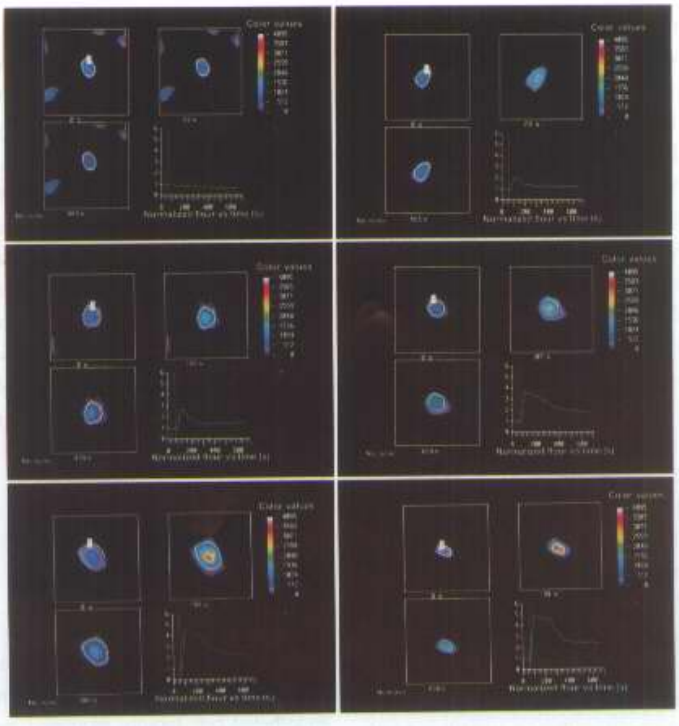

Fig 1. The confocal scanning image shows the effect of TNFα on $[Ca^{2+}]_i$ in a single endothelial cell. The color scanning picture could directly reflect the changes of single endothelial cell $[Ca^{2+}]_i$ in various typical phases. The color index at right-upper corner indicates that the different colors correspond to the different fluorescent intensity. The right-lower corner is the curve of the fluorescent intensity of $[Ca^{2+}]_i$ with time. A: saline control; B: TNFα 0.3 nmol/L; C: TNFα 0.6 nmol/L; D: TNFα 1.8 nmol/L; E: TNFα 2.4 nmol/L; F: TNFα 3 nmol/L.

2 μmol/L, Cyp 30 or 60 μmol/L, and Ani 20 or 40 μmol/L. The peak values of fluorescent intensity of $[Ca^{2+}]_i$ elevation induced by TNFα 1.2 nmol/L in a single endothelial cell was obviously reduced in comparison with TNFα 1.2 μmol/L alone. It suggested that Ver, Cyp, and Ani have obvious antagonistic effects on TNFα-induced $[Ca^{2+}]_i$ elevation in a single endothelial cell (Tab 2, Fig 2).

Tab 2. Effect of verapamil, cyproheptadine, and anisodamine on TNFα (1.2 nmol/L)-induced $[Ca^{2+}]_i$ elevation in single cultured endothelial cell. $n = 6$ samples. $\bar{x} \pm s$. $^cP < 0.01$ *vs* TNFα.

Drugs/μmol·L^{-1}		Percentage of increase in fluorescent intensity/%
TNFα		216 ± 34
TNFα + Ver	1	104 ± 17^c
	2	69 ± 14^c
TNFα + Cyp	30	117 ± 19^c
	60	85 ± 16^c
TNFα + Ani	20	134 ± 21^c
	40	96 ± 18^c

DISCUSSION

Intracellular free Ca^{2+} as a second messenger plays an important role in cellular various functions such as cell division, differentiation, gland secretion, and neurotransmitter release. Control of $[Ca^{2+}]_i$ homeostasis includes maintaining Ca^{2+} gradient on both sides of membrane, mediating celluar response on external stimulus, and performing message transduction across membrane[8,9]. $[Ca^{2+}]_i$ is increased in many pathophysiologic processes including shock. Many of the invasive stimuli known to cause sepsis, such as endotoxin and enterotoxin, promote macrophages to release TNFα. As TNFα binds to tumor necrosis factor receptors on cellular membrane, phospholipase C is activated and catalyzes phosphatidylinositol

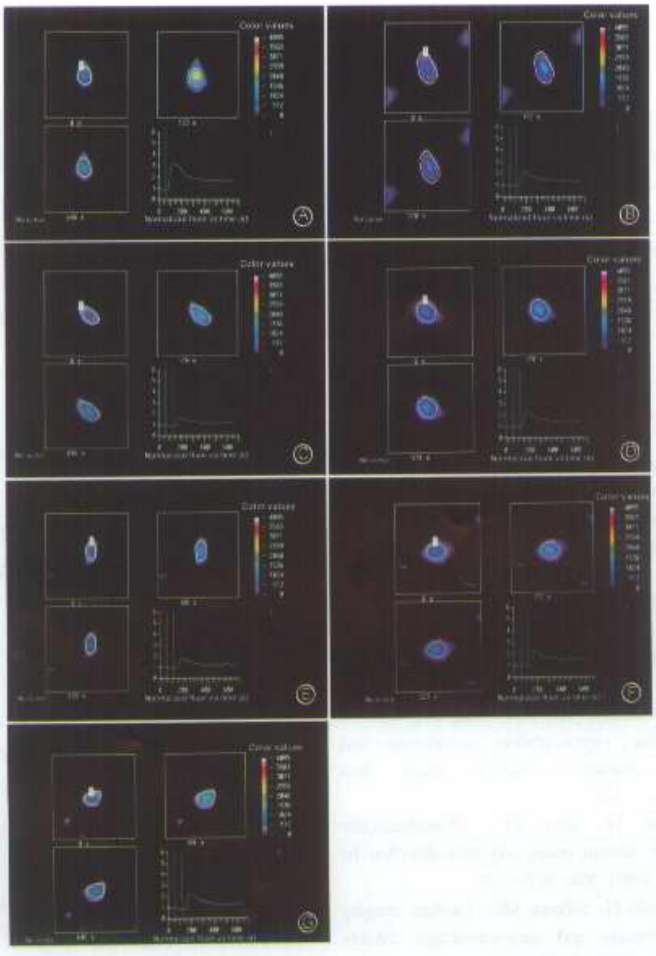

Fig 2. Inhibitory effects of verapamil, cyproheptadine, and anisodamine on $[Ca^{2+}]_i$ elevation induced by TNFα in cultured endothelial cell. A: TNFα 1.2 nmol/L; B: Ver 1 μmol/L + TNFα 1.2 nmol/L; C: Ver 2 μmol/L + TNFα 1.2 nmol/L; D: Cyp 30 μmol/L + TNFα 1.2 nmol/L; E: Cyp 60 μmol/L + TNFα 1.2 nmol/L; F: Ani 20 μmol/L + TNFα 1.2 nmol/L; G: Ani 40 μmol/L + TNFα 1.2 nmol/L.

4,5-diphosphate into 1,4,5-inositol triphosphate (IP_3). IP_3 leads to the intercellular Ca^{2+} release and extracellular Ca^{2+} influx[10]. $[Ca^{2+}]_i$ elevation could disturb the process of oxidative phosphorylation in mitochondria, reduce adenosine triphosphate (ATP) production, activate certain phospholipases, and seriously damage cellular and subcellular organelles membranes. In the meantime, the $[Ca^{2+}]_i$ overload might directly activate certain proteinases to produce a great amount of free radicals, and promote the expression of the genes to produce the inflammaory mediators such as TNFα and interleukin-1 β which are important factors of septic shock. Therefore, the $[Ca^{2+}]_i$ overload is the common pathway for cell death, and closely related to the development of shock.

At present LSCM is the best means to study the spatial distribution and the dynamic changes of $[Ca^{2+}]_i$ in single cell. This results showed that TNFα markedly induced $[Ca^{2+}]_i$ elevation. The $[Ca^{2+}]_i$ elevation induced by TNFα was more obvious in nuclear than in cytoplasma, it may be the event released from Ca^{2+} pool in nucleus[11]. The increase of nuclear calcium promotes the gene transcription and expression of certain shockgenic cytokines such as TNFα and interleukin-1 β. It may be an important mechanism of TNFα-induced shock and tissue injury. Verapamil, cyproheptadine, and anisodamine have an agtagonistic effects on TNFα-induced

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$[Ca^{2+}]_i$ elevation. Verapamil, a Ca^{2+} channel specific antagonist, inhibit elevation of $[Ca^{2+}]_i$ induced by TNFα, suggesting that TNFα-induced $[Ca^{2+}]_i$ elevation also result from the increase of calcium influx. Our previous studies found that Cyp and Ani strongly inhibited LPS-induced TNFα production, and had a beneficial anti-endotoxic shock effects. This study indicated that antishock mechanism of Cyp and Ani may be related to inhibiting the $[Ca^{2+}]_i$ elevation induced by TNFα.

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维拉帕米、噻庚啶和山莨菪碱拮抗 TNFα 诱导单个内皮细胞胞内游离 Ca^{2+} 浓度的增高[1]

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关键词　肿瘤坏死因子；钙；内皮；维拉帕米；噻庚啶；山莨菪碱；培养的细胞；共聚焦显微镜检查

目的：研究肿瘤坏死因子(TNFα)对单个内皮细胞胞内游离 Ca^{2+} 浓度($[Ca^{2+}]_i$)的影响及维拉帕米(Ver)、噻庚啶(Cyp)和山莨菪碱(Ani)对 TNFα 诱导 $[Ca^{2+}]_i$ 变化的影响，以探讨 TNFα 介导休克和 Cyp、Ani 的抗休克的机制．**方法**：人脐静脉内皮细胞株(ECV304)接种于 35 mm 含有 2 mL DMEM 培养基的组织培养盘中培养．Fluo-3/AM 负载细胞，激光扫描共聚焦显微技术测定单个内皮细胞 $[Ca^{2+}]_i$．**结果**：TNFα 使单个内皮细胞 $[Ca^{2+}]_i$ 呈剂量依赖性升高，在 60 s 内达到峰值，然后下降并保持在基础水平之上．共聚焦扫描图像显示细胞核区 $[Ca^{2+}]_i$ 升高比胞浆区明显，下降比胞浆区慢．维拉帕米 1 和 2，噻庚啶 30 和 60 或山莨菪碱 20 和 40 μmol/L 均能显著抑制由 TNFα 1.2 nmol/L 诱导的单个内皮细胞 $[Ca^{2+}]_i$ 升高．**结论**：TNFα 显著诱导内皮细胞 $[Ca^{2+}]_i$ 升高，可能是 TNFα 介导休克的重要机制；维拉帕米、噻庚啶和山莨菪碱对 TNFα 诱导的 $[Ca^{2+}]_i$ 升高有拮抗作用，可能是噻庚啶和山莨菪碱抗休克作用的机制之一．

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