

Original Paper

Ethylenediaminetetraacetic Acid Inhibits *Vibrio Vulnificus*-Induced Dendritic Cell Apoptosis by Lowering $[Ca^{2+}]_i$

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Key Words

Vibrio vulnificus • Dendritic cell • EDTA • $[Ca^{2+}]_i$ • STAT3 • m-Tor • Bcl-2

Abstract

Background/Aims: *Vibrio vulnificus* (*V. vulnificus*) is a Gram-negative marine bacterium that can cause life-threatening primary septicemia, especially in the innate immune system. But how *V. vulnificus* affects and acts on dendritic cells (DC) is not well understood. The aim of the present study is to investigate $[Ca^{2+}]_i$ change and the expression of the mTor-STAT3-Bcl-2 signaling pathway in *V. vulnificus* B2-induced DC apoptosis, and explore the protective effect of ethylenediaminetetraacetic acid (EDTA) against DC apoptosis in a *V. vulnificus* B2 and DC2.4 cell coculture infection model, using EDTA as an intervenient. **Methods:** The apoptosis rate, $[Ca^{2+}]_i$, and the expression of STAT3, m-Tor and Bcl-2 were detected by cytometry, Fluo-8-AM and Western blotting respectively. **Results:** The results demonstrated that EDTA inhibited the increase of $[Ca^{2+}]_i$, upregulated the expression of m-Tor-STAT3-Bcl-2 signaling pathway, and protected DC against *V. vulnificus* B2-induced apoptosis. **Conclusions:** EDTA inhibits *V. Vulnificus*-induced DC apoptosis by lowering $[Ca^{2+}]_i$ via m-Tor-STAT3-Bcl-2 signaling pathway.

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Introduction

Vibrio vulnificus (*V. vulnificus*) is a species of Gram-negative, motile, curved and rod-shaped (bacillus) pathogenic bacteria of the genus *Vibrio* and present in marine environments such as estuaries, brackish ponds, or coastal areas [1]. Infection with *V. vulnificus* leads to rapidly expanding cellulitis (acute gastroenteritis and necrotizing wound infections) or invasive septicemia [2]. *V. vulnificus* infection is known to stimulate the production of several pro-inflammatory cytokines, which are associated with inflammatory responses mediated predominantly by dendritic cells (DCs), functioning as antigen-presenting cells of

the mammalian immune system to process antigen materials and present them on the cell surface to T cells of the immune system [1, 3].

DCs act as messengers between the innate and the adaptive immune systems. DCs are present in tissues in close contact with the external environment such as the skin (where there is a specialized DC type called Langerhans cell) and the inner lining of the nose, lungs, stomach, and intestines [4]. They can also be found in an immature state in the blood. Once activated, they migrate to lymph nodes where they interact with T cells and B cells to initiate and shape the adaptive immune response [5]. At certain developmental stages, they grow branched projections known as dendrites. Stimulating DCs with microbial extracts *in vivo* caused DCs to rapidly begin producing IL-12, a signal that helps send naive CD4⁺ T cell towards a T_H1 phenotype [6]. The ultimate consequence is priming and activation of the immune system for attack against the antigens that DCs present on their surface [7]. Previous studies demonstrated that *V. vulnificus* could get access into DCs by pinocytosis and localized at 1-2 µm of the inner side membrane [8], directly causing disruption of the DCs cytoskeleton structure.

Ethylenediaminetetraacetic acid (EDTA), an aminopolycarboxylic acid and a colorless, water-soluble solid with a hexadentate ligand and chelating agent, is able to sequester metal ions such as Ca²⁺ and Fe³⁺ [9]. After being bound by EDTA into a metal complex, metal ions remain in solution but exhibit diminished reactivity. EDTA is a slime dispersant and has been found to be highly effective in reducing bacterial growth during implantation of intraocular lenses [10]. Studies found that the surfactant protein A (SP-A), an important innate immune factor of the lung, bound to human immunodeficiency virus (HIV) in a calcium-dependent manner, and this process could be inhibited by EDTA [11]. EDTA could inhibit the enzyme activity of the extracellular metalloprotease of *V. vulnificus* [12], but the mechanism underlying its antibacterial activity remains unclear.

In the present study, we sought to investigate the antibacterial mechanism of EDTA in *V. vulnificus*-infected DCs, and discovered that the intracellular Ca²⁺ concentration ([Ca²⁺]_i) signaling was a critical step in the pathogenesis of *V. vulnificus*-induced DC apoptosis.

Materials and Methods

Cell and *V. vulnificus* Culture

Murine DC 2.4 cells (Suer, Shanghai, China) maintained in Roswell Park Memorial Institute (RPMI) 1640 minimum essential medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified incubator with 5 % CO₂ were seeded onto 96-well plate at a density of 5.6×10⁵/mL.

V. vulnificus strain B2 was cultured in Columbia blood agar base medium at 37°C for 24 h until the late logarithmic growth phase. Bacteria were collected, washed with phosphate buffer saline (PBS), and then suspended with RPMI 1640 medium (2.5×10⁷ colony-forming units/mL [CFU/mL]) [13]. *V. vulnificus* B2 and DC 2.4 cells (80:1) were co-cultured at 37°C for 4h. A total of 5 µM EDTA was used to chelate extracellular Ca²⁺. DC 2.4 cells cultured in normal culture medium without bacteria were used as negative control.

Analysis of Apoptosis by Flow Cytometry

The apoptosis rate in different groups was analyzed by Annexin V-FITC/PI double stain assay using Cytomics FACSVerse (BD, USA) according to the manufacturer's instructions (Zoman, Beijing, China).

[Ca²⁺]_i Measurement

[Ca²⁺]_i was measured with a Fluo-8-AM fluorescent probe (Invitrogen, CA). Cells were washed three times after bacterial contagion and then incubated with 5 µM Fluo-8-AM for 40 min at 37°C. Ca²⁺-dependent fluorescence was monitored under a fluorescence microscope with the emission wavelength set at 527 nm and the excitation wavelength at 488 nm. For analysis, a region of interest (ROI) that applied to all images was determined by threshold foreground pixels, and these foreground pixels were spatially averaged to

calculate F for each cell. The background was calculated as F0. Levels of $[Ca^{2+}]_i$ were expressed as F/F0 ratios.

Western Blotting

The western blotting analysis was performed as previously described [14, 15]. Briefly, an equal amount of proteins was loaded and fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, MA). Blots were blocked with 5% dried skimmed milk and then incubated with primary antibody in Tris-buffered saline containing 0.1% Tween 20 (TBST) at 4 °C overnight. After being rinsed in milk-TBST, blots were incubated in the horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), reacted with chemiluminescent substrate (Pierce, IL), and then exposed to film (Kodak, NY). Film signals were digitally scanned and quantitated using Image J (NIH, MD).

Statistical Analysis

Data are expressed as mean \pm standard error of mean (SEM). Statistical differences were determined using one-way analysis of variance (ANOVA) test. A P-value <0.05 was considered statistically significant.

Results

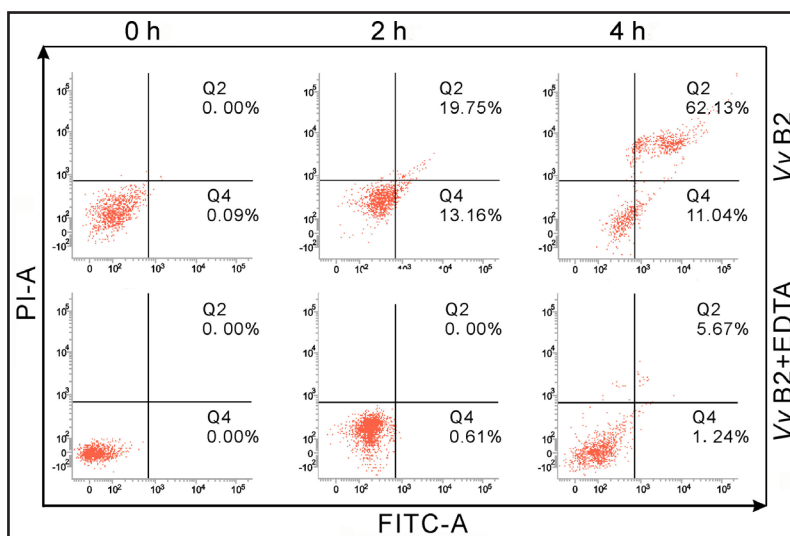
EDTA Inhibits *V. vulnificus* B2-induced DC2.4 Cell Apoptosis

V. vulnificus B2 has high cytotoxicity to DC2.4 cells compared with the environmental isolates [8]. As shown in Fig. 1, *V. vulnificus* B2 caused apoptosis of DC2.4 cells in a time-dependent manner. DC2.4 cell apoptosis was increased markedly 2 and 4 h after bacterial co-culture compared with that at 0 h. In contrast, DC2.4 cell apoptosis remained unchanged significantly after addition of EDTA, indicating that EDTA inhibited *V. vulnificus* B2-induced DC2.4 cell apoptosis.

EDTA Inhibits Ca^{2+} Influx during *V. vulnificus* B2 Contagion in DC2.4 Cells

Knowing that extracellular Ca^{2+} is an important source to raise $[Ca^{2+}]_i$ and increased $[Ca^{2+}]_i$ can trigger and accelerate apoptosis [16], we detected the $[Ca^{2+}]_i$ change after bacterial contagion and EDTA incubation using the Fluo-8-AM to see whether EDTA could inhibit extracellular Ca^{2+} influx. As shown in Fig. 2, the fluorescent intensity was increased significantly in *V. vulnificus* B2 contagion, while this increase was inhibited by EDTA. There was no significant change in $[Ca^{2+}]_i$ at different time points when *V. vulnificus* B2 was co-

Fig. 1. Effect of EDTA on *V. vulnificus* B2-induced DC2.4 cell apoptosis. After treatment, cells were incubated with FITC-A and PI-A and subjected to flow cytometric analysis. The apoptosis cells were detected as FITC-A positive (Q2 and Q4). Vv B2 represents DC 2.4 cells co-cultured with *V. vulnificus* B2; Vv B2+EDTA represents the addition of 5 μ M EDTA to the co-cultured cells.



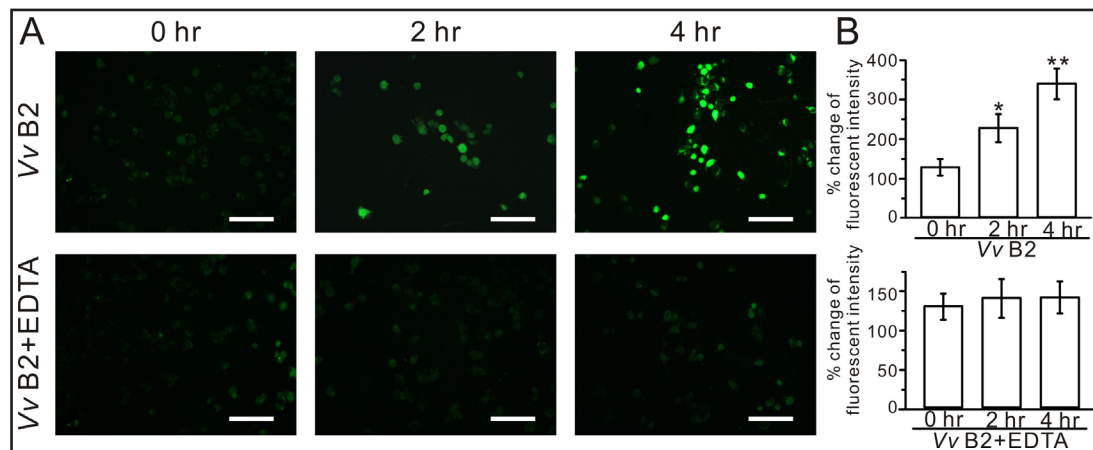
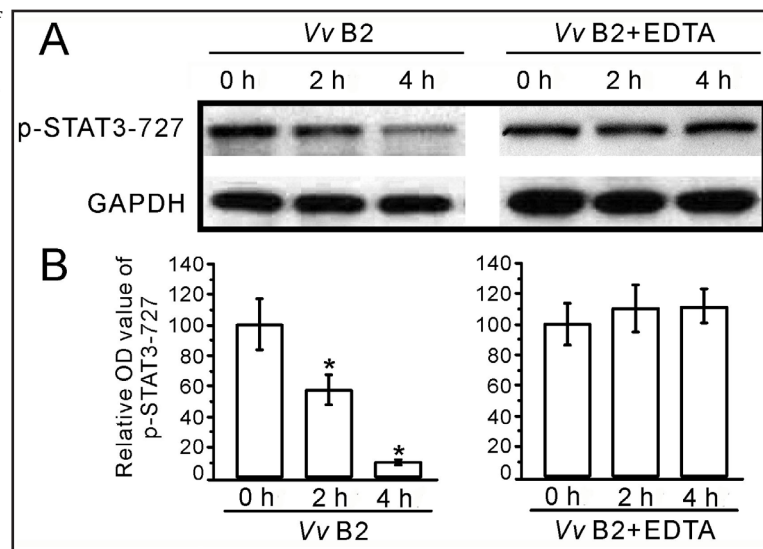


Fig. 2. EDTA inhibited Ca^{2+} influx during *V. vulnificus* B2 infection. (A) Cell fluorescence; (B) Quantitative analysis. Fluo-8-AM was used to detect the change of $[\text{Ca}^{2+}]_i$. Fluorescent intensity of Ca^{2+} was increased after the co-culture with *V. vulnificus* B2, but there was no notable difference in *V. vulnificus* B2 and EDTA co-culture. Scale bar: 100 μm.

Fig. 3. Expression of p-STAT3-727 in DC 2.4 cells at the different time points. * $P < 0.05$ vs. 0 h.

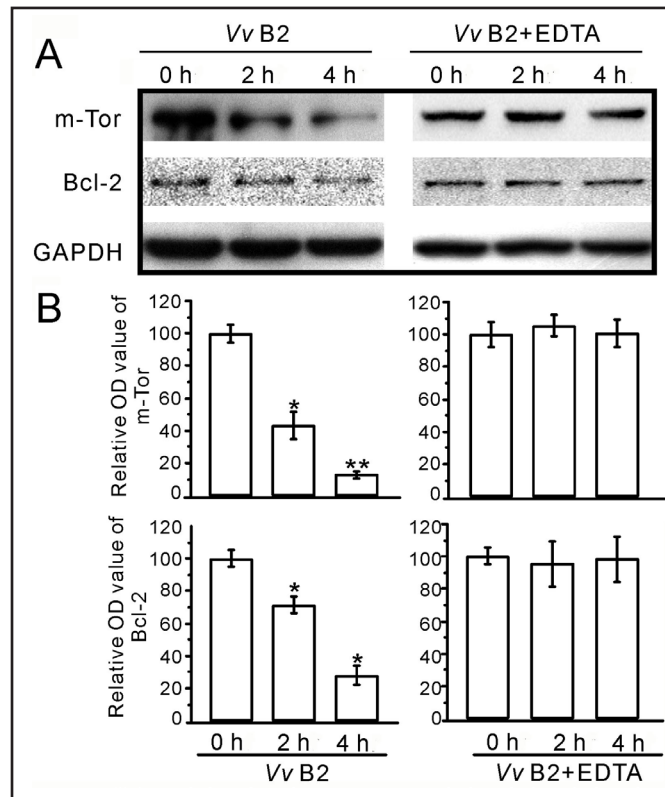


cultured with EDTA, suggesting that EDTA blocked Ca^{2+} influx during *V. vulnificus* B2 contagion in DC2.4 cells and inhibited cell apoptosis.

STAT3 was Involved in *V. vulnificus* B2-induced DC2.4 Cell Apoptosis

Recent studies demonstrated that STAT3, a key target of many anticancer drugs, was constitutively activated in the anti-apoptotic possesses [17-19], and STAT3 molecular was involved in the apoptosis induced by environmental isolation of *V. vulnificus* [13], we, therefore, hypothesized that STAT3 molecular may also be involved in *V. vulnificus*-induced DC2.4 cell apoptosis. We detected the expression of phosphorylation of STAT3 at Ser727 (p-STAT3-727) which regulated the transcriptional activation and found that the expression of p-STAT3-727 was significantly decreased during *V. vulnificus* B2 contagion. However, after EDTA chelating extracellular Ca^{2+} , the expression of p-STAT3-727 remained unchanged significantly at 2 h and 4 h compared with the control (Fig. 3), suggesting that STAT3 may play an important role in the process of *V. vulnificus*-induced DC2.4 cell apoptosis, and the inhibited Ca^{2+} influx blocked the decrease of p-STAT3-727 expression.

Fig. 4. *V. vulnificus* B2 regulated m-Tor and Bcl-2 expression in the apoptosis processes. * $P < 0.05$, ** $P < 0.01$ vs. 0 h.



EDTA Inhibited Bcl-2 through m-Tor Signaling Pathway

Knowing that phosphorylation at Ser727 of STAT3 was regulated by m-Tor signaling pathway [20], we detected the expression of m-Tor at a different time of *V. vulnificus* B2 contagion to determine whether m-Tor signaling pathway participated in *V. vulnificus*-induced DC2.4 cell apoptosis. As shown in Fig. 4, m-Tor expression was dramatically reduced after co-culture with *V. vulnificus* B2 and DC2.4 cell, while the expression of m-Tor did not show any significant change compared with that in co-culture with *V. vulnificus* B2 and EDTA.

Several studies have shown that the STAT3 signaling pathway regulated the expression of apoptosis-related proteins such as Bcl-2 family members [21]. We further detected Bcl-2 expression. It was found that the expression of Bcl-2 was decreased after *V. vulnificus* B2 contagion, while the level of Bcl-2 had no notable change after the use of EDTA (Fig. 4). These findings indicate that the increased $[Ca^{2+}]_i$ was associated with the down-regulation of m-Tor, STAT3 and Bcl-2 during the *V. vulnificus* B2-induced DC2.4 cell apoptosis, and EDTA could inhibit this process through blocking Ca^{2+} influx.

Discussion

V. vulnificus is highly invasive, causing rapidly damage to the inherent immunity following apoptosis of lymphocyte, macrophagocyte and DCs [13, 22-25]. These cells are critical parts of the barrier in the immune system, and also closely associated with adaptive immunity [3, 26-28]. Therefore, the knowledge about how to make this bacterium successfully induce cell death may help better understand the virulence of this significant human pathogen. Accumulating evidence indicates that virulence factors of *V. vulnificus* are responsible for the remarkable disease process [2, 18, 29-32], and many kinds of them induce the increase of $[Ca^{2+}]_i$, suggesting that alternations in calcium levels contribute to cell death [33, 34]. EDTA is used extensively as an anticoagulant for blood samples in blood biochemical analysis. However, EDTA exhibits low acute toxicity with the rat median lethal dose (LD50) of 2.0-2.2

g/kg [35]. It has been found to be both cytotoxic and weakly genotoxic in laboratory animals. Excessive exposures of EDTA have been noted to cause reproductive and developmental effects [36].

In the present study, we examined the characteristics initiated by Ca^{2+} in *V. vulnificus* B2-induced DC 2.4 cell apoptosis and found that *V. vulnificus* B2 caused calcium influx and induced cellular apoptosis, and this process could be inhibited by EDTA. Therefore, an increase in $[\text{Ca}^{2+}]_i$ is an initiator of apoptosis in *V. vulnificus* B2 infection.

Ca^{2+} regulates many signaling pathways such as protein kinase C (PKC), extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), which can mediate apoptosis [37, 38]. We found that m-Tor and STAT3 signaling pathway were involved in DC apoptosis induced by *V. vulnificus* B2, where m-Tor expression was inhibited, while p-STAT-727 expression was activated, eventually downregulating Bcl-2 expression. Disequilibrium of Ca^{2+} seems to be a trigger and m-Tor-STAT3-Bcl-2 signaling pathway may participate in cell apoptosis. EDTA could sufficiently inhibit Ca^{2+} influx and the inactivation of the m-Tor-STAT3-Bcl-2 signaling pathway.

In summary, blocking excessive $[\text{Ca}^{2+}]_i$ has a protective effect during the apoptosis initiation, and therefore the m-Tor-STAT3-Bcl-2 signaling pathway regulated by $[\text{Ca}^{2+}]_i$ may prove to be a potential therapeutic target for acute *V. vulnificus* B2-induced DC apoptosis and death.

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Disclosure Statement

All the authors declare no commercial or financial conflicts of interest.

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