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Role of potassium channels in chlorogenic acid-induced apoptotic volume decrease and cell cycle arrest in *Candida albicans*

JiEun Yun, Dong Gun Lee *

School of Life Sciences, BK 21 Plus KNU Creative BioResearch Group, College of Natural Sciences, Kyungpook National University, 80 Daehakro, Bukgu, Daegu 41566, Republic of Korea.

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ABSTRACT

Background: Chlorogenic acid (CRA) is an abundant phenolic compound in the human diet. CRA has a potent antifungal effect, inducing cell death in *Candida albicans*. However, there are no further studies to investigate the antifungal mechanism of CRA, associated with ion channels.
Methods: To evaluate the inhibitory effects on CRA-induced cell death, *C. albicans* cells were pretreated with potassium and chloride channel blockers, separately. Flow cytometry was carried out to detect several hallmarks of apoptosis, such as cell cycle arrest, caspase activation, and DNA fragmentation, after staining of the cells with SYTOX green, FITC-VAD-FMK, and TUNEL.
Results: CRA caused excessive potassium efflux, and an apoptotic volume decrease (AVD) was observed. This change, in turn, induced cytosolic calcium uptake and cell cycle arrest in *C. albicans*. Moreover, CRA induced caspase activation and DNA fragmentation, which are considered apoptotic markers. In contrast, the potassium efflux and proapoptotic changes were inhibited when potassium channels were blocked, whereas there was no inhibitory effect when chloride channels were blocked.
Conclusions: CRA induces potassium efflux, leading to AVD and G2/M cell cycle arrest in *C. albicans*. Therefore, potassium efflux via potassium channels regulates the CRA-induced apoptosis, stimulating several apoptotic processes.
General significance: This study improves the understanding of the antifungal mechanism of CRA and its association with ion homeostasis, thereby pointing to a role of potassium channels in CRA-induced apoptosis.

1. Introduction

Many studies have shown that consumption of foods with high phenolic content helps to prevent several human diseases. Chlorogenic acid (CRA), formed via esterification of caffeic and quinic acids, is an abundant phenolic compound in the human diet and is present in apples, pears, berries, and coffee [1]. The health effects of CRA have been proved in vivo, where the absorbed fraction of CRA enters the blood circulation and triggers several biological effects [2]. CRA inhibits carcinogenesis in the colon, liver, and tongue and reduces the risk of cardiovascular disease by decreasing the oxidation of low-density lipoprotein cholesterol and total cholesterol [1]. In addition, CRA has a broad range of biological activities such as antiobesity, antimutagenic, antioxidant, and antimicrobial [2,3]. Its antifungal effect, in particular, has been in the spotlight for several years because of its efficacy against fungal infections. Lee et al. studied the antiarthritic effect of CRA in mice with septic arthritis caused by *Candida albicans*, and Sung et al. demonstrated the antifungal mechanism of action of CRA mediated by membrane disruption [4,5].

Regulation of cytoplasmic and intraorganellar ion concentration is essential for the maintenance of membrane potential and control over cell volume. Ion channels, including potassium, calcium, sodium, and chloride channels, play a major role in the regulation of ionic homeostasis and are located within the plasma membrane [6]. Many studies have shown that potassium and chloride channels are involved in several physiological processes, such as cell volume regulation, acidification, cell cycle progression, and apoptosis in eukaryotic cells [7]. The presence or inhibition of ion channels mainly affects the induction of apoptosis, which is a fundamental cellular mechanism for elimination of unwanted cells and for cellular homeostasis [6]. Andres et al. demonstrated the role of ion channels in the apoptosis of *C. albicans*. Cell death induced by human lactoferrin was found to be associated with a release of high concentrations of potassium. In contrast, blocking the potassium and chloride channels inhibits apoptosis, and the cell volume reduction is attenuated [7].

An appropriate cell size is crucial for the health of cells. Hence, maintaining ionic concentration gradients is important for adaptation to anisotonic conditions. Usually, most cells have inherent volume-regulatory mechanisms known as regulatory volume decrease (RVD) and

Abbreviations: AVD, apoptotic volume decrease; CRA, chlorogenic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt; TEA, tetraethylammonium chloride.

* Corresponding author.
E-mail address: dglee222@knu.ac.kr (D.G. Lee).

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regulatory volume increase (RVI) responses, which control and maintain the normal cell size. In addition, they return the cell volume to normal size after cellular processes influencing the cell size [8,9]. Nonetheless, cells can undergo a unique and irreversible cell volume decrease under anisotonic conditions. The apoptotic volume decrease (AVD), characterized by the loss of cell volume or by cell shrinkage, is the physiological and morphological hallmark of apoptosis [10]. Cell volume homeostasis is maintained by a constant ratio of positive and negative ions, which makes the cell electrically neutral. Therefore, both monovalent cations and anions participate in AVD. Induction of AVD by the loss or gain of potassium and chloride ions has been demonstrated in several studies [8]. AVD usually occurs within 0.5–2.0 h after apoptotic stimulation and triggers a cytochrome *c* release, caspase activation, and DNA fragmentation [9].

Our previous study has revealed that CRA induces membrane depolarization in *C. albicans* [5]. On the basis of this finding, we hypothesized that CRA induces a decrease in cell volume, with the involvement of ion channels, during apoptosis. Therefore, the function of ion channels in CRA-induced cell death and several other hallmarks of apoptosis such as AVD, cell cycle arrest, caspase activation, and DNA fragmentation were investigated in this study by pretreatment of *C. albicans* cells with ion channel blockers.

2. Material and methods

2.1. CRA

CRA was obtained from Sigma (St. Louis, MO, USA), and its purity was higher than 98%. Prior to its use in experiments, CRA was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/mL.

2.2. C. albicans and culture conditions

C. albicans (ATCC 90028) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *C. albicans* cells were grown on yeast extract peptone dextrose (YPD; Difco, Sparks, MD, USA) agar plates and cultured for 15 h at 28 °C in YPD broth before use in experiments.

2.3. Antifungal activity of CRA

C. albicans cells (1×10^6 cells/mL) were inoculated into YPD and then dispensed into microtiter plates. The minimum inhibitory concentration (MIC) was determined by a standard microdilution method. After incubation with CRA for 12 h, the growth was measured using a microtiter ELISA Reader (Molecular Devices Emax, CA; Table 1).

2.4. Ion channel blockers and CRA treatment

Cells were washed with phosphate buffered saline (PBS). Tetraethylammonium chloride (TEA) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt (DIDS) were used as potassium and chloride channel blockers, respectively (Sigma, St. Louis, MO, USA). The cells, preincubated with TEA and DIDS for 15 min at 28 °C were washed with PBS, and then treated with CRA at 4 × MIC (320 μg/mL) for 2 h at 28 °C [7].

Table 1
The antifungal activity of chlorogenic acid on *Candida albicans*.

	MIC (μg/mL)	
Fungal strain	Chlorogenic acid	H_2O_2
Candida albicans ATCC 90028	80	20

2.5. Determination of cell viability

To evaluate the effects of ions, the cells were preincubated with 2.5, 5, 10, and 20 mM TEA and 0.25, 0.5, 1, and 2 mM DIDS. After incubation with CRA, the samples were serially diluted and spread on YPD agar plates. The plates were incubated for 24 h at 28 °C, and cell viability was expressed in colony-forming units [11].

2.6. Detection of external potassium levels

To detect the external potassium levels, the samples were centrifuged after incubation with CRA for 30-min intervals and the supernatants were transferred to 24-well plates. Potassium ionic strength adjuster and distilled water were added up to 3 mL. After 10-min incubation for stabilization, potassium voltage was measured using an ion-selective electrode meter (Orion Star A214; Thermo Scientific, Singapore), and the external potassium level (expressed in percentage points) was calculated by means of the following formula: External potassium level (%) $= 100 \times ([K^+] - [K^+]_0) / ([K^+]_t - [K^+]_0)$, where $[K^+]$ represents the potassium voltage of the treated samples, and $[K^+]_0$ and $[K^+]_t$ denote potassium voltage of the untreated samples and sonicated samples, respectively. Sonication was performed to determine total external potassium voltage [12].

2.7. AVD

To confirm the decrease in cell volume by CRA, the changes in forward scatter (FS) and side scatter (SS) were analyzed by flow cytometry as follows. After incubation with CRA, the cell samples were washed with PBS. Unstained live cells were analyzed in each sample by determining their position on FS versus SS contour plots using a FACSVerse flow cytometer (Becton Dickinson, NJ, USA) [13].

2.8. Detection of cytosolic calcium levels

To detect the cytosolic calcium levels, the calcium-sensitive fluorescent dye Fura-2AM was used. After incubation with CRA, the samples were washed twice with Krebs buffer (pH 7.2), and resuspended in a buffer consisting of Krebs buffer containing 0.01% Pluronic F-127 and 1% bovine serum albumin. The samples were then incubated with Fura-2AM for 40 min and washed thrice with calcium-free Krebs buffer (pH 7.2). The fluorescence intensity was measured on a spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu, Kyoto, Japan) [14].

2.9. DNA content analysis

To confirm the cell cycle arrest caused by CRA, DNA content of the cells was analyzed on the FACSVerse flow cytometer (Becton Dickinson). After incubation with CRA, the cell samples were washed with PBS and fixed overnight at 4 °C in 70% ethanol. The cells were then washed twice with 50 mM sodium citrate buffer and treated with 0.2 g/mL RNase A. After that, the samples were incubated for 2 h at 37 °C with constant shaking and washed twice with sodium citrate buffer. The DNA-sensitive fluorescent probe SYTOX green was used for DNA staining [15].

2.10. Measurement of caspase activity

To evaluate the caspase activity, a fluorescein isothiocyanate (FITC)-conjugated pancaspase inhibitor, VAD-FMK, was used. After incubation with CRA, the cell samples were washed twice and incubated with CaspACE FITC-VAD-FMK for 20 min. The fluorescence intensity was analyzed on the FACSVerse flow cytometer (Becton Dickinson) [16].