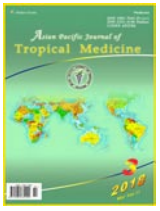

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Chloride channel involved in the regulation of curcumin-induced apoptosis of human breast cancer cells

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ABSTRACT

Objective: To investigate the role of ClC-3 chloride channel in the proliferation of breast cancer cell line Mcf-7 treated with curcumin and its specific mechanism. **Methods:** MTT assay was used to detect the effect of chloride channel blocker (DIDS) and curcumin on Mcf-7 and human normal cell viability. Patch-clamp technique was used to determine the current density before and after drug treatment. Apoptosis assay by flow cytometry was performed for further examination of cell apoptosis. **Results:** Curcumin had toxicity on Mcf-7 and HUVEC cells and DIDS reduced the survival rate of Mcf-7 cells by inhibiting proliferation. Curcumin could activate the chloride ion current on MCF-7 cell membrane, which would be inhibited by DIDS. Finally, curcumin in low concentration combined with DIDS could significantly promote the MCF-7 cells apoptosis. **Conclusions:** Our results suggest that ClC-3 protein is involved in the regulation of curcumin induced proliferation inhibiting in breast cancer cells through inducing cell apoptosis. ClC-3 may be a potential target of tumor therapy.

1. Introduction

Breast cancer is the main health problem of women around the world, which is the main cause of cancer death in women[1]. Many breast cancer patients fail in the face of conventional treatment strategies: chemotherapy, radiation and anti-estrogen therapy[2]. Therefore, it is essential to search for new effective drugs and targets for the treatment of breast cancer. There is a need to study molecular pathways and biomarkers associated with breast cancer development to find successful treatments.

As we all know, traditional Chinese medicine curcumin has hypolipidemic, antioxidant, anti-inflammatory, anti-atherosclerosis and other pharmacological activities[3,4]. In addition, curcumin can induce cell apoptosis. For example, Sha Jian *et al*. reported that curcumin could induce cell apoptosis in hormone independent prostate cancer DU-145 cells[5]. Curcumin can also lead to mitochondrial swelling, calcium release, respiratory injury and mitochondrial membrane potential collapse. And these series of events gave rise to mitochondrial released of apoptosis factors and lead to cell apoptosis[6]. In recent years, anti-tumor effect is another major pharmacological activity of curcumin, which has become a recent research focus on anti-cancer drug research and development[7,8]. Studies have shown that curcumin can regulate the

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expression of I κ B α , c-Jun and androgen receptors in prostate cells to induce cell cycle arrest and cell apoptosis[9]. Li *et al*. reported that curcumin exerts its anti-cancer effect in human breast cancer cells through cell cycle arrest in G1 phase[10]. However, the specific mechanism ofcurcumin to cause tumor cell cycle arrest and cell apoptosis is not yet fully understood.

In the process of tumor development, ion channels expression and activity change and this abnormal expression and activity changes are closely related to tumor cell proliferation and apoptosis. Chloride channel (ClC) is an anion channel, which is a channel protein, distributed in the cell and organelles membrane and presented permeability to the chloride ion or other anion. ClC type chlorine channels refer to the voltage-gated chloride channels, which are widely presented in the body's cell membrane and organelles membrane, involved in a variety of cell activity and functional regulation process. For example, ClC-3 plays a major role in hyperglycemia induced hippocampal neuronal apoptosis with its increased expression[11]. Furthermore, ClC-3 chloride channel isan important regulator of cell apoptosis in human airway epithelial cells[12]. Studies have confirmed that ClC-3 affects tumor cell proliferation in tumor cells[13]. The pathogenesis of the tumor is complicated, and the entry points of the tumor study are numerous. Abnormally expression of potassium, calcium and chloride channels in tumor cellsis closely related to the development of tumor. The possible mechanism is to regulate cell membrane potential, cell cycle, cell volume, intracellular calcium concentration and cytoplasmic pH to control tumor cell proliferation and cell apoptosis. Studies have shown that the treatment of cancer can be innovative to the perspective on the ion channel. In certain specific tumors (including breast cancer), this method of tumor treatment has achieved significant results[14]. Studying the tumor through the ion channel perspective has become a research hot spot at home and abroad. The relationship between chloride channel and tumor has aroused people's attention and there has beena better understanding of the role of chloride channel in tumor formation, progression and metastasis. It not only helps to better elucidate the pathogenesis of tumor, but may also provide new target and new strategy for tumor prevention and treatment of biological intervention.

Combined with the work of predecessors, curcumin antitumor activity is likely to be related to chloride channels. Therefore, the purpose of this study is to investigate the role of ClC-3 chloride channel in the proliferation of Mcf-7 cells treated with curcumin in order to clarify the effect of curcumin on breast cancer cell line Mcf-7 cells and its specific mechanism.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell line MCF-7 was maintained at 37 ℃ under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin. The cell line was purchased from the American Type Culture Collection. Cells were observed by inverted microscope and passaged about 2-3 d. Then the cells in logarithmic growth phase were taken for formal experiments.

2.2. MTT assay

The logarithmic growth phase cells were selected and DMEM complete culture medium prepared into cell suspension, per well 100 μL, 2×10^4 / mL density inoculated in 96-well culture plates. After the cells completely adhered to the wall, different reagents were added and cultured for 24 h and 48 h. MTT 10 μL (5 mg/mL) was added to each well and incubated for another 4 h until the formation of granular blue-purple crystal. Supernatant was aspirated carefully and added DMSO 100 μL/well to dissolve the crystals, shaking in the micro-oscillator for 5 min. Then the absorbance (expressed as optical density, OD) was recorded at 570 nm by a microplate reader. Each experiment was repeated three times. Cell survival rate = OD value (drug treatment group) / OD value (control group) × 100%.

2.3. Electrophysiology

As described in Chen *et al*.[15] and Bai *et al*.'s[16] work, whole-cell chloride currents were recorded using the patch-clamp technique. When filled with the pipette solution, the patch-clamp pipettes showed a 4–5 MΩ resistance. And the liquid junction potential was corrected while the pipette entering the bath and the access resistance was compensated. After adjusting and minimizing the capability transients in response to a 20 mV voltage step, the whole-cell capacitance was determined. Cells were held at the Cl-equilibrium potential (0 mV) and then stepped in sequence to ±80, ±40, 0 mV repeatedly, with a 200 ms duration for each step and 4 s intervals between steps. Using a laboratory interface (CED 1401, Cambridge, UK), datawas sampled at 3 kHz and collected. Through dividing the whole-cell current with the membrane capacitance in each individual cell, the current density was determined.

2.4. Apoptosis assay by flow cytometry

Suspension cells were collected directly into 10 mL centrifuge tubes, and the number of cells was (1-5) $\times 10^6$ /mL. Then centrifuge was carried out in 2.500-1 000 r / min for 5 min to discard the culture medium. Cells were washed once with incubation buffer and centrifuged for another 5 min, then resuspended with 100 uL labeled solution. Incubated at room temperature for 10-15 min, the cell suspension was centrifuged for 5 min in 500-1 000 r/min and the cell sedimentation was washed with incubation buffer. Fluorescence solution (SA-FLOUS) was addedand flow cytometry was used to detect FITC fluorescence with a bandpass filterand PI fluorescencewith another filter.

2.5. Chemicals and solutions

The patch clamp pipette solution contained (in mmol/L): 70 N-methyl-D-glucosamine-chloride, 1.2 $MgCl_2$, 10 HEPES, 1 EGTA,

140 D-mannitol and 2 ATP. The isotonic bath solution contained (mmol/L): 70 NaCl, 0.5 $MgCl_2$, 2 $CaCl_2$,10 HEPES and 140 D-mannitol. The osmotic pressure of the pipette and isotonic bath solution was adjusted to 300 mOsmol/L with D-mannitol. 47% of the hypotonic solution (160 mOsmol/L) was obtained by omitting 140 D-mannitol from the isotonic bath solution. Respectively, the pH of the pipette and bath was adjusted to 7.25 and 7.4. Curcumin and the chloride channel blockers 4,4'-disothiocya-nostibibene-2,2'-disulfonic acid (DIDS) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 12.5 and 100 mmol/L. All chemicals were available from Sigma.

2.6. Statistical analysis

Data were analyzed using independent sample *t* tests or two-way ANOVA when appropriate. For all quantitative data, statistical analyses were performed using SigmaPlot software. All data are expressed as the mean ± SEM. Values of $P< 0.05$ were considered significant.

3. Results

3.1. Curcumin had toxicity on Mcf-7 and HUVEC cells, which reduced cell viability by inhibiting proliferation

The results showed that curcumin significantly inhibited the viability of MCF-7 and human umbilical vein endothelial cells (HUVEC) in a dose-dependent manner. Here we regarded HUVEC as human normal cell to study. The inhibitory rate of curcumin at the concentration of 25 μmol/L on MCF-7 cells was significantly different from the control group in 24 h and 48 h ($P<0.01$) (Table1). In HUVEC, cell viability in curcumin group at the concentration of 12.5 μmol/L in 24 h was significantly different from the control group ($P<0.05$), and the difference in concentration was highly significant in 48 h ($P<0.01$) (Table 1).

Table 1

Cell viabilities of the curcumin on Mcf-7 proliferation and HUVEC cell proliferation (100%).

Group		Percentage change for control on MCF-7		Percentage change for control on HUVEC	
		24 h	48 h	24 h	48 h
Control		100.00	100.00	100.00	100.00
Curcumin (μmol/L)	6.25	95.06	88.11	98.75	94.52
	12.5	94.37	80.42	94.80*	88.80**
	25	52.62**	38.45**	77.31**	85.69**
	50	33.84**	19.03**	58.16**	72.05**
	100	19.29**	10.44**	22.58**	28.13**

Relative cell number was detected by the MTT assay and expressed as the percentage (percentage change of cell viability for control: presents the percentage change for control of curcumin at the concentration of 6.25, 12.5, 25, 50 and 100 μmol/L in Mcf-7 cellsand HUVEC cells in 24 h and 48 h. Data in the table are expressed as mean±SE of four experiments.*$P< 0.05$, **$P< 0.01$ compared to control group.

3.2. DIDS reduced the survival rate of Mcf-7 cells by inhibiting proliferation, but it had no significant effect on HUVEC cells

Within 24 h, cell viability of DIDS in Mcf-7 cells was close to 80% at the concentration of 100 μmol/L, which was close to 60% at that concentration within 48 h. Both of them were significantly different from that of the control group ($P<0.01$) (Table 2). DIDS had no significant effect on HUVEC cells, and there was no significant difference at each time point (Table 2). According to the results above, we have learnt that curcumin significantly inhibited the survival rate in MCF-7 cells in a dose-dependent manner, but at the same time curcumin had the same killing effects on normal cells. Therefore, we selected curcumin at a low dose (12.5 μmol/L) to explore the MCF-7 cell apoptosis at the presence of DIDS (100 μmol/L). At a dose of 100 μmol/L, DIDS had an effect on MCF-7 cells, but most of the cells were still viable.

Table 2

Cell viabilities of the DIDS on Mcf-7 cell proliferation and HUVEC cell proliferation (100%).

Group		Percentage change for control on MCF-7		Percentage change for control on HUVEC	
		24 h	48 h	24 h	48 h
Control		100.00	100.00	100.00	100.00
DIDS (μmol/L)	25	92.17	79.86**	104.21	98.26
	50	91.89	69.98**	106.33	100.64
	100	87.61*	61.50**	98.51	100.09
	200	71.12**	42.10**	97.72	103.81
	400	59.70**	31.53**	92.98	104.36

Relative cell number was detected by the MTT assay and expressed as the percentage (percentage change of cell viability for control): presents the percentage change for control of DIDS at the concentration of 25, 50, 100, 200 and 400 μmol/L in Mcf-7 cellsand HUVEC cells within 24 h and 48 h. Data in the table are expressed as mean±SE of four experiments.*$P< 0.05$, **$P< 0.01$ compared to control group.

3.3. Curcumin can activate the chloride ion current on MCF-7 cell membrane, which would be inhibited by DIDS

We studied the chloride currents situation on MCF-7 cell membrane before and after the addition of curcumin. As shown in Figure 1, the initial currents of the cells were very small when the isotonic solution was perfused in Mcf-7 cells. The outward currents density was (5.69 ± 2.60) pA / pF at + 80 mV while the inward currents density was (-5.21 ± 2.70) pA / pF at - 80 mV (Figure 1B). According to Figure 1A, the currents were significantly activated when using 12.5 μmol/L curcumin solution to perfuse cells. And the reversal potential of this currents was (-3.2 ± 0.5) mV (n= 18), which was close to the theoretical value of chloride ion equilibrium potential (-0.9 mV) in this experimental condition. This result suggested that the currents activated by curcumin were chlorine currents. DIDS is a commonly used chlorine channel blocker, and this experiment observed the

role of DIDS on curcumin activation currents. As shown in Figure 1A, after activation with curcumin in Mcf-7 cells, the extracellular perfusion of 100 μmol/L DIDS completely inhibited the currents. Under different clamping voltage conditions, the currents density before and after DIDS used is shown in Figure 1B, and the curcumin-activated currents decreases by (59.5 ± 11.3)% at +80 mV and (58.6 ± 12.4)% at -80 mV (n= 8; P<0.05, compared to the control). The suppression effect of DIDS on the internal and external currentswas the same. Figure 1D and 1E showed the instantaneous currents of Mcf-7 cells in perfusion of curcumin, curcumin and DIDS, respectively. The final concentration of DMSO, which used to formulate DIDS storage solution in the perfusate, was less than 1 ‰. It was said that DMSO had no effect on the activation currents of curcumin. The results further confirmed that the curcumin-activated currents were chlorine channel-mediated chlorine currents.

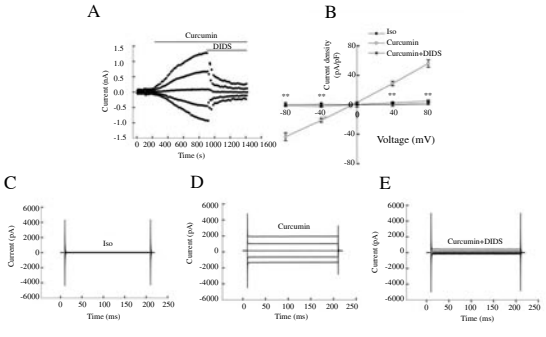

Figure 1. Activation of curcumin-induced chloride currents and inhibition of the currents by the chloride channel blockers DIDS in Mcf-7 cells.
(A & B) Cells were held at 0 mV and then stepped in sequence to ±80, ±40 and 0 mV repeatedly. Curcumin activated a chloride current which was inhibited by 100 μmol/L DIDS. (C) Traces of background currents recorded in the isotonic solution (Iso); (D)Traces of curcumin-induced currents. (E) Current traces showing inhibition of curcumin-activated currents by DIDS. Data in (B) are mean ±SE of Mcf-7 cells. $^{*}P$< 0.05, $^{**}P$< 0.01.

3.4. Curcumin in low concentration combined with DIDS could significantly promote the MCF-7 cells apoptosis

We investigated the apoptosis of Mcf-7 cells treated with curcumin and DIDS by flow cytometry. Apoptotic cells are not resistant to all dyes such as PI for cell viability, and necrotic cells cannot. Cell membrane damage cells can be stained with PI red fluorescence, and cell membrane remains intact cells will not have red fluorescence. Thus, PI is not stained early in the early stage of apoptosis without a red fluorescence signal. Normal live cells are similar (FITC- / PI-); the upper left quadrant is non-living cells, *i.e.*, necrotic cells(FITC- / PI +); and the right quadrant is apoptotic cells, appearing (FITC + / PI±).We regarded early apoptosis (upper right corner) as a reference of cell apoptosis. According to Table 3, the effects of respective use of curcumin and DIDS on the cell apoptosis in MCF-7 cells was not significant no matter in 24 h or in 48 h. However, the combination uses of curcumin and DIDS appeared a significant increase in apoptotic cells, which indicated that curcumin combined with DIDS could significantly promote the induction on Mcf-7 cell apoptosis.

Table 3

The anti-apoptosis effect in Mcf-7 cells of different treatment (control, 12.5 μmol/Lcurcumin, 100 μmol/L DIDS and curcumin+DIDS) was analyzed in a flow cytometry assay.

Treatment	Percentage of apotosis (%)	
	24 h	48 h
Control	4.71	4.46
Curcumin	5.77$^{\#\#}$	7.58$^{\#\#}$
DIDS	7.66$^{\#\#}$	12.64$^{\#\#}$
Curcumin+ DIDS	15.60**	33.82**

Percentage of apoptotic cells by quantitative analysis of curcumin and DIDS on apoptosis within 24 h and 48 h. The combinationuses of curcumin and DIDS significantly induced the cell apoptosis compared to control group, whereas, the separate treatment with curcumin and DIDS did not show the significantly different effects on cell apoptosis. $^{**}P$<0.01 versus control group, $^{\#\#}P$<0.01 versus combination group, n = 3.

4. Discussion

To correctly prove the role of Cl- channel in apoptotic process, we studied the role of ClC-3 channel by using ClC-3 blockers (4,4'-diisothiocyanostibibene-2,2'-disulfonic acid, DIDS) and the patch-clamp technique. ClC-3 chloride channel has the characteristics of outward rectification, and it plays an important role in synaptic vesicles and organelles acidification[17]. In this study, we found that curcumin affected the proliferation of Mcf-7 in a dose-dependent manner, but high doses of curcumin inhibitedproliferation of Mcf-7and had toxic effects on human normal cells at the same time. According to our observation, the use of low-dose curcumin or DIDS alone on MCF-7 did not have significant impact on cell apoptosis. To our surprise, the combination uses of curcumin and DIDS appeared a significant cellapoptosis induction. Previous study has shown that ClC-3 involved inBcl-2-dependent modulation of cell apoptosis in human prostate cancer epithelial cells[18]. Therefore, we believed that the tolerance of MCF-7 to curcumin at low doses may be related to chloride channel. The results of the patch clamps had initially confirmed our conjecture that curcumin treatment indeed activated the chloride currents. The currents could be inhibited after the addition of DIDS, which indicated that the inhibitory effect of curcumin combined with DIDS on breast cancer cell lines was probably because the activity of the chloride channelswere suppressed. The data of western blot further confirmed

our conjecture that DIDS couldindeed down-regulate the chloride channels family ClC-3 expression on the protein level. At the same time, we observed that the expressions of apoptosis-related proteins were related to the expression of ClC-3.

As we know, the mechanisms of apoptosis are very complicated. Different factors may be associated with different apoptotic signal transduction pathways to induce cell apoptosis[19]. It is widely acknowledged that death receptor-mediated pathway and the mitochondrion dependent pathway are two basic apoptotic pathways that mediate the process of cell apoptosis[20]. In basilar artery smooth muscle cells, ClC-3 chloride channel prevents apoptosis induced by hydrogen peroxide through mitochondria dependent pathway[21].

We concluded that curcumin inhibited the proliferation of MCF-7 cells in a dose-dependent manner, and the combination of curcumin and DIDS significantly enhances the induction of MCF-7 cell apoptosis and the ability to down-regulate the activation of chloride channels by curcumin. ClC-3 are involved in the regulation of curcumin-induced proliferation inhibiting in breast cancer cells through inducing apoptosis, which suggested that it could be a potential strategy for cancer therapy.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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