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ORIGINAL ARTICLE

The role of calcium, P38 MAPK in dihydroartemisinin-induced apoptosis of lung cancer PC-14 cells

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Abstract
Introduction Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin isolated from the traditional Chinese herb *Artemisia annua*, is an effective novel antimalarial drug. Recent studies suggest that it also has anticancer effect.
Purpose The present study was designed to investigate the effects of DHA on cultured human lung cancer cells (PC-14 cells) to better understand its apoptosis and apoptosis-related factors in vitro.
Methods The cell viability was measured by MTT assay. The apoptosis induction was examined by DNA ladder and flow cytometry. The intracellular-free calcium concentration in the lung cancer cells were evaluated by laser scanning confocal microscopy with Fura-3/AM as probe. The associated gene expression was examined by Western blot.
Results After treatment with DHA, a decrease in the viability of PC-14 cells and apoptosis were observed. DHA-induced apoptosis were accompanied by an increase of Ca^{2+} and activation of p38. Deleted levels of Ca^{2+} by BAPTA-AM 20 μM or inhibition of p38 by its selective inhibitor SB202190 then led to decreased DHA-induced apoptosis.
Conclusions These results demonstrated that DHA can induce apoptosis of lung cancer cell line PC-14 cells and calcium and p38 play important roles in the apoptotic signalling pathways.

Keywords Dihydroartemisinin · PC-14 cell · Intracellular calcium concentration · p38 MAPK · Apoptosis

Introduction

Lung cancer is a major public health problem worldwide. Despite recent advances in chemotherapy, lung cancer remains incurable. The effectiveness of chemotherapeutic agents is often limited by the side effects of drug treatment. There is a great necessity to discover novel agents with less severe side effects.

Apoptosis is a kind of cell death, which causes specific morphological changes and DNA fragmentation that are considered as the major cytopathologic hallmarks of the apoptotic process [1, 2]. Many current chemotherapeutic strategies are designed to induce apoptosis of cancer cells [3]. Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, isolated from the traditional Chinese herb *Artemisia annua*, is an effective novel anti-malarial drug with low toxicity [4]. In recent years, artemisinin and its derivatives such as DHA and artesunate were found capable of inducing apoptosis in breast cancer cells and also killing radiation-resistant breast cancer cells but the underlying mechanism calls for further investigation [5–7].

Elevation in intracellular Ca^{2+} trigger apoptosis in in vivo and in in vitro systems have been reported [8, 9], however the role of Ca^{2+} on the apoptosis caused by DHA remains to be evaluated.

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MAPKs participate in diverse cellular functions such as cell proliferation, cell differentiation and cell death. Some articles supported that p38 kinase, activated by extracellular stress signals, are involved in apoptosis [10, 11]. Whether p38 involved in DHA-induced apoptosis needs investigation.

In this study, human lung cancer PC-14 cells were incubated with dihydroartemsinin and apoptosis of PC-14 cells was analysed by DNA ladder and flow cytometry. Intracellular calcium concentration in the cell line was evaluated by laser copolymerization using Fura-3/AM as probe. The protein expression level of p38 in the cell line was assayed by Western blot. The aim of this study was to probe the mechanisms underlying DHA-induced cytotoxicity and the role of Ca^{2+} and p38 on the apoptosis.

Materials and methods

Chemicals and antibodies

RPMI-1640 medium and fetal bovine serum were from Gibco BRL. (Grand Island, NY, USA). DHA was purchased from Huisheng Pharmaceutical Corporation (Xi'an, Shaanxi Province, China). The dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fura-3/AM, propidium iodide (PI), Annexin V-FITC and p38 selective inhibitor SB202190 were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Rabbit polyclonal antibody to p38 and to phospho-p38, HRP-conjugated goat anti-rabbit Ig were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). EGTA was from Amresco, Inc. (Solon, OH, USA). BAPTA-AM was from DOJINDO Laboratories (Kumamoto, Japan). Enhanced chemiluminescence (ECL) detection reagents were from Pierce Biotechnology Inc. (Rockford, IL, USA). Bio-Rad protein assay kit was from BIO-RAD Laboratories (Hercules, CA, USA).

Cell culture

The human lung cancer cell line PC-14 used in this study was kindly gifted by Dr Junqing Zhang of Oncology Department of Tangdu Hospital. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C, 5% CO_2 in air. The DHA was dissolved in dimethylsulfoxide to make a stock solution of 100 mM. The solution was diluted at least 1,000-fold in the growth medium so that the final concentration of DMSO would have no effect on the proliferation of PC-14 cells. All the experiments were performed with cells in the logarithmic growth phase.

MTT assay

The growth inhibitory effect of DHA on cells was assessed by the MTT method [12]. The cells were seeded at a density of 5×10^3cells/well into 96-well culture plates (Nunc, Roskilde, Denmark) and incubated with 5, 10, 20, 40, 80, 160, 320 μM DHA. After 48 h of incubation, MTT solution was added to attain a final concentration of 5 mg/ml. After incubation at 37°C for an additional 4 h, the supernatant in each well was removed carefully. Formazan crystals were dissolved in DMSO for 10 min with shaking. The absorbance of each well was then read at 570 nm using an enzyme-linked immunosorbent assay reader. All of the experiments were performed thrice, and the mean and S.D. of IC_{50} value were calculated. Then the PC-14 cells were treated with DHA at IC_{50} concentration for 12, 24, 48, 72, 96 h and the cell viability was measured by MTT assay described above.

Analysis of DNA fragmentation

DNA fragmentation is a distinctive feature of apoptosis at the biochemical level. To determine the occurrence of apoptosis, a method for measuring DNA fragmentation was performed [13]. Briefly, the culture medium was removed and centrifuged at $3{,}000 \times g$ for 5 min to collect the detached cells. The adherent cells were lysed with a hypotonic lysis buffer (10 mM Tris–HCl, pH 8.0, containing 10 mM EDTA and 0.5% Triton X-100) and then pooled with pellets made of the detached cells. RNA and proteins were digested using 0.1 mg/ml RNase at 37°C for 1 h, followed by 0.5 mg/ml proteinase K digestion for 2 h for 56°C. Total DNA was isolated by phenol/chloroform extraction and by absolute ethanol precipitation. The ethanol was then removed, and the DNA pellet was suspended in 70% ethanol, and centrifuged at $6{,}000 \times g$ for 5 min at 4°C. The supernatant was removed gently, and the DNA pellet was briefly air-dried at room temperature. The DNA pellet was dissolved in TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). Finally, genomic DNA was electrophorsed on 1.5% agarose gel, stained with ethidium bromide and then visualized by a gel imaging system.

Pre-treatment or treatment of PC-14 cells by DHA, BAPTA-AM, EGTA, SB202190

RPMI 1640 containing 10% serum plus IC_{50} value concentration DHA was used to replace the culture media for 48 h incubation. For pretreatment, the RPMI 1640 containing 10% serum + 20 μM BAPTA-AM or 3 μM EGTA was used to replace the culture media for 1 h incubation before the PC-14 cells were subjected to the treatment by IC_{50} value concentration DHA for 48 h.

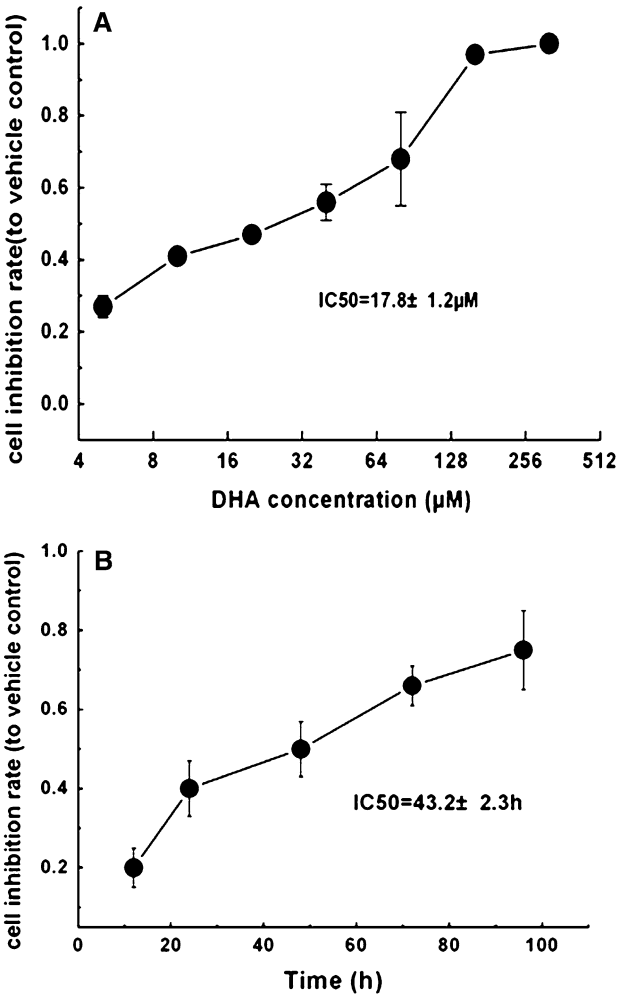

Fig. 1 Effect of DHA on proliferation of PC-14 cells. **a** PC-14 cells (5×10^3cells/well) were seeded in 96-well plates and incubated with various concentrations of DHA for 48 h and then cell viability was detected with the MTT assay. **b** PC-14 cells were incubated with 18 μM DHA for 12, 24, 48, 72, 96 h and the cell viability was measured with MTT assay. Results of experiments in triplicate were expressed as the ratio to vehicle control (mean ± S.D)

Intracellular calcium concentration detection

Concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured using Ca^{2+} indicator fura-3/AM as probe. In brief, the PC-14 cells were seeded into plates at a density of 4×10^4 cells/plate and treated or pre-treated with DHA, EGTA, BAPTA-AM for the indicated time. Then the cells were washed with D-hank's solution thrice, loaded with fura-3/AM (final concentration 10 μmol/l) at 37°C, 30 min. The cells were washed again for another thrice with D-hank's solution. Then the dynamics of intracellular Ca^{2+} concentration was assessed with cells in the microincubation chamber (37 ± 0.2°C). The images were captured using 100× oil-immersion objective (488 nm excitation, 522 nm emission). The analysis was controlled with Time-course/ratiometric software. The change of fluorescent intensity in the cells could represent the change of intracellular Ca^{2+} concentration.

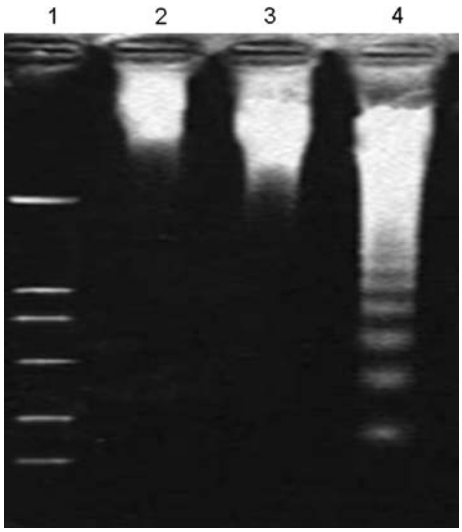

Fig. 2 Agarose gel electrophoresis of DNA extracted from DHA-treated PC-14 cells. PC-14 cells were incubated with 18 μM DHA for 48 h, then DNA was extracted, fractionated with 1.5% agarose gel electrophoresis. All of experiments were performed thrice. *Lane 1* DNA marker ladder, *lanes 2 and 3* control, *lane 4* cells were treated with 18 μM DHA for 48 h

Western blot analysis

The cells were pre-treated with BAPTA-AM following by treatment of DHA or treated only with DHA for indicated time. For western blot, cells were lysed in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton-100, 0.1% SDS, 1% Na-deoxycholate and 1 mM PMSF) and the protein content was determined with the Bio-Rad protein assay kit. Equivalent amounts of total protein were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore Co., Billerica, MA, USA) following by conventional protocols. Before being immunoblotted, the membranes were blocked in 5% nonfat milk in TBST buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The rabbit polyclonal anti-P38 and anti-phospho-P38 were used at a dilution of 1:1,000 for 2 h at room temperature and secondary anti-rabbit IgG-HRP was used at 1:5,000 for 1 h. Immunoreactive bands were visualized using an ECL + western blotting detection reagent according to the manufacture's protocol.

Flow cytometry

Quantification of apoptosis was done by PI and Annexin V-FITC double-staining. Briefly, PC-14 cells were treated and pretreated with DHA, BAPTA-AM, SB202190 for indicated time and then lifted with trypsin/EDTA and

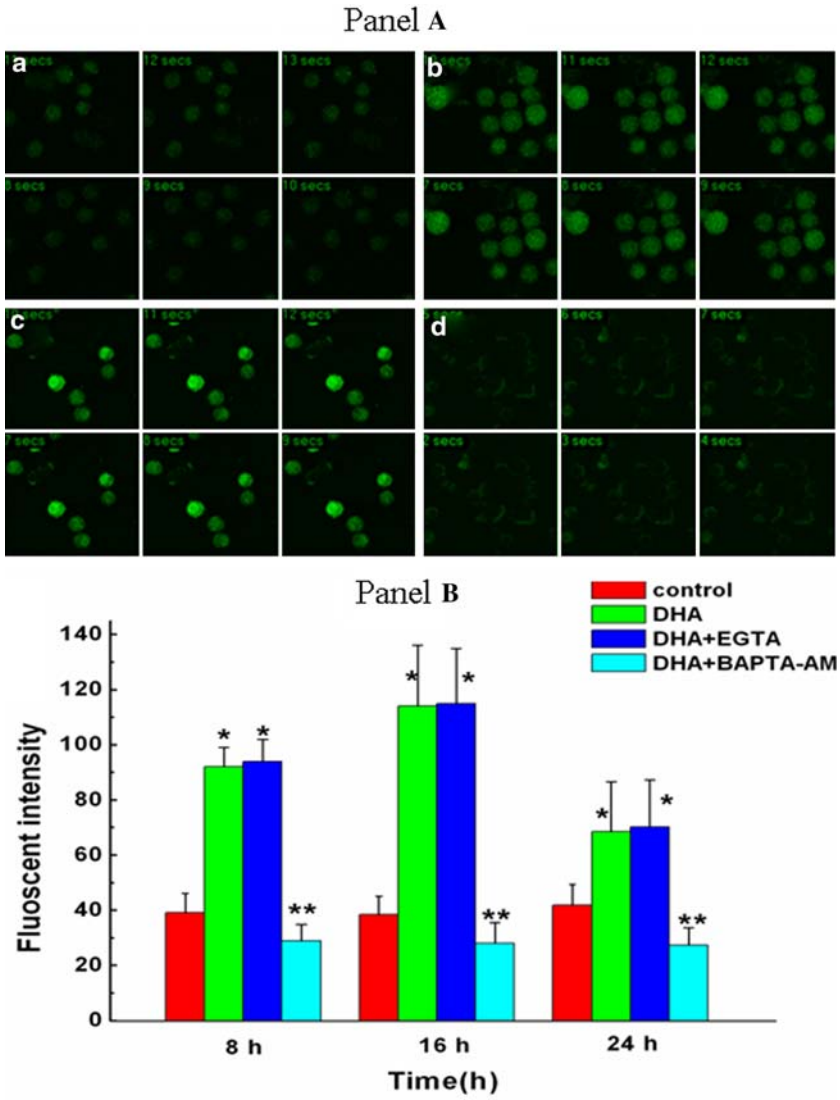

Fig. 3 Effect of DHA, EGTA and BAPTA-AM on $[Ca^{2+}]_i$ in fura-3/AM loaded PC-14 cells. Panel **a**: *a* control group; *b* DHA group; *c* DHA + EGTA group; *d* DHA + BAPTA-AM group. Panel **b**: After PC-14 incubated for 8, 16 and 24 h, the fluorescence intensity increased significantly and reached the peak at 16 h time point (*green bar*). Pretreated with EGTA and incubated with DHA as described previously, fluorescence intensity was not decreased but slightly increased (*blue bar*). Pretreatment of BAPTA-AM did attenuate the DHA-induced intracellular calcium increase (*cyan bar*). Data were mean ± S.D of 4–7 separate cells. Experiments were repeated thrice. *Asterisk*: vs control, $P < 0.05$; *double asterisk*: vs DHA group, $P < 0.05$

washed twice with ice-cold PBS. The cells were incubated with 10 μl Annexin V-FITC and 5 μl PI for 30 min at room temperature. FACs analysis was performed by flow cytometry. For each sample, 5,000 cells were analyzed. All of the experiments were performed thrice.

Data analysis

Throughout the test, data were expressed as mean ± S.D. and evaluated for significant levels with one-way ANOVA. P values less than 0.05 were considered to be statistically significant.

Result

Dihydroartemisinin-induced cytotoxicity. Treated with DHA at concentrations from 5 to 320 μM for 48 h PC-14 cells growth was inhibited in a concentration-dependent manner, the IC_{50} value of concentration was 17.8 ± 1.2 μM (Fig. 1a). Treated with DHA 18 μM for 12, 24, 48, 72, 96 h, PC-14 cells growth was inhibited in a time-dependent manner, the IC_{50} value of time was 43.2 ± 2.3 h (Fig. 1b). Hence 18 μM and 48 h treatment of DHA was adopted for our following research.

DHA-induced apoptosis in PC-14 cells

Artemisinin and its derivatives such as artesunate and DHA induce apoptosis in several cell types. DNA ladder measures DNA strand breaks typical of apoptosis while flow cytometry measured the percentage of cells undergoing apoptosis. We tested whether DHA induces apoptosis in the human lung cancer cell line PC-14 cells by DNA ladder assay and by flow cytometry. When PC-14 cells treated with 18 μM DHA for 48 h, typical DNA ladders appeared on a DNA electrophoresis gel (Fig. 2) with about 45% of them undergone apoptosis (see Fig. 5).

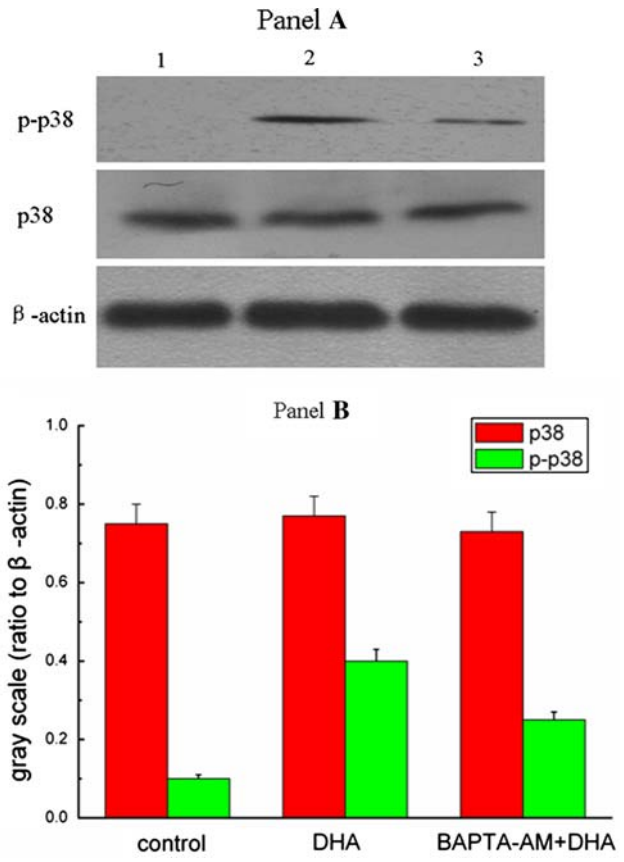

Fig. 4 Activation of p38 MAPK was stimulated by the DHA-induced intracellular calcium increase. Treatment of DHA induced an increase of phospholation of p38 MAPK but had little influence on total p38. *β*-actin was used for normalization. All of the experiments were performed thrice. Panel **a**: Intracellular calcium chelator BAPTA-AM attenuated the DHA-induced activation of p38. *Lane 1* control, *lane 2* treatment of DHA, *lane 3* pre-treatment of BAPTA-AM followed treatment of DHA for indicated time. Panel **b**: Diagram with qualified data based on the results of western blot. The *y*-axis represents the ratio of the *grey scale* of p38 or p-p38 to that of *β*-actin

DHA-induced $[Ca^{2+}]_i$ increase

The fluorescence intensity significantly increased after PC-14 cells were stimulated by DHA. Incubated with DHA for 16 h, the intracellular calcium reached the peak and then began to lower as the time of incubation prolonged (Fig. 3, green bar). Pre-treated with 3 μM EGTA did not prevent the $[Ca^{2+}]_i$ increase (Fig. 3, blue bar) while pre-treated with BAPTA-AM did prevent the $[Ca^{2+}]_i$ increase (Fig. 3, cyan bar) which indicated that the calcium was not from extracellular calcium but from intracellular store instead.

Effect of DHA and BAPTA-AM on p38 activation

To examine if p38 activation was associated with DHA-induced cell apoptosis, we examined the effect of DHA on p38 activation. As shown in Fig. 4, DHA caused an increase in p38 phosphorylation while with little change in total p38 protein in PC-14 cells. To determine if the activation of p38 was a consequence of DHA-induce intracellular calcium increase, we used calcium chelator BAPTA-AM to pre-treate PC-14 cells for 1 h and then PC-14 cells were treated with 18 μM DHA for 48 h. As shown in Fig. 4 lane 3, p38 activation was attenuated and this tended to indicate that activation of p38 was at the down stream of intracellular calcium increase.

Effects of BAPTA-AM and SB202190 on DHA-induced cell apoptosis

To determine whether intracellular calcium increase and p38 were essential for DHA-induced cell apoptosis, we examined the effects of calcium chelator BAPTA-AM and p38 selective inhibitor SB202190 on DHA-induced cell apoptosis using flow cytometry assay. As shown in Fig. 5, calcium chelator BAPTA-AM and p38 inhibitor SB202190 attenuated the DHA-induced apoptosis.

Discussion

Artemisinin, isolated from the traditional Chinese herb *A. annua*, is an effective novel anti-malarial drug with low toxicity. No toxicity has been reported with oral administration [4].

In recent years, it was found that artemisinin and its derivatives also possessed anti-tumour nature. The present study showed that DHA inhibited the proliferation of PC-14 cells in a concentration and time-dependent manner. By DNA ladder assay and flow cytometry analysis, DHA can induce PC-14 obvious apoptosis significantly.

Although many experts have observed the anti-tumour effect of artemisinin and its derivatives, the underlying mechanism is not fully elucidated. Many studies have found that artemisinin and its derivates can induce apoptosis in many different kinds of cancer cells [5, 14–17]. The endoperoxide function is at least in part responsible for the growth inhibitory activity of artemisinins, since the reduction to an ether bond greatly reduces cytotoxicity [18]. Other reports however, point out that the endoperoxide is not absolutely necessary for the effects against tumour cells [19, 20].

Calcium signal is one of the most important second messages. Calcium signals have been implicated in a vast array of cellular functions from short-term response, such as contraction and secretion, to long-term control of transcription, cell division and cell death. In most cells, the generation of cytosolic calcium signals is complex and involves two interdependent and closely coupled components: the

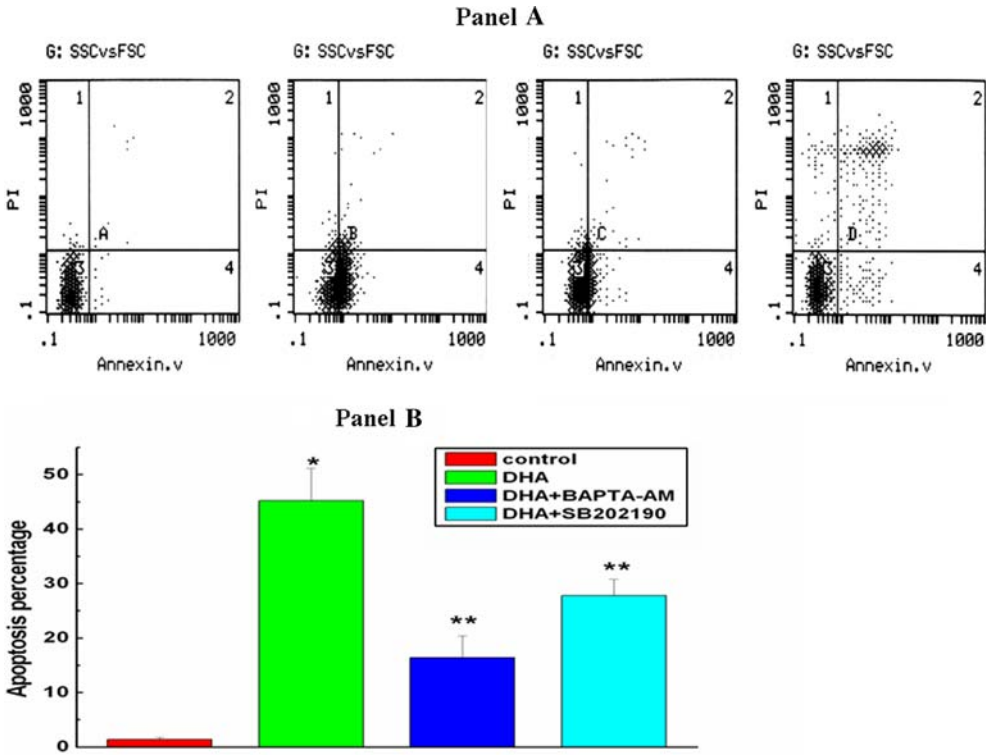

Fig. 5 Effects of DHA, BATPA-AM and SB202190 on the apoptosis in PC-14 cells. Panel **a**: Flow cytometrical analysis of the BAPTA-AM or SB202190 on DHA-induced apoptosis. *a* Apoptotic analysis of PC-14 cells incubated without DHA. *b* Apoptotic analysis of PC-14 cells after incubation with 30 μM DHA for 48 h. *c* Apoptotic analysis of PC-14 cells pre-treated with SB202190 then treated with DHA. *d* Apoptotic analysis of PC-14 cells pre-treated with BAPTA-AM then treated with DHA. Panel **b**: DHA induced a significant increase of the apoptosis percentage in PC-14 cells (*green bar*). BAPTA-AM and SB202190 both could attenuate the DHA-induced apoptosis (*blue bar* and *cyan bar*, respectively) and the attenuation effect of BAPTA-AM was more significant than that of SB202190. *Asterisk*: vs control, $P < 0.05$; *double asterisk*: vs DHA, $P < 0.05$

release of calcium from stores in the endoplasmic reticulum and influx of extracellular calcium [21]. Cytosolic Ca^{2+} elevation has been proposed to play an important role in the triggering of apoptotic signals [22]. The present study showed that DHA-induced the intracellular Ca^{2+} increase. Removal of extracellular Ca^{2+} by EDTA did not prevent the DHA-induced $[Ca^{2+}]_i$ increase. On the contrary, incubation with BAPTA-AM did prevent the DHA-induced $[Ca^{2+}]_i$ increase and attenuate the DHA-induced apoptosis. These facts seemed to indicate that DHA-induced increase of intracellular Ca^{2+} was from intracellular store and contributed to the downstream apoptosis.

The role of MAPK cascades as an apoptotic signal transduction pathway has attracted considerable attention. The role of p38 in cell survival and apoptosis has been extensively examined, but the results of these studies are inconsistent. Some data indicate that p38-dependent pathways promote apoptosis and cell death [10, 23, 24], but it has also been reported that p38 signalling promotes cell survival and growth [25, 26]. These findings indicate a dual role for p38 in the cellular response that appears to be specific to the type of cell and apoptotic stimulus studied. In the present study, we found that after incubation with 18 μM DHA for 48 h, the phosphorylated p38 content had an obvious increase but the total p38 underwent little changes. Pre-treatment with BAPTA-AM followed by treatment of DHA could decrease the phosphorylated p38 content, which indicated that intracellular calcium elevation mediated the DHA-induced activation of p38. Interestingly, BAPTA-AM did not block completely the activation of p38, so there should be other mediators except calcium in the link of DHA and activation of p38. P38 selective inhibitor SB202190 attenuated the DHA-induced apoptosis (Fig. 5 cyan bar) which indicated that p38 participated in the induction of apoptosis by DHA.

To sum up, it seemed that DHA could induce apoptosis in lung cancer cell line PC-14 cells. Calcium and p38 might have participated in the pathway signalling, and calcium might be at the upstream of activation of p38.

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