Apigenin Induces Alterations of Reactive Oxygen Species, Ca(superscript +2) and Mitochondria Membrane Potential in Human Lung Cancer H460 and A549 Cells

芹菜素誘發人類肺癌H460與A549細胞株之活性氧、鈣離子與粒線體膜電位改變

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Apigenin induces alterations of reactive oxygen species, Ca^{+2} and mitochondria membrane potential in human lung cancer H460 and A549 cells

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Abstract

Lung cancer is a leading cause of cancer death in the United States and Taiwan. Currently, the mortality rates in lung cancer patients remain high. Numerous evidences have demonstrated that natural compounds contain chemopreventive and chemotherapeutic functions. Apigenin (4,5,7-trihydroxyflavone), a promising chemopreventive agent presented in fruits and vegetables, has been shown to induce cell cycle arrest and apoptosis in many types of human cancer cell lines. In the present studies, H460 and A549 cells were treated with apigenin and then were analyzed for alterations of reactive oxygen species (ROS), Ca^{+2} and mitochondria membrane potential (MMP). The results indicated that H460 cells treated with 120 μM apigenin for 10 min to 3 hr led to the ROS production up to 3 h treatment. We also found that ROS for A549 cells was higher in the apigenin treated groups. The ROS production was maximal at 24hr after treatment. H460 cells treated with 120 μM apigenin for 0–4 h will lead to cytoplasmic Ca^{+2} increased for up to 2 h treatment. Increasing the time of apigenin treatment from 24 to 72 hr led to slightly increase in the Ca^{+2} production in A-549 cells. The MMP was led to slightly reduction in the apigenin-treated groups for 6hr than in the control in H460 cells. Increasing the reaction periods of time of apigenin led to greater reduction of the MMP in A-549 cells during 1 hr. We may conclude that apigenin treatment decreased the levels of MMP and increased the productions of ROS and Ca^{2+} in H460 and A549 cells.

Keywords: chemopreventive, chemotherapeutic, apigenin

Introduction

Lung cancer is a disease of uncontrolled cell growth in tissues of the lung. This growth may lead to metastasis, which is the invasion of adjacent tissue and infiltration beyond the lungs. Lung cancer, the most common cause of cancer-related death in men and women, is responsible for 1.3 million deaths worldwide annually (Emami B, Graham MV, Purdy JA, 1994). In Taiwan, lung cancer is the number one cause of cancer-related deaths in men and women

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with about 34.5 persons per 100 thousand dying annually from lung cancer, based on the report in 2009 from the Department of Health, Executive Yuan, R.O.C. (TAIWAN) (http://www. doh.gov.tw/EN2006/ index_EN. aspx.). The treatment of lung cancer includes surgery, radiotherapy and chemotherapy. However, it is not satisfying. Therefore, the mortality in lung cancer patients remains high. Numerous evidences have demonstrated that naturally occurring compounds contain chemopreventive and chemotherapeutic functions (Hashibe M, Brennan P, Benhamou S et al., 2007)(Parkin DM, Pisani P, Ferlay J, 1999)(Rahman M, Sakamoto J, Fukui T., 2005). Lots of evidences also showed that consumption of a phytochemical-rich diet including fruits and vegetables can reduce the risk of certain types of human cancers (Meyskens FL, Jr., Szabo E., 2005) (Steinmetz KA, Potter JD., 1991). Moreover, the using of drugs cancer patients in clinical treatment is derived from natural plant species (Room R, Babor T, Rehm J., 2005)(Balaram P, Sridhar H, Rajkumar T et al., 2002).

Apigenin (4',5,7-trihydroxyflavone), is abundantly present in common fruits and vegetables such as Chinese cabbage, bell pepper, garlic, celery, and guava, contain anticancer function and act as a chemopreventive agent (Miean KH, Mohamed S., 2001)(Patel D, Shukla S, Gupta S., 2007)(Ren W, Qiao Z, Wang H et al., 2003). Apigenin inhibited tumor initiation in animal and suppressed angiogenesis in melanoma and carcinoma of the breast, skin, and colon (Wei H, Tye L, Bresnick E et al., 1990)(Atsuta A, Iishi H, Baba M et al., 2000)(Liu LZ, Fang J, Zhou Q et al., 2005)(Fang J, Xia C, Cao Z et al., 2005)(Trochon V, Blot E, Cymbalista F et al., 2000)(Schindler R, Mentlein R., 2006). Furthermore, apigenin sensitizes tumor cells to TNF-α-induced apoptosis through inhibition of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), induces apoptosis in solid tumors through up-regulation of IGFBP-3 (insulin growth factor binding protein-3) and induced suppression of tumor proliferation correlates with down-regulation of cyclin D1 (Shukla S, Gupta S., 2004)(Shukla S, Mishra A, Fu P et al., 2005)(Shukla S, Gupta S., 2006). Recently, apigenin has been reported to induce prostate cancer cell death via reactive oxygen species (ROS) production and p53 activation (Shukla S, Gupta S., 2008). Apigenin is capable of selectively inhibiting cell growth and inducing apoptosis in cancer cells, but it will not affecting normal cells (Hsia TC, Yang JS, Chen GW et al.,2009).

However, there is not much information to address apigenin affecting human lung cancer cells. Herein, we investigated the effects of apigenin in human lung cancer H460 and A549 cells and results showed that apigenin induced alterations of reactive oxygen species, Ca^{+2} and mitochondria membrane potential (MMP).

Materials and Methods

Materials, chemicals and cell cultures

Apigenin were purchased from Sigma Chemical. (St. Louis, MO, USA). The fluorescent probe 2', 7'-dichlorofluorescin diacetate (DCFH-DA), Flou-3/AM and $DiOC_6$ for ROS, Ca^{+2} and mitochondrial membrane potential were purchased from Molecular Probes/Invitrogen Corp. (Eugene, OR, USA).

Human lung cancer H460 and A549 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The H460 and A549 cells were placed in 75 cm^2 plate with RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA), 100 units/ml penicillin and 100 μg/ml streptomycin and 2 mM L-glutamine and then were cultured under a humidified 5% CO_2 and 95% air grown at 37℃ and one atmosphere in an incubator (Chen GW et al., 2009) (Yang JS, Chen GW, Hsia TC et al., 2009).

Detection of Reactive Oxygen Species (ROS) in H460 or A549 Cells after Treatment with 120μ M Apigenin by Flow Cytometry

Approximated $2x10^5$ cells/well H460 cells or A549 cells were plated onto 12-well plates and treated with 120 μM of apigenin for different periods of incubation time (10 min, 30 min, 1 hr and 3 hr for H460; 0, 3, 6, 12, 24hr for A549) before being harvested, washed twice, and re-suspended in the 500μL of ROS indicator 2, 7-dichlorodihydrofluorescein diacetate (H2DCF-DA)(10 μM). After the above procedures, the products were incubated at 37℃ for 30 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur).

Detection of Ca^{+2} Concentrations in H460 or A549 Cells Treated with 120μ M Apigenin by Flow Cytometry

The levels of Ca^{+2} of the H460 cells or A549 cells were determined by flow cytometry (Becton Dickinson FACS Calibur), using the Indo 1/AM (Calbiochem

; La Jolla, CA). Approximated 2 x 10^5 cells/well were plated onto 12-well plates and treated with 120 μM apigenin for different periods of incubation time (15 min, 1 hr, 2hr and 4 hr for H460; 3, 24, 36 and 72hr for A549) and cells were harvested and washed twice. After the above procedures, the products were incubated at 37℃ for 30 min and analyzed by flow cytometry.

Detection of Mitochondrial Membrane Potential (MMP) in H460 or A549 Cells Treated with 50 lM Baicalein by Flow Cytometry

Approximately 2×10^5 cells/well of H460 or A-549 cells in 12-well plates were treated with 120 μM apigenin for different periods of incubation time (6 hr, 12hr and 24 hr for H460; 15, 30 and 60 min for A549) to detect the changes in ΔΨm. The cells were harvested and washed twice, re-suspended in 500μl of indicator 3, 3'-Dihexyloxacarbocyanine iodide (DiOC6; 4μ mol/l) and incubated at 37˚C for 30 min. The levels of cell ΔΨm in H-460 or A-549 were determined by flow cytometry (Becton Dickinson FACS Calibur).

Statistical analysis

Student's *t*-test was used to analyze differences between apigenin-treated and control groups. *p<0.05, **p<0.01, ***p<0.001

Results

For investigating apigenin induced the production of ROS and Ca^{+2} and loss of $\Delta\Psi_m$ in H460 and A549 cells, the cells were treated with apigenin for various time periods. H460 and A549 cells were harvested for examining the ROS and Ca^{2+} productions and the levels of $\Delta\Psi_m$. The results from flow cytometric analysis indicated that H460 cells were treated with 120 μM apigenin for 10 min to 3 h led to the ROS production up to 3 h treatment (Fig. 1A) compared with control group. The result showed that the percentage of ROS for A549 cells was higher in the apigenin treated groups than in the control. The ROS production was maximal at 24hr after treatment (Fig. 1B).

Fig. 1A

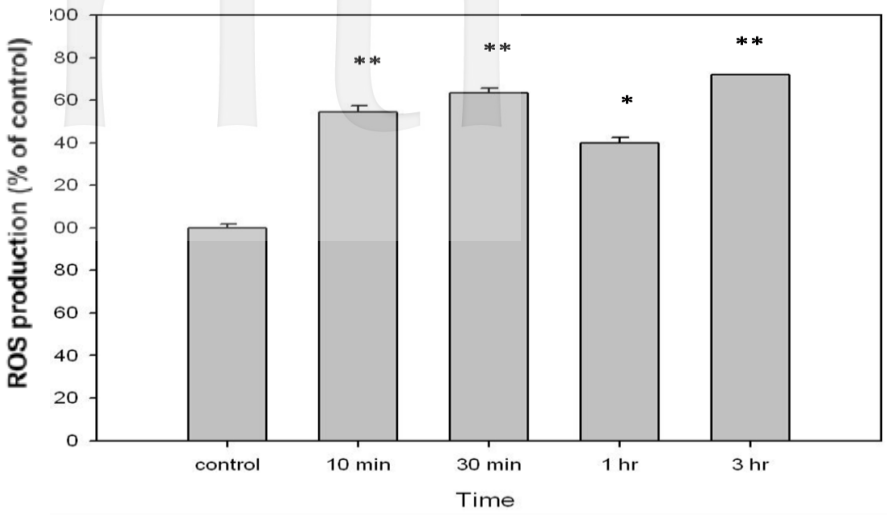

Fig. 1B

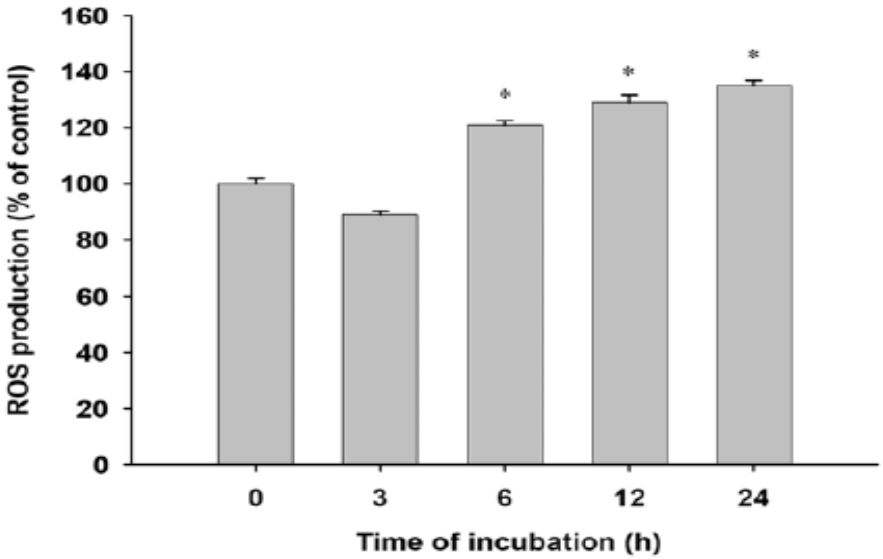

Fig. 1 Apigenin induced ROS productions in (A) H460 cells and (B) A549 cells. Cells were exposed to 120 μM of apigenin for different periods of time, and the cells were harvested for examining the ROS as described in Materials and Methods.

The results from flow cytometric analysis indicated that H460 cells were treated with 120 μM apigenin for 0–4 h led to the levels of cytoplasmic Ca^{+2} increased for up to 2 h treatment but after 4 hr treatment led to decrease the levels of cytoplasmic Ca^{+2} (Fig. 2A).

The percentage of Ca^{+2} production was not significantly higher in the apigenin-treated groups for 3hr than in the control. Increasing the time of apigenin treatment from 24 to 72 hr led to slightly increase in the Ca^{+2} production in A-549 cells (Fig. 2B).

Fig. 2A

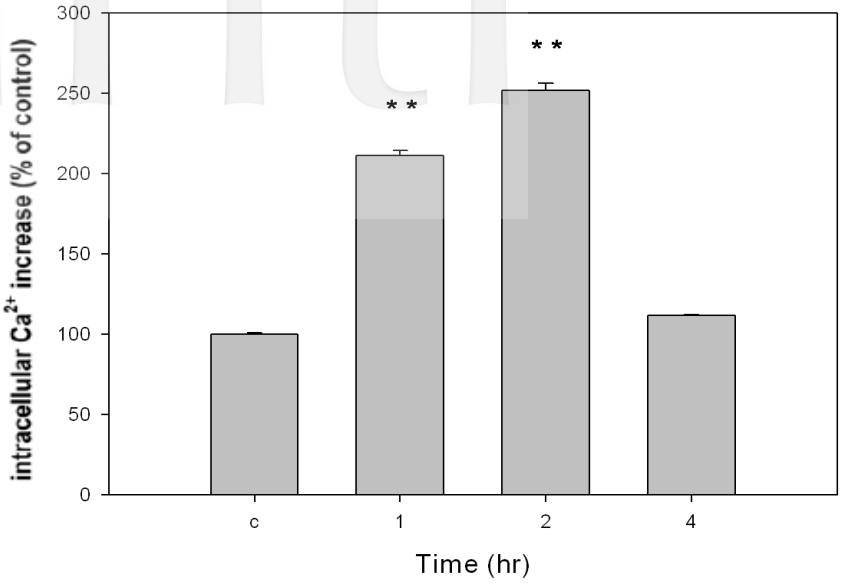

Fig. 2B

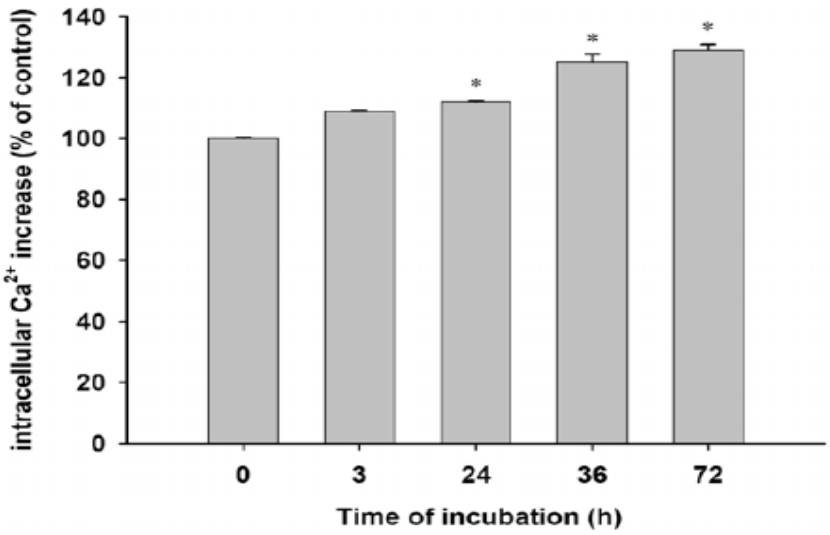

Fig. 2 (A) H460 cells and (B) A549 cells were exposed to apigenin (120 μM) for various intervals of time. The zero hour was defined as untreated control. The percentage of cells in cytosolic Ca^{2+} levels were stained by specific dyes and determined by flow cytometry as described in Materials and Methods.

The ΔΨm was led to slightly reduction in the apigenin-treated groups for 6hr than in the control but the increase of the ΔΨm for 12 and 24 hr in H460 cells (Fig. 3A). The ΔΨm was significantly lower in the apigenin -treated groups than in the control. Increasing the reaction periods of time of apigenin led to greater reduction of the ΔΨm in A-549 cells (Fig. 3B).

Fig. 3A

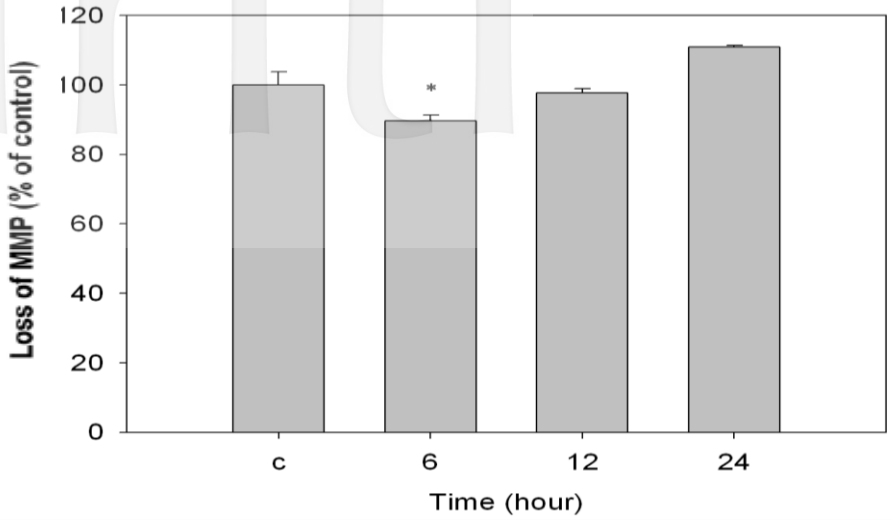

Fig. 3B

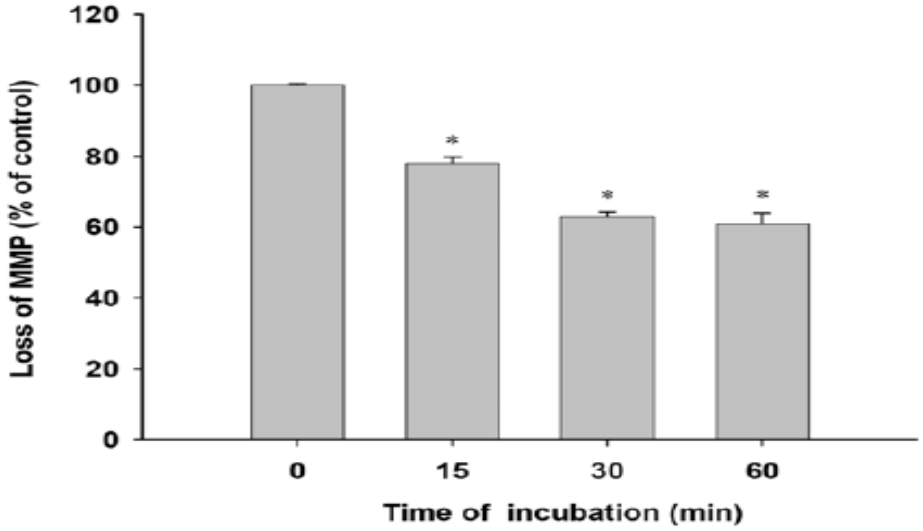

Fig. 3 The effects of apigenin were on the production of ΔΨm levels in (A) H460 cells and (B) A549 cells. Cells were incubated with 120 μM apigenin for various time periods. The percentage of cells stained by DiOC6 for ΔΨ m were examined and quantitated by flow cytometry as described in the Materials and Methods. *, **, *** Significantly different from the control at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Discussion

Reactive oxygen species (ROS) is a phrase used to describe a variety of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen. Strong oxidants like the various and excessive production of ROS can not only lead to oxidative stress but also damage other molecules and the cell structures of which they are a part and then loss of cell function, and finally result in

ultimately apoptosis or necrosis. A balance between oxidant and antioxidant intracellular systems is hence vital for cell function, regulation, and adaptation to diverse growth conditions (Nordberg J, Arnér ES., 2001). Among the most important of these are the actions of free radicals on the_fatty acid side chains of lipids in the various membranes of the cell, especially mitochondrial membranes (Bai J, Rodriguez AM, Melendez JA, Cederbaum AI, 1999). It is recognized that the intracellular generation of H_2O_2 (the most stable ROS) was an important mediator of intracellular signaling cascades of apoptosis including exogenous addition of H_2O_2 that is a potent activator of the apoptotic machinery (Bai J, Rodriguez AM, Melendez JA, Cederbaum AI, 1999) and some of the added H_2O_2 diffuses into the mitochondria and perhaps causes damage to the mitochondrial membrane (Yang JS, Chen GW, Hsia TC et al., 2009). It is well known that mitochondria can act as a nodal point for execution of apoptosis and mitochondria also play an essential role in death signal transduction by the permeability transition pore opening and collapse of the $\Delta\Psi_m$, resulting in the rapid release cytochrome c into the cytoplasm before binding to Apaf-1 and activating caspase-3 via caspase-9, culminating in cell death (Robertson JD, Orrenius S, 2000)(Zou H, Li Y, Liu X, Wang X, 1999), and AIF or Endo G release from mitochondria into the nuclei for causing apoptosis (Kim J, Soh J, 2009). Therefore, apoptosis can be divided into mitochondrion-dependent and independent pathways.

Another factor that we may suggest is that mitochondrial produced H_2O_2 diffuses into the cytosol where it may exert cytotoxic effects. Our results showed that apigenin increase the ROS and Ca^{+2} in H460 and A549 cells and decreased the levels of $\Delta\Psi_m$ in A549 cells but not significant in H460 cells. Thus, a mitochondrial damage-dependent pathway might be involved in apigenin induced apoptosis in H460 and A549 cells.

The ROS production in A549 cells after treated with apigenin did not occur in earlier time at 3h, but after 6-24 h, the ROS was elevated in these examined cells. Although it was reported that the low levels of ROS enhanced cell proliferation (Kamata H and Hirata H, 1999), it was reported that the production of ROS may contribute to

tumor progression because ROS levels are significantly low in non-tumor cells when compared to the tumor cells (Szatrowski TP and Nathan CF, 1991). The interesting point is that antioxidants scavenge intracellular ROS which led to suppress proliferation of transformed cells and colony formation (Urgensmeier JM, Panse J, Schafer R and Bauer G, 1997).

Although the anticancer function of apigenin is still unclear, our results clearly showed that apigenin induced ROS and Ca^{2+} production in H460 and A549 cells. These results are in agreement with other investigators who demonstrated that ROS plays an important role in apoptosis which induced by agents including certain chemopreventive agents (table 1) through the engagement of downstream proteins involved in the execution of apoptosis (Trachootham D, Zhou Y, Zhang H et al., 2006) (Singh SV, Srivastava SK, Choi S et al., 2005) (Dini L., 2005).

Table 1 Apigenin induced ROS and Ca^{2+} production in H460 and A549 cells. There results are in agreement with other certain chemopreventive agents.

			10min	15min	30min	1h	2h	3h	4h	6h	12h	24h	36h	72h	REF
Apigenin	H460	ROS ↑	**		**	*		**							
		Ca^{+2} ↑				**	**		NS						
		MMP ↓								*	NS	NS			
	A549	ROS ↑						NS		*	*	*			
		Ca^{+2} ↑						NS				*	*	*	
		MMP ↓		*	*	*									
Baicalein	N18	ROS ↑				*	*	*	*	*					(Li et al, 2009)
		Ca^{+2} ↑				*	*	*	*	*					
		MMP ↓				NS	NS		*		*				
Danthron	GBM8401	ROS ↑						***		***	***	***			(Lu et al, 2010)
		Ca^{+2} ↑						*		**	*	**			
		MMP ↓						**		**	***	***			
Morin	HL60	ROS ↑			**	**	**		**						(Kuo et al, 2001)

Student's *t*-test was used to analyze differences between apigenin-treated and control groups. *p<0.05, **p<0.01, ***p<0.001, NS; not significant. The arrow bar "↑" indicated elevation and '↓'means decline.

ROS is one of the factors for ER stress which may cause Ca^{+2} to release. The majority of Ca^{+2} stayed in ER if there are ER stress then it will led to the release of Ca^{+2}. Apigenin had shown to induce ROS production in H460 and A549 cells that may be a factor for caused ER stress then led to Ca^{+2} release. These effects are almost time-dependent.

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芹菜素誘發人類肺癌 H460 與 A549 細胞株之活性氧、鈣離子與粒線體膜電位改變

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摘要

美國與台灣的肺癌死亡率名列前茅，許多研究證明某些天然成分具防癌或化學療效。芹菜素(4,5,7-trihydroxyflavone)被認為與一般的蔬菜與水果一樣具防癌，並且可造成各種癌細胞株的細胞生長停滯與凋零。本研究以芹菜素與兩種人類肺癌 H460 與 A549 細胞株作用，檢測活性氧、鈣離子與粒腺體膜電位改變的情形。結果顯示以濃度 120 μM 的芹菜素與 H460 作用，經過 10 分鐘至 3 小時後，導致活性氧的大量產生。然而 A549 細胞與芹菜素需做用到 24 小時才會達到高峰。針對細胞質鈣離子而論，H460 細胞與 120 μM 的芹菜素作用 0-4 小時，當作用時間 2 小時為釋出鈣離子的高峰期。至於 A549 細胞，釋出鈣離子的濃度隨著作用時間的增加，呈正相關逐漸增加。另外 H460 細胞與芹菜素作用，於反應 6 小時，MMP 僅呈小幅微降。然而 A549 細胞之 MMP 卻在 1 小時內劇烈下降。吾輩認為芹菜素可使兩種人類肺癌 H460 與 A549 細胞株之活性氧與鈣離子濃度升高，並使粒腺體膜電位降低。

關鍵字： 防癌、化學療效、芹菜素

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