國立中山大學生物科學系

碩士論文

Department of Biological Sciences

National Sun Yat-sen University

Master Thesis

白藜蘆醇對人類口腔癌細胞鈣離子濃度和凋亡的影響

Effect of resveratrol on $[Ca^{2+}]_i$ rises and apoptosis in human oral cancer cells

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中華民國 102 年 6 月

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國立中山大學研究生學位論文審定書

本校 生物科學系 研究所 碩士班

研究生 洪慈翊 (學號：N002010003) 所提論文

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於中華民國 102 年 6 月 21 日經本委員會審查並舉行

口試，符合碩士學位論文標準。

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指導教授簡崇仁＿＿＿＿＿＿

誌謝

進修研究所是想增廣見聞，透過不斷地學習來提升自我的競爭力。兩年半前離開台東馬偕醫院到高雄工作時，馬偕的主管劉玉珍主任提醒我，醫學的進步是很迅速的，如果到新的環境，必須擴展自己的視野，要不斷地學習，不要當個井底之蛙！她的話語激勵我踏上研究所這條路。如今順利地取得學位，來自許多人的幫忙與支持，在此我要感謝曾經給予指導與協助的師長、前輩、夥伴與家人。

首先我要感謝我的指導教授—劉仲康老師，在這段期間對我的指導與協助，讓我自主地選擇研究方向，但仍不時提供必要的建議與指導，且不斷關心我、鼓勵我，讓我順利完成論文。

感謝共同指導教授—簡崇仁老師，帶領我走進細胞藥理學中「鈣離子」研究的領域，實驗室並提供完善的研究設備。簡老師在論文設計、研究方法與實驗技術上的指導，讓我可以在這個優良的環境完成實驗及論文。感謝論文口試委員—中山大學生物科學系梁維陞博士、高雄榮總林克隆醫師對於論文內容的修正。

初進入研究所時，感謝在我任職醫院(高雄國軍總醫院左營分院)的方譯謙醫官引薦，讓我能順利到簡老師實驗室做研究，由於長官的支持與肯定，使院內臨床工作與實驗室研究能順利進行。

進修期間，非常感謝實驗室的維陞(阿猴)學長，在學業上，不斷地叮嚀和指導；在實驗上，從實驗的基礎原理到操作，每個環節都很細心的指導和教導我。在學長的耐心帶領下，從一個完全未知細胞藥理實驗到現在能獨力完成論文實驗，使我的實驗技術精進不少。也很感謝一起在研究室的夥伴們：金滿姐、金菊、晏岑、佳蓉(小白)的幫助；感謝一起在課業上研讀的同學們：宜倩、士婷，沒有他們的幫忙，我的研究之路不會如此順遂。

最後要感謝我的父母，因為有他們的養育和提攜，給予我正向的人生觀，讓我有機會能完成碩士學位。以及我親愛的姐姐、妹妹們給我精神上的支持鼓勵。也感謝我的公公、婆婆、大姑、二姑、小叔的愛護體恤，讓我無後顧之憂，可以全心全意完成學業。最後必須要感謝老公張家豪，從結婚以來一直在背後默默地關懷，陪我度過這段充實忙碌的日子。感謝身邊幫助鼓勵我的朋友們和家人們，願大家幸福健康，讓我完成這艱辛的任務。僅以此論文獻給我的恩師、家人以及幫助過我的朋友們。

中文摘要

本研究探討天然產物白藜蘆醇(resveratrol)對人類口腔癌細胞株(human oral cancer cells, OC2)細胞內鈣離子濃度和細胞存活率的影響。本研究利用鈣離子螢光染劑 fura-2 來偵測胞內鈣離子濃度，人類口腔癌細胞株 OC2 加入濃度 5-20 μM 之 resveratrol 後，其鈣離子的濃度的變化隨 resveratrol 的濃度增加而上升。當移去細胞外培養液中的鈣離子後，鈣離子的訊號則部分下降。由 resveratrol 誘發細胞外之鈣離子信號內流，受 nifiedipine 和蛋白質激酶(protein kinase C, PKC)抑制劑（GF109203X）抑制所影響。當去除細胞外鈣離子，培養在不含鈣離子的細胞培養液中，細胞加入 2,5-di-tert-butylhydroquinone (BHQ) (一種內質網上鈣離子幫浦抑制劑)完全抑制 resveratrol 誘導鈣離子濃度的上升。反之，加入了 resveratrol 的細胞培養液中，抑制大部分 BHQ 誘導的鈣離子濃度上升。磷脂酶 C (phospholipase C, PLC)抑制劑(U73122)抑制大部分 resveratrol 誘導的鈣離子濃度上升。Resveratrol 在濃度 20-100 μM 下，以濃度所依賴的方式造成細胞毒殺性死亡。在細胞液中加入 BAPTA/ AM (鈣離子螯合劑)，這種細胞毒殺作用並沒有改變。Annexin V/Propidium (PI) (偵測細胞凋亡的螢光染劑)的染色數據表示，resveratrol 在濃度 20 μM 和 40 μM 誘導細胞凋亡。另一方面，在濃度 20 μM 和 40 μM 的 resveratrol 處理下也引起細胞週期的阻滯。總之，在人類口腔癌細胞株中加入 resveratrol 後誘導鈣離子濃度上升，是藉由內質網釋放之磷脂酶 C 依賴路徑，且鈣離子經由蛋白質激酶 C 和 nifidepine 影響的鈣離子通道進入而上升。Resveratrol 誘導細胞死亡可能涉及細胞凋亡。

關鍵字：白藜蘆醇，鈣離子，人類口腔癌細胞，細胞凋亡

Abstract

This study examined whether the natural product resveratrol altered cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and viability in OC2 human oral cancer cells. The Ca^{2+}-sensitive fluorescent dye fura-2 was applied to measure $[Ca^{2+}]_i$. Resveratrol at concentrations of 5-20 μM induced a $[Ca^{2+}]_i$ rise in a concentration-dependent fashion. The response was decreased partially by removal of extracellular Ca^{2+}. Resveratrol-induced Ca^{2+} signal was inhibited by nifedipine and protein kinase C (PKC) inhibitor GF109203X. When extracellular Ca^{2+} was removed, incubation with the endoplasmic reticulum Ca^{2+} pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) abolished resveratrol-induced $[Ca^{2+}]_i$ rise. Conversely, incubation with resveratrol largely inhibited BHQ-induced $[Ca^{2+}]_i$ rise. Inhibition of phospholipase C (PLC) with U73122 largely inhibited resveratrol-induced $[Ca^{2+}]_i$ rise. At concentrations of 20-100 μM, resveratrol caused cytotoxicity in a concentration-dependent manner. This cytotoxic effect was not changed by chelating cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM). Annexin V/propidium iodide (PI) staining data suggest that resveratrol between 20 μM and 40 μM induced apoptosis. At concentrations of 20 μM and 40 μM, resveratrol also caused cell cycle arrest. Collectively, in human oral cancer cells, resveratrol induced a $[Ca^{2+}]_i$ rise by inducing PLC-dependent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry via PKC-sensitive, nifidepine-sensitive Ca^{2+} channels. Resveratrol induced cell death that might involve apoptosis.

Keywords: resveratrol, Ca^{2+}, human oral cancer cells, apoptosis

List of abbreviations

AIF	apoptosis inducing factor
ATP	adenosine triphosphate
AV/PI	annexin V/propidium iodide
BAPTA/AM	1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl ester
BHQ	2,5-di-tert-butylhydroquinone
DISC	death-inducing signal complex
FasL	Fas ligand
FADD	Fas-associated death domain protein
IAP	inhibitor of apoptosis
IP_3	inositol 1,4,5-trisphosphate
OC2	human oral cancer cells
OSCC	oral squamous cell carcinoma
PBS	phosphate buffered saline
PCD	programmed cell death
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
TRPM8	transient receptor potential cation channel subfamily M member 8
VOCC	voltage-gated Ca^{2+} channels
WST-1	4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1, 3-benzene disulfonate]

目　錄

1. Introduction

1.1 Resveratrol

Natural products are an abundant source of polyphenolic compounds. These polyphenolic compounds constitute a large class of resveratrol derivatives-including monomers, dimers, and oligomers-resulting from different oxidative condensations of the individual monomers (Richard et al 2011). Resveratrol (3,4′,5-trihydroxy-stilbene) is a polyphenolic compound abundant in grapes, red wine, peanuts, and a variety of food sources (Figure. 1-1). It has been shown that resveratrol affected cell division, growth, inflammation, angiogenesis, or metastasis in different cell models (Baur and Sinclair 2006), including human lung adenocarcinoma cells (Li et al 2012), human breast cancer cells (Osman et al 2012), human colon cancer cells (Vanamala et al 2010) and human prostate cancer cells (Fang et al 2012). Furthermore, resveratrol induced apoptosis in many cancer cell lines including human lung adenocarcinoma cells (Li et al 2012), human breast cancer cells (Vanamala et al 2010), and human colon cancer cells (Sareen et al 2007).

In a previous study, intracellular Ca^{2+} rises played an essential role in the resveratrol-induced cell death of breast cancer (Sareen et al 2007) or rat primary neuron (Gong et al 2007). However, the exact molecular mechanisms underlying resveratrol's chemopreventive and anticancer effects are not completely understood, and no studies have explored the specific signaling pathways involved in cyotoxicity of resveratrol in oral cancer cells. Therefore, establishing these pathways may be an important step in discovering methods of alleviating toxic outcomes in patients exposed to resveratrol.

1.2 Ca^{2+} as a pivotal intracellular signal in cells

Ca^{2+} signal is precisely yet flexibly controlled inside cells to mediate fundamental biological processes including gene transcription, cell proliferation, differentiation and

apoptosis. Ca^{2+} homeostasis inside cells has been achieved by the exquisite choreography of a repertoire of Ca^{2+} signaling toolkit including cellular membrane receptors, Ca^{2+} channel, pumps and exchangers across the membrane (Li et al 2012). Aberrant Ca^{2+} signaling is implicated in tumor growth and metastasis, cardiovascular diseases, and the pathogenesis of immunodeficiency, allergy, autoimmune and inflammatory disorders. Targeting Ca^{2+} signaling pathway may hold therapeutic potential in the treatment of these human diseases (Kao et al 2012; Ali et al 2012).

A rise in intercellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) is a key signal for many pathophysiological processes in different cells (Bergner and Huber 2008; Courjaret and Machaca 2012). However, an abnormal $[Ca^{2+}]_i$ rise is cytotoxic and can lead to apoptosis, dysfunction of proteins, interference of ion flux, etc. (Pinton et al 2008; Xu et al 2013). Ca^{2+} ion serve as a ubiquitous second messenger in all eukaryotic cells (Clapham 1995). The resting $[Ca^{2+}]_i$ is maintained at level less than 0.1 μM, about four orders of magnitude lower than in the extracellular solution (1.8 mM), but cellular excitation induces a transient $[Ca^{2+}]_i$ rise up to several μM, or to even higher levels in tiny cellular compartments.

Transient fluctuations of $[Ca^{2+}]_i$ (termed "Ca^{2+} signal") triggered or regulated various intracellular events. Usually, the generation of Ca^{2+} signal is determined by interaction of 1] external Ca^{2+} entry, 2] Ca^{2+} from interacellular compartments (Ca^{2+}stores), 3] cytoplasmic Ca^{2+} buffering by Ca^{2+} binding proteins, and 4] subsequent Ca^{2+} removal from the cytoplasm due to transmembrane Ca^{2+} efflux or sequestration by intracellular Ca^{2+} stores located in organelles (Blaustein 1988). Ca^{2+} entry in excitable cells such as chromaffin cells and neurons is mainly mediated by voltage-gated Ca^{2+} channels (VOCC). While in nonexcitable cells such as kidney cell, prostate cells and liver cells, VOCC do not exist, in this case, Ca^{2+} signaling is mainly initiated by the release of

internal Ca^{2+}. Most cells have two major mechanisms available for regulation of the release of internal Ca^{2+}.In one case, external signals acting in receptors at the cell surface generate the second messenger inositol 1,4,5-trisphosphate (IP_3), which diffuses into the cytoplasm and release Ca^{2+} by activating IP_3 receptors on the endoplasmic reticulum (Berridge 2009; Molnar et al 2012). IP_3 is formed by phospholipase C (PLC) which is activated by a cascade of events, including G protein activation initiated by receptor stimulation on the plasma membrane (Figure. 1-2) (Rong et al 2006).

Previous studies showed that resveratrol increased $[Ca^{2+}]_i$ in different cell types, including breast cancer cells (Sareen et al 2007), HepG2 cells (Ma et al 2007), rat primary neuron (Gong et al 2007), rat liver cell (Tian et al 2007), and human endothelial cells (Elíes et al 2012) etc.. However, the effect of resveratrol on $[Ca^{2+}]_i$ in oral cancer cells has not been explored.

1.3 Apoptosis

Apoptosis, or programmed cell death (PCD), is a highly regulated process that allows a cell to self-degrade in order for the body to eliminate unwanted or dysfunctional cells. During apoptosis, the genome of the cell will fracture, the cell will shrink and part of the cell will disintegrate into smaller apoptotic bodies (Figure. 1-3) (Raff and Martin 1998). Unlike necrosis, where the cell dies by swelling and bursting its content in the area, which causes an inflammatory response, apoptosis is a very clean and controlled process where the content of the cell is kept strictly within the cell membrane as it is degraded (Raff and Martin 1998; Fuchs and Steller 2011; Indran et al 2011). The apoptotic cell will be phagocytosed by macrophages before the cell's contents have a chance to leak into the neighbourhood (Raff and Martin 1998; Degterev and Yuan 2008). Therefore, apoptosis can prevent unnecessary inflammatory response.

Apoptosis is triggered by two major apoptotic pathways in mammalian cells: extrinsic

pathway and the intrinsic pathway (Figure. 1-4). The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as the Fas receptors, located on the cell membrane (Kurokawa and Kornbluth 2009).

In the extrinsic pathway, signal molecules known as ligands, which are released by other cells, bind to transmembrane death receptors on the target cell to induce apoptosis. For example, the immune system's natural killer cells possess the Fas ligand (FasL) on their surface (Csipo et al 1998; Kurokawa and Kornbluth 2009). The binding of the FasL to Fas receptors (a death receptor) on the target cell will trigger multiple receptors to aggregate together on the surface of the target cell. The aggregation of these receptors recruits an adaptor protein known as Fas-associated death domain protein (FADD) on the cytoplasmic side of the receptors. FADD, in turn, recruits caspase-8, an initiator protein, to form the death-inducing signal complex (DISC). Through the recruitment of caspase-8 to DISC, caspase-8 will be activated and it is now able to directly activate caspase-3, an effector protein, to initiate degradation of the cell. Active caspase-8 can also cleave BID protein to tBID, which acts as a signal on the membrane of mitochondria to facilitate the release of cytochrome c in the intrinsic pathway (Matthews et al 2012; Kaufmann et al 2012).

In the intrinsic pathway, it is triggered by cellular stress, specifically mitochondrial stress caused by factors such as DNA damage and heat shock (Matthews et al 2012). Upon receiving the stress signal, the proapoptotic proteins in the cytoplasm, BAX and BID, bind to the outer membrane of the mitochondria to signal the release of the internal content. However, the signal of BAX and BID is not enough to trigger a full release. BAK, another proapoptotic protein that resides within the mitochondria, is also needed to fully promote the release of cytochrome c and the intramembrane content from the mitochondria (Hague and Paraskeva 2004; Kaufmann et al 2012). Following the release, cytochrome c forms a complex in the cytoplasm with adenosine

triphosphate (ATP), an energy molecule, and Apaf-1, an enzyme. Following its formation, the complex will activate caspase-9, an initiator protein. In return, the activated caspase-9 works together with the complex of cytochrome c, ATP and Apaf-1 to form an apoptosome, which in turn activates caspase-3, the effector protein that initiates degradation. Besides the release of cytochrome c from the intramembrane space, the intramembrane content released also contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP) (Hague and Paraskeva 2004; Kurokawa and Kornbluth 2009; Pradelli et al 2010).

Previous studies showed that resveratrol induced apoptosis in different type cancer cells, including PC3 human prostate cancer cells, HepG2 human hepatic cancer cells, MCF-7 human breast cancer cells and HEK293T human embryonic kidney epithelial cells (Khan et al 2013); however, the detailed mechanism of resveratrol-induced apoptotic pathway in oral cancer is still waiting to be elucidated.

1.4 The OC2 human squamous cell line

In Taiwan, oral cancer is even the fourth leading cause of cancer death for males (Chung et al 2011). Oral squamous cell carcinoma (OSCC) is the most common neoplasia and is found frequently in oral cavity such as cheek, gum, and tongue (Lee et al 2012). Although cigarette and alcohol are considered as two major risk factors of oral carcinogenesis (Jemal et al 2011), occurrence of oral cancer was proved to be tightly associated with betel quid chewing in Taiwan and in south-east Asia (Jemal et al 2011; Trivedy et al 2002). Treating patients with oral cancer should focus on rendering the patients free of disease. In general, early-stage lesions may be treated by surgery or radiation with comparable results, and more advanced cancers are best approached with combined therapy, including surgery, radiotherapy and chemotherapy (Zhang and

Reichart 2007; Lee et al 2012). So it is of interest to know how different chemicals alter viability of oral cancer cells for developing of new pharmacological therapies. OC2 human squamous cell line is a useful model for oral research. OC2 was derived from a Chinese male. This cell line grows as adherent monolayers and has epitheloid morphology (Wong et al 1990). It has been shown that in this cell, $[Ca^{2+}]_i$ can increase in response to the stimulation of various compounds, such as m-3m3FBS (Chi et al 2012), Diethylstilbestrol (DES) (Hu1 et al 2012), and carvacrol (Liang et al 2013).

2. Aims

This thesis was to explore the effect of resveratrol on Ca^{2+} movement and viability in OC2 human oral cancer cells. Using fura-2 as a flurorescent Ca^{2+}–sensitive dye, here we show that resveratrol induced concentration-dependent $[Ca^{2+}]_i$ rise both in the presence and absence of extracellular Ca^{2+}. The $[Ca^{2+}]_i$ rise were characterized, the concentration-response relationships in the presence and absence of extracellular Ca^{2+} are established, and the pathways underlying resveratrol-induced Ca^{2+} influx and Ca^{2+} release are evaluated. The effect of resveratrol on cell viability, apoptosis, and the relationship to Ca^{2+} was also explored.

3. Materials and methods

3.1 Chemicals

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). Resveratrol and other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

3.2 Cell culture

OC2 human oral cancer cells obtained from Bioresource Collection and Research Center (Taiwan, ROC) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.

3.3 Solutions used in $[Ca^{2+}]_i$ measurements

Ca^{2+}-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose. Resveratrol was dissolved in dimethyl sulfoxide as a 10^{-1} M stock solution. The other agents were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the solution used in experiments did not exceed 0.1%, and did not alter viability or basal $[Ca^{2+}]_i$.

3.4 $[Ca^{2+}]_i$ measurements

Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6/mL. Cells were subsequently loaded with 2 μM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca^{2+}-containing medium twice and was made into a suspension in Ca^{2+}-containing medium at a density of 10^7/mL. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 mL of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 mL cell suspension was added to 0.9 mL Ca^{2+}-containing or Ca^{2+}-free

medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 sec to open and close the cuvette-containing chamber. For calibration of $[Ca^{2+}]_i$, after completion of the experiments, the detergent Triton X-100 and 5 mM $CaCl_2$ were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was subsequently added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. $[Ca^{2+}]_i$ was calculated as previously described (Grynkiewicz et al 1985). Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 1 min before starting the fluorescence recoding. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously (Merritt et al 1989).

3.5 Cell viability assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases (Ishiyama et al 1996). Augmentation in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions designed specifically for this assay (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a density of 10,000 cells/well in culture medium for 24 h in the presence of 0-100 μM resveratrol. The cell viability detecting reagent 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1, 3-benzene disulfonate] (WST-1; 10 μM pure solution) was added to samples after resveratrol treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM ([1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl ester)]) to chelate cytosolic Ca^{2+}, fura-2-loaded cells were

treated with 5 μM BAPTA/AM for 1 h prior to incubation with resveratrol. The cells were washed once with Ca^{2+}-containing medium and incubated with or without resveratrol for 24 h. The absorbance of samples (A_{450}) was determined using enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

3.6 Alexa® Flour 488 Annexin V/PI staining for detection of apoptosis

Annexin V/propidium (PI) staining assay was employed to further detect cells in early apoptotic stage. Cells were exposed to resveratrol at concentrations of 20 μM and 40 μM for 24 h. Cells were harvested after incubation and washed in cold phosphate buffered saline (PBS). Cells were resuspended in 400 μl reaction solution with 10 mM of HEPES, 140 mM of NaCl, 2.5 mM of $CaCl_2$ (pH 7.4). Alexa Fluor 488 Annexin V/PI staining solution (Probes Invitrogen, Eugene, OR, USA) was added in the dark. After incubation for 15 min, the cells were collected and analyzed in a FACScan flow cytometry analyzer. Excitation wave was at 488 nm and emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using 530 nm and 575 nm band pass filters, respectively. A total of at least 10,000 cells were analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity was on a logarithmic scale. The amount of apoptosis was determined as the percentage of AV^+/PI^- or AV^+/PI^+cell.

3.7 Measurements of subdiploidy nuclei by flow cytometry

This protocol was based on previous reports (Lovborg et al 2005). After treatment with 0-40 μM resveratrol for 24 h, cells were collected from the media, and were washed with ice-cold Phosphate Buffered Saline (PBS) twice and resuspended in 3 mL of 70% ethanol at -20°C. The cells were centrifuged for 5 min at 200xg. Ethanol was decanted and the cell pellet was washed with ice-cold medium twice, and was

suspended in 1 mL propidium iodide solution (1% Triton X-100, 20 μg PI, 0.1 mg/mL RNAse). The cells were washed once with PBS and incubated with or without resveratrol for 24 h. The cell pellet was incubated in the dark for 1hr at room temperature. Cell fluorescence was measured in the FACScan flow cytometer (Becton Dickinson immunocytometry systems, San Jose, CA, USA) and the data were analyzed using the flow cytometer analysis software WinMDI 2.8 (by Joe Trotter, freely distributed software) by gating 10^2-10^4 areas of the X and Y coordinates.

3.8 Statistics

Data are reported as typical or means±S.E.M. of three experiments. Data were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significant difference) procedure. A P-value less than 0.05 was considered significant.

4. Results

4.1 Effect of resveratrol on $[Ca^{2+}]_i$

Figure 2-1 (A) shows that the basal $[Ca^{2+}]_i$ level was approximately 50 nM in OC2 cells. At concentrations between 5 μM and 20 μM, resveratrol evoked $[Ca^{2+}]_i$ rises in a concentration-dependent manner in Ca^{2+}-containing medium. The $[Ca^{2+}]_i$ rise induced by 20 μM resveratrol attained to 356±2 nM followed by a sustained phase. The Ca^{2+} response saturated at 20 μM resveratrol because at a concentration of 25 μM, resveratrol induced a similar response as that induced by 20 μM. The $[Ca^{2+}]_i$ rises induced by 5-20 μM resveratrol in Ca^{2+}-free medium are shown in Figure 2-1 (B). At 0.1 and 1 μM, resveratrol did not cause a $[Ca^{2+}]_i$ rise. At a concentration of 20 μM, resveratrol induced a $[Ca^{2+}]_i$ rise by 198±2 nM above baseline followed by a gradual phase. The concentration-response plot of resveratrol-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing or Ca^{2+}-free medium is shown in Figure 2-1 (C). The EC_{50} value is approximately 5±2 μM in Ca^{2+}-containing or Ca^{2+}-free medium. These data suggest that the resveratrol-induced $[Ca^{2+}]_i$ rises in OC2 cells were cause from extracellular Ca^{2+} influx and intracellular Ca^{2+} release.

4.2 Effect of resveratrol on Mn^{2+} influx

Experiments were performed to confirm resveratrol-induced $[Ca^{2+}]_i$ rise involved Ca^{2+} influx in OC2 cells. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (Merritt et al 1989). Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} implies Ca^{2+} influx. Figure 2-2 shows that 20 μM resveratrol induced an immediate decrease in the 360 nm excitation signal (compared to trace a). This suggests that resveratrol-induced $[Ca^{2+}]_i$ rise involved Ca^{2+} influx. The decrease attained to a maximum of 88 units at the time point of 256 sec.

4.3 Effect of resveratrol-induced Ca^{2+} influx pathways

Experiments were performed to explore the Ca^{2+} entry pathway of the resveratrol-indcued response in OC2 cells. In modulator-treated groups, the reagent was added 1 min before resveratrol (1 or 20 μM). Figure 2-3 (A) shows that in Ca^{2+}-medium, the concentration of reagents were 10 nM for phorbol 12-myristate 13-acetate (PMA), 2 μM for GF109203X, 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SK&F96365. Resveratrol-induced Ca^{2+} signal was inhibited by nifedipine and PKC inhibitor GF109203X. Figure 2-3 (B) shows that in Ca^{2+}-free medium, resveratrol-induced Ca^{2+} signal was partially inhibited by the PKC inhibitor GF109203X.

4.4 Intracellular Ca^{2+} store of resveratrol-induced rise $[Ca^{2+}]_i$

Previous reports have shown that the endoplasmic reticulum was the major Ca^{2+} store in OC2 cells (Liu et al 2008; Yeh et al 2006; Wang et al 2005). Experiments were used by using inhibitor of endoplasmic reticulum Ca^{2+} pumps, 2,5-di-tert-butylhydroquinone (BHQ) (Wassenberg et al 1997; Shideman et al 2009). Figure 2-4 (A) shows that 20 μM resveratrol added after pretreatment with BHQ (50 μM), partially induce a $[Ca^{2+}]_i$ rise in OC2 cells. In contrast, Figure 2-4 (B) shows that BHQ induced a $[Ca^{2+}]_i$ rise of 110±2 nM. Subsequently added resveratrol (20 μM) induced a $[Ca^{2+}]_i$ rise of 7±2 nM. BHQ abolished resveratrol-induced $[Ca^{2+}]_i$ rise.

4.5 A role of PLC in resveratrol-induced rise $[Ca^{2+}]_i$

PLC-dependent formation of inositol 1,4,5-trisphosphate is a key step for releasing Ca^{2+} from the endoplasmic reticulum (Florenzano et al 2006). Because resveratrol was able to release Ca^{2+} from the endoplasmic reticulum, the role of PLC in this release was examined. U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione), a PLC inhibitor (Thompson et al 1991), was applied to see whether this enzyme was required for resveratrol-induced Ca^{2+} release. Figure 2-5 (A) shows that ATP (10 μM) induced a $[Ca^{2+}]_i$ rise of 89±2 nM in OC2 cells. ATP is a PLC-dependent agonist of

$[Ca^{2+}]_i$ rise in most cell types (Florenzano et al 2006). Figure 2-5 (B) shows that incubation with 2 μM U73122 did not change basal $[Ca^{2+}]_i$ but largely inhibited ATP-induced $[Ca^{2+}]_i$ rises. This suggests that U73122 effectively suppressed PLC activity in OC2 cells. Figure 2-5 (B) also shows that incubation with addition U73122 and ATP abolished the 20 μM resveratrol-induced $[Ca^{2+}]_i$ rise.

4.6 Relationship between resveratrol-induce $[Ca^{2+}]_i$ rise and cell death

Given that acute incubation with resveratrol induced a substantial $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rise often alters cell viability (Bootman et al 2002), experiments were performed to examine the effect of resveratrol on viability of OC2 cells. Cells were treated with 0-100 μM resveratrol for 24 h, and the tetrazolium assay was performed. In the presence of 20-100 μM resveratrol, cell viability decreased in a concentration-dependent manner (Figure 2-6). Because resveratrol induced $[Ca^{2+}]_i$ rises and cell death, it would be interesting to know whether the death occurred in a Ca^{2+}-dependent manner. The intracellular Ca^{2+} chelator BAPTA/AM (Tsien 1980) was used to prevent a $[Ca^{2+}]_i$ rise during resveratrol pretreatment. Figure 6 shows that 5 μM BAPTA/AM loading did not alter control cell viability. BAPTA/AM at 5 μM has been shown to effectively prevent a $[Ca^{2+}]_i$ rise in several cell types including OC2 cells. (Chi et al 2012). In the presence of 20 and 40 μM resveratrol, BAPTA/AM loading failed to alter resveratrol-induced cell death (n=3; $P < 0.05$). This result suggests that resveratrol-induced cell death was not triggered by a preceding $[Ca^{2+}]_i$ rise.

4.7 The role of apoptosis in resveratrol-induced cell death

Annexin V/PI staining was applied to detect apoptosis cells after resveratrol treatment. Figure 2-7 (A) (B) shows that resveratrol between 20 μM and 40 μM induced apoptosis. At 40 μM resveratrol also induced necrosis. PI staining was also used to explore the role of apoptosis in resveratrol-induced cell death. The percentage of cells that underwent apoptosis was analyzed by flow cytometry via measuring subdiploidy nuclei, a hallmark

of apoptosis, after cells were treated with 20 μM and 40 μM resveratrol for 24 h. As shown in Figure 2-8 (A) (B), concentration-dependent apoptosis was observed in 20 and 40 μM resveratrol-treated groups. The sub G1 phase was 2.63% in control. After incubation with 20 μM and 40 μM resveratrol, sub G1 phase increased to 5.41% and 28.51%. Therefore, the data suggests that resveratrol-induced cell death may be through apoptosis.

5. Discussion

Our study showed that the natural product resveratrol induced a $[Ca^{2+}]_i$ rise in a cultured oral cancer cell line. Resveratrol increased $[Ca^{2+}]_i$ by depleting intracellular Ca^{2+} stores and causing Ca^{2+} entry from extracellular milieu because removing extracellular Ca^{2+} reduced 40% of resveratrol-induced $[Ca^{2+}]_i$ rise. Removal of extracellular Ca^{2+} reduced the resveratrol-induced $[Ca^{2+}]_i$ rise throughout the measurement period, suggesting that Ca^{2+} entry occurred during the whole stimulation interval.

The mechanism of resveratrol-induced Ca^{2+} influx was explored. Because resveratrol-induced $[Ca^{2+}]_i$ rise was partially inhibited by nifedipine, the results suggested that resveratrol might cause Ca^{2+} entry via store-operated Ca^{2+} entry. Nifedipine was originally thought to be a selective blocker of L-type voltage-gated Ca^{2+} channels. Recent evidence shows that nifedipine also blocks store-operated Ca^{2+} channels in different cell types such as breast cancer cells (Davis et al 2012), colon cancer cells (Sun et al 2012) and hepatocarcinoma cell (Yang et al 2013). Therefore, the Ca^{2+} entry pathways involved in resveratrol-induced $[Ca^{2+}]_i$ rise may be through store-operated Ca^{2+} channels. On the other hand, it has been shown that blockade of transient receptor potential cation channel subfamily M member 8 (TRPM8) activity reduced the invasion potential of oral squamous carcinoma cell lines (Okamoto et al 2012). Because TRP Ca^{2+} channels have been shown to exist in oral cells, it is possible that TRP Ca^{2+} channels may mediate resveratrol-induced $[Ca^{2+}]_i$ rise in our study.

In this study, BHQ pretreatment nearly abolished resveratrol-induced $[Ca^{2+}]_i$ rise; and conversely, pretreatment with resveratrol nearly abolished BHQ-induced $[Ca^{2+}]_i$ rise. Therefore, regarding the Ca^{2+} stores involved in resveratrol-induced Ca^{2+} release, the BHQ-sensitive endoplasmic reticulum stores might be the dominant one.

Because activation of PLC produces IP_3 and diacylglycerol, which stimulates PKC, the effect of regulation of PKC activity on resveratrol-induced $[Ca^{2+}]_i$ rise was explored. Both activation and inhibition of PKC altered resveratrol-induced $[Ca^{2+}]_i$ rise. Regulation of PKC activity has been shown to modulate Ca^{2+} signaling in different systems. Activation or inhibition of PKC by different agents has been shown to regulate store-operated Ca^{2+} channels in OC2 cells such as paroxetine (Fang et al 2011), carvedilol (Hsieh et al 2011), and setraline (Chien et al 2011). It seems that PLC-dependent pathways played a role in resveratrol-induced Ca^{2+} release, since the response was abolished when PLC activity was inhibited by U73122. Therefore it appears that resveratrol-induced Ca^{2+} release was caused by an IP3-dependent Ca^{2+} release from the endoplasmic reticulum and other stores.

Resveratrol was found to be cytotoxic to OC2 cells in a concentration-dependent manner. Our data showed that resveratrol between 5 μM and 20 μM increased $[Ca^{2+}]_i$; whereas at a concentration of 40 μM, resveratrol had caused death of 40% of cells. These data are not contradictory because $[Ca^{2+}]_i$ measurements were completed within 250 sec after addition of resveratrol, but viability was assayed after 24h treatment with resveratrol.

Because resveratrol induced both $[Ca^{2+}]_i$ rises and cell death, it would be interesting to know whether the death occurred in a Ca^{2+}-dependent manner. In this study, BAPTA loading for 25 h did not reverse resveratrol-induced cell death in the presence of 20-100 μM resveratrol. Therefore, it is suggested that resveratrol-induced cell death was not triggered by a $[Ca^{2+}]_i$ rise. Emptying of intracellular Ca^{2+} stores and/or influx of extracellular Ca^{2+} can modulate cell viability in many cell types (Bootman et al 2002). However, Ca^{2+}-independent cell death could be found in some cell types such as prostate cancer cells (Tsai et al 2010) and cortical neuron cells (Zhang et al 2013).

Resveratrol-induced cell death was found to involve apoptosis. In our studies,

Annexin/PI staining data suggest that apoptosis was involved in resveratrol between 20 μM and 40 μM induced cell death. Furthermore, cells were treated with resveratrol for 24 h in order to obtain measurable changes in apoptosis. Similarly, resveratrol between 10 μM and 200 μM induced apoptosis in including human hepatocarcinoma cells (Du et al 2012), human gastric cancer cells (Wang et al 2012), and human colon carcinoma cells (Fang et al 2012). Therefore, the concentration range of resveratrol that caused apoptosis in our study was reasonable; and it appears to be significantly different between oral cancer cells and different cancer cells.

Previous studies have explored the plasma concentration of resveratrol after oral ingestion. BioResponse resveratrol (BR-resveratrol)-related adverse effects were reported at doses up to 5 g. A single 5 g dose of BR- resveratrol resulted in a mean of the maximum concentration (C_{max}) of ~20 μM after 24 h (Scott et al 2012). Consistently, our data show that resveratrol at 20 μM caused a $[Ca^{2+}]_i$ rise and cytotoxicity in OC2 cells. In addition, this study also showed that in elderly or impaired oral patients, the plasma concentration of resveratrol after oral administration might be 3~8 fold higher than in healthy adults (Scott et al 2012). The local concentrations in the oral cavity may be even much higher than in the plasma. Therefore, our study may have clinical relevance.

6. Conclusion

Collectively, the results show that resveratrol induced Ca^{2+} release from endoplasmic reticulum in a PLC-dependent manner and also caused Ca^{2+} influx *via* store-operated Ca^{2+} entry in OC2 human oral cancer cells. Resveratrol also induced apoptosis in a Ca^{2+}-independent manner.

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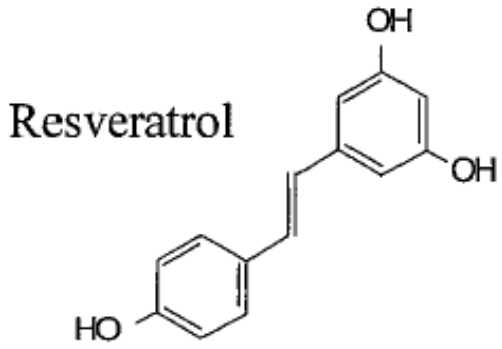

Figure. 1-1 Structure of resveratrol Resveratrol (trans-3,4′,5-trihydroxystilbene) was purchased from Calbiochem (San Diego, CA). The protein substrates protamine sulfate and histone III-S were purchased from Sigma Chemical Co (Stewart et al 1999).

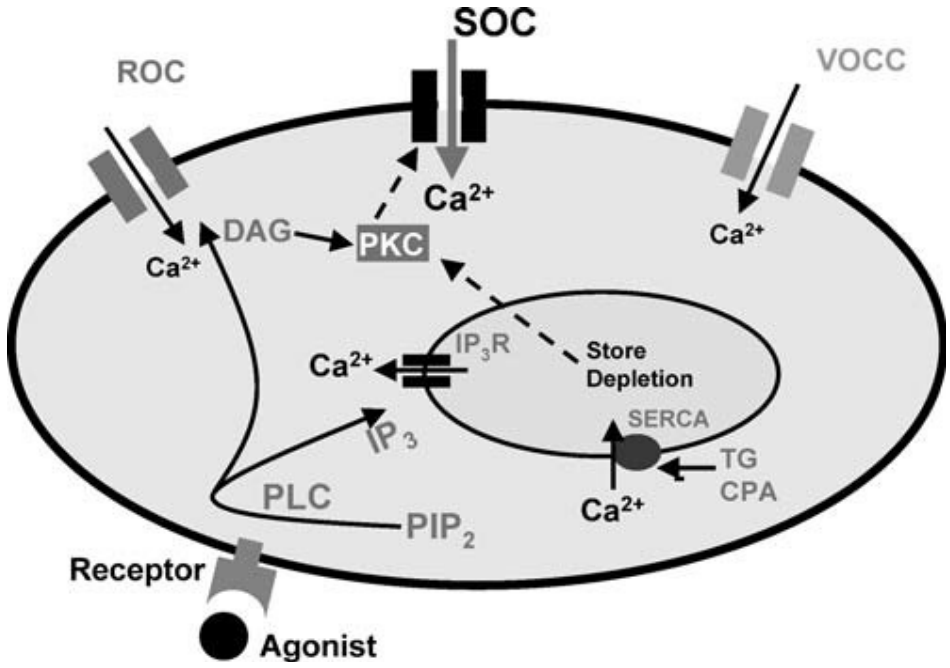

Figure 1-2. Major Ca^{2+} entry pathways (Rong et al 2006)

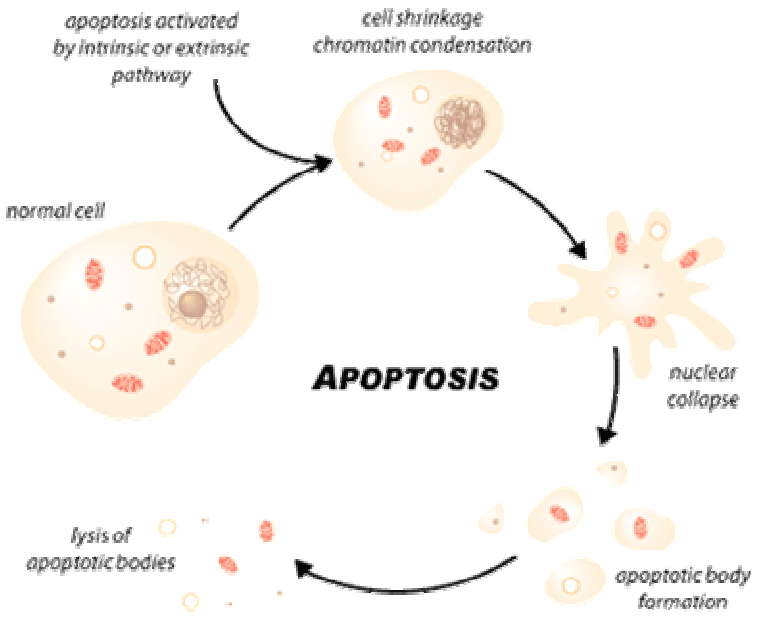

Figure. 1-3 Apoptosis (programmed cell death) (Raff and Martin 1998)

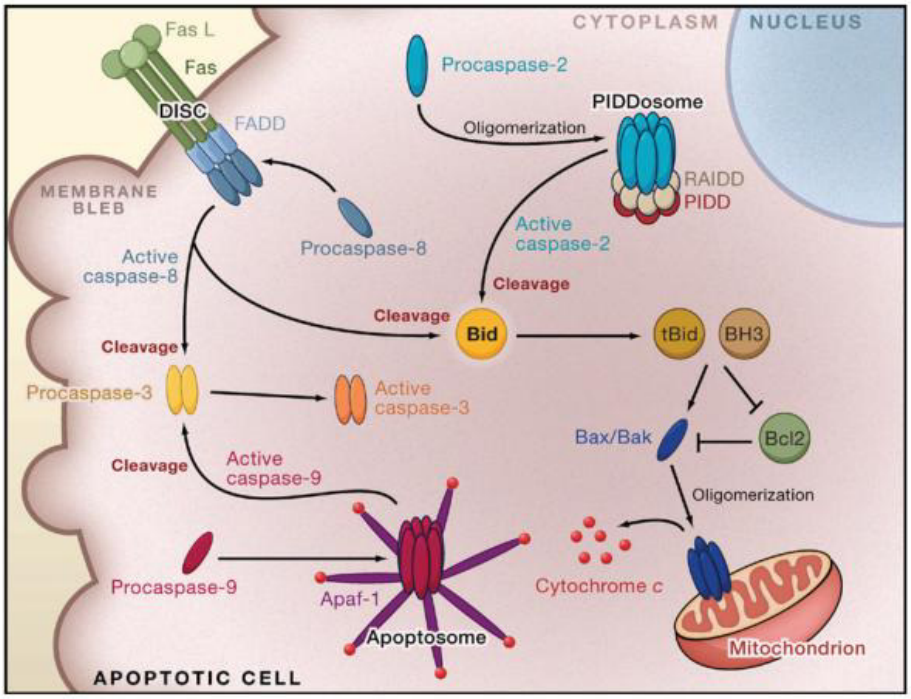

Figure. 1-4 Extrinsic and intrinsic apoptotic pathways (Kurokawa and Kornbluth 2009)

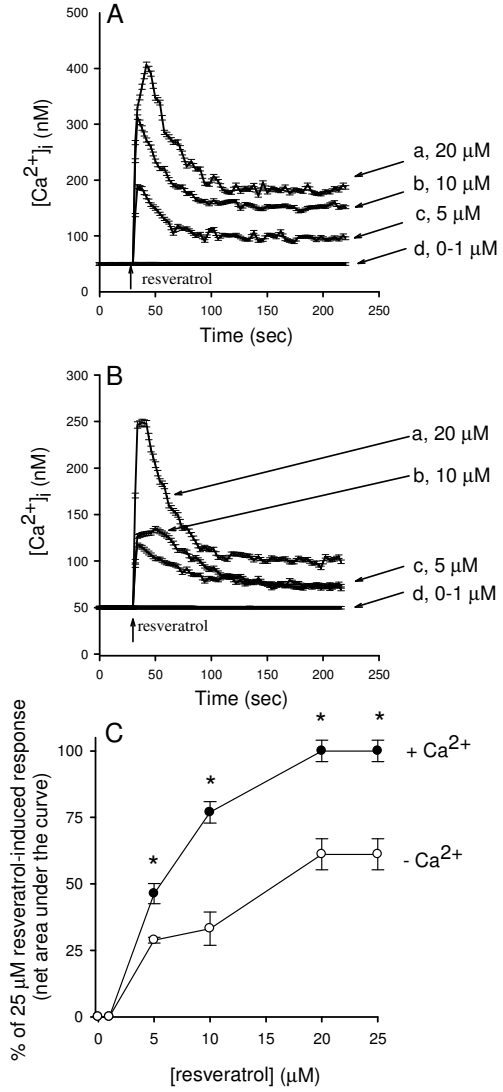

Figure. 2-1 Resveratrol induced a $[Ca^{2+}]_i$ rise in a concentration-dependent manner in OC2 cells. (A) Effect of resveratrol on $[Ca^{2+}]_i$ in fura-2-loaded OC2 cells. Resveratrol was added at 25 sec. The concentration of resveratrol was indicated. The experiments were performed in Ca^{2+}-containing medium. (B) Effect of resveratrol on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Resveratrol (5-20 μM) was added at 25 sec in Ca^{2+}-free medium. (C) Concentration-response plots of resveratrol-induced $[Ca^{2+}]_i$ rises in the presence or absence of extracellular Ca^{2+}. **P*<0.05 compared to open circles. Data are mean ± S.E.M. of three experiments.

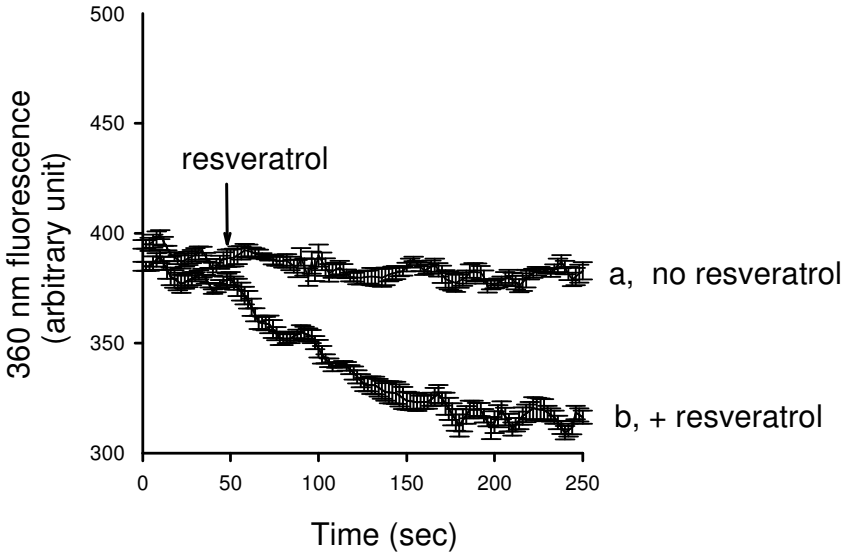

Figure. 2-2 Effect of resveratrol on Ca^{2+} influx by measuring Mn^{2+} quenching of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm.

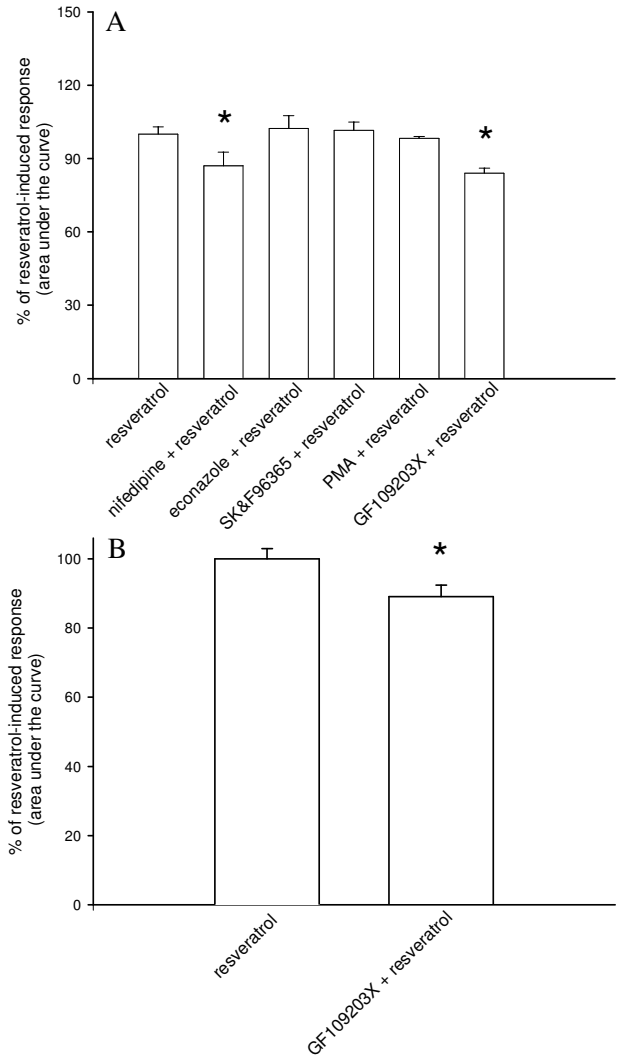

Figure. 2-3 Effect of Ca^{2+} channel modulators on resveratrol-induced $[Ca^{2+}]_i$ rise. (A) In modulator-treated groups, the reagent was added 1 min before resveratrol (1 or 20 μM). The concentration was 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SK&F96365, 10 nM for phorbol 12-myristate 13-acetate (PMA), 2 μM for GF109203X . Data are expressed as the percentage of control (1st column) that is the area under the curve (25-200 sec) of 20 μM resveratrol-induced $[Ca^{2+}]_i$ rise, and are mean ± S.E.M. of three experiments. Three experiments are independent biological replicates. **P*<0.05 compared to the 1st column. (B) Recording of resveratrol-induced responses in the presence or absence of GF109203X.

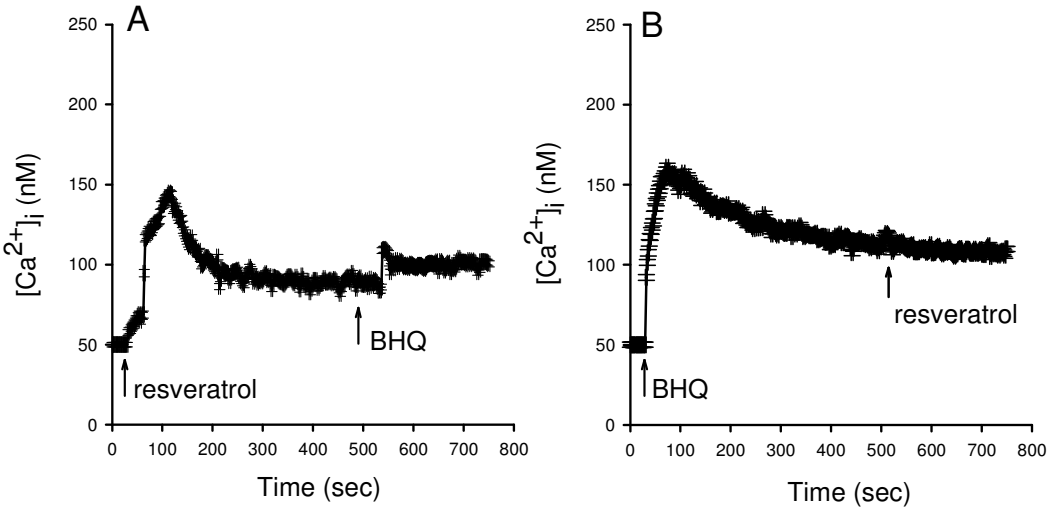

Figure. 2-4 Intracellular Ca^{2+} stores of resveratrol-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) (B) resveratrol (20 μM), BHQ (50 μM) were added at time points indicated. (BHQ are endoplasmic reticulum Ca^{2+} pump inhibitor).

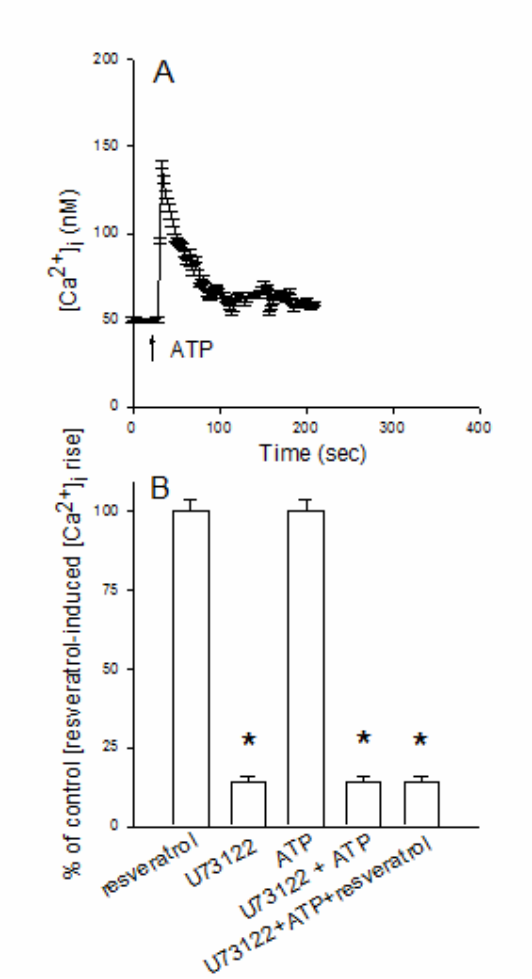

Figure. 2-5 Effect of U73122, a PLC inhibitor, on resveratrol-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added as indicated. (B) U73122 (2 μM), ATP (10 μM), and resveratrol (20 μM) were added as indicated. **P*<0.05 compared to first bar (control). Data are mean ± S.E.M. of three experiments. Control is the area under the curve of 20 μM resveratrol-induced $[Ca^{2+}]_i$ rise (25-250 sec).

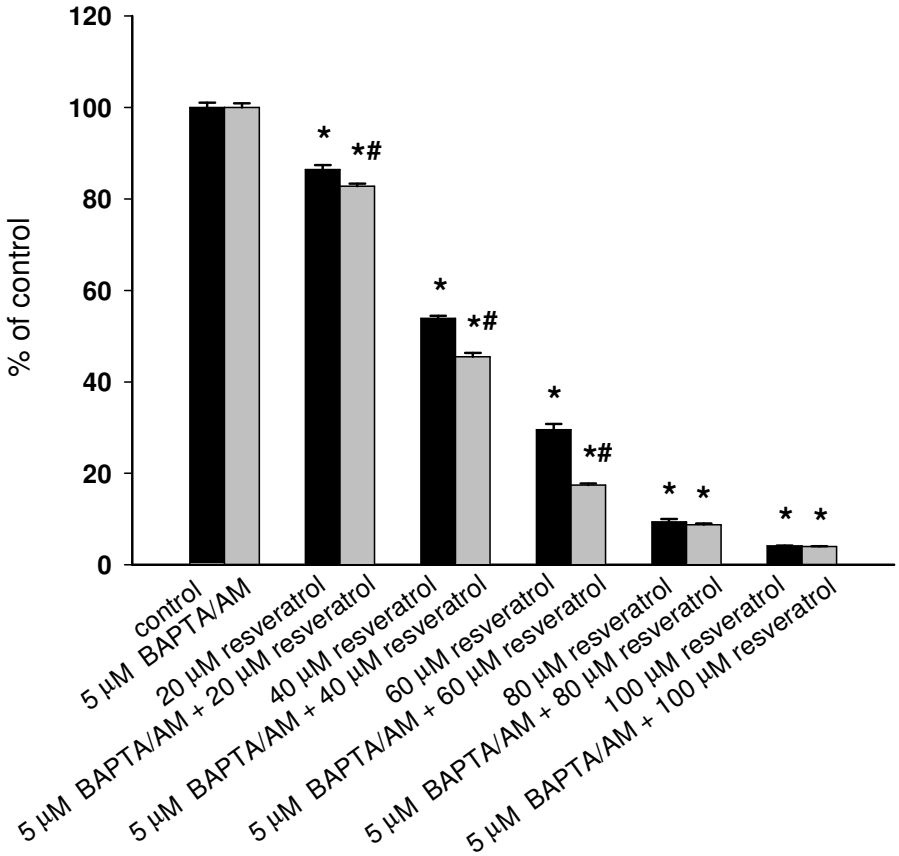

Figure. 2-6 Resveratrol reduced cell viability in a concentration dependent manner and BAPTA-AM loading did not reverse resveratrol-induced cytotoxicity in OC2 cells. Cells were treated with 0-100 μM resveratrol for 24 h, and the cell viability assay was performed. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to fura-2-loaded cells followed by treatment with resveratrol in Ca^{2+}-containing medium. **P*<0.05 compared to control. Data are mean ± S.E.M. of three experiments.

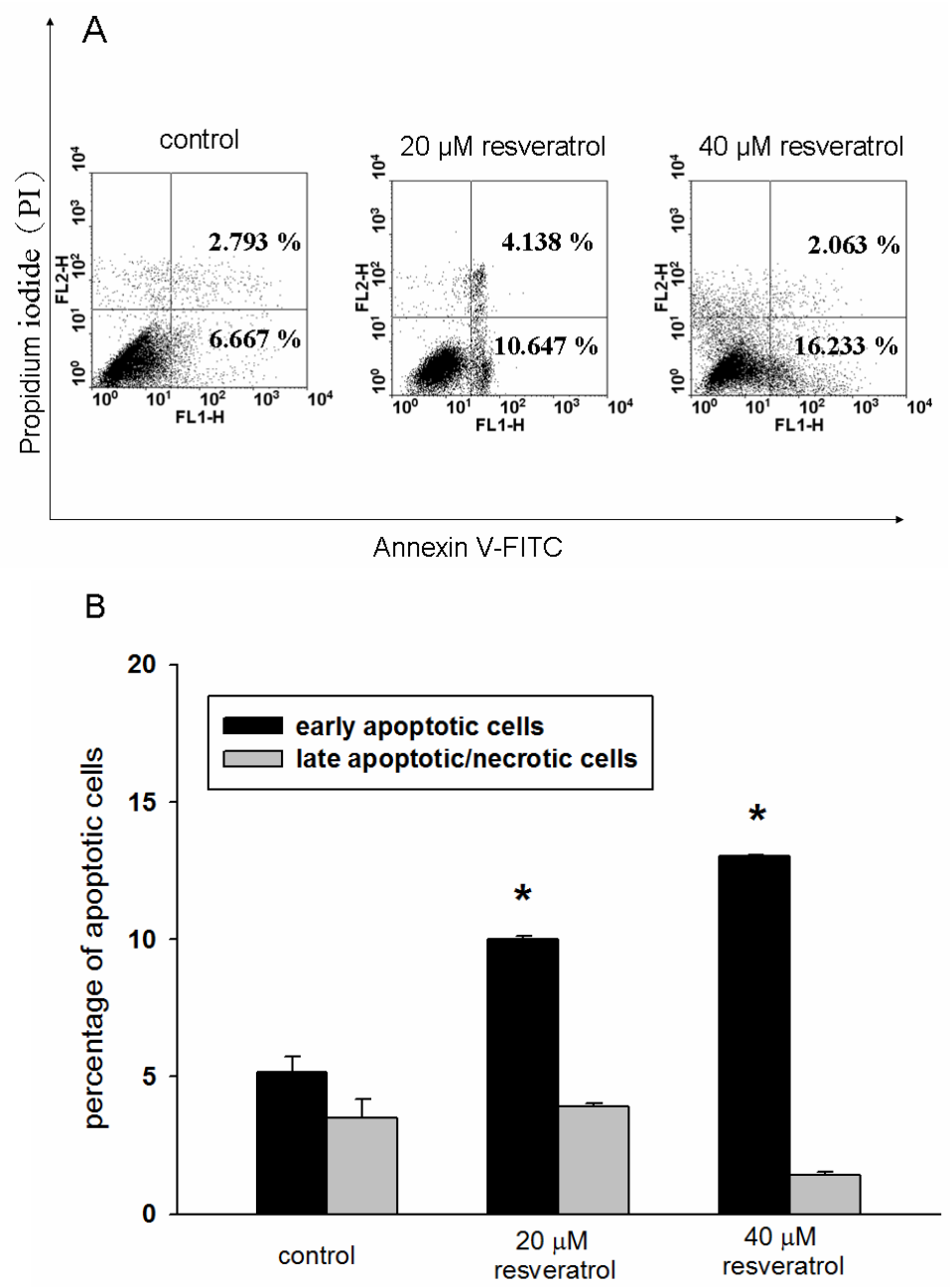

Figure. 2-7 Apoptosis induced by resveratrol measured by Annexin V/PI staining. (A) Cells were treated with 20 μM and 40 μM resveratrol, respectively, for 24 h. Cells were then processed for Annexin V/PI staining and analyzed by flow cytometry. (B) The percentage of apoptotic cells. **P*<0.05 compared with control. Data are mean ± S.E.M. of three experiments.

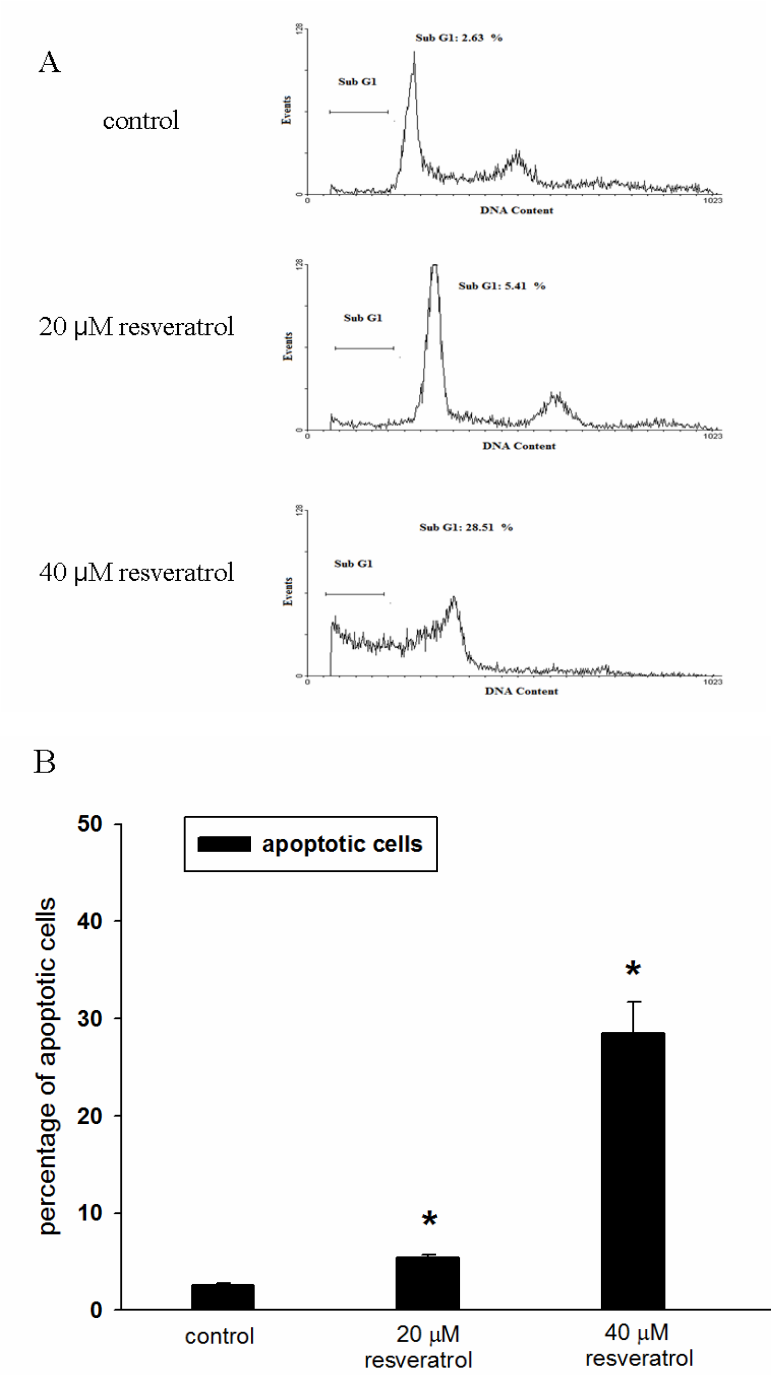

Figure. 2-8 Resveratrol between 20 μM and 40 μM caused cell cycle arrest in OC2 cells. (A) Cells were treated with 20 μM and 40 μM resveratrol, respectively, for 24 h. Cells were then processed for PI staining and analyzed by flow cytometry. (B) The percentage of apoptotic cells. **P*<0.05 compared with control. Data are mean ± S.E.M. of three experiments.