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以動物模式探討烹調油煙引起之慢性發炎反應在女性肺腺癌形成之角色研究(3/3)

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以動物模式探討烹調油煙引起之慢性發炎反應在女性肺腺癌形成之角色研究

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1、中文摘要

過去許多流行病學研究結果都發現廚房油煙 (cooking oil fumes, COF) 暴露與台灣女性肺癌之形成有關。本研究室的研究顯示 COF 會誘發肺腺癌 CL-3 細胞株經由 NF-kB 路徑使 Cox-2 基因過度表現，這可能是 COF 會引起 ROS 的形成所致。這結果亦可由女性肺腫瘤組織之 Cox-2 蛋白表現與 8-OH-dG 之表現量有正相關性得到印證。最近研究顯示 NF-kB 活化會引起發炎反應，且發炎反應又與人類腫瘤形成可能有關。但其中之分子機轉並不清楚。為了探討此機制和肺腫瘤形成之相關性本研究第一年以氣管內注入法將已知致癌物 DMBA 處理單一劑量做為誘發腫瘤形成之起始劑 (initiator)，然後每週以氣管注入 COF 一次，造成大鼠肺部持續性的發炎狀態做為啟動劑 (promoter)，來探討慢性發炎和肺腫瘤形成間之相關性。經過連續刺激二、六及八個月結果發現長期 COF 暴露所引起發炎反應會促進支氣管及肺泡上皮細胞增生，且此現象在 COF 刺激早期 (二個月) 即可觀察到，然而此類增生現象是否為引起肺腫瘤化之前期變化，在本研究八個月的暴露時間仍無法誘發成功。因此本計畫改以肺腺癌 A549 肺癌細胞為研究模式，探討 COF 是否會促進肺癌細胞存活與生長，並了解其可能之訊號路徑及其作用分子機轉。結果發現 COF 暴露會增加細胞 S phase 之分布比率；且觀察到 IAP-2 蛋白會隨著 COF 濃度增高，增強 IAP2 蛋白的表現量。當同時處理 NF-κB 抑制劑 BAY，則會阻斷 COF 誘導 IAP2 蛋白之能力。這顯示 NF-κB 路徑可能參與 COF 誘導 IAP2 蛋白之表現。COF 除了會誘導 IAP2 表現外，是否亦會誘發其他三種 IAPs family 蛋白的表現 （IAP1、survivin 以及 XIAP）？這些蛋白之表現與 COF 所誘導肺腺癌細胞增生是否有關？結果顯示，IAP1 及 survivin 蛋白之表現層次可以被 COF, BaP，及 2,4-DDE 所誘導，但是以 IAP2 蛋白之表現較具敏感性。相反的是，XIAP 蛋白卻可以被 COF 及 2,4-DDE 所抑制，但是 BaP 則不具影響。雖然 COF 可以對 IAPs 中 IAP2 及 XIAP 蛋白產生不同之調控效果，但是凋亡蛋白 caspase-3 之活性卻可以被 COF 所抑制。本研究進一步將三種不同訊息路徑之抑制劑（SB203580，PD98059，LY294002）分別與 COF 同時處理細胞，IAP2 蛋白表現可被 PI3K 之抑制劑 LY294002 抑制，而 SB203580 (p38 MAPK) 及 PD98059 (ERK) 則無此能力。在細胞週期 sub-G1phase 與 TUNEL assay 之結果顯示，LY294002 會促進肺癌細胞之凋亡，因此除了 NF-κB 路徑之外，PI3K 在 COF 所誘導肺腺癌細胞增生與存活可能亦扮演重要之角色。爲了解 COF 誘導 A549 細胞增生是否與細胞週期相關之調控蛋白有關，本研究同樣以西方墨點法得知，COF 確實會促進 cyclin D1 的表現，卻會顯著抑制 p21 蛋白的表現。由以上之結果得知，活化 IAP1, IAP2, survivin 以及 cyclin D1 蛋白之表現與抑制 XIAP，caspase-3，以及 p21 蛋白之表現，可能部份解釋 COF 誘導肺腺癌細胞之存活與增生的分子機轉。至於 COF 引起肺癌細胞增生是否與 ROS 產生有關？本研究以 DCF-dA 分析 ROS 之形成，發現 COF 處理肺腺癌細胞會產生 ROS，並發現 COF 所誘導 ROS 之產生可能是經由 PI3K 活化，進而誘導 p-Akt 之表現，促進肺腺細胞之增生。總之，本研究結果發現 COF 暴露會促進肺腺癌細胞之存活與增生，有助於了解廚房油煙暴露與台灣女性肺癌的形成之分子機轉，在台灣不抽菸女性肺癌之預防與治療策略上會有些許助益。

2、英文摘要

Accumulated epidemiological data indicated that exposure of cooking oil fumes (COF) was associated with lung cancer among Taiwanese women. Our previous study showed that Cox-2 was up-regulated by COF treatment in lung adenocarcinoma CL-3 cells. In addition, reactive oxygen species generated by COF treatment may be involved in the activation of NF-kB pathway to cause Cox-2 expression in lung cancer cells. These results were supported by our preliminary data from lung cancer patients showing that Cox-2 protein expression was significantly correlated with 8-OH-dG expression levels. Thus, we hypothesized that chronic inflammation induced by COF may be involved in DMBA carcinogen-initiated lung carcinogenesis. In this study, the intra-tracheal instillation of COF and/or DMBA have been performed weekly for two, six and eight months. In two months experiment, the epithelial cells of small tracheals of DMBA or COF treated rats and COF+DMBA combined treated-rats were observed to have the phenomenon of tracheal and alveolar epitheial cell proliferation. The phenomenon of cell proliferation was more obvious in the rats treated with COF+DMBA than that of COF or DMBA alone treatment. Thus, COF appears to promote the cell proliferation of tracheal epithelial cells. But in six and eight months experiment, the result were not more significant than two months. We had to use A549 lung cancer cell line model to explore the role of cooking oil fume in lung tumorigenesis. The MTT assay data showed that the cell viability of A549 was significantly increased in a concentration-dependent manner by COF treatment for 48 h. Flow cytometery results indicated that the proportion of A549 cells at S-phase was markedly increased after exposure of COF. To elucidate whether the antiapoptotic c-IAP2 (IAP2) was involved in COF-improved cell survival, IAP2 protein levels was determined by western blot and the results showed it was significantly induced by COF in a concentration-dependent manner. Moreover, the suppression of BAY, a NF-κB binding inhibitor, on the COF-induced IAP2 protein levels indicated that NF-κB activation by COF may partly be involved in IAP2 induction. These results showed that the positive impact of COF on cell survival and proliferation of A549 lung tumor cells may be through an induction of IAP2 overexpression. In this study, the other three IAP family proteins, c-IAP1 (IAP1), XIAP and survivin were investigated to understand whether these proteins were involved in COF-induced cell survival. In this study, to verify whether other antiapoptotic proteins were also associated with lung cancer cell survival and proliferation, the other three IAP family proteins, including IAP1, XIAP and survivin expressions, were evaluated by Western blotting after the cells were treated with COF and its two major components, BaP and 2,4-DDE. Our data shows that IAP1 and survivin expression levels were increased by COF, BaP, and 2,4-DDE, but not like IAP2 induction in a dose-dependent manner, on the contrary, XIAP surprisingly decreased by COF and 2,4-DDE, but not by BaP. Even though different effects of COF on IAP2 and XIAP protein expressions, the apoptotic caspase-3 expression was diminished by COF. Therefore, to mimic the effects of complex mixture of COF exposure on Chinese female lung cancer development, the role of IAPs attenuated by COF on lung cancer cell proliferation was investigated. To further verify which signaling pathways may be involved in IAP2 induction by COF, SB203580, PD98059, BAY and LY294002, for inhibitors respectively specific for p38 mitogen-activated protein kinase (p38 MAPK), extracellular-regulated kinase (ERK), nuclear factor-kappa B (NF-κB) and phosphatidylinositol-3-kinase (PI3K)/Akt pathway were used. Our data showed that IAP2 protein expression levels were markedly attenuated by LY294002 and BAY, but not by SB203580 and PD98059. Meanwhile, TUNEL assay results showed that the percentage of apoptotic cells increased by LY294002 was more obvious than by BAY. Additionally, we also investigated that the cell cycle regulated of A549 cells proliferation was associated of induction of cyclin D1 and reduction of p21 protein expression with the lung tumor cell progression. Additionally, we also investigated that COF could induce ROS generation in a dose-dependend manner and it may be through the PI3K pathway to involve the phosphorylation of Akt. Taken together, increase of IAP1, IAP2, survivin, cyclin D1 and ROS, and decrease of XIAP, caspase-3, and p21 proteins might partly contribute to cell survival and proliferation after COF exposure.

關鍵字: Cooking oil fumes (COF) ， Inhibitor of apoptosis proteins (IAPs)，Lung cancer

目錄

1、中文摘要 2
2、英文摘要 3
3、報告內容 5
4、前言與研究目的 5
5、文獻探討 6
6、研究方法 12
7、結果 16
8、討論 21
9、參考文獻 24
10、表與圖 31

3、報告內容

3.1. 前言與研究目的

過去許多流行病學研究結果都發現廚房油煙 (cooking oil fumes, COF) 暴露與台灣女性肺癌之形成有關。在流行病學上已有許多研究證實中國女性肺癌發生與烹調油煙暴露有關。因此本研究室早在十年前即開始研究烹調油煙之致癌物分析。在1998 年首次以MS 分析確定煎魚油煙中主要基因毒物為 MeIQx 異環胺類化合物 (Yang et al., 1998)。在2000 年，本研究室以 LC-MS 確定煎魚油煙處理之肺腺癌CL-3 細胞中主要形成之DNA adduct 為 BPDE-N2-dG (Yang et al., 2000)。此DNA adduct 也是香菸引起p53 基因突變之主要 DNA adduct，但是我們並沒有發現台灣女性肺癌患者有與香菸一樣之 p53 基因突變輿圖，即 G->T mutation pattern 在一定的 hot spot 上。然而，本實驗是先前結果發現不抽煙之女性肺癌患者之 DNA adduct levels 確實高於同樣不抽煙之男性患者。而此現象並不是多環芳香烴代謝有關基因CYPA1 與GSYM1 之多形性所造成，而可能是女性對環境污染物之感受性較男性為高 (Cheng et al., 2001)。因此推測女性對環境致癌物暴露，包括 COF 暴露所引起之 BPDE-N2 dG adduct 以及其他 PAH-DNA adduct levels 高，可能都在肺癌之晚期發生，似乎可用來解釋為何台灣女性為何不抽煙有較高之罹患肺癌之危險性。

本研究室的研究顯示 COF 會誘發肺腺癌 CL-3 細胞株經由NF-kB 路徑使 Cox-2 基因過度表現，這可能是 COF 會引起 ROS 的形成所致。這結果亦可由女性肺腫瘤組織 Cox-2 蛋白表現與 8-OH-dG 之表現量有正相關性得到印證。過去本研究室已發現 COF 會經由 NF-kB 路徑誘發 Cox-2 之轉錄活化與蛋白表現，且發現COF 引起 Cox-2 表現之能力遠高於 COF 之主要致癌成份 benzo[a]pyrene (BaP) (Lin et al., 2002)。又由於會誘發Cox-2 基因表現，因此亦發現其所產生之 PGE2 與 lipid peroxidation 終產物 maldialdehyde 之產生呈正相關性 (Lin et al., unpublished data)。最近研究之結果發現 COF 會經由 PI3K 誘發抑制細胞程序性死亡之 IAP1 與 IAP2 蛋白表現，又由 flow cytometry 之數據顯示 COF 處理後不僅程序性死亡之細胞數減少，且 G1/S transition 細胞數大增 (Hung et al., 2007) 實驗之結果均顯示COF 不僅能使細胞存活且會促進細胞增生。

發炎反應與人類癌症之相關性早在1863年的病理學報告中，即假設人類腫瘤可能起源於慢性發炎之區域，這假設主要依據有些不同之刺激，會引起組織受傷而發炎，進而促進細胞增生。已知只有細胞增生並不會產生腫瘤，但若持續在含有許多發炎細胞，生長因子，活化之基質細胞以及引起DNA 傷害之促進劑之環境，則促進腫瘤發生之危險性即大增。當一基因變異之起始細胞持續在上述之細胞增生的微環境(microenvironment)，則腫瘤會經由傷口無法癒合而形成。近年來，發炎、免疫與腫瘤間之相關性已漸被接受，但是其間之分子機轉至今未明 (Coussen and Werb, 2002)。

發炎反應在腫瘤形成之過程中可能扮演啟動者 (promoter)之角色，而非如化學致癌物引起致癌基因或抑癌基因突變而起始腫瘤形成之起始者 (initiator)角色。腫瘤形成之啟動期可能是暴露化學啟動劑，例如 phorbol esters, 傷口，器官部分切除以及慢性刺激和發炎反應所釋放出來之因子。在慢性發炎疾病，例如風濕性關節炎與發炎性腸疾病 (inflammatory bowel disease)常發現p53 基因突變 (Yamanishi et al.,2002)。例如慢性發炎反應與癌症具有很強相關性的結腸癌，就是由於有長期之發炎性腸疾病。又如慢性胃潰瘍與胃癌之關係以及肝炎病毒C 和B 型感會引起肝癌。另外血吸蟲病(schistosomiasis)與膀胱與結腸癌有關。慢性*Helicobacter pylori* 感染與胃癌發生有關，以上都是典型的經由慢性發炎反應而造成腫瘤之發生 (Coussens and Werb, 2002)。

最近研究顯示 NF-kB 活化會引起發炎反應，且發炎反應又與人類腫瘤形成可能有關。但其中之分子機轉並不清楚。由以上之研究結果顯示油煙與女性肺癌之關係有可能是經由油煙刺激造成肺組織細胞氧化性壓力經由 NF-kB 訊號傳遞路徑造成刺激組織部位持續性的發炎反應，進而改變刺激部位的免疫微環境轉為利於腫瘤生長的環境，促進肺腫瘤的形成。

3.2. 文獻探討

3.2.1 烹調油煙 (Cooking oil fumes; COF) 與女性肺癌之相關性

3.2.1.1、COF 的來源

中國婦女在煮菜前，會先將烹調用油加熱到 250°C，經由高溫作用會產生煙霧，加上其多以煎、炒、炸等方式烹調食物，因而與肺癌之形成有關 (Gao et al., 1987)。已知廚房室內之污染原有兩種，一類與烹調時所用之爐具燃料所產生之芳香烴類致癌物有關，另一類為烹調時所產生之油煙 (Wu-Williams et al., 1990)。烹調時肉類經由高溫作用會產生梅納氏反應或非酵素性核變反應，使蛋白凝結。當表面溫度升高時，相對的溼度就會減低，因化學及物理變化而形成外皮，隨著溫度從 100°C 持續增加時，表皮不斷的脫水，再加上化學反應，外皮會形成多孔的絕緣體，原本在外皮下之水蒸氣會往內跑，但因蛋白質經加熱而破壞結構並皺縮時，水分及油汁就會往外流出，於高熱下形成蒸氣而揮發為油煙 (Halliwill and Skjoldebrand, 1983)。COF 是一種混和性的產物，包含上千種以上的化合物。目前已知 COF 產生的汙染物，可以區分為粒狀及氣狀污染物兩大類。粒狀污染物主要之基因毒物已知有多環芳香烴 (polycyclic aromatic hydrocarbons, PAHs)、硝基多環芳香烴 (Nitro-polycyclic aromatic hydrocarbons, Nitro-PAHs) 等化合物。氣狀污染物則有甲醛、乙醛、丙烯醛及 1,3-丁二烯 (1,3-butadiene) 等。

3.2.1.2、COF 之生物毒性、基因毒性與致癌性

國人常用的食用油不論是沙拉油、豬油或是花生油等在烹調過程中，均可分解出各種油煙成份。這些食用油在 250°C 下攪拌加熱，產生的油煙微粒粒徑介於 0.15～0.56 µM，正是容易吸入肺泡的粒徑。烹調各類肉製食品所生成油煙之致突變物和烹調時間有正相關，且和烹調溫度有關，隨著溫度升高，油煙內間接致突變物有增加趨勢 (Berg et al., 1988)。研究發現沙拉油油煙微粒中含有十多種多環芳香烴，且其毒性高於相當濃度之花生油及豬油 (Hsu, 1999)。Wu 等人指出，將豬油、沙拉油和花生油加熱到 250 ± 10°C 時所產生的油煙會使 CHO-K1 細胞產生姐妹染色體交換 (SCE) 之基因毒性反應 (Wu et al., 1998)。研究指出豬油加熱到 200°C 以及沙拉油加熱到 300°C 時所產生之油煙，對於加有 S9 代謝活化之沙門氏桿菌 TA98 具有致突變 (Chiang et al., 1998)。油煙中致突變物含量與烹調時間呈現正相關性 (Lofroth et al., 1991)。在 Ames test 以及 SOS chromtest 中，油煙中所測得的多環芳香烴已被證明具有基因毒性 (McCann et al., 1975)。Qu 等人也指出，加熱棉子油或大豆沙拉油所收集到的揮發性氣體，具有突變性，會造成老鼠骨髓細胞產生姐妹染色體交換等異常現象，然而在抗氧化劑 BHA 共存下的氫化油，產生的油煙則不具致突變性 (Qu et al., 1992)。由於多環芳香烴的種類很多，其表現也不盡相同，例如：B[a]A、chrysene、BaP、DB[*a,h*]A 等具有間接致突變性，而 phenanthrene、B[e]P、naphthalene、fluorene 等則不具有致突變性 (McCann et al., 1975)。COF 內所含的多環芳香烴主要為 benzo[a]pyrene (BaP) (Chiang et al., 1998；Li et al., 1994；Yang et al., 2000)。多環芳香烴在生物體內首先會經由微粒體單氧合酶 (monooxygenase) 或前列腺素 H 合成酶 (prostaglandin H synthase; cyclooxygenase) 代謝活化成環氧化物 (epoxide)，然後再水解變成二酚 (diols) 型的活化中間產物，與 DNA 共價鍵結形成 DNA 鍵結物 (DNA adduct)，造成基因突變而引起癌症 (Dipple et al., 1987)。BaP 有許多代謝活化路徑，主要是經由細胞色素 P-450 1A1 (cytochrome P-4501A1 ； CYP1A1) 酵素系統代謝活化，首先生成

(-)-trans-7,8-dihydroxy-7,8-dihydro-benzo[a]pyrene，然後再繼續氧化形成 anti-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9, 10-tetrahydro-benzo[a]pyrene (BPDE)，它會直接攻擊 DNA 上 dG 之 N^2 位置 (Jeffrey., 1976; 1977)。另一個代謝路徑是單電子氧化作用，使 BaP 產生自由基陽離子 (radical cation)，它會攻擊 DNA 上 dG 之 N^7 胺基位置，進而形成 DNA 鍵結物 (Cavalieri and Rogan, 1992)。人類許多器官組織中都能偵測到 DNA 鍵結物的存在，例如肺臟、支氣管、皮膚、胰臟、口腔黏膜、子宮頸和乳房組織等 (Dunn and Stich, 1986; Phillips et al., 1988; Phillips et al.,1990; Everson et al., 1986; Manchester et al., 1988; Seidman et al., 1988; Schoket et al., 1990)。Cherpillod 和 Amstad 的研究結果顯示，BaP 會造成 p53 抑癌基因之第 248 及 249 密碼發生 G→T 的 transversion 突變，甚至可能引起人類癌症 (Cherpillod and Amstad, 1995)。這些結果證實 BaP 確實會引起人類和動物細胞基因發生突變，甚至可能引起人類癌症。

3.2.1.3、油煙成分與女性肺癌之關係

流行病學研究指出，烹調時所產生的油煙與鼻咽癌、口腔癌、膀胱癌和肺癌具有相關性 (Cole et al., 1972; Hederson and Louie, 1978; Dickson, 1981; Hargraves and Pariza, 1984)。研究更證實台灣婦女罹患肺癌可能和暴露廚房油煙有關 (Gao et al., 1988; Ko et al., 1997)。上海中醫藥大學何裕民教授公佈一項長達五年的肺癌流行病學調查，結果顯示中青年女性在廚房接觸高溫油煙，包括路邊燒烤，罹患肺癌危險性增加二至三倍，成為繼吸煙後另一個肺癌元兇。衛生署的研究指出，廚房油煙確實含有 dinitropyrene (DNP) 的致癌物質，而炒菜所產生的 DNP 含量，遠超過室外空氣的 188 倍。此外，沒有使用抽油煙機的煮婦，罹患肺癌的危險性也比使用油煙機的婦女高。研究還發現，油煙的致癌性，不僅反映在家庭主婦上，也發現廚師有較高的比率發生肺腺癌 (Dubrow and Wegman, 1984)。學者對中國大陸之研究調查指出，肺癌之發生與其烹調方法、餐數、油煙之大小、食用油之種類均具有相關性 (Gao et al., 1987)。更有研究指出，當蔬菜油和沙拉油加熱到一定溫度時，所生成之油煙中具有高含量之 BaP 和 DB[*a,h*]A，因而推論此兩種致癌物與中國婦女罹患肺癌具有相關性 (Li et al., 1994)。

3.2.2 IAP2 與女性肺癌成因之相關性

3.2.2.1、IAP2 之生理功能與作用機制

Inhibitory of apoptosis proteins (IAPs) 為一持續表現於哺乳類及昆蟲生物體抗凋亡蛋白之基因族群。IAPs主要包含有IAPs、Cp-IAP和Op-IAP三種同源性蛋白細胞凋亡抑制蛋白 p35 所界定出，其主要功能為抑制caspase family 中和死亡具有相關protease 的活性 (Deveraux and Reed., 1999)。IAP 蛋白主要包含 baculoviral IAP repeat (BIR)，此種重複性功能區塊有約70～80個 baculoviral amino acids。目前 IAP protein 於生物體的作用機轉主要為抑制 caspase 活性以及媒介接受體對於細胞週期的訊息調控機制。文獻證實，BIR domain為目前已知 IAP protein 的主要作用功能位，具有抑制 caspase 的活性之功能 (Rothe et al., 1995; Duckett et al., 1996; Gordon et al., 2002; Hasegawa et al., 2003)。目前已知有 8 種 IAPs 被界定出，並且可以區分為三大族群。Class I：為一種包含 BIR domain 及 RING finger motif 之同源性蛋白。X-linked IAP [XIAP (又稱為 hILP, MIHA, 和BIC4)]，為最早被界定出之 IAPs，它包含有三個 BIR domains 及一個 RING finger 之功能結合位，可抑制 caspase-3, 7, 和 9 之活性。cIAP1 (又稱為 MIHB, hIAP2, BIRC2) 和 cIAP2 (又稱為 MIHC, hIAP1, 和 BIRC3) 其功能結構和 XIAP 相同。cIAP1 和 cIAP2 可被表現於許多組織中，並且可以抑制 caspase-3, 7 的活性。ML-IAP (又稱為 livin，KIAP, 和 BIRC7) 和 ILP-2 則包含單一個 RING finger 和 BIR3 domain, 且其功能性表達和 XIAP, cIAP1, cIAP2 具有高度之同源性，而 ML-IAP 主要為抑制 caspase-3 和 caspase-9 之活性。Class II：NAIP 為 1995 年由 Roy et al. 所界定出 (Roy et al., 1995)。主要包含有三個 BIR domain，但是不包含有 RING finger motif。它可以高度表達於人類之肝臟、胎盤和中樞神經系

統，其主要功能為抑制 caspas-3, 7 和 9 之活性。Class III：Survivin 為 class III family 之主要 protein，包含有單一個 BIR domain 而不具有 RING finger motif。Survivin 可以表達於胎兒之肝臟、腎臟、肺臟和腸胃道，而不表達於大部分正常成人之組織中 (Schimmer, AD., 2004)。

3.2.2.2、IAPs 與癌症發生之相關性

細胞凋亡的機制可分為兩種模式。其一，死亡訊息的傳遞是透過細胞表面死亡器接受器 (death receptor) 的活化。當死亡接受器如 TNF-R、Fas、DR-4、DR-5 等相對應之配子 (ligand) 結合，活化受器位於細胞內的凋亡區域 (death domain) 與下游之 adaptor protein 進而活化 caspase-8，而活化態之 caspase-8 繼續活化下游之 caspase- 3 最終導致細胞凋亡的發生 (Zhang et al., 2004)。細胞凋亡可以區分為外生型和內生型作用方式。內生型主要為粒線體內 cytochrome c 和 smac 蛋白釋放置細胞質中。Cytochrome c 可以直接和 Apaf-1 結合，加速活化 casapase-9 進而再活化 casapse-3。反之， smac protein 可以抑制 IAPs 對 caspase-3 的阻斷活性。外生形之路徑主要是經由 caspase-8 活化 caspase-3 之活性。

當細胞內 pro-caspase-3, 7, 和 9 過度表現時會誘導凋亡機制之進行，反之，抗凋亡蛋白族群 XIAP，IAP1，IAP2，和 survivin 則會抑制此過程之進行 (Deveraux et al., 1997; Roy et al., 1997)。此外，當細胞凋亡機制亦可被 Fas (CD95)，mitochondria 或是 TNFR1 (CD120a) 所活化而啟動。而目前已知 Tumour necrosis factor (TNF) receptor-associated factors (TRAFs) 可以經由 Tumor necrosis factor (TNF) receptors (TNF-R) -1 和 -2 兩種 cytokine receptor 活化 NF-κB 及 c-Jun N-terminal kinase (JNK) 訊息路徑調控 IAPs 蛋白之表現 (Deveraux et al., 1999)。

目前已知 IAPs 與許多癌症中的發生有關，包括：結腸癌 (Wang at al., 2003)、前列腺癌 (Krajewska et al., 2003)、皮膚癌 (Bush et al., 2001)、乳癌 (Ferguson et al., 2003)、子宮癌 (Frost et al., 2002)、淋巴癌 (Chauhan et al., 2001) 以及肺癌 (Okouoyo et al., 2004)。MIHC/c-IAP2 (IAP2) 又稱為 Inhibitor of apoptosis-1 (IAP-1)，當細胞經由凋亡訊息刺激後，可抑制凋亡機制之進行，促進腫瘤細胞存活之基因族群之表現。文獻指出 IAP2 可以增加肺癌細胞對 Gemcitabine 產生抗藥性機制 (Bandala et al., 2001)。目前有許多對癌症細胞研究，包括有：肺癌 (Denilinger et al., 2004)、結腸癌 (Guo et al., 2004)、胃癌 (Liu et al., 2004)、前列腺癌 (Uzzo et al., 2002)，發現當 NF-κB 的活化機制被阻斷時可以相對抑制 IAP2 蛋白之活化，促進腫瘤細胞產生凋亡過程，減少其生長，進而增加化學療法之敏感性。而於許多對腫瘤細胞的研究也已證實 ionizing radiation 可以經由 NF-κB 的活化作用，誘導 IAP2 蛋白的產生 (Ueda et al., 2001a)。當 NF-κB 的次單位 p50、p65 以及 c-Rel 被過度活化時，可以相對誘導 XIAP、IAP1、IAP2 以及 survivin 基因之表現 (Chen et al., 2003)。對 MDA-MB-231 細胞株的研究中指出，NF-κB 主要是藉由調控 TRAF1、TRAF2、IAP1、IAP2 等基因轉錄層次的表現，抑制凋亡機制的進行 (Swinney et al., 2002)。Wang 等人提出 TNF receptor 可藉由與 TRAF-1/TRAF-2 之交互作用，進而促進 IAP1 以及 IAP2 執行抑制 caspase-8 的活性功能 (Wang et al., 1998a)。文獻指出，在許多細胞中，經由 TNF、IL-1β、CD40、lipopolysacharide 及 etoposide 刺激後，可以誘導 NF-κB 的表現，進而活化 IAP2 (Hasegawa et al., 2003)。目前已有許多文獻證實，IAP2 所參與之一些相關之訊息調控機制，除了經由 caspase-dependent 作用機制外，目前已證實 IAP2 可以參與訊息轉錄因子 NF-κB 之正回饋調控機制 (Chu et al., 1997)。Rel A 次單位的角色功能如同生存因子一般，可以促進 IAP2 之基因表現，進而抑制 DR4/DR5 及 caspase-8 的活性表現 (Chen et al., 2003)。CD30 可以誘導 Lymphoma 細胞中 IAP2 蛋白之過度活化 (Hubinger et al., 2004)。PI3K 的抑制劑 LY294002 可以抑制肺癌及食道癌細胞中 PI3K/Akt 並且可以伴隨抑制 NF-κB 誘導 IAP2 之活化路徑，增加腫瘤細胞對化學治療藥物之作用敏感性，進而增加治療效果 (Nguyen et al, 2004) 在。慢性 leukemia 的研究中亦發現 IAP2 蛋白有持續被過度活化的現象 (Hasegawa et al.,

2003)。Cisplatin 可以抑制 IAP2 蛋白之活化，促進凋亡機制之進行，進而減少 MPM (malignant pleural mesotheloma) 細胞存活率之降低，進而增加化學治療之療效 (Gordon et al., 2002)。AKT [又稱為 protein kinase B (PKB)] 為一種 serine/threoine protein kinase，是 PI3K 活化癌訊息路徑中之下游標的基因，它可藉由磷酸化作用，誘導調控細胞週期相關凋亡基因之表現，提供腫瘤生長生存訊息之傳遞 (Woo et al., 2004)。Neri 等人於對 leukemia 細胞 (HL-60) 的研究中發現，IAP2 的蛋白表現層次和 PI3K/Akt 的活化有關 (Neri et al., 2003)。當處理 HL60AR (HL60-leukemia cell clone) 細胞 LY294002、AKT 抑制劑以及 NF-κB 抑制劑 SN50 時，可以抑制 IAP2 之活性表現 (Martelli et al., 2003)。

3.2.3 廚房油煙與女性肺癌形成相關訊息調控路徑之探討

氧化緊迫可促進 NF-κB 之活化作用，誘發 Cox-2 蛋白之表現 (Nie et al., 1998)。NF-κB 轉錄因子 family 是由 p50、p52、p65 和 Rel-B 及 C-Rel 所組成之二元體 (dimer)。正常條件之下，存在於細胞質內不活化型的 NF-κB，係 p50 / p65 與 IκB 抑制劑次單元蛋白結合，當有氧化劑、紫外光照射、生長因子或是發炎性細胞激素等存在下，例如：TNF-α、TPA、LPS，促使 I-κB 蛋白快速磷酸化而裂解，釋放 p50/p65 而轉移到細胞核，隨即產生 NF-κB 之活化作用 (Renard et al., 2000; Pahl, 1999)。Cox-2 是促發炎性類前列素合成作用之關鍵酵素 (Kim et al.,2000)。研究顯示 BaP 可以經由 NF-κB 調控 Cox-2 基因之表現 (Yan et al., 2000)。COF 中之主要多環芳香烴為 BaP (Chiang et al., 1997; Li et al., 1994; Yang et al., 2000)。先前結果顯示，當添加羥自由基捕捉劑 (D-mannitol：甘露醇) 和超氧歧化酶 (SOD)，能顯著減少 COF 所誘發 Cox-2 蛋白之表現量 (Lin et al., 2002)。此外，更發現 COF 會誘發 CL-3 細胞產生 MDA-DNA 鍵結物，並促使 OGG1 基因之表現 (Cherng et al., 2002)，因而推論 COF 與誘導活性氧之產生，以及 DNA 修補之機制具有相關性。 已知 NF-κB loci 具有 Cox-2 promoter 之鍵結部位，可促進 NF-κB 之訊息系統參與活性氧之反應 (Yan et al., 2000; Roman et la., 2000)。研究結果發現，槲皮桐會經由活化多環芳香烴感受性路徑及 NF-κB，一方面可以有效抑制煎魚油煙誘發的 AhR-Arnt 複合體與 DRE 之鍵結作用，一方面則可以抑制 NF-κB 與 DNA 之鍵結能力，因而抑制 CYP1A1 與 Cox-2 基因之表現，減少 COF 所誘發 DNA 之傷害。因而推論 COF 會促使 CL-3 細胞產生活性氧而造成氧化性緊迫，一方面誘發代謝活化酵素 CYP1A1 之活性，一方面啟動轉錄因子 NF-κB，進而誘發 Cox-2 基因之表現，使得細胞增加對發炎性前列腺素的分泌，造成細胞具有免疫抑制力，讓癌細胞逃脫免抑捕殺作用而繼續增殖，而不抽菸之台灣女性患者可能係長期暴露 COF，使 DNA 傷害之修補能力減弱，以及體內產生過多活性氧，改變體內氧化作用機制之平衡，誘發 CYP1A1 及 Cox-2 基因之表現，增加 MDA 和促發炎性 PGE2、IL-6 之表現，改變體內之免疫微環境，增加不抽菸台灣女性罹患肺癌之危險性。

4、研究方法

4.1 COF 的製備與萃取

COF 的製備與萃取得自於先前實驗 (Yang et al., 2000)。首先取四條白鯧魚共重 1042 克，每次以 15 ml 沙拉油煎一條魚，所生成之油煙先用排油煙機過濾油脂，然後在排氣口處裝設空氣採樣器採得樣品。將採得樣品之玻璃纖維濾紙剪成一公分見方的小塊泡在 200 ml 丙酮中，以震盪器震盪一小時，然後再以 Whatman No.1 濾紙過濾取得丙酮萃取液，以上述方法重複三次，將所有的丙酮萃取液集中起來，加入無水硫酸鈉脫水、過濾，再經減壓濃縮，所得濃縮殘留物即為 COF 懸浮微粒丙酮萃取物，溶於一定體積之有機溶劑 DMSO 中，以此為樣品進行各項分析。

4.2 樣品之純化

煎魚所取得之粗萃取物以 Baker-10 Solid Phase Extraction system 純化，所使用的管柱為 octadecyl (C_{18})。將 10 mg 之油煙丙酮萃取物以氮氣吹乾，先溶於 3.5 ml 異丙醇 (isopropanol)，再加入 20 ml 水。購得之 Baker-10 先以 6 ml 甲醇濕潤後，再以 9 ml 異丙醇/水 (15 / 85, v/v) 將管柱活化，並使溶劑保持在管柱固相物的上方，然後注入樣品直至所有溶液通過管柱，然後再以幫浦抽五分鐘，直至管柱全乾為止。接著以 6 ml 甲醇沖堤出結合在管柱上的物質，沖堤液以氮氣吹乾後，溶於 1 ml 的甲醇中並儲存於 -20°C 冰箱中備用，此即稱為 COF 粗萃取物之 Baker-10 多環芳香烴純化物 (COF-B)。

4.3 肺腺癌 A549 之培養

肺腺癌 A549 細胞購自 American Type Culture Collection (ATCC)。A549 細胞在形態學上屬於非小細胞型肺癌細胞。以含有 10 % 胎牛血清 (fetal bovine serum; FBS, Gibco)，0.03 % penicillin / streptomycin (100 U /ml)，5.5 % 重碳酸鈉 (sodium bicarbonate) 之 DMEM medium (Gibco) 培養於 95°C 濕度、5% CO_2 的 37°C 培養箱。台灣肺腺癌 CL-3 細胞是台大醫院楊泮池醫師殖株培養成功之人工培養系細胞株，CL-3 細胞於型態上是屬於 epithelial-like cell。以含有 10 % 胎牛血清 (fetal bovine serum; FBS, Gibco)，0.03 % penicillin / streptomycin (100 U /ml)，5.5 % 重碳酸鈉 (sodium bicarbonate) 之 RPMI medium (Gibco) 培養於 95°C 濕度、5 % CO_2 的 37°C 培養箱中。A549 (p53-knock down) 細胞株來源取得來自中山醫學大學分子醫學毒理學研究所蔡菁華老師研究室。以含有 10 % 胎牛血清 (fetal bovine serum; FBS, Gibco)、0.03 % penicillin/streptomycin (100 U /ml)、5.5 % 重碳酸鈉 (sodium bicarbonate) 之 RPMI medium (Gibco) 培養於 95°C 濕度、5 % CO_2 的 37°C 培養箱中。

4.4 細胞生長曲線之分析

4.4.1 細胞數的計算

將培養皿中的細胞均勻沖散，取 100 μl 細胞液到 micro-centrifuge tube，並加入同體積 trypan blue 均勻混和。將混合液吸到血球計數器(Homocytometer) 於倒立顯微鏡下計算細胞數目。死亡的細胞會被 trypan blue 染成藍色，活細胞則否，由此可以計算出死細胞和活細胞之數目。

4.4.2 細胞生長曲線及增殖速度的計算

取 1×10^5 cells / ml 置於 6-well 培養皿中 (Nunc，Denmark)，加入低濃度之 COF (12.5 μg/ml)，未添加者為控制組，實驗組及控制組皆以 3 重複條件處理，每隔 24 小時取一盤進行細胞數目的計算，連續進行 6 天，及可以做出細胞生長曲線。一般之生長曲線，由遲滯期 (lag phase)，指數增殖期 (log phase)，穩定期 (stationary phase)，死亡期
(death phase) 所構成。本實驗以指數增殖期作為細胞增殖速度的計算，細胞增殖速度以兩倍時

間 (doubling time; D.T.) 表示，兩倍時間由 D.T.＝ $(t-t_0)\log 2/\log N-\log N_0$ 公式求得，期中 t、t_0 為計算細胞數目的時間，N_0 為 t_0 時之細胞數目，N 為 t 時之細胞數目。以實驗組之兩倍時間與控制組之兩倍時間加以比較 (設實驗組為 100 %) 及得相對生長速度。

4.4.3 MTT 分析法檢測細胞生長之速率

3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT)，其原理是利用細胞內脫氫酶酵素 (dehydrogenase) 可將四錯鹽 (tetrazolium salt) 還原反應成橘紅色螢光的 formazan 的現象，此螢光物質在 450 nm 有最大之吸光值。實驗首先將細胞培養於 96-well 平底 microtitration plate 中 (1×10^4 cells/well) 分別以 triplicate 加入不同稀釋濃度濃之 COF，於 37°C，5 % CO_2 及飽合溼度環境下,培養 48 小時後，每 well 加入 100 ul MTT labeling reagent，並置於
37°C，5 % CO_2 恆溫培養箱中反應 4 小時。

4.5 流式細胞分析儀測定細胞週期之分佈比率

將經藥物刺激後的細胞自培養皿中取出，經 PBS 清洗後，進行細胞週期之分析。細胞首先加入含有 Trypsin 之溶液，37°C 下作用，離心 7000 rpm，2 分鐘，再以含 Trypsin 抑制劑與 RNase A 之溶液，室溫下反應 10 分鐘，以終止 Trypsin 的作用並破壞 RNA，繼以 propidium iodide (PI) 進行 DNA 染色，然後以流體細胞儀分析，並配合 CellQuest 軟體來分析細胞週期之分佈。分析利用 Agaron laser 於 493 nm absorption；630 nm emission 偵測 PI 螢光，以 Forward light scatter (FSC) 參數分析細胞大小以及 Sideward light scatter (SSC)；PMT4 等參數分析細胞顆粒性及螢光強度之關係，將上述參數可將 sub-G1、G0/G1、S 及 G2/M 細胞週期的細胞經由 10^4 的細胞族群中分別出來，分析各細胞週期之分佈比率。

4.6 西方墨點分析法 (Western Blot)

4.6.1、總蛋白質之製備

將 2×10^5 個細胞種於 10 cm^2 之培養皿中，經 24 小時後，加藥處理適當時間，然後將細胞收下，加入適量新鮮配置的消化緩衝液 (1 % SDS, 100 mM Tris-HCl, pH＝8.0)，置於 94°C 的乾式加熱器 (dry bath) 反應 30 分鐘，以 12,000 rpm 離心 20 分鐘，收取上清液即為總蛋白質，並取 5 µl 用作蛋白定量。分析時取一定量蛋白質加 5 µl 樣品緩衝液 (sample buffer)，置於 94 °C 之乾式加熱器反應 10 分鐘再使蛋白質變性，取出置於水浴中而後裝載入膠中。

4.6.2、SDS-PAGE 的製備

本實驗選用 Hoefer 之器材製備 SDS-PAGE，其步驟如下：首先製備下膠 10 % SDS 膠片，依序加入 7.4 毫升二次水、4 毫升 1.5 M Tris pH 8.8、4 毫升、40 % 丙醯胺：bis-丙醯胺 (acrylamide-bisacrylamide；29：1)、0.2 毫升 10 % SDS、0.4 毫升 10 % ammonium persulafate (APS)、0.01 毫升 TEMED 混和均勻後，然後緩緩加入 1.5 mm 厚度的直立式電永槽膠台座中，加到約佔膠台四分之三高度時，加水壓平下膠並靜置 30～40 分鐘後，再開始置備 5 % 的上膠，依序加入二次水 3.54 毫升，0.5 M pH 6.8 1.5 毫升、40 % 丙醯胺：bis-丙醯胺 (acrylamide-bisacrylamide；29:1) 0.6 毫升、10 % SDS 0.06 毫升、10 % APS 0.3 毫升、TEMED 0.015 毫升混和均勻後，緩緩加入直立式電永槽膠台座中，然後將梳狀膠片 (comb) 放入，待凝固後小心取出梳狀膠片，所留下的凹槽可作為樣品裝載之用。

4.6.3、SDS-PAGE 之電泳操作與轉漬

將置備好的膠，先用 1 × Tris /Glycine 電泳緩衝液填滿，之後須先將樣品 (細胞萃取蛋白) 以 95°C 5 分鐘之加熱變性 (denature) 處理後，再以每格 loading 20 微克 (µg/ml) 的樣品，先以 90

voltage 跑 10 分鐘，再用 120 voltage 跑約 80 分鐘 ，直到樣品接近底部及可停止。在膠塊跑完前 10 分鐘，準備一張大小恰當的 PVDF 膜 (Tropix)，先用甲醇清洗一下 (約 15 秒)，再換成清水震盪 5 分鐘。將膠卸下後，同時與 PVDF 膜及兩片濾紙浸在轉漬溶液中，震盪 5 分鐘。利用半乾式轉漬槽以 150 mA 轉漬 120 分鐘，將膠上之蛋白以負極往正極方式轉移到 PVDF 膜上。將轉漬好的 PVDF 膜浸在 5 % 脫脂奶粉的 1 × TTBS 緩衝溶液 (50 mM Tris, 0.2 % Tween 20, 150 mM NaCl, pH 7.5) 中，置於室溫下緩慢搖晃 60 分鐘。加入可以辨識欲觀察之蛋白的一次抗體，於 4^oC 下經過一夜震盪或室溫震盪 1.5 小時，抗體皆由 1 × TTBS 之脫脂奶粉稀釋，加入 1/100 的 2 % sodium azide (NaN_3) 後保存於 4^oC，可以回收再利用。將處理的一次抗體之 PVDF 膜用含 5 % 之脫脂奶粉的 1 × TTBS 緩衝溶液清洗兩次，接著在室溫下用二次抗體反應 1 小時。將處理完的二次抗體之 PVDF 膜用含有 5 % 脫脂奶粉之 1 × TTBS 緩衝溶液 (50 mM Tris, 0.5 % Tween 20, 300 mM NaCl, pH 7.5) 清洗兩次，用不含脫脂奶粉 1 × PBS 緩衝溶液 (0.8 % NaCl, 0.002 % Cl, 0.14 % $Na_2H_2PO_4$, 0.02 % KH_2PO_4) 稍為洗一下，即可利用 ECL 呈色劑 (Amersham RPN 2106)，將經過抗體作用之蛋白影像呈現在 X-光底片 (Kodak Science Imaging Film) 上，約 5～30 分鐘。

4.7 TUNEL 分析法檢測細胞凋亡之程度

將經加藥刺激後的細胞以 1 × PBS 清洗兩次，加入 Trypsin 作用後，離心 12000 rpm、4^oC、10 分鐘，移除上清液，將 pellete 溶於 0.5 ml 的 PBS。之後，加入 5 ml 的 1 % methanol-free formaldehyde 並置於冰上固定 20 分鐘。離心 12000 rpm、4 ^oC、10 分鐘。移除上清液，再將細胞溶於 5 ml PBS，再以 12000 rpm 離心 10 分鐘，並將細胞輕輕拍散，溶於 0.5 ml PBS 中。加 入 5 ml ice-cold ethanol 固定細胞，並置於 -20^oC 至少 4 小時。取出細胞，加入 5 ml PBS，離心 12000 rpm，10 分鐘，再重複一次離心步驟，移除細胞上清液後，將細胞溶於 1 ml 的 PBS，並將細胞 transfer 到 1.5 ml microcentrifuge tube，離心 12000 rpm、10 分鐘，移除上清液。再以 DeadEND^{TM} Fluorometeric TUNEL System 分析細胞凋亡凋亡程度。將 pellete 溶於 80 ul Equilibratin buffer，並置於室溫下作用 5 分鐘，加入 5 ul Nucleotide mix 以及 1ul TdT 酵素，並置於 37^oC 溫水浴下作用 60 分鐘，並且間隔 15 分鐘輕拍細胞一次。再加入 20 ul mM EDTA 終止此反應。離心 12000 rpm、10 分鐘，移除上清液，加入 1 ml 內含有 0.1 % Triton ^{R} X-100 及 5 mg/ml BSA，離心 12000 rpm、10 分鐘，再重複 wash 步驟一次。離心後移除上清液，加入 0.5 ml 5 μg/ml propidium iodide solution 及 DNase-free RNase A 的 PBS 溶液，將細胞置於室溫下 30 分鐘 (此步驟須避光)。再以流式細胞分析儀分析細胞凋亡程度。以 FL-1 (520 ± 20 nm bandpass filter) 的綠色螢光參數分析 (fluorescencein-12 dUTP) 及 FL-2 (＞620 nm) 的紅色螢光分析 propidium iodide 的細胞螢光強度 。

4.8 統計分析

本實驗結果皆經由三次個別實驗結果得到之平均值及標準偏差，再經由統計分析系統 (SPSS version 10.0) 進行統計分析，以 One-Way ANOVA 進行變異分析，並且以 *Scheffe-test* 測其顯著性差異比較，當 p 值小於 0.05 代表具有統計上之意義。

5、結果

5.1　油煙慢性刺激所引發動物之慢性發炎反應

本實驗是由國科會動物中心購得之雌性 Sprague-Dawley (SD) 大白鼠(5 週齡)，動物入室後觀察兩週，待動物適應後將實驗動物分成四組，第一組僅投與玉米油，當作溶劑對照組(Corn oil -100ul/100g BW, once per week)；第二組每週單獨投與 COF (10mg/kg BW, once per week)；第三組將 DMBA 10mg 分四次注射給予後；每週投與玉米油一次 (0.1cc Corn oil,once per week)；第四組將 DMBA 10mg 分四次注射給予後後，每週投與 COF 一次 (10mg/kg BW,once per week)。動物以 Zoletil (Tiletamine/Zolazepam)， Xylazine 及 Atropine 以1:1:1的體積比混和後以腹腔注射進行麻醉，待動物麻醉完全後進行氣管內注射法 (intratracheal instillation)。將溶於玉米油之樣品 (每隻大鼠注入總體積為 0.1cc) 以微量注射器深入大鼠氣管底部，然後快速的注射入樣品，以此方式分別連續暴露二、六、八個月，以腹腔動脈放血的方式犧牲動物，將取得之老鼠肺臟進行拍照。後分別將大鼠五片肺葉取出，一部份以福馬林固定、修片、包埋、切片染色以進行組織病理判讀；，另一部分組織儲存於-80℃以利進行後續之研究。

本研究初步結果發現，以氣管注射技術分別將 DMBA 及 COF 以上述分組處理方式，每週注射一次的頻率打入大鼠肺部結果發現無論二、六、八個月的刺激過程中各組間老鼠體重並無明顯差異(Fig. 1, 2, 3)。在兩個月的處理中所造成的傷害主要集中在支氣管上皮細胞處，主要造成大鼠肺部支氣管炎(bronchitis)(Fig.4)，較嚴重的甚至有濃瘍(pus)(Fig.5)的現象。其中 DMBA + COF 組及 DMBA only 組發現支氣管上皮細胞有過度角質化 (hyperkeratization) 的現象，並可觀察到大量分泌角質素(keratin)的現象(Fig. 9)，且其周圍支氣管上皮細胞可觀察到不正常的增生(Fig. 6) 以及化生(metaplasia)(Fig.9) 現象；在比較各組間的差異後發現由於長期的刺激可發現各組皆有持續性發炎的現象，但僅在 DMBA + COF 組及 DMBA only 組可觀察到支氣管上皮細胞不正常的增生現象，而增生的程度尤以 DMBA + COF 組較 DMBA only 組顯著，但未達統計上的差異。另一方面，在 COF only 組、DMBA only 組以及 DMBA + COF 組皆可發現肺泡細胞有輕微增生現象，此現象在 COF only 及 DMBA + COF 兩組相較於溶劑對照組其傷害嚴重程度在統計學上達到顯著的差異(COF only/Solvent control; $p < 0.05$; DMBA+COF /Solvent control; $p \leqq 0.01$, T test)。由此發現推測 DMBA 刺激可造成肺支氣管上皮細胞增生，而 COF 持續性暴露刺激似乎可促進 DMBA 所造成之細胞傷害及增生現象；另一方面發現 COF only 組其所造成巨噬細胞聚集的程度較其他三組顯著，且與溶劑組相比達到統計上的差異($p \leqq 0.01$, T test)，由以上觀察初步結果推測油煙的暴露會增強慢性發炎的程度並且加強 DMBA 所造成的傷害。以上有關於兩個月實驗的結果列於 Table.1。然而在六個月的實驗結果則發現除了支氣管炎亦可觀察到之外，支氣管上皮細胞過度角質化並大量分泌角質素，且其周圍支氣管上皮細胞可觀察到不正常的增生或化生現象則有降低。另一方面，在 DMBA + COF 組或 COF only 之組別以及較少數 DMBA only 組別之大鼠則可觀察到肺泡上皮細胞有增生的現象(alveolar epithelial hyperplasia) (Fig. 7)，其中DMBA + COF 組增生的情形更達到統計上的差異(DMBA + COF/Solvent control, $p \leqq 0.01$; DMBA + COF/DMBA, $p < 0.05$, T test)，並發現肺泡周圍亦有巨嗜細胞聚集的現象，尤以 DMBA+ COF 組最明顯(Fig. 8)，以上有關於六個月的實驗結果列於 Table 2.。然而有趣的是為何二個月及六個月的刺激卻看到不同部位的實驗結果，仍須更近一步作探討。

在八個月的實驗結果更進一步發現，無論是造成支氣管上皮細胞增生或化生、肺泡細胞的增生以及巨噬細胞聚集等病理傷害皆較前面兩組實驗更為顯著，其中在DMBA + COF 組更可觀察到一隻老鼠有鱗狀上皮癌的發生，由以上結果發現 COF 長期刺激有增強DMBA 這個致癌物質所造成之傷害的能力，使原本 DMBA 所造成支氣管上皮細胞輕微化生 (metaplasia) 的程度更加嚴重，雖然在 DMBA+ COF 組中僅有一隻老鼠有鱗狀上皮癌的發生，但由此可知 COF 確實有促進細胞增生走向癌化傾向。此外，在 COF only 及DMBA+ COF 這兩組在二、六、八個

月皆可發現肺泡細胞增生的現象，其程度相較於溶劑對照組達到統計上顯著差異 ($p<0.05$)，但此兩組之間並無顯著差異，由此可知單獨油煙刺激似乎即可造成肺泡細胞的增生，且在刺激早期即可發生。另一個有趣的現象為巨噬細胞的聚集，此現象在 DMBA + COF 組最顯著，隨著刺激時間愈長巨噬細胞聚集的程度愈顯著，和 DMBA only 組相比亦有增強的情形，由此推測 COF 的刺激會造成巨噬細胞的聚集。在過去的研究發現慢性發炎反應與癌症形成機制之相關性中巨噬細胞的聚集是參與癌症發生過程之一個重要因子(Chen et al., 2005)，因此 COF 刺激所造成巨噬細胞的聚集在此所扮演的角色為何？值得我們進一步去探討。

5.2 油煙刺激所誘導 A549 細胞增生現象及可能機制探討

5.2.1、A549 細胞 doubling time 之分析

本文之研究主題是想了解以長時間低濃度處理肺腺癌細胞 COF，探討女性肺腺癌與 COF 相關之成因。以三重複條件處理 A549 細胞低濃度之 COF (12.5 μg/ml) (實驗組)，和控制組 (未處理) 後，每隔 24 小時取一盤進行細胞數目的計算，連續進行 6 天，即可做出細胞生長曲線。一般的生長曲線，由遲滯期 (lag phase)，指數增殖期 (log phase)，穩定期 (stationary phase)，死亡期 (death phase) 所構成。本實驗以指數增殖期作為細胞增殖數度的計算，增殖速度以兩倍時間 (doubling time；D.T.) 表示。二倍時間由 $D.T.=(t-t_0)\log 2/\log N-\log N_0$ 公式求得，其中 t、t_0 為計算細胞數目的時間，N_0 為 t_0 時之細胞數目。並與控制組比較相對生長速率 (relative growth rate)，控制組相對生長素率定為 100 %，結果顯示低濃度之 COF (12.5 μg/ml) 和控制組相較，其 doubling time 較短，且其相對生長速率也較快 (Fig. 11)。

5.2.2、A549 細胞存活率之分析: MTT assay

先前實驗室在肺腺癌 CL-3 細胞的研究結果中指出，COF 會造成細胞之總傷害程度，且隨著 COF 濃度的增加而加長，但在較高的劑量範圍下 (250～500 μg/ml)，COF 具有較強的細胞毒殺性。本實驗以 MTT 方法更進一探討低濃度 (0、12.5、25、50 μg/ml) COF 處理 A549 細胞 48 小時，分析 COF 對 A549 細胞生長之影響程度。研究結果顯示 COF 可造成 A549 細胞存活率之增加，且其隨著 COF 處理濃度 (0、12.5、25、50 μg/ml) 之增加而增加 (Fig. 12)。

5.2.3、A549 細胞細胞週期 (S-phase) 之分析

先前研究指出 COF 能產生各種活性氧，誘發氧化敏感性基因 Cox-2 之表現，增加促發炎性前列腺素 PGE2 的分泌作用，提升細胞免疫抑制能力，使癌細胞能順利逃脫免疫毒殺作用而繼續增殖生長。為更了解 COF 誘發人類肺癌細胞增生之相關機制，本研究以另一株肺腺癌細胞株 A549 為實驗模式，探討 COF 造成肺癌細胞進展之相關癌化作用。以流式細胞分析儀分析以低濃度之 COF (0、12.5、25、50 μg/ml) 處理 A549 細胞 48 小時是否會影響 A549 細胞週期中 S-phase 之分佈比率。結果顯示隨著 COF 處理濃度之增加，其 S-phase 呈現線性增加之趨勢，其增加比率分別為 (0：34.21 %、12.5：48.45 %、25：56.38 %、50：61.48 %)，相對於未處理控制組之 34.21 %，其 S-phase 之分佈比相對提高之趨勢 (Fig. 13)。

5.2.4、比較 COF 與其他兩種主要成分 (BaP、2,4-DDE) 對 IAP2 蛋白之分析

本實驗以 Benzo[a]Pyrene (BaP) 和醛類物質 2,4-decadiental (2'4-DDE)以及處理 COF，同時比較 IAP2 之表現程度是否有所差異。研究結果指出，不同濃度之 COF (0、12.5、12.5、50 μg/ml)；BaP (0、2.5、5、10 μM)；2,4-DDE (0、5、10、25 μM)，IAP2 蛋白之活性皆可以被此三種物質所誘發表現，但是其誘導強度則以 2,4-DDE 之誘發強度較為明顯 (Fig. 14)。

5.2.5、COF 可以經由 NF-κB 之活化路徑誘發 IAP2 蛋白之表現

先前實驗室結果指出 COF 可以經由 NF-κB 之活化路徑活化 Cox-2 蛋白之活化表現。因此為更近一步了解除了 Cox-2 基因可被 NF-κB 誘導外，IAP2 之表現是否經由相同之活化路徑，因此使用 NF-κB 之特定部位 (site-specific) 抑制劑，BAY，進一步釐清 IAP2 蛋白之過度表現，是否也相同受到 NF-κB 之調控。結果顯示，當同時處理 A549 細胞 COF (25 μg/ml) 和 BAY (2、10 μM) 作用 48 h 後，發現 BAY 確實會減弱 COF 所誘導 IAP2 蛋白之表現層次 (Fig. 15)。

5.2.6、BAY 抑制 COF 誘導 NF-κB 所調控之細胞存活

為了進一步證實 BAY 可以抑制 COF 誘導 NF-κB 所調控之抗凋亡及細胞存活機制，我們以 TUNEL assay 以及細胞週期之分析法檢測此訊息路徑。結果顯示，同時處理 A549 細胞 COF (25 μg/ml) 和 BAY (10 μM)，可以顯著降低 A549 細胞之凋亡比率為 18.34 % 相較於單獨處理 BAY 之 30.69 % (Fig. 17)。同時於細胞週期之分析結果中，發現 sub-G1 之分佈比率可以降低從 58.36 % (單獨 BAY 處理) 至 42.26 % (同時處理 BAY 及 COF) (Fig. 16)。這些結果顯示，NF-κB 之訊息傳遞路徑於 COF 所誘導 A549 存活以及抗凋亡機制中，在台灣女性肺腺癌之形成過程中可能調控細胞存活與凋亡之作用機制具有重要之相關性。

5.3 廚房油煙誘發肺腺癌細胞增生之分子機轉研究

5.3.1 、BaP, 2,4-DDE , COF 造成四種 IAPs (IAP1、IAP2、XIAP、survivin) 蛋白程度表現層次之比較

本實驗以 Benzo[a]Pyrene (BaP) 和醛類物質 2,4-decadiental (2’4-DDE)以及處理 COF，同時比較 IAPs 之表現程度是否有所差異。研究結果指出，不同濃度之 COF (0、12.5、12.5、50 μg/ml)；BaP (0、2.5、5、10 μM)；2,4-DDE (0、5、10、25 μM)，IAP2 蛋白之活性皆可以被此三種物質所誘發表現，但是其誘導強度則以 COF 之誘發強度較具敏敢性 (Fig. 4)，亦發現 IAP1，IAP2 和 survivin 蛋白，皆可以被 COF，BaP 和 2,4-DDE 所誘導。但是 IAP2 蛋白之表達與處理 COF 時表現較具有敏感性。此外， XIAP 蛋白之表達卻可以被 COF，2’4-DDE 所抑制，而 BaP 則較不具影響性 (Fig. 18)。

5.3.2 COF 誘導 IAP2 蛋白之表現 (time and dose-dependent manner)

研究結果指出 COF 對肺腺癌 A549 細胞中 IAP2 蛋白之過度活化之誘導效果較具敏感性。更進一步更觀察到 IAP2 蛋白可被油煙濃度為 25 μg/ml 以時間點之增加方式所產生 (0、2h、24h、48h)。並且觀察到當 COF 作用時間達 48 h 後，IAP2 蛋白之層次亦以不同濃度比增加（0、12.5、25、50 μg/ml）而增加之現象 (Fig. 19)。

5.3.3 比較 A549 細胞 p53-knock down 以及其他相關肺腺癌 CL-3 細胞株以油煙處理 c-IAP2 蛋白之表現層次

本實驗以另外一株肺腺癌細胞，CL-3，探討處理相同濃度點及時間點之 COF 對誘導 IAP2 基因表現之影響程度。結果顯示，作用不同濃度 COF（0、12.5、25、50 μg/ml）48 h 後相同可以誘導 IAP2 蛋白之表現程度 (Fig. 20)。p53-knock down 之 A549 細胞為實驗模式，比較 COF 誘發 IAP2 之表現程度。實驗結果得知，當處理 A549 細胞不同濃度之 COF (0、12.5、25、50 μg/ml) 經過 48 小時培養後，發現 COF 皆可以誘 p53-knock down A549 細胞 IAP2 蛋白之表現，且都呈現劑量比增加之趨勢 (Fig. 21)。因而推論 IAP2 之活化，可能和 p53 基因所所調控之訊息路徑

較無相關性。此一結果也許可以解釋為何台灣女性肺癌之 p53 突變率偏低可能與環境污染物 COF 所誘發之成因更具有相關性。

5.3.4 COF 對 caspase-3, caspase-8 蛋白活性表現之影響

IAPs 的主要功能為可以直接抑制 Caspase-3、7、9 之活性。以不同濃度 (0、12.5、25、50 μg/ml) 之 COF 處理 A549 細胞 48 小時，結果發現，隨著 COF 處理濃度之增高，相對伴隨 caspase-3 活性表現之降低，但是 caspase-8 之表現則不受影響。而 IAPs family 所產生之抗凋亡效應主要為抑制 caspase 之活化，因此推測 COF 誘導 IAP2 所產生的抗凋亡機制，可能是經由抑制 caspase-3 之活性，而與 caspase-8 之表現則不具相關性 (Fig. 22)。

5.3.5 四種傳遞因子抑制劑 (SB203580、 PD98059、BAY、LY294002) 對 IAP2 相關之作用機制

已知 COF 為一成分複雜之化合物，更進一步了解，除了 NF-κB 所活化之外，是否有其他相關之訊息路徑調控肺腫瘤細胞之增生與惡化？我們選用其他三種相關之細胞增生相關之訊息因子抑制劑：p38 MAPK (SB203580)、ERK (PD98059)、以及 PI3K (LY294002) 與 COF (25 μg/ml) 同時處理 A549 細胞 48 小時，探討 COF 誘發 IAP2 此種抗凋亡蛋白之相關分子作用機制。結果發現 BAY (2、10 μM) 及 LY294002 (5、25 μM) 可以有效抑制 IAP2 蛋白之表現，而 SB203580 (1、5 μM) 和 PD98059 (5、20 μM) 則無明顯效果，且反而呈現促進作用。因此推論 COF 誘發 IAP2 蛋白抗凋亡機制之生成，可能是同時經由 NF-κB 和 PI3K 兩種訊息路徑所傳導 (Fig. 23)。

5.3.6 COF 誘發 p-Akt 、total-Akt 基因表現之影響

先前研究結果顯示，LY294002 以及 BAY 可以有效抑制 IAP2 蛋白之表達。已知 Akt 為 PI3K 活化之下游標的基因，因此，為更進一步證實 PI3K 是經由活化 Akt 之活性，誘導 IAP2 蛋白之表現，實驗加入不同濃度之 COF (0、12.5、 25、50 μg/ml) 處理 A549 細胞 48 小時，觀察 p-Akt 蛋白之表現。結果發現，加入 COF 25～50 μg/ml 後可以促進 p-Akt 之活化 (Fig. 24)，此一結果與以 COF 處理 A549 細胞 48 小時後在 COF 濃度範圍為 25～50 μg/ml 中，其 IAP2 之表現最具明顯性之表現一致。因此推論 PI3K 可以經由活化 Akt 之活性，誘導 IAP2 蛋白之表現。

5.3.7 LY29002 抑制 COF 誘導 p-Akt 基因表現之影響

已知 Akt 為 PI3K 所調控之下游基因，因此為更進一步釐清 COF 所誘導 p-Akt 之活化確實是經由 PI3K 路徑所活化，同時處理 A549 細胞 COF (25 μg/ml) 及 LY294002 (25 μM) 48 小時後，結果發現 LY294002 確實可以完全抑制 COF 所誘導 p-Akt 之活化現象，而 total Akt 之表現則不受影響 (Fig. 25)。

5.3.8 LY294002 可以抑制 COF 誘導調控 A549 細胞之抗凋亡及細胞存活機制 (TUNEL assay+sub-G1 phase analysis)

先前研究指出，NF-κB 之 site-specific 抑制劑 (BAY) 可以抑制 COF 所誘導 A549 細胞中相關之存活路徑，並且造成細胞凋亡。為更進一步證實 PI3K 確實與肺癌細胞內抗凋亡訊息之傳遞與細胞之增殖與存活有關，分析細胞週期中 sub-G1 phase 及 TUNEL assay 之方法來界定。首先，以流式細胞儀 PI staining 之分析方法，檢測 sub-G1 DNA content 之分布比率。結果顯示 A549 細胞之凋亡現象在同時處理 COF (25 μg/ml) 及 LY294002 (25 μM) 後，可以明顯減低為 46.4 % 相

對於單獨處理 LY294002 (25 μM) 之 65.2 % (Fig. 26)。此外，隨著 sub-G1 比率之增加，S-phase 之分布比率也隨之減少。再以 TUNEL assay 分析凋亡細胞，以相同實驗條件之處理後，發現凋亡細胞之分佈比率可以從 36.92 % (單獨處理 LY294002 25 μM) 減少為 21.65 % (同時處理 COF 及 LY294002) (Fig. 27)。因此推論 COF 所誘導之抗凋亡機制及細胞存活機制中，除了 NF-κB 之外，PI3K/Akt 訊息路徑亦扮演重要之角色。

5.3.9 COF 對 p21 及 cyclin D1 蛋白之表現層次

先前研究指出，COF 可以誘導肺腺癌 A549 細胞中 S-phase 之分布比率之增加，並且相對可以減少 G0/G1-phase 之分布比率 (Fig. 28A)，因而推論 COF 之暴露可能與肺腺癌之增生有關。因此，更進一步想了解，此增生機制是與細胞周期中之何種蛋白調控有關？結果顯示，當處理 A549 細胞 COF 48 h 後，可以促進 cyclin D1 蛋白之活化，反之卻可以抑制 p21 蛋白之表現，因而推論此兩種蛋白可能與其增生機制具有相關性 (Fig. 28B)。

5.3.10 同時處理 A549 細胞 COF 及 LY294002 對 cyclin D1 和 p21 蛋白表現層次之影響

為了更進一步釐清 PI3K 之活化路經以及其所誘發肺腺癌細胞中之存活機制，是否與調控 cyclin D1 和 p21 蛋白具有相關性，因此同時處理 A549 細胞 LY294002 (25 μM) 和 COF (25 μg/ml) 48 小時後，發現 LY294002 相對會促進 p21 蛋白之活化並且抑制 cyclin D1 蛋白之表現 (Fig. 28C)。根據此依結果，推論 PI3K 訊息路徑之活化，可能與肺腺癌細胞周期中之增生機制中扮演某種重要之調控角色。

6、討論

本實驗室先前研究指出，高劑量之 COF (250～500 μg/ml) 會造成肺腺癌細胞 CL-3 DNA 傷害及細胞之毒殺性。但是女性肺癌之形成係因長時間的暴露於 COF 之中，逐漸誘導肺癌之形成。因此，本實驗開始之研究動機，主要是想了解以低濃度之 COF，長時間處理肺腺癌細胞，觀察腫瘤細胞之生長是否會更加惡化。在細胞生長曲線之分析結果中，我們意外發現以低濃度之 COF (12.5 μg/ml) 連續處理 A549 細胞 6 天後並計算細胞生長曲線，處理 COF 之實驗組別其 doubling time 較未處理之控制組為快，且相對之細胞數目也增加較多 (Fig.11)，同時並發現低濃度之 COF 可以促進細胞之存活率 (Fig.12)。根據此一結果，更進一步想探討低濃度之 COF 與肺腺癌細胞之增生是否具有相關性？NF-κB 主要為各種壓力反應之調控者，例如；DNA 傷害 (Yamamoto and Gaynor, 2004)。實驗室先前研究指出，經由 COF 暴露後，BPDE-N2-dG 為肺腺癌 CL-3 細胞之主要 DNA adduct (Yang et al., 2000)；經由 Comet assay 的檢測過程中，bulk-like DNA adducts 為 COF 誘導之 DNA 傷害之主要產物，此外 COF 所誘發之 Cox-2 蛋白之表現也和 NF-κB 之活化調控有關 (Lin et al., 2002)。於此研究中，我們發現 COF 可以誘發 A549 細胞中 NF-κB 的活化，IAP2 蛋白之表現。於細胞週期之分析結果中，發現低濃度之 COF 可以促進細胞週期中 S-phase 之分佈比率 (Fig.13)。因此，推論當肺腺癌 A549 細胞暴露於低濃度 COF 處理條件時，可能和細胞增生具有相關性，導致癌細胞更加腫瘤化。Bandala 等人以 gemcitabine 處理 A549 細胞後，發現 NF-κB 的活化可以調控 IAP2 蛋白之表現 (Bandala et al., 2001)。更有研究指出當 A549 細胞經由 ionoizing radiation 刺激後，IAP2 確實可被 NF-κB 之活化機制所誘導 (Ueda et al., 2001a)，此結果與我們發現 COF 確實會經由 NF-κB 路徑活化 IAP2 蛋白之表現相同 (Fig.15)。本實驗室先前研究指出，BaP 可以作為誘發 DNA 股斷裂之促進因子，但換算成 COF 所含有之 BaP 濃度時，發現 1000 μg/ml COF 相當於含有 0.74 μM BaP，而 1000 μg/ml 所造成之彗星體長度是 0.74 μM BaP 的 1.7 倍左右，顯示 COF 中除了有經代謝活化之 BaP 能攻擊 DNA 造成傷害之外，可能還有其他成分參與 DNA 單股之斷裂。實驗結果中，我們發現 COF 中之其它相關之兩種成分 BaP 和 2’4’-DDE 皆會誘導 IAP2 蛋白之表現層次，但是表現強度以 2’4-DDE 較為顯著 (Fig.14)。COF 確實為一複雜之化合物，當婦女烹調之所需而暴露於 COF 刺激之作用環境中時，相對也增加致癌性發生之可能性。此外，根據先前實驗室之研究結果中，我們已得知，COF 造成肺腺癌細胞產生之氧化性傷害是和 NF-κB 之活化路徑有關，在此結果中我們證實，IAP2 所產生之抗凋亡機制是經由 NF-κB 所調控 (Fig.16; Fig.17)。因此推論，COF 誘發台灣女性肺腺癌增生之分子機轉中，除了先前研究發現產生氧化性壓力之傷害外，可能與腫瘤細胞之增生與所活化之抗凋亡機制有關。

當以 COF 處理後，雖然 IAP2 可以同時被誘導於 A549 及 CL-3 兩株肺腺癌細胞中 (Fig. 20)，但是只有 IAP2 蛋白可以以時間比與濃度比之增加趨勢表現於 A549 細胞中 (Fig.19)。因此，根據此一結果，更進一步想要探討 IAP2 蛋白在肺腺癌 A549 細胞中之生存與增殖機制中所扮演之角色。研究結果顯示，COF 和 2,4-DDE 可以同時誘導 IAP2 蛋白及抑制 XIAP 蛋白之表現，但是有趣的是我們亦發現，COF 對 XIAP 蛋白卻呈現劑量比之抑制效果 (Fig.18)，此一結果和 Ferreira 等人對非小細胞性肺癌之研究結果中指出 XIAP 表現高之病人其存活率相對更高，可作為一種 proliferation marker，以提供對肺癌治療之診斷工具（Ferreira et al., 2001）。Cummins 等人研究指出當結腸癌細胞中 XIAP 蛋白被 knock-out 時並不會影響其細胞存活率 (Cummins et al., 2004)。XIAP-null mice 之研究結果中無法偵測到 apoptosis 之作用機制 (Harlin et al., 2001)。更有研究指出，XIAP 蛋白之過度表現可以抑制 cyclin A 和 D1 之活化，並且會促進內皮細胞中 $p21^{Cip1/Waf1}$ and $p27^{kip1}$ 蛋白之表現 (Levkau et al., 2001)。於此研究中發現，未經由 COF 處理之 A549 細胞具其 XIAP 蛋白之表現程度明顯高於其他三種抗凋亡蛋白。然而，XIAP 蛋白之抑制

效果與 IAP2 蛋白之過度活化可能與 COF 所誘導 A549 細胞中 S-phase 之分布比率增加，細胞增殖效應具有相關性。

p53 基因是一已知的抑癌基因，在腫瘤發生之過程中 p53 是最常發生基因突變或蛋白表現異常之基因 (Greenblatt et al., 1994)。但是台灣女性肺癌 p53 之突變率偏低，且有不同於其他國家之突變圖譜 (Wang et al., 1998)，因此就 p53 之突變頻率和型式推測，台灣女性肺癌之成因可能與環境暴露之致癌物，例如，COF，較具有相關性。實驗結果得知，COF 皆可以誘導 p53 wild type 及 p53 knock down A549 細胞 IAP2 蛋白之表現 (Fig. 21)，因此推論，IAP2 蛋白之活化，可能不是經由p53 基因所誘導。此一結果也許可以解釋為何台灣女性肺癌之p53 突變率偏低之現象。目前已知細胞凋亡可以經由外生型 (extrinsic)及內生型 (intrinsic)兩種途徑所傳遞。外生型之細胞凋亡機制主要可以經由一些細胞表面之接受體，例如，TNFR 並且活化 casapse-8 以傳遞凋亡訊息。而內生型機制之媒介，則是藉由粒線體內 cytochrome c 的釋放，並且藉由活化 caspase-3，起始凋亡訊息 (Kiechle and Zhang, 2002)。目前已知，IAP2 蛋白之主要功能為抑制 caspase 之活性，特別是 caspase-3、7 之活性 （LaCasse et al., 1998; Deveraux et al., 1997; Roy et al., 1997）。Zou 等人研究指出，polyamide 之 depletion 可以藉由活化小腸上皮細胞中 NF-κB 之活化，誘導 IAP2 蛋白之表現，並且抑制 caspase-3 之表達，進而對 TNF-α/CHX 所誘導之凋亡機制產生拮抗性 (Zou et al., 2004)。於此研究中，我們亦觀察到 COF 可以以濃度比的方式抑制 caspase-3 之活性，但是對 caspase-8 則不具影響效果 (Fig. 22)。我們之研究結果顯示，COF 所誘導 IAPs 所產生之抗凋亡機制，可能是與細胞內生型之抑制凋亡蛋白 caspase-3 之活性表達有關，以調控台灣不抽菸女性肺癌細胞之增生 (Figure. 12)。

Lin 等人於對人類肺腺癌細胞 CL1.0 細胞株之研究中發現 PI3K/Akt 機制之活化可以拮抗由 ultrtaviolet B 所誘導之細胞凋亡現象 (Lin et al., 2001)。而於對肺癌細胞之抗腫瘤策略中發現到抑制 PI3K/Akt 之活化機制可以有效預防癌症細胞之過度增殖 (Nakashio et al., 2000; Krystal et al., 2002）。研究指出 cyclic AMP 可以經由 ERK1/2 及 p38 MAPK 活化路徑，誘導 IAP2 蛋白之表現於許多不同之細胞株中（Liu et al., 2004; Nishihara et al., 2004）。根據我們最近的研究結果推論 COF 可以經由 NF-κB 之活化調控 A549 細胞中 IAP2 蛋白之表現 (Hung et al., 2005)。再更進一步使用三種調控 IAP2 蛋白表現之相關訊息作用因子，包括有：SB203580 (p38 MAPK 抑制劑)、PD98059 (ERK 抑制劑) 以及 LY294002 (PI3K 抑制劑)，以釐清 IAP2 所被調控之上游機制。結果顯示，PI3K 抑制劑亦可抑制 IAP2 蛋白之表現，但是 p38 MAPK 和 ERK 之抑制劑卻沒有相同之抑制效果 (Fig. 23)。這些結果顯示，PI3K 在 COF 所誘導 IAP2 產生的抗凋亡機制中，除了 NF-κB 外，可能較具重要性。Serine/threoine kinase (Akt)（又稱為 protein kinase B）為 PI3K 媒介存活訊息機制預防凋亡機制進行之主要下游基因 (Krasilnikov, 2000; Vivanco and Sawyers, 2002)。對 leukemia 細胞之研究中指出，Akt 之活化與 IAP2 蛋白之表現具有密切關係 (Martelli et al., 2003; Neri et al., 2002)。我們的研究結果指出，PI3K 為調控 IAP2 蛋白表現之上游基因，並且可以調控 COF 誘導 A549 之細胞存活與凋亡機制之進行 (Fig. 26; Fig. 27)。而 PI3K 之下游基因 p-Akt 亦可被 COF 處理 48 h 後後所誘導 (Fig. 14)，p-Akt 蛋白之表達卻可以被 LY294002 所抑制 (Fig. 27)。

Cyclins 和其相關 cyclin-dependent kinas (CDK) 為調控細胞週期進展之主要相關分子。當細胞週期中 G1-S transition 產生 arrest 之作用時會造成細胞產生增殖控制失調之作用機轉。Cyclin D1 為目前已知之一種可以調控細胞週期之重要 protocogene (Singhal et al., 2005)。Jim 等人之研究指出 cyclin D1 之過度表達與 p16 蛋白功能之缺失會造成肺腺癌之病人具有較短之存活率 (Jin et al., 2001)。CDK inhibitor, p21 (又稱為 WAF1, CIP1)可以經由抑制數種分子作用機轉抑制細胞週期之進行。它可以抑制早期 G1-phase 中 cyclin D/CDK4 以及 cyclin E/CDK2 之表現。因此，綜

合以上之研究結果，我們結論 COF 之暴露可能與 IAP2, p-Akt, 以及 cyclin D1 (Fig. 28B) 蛋白之活化，以及抑制 XIAP 和 p21 蛋白之表達 (Fig. 28C) 可能與肺腺癌細胞之存活與增生具有相關性。這些研究結果也指出，PI3K/Akt 之活化路徑可能與肺腺癌之病程進展中扮演某種重要之調控角色，並且對台灣女性肺癌病人之分子作用機轉提供些許新的治療策略。

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8、表與圖

Table 1. Pathological changes of rats in two months experiment.

2 months

	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ
Number of animals	7	7	6	7
Pus	0	0	0	4(1.29)*#
Bronchi:				
Bronchitis	7(1.43)	7(2.00)	7(2.50)	7(2.86)
Bronchial lymphoid proliferation	6(1.00)	7(1.29)	4(0.67)	6(1.86)
Bronchiol epithelial hyperplasia	0	0	1(0.33)	3(0.71)
Metaplasia (or dysplasia)	0	0	1(0.17)	2(0.43)
macrophage infiltration	3(0.43)	7(1.29)**	4(1.17)	3(0.71)
Hyperkeratization	0	0	2(0.50)	2(0.43)
Alveoli:				
Alveolar hyperplasia	2(0.29)	6(0.86)*	3(0.67)	6(1.71)**

◆ Average severity grade of lesion in animals: 1=minimal, 2=mild, 3=moderate, 4=marked
minimal:<10%; mild:10%-50%; moderate:50%-75%; mark:>75%

* Indicates average severity grade (mean±SE) of lesion significantly different (p≦0.05) from solvent control group by T test.

** Indicates average severity grade (mean±SE) of lesion significantly different (p≦0.01) from solvent control group by T test.

# Indicates Group 4 (DMBA + COF) average severity grade of lesion significantly different (p≦0.01) from Group 3 (DMBA only) by T test.

Table 2. Pathological changes of rats in six months experiment.

6 months

	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ
Number of animals	6	8	7	10
Bronchi:				
Bronchitis	6(1.67)	8(1.63)	7(1.71)	10(1.90)
Bronchial lymphoid proliferation	6(1.50)	8(1.38)	7(1.29)	10(1.60)
macrophage infiltration	0	3(0.38)	2(0.29)	9(1.40)**#
Hyperkeratization	0	0	1(0.14)	2(0.20)
granulation(proteinosis)	0	1(0.13)	0	9(1.20)**
Alveoli:				
Alveolar hyperplasia	3(0.50)	7(1.25)	6(0.86)	9(1.90)**#

◆ Average severity grade of lesion in animals: 1=minimal, 2=mild, 3=moderate, 4=marked

minimal:<10%; mild:10%-50%; moderate:50%-75%; mark:>75%

* Indicates average severity grade (mean±SE) of lesion significantly different ($p<0.05$) from solvent control group by T test.

** Indicates average severity grade (mean±SE) of lesion significantly different ($p \leqq 0.01$) from solvent control group by T test.

# Indicates Group 4 (DMBA + COF) average severity grade of lesion significantly different ($p \leqq 0.01$) from Group 3 (DMBA only) by T test.

Table 3. Pathological changes of rats in eight months experiment.

Pus	0	0	3(0.90)	3(0.82)
Bronchi:				
Bronchitis	9(1.56)	8(2.00)	10(2.30)*	11(2.63)**
Bronchial lymphoid proliferation	9(1.56)	8(1.12)	10(1.80)	11(1.91)
Bronchiol epithelial hyperplasia	0	0	3(0.60)	3(0.82)
Metaplasia (or dysplasia)	0	0	1(0.2)	5(0.73)*
Squamous cell carcinoma	0	0	0	1(0.18)
macrophage infiltration	9(1.00)	8(1.26)	10(1.4)*	11(2.18)**#
Hyperkeratization	0	0	2(0.40)	3(0.73)
Alveoli:				
Alveolar hyperplasia	0	7(0.88)**	5(0.50)*	7(0.73)**

◆ Average severity grade of lesion in animals: 1=minimal, 2=mild, 3=moderate, 4=marked
minimal:<10％; mild:10％-50％; moderate:50％-75％; mark:>75％

* Indicates average severity grade (mean±SE) of lesion significantly different ($p<0.05$) from solvent control group by T test.

** Indicates average severity grade (mean±SE) of lesion significantly different ($p \leqq 0.01$) from solvent control group by T test.

# Indicates Group 4 (DMBA + COF) average severity grade of lesion significantly different ($p \leqq 0.01$) from Group 3 (DMBA only) by T test.

Figure 1. The average weight of each group experimental animals (2 months)

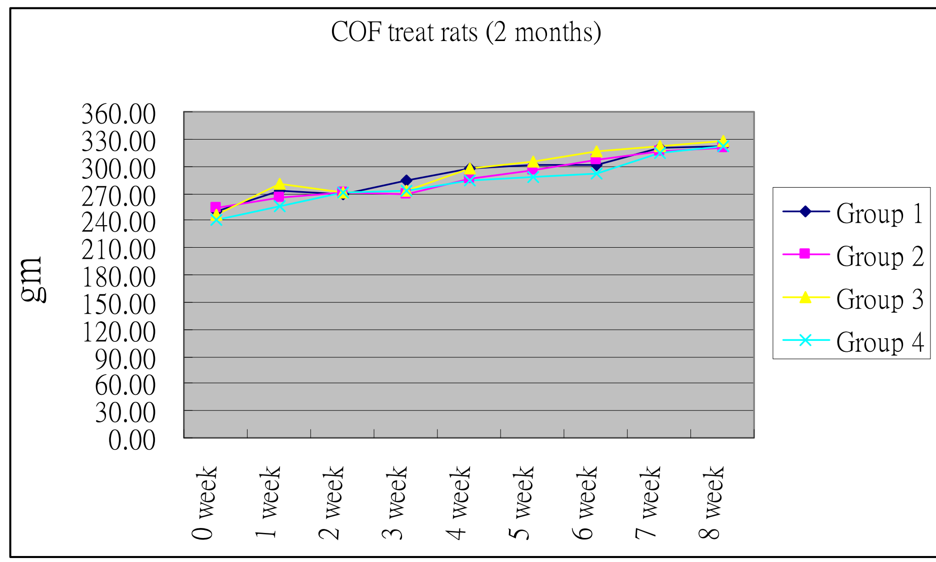


Figure 2. The average weight of each group experimental animals (6 months)

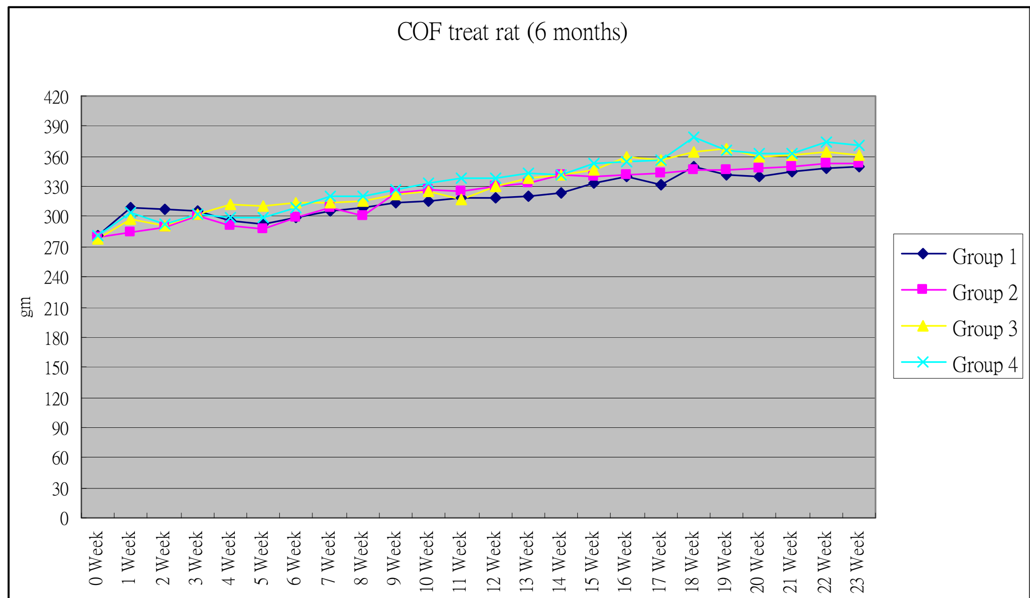


Figure 3. The average weight of each group experimental animals (8 months)

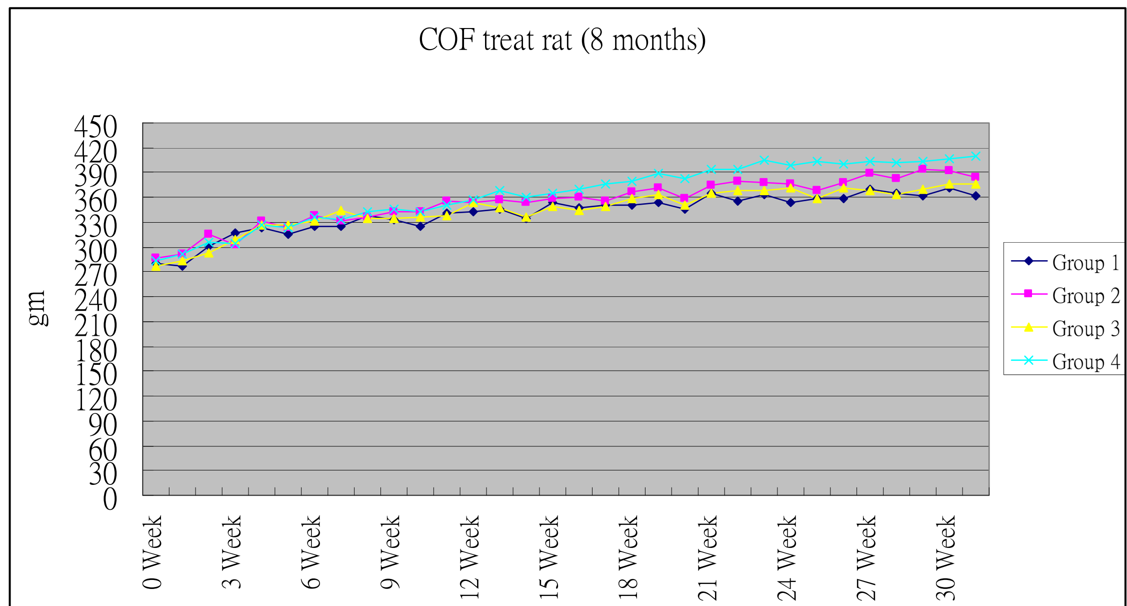
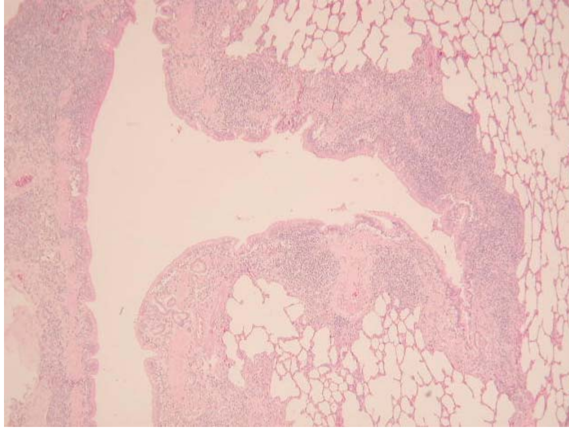


Figure 4. Bronchitis of rats induced by Corn oil, COF, DMBA and COF+DMBA.

40X



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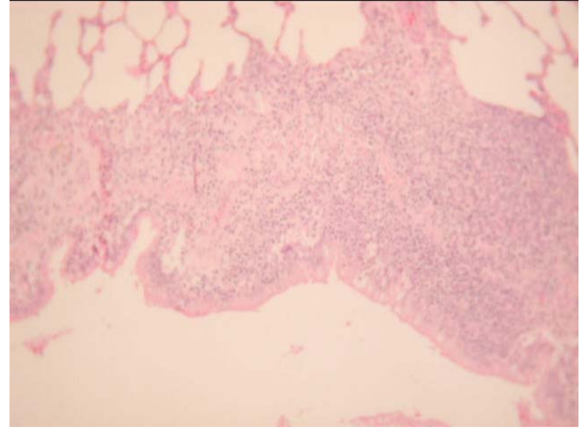

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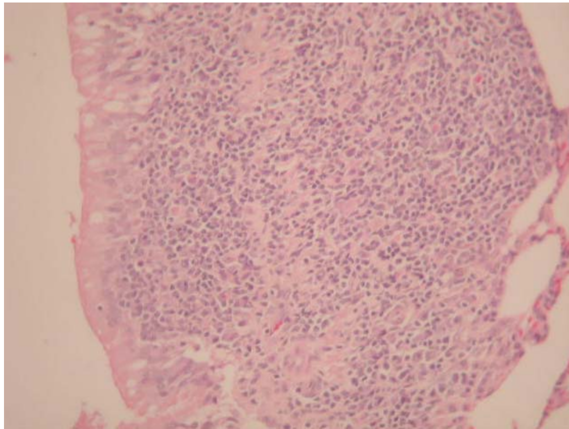


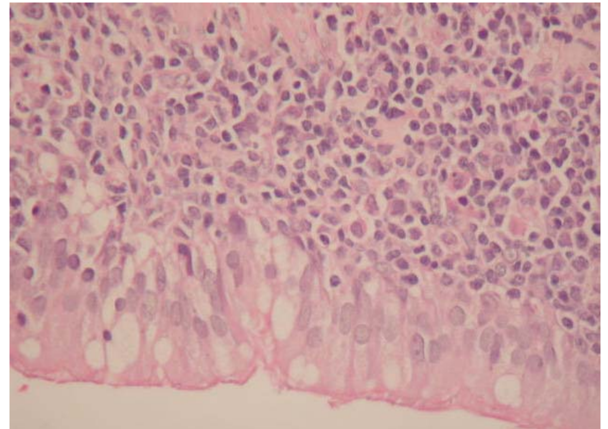
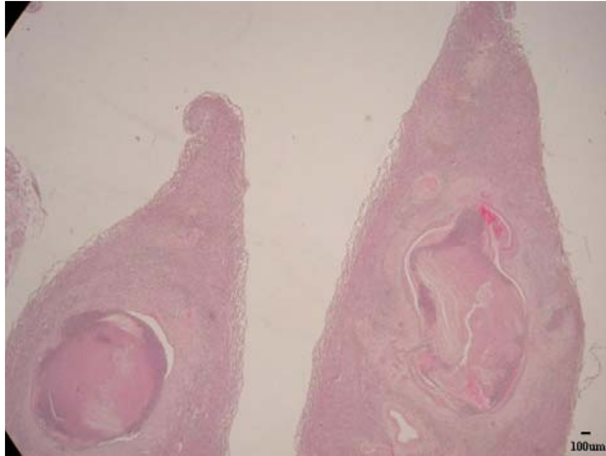


Figure 5. Pus of rats induced by DMBA+ COF and DMBA only.

Pus (20×)



Pus (40×)

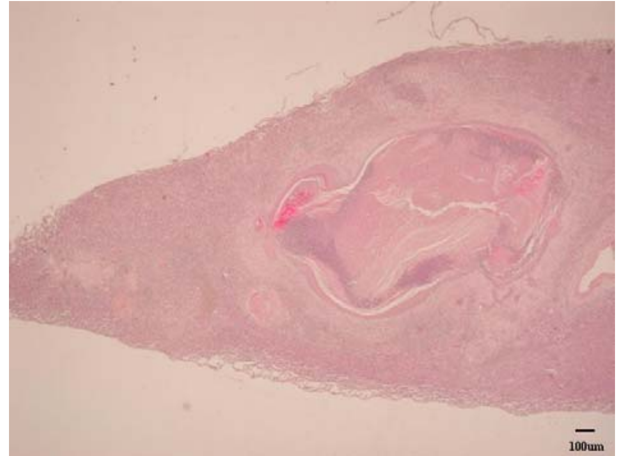
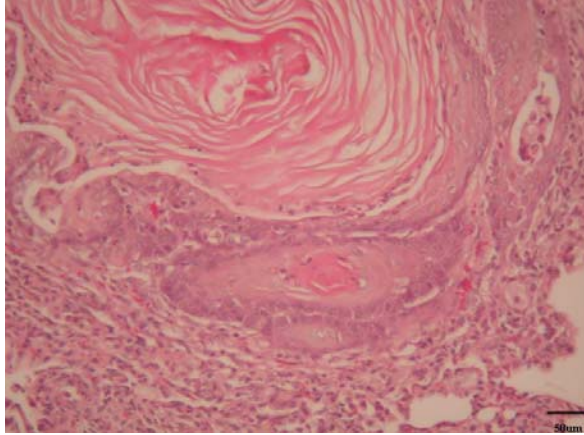
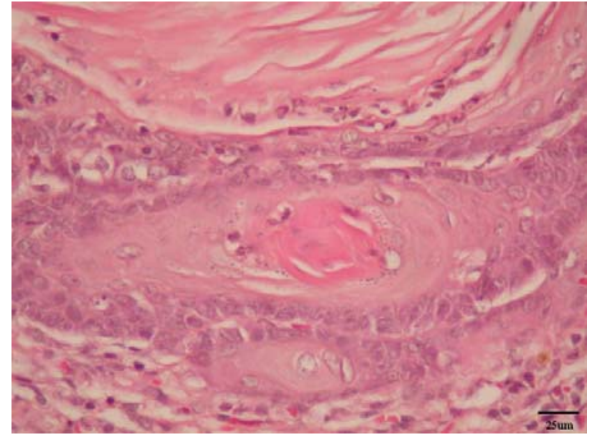


Figure 6. Atypical hyperplasia of rats induced by DMBA+ COF and DMBA only.

Atypical Hyperplasia around bronchi

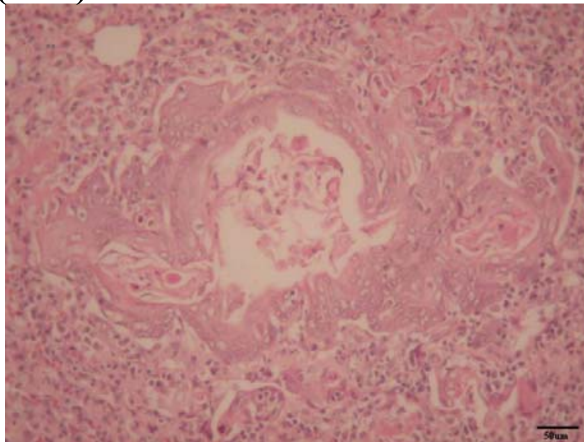
(200×) (400×)





Atypical Hyperplasia around bronchiol

(200×) (400×)



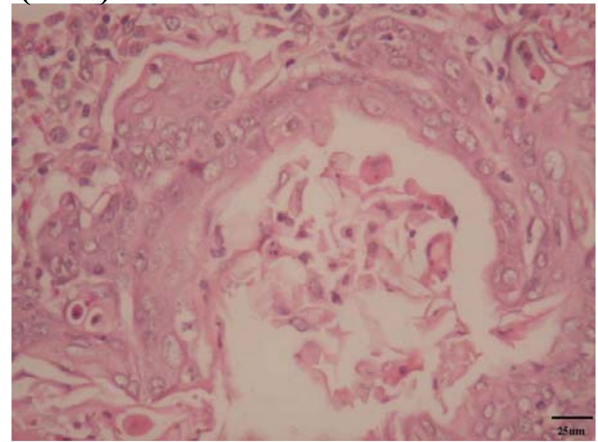
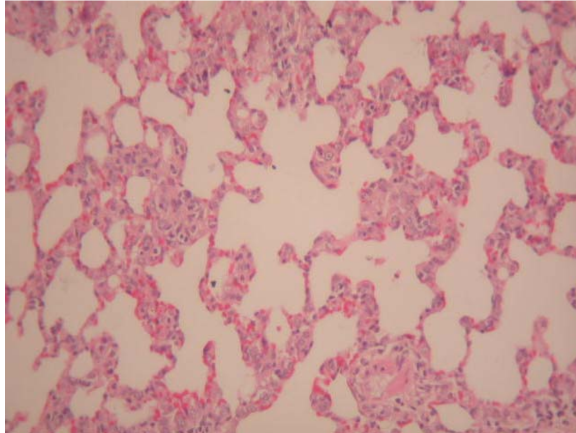


Figure 7. Alveolar epithelial hyperplasia of rats induced by DMBA+ COF, COF only and DMBA only.

200x



400x

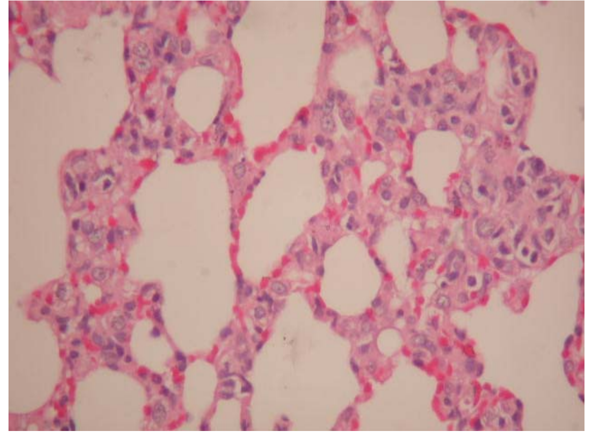
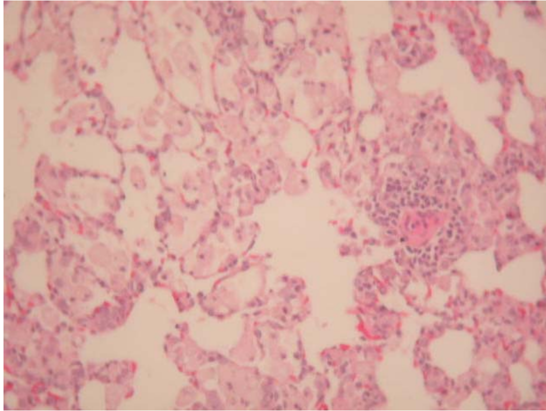


Figure 8. Macrophage aggregation in lung of the rats .

(200x)



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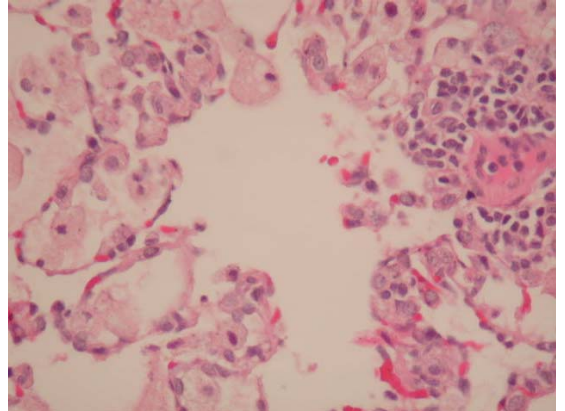
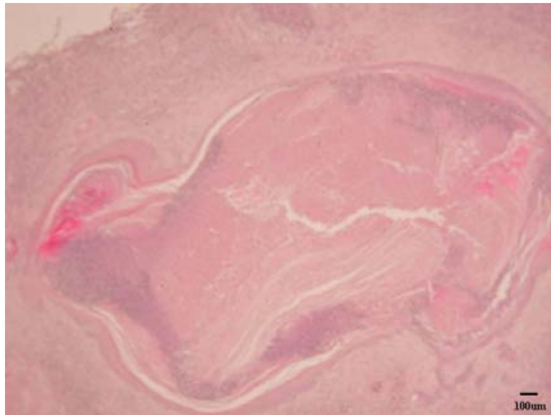


Figure 9. Hyperkeratization and metsplasia of rats induced by DMBA+ COF and DMBA only.

(40x)



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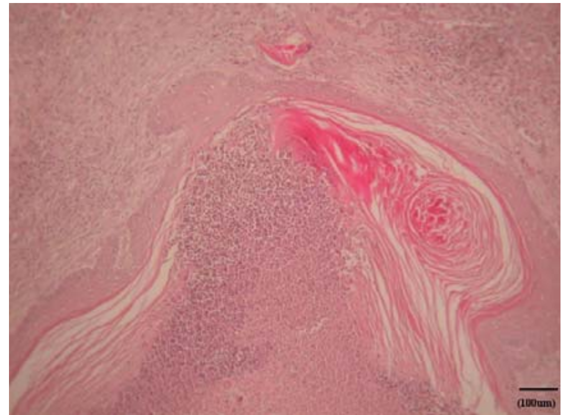

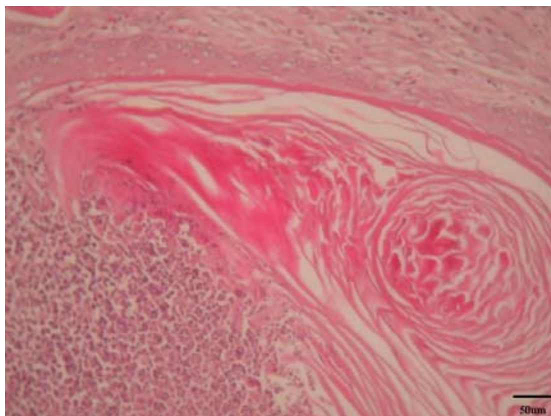

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metaplasia

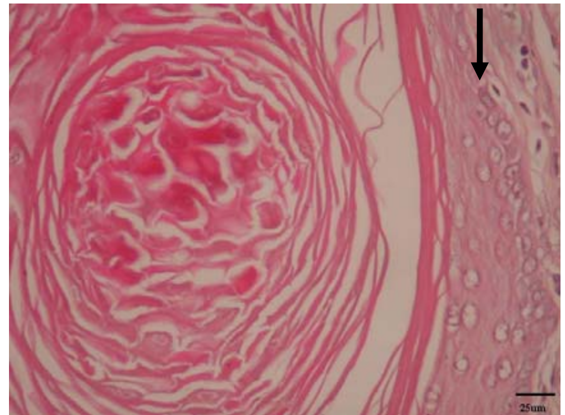
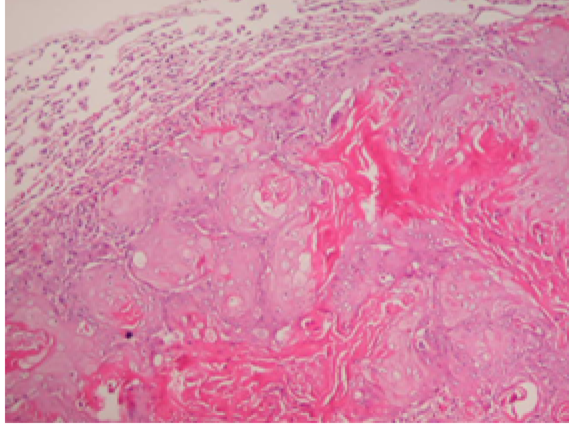


Figure 10. Squamous cell carcinoma of rats induced by DMBA+ COF in eight months treatment experiment.

(100x)



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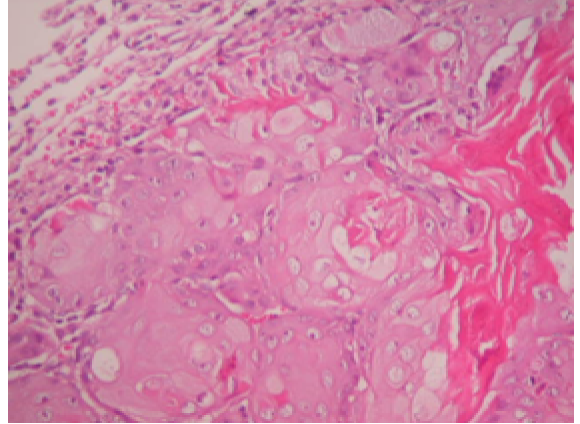

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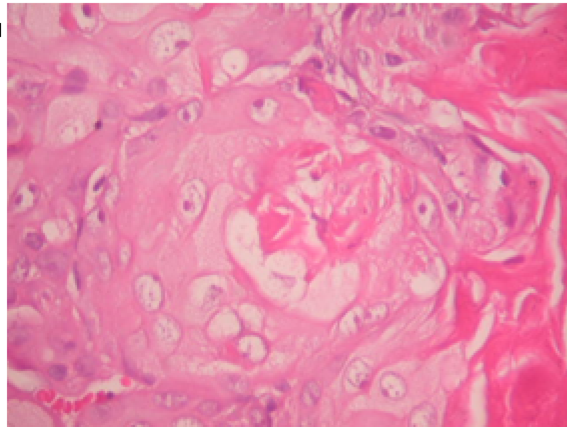


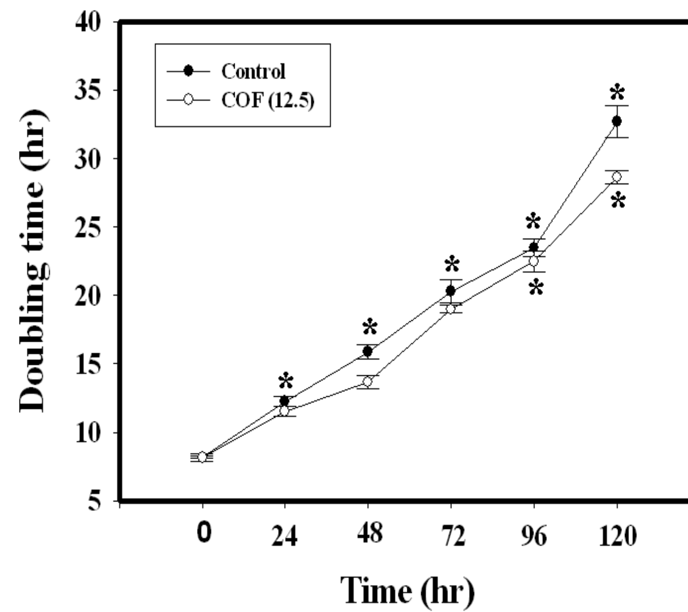


Fig.11. Effect COF at concentration of a 12.5 μg/ml to investigate the cell growth curve on lung adenocarcinoma A549 cells. A549 cells were diluted with at concentration of 1×10^5 cells/ml，culture with 10 % FBS condition medium, after each 24 h treatment, stain with trypan blue, count the whole live cell number for growth curve measurement. The cell proliferation rate was indicated at the doubling time (D.T). The D.T. equation was determined as：D.T.＝ (t－t0) log2 / logN－logN0. Results are the mean of three different experiments ± SD. * (P＜0.05; one-way ANOVA, Scheffe-test).

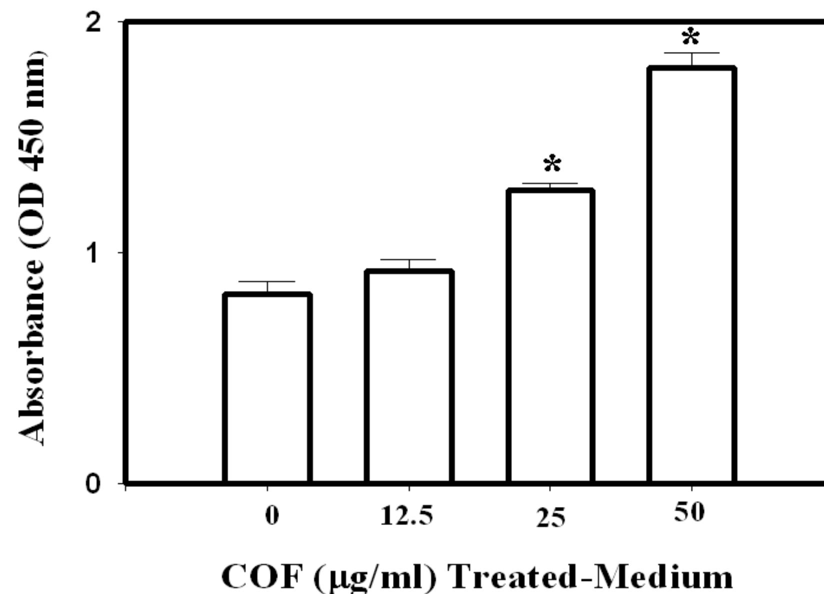
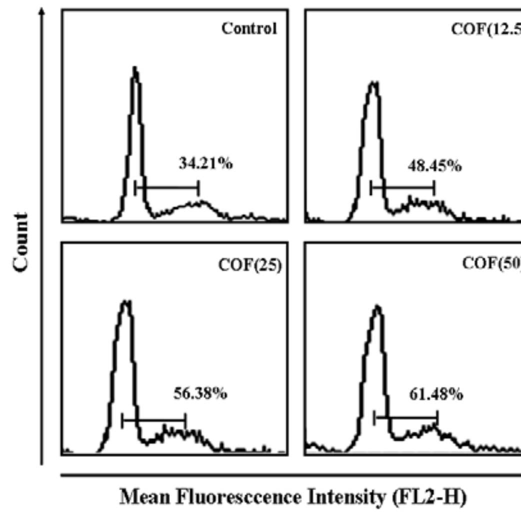


Fig. 12. Effect COF on A549 cells induction for cell viability. The graph show change in cell viability of A549 following a treatment with various concentrations of COF. Viable cells were detected based on mitochondria MTT metabolism and measured at 570 nm using a Micro plate reader (Bio－Rad). Cell viability was then calculated by the equation：percentage cell viability＝(absorbance of treated culture－absorbance of background control) / (absorbance of control cell－absorbance of background control)×100. Note the extremely significant induction of viable cells in the COF-treated group as well as increased percentage of viable cells with increased COF concentrations. Results are the mean of three different experiments ± SD. * ($P < 0.05$; one-way ANOVA, Scheffe-test).



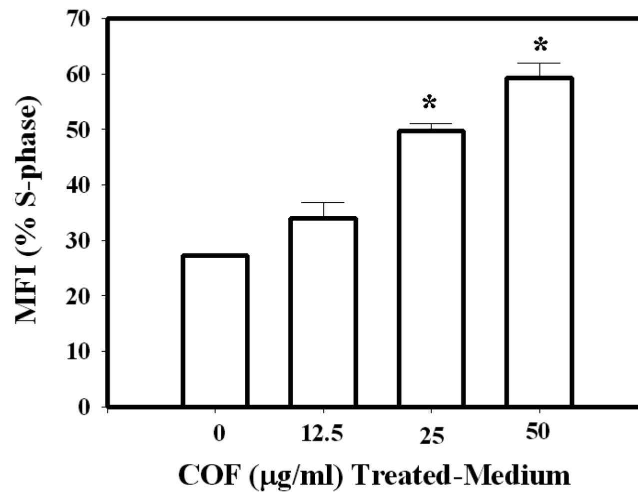


Fig. 13. The effects of COF induce S-phase increase in A549 cells. The number inside each box indicated the calculated % fraction of each S-phase. Cells were treated with COF (0, 12.5, 25, 50 μg/ml); control contains DMSO (< 0.01 %). A total of 10,000 cells were counted per sample and the data were processed using the CellQuest software (Becton Dickinson). The % of S phase were (0:34.21 %), (12.5: 48.45 %), (25: 56.38 %), (50: 61.48 %) respectively. Results are the mean of three different experiments ± SD.* ($P < 0.05$; one-way ANOVA, Scheffe-test).

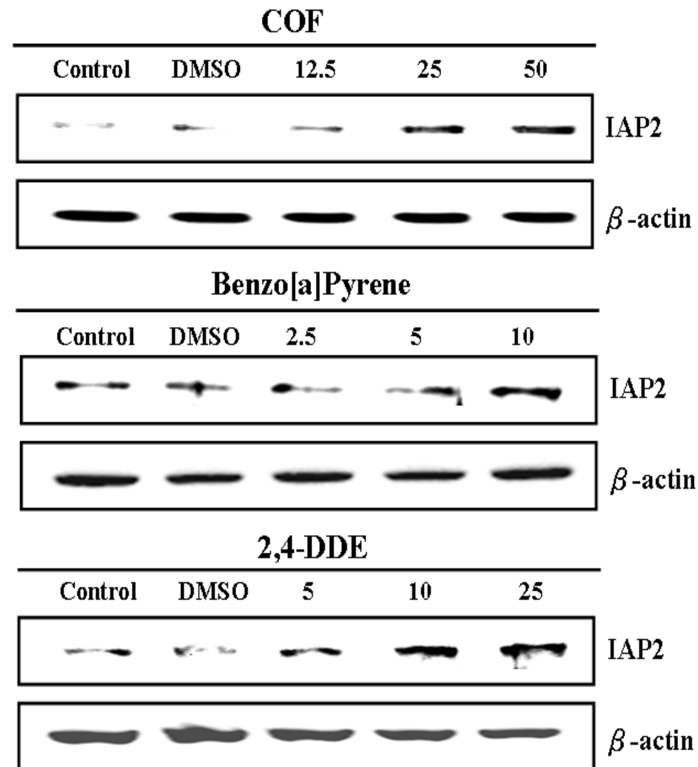


Fig. 14. Immunoblot analysis for IAP2 expression in A549 cells with COF, Benzo[a]Pyrene and 2,4-DDE treatment. After cells being treated with various doses of COF (0, 12.5, 25, 50 μg/ml), Benzo[a]Pyrene (0, 2, 5, 10 μM) and 2,4-DDE (0, 5, 10, 25 μM) for 48 h, cell extracts were prepared and a prepared sample with a total protein of 20 μg was load in each lane, resolved on a 10 % SDS-PAGE gel, and probed with a 1∶500 dilution of anti-IAP2 antibody. DMSO (＜0.01 %) was used as a solvent control. Results are from three independent experiments.

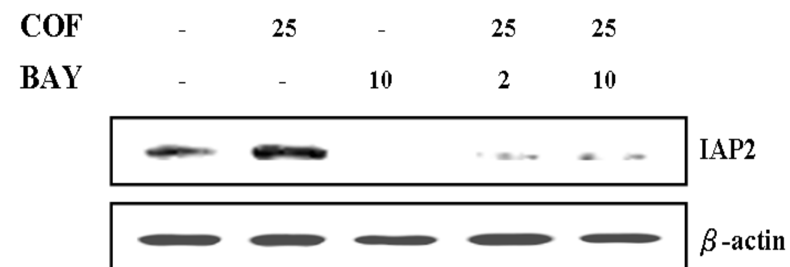


Fig. 15. Effects BAY on expression of IAP2 in A549 cells stimulated by COF. A549 cells were preincubated with BAY (2 or 10 μM) for 1 hour, and thereafter cultivated in the presence of COF (25 μg/ml) for 48 h. DMSO (< 0.01 %) was used as a solvent control. Results are from three independent experiments.

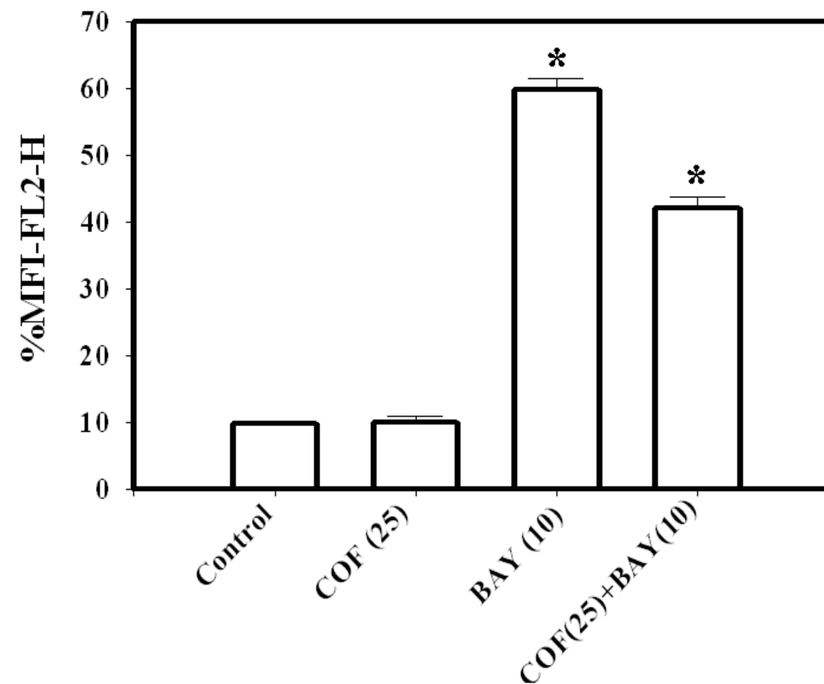
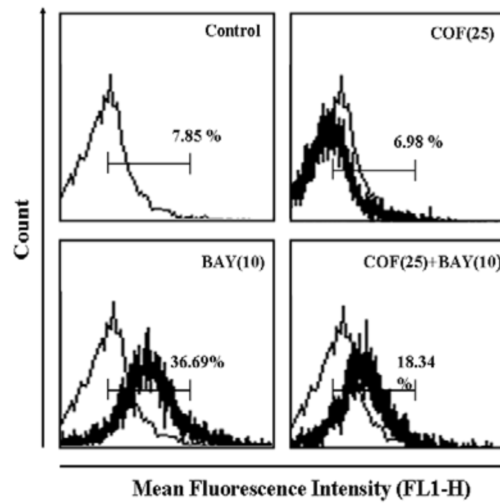


Fig.16. BAY induced apoptosis on A549 cells. Cells were pretreated with BAY (10 μM) for 1 h, and then treated with COF at concentration of 25 μg/ml for 48 h, respectively. BAY treatment leads to increase the sub-G1 phase. The percent of sub-G1 phase was determined by PI staining. The mean of fluorescence intensity (MFI) was analyzed by CellQuest software. Results are the mean of three different experiments ± SD. * ($P < 0.05$; one-way ANOVA, Scheffe-test).



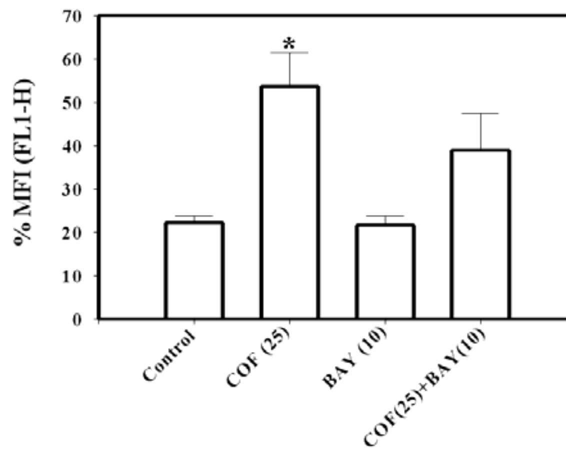


Fig.17. BAY induced apoptosis on A549 cells. Cells were pre-treated with BAY (10 μM) for 1 h, and then treated with COF at a concentration of 25 μg/ml for 48 h as specified in figures. Fluorescein-positive cells were analyzed by TUNEL assay and detected through flow cytometery. The mean of fluorescence intensity (MFI) was analyzed by CellQuest software. Results are the mean of three different experiments ± SD. * ($P < 0.05$; one-way ANOVA, Scheffe-test).

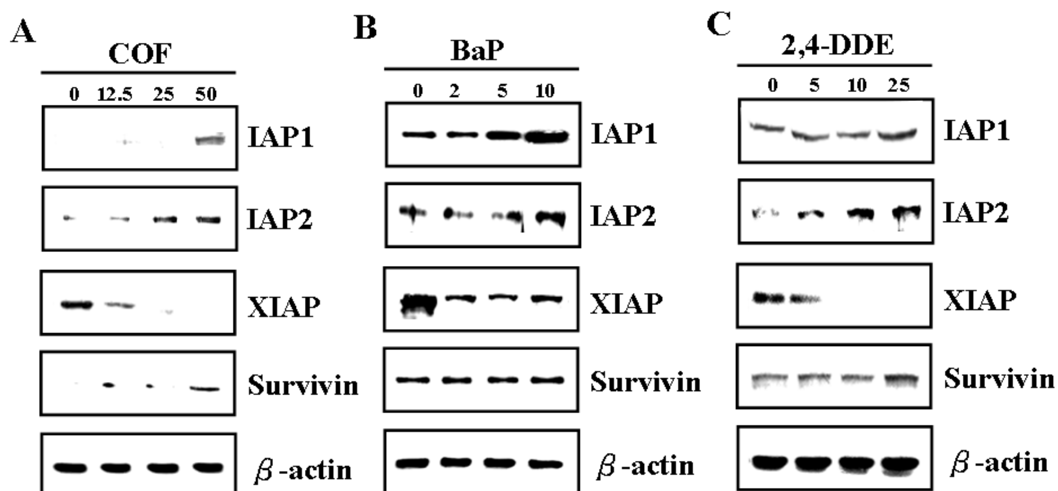


Fig. 18 COF, BaP, 2'4-DDE induced expression of apoptotic related proteins in A549 cells. Cells were incubated in contain various concentration of COF (μg/ml), BaP (μM), 2,4-DDE (μM) as indicated time for 48 h. Western blotting analysis of cell extracts (25 μg/lane) probed for anti-IAP1, IAP2, survivin, and XIAP antibodies, equal amounts of protein were subjected to immunoblotting, the protein bands were detected by enhanced chemiluminescence (ECL). Cell treated with DMSO (< 0.01 %) was used as the control for each experiment. Results are from three independent experiments. β-actin was used as a loading control.

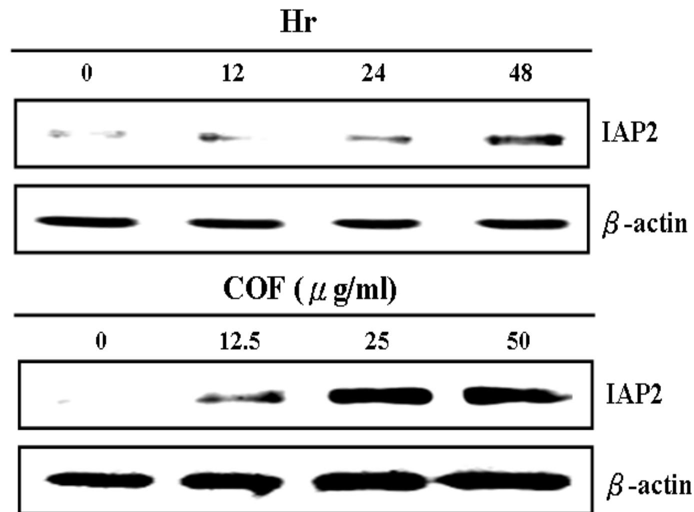


Fig. 19 The time course and dose dependent manner of IAP2 protein activation with COF stimulate. A549 cells were serum starved overnight and then either not stimulated or stimulated with COF (25 μg/ml). Whole cell lysates were extracted at the indicated intervals after stimulation and analyzed by Western blotting with anti-IAP2 antibody. (A) A time course (0、12, 24, 48 h) performed simultaneously and electrophoretically analyzed on A549 cells. (B) A dose-dependent manner after stimulation with various concentrations of COF (0, 12.5, 25, 50 μg/ml) for 48 h. Equal amounts of protein were subjected to immunoblotting. Cell treated with DMSO (< 0.01 %) was used as the control for each experiment. Results are from three independent experiments. β-actin was used as a loading control.

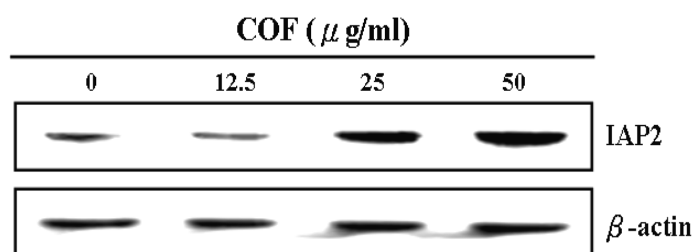


Fig. 20 Effect various concentrations of COF on lung adenocarcinoma CL-3 cells for 48 h to investigate the IAP2 protein expression level. β-actin was used as a loading control. Results are from three independent experiments.

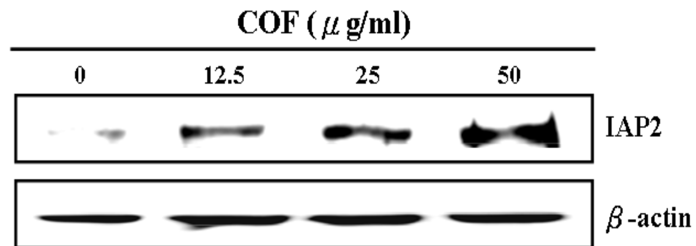


Fig. 21. Effect various concentrations of COF on lung adenocarcinoma A549 cells (p53 knock down) for 48 h to investigate the IAP2 expression level. β-actin was used as a loading control. Results are from three independnet experiments.

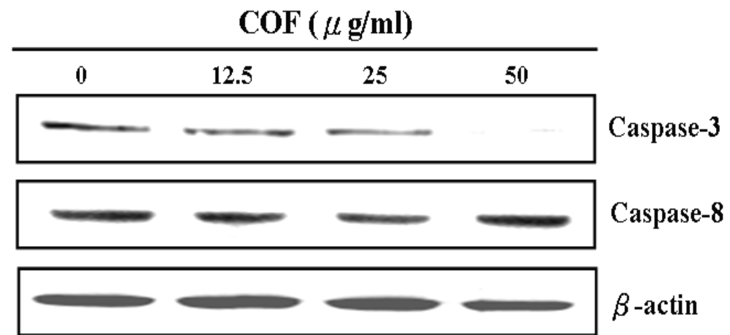


Fig. 22. Effect various concentrations of COF on lung adenocarcinoma A549 cells for 48 h to investigate the caspase-3, caspase-8 proteins expression level. β-actin was used as a loading control. Results are from three independent experiments.

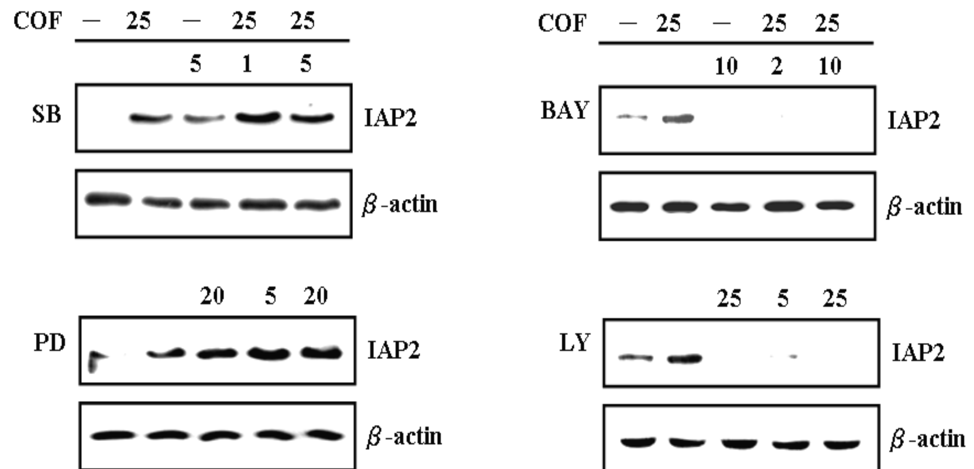


Fig. 23. Signal requirements for COF-stimulated IAP2 activation. A549 cells were pretreated with the indicated concentrations of SB203580 (SB) (1,5 μM), PD98059 (PD) (5, 20 μM), BAY (2, 10 μM) and LY294002 (LY) (5, 25 μM) and treated with or without COF (25 μg/ml) for 48 h. Whole cell lystates were prepared and used for IAP2 Western with respective antibody. Cell treated with DMSO (< 0.01 %) was used as the control for each experiment. Results are from three independent experiments. β-actin levels were used as a loading control.

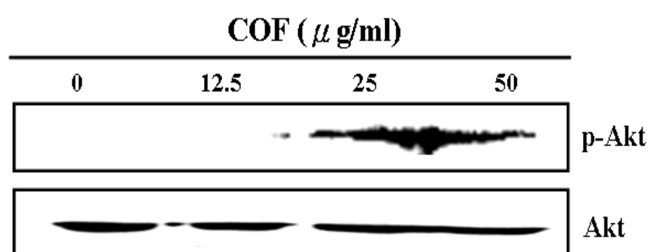


Fig. 24. Effect various concentrations of COF on A549 cells to investigate the p-Akt expression level. Akt phosphorylation was response to control, COF (0, 12.5, 25, 50 μg/ml) induction for 48 h and cell extracts (25 μg/lane) were analyzed by probing for Ser 473 phosphorylated Akt and total Akt levels, and determined by western blot. Cell treated with DMSO (< 0.01%) was used as the control for each experiment. Results are from three independent experiments. Total Akt levels were used as a loading control.

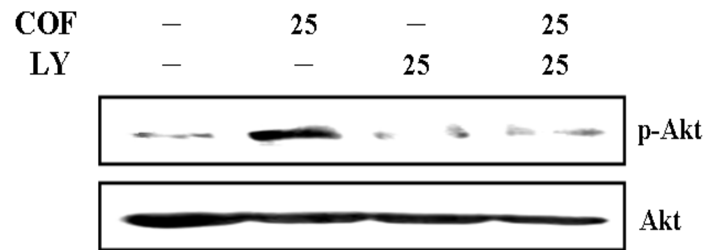
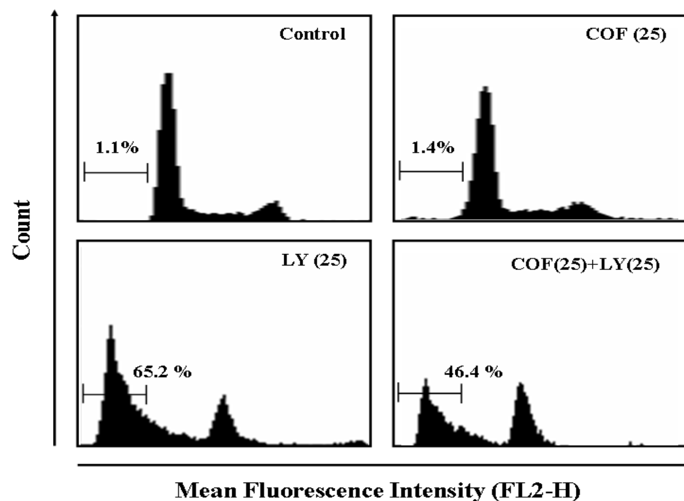


Fig. 25. Effect of LY294002 (LY) on cell survival in COF treated A549 cells. Responses of Akt phosphorylation by the PI3K inhibitor LY294002 in COF-induced A549 cells. Cells were pretreated with LY294002 (25 μM) for 1 hr before exposure to COF (25 μg/ml), and cell extracts (25 μg/lane) were analyzed by probing for Ser 473 phosphorylated Akt and total Akt levels, and determined by western blot. Cell treated with DMSO (< 0.01%) was used as the control for each experiment. Results are from three independent experiments. Total Akt levels were used as a loading control.



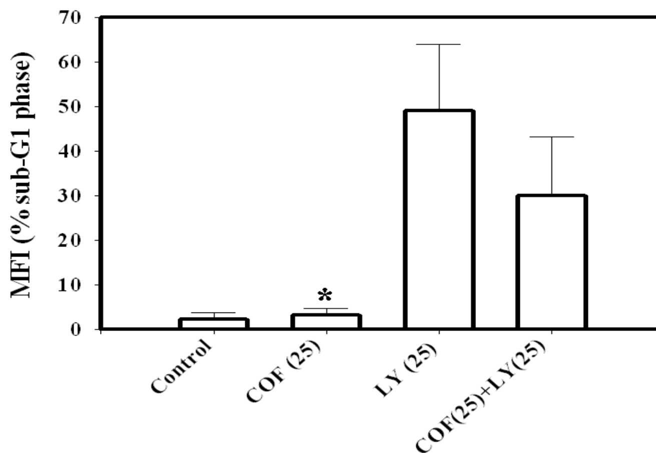
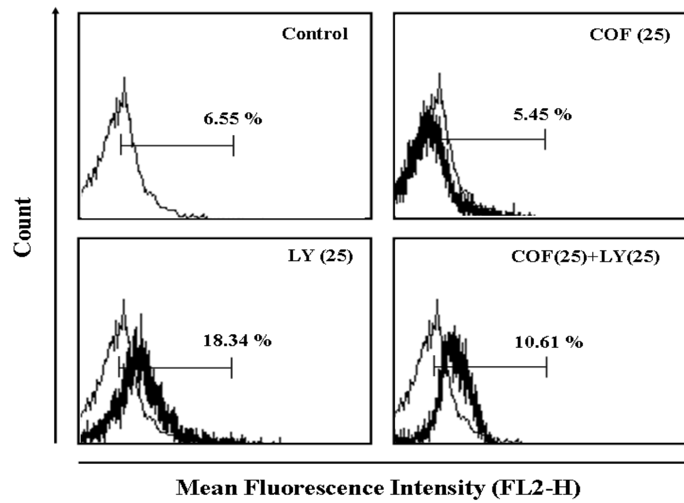


Fig. 26. COF mediated a prolongation of cell cycle progression in sub-G1 phase in A549 cells. Cells were co-treated with COF at concentration of 25 μg/ml and LY294002 (25 μM) after 48 h treatment. Distribution of A549 cells in the cell cycle analyzed by propidium iodide staining. The percentage of cells in sub-G1 phase was shown. A total of 10,000 cells were counted per sample and the data were processed using the CellQuest software (Becton Dickinson). A total of 10,000 cells were counted per sample and the data were processed using the percentage of each phase was displaying increased mean of fluorescence intensity (FL2-H) compare with control. Values are the mean S.D. for three independent experiments. * ($P < 0.05$; one-way ANOVA, Scheffe-test).



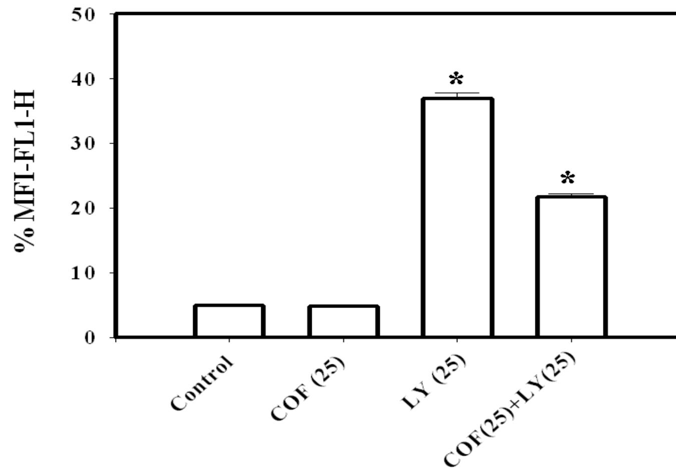


Fig. 27. LY294002 induced apoptotic effect in A549 cells after COF treatment for 48 h. Cells were treated with COF at concentration of 25 μg/ml and LY294002 (25 μM) after 48 h treatment. Distribution of A549 cells in the cell cycle analyzed by TUNEL staining. A total of 10,000 cells were counted per sample and the data were processed using the CellQuest software (Becton Dickinson). The percentage of each phase was displaying increased mean of fluorescence intensity (MFI：FLI-H) compare with control. Values are the mean S.D. for three independent experiments. * (P＜0.05; one-way ANOVA, Scheffe-test).

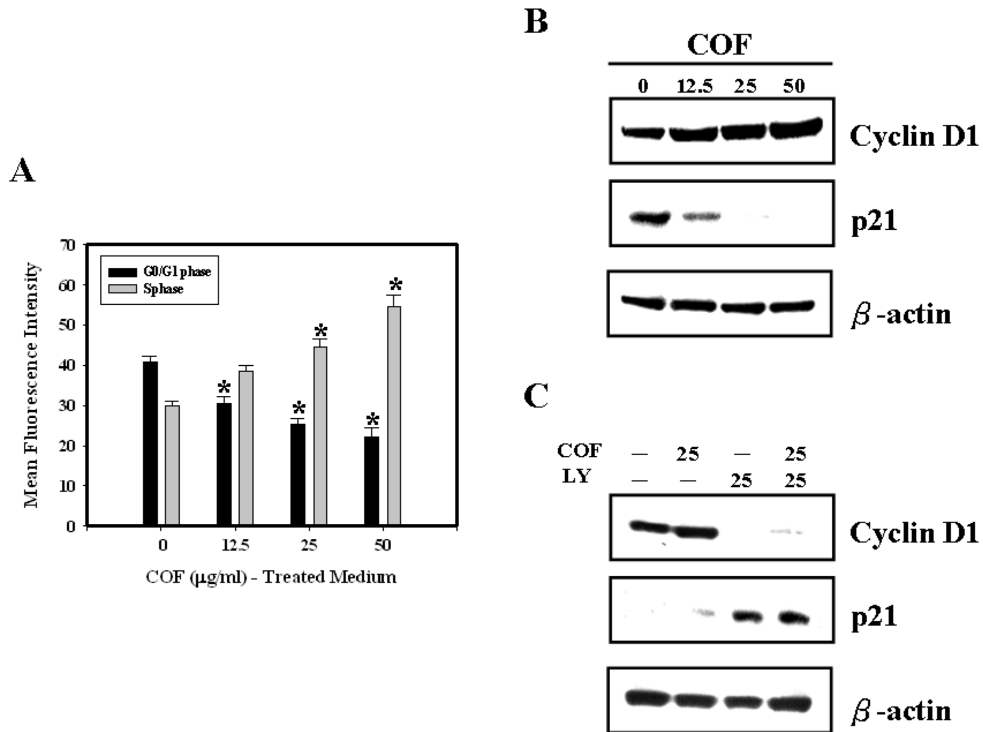


Fig. 28. Effect of LY294002 on A549 cells survival event with COF treated A549 cells. (A) Flow cytometry was analyzed for DNA content by propidium iodide staining on cells. Result was indicated by the percentage of sub-diploid DNA content (sub-G1) and was expressed as absolute level of fluorescence intensity (FL2-H). (B) Apoptosis event was assessed by TUNEL assay. Fluorescence-positive cells in TUNEL assay was detected with FACSCaliber cytometer. Percentage of apoptotic cells displaying increased fluorescence (FL1-H) compared with control is indicated above the marker bar in each panel are shown. Data were processed using the CellQuest software (Becton-Dickinson). Experiments are from three independent experiments. * ($P < 0.05$; one-way ANOVA, Scheffe-test).

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Association of cooking oil fumes exposure with lung cancer: Involvement of inhibitor of apoptosis proteins in cell survival and proliferation *in vitro*

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Abstract

Cooking oil fumes (COF) have been shown to be associated with lung cancer incidence in Chinese women. Our recent report indicates that inhibitor of apoptosis protein 2 (IAP2) induced by COF may contribute to the survival and proliferation of A549 lung cancer cells. In this study, to further verify whether other antiapoptosis proteins including IAP1, X-linked IAP (XIAP), and survivin, were linked with lung cancer cell survival and proliferation, these IAPs expressions in A549 cells after treatment with COF and its two major components, benzo[*a*]pyrene (BaP) and 2,4-decadienal (2,4-DDE) were evaluated by Western blotting. Our data showed that IAP2 was significantly induced by COF, BaP, and 2,4-DDE, but XIAP was decreased by COF and 2,4-DDE, but not by BaP. Even though different effects of COF and 2,4-DDE on IAP2 and XIAP protein expressions were observed, the caspase-3 expression was diminished by COF and 2,4-DDE. In addition, induction of IAP2 and phosphorylated Akt proteins by COF and 2,4-DDE were simultaneously abolished by LY294002. Flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis showed that the proportion of A549 cells at the S-phase was increased significantly after treatment with COF or 2,4-DDE. The cell proliferation induced by COF is associated with the attenuation of $p21^{Cip/Waf1}$ expression. Therefore, increases of IAP1, IAP2, survivin, and cyclin D1 expressions and decreases of XIAP, caspase-3, and p21 expressions might partly contribute to the survival and proliferation of lung cancer cells after exposure to 2,4-DDE and COF. In conclusion, the lung cancer cell growth promoted by COF might support previous epidemiological reports indicating that exposure of COF was associated with lung cancer development among Chinese women.
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Keywords: 2,4-DDE; Cooking oil fumes (COF); Inhibitor of apoptosis proteins (IAPs); Lung cancer; Cell survival and proliferation

1. Introduction

Since 1982, lung cancer has been the leading cause of cancer death in Taiwanese women. However, this phenomenon cannot be ascribed to cigarette smoking, because less than 10% of the Taiwanese women are smokers [1,2]. Cooking oil fumes (COF)

Abbreviations: COF, cooking oil fumes; BaP, benzo[*a*]pyrene; 2,4-DDE, 2,4-decadienal; IAPs, inhibitor of apoptosis proteins; XIAP, X-linked IAP; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

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has been shown to be associated with lung cancer incidence in Chinese women [3,4]. COF contains two major classes of compounds. One class consists of polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene, benzo[*b*]fluoranthene, fluoranthene, and benzo[*g*,*h*,*i*]perylene [5]. Compounds in the other class are aldehydes, such as formaldehyde, acetaldehyde, 1,3-butadiene, and 2,4-decadienal (2,4-DDE) [6,7]. 2,4-DDE has been shown to contribute to the genotoxicity of COF [8] and to induce proliferation and cytokine production in human bronchial epithelial cells, possibly through generation of reactive oxygen species (ROS) [9].

We have previously identified benzo[*a*]pyrene triol-N2-deoxyguanine as a major DNA adduct in lung cancer cells after exposure to COF [5]. Furthermore, this adduct had been identified to cause the hotspot mutation of the p53 gene in smoking lung cancer patients. We also reported that DNA damage induced by COF cannot be solely attributed to benzo[*a*]pyrene, indicating a role of oxidative reactions in COF-induced DNA damage [10]. The oxidative DNA damage and cycloxygenase-2 expression induced by COF through NF-κB can be concomitantly inhibited by quercetin, a naturally occurring flavonoid which was used as an ROS scavenger and antioxidant [10]. Our recent report indicated that two major components contained in COF, BaP and 2,4-DDE, had a similar effect in the induction of an inhibitor of apoptosis proteins (IAPs), IAP2, to contribute A549 lung cancer cell survival and proliferation mediated through NF-κB pathway [11].

The IAPs are defined by a domain of about 70 amino acids named as the baculovirus IAP repeat (BIR), which bind directly to caspases and are absolutely required in order for IAPs to suppress apoptosis [12–14]. In mammalian cells, the IAP family of proteins that have been identified include c-IAP1 (IAP1), c-IAP2 (IAP2), X-linked IAP (XIAP), neuronal apoptosis inhibitory protein (NAIP), survivin, BRUCE, and melanoma-associated IAP (ML-IAP) Livin [15]. Our recent report indicates that IAP2 induced by COF mediates lung cancer cell survival [11]. IAP2 is one of the IAPs that binds and inhibits caspases-3, 7, and/or 9, but not caspase-8 [16]. IAPs also modulate cell division, cell cycle progression, and signal transduction pathways. Depletion of survivin in human cells has been reported to cause apoptosis and pleiotropic defects in cell division [17]. A recent report suggests that XIAP interacts with checkpoint kinase 1 (Chk1), a protein that prevents progression of cell cycle into mitosis in the presence of DNA damage, and may modulate apoptosis during the DNA-damage activated G2/M checkpoint [18]. IAP1-overexpressing cells also exhibited cytokinesis defects over 10 times more often than control cells and displayed a mitotic checkpoint abnormality with production of polyploidy cells when exposed to microtubule-targeting drugs [19]. More interestingly, XIAP can activate the transcription factor NF-κB, a known survival factor for human endothelial cells and it can also inhibit cell proliferation via down-regulation of cyclins A and D1 and induction of the cyclin-dependent kinase inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ [20].

As an important regulator of cellular survival and proliferation, the phosphoinositide 3-kinase (PI3K) signaling pathway plays a central role in the development of various human cancers [21]. Notably, IAP2 up-regulation through the PI3K/Akt pathway was reported in human leukemia cells [22,23]. It is well supported that PI3K and its mediator Akt have an important function in tumorigenesis and the inhibitors of PI3K and/or Akt will be potentially useful as novel therapeutic agents in the treatment of lung cancer cells [24–26]. The inhibition of PI3K activity by LY294002 suppresses prostate cancer cell proliferation and induces G1 phase arrest which is accompanied by a decreased expression of G1-associated proteins including cyclin D1, CDK, and Rb. An exception to this is the cyclin kinase inhibitor, $p21^{Cip1/Waf1}$, which is increased [27]. Although IAP2 induction by COF has been shown to be related to A549 lung cancer cell survival and proliferation, the molecular mechanism of COF-induced lung cell proliferation is largely unclear.

Based on these recent information and findings, in this study we have now explored the mechanism of COF-induced lung cell survival and proliferation, using an *in vitro* cell culture model.

2. Materials and methods

2.1. Collection and preparation of COF

The detailed procedure for COF preparation has been described by Yang et al. [5]. Briefly, oil smog particulates from frying a pomfret were collected, filtered with high-purity glass filters (EPM 1000, Whatman), and extracted with acetone in a shaker. The acetone extracts, that is COF, were weighed and redissolved in the original solvent and stored at −80 °C.

2.2. Chemicals

Anti-IAP1, IAP2, XIAP, survivin, and p-Akt (ser 473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Anti-Akt polycolonal antibody was purchased from Cell Signaling Technology (Beverly, MA). RNase A, propidium iodide (PI), ethylene diaminetetraacetic acid (EDTA), Triton X-100, benzo[*a*]pyrene (BaP), *trans-trans*-2,4-

decadienal, 2-(4-morpholinyl)-8-phenyl-1-4H-benzopyran-4-one-hydrochloride (LY294002) were obtained from Sigma Chemical (St. Louis, MO); DeadEnd^{TM} fluorometric TUNEL system from Promega (Corp. Madison, WI); Cell culture medium, penicillin-streptomycin, and fetal bovine serum from GIBCO BRL (Eggenstein, Germany).

2.3. Cell culture

A549 cells were purchased from the American Type Culture Collection (ATCC). Parental cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, within a humidified atmosphere with 5% CO_2 at 37 °C.

2.4. Cell cycle analysis of COF-treated A549 cells

Distribution of the cells in the cell cycle was determined by propidium iodide (PI) staining [28]. In brief, 2×10^5 ml^{-1} cells were fixed with PBS containing 80% ethanol, then incubated at 4 °C in 0.2 ml PBS solution containing 0.05 mg/ml propidium iodide, 1 mM EDTA, 0.01% Triton X-100, and 1 mg/ml RNase A for 1 h. Analysis was performed with a FACSCaliber cytometer (Becton Dickinson). Cells with subdipolid DNA content were considered apoptotic cells. Cell cycle distributions were analyzed by CellQuest software.

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Analysis of apoptosis was performed by using TUNEL assay [28]. DNA fragmentation was assayed using the DeadEnd fluorometric TUNEL system (Promega). The assay was based on TdT-mediated dUTP nick end labeling. TdT catalyzes incorporation of fluorescein-dUTP at the free 3′-hydroxyl ends of fragmented DNA. Fluorescein-labeled DNA was detected via flow cytometry. Attached cells were dispersed with trypsin-EDTA, pelleted, washed, and fixed in 1% paraformaldehyde for 30 min on ice, and resuspended in 50 μl TdT incubation buffer containing a nucleotide mix. TdT-mediated incorporation of labeled dUTP into DNA fragments was performed by incubating the tube at 37 °C in the water bath for 60 min, terminating the reaction by adding 1 ml of 20 mM EDTA, and then analyzing by FACSCaliber cytometer (Becton Dickinson). Fluorescein-positive cells were processed using the CellQuest software.

2.6. Cell lysate preparation and Western blot analysis

Immunoblotting analysis was performed according to a previously described method [29]. Cells pellets were washed twice with PBS, resuspended with a sodium dodecyl sulfate (SDS) sample buffer (containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 40 mM Tris–HCl, pH 6.8), incubated at 95 °C for 5 min, and immediately stored on ice. The lysate was then centrifuged at 13,000 rpm for 10 min. The protein concentration in the supernatant was quantified by using a protein assay kit (Bio-Rad Labs, Hercules, CA), and 25 μg of each sample was subjected to SDS-PAGE. For immunoblotting, separated proteins were transferred onto a nitrocellulose membrane by a semi-dry blotting technique. The membrane was blocked in 5% non-fat dry milk with PBS for 1 h at room temperature before overnight incubation at 4 °C with the primary antibodies. Anti-actin antibody was used as a control to ensure for uniformity of loading. After incubation with the primary antibodies, the membrane was washed, incubated for 1 h with peroxidase-conjugated secondary antibodies, and then treated with the ECL Western blotting detection system (Amersham Life Science), according to the manufacturer's instructions.

2.7. Statistical analysis

Statistical analysis was performed by SPSS software (Version 10.0). Differences between mean values were determined by a one-way ANOVA followed by a Scheffe test. Probability values (p) <0.05 were considered to represent significant differences between group values.

3. Results

3.1. Different expression profiles of IAPs and caspases after treatment with COF, BaP, and 2,4-DDE

Our previous report has indicated that IAP2 protein induced by COF may contribute to the cell survival and proliferation of A549 lung cancer cells [11]. In this study, the other three IAP family proteins including IAP1, XIAP, and survivin expressions were evaluated by Western blotting after the cells were treated with various concentrations of COF, BaP, and 2,4-DDE to understand whether other anti-apoptotic proteins were involved in lung cancer cell survival and proliferation. As shown in Fig. 1A, IAP1, IAP2 and survivin protein expression levels were increased by COF and its two active components, BaP and 2,4-DDE, although survivin was not induced by BaP. Additionally, a similar dose-dependent IAP2 induction was observed among COF, BaP, and 2,4-DDE, but IAP2 induction was more remarkable than IAP1 induction. In contrast, XIAP expression level was significantly reduced by COF and 2,4-DDE, but not by BaP. To verify whether the different antiapoptotic protein expression profiles are induced by COF and 2,4-DDE, and are responsible for the differential reduction of caspase activity to cause anti-apoptosis, experiments were conducted and the results are summarized in Fig. 1B. These results revealed that anti-apoptosis response was still observed in lung cancer cells despite the reverse

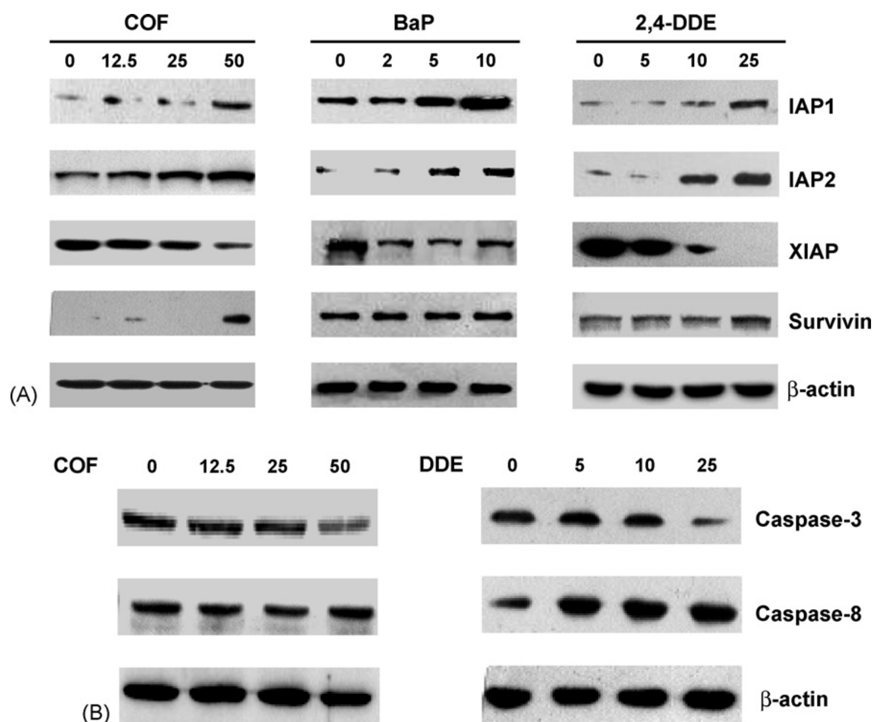


Fig. 1. Different expression profiles of IAPs in A549 cells after treatment of COF (μg/ml), benzo[*a*]pyrene (BaP) (μM), and 2,4-decadienal (2,4-DDE) (μM), respectively. Cells were incubated with various concentrations of COF, BaP, and 2,4-DDE for 48 h. Western blotting analysis of cell extracts (25 μg/lane) probed with the indicated primary antibodies (A) IAPs (IAP1, IAP2, XIAP, and survivin); (B) caspase-3 and caspapse-8 (cleaved form), the protein bands were detected by enhanced chemiluminescence. Equal amounts of protein were subjected for the immunoblotting and β-actin was used as a loading control. Cells treated with DMSO (<0.01%) were used as the solvent control for each experiment. All results are representative of one of three independent experiments.

effect of IAP2 and XIAP that occurred after a treatment with COF and 2,4-DDE. Based on the above results, the predominant effect of COF on IAP2 and XIAP was quite similar to that of 2,4-DDE, but different to that of BaP. Thus, the following studies were performed to understand whether 2,4-DDE was the active ingredient of COF involved in lung cancer cell survival and proliferation.

3.2. IAP2 induction by COF and 2,4-DDE through PI3K and NF-kB pathway

The induction of IAP2 was more prominent than that of other IAPs (Fig. 1). To verify which signaling pathways were involved in IAP2 induction by COF and its active ingredient 2,4-DDE, A549 cells were treated with specific inhibitor of four signaling pathways, SB203580 (p38 MAPK), PD98059 (ERK), BAY (NF-κB), and LY294002 (PI3K). As shown in Fig. 2A and B, IAP2 induction by COF and 2,4-DDE was significantly abolished by the addition of BAY (10 μM) and LY294002 (25 μM). However, IAP2 expression did not change by treatment with SB203580 (5 μM) or PD98059 (20 μM). Thus, both NF-κB and PI3K signaling pathways could be involved in IAP2 induction by COF and 2,4-DDE. These results were consistent with our previous finding that showed NF-κB pathway may be involved in IAP2 induction by COF [11]. Therefore, the following studies were performed to evaluate the role of PI3K on IAP2 induction by COF and 2,4-DDE.

3.3. The involvement of PI3K/Akt pathway in COF- and 2,4-DDE-induced cell survival

Accumulated evidence revealed the PI3K/Akt pathway was involved in the process of cancer cell survival. To explore if PI3K/Akt pathway was involved in COF- and 2,4-DDE-induced cell survival and proliferation, immunoblotting was performed with antibodies specific for serine 473 phosphorylation Akt (p-Akt), the active form of Akt. As shown in Fig. 3A, COF and 2,4-DDE at various concentrations could induce Akt phosphorylation without altering the Akt protein level. In addition, after the cells were concomitantly treated with LY294002, as shown in Fig. 3B, the increase of

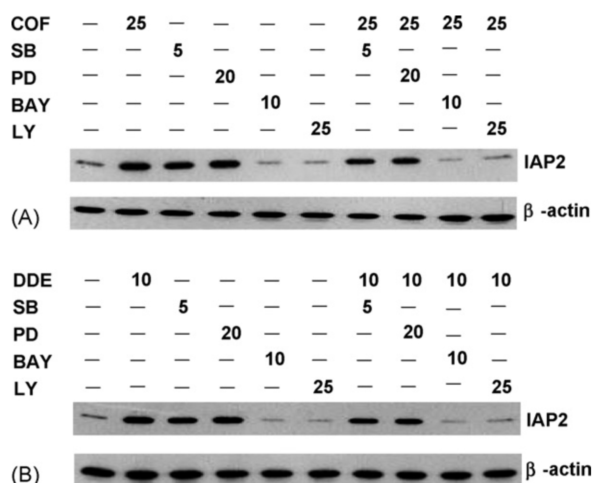


Fig. 2. IAP2 induction by COF and 2,4-DDE through PI3K and NF-κB pathway. A549 cells were pretreated with the indicated concentrations of SB203580 (SB) (5 μM), PD98059 (PD) (20 μM), BAY (10 μM) or LY294002 (LY) (25 μM) and treated with or without (A) COF (25 μg/ml) and (B) 2,4-DDE (10 μM) for 48 h. Whole cell lysates were prepared and probed with anti-IAP2 antibodies to detect IAP2 protein expressions. Cells treated with DMSO (<0.01%) were used as the control for each experiment. β-actin levels were used as a loading control. All results are representative of one of three independent experiments.

p-Akt expression caused by COF and 2,4-DDE was significantly decreased by LY294002. However, p-Akt induction by COF and 2,4-DDE could not be repressed by BAY (data not shown). These results showed that the PI3K/Akt pathway may play an important role in COF- and 2,4-DDE-induced cell survival.

To verify whether PI3K/Akt pathway is involved in cell survival and proliferation induced by COF and 2,4-DDE, flow cytometry analysis and TUNEL assay were employed to evaluate the proportions of apoptotic cells. The percentage of cells with sub-G1 DNA content was determined by PI staining followed by flow cytometry analysis after the cells were treated with COF +LY294002 or 2,4-DDE +LY294002. As shown in Fig. 4A, the proportion of apoptotic cells was changed to 46.4% (COF +LY294002) and 48.4% (2,4-DDE +LY294002) compared with 65.2% (LY294002 alone). TUNEL assay revealed that the proportion of apoptotic cells was reduced to 21.7% (COF +LY294002) and 22.1% (2,4-DDE +LY294002) compared with 36.9% (LY294002 alone) (Fig. 4B). These results indicate that the PI3K pathway may contribute to COF- and 2,4-DDE-induced resistance of apoptosis.

3.4. The effect of COF and 2,4-DDE on the cell cycle of A549 cells

The effect of COF and 2,4-DDE on the cell cycle of A549 cells was further investigated by flow cytometry. As shown in Fig. 5A, the proportion of A549 cells at S-phase was increased significantly from 38.6% to 54.4% by various concentrations of COF compared with that

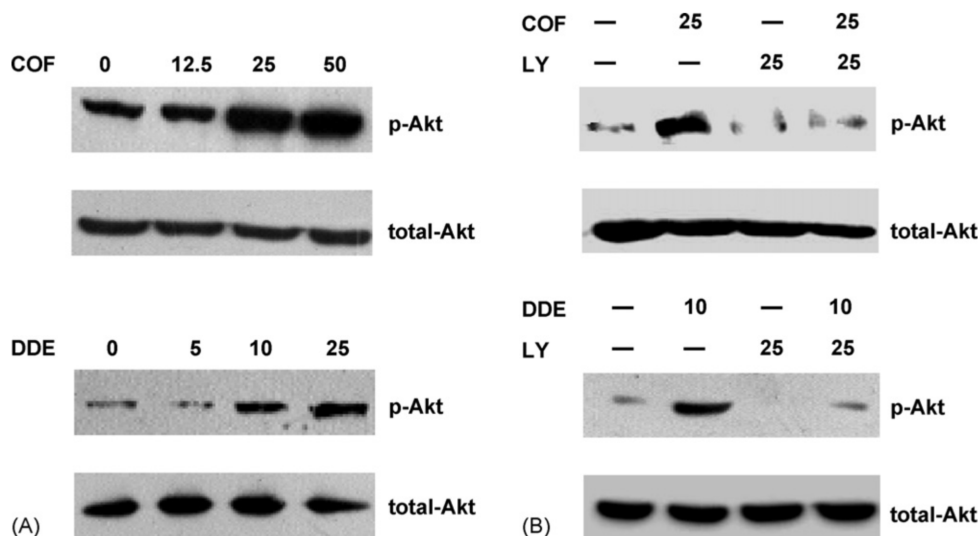


Fig. 3. The contribution of phosphorylated Akt to COF and 2,4-DDE induced cell survival. Western blotting analysis of cell extracts (25 μg/lane) probed with anti-Ser473 phosphorylated Akt and total Akt antibodies. (A) Akt phosphorylation responses to COF (0, 12.5, 25, 50 μg/ml) were detected after COF and 2,4-DDE treatment for 48 h. (B) Responses of Akt phosphorylation by the addition of LY294002 (PI3K inhibitor) in COF (25 μg/ml) and 2,4-DDE (10 μM) treated A549 cells were also detected. Cells were pretreated with LY294002 (25 μM) for 1 h before exposure to COF (25 μg/ml) and 2,4-DDE (10 μM). The protein bands were detected by enhanced chemiluminescence. Cells treated with DMSO (<0.01%) were used as the control for each experiment. Total Akt levels were used as the loading control. All results are representative of one of three independent experiments.

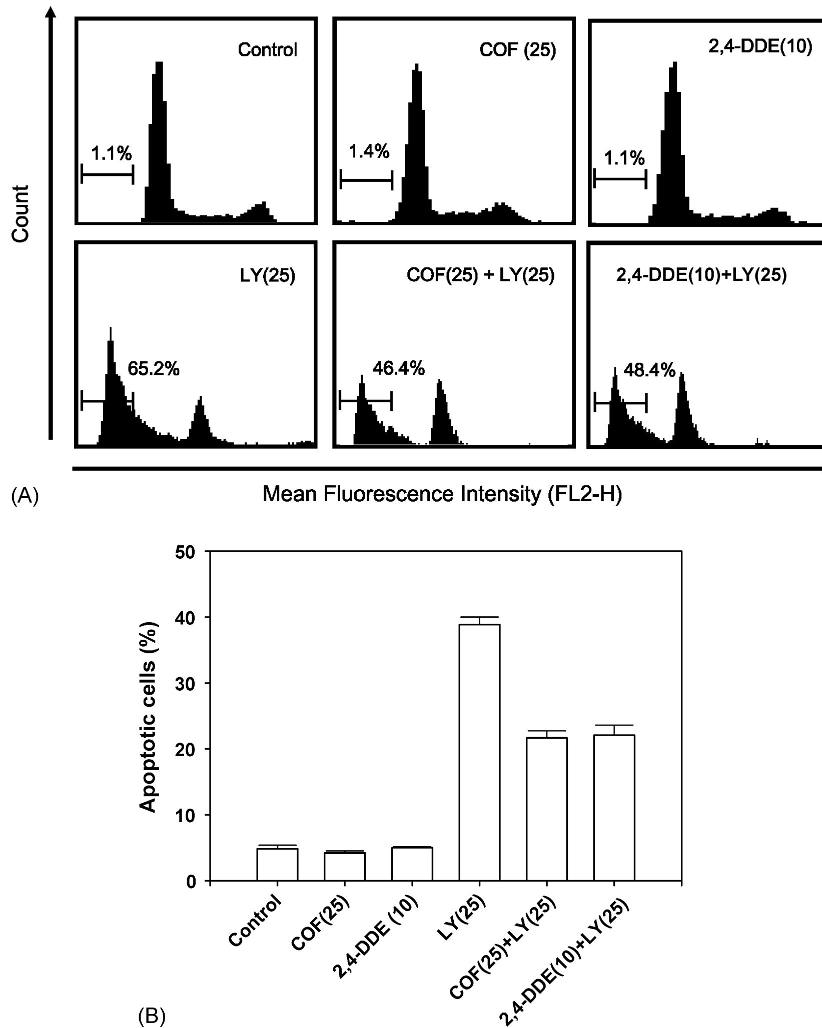


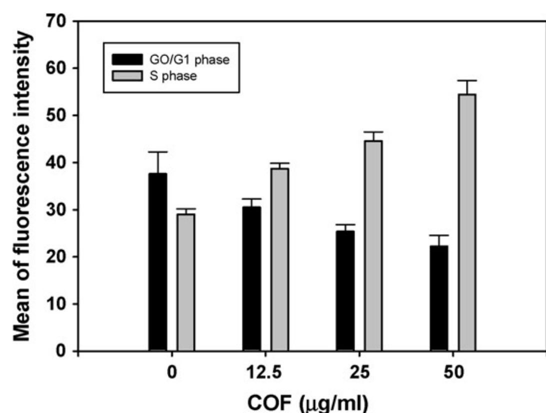
Fig. 4. The contribution of PI3K pathway on apoptosis resistance induced by COF and 2,4-DDE. Effect of LY294002 (LY) on cell survival in COF and 2,4-DDE treated A549 cells. (A) Flow cytometric analysis for DNA content with propidium iodide staining. The result is indicated by the percentage of sub-diploid DNA content (sub-G1 phase) and is expressed as the absolute level of fluorescence intensity (FL2-H). (B) An apoptosis event was assessed by TUNEL assay. Fluorescence-positive cells in the TUNEL assay were detected with a FACSCaliber cytometer. The percentage of apoptotic cells displaying increased fluorescence intensity compared with the control is indicated above the marker bar in each panel. Data were processed using CellQuest software (Becton-Dickinson). All results are representative of one of three independent experiments. The percentages of apoptotic A549 cells exposed to COF+LY and 2,4-DDE+LY group are statistically different from LY exposed group ($p < 0.05$).

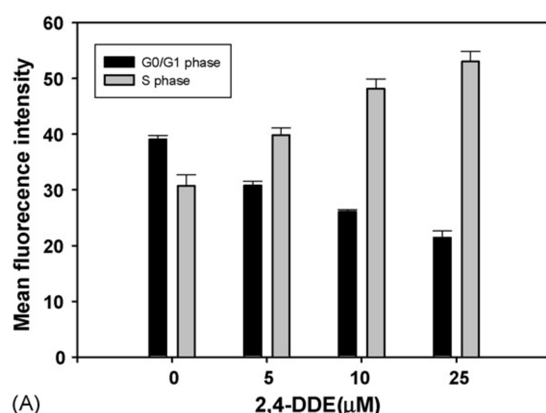
of untreated cells (29.1%). Meanwhile, the increased S-phase proportion of A549 cells was enhanced from 39.9% to 53.1% by a treatment with two different concentration levels of 2,4-DDE compared with that of untreated cells (31.7%). In contrast, the proportion of A549 cells at G0/G1-phase was decreased from 30.5% to 22.2% by COF (two levels), compared with that of untreated cells (40.9%), and cell proportion at G0/G1-phase was also decreased from 30.8% to 21.4% by 2,4-DDE compared with that of untreated cells (39.1%). Previous report has indicated that increased XIAP protein expression could induce cell cycle arrest through the reduction of cyclin D1 and activation of $p21^{Cip/Waf1}$ protein expressions [10]. As shown in Fig. 5B, cyclin D1 was induced in a dose-dependent manner after COF and 2,4-DDE exposure. Conversely, the $p21^{Cip/Waf1}$ protein was subsequently decreased by COF and 2,4-DDE treatment. To explore the mechanism by which the inhibition of PI3K activity induces cell cycle arrest at the G1 phase in A549 cells, the expressions of cyclin D1 and $p21^{Cip/Waf1}$ were evaluated after a co-treatment of COF and 2-4-DDE with LY294002, respectively. As shown in Fig. 5C, the induction of cyclin D1 by COF and 2,4-DDE was significantly reduced by a co-treatment with

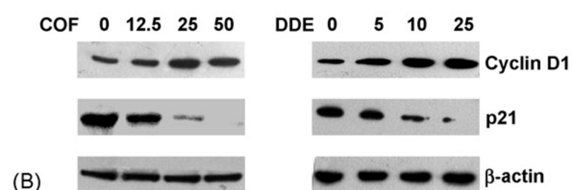
LY294002. In contrast, the reduction of p21$^{Cip/Waf1}$ by COF and 2,4-DDE was markedly increased by a co-treatment with LY294002. These results indicated that enhancement of cyclin D1 expression and attenuation of p21$^{Cip/Waf1}$ expression mediated through PI3K/Akt pathway may be involved in cell proliferation induced by COF and 2,4-DDE.

4. Discussion

In this study, three events were clearly observed after a treatment of the cells with COF and 2,4-DDE: (1) the reduction of XIAP and the induction of IAP2, (2) the decreases of caspase-3 and p21$^{Cip/Waf1}$, and the increase of cyclin D1, (3) the involvement of PI3K/Akt pathway in the cell survival and proliferation. Our previous report indicated that IAP2 could be induced by two major components of COF, 2,4-DDE and BaP, although the 2,4-DDE had a greater induction capability than BaP [11]. In addition, a similar reduction capability of XIAP expression was observed in the COF and 2,4-DDE treated group (Fig. 1A). Thus, the modulation of IAP2 and XIAP caused by 2,4-DDE were quite similar to those induced by COF. This result suggests that 2,4-DDE in COF may make a more important contribution than BaP to the cell survival and proliferation of A549 lung cancer cells. It is well known that apoptosis and necrosis represent two distinct types of cell death. Apoptosis possesses unique morphological and biochemical features which distinguish this mechanism of programmed cell death from necrosis. Extrinsic apoptotic cell death is receptor linked and initiates apoptosis by activating caspase-8. Intrinsic apoptotic cell death is mediated by the release of cytochrome *c* from mitochondria and initiates apoptosis by activating caspase-3 [30]. Studies have shown that IAPs binds and inhibits casapse-3, 7, and 9, but not caspase-8 [13,14]. Our results indicated that COF and 2,4-DDE could induce an anti-apoptotic effect, which may be mediated through the reduction of caspase-3 (Fig. 1B). It is well known that the NF-κB, a downstream effector regulated by Akt is known to modulate the intracellular signals. Previous reports showed that IAP2 was identified as target gene of transcriptional activity of NF-κB, and required to fully suppress TNF-induced







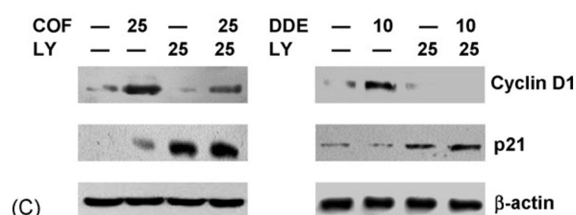


Fig. 5. The effect of COF and 2,4-DDE on the cell cycle of A549 cells. (A) Cell cycle analysis of COF and 2,4-DDE treated A549 cells. The graph shows the calculated percentage of the cell cycle for the G0/G1 and S phases. Cells were treated with various concentrations of COF (0, 12.5, 25, 50 μg/ml) and 2,4-DDE (0, 5, 10, 25 μM), respectively. In totality, 10,000 cells were counted per sample and the data were processed using CellQuest software (Becton Dickinson). The proportions of cells in the G0/G1 phase and S-phase were calculated, respectively. (B) Cyclin D and p21 expressions were detected after exposure to COF (0, 12.5, 25, 50 μg/ml) and 2,4-DDE (0, 5, 10, 25 μM) for 48 h. Cyclin D1 and p21 expressions with the addition of (C) LY294002 (PI3K inhibitor) in COF (25 μg/ml) and 2,4-DDE (10 μM) treated A549 cells were also detected. Cells were pretreated with LY294002 (25 μM) for 1 h before exposure to COF (25 μg/ml) and 2,4-DDE (10 μM). The protein bands were detected by enhanced chemiluminescence. Cells treated with DMSO (<0.01%) were used as the control for each experiment. β-Actin levels were used as the loading control. All results are representative of one of three independent experiments.

apoptosis in leukemia cell lines [31]. NF-κB activates IAP2 to suppress TNF-induced apoptosis and functions more distally to inhibit genotoxic agent-mediated apoptosis [32]. Our previous and present results showed that IAP2 induced by COF and 2,4-DDE could promote cell survival of A549 cells and the induction of IAP2 by COF and 2,4-DDE could be suppressed by BAY (Fig. 2) [11]. Additionally, COF could inhibit TNF-α expression in A549 cells (data not shown). Thus, we suggest that NF-κB could contribute to IAP2 induction by COF and 2,4-DDE to suppress TNF-induced apoptosis.

Cummins et al. indicated that cell survival was not affected by XIAP knock-out colorectal cancer cells compared to their parental cells [33]. XIAP-deficient mice are completely normal and have no detectable effects on apoptosis. In these mice, there is a compensatory up-regulation of IAP1 and IAP2, implying that XIAP may be redundant like other IAP family members [34]. Interestingly, overexpression of XIAP leads to down-regulation of cyclin A and D1 and up-regulation of two cyclin-dependent kinase inhibitors, $p21^{Cip1/Waf1}$ and $p27^{kip1}$ in endothelial cells [20]. Thus, XIAP seems to exhibit an anti-proliferation effect at the G1/S phase of the cell cycle. In this study, untreated A549 lung cancer cells had a higher XIAP expression than the other three IAPs. However, XIAP reduction could be a compensatory upregulation of IAP2 after COF and 2,4-DDE treatment. Thus, it is conceivable that XIAP reduction and IAP2 induction might contribute to COF- or 2,4-DDE-induced A549 cell survival and proliferation. Zou et al. indicated that polyamide depletion increased expression of IAP2 and XIAP by activating NF-κB in intestinal epithelial cells which could induce the resistance to TNF-α/cycloheximide-induced apoptosis through inhibition caspase-3 activity [35]. One report pointed out that the resistance to apoptosis in gastric cancer cells with elevated $p21^{Cip1/Waf1}$ is in a p38 MAPK- and ERK-mediated pathway with increased IAP2 expression and decreased caspase-3 activity and that this pathway is sensitive to the inhibition of NF-κB [36]. Huang et al. revealed that IAP2 functions as an ubiquitin-protein ligase and promotes *in vitro* monoubiquitation of caspase-3, and -7 [37]. In this study, we observed that caspase-3 activity was reduced by COF and 2,4-DDE treatment in a dose-dependent manner, but it had no effect on caspase-8 (Fig. 1B).

Lin et al. specified that the Mcl-1-dependent survival mechanism induced by Cox-2 expression, resulting in resistance to apoptosis in human lung adenocarcinoma CL1.0 cells mediated through the PI3K/Akt-dependent pathway [24]. The serine/threonine kinase Akt is a downstream effector of PI3K that is recognized as the major mediator of survival signals that protect cells from undergoing apoptosis [38,39]. A close relationship between the activation of the PI3K/Akt pathway and IAP2 expression of leukemia cells has been demonstrated [22,23].

The intracellular redox state may regulate hepatoma cell growth through Akt phosphorylation, resulting in a broad array of responses from cellular proliferation to apoptosis in various cancer cells [40,41]. It has also been known that *N*-acetyl-cysteine (NAC) is a scavenger of hydroxyl radical ($OH^{\bullet}$) [42]. Menadione has been shown to be an inhibitor of aldehyde oxidase. Previous report indicated that antiapoptosis through increased p-Akt level could be modulated by menadione in rat aortic smooth muscle cells [43]. The PI3K/Akt signal transduction cascade has been extensively investigated on oncogenic transformation. More evidence has indicated that PI3K/Akt pathway may be involved in cell cycle progression. There was a tight relationship between the activation of the PI3K/Akt1 axis and the expression of IAP1 and IAP2 proteins of the survival mechanism in leukemia cells [22,23]. Our preliminary data showed that NAC and menadione significantly decrease the p-Akt and IAP2 protein expressions and then cause apoptosis of A549 cells (data not shown). Thus, we suggest that $OH^{\bullet}$ might play an important role in COF- and 2,4-DDE-induced A549 cell survival and proliferation mediated through induction of p-Akt and IAP2 expressions.

The present study indicates that the induction of cyclin D1 and the decrease of $p21^{Cip/Waf1}$, which are mediated through PI3K/Akt pathway may contribute to the COF- and 2,4-DDE-induced lung cancer cell survival and proliferation (Figs. 3 and 5). Moreover, inhibition of cyclin D1 and IAP2 and induction of $p21^{Cip/Waf1}$ after LY294002 treatment suggests that PI3K/Akt might be involved in the regulation of G1/S checkpoint in A549 cells.

Cyclins and their associated cyclin-dependent kinases (CDKs) are the central machinery that control cell cycle progression. Failure of cell cycle arrest at the G_1-S transition can cause uncontrolled cellular proliferation. Up-regulation of the cyclin D1 protocogene is known to be important in the regulation of the cell cycle [44]. Jin et al. demonstrated that the expression of cyclin D1 with the loss of p16 function was associated with a shorter survival rate and the worst prognosis in non-small cell lung cancer (NSCLC) patients [45]. The CDK inhibitor $p21^{cip1/waf1}$ inhibits progression through the cell cycle via several mechanisms. It can inhibit the cyclin D/CDK4 and cyclin E/CDK2 complexes early in the G1 phase [46]. A previous study has indicated that XIAP can inhibit cell proliferation via down-regulation of cyclins A and D1 and induction of the cyclin-dependent kinase inhibitors $p21^{cip1/waf1}$ and $p27^{kip1}$ [20].

The increased cyclin D1 and decreased p21 observed in the present study might be at least in part mediated through the reduction of XIAP by COF and 2,4-DDE to cause cell survival and proliferation. This speculation needs to be addressed by further investigation to provide direct evidence. XIAP has been shown to restrain cell death in cancer cells through a mechanism initially involving only inhibition of the effectors caspase-3 and -7 [47]. However, this study showed that only capase-3 was reduced by COF and 2,4-DDE (Fig. 1B). The result was consistent with a previous report showing that higher expressions of XIAP correlate with lower tumor proliferation in lung tumors [48]. Moreover, higher expressions of XIAP may act as an independent positive prognostic factor for survival in radically resected NSCLC patients [41]. The unexpected inverse correlation between XIAP and tumor proliferation has implied a more complex role of XIAP in tumor biology.

In summary, increases of IAP1, IAP2, survivin, and cyclin D1 expressions and decreases of XIAP, caspase-3, and p21 expressions might partly contribute to lung cancer cell survival and proliferation after a treatment of 2,4-DDE, a component in COF. These results may be helpful to understand the possible mechanism in COF-associated lung cancer development among Chinese women living in Taiwan.

Acknowledgements

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COOKING OIL FUMES IMPROVE LUNG ADENOCARCINOMA CELL SURVIVAL THROUGH c-IAP2 INDUCTION

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Cooking oil fumes (COF) exposure was demonstrated to be associated with lung cancer development in Taiwanese nonsmoking women. Previous studies identified Cox-2 overexpression and oxidative DNA damage in lung adenocarcinoma cells after exposure to COF. Involvement of COF in lung tumorigenesis may be associated with cell survival, as well as proliferation of lung adenocarcinoma. To test this hypothesis, A549, a lung adenocarcinoma cell line, was used, and MTT assay data showed that the cell viability of A549 was significantly increased in a concentration-dependent manner by COF treatment for 48h. Flow cytometery results indicated that the proportion of A549 cell at S-phase was markedly increased after exposure of COF. To elucidate whether the antiapoptotic c-IAP2 (IAP2) was involved in COF-improved cell survival, IAP2 protein levels was determined by Western blot, and the results showed it was significantly induced by COF in a concentration-dependent manner. Moreover, the suppression of BAY, a nuclear factor (NF)-κB binding inhibitor, or the COF-induced IAP2 protein levels indicated that NF-κB activation by COF may partly be involved in IAP2 induction. These results showed that the positive impact of COF on cell survival and proliferation of A549 lung tumor cells may be through an induction of IAP2 overexpression.

Accumulated epidemiological data showed that cooking oil fumes (COF) exposure was associated with lung cancer incidence in Chinese women (Chen et al., 1990; Ko et al., 2000a, 2000b, 2000c; Gao et al., 1998; Zhong et al., 1999). Our previous reports indicated that benzo[a]pyrene 7,8-diol 9,10-epoxide *N*-2-dexyguansine (BPDE-N-2-dG) adduct was the major DNA adduct in human lung adenocarcinoma CL-3 cells after treatment with COF (Yang et al., 2000). The same adduct was found to induce p53 mutations at hot spots in smoking lung cancer patients (Hussain et al., 2001; Denissenko et al., 1996). However, a higher benzo[a]pyrene (BaP)-like DNA adduct levels seemed not to be correlated with a relatively lower p53 mutation frequency in female lung cancer patients in Taiwan (Y. C. Wang et al., 1998; Ko et al., 2000a, 2000b, 2000c). Thus, it was hypothesized that altered certain gene(s)

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other than p53 may be linked with the development of COF-associated lung cancer.

The inhibitors of antiapoptotic proteins (IAPs) are a family of antiapoptotic proteins that regulate both the mitochondria and death receptor pathway for anti-apoptosis (Guzey et al., 2002). The IAPs have been attributed to the conserved baculovirus IAP repeat domain, which is found in all members of this protein family (Deveraux & Reed, 1999). Some human IAPs, including XIAP, IAP1, and c-IAP2 (IAP2), were shown to (1) directly bind procaspase-9, (2) prevent its response to cytochromsome *c* and (3) also directly suppress the protease activity of the proteases caspase-3 and caspase-7 (Gorden et al., 2002; Hasegawa et al., 2003). Overexpression of IAPs was shown to suppress apoptosis induced by a variety of stimuli, including tumor necrosis factor (TNF), Fas, menadione, staurosporin, etoposide (VP16), taxol, and growth-factor withdrawal (Hasegawa et al., 2003). Previous reports indicated that this single BIR-containing IAP was highly expressed in many types of cancers, including colon (Wang et al., 2003), prostate (Krajewska et al., 2003), skin (Bush et al., 2001), breast (Ferguson et al., 2003), uterus (Frost et al., 2002), lymphoma (Chanhan et al., 2001), and lung cancers (Okouoyo et al., 2004). It has been reported that TNF upregulates human IAP1, also known as IAP2, through activation of nuclear factor-κB (NF-κB) in Jurkat cells (Swinney et al., 2002). Previous reports showed that IAP2 was identified as gene targets of NF-κB transcriptional activity and was required to fully suppress TNF-induced apoptosis in leukemia cell lines (Kreuz et al., 2001). NF-κB activates IAP2 to suppress TNF-induced apoptosis and functions more distally inhabit genotoxic agent-mediated apoptosis (Lee et al., 1999). Therefore, in this study, it was of interest to investigate whether (1) antiapoptotic IAP2 was induced by COF, (2) IAP2 induction was mediated through the NF-kB pathway, and (3) IAP2 was involved the survival and proliferation of lung adenocarcinoma A549 cells.

MATERIAL AND METHODS

Collection and Preparation of COF

The detailed procedure for COF preparation was described by Yang et al. (2000). Briefly, oil smog particulates from frying a promfret were collected, filtered with high-purity glass filters (EPM 1000, Whatman), and extracted with acetone in a shaker. The acetone extracts, that is, COF, were weighted and redissolved in the original solvent and stored at −80°C.

Chemicals and Reagents

An anti-cIAP2 polyclonal antibody (H-85, used at a dilution of 1:500), was purchased from Santa Cruz Biotechnology, Inc. Propidium iodide (PI), RNase A, benzo[a]pyrene (BaP), 24-DDE, tetrazolium salt 4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), and *E*-3, 4-*t*-butylphenylsulfonyl-2-propenentitrile (BAY) were purchased from Sigma Chemical (St. Louis, Mo).

DeadEnd fluorometric TUNEL system was purchased from Promega (Corp., Madison, WI). Cell culture medium, penicillin–streptomycin, and fetal bovine serum were purchased from GIBCO BRL (Eggenstein, Germany). All other chemicals and biochemicals were of the highest quality available from commercial sources.

Cell Culture

A549 cells were purchased from the American Type Culture Collection (ATCC). Parental cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS, GIBCO, BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin within a humidified atmosphere of 5% CO_2 at 37°C.

MTT Assay for Cell Viability

MTT assay was performed by following the procedures described by Luan and Diekwisch (2002). A549 cells were seeded into 96-well plated at a density of 1×10^5 cells. After a 48-h incubation, the MTT assay stock solution was added to a final concentration of 0.5 μg/ml. To control background absorbance, 8 wells of cells were lysed by the addition of Triton X-100 to a final concentration of 0.1% v/v immediately prior to the addition of MTT reagent. After a 3-h incubation, the insoluble product was dissolved in 100 μl of cell lysis buffer (20% w/v SDS, 50% v/v dimethyformamide, pH 4.7), and absorbance at 570 nm was measured using a microplate spectrophotometer (Bio-Rad). Cell viability was then calculated by the equation: Percent of cell viability = (absorbance of treated cultures – absorbance of background control)/(absorbance of control cultures – absorbance of background control) × 100.

Cell Cycle Analysis of COF-Treated A549 Cells

The assay was performed by propidium iodide-based flow cytometry (Erez et al., 2004) to examine the effect of various concentrations of COF (0, 12.5, 25, and 50 μg/ml) for 48 h. Cell cycle analysis by flow cytometery was performed after detaching cells from culture plates with trypsin, washing 2 times with phosphate-buffered saline (PBS), and fixing in 80% ethanol with PBS. After 30 min of incubation at 4°C, cells were centrifuged for 5 min at 3000 × g. The cell pellet was resuspended and incubated for 30 min in 0.05 mg/ml PI, 1 m*M* EDTA, 0.1% Triton X-100, and 1 mg/ml RNase A in PBS. The ratio of cells in the S and sub-G1 phases of cell cycle were determined by their DNA content then analyzed on a Becton Dickinson FACScan system.

Determination of Apoptotic Cells by TUNEL Assay

DNA fragmentation was assayed using the DeadEnd fluorometeric TUNEL system (Promega). The assay was based on TdT-mediated dUTP nick end labeling. TdT catalyzes incorporation of flourescein-dUTP at the free 3′-hydroxyl ends of fragmented DNA. Fluorescein-labeled DNA was detected via

flow cytometry. In brief, cells were incubated in 6-well plates in the presence or absence of cotreatment of BAY (10 μ*M*) and COF (25 μg/ml) for 48 h. Attached cells were dispersed with trypsin–ethylenediamine tetraacetic acid (EDTA), pelleted, washed, and fixed in 1% paraformaldehyde for 30 min on ice, and resuspended in 50 μl TdT incubation buffer containing a nucleotide mix. TdT-mediated incorporation of labeled dUTP into DNA fragments was performed by incubating the tube at 37°C in a water bath for 60 min, terminating the reaction by adding 1 ml of 20 m*M* EDTA, and then analyzing by flow cytometery.

Western Blot Analysis

Immunoblotting analyzing was performed according to previously described methods (Lee et al., 1996). Samples containing 20 μg of total protein were heated in a final volume of 20 μl with sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 40 m*M* Tris-HCl, pH # 6.8) for 5 min at 95°C and then chilled on ice. Supernatants were subjected to SDS–polyacrylamide gel electrophoresis (5% acrylamide stacking gel and 10% acrylamide separating gel). For immunoblotting, separated proteins were transferred onto a nitrocellulose membrane by semidry blotting technique. The membrane was blocked with 5% blocking reagent [5% (w/v) nonfat dry milk powder and 0.1% Tween 20 in PBS] and then probed with antibody preparations. Immunoreactive proteins were visualized by the enhanced chemiluminesence detection method. To ensure a quantitatively equal loading, blots were also probed with an antibody to β-actin.

Statistical Analysis

Statistical evaluations were performed by analysis of variance followed by Duncan's test for dose-response data, with $p < .05$ being considered to be statistically significant.

RESULTS

Cell Survival and Proliferation Promoted by COF

Our previous report indicated that Comet DNA damage was induced by COF in lung adenocarcinoma cells. In this study, whether COF affected A549 cell viability by MTT assay was further evaluated. Surprisingly, the cell viability of A549 was significantly increased in a concentration-dependent manner by COF treatment for 48 h (Figure 1). The effect of COF on the cell cycle of A549 cells was further investigated by flow cytometery. As shown in Figure 2, the proportion of S-phase of A549 cells was significantly increased from 48.45% to 61.48% by the treatment with COF, compared with 34.21% untreated cells. These results showed that cell survival and proliferation of A549 lung tumor cells were promoted by treatment with COF.

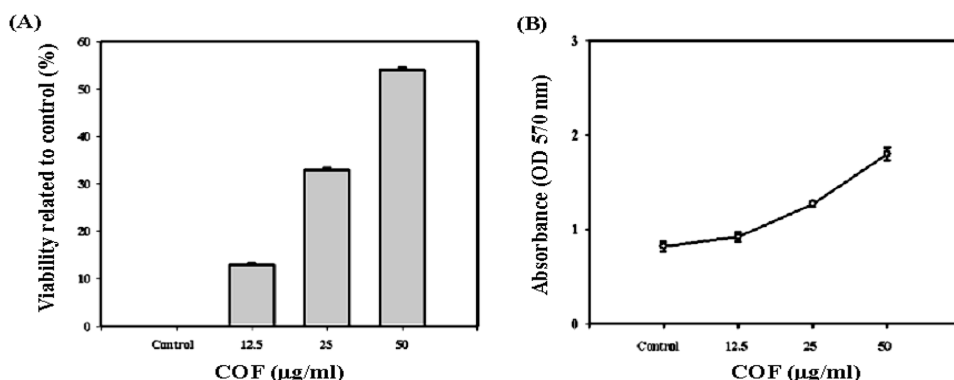

FIGURE 1. Effects of COF induction on cell viability. The graph shows changes in cell viability of A549 following a treatment with various concentrations of COF. Viable cells were detected based on mitochondria MTT metabolism and measured at 570 nm using a microplate reader (Bio-Rad). Cell viability was than calculated by the equation: Percent cell viability = (absorbance of treated culture – absorbance of background control)/(absorbance of control culture – absorbance of background control) × 100. (A) Viability related to control. (B) Concentration-dependent curve. Results are the mean of three different experiments ± SD.

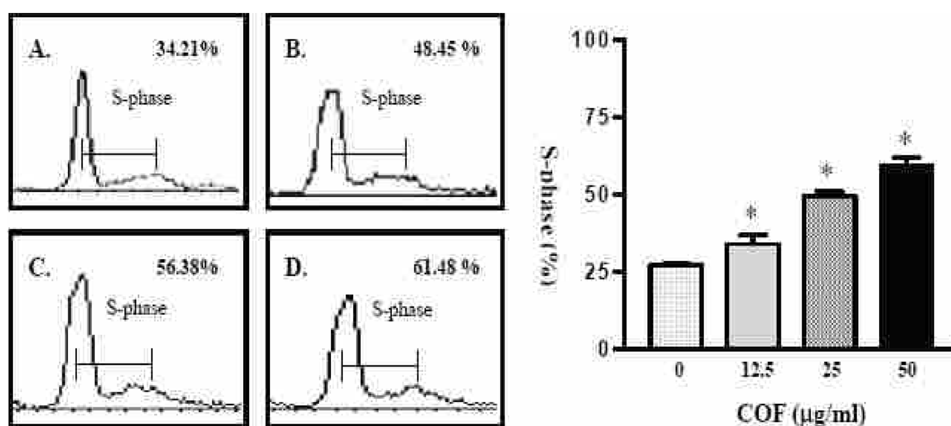

FIGURE 2. Effects of COF, inducing S-phase increase in A549 cell. The number inside each box indicates the calculated percent of cells in each S-phase. (A) Control, contains DMSO (<0.1%). (B–D) Cells were treated with COF (12.5, 25, 50 μg/ml, respectively. In total, 10,000 cells were counted per sample and the data were processed using the CELL QUEST software (Becton Dickinson). The proportions of cells in S-phase in (A)–(D) were 34.21%, 48.45%, 56.38%, and 61.48%, respectively. Values are the mean SD for three independent experiments. Asterisk indicates significance at $p < .05$ compared to control cells.

IAP2 Induction by COF and Its Two Major Components

It has been shown that the antiapoptotic effects of NF-κB are to enhance the production of IAPs, particularly IAP2. These proteins inhibit the activity of diverse caspases involved in the apoptotic signaling cascade. It was thus suspected that the

effect of COF on IAP2 overexpression may be due to the activation of the NF-κB pathway. To test this possibility, IAP2 protein levels were measured by Western blotting after an exposure to COF. Our data showed that IAP2 protein levels were markedly induced by COF in a concentration-dependent manner (Figure 3A). To verify which component of COF are involved in IAP2 induction, the capabilities to induce IAP2 of two major components of COF, BaP, and 2,4-DDE were evaluated. Our data showed that IAP2 protein expression was upregulated by both components, and the induction capability of 2,4-DDE may be more potent than that of BaP (Figure 3, B and C). These results indicated that IAP2 overexpression induced by COF and two major components of COF may contribute to the cell survival and proliferation of A549 cells after treatment with COF.

IAP2 Induction by COF Mediated Through NF-κB Pathway

Previous data indicated that Cox-2 protein was significantly induced by COF through the NF-κB signaling pathway. Thus, a site-specific NF-κB inhibitor (BAY) was used to verify whether IAP2 induction by COF was mediated through the NF-kB pathway. As shown in Figure 4, IAP2 protein levels were decreased when the cells were cotreated with COF and BAY. This result

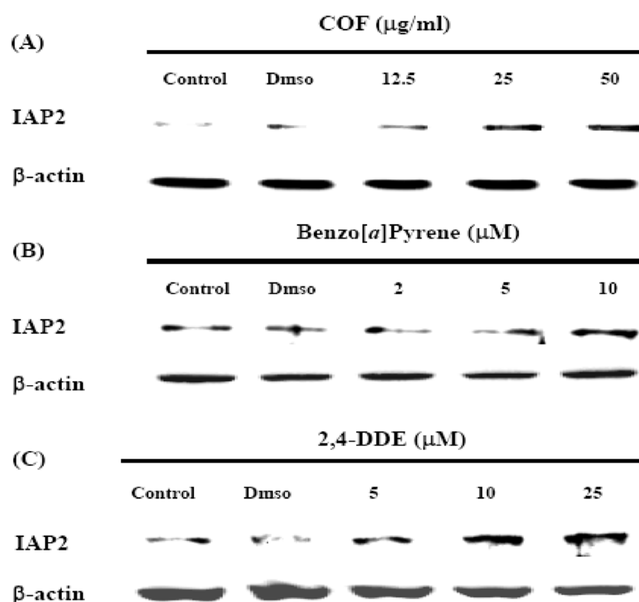

FIGURE 3. Immunoblot analysis for IAP2 expression in A549 cells with COF, benzo[a]pyrene, and 2,4-DDE treatment. After cells were treated with various doses of COF (0, 12.5, 25, 50 μg/ml), benzo[a]pyrene (0, 2, 5, 10 μ*M*), and 2,4-DDE (0, 5, 10, 25 μ*M*) for 48 h, cell extracts were prepared and a prepared sample with 20 μg total protein was load in each lane, resolved on a 10% SDS-PAGE gel, and probed with a 1:500 dilution of anti-IAP2 antibody. DMSO (<0.1%) was used as a solvent control. Data are presented as means of three experiments.

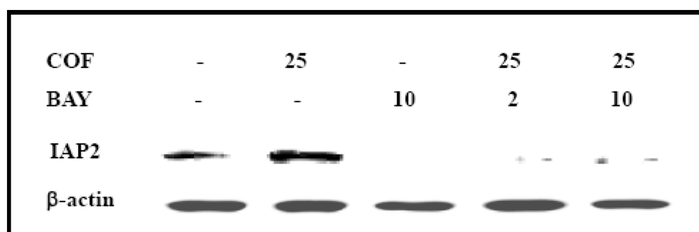

FIGURE 4. Effects of BAY on expression of IAP2 in A549 cells stimulate by COF. A549 cells were preincubated with BAY (2 or 10 μ*M*) for 1 h, and thereafter cultivated in the presence of COF (25 μg/ml) for 48 h. DMSO (<0.1%) was used as a solvent control. Data are presented as means of three experiments.

supported the previous studies showing that NF-kB activation was at least in part involved in IAP2 induction by COF.

The Inhibitory Effect of BAY on COF-Induced NF-κB-Dependent Antiapoptosis and Cell Survival

To examine whether BAY could inhibit anitapoptosis and cell survival induced by COF, TUNEL assay and cell cycle analysis were performed to confirm the results already described showing that COF-induced antiapoptosis and cell survival were partly through the NF-κB signaling pathway. As shown in Figure 5, A and B, after cotreatment with COF (25 μg/ml) and BAY (10 μ*M*), the apoptotic A549 cells were significantly decreased to 18.34% as compared with BAY treatment alone (30.69%). Additionally, the proportion of sub-G1

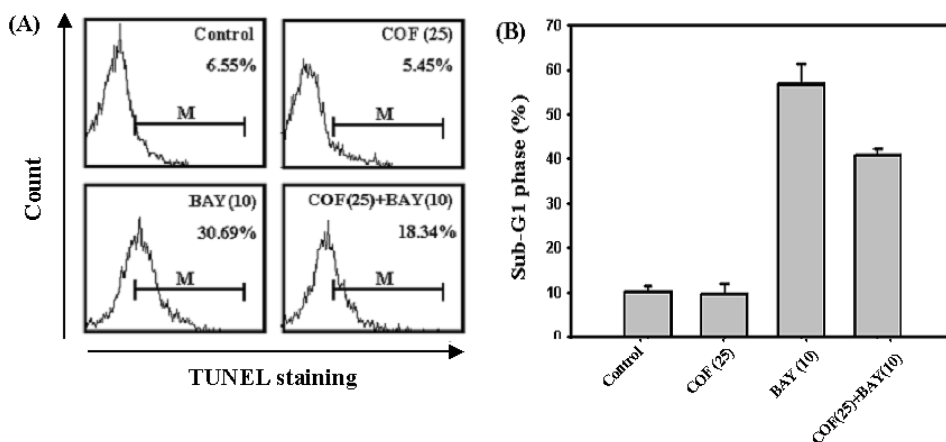

FIGURE 5. BAY-induced apoptosis on A549 cells. Cells were pre-treated with BAY (10 μ*M*) for 1 h and then treated with COF at a concentration of 25 μg/ml for 48 h as specified in figures. (A) Fluorescein-positive cells were analyzed by TUNEL assay and detected through flow cytometery. (B) Q BAY treatment resulted in an increase in the cell portion at sub-G1 phase. The proportion of cells at sub-G1 phase was determined by PI staining. Results were reproduced from three independent experiments.

phase cells was reduced from 58.36% (BAY treatment alone) to 42.26% (BAY + COF cotreatment). These results suggested that the NF-kB signaling pathway may play a role in COF-induced antiapoptosis and cell survival of A549 cells.

DISCUSSION

NF-κB has been reported to be a primary regulator of the response to various stresses including DNA damage (Yamamoto & Gaynor, 2004). Our previous report demonstrated that BPDE-N2-dG was the major DNA adduct in lung adenocarcinoma CL-3 cells after an exposure to COF (Yang et al., 2000). In addition, oxidative DNA damage induced by COF, compared to that of bulky BaP-like DNA adducts, may contribute more to DNA damage, evaluated by Comet assay in CL-3 cells. Moreover, NF-κB activated by COF was responsible for Cox-2 expression (Lin et al., 2002). Our preliminary data showed that IAP1, IAP2, and survival in 4 different cell lines, A549, CL-3, H23, and H1355, were unregulated by a 48-h treatment with COF; however, only IAP2 was gradually induced in a concentration-dependent manner (data not shown). These results led us to investigate the role of IAP2 in cell survival and proliferation of A549 cells. In this study, it was revealed that NF-κB activation by COF may be responsible for IAP2 induction in A549 cells. In fact, a similar IAP2 induction by COF was also observed in another lung cancer cell line, CL-3 cells (data not shown). Based no previous and present studies, it may be concluded that NF-kB activation by COF and successive IAP2 expression may contribute to the cell survival and proliferation of A549 cells.

Our previous data indicated that no cytotoxicity was observed for a 1- to 4-h exposure to COF, up to 50 μg/ml, for CL-3 and A549 cells; therefore, the concentration range of COF used in this study was determined to be 12.5–50 μg/ml. For cell proliferation, the exposure time of COF was extended to 48 h. Our data showed that S-phase cell portion was significantly increased after exposure of COF. This observation was similar to the finding in A549 cells exposed to gemcitabine (Bandala et al., 2001). In addition, overexpression of IAP1 in A549 cells after exposed to gemicitabine was consistent with the activation of NF-κB. A similar result also indicated that IAP2 was induced by ionizing radiation in A549 cells through NF-κB binding sites (Ueda et al., 2001). In fact, IAP1, similar to IAP2, was induced by COF in A549 cells (data not shown). In the present study, IAP2 protein levels induced by COF were markedly suppressed by the addition of BAY, an inhibitor of the NF-κB binding site. This result suggested that NF-κB activation by COF was responsible for IAP2 induction. Recent studies in various cell types suggested that IAP2 induction was one of the NF-κB-mediated anti-apoptotic function and blocked the activation of caspase-8 (C. Y. Wang et al., 1998; Chen et al., 2003). In this study, BAY indeed abrogated the cell survival and proliferation induced by COF (Figure 5).

Endogenous reactive oxygen species (ROS) are generated in cells by reducing antioxidants, or directly induced by oxidants/oxidases. A link bet

ween intracellular redox balance and growth control has long been implied (Dong et al., 2003). Our previous report indicated that ROS generated from COF exposure may be involved in NF-κB activation based on the addition of ROS scavengers to suppress NF-κB activation and Cox-2 protein expression levels in CL-3 cells (Lin et al., 2002). Thus, it is conceivable that ROS generated from COF in lung tumor cells may at least be in part involved in IAP2 induction.

The evidence on cell growth indicated that the serine-threonine kinase Akt/protein kinase B (PKB) is a critical enzyme in a cell survival pathway (Zhang et al., 2004). Akt-activated phosphorylation has been shown to protect cells from apoptosis that is induced by external stimuli in different cell types. The Akt pathway has been shown to play an important role in the regulation of cell growth (Woo et al., 2003). The phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin inhibited Akt phosphorylation induced by ROS (Dong et al., 2003). The hepatoma cell growth is ROS dependent, and fluctuation of the intracellular redox state may regulate hepatoma cell growth through Akt phosphorylation and the PI3K/Akt pathway, resulting in a broad array of responses from cellular proliferation to apoptosis (Suliman et al., 2003; Lee et al., 2003). Our preliminary data showed that the PI3K/Akt pathway may play an important role in IAP2 by COF in A549 cells. Whether the induction of COF directly targeted the PI3K/Akt pathway by ROS or secondarily mediated it by cytokines such as interleukin (Il)-6 and tumor necrosis factor (TNF)-alpha, or others is being investigated.

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