行政院國家科學委員會補助專題研究計畫成果報告

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※Wogonin 與 Fisetin 誘導 HL-60 血癌細胞凋亡機制探討※
※:Caspase 與 endonuclease 活化角色之研究 ※

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中文摘要

本計劃探討 wogonin 與 fisein 誘導血癌細胞 HL-60 凋亡之機轉。研究結果證實 wogonin 與 fisein 能有效誘導血癌細胞 HL-60 走向細胞凋亡，其過程會伴隨著 DNA 階梯斷裂、凋亡小體之出現與細胞週期不正常變化。我們同時也發現 caspase 3 (not caspase 1)被活化。PARP 被切斷與 endonuclease 活化。此 endonuclease 為 Ca+2 depedent 且其適合之活性 pH 值為 6.5。以 caspase3 抑制劑處理能抑制 wogonin 與 fisein 誘導血癌細胞 HL-60 凋亡與 endonuclease 活化。此結果顯示 caspase 3 與 endonuclease 在 wogonin 與 fisein 作用機轉中很重要。在探討 Bcl-2 family proteins 表現方面，wogonin 與 fisein 誘導 Bax 蛋白質產生同時抑制 MCl-1 蛋白質表現。NAC 或 catalase 能有效抑制 H2O2 誘導細胞凋亡，然而無法有效抑制 wogonin 與 fisein 誘導之細胞凋亡，推測自由基產生不包含在其作用機轉。以 DCHF-DA 偵測細胞內 peroxide 量之變化，結果指出 wogonin 與 fisein 能明顯減少細胞內之 peroxide 量。本結果證實 wogonin 與 fisein 能經由活化 caspase 3 與 endonuclease 而殺死血癌細胞，且此活性與降低細胞內 ROS 量有相關。（accepted by Biochemical Pharmacology）

Abstract

Seven structurally related flavonoids including luteolin, nobiletin, wogonin, baicalein, apigenin, myricetin and fisetin were used to study their biological activities on the human leukemia cell line, HL-60. On MTT assay, wogonin, baicalein, apigenin, myricetin and fisetin showed obvious cytotoxic effects on HL-60 cells, with wogonin and fisetin being the most-potent apoptotic inducers among them. The cytotoxic effects of wogonin and fisetin were accompanied by the dose- and time-dependent appearance of characteristics of apoptosis including DNA fragmentation, apoptotic bodies and the sub-G1 ratio. Treatment with an apoptosis-inducing concentration of wogonin or fisetin causes rapid and transient induction of caspase 3/CPP32 activity, but not caspase 1 activity. Further, cleavage of poly(ADP-ribose) polymerase (PARP) and decrease of pro-caspase 3 protein were detected in wogonin and fisetin treated HL-60 cells. An increase in the pro-apoptotic protein, bax, and a decrease in the anti-apoptotic protein, Mcl-1, were detected in fisetin- and wogonin- treated HL-60 cells. However, Bcl-2, Bcl-XL, and Bad all remained unchanged in wogonin- and fisetin-treated HL-60 cells. In vitro chromatin digestion revealed that endonuclease activity was profoundly enhanced in wogonin- and fisetin-treated HL-60 cells, and the addition of EDTA or EGTA into the reaction blocked endonuclease activation and at an optimum pH of 7.5. The caspase 3 inhibitor, Ac-DEVD-CHO, but not the caspase 1 inhibitor, Ac-YVAD-CHO, attenuated wogonin- and fisetin- induced DNA ladders, PARP cleavage, and endonuclease activation. Pre-treatment of HL-60 cells with N-acetyl-cysteine or catalase efficiently inhibited H2O2 (200 μM)-induced apoptosis, but showed no inhibitory effect on wogonin- and fisetin-induced DNA ladders, caspase 3 activation, or bax protein induction. Decrease in endogenous ROS production was detected in wogonin- and fisetin-treated HL-60 cells by DCHF-DA assay. In conclusion, our experiments indicate that a decrease in intracellular peroxide level was involved in wogonin- and fisetin-induced apoptosis; activation of caspase 3 and endonuclease, induction of bax protein and suppression of Mcl-1 protein were detected in the process.

Keywords: flavonoids, apoptosis, caspase 3, endonuclease, PARP, Bax, Mcl-1

緣由與目的

Polyphenolic compounds are widely distributed in plants and are common components of human diets [1-3]. Flavonoids are one type of polyphenols including flavones, isoflavones, and flavanones, and some of them have been found to possess antilipoperoxidant, antitumoral and antiplatelet and anti-inflammatory activities [4-6]. Several recent studies demonstrated that flavonoids are able to inhibit the activities of several enzymes including lipoxygenase, cyclooxygenase and xanthine monooxygenase [7-9]. In contrast to the beneficial effects, flavonoids have also been found to be mutagenic. These harmful effects were suspected to

result from the prooxidant rather than the antioxidant action of the related flavonoids [10-11]. Therefore, the biological and pharmacological effects of a flavonoid compound may dependent upon its behavior as either an antioxidant or a proxidant. However, the differential biological effects of proxidant and antioxidant of flavonoids are still undefined.

Two distinct modes of cell death, termed apoptosis and necrosis, have been recognized. Morphological and biochemical studies have shown the differences between these two forms of cell death [12-13]. Apoptosis is characterized by cell shrinkage, blebbing of the plasma membrane, and chromatin condensation that are associated with DNA cleavage into ladders [14, 15]. Previous studies indicated that cells from a variety of human malignancies have a decreased ability to undergo apoptosis in response to some physiological stimuli [16-18]. Therefore, developing various kinds of effective agents that can enhance the extent of apoptosis might be a promising strategy in the treatment of cancer. Apoptosis signal transduction and execution require the action of the cascade of caspases [19, 20]. Human caspases-1 to –10 have been described, and several previous study has demonstrated that activation of caspase cascade is involved in chemicals- and agents-induced apoptosis [21]. Recent studies have shown that Apaf-1, a homolog of CED-4, binds to initiator caspase 9 and causes caspase 9 activation through inducing its oligomerization [22, 23]. Activated caspase 9 in turn cleaves and activates executioner caspase 3 [24]. Caspase 3 usually exists in the cytoplasm as an inactive pro-caspase 3 that becomes proteolytically activated by multiple cleavages of its precursor 32 kDa to generate the 20/11 or 17/11 kDa active forms in cells undergoing apoptosis [25]. Downstream of caspase 3 activation, some specific substrates are cleaved that are important for the occurrence of apoptosis such as PARP, actin, and Bid protein. PARP is required for the DNA repair and activated caspase 3 cleaves PARP at Asp 216 to generate the 85 kDa and 31 kDa apoptotic fragments in coordination with DNA fragmentation during apoptosis [26].

Digestion of nuclear DNA into a nucleosomal ladder is an important biochemical hallmark of apoptosis. Several previous studies characterized the endonucleases including DNase I, DNase II, Ca^{+2}/Mg^{+2}-dependent endonuclease and Ca^{+2}/Mn^{+2}-dependent endonuclease that are responsible for the DNA fragmentation in apoptosis [27, 28]. In our previous study, we demonstrated that activation of Ca^{+2}-dependent endonuclease is involved in curcumin-induced apoptosis [29]. These data indicated that endonuclease activation is important in the process of apoptosis. However, the mechanisms of activation of apoptotic endonuclease and the key role of the caspase cascade played in the endonulcease activation remain unclear.

Human myelogenous leukemia HL-60 cells are very sensitive to apoptosis in the presence various stimuli. The results of the present study show that wogonin and fisetin exhibit significant apoptosis inducing activities in HL-60 cells among seven structurally related flavonoids tested as demonstrated by morphology, DNA fragmentation and flow cytometry assay. Decrease of intracellular ROS, activation of endonuclease, and caspase 3 activity, decrease in the Mcl-1 protein, and increase in the bax protein were associated with the apoptotsis induced by wogonin and fisetin.

結果與討論

Induction of Apoptosis by Wogonin and Fisetin

Flavonoids are diphenylpropanes that commonly occur in plants, and more than 4000 flavonoids have been found and are frequently components of the human diet. However, several biological activities of flavonoids are still undefined. To obtain information on whether flavonoids exert inhibitory effects on human leukemia cells, seven structurally related compounds including luteolin, nobiletin, wogonin, baicalein, apigenin, myricetin, and fisetin were first used to study their biological activities here. The chemical structures of the seven flavonoids used in the present study are shown. 1, and the effects of these compounds on the viability of cells were investigated by MTT assay. When HL-60 cells were treated with various concentrations of each indicated compound (20, 40, and 80 μM) for 12 h, significant

concentration-dependent inhibition on the viability of HL-60 cells was detected in the presence of wogonin, baicalein, apigenin, myricetin, and fisetin, but this was not obvious in luteolin- and nobiletin-treated cells. Among them, wogonin and fisetin showed the most-potent cytotoxicity on HL-60 cells. DMSO, even at the highest dose of 0.5%, showed no effect on cellular viability of HL-60 cells. To characterize cell death induced by flavonoids, the integrity of genomic DNA, occurrence of apoptotic bodies, and the ratio of sub-G1 peak were examined. On analysis of DNA integrity by agarose electrophoresis, the results show that addition of luteolin, nobiletin, wogonin, baicalein, apigenin, myricetin and fisetin caused the digestion of genomic DNA into ladders in dose-dependent manners, associated with a decrease in intact DNA. However, luteolin and nobiletin, only at 80 μM, produced few DNA ladders in this assay. In the same part of the experiment, apoptotic bodies by microscopic observation and increase of sub-G1 ratio were detected in wogonin- and fisetin-treated HL-60 cells. No obvious DNA ladders, apoptotic bodies, or sub-G1 peak were detected in DMSO-treated HL-60 cells (data not shown).

Stimulation of Caspase 3-like Activities, not Caspase 1-like Activities, in Wogonin and Fisetin-induced Apoptosis

Caspases are believed to play a central role in mediating various apoptotic responses and are activated in a sequential cascade of cleavages from their inactive forms. After activation, caspases can cleave their substrates at specific sites such as DXXD↓X for caspase 3 and YXXD↓X for caspase 1. To detect the enzymatic activity of caspases during wogonin- and fisetin-induced apoptosis, two colorimetric substrates, Ac-DEVA-pNA for caspase 3-related activities and Ac-YVAD-pNA for caspase 1 related activities, were used in this study. As illustrated, wogonin and fisetin induced a dramatic increase in DEVD-specific caspase activity in treated HL-60 cells. Adding the caspase 3 inhibitory peptide, Ac-DEVD-CHO, into the reaction mixture containing cell lysates from 80 μM wogonin-treated HL-60 cells inhibited the increase of caspase 3 like activities. In contrast to the increase in DEVD-specific activity, no significant induction of YVAD-specific activity was observed. Thus, induction of caspase 3-like activity might be involved in wogonin- and fisetin- induced apoptosis.

Involvement of PARP Degradation, Caspase 3 Activation, and Bcl-2 Family Proteins in Wogonin and Fisetin induced Apoptosis

Activation of caspase 3 leads to the cleavage of a number of proteins, one of which is PARP, another hallmark of apoptosis. Figure 5 shows that exposure of HL-60 cells to wogonin and fisetin caused the degradation of 116-kDa PARP into 85-kDa fragments in a concentration-dependent manner, associated with the activation of caspase 3 brought about by its cleavage, represented here as a decline in its pro-level on the Western blot. Bcl-2 family proteins act as important regulators in apoptosis and are located at the upstream of caspase activation. In wogonin- and fisetin-treated HL-60 cells, decrease in the Mcl-1 protein and increase in the bax protein were detected in dose dependent manners. In contrast to the expression of bax and Mcl-1 proteins, Bcl-2, Bcl-XL, and Bad proteins remained unchanged in HL-60 cells treated with or without different concentrations of wogonin and fisetin.

Activation of Endonuclease Activity in Wogonin and Fisetin-treated HL-60 Cells.

Internucleosomal DNA fragmentation is thought to be a result of activation of endogenous endonuclease. In vitro chromatin digestion provides a sensitive way to analyze the activity of apoptotic endonuclease. Incubation of the isolated nuclei with cell lysates from untreated HL-60 cells failed to produce DNA fragmentation. When cell lysates extracted from wogonin- and fisetin-treated HL-60 cells were incubated with isolated intact nuclei, the intact DNA was cleaved into fragments. Other flavonoids such as luteolin, nobiletin, baicalein, apigenin, and myricetin that induced DNA ladders in Figure 2 showed only slightly detectable endonucleolytic activity in this assay. Further examination of the properties of endonuclease induced by wogonin and fisetin in HL-60 cells revealed that this endonuclease might be Ca^{+2}-dependent since DNA fragmentation disappeared in calcium free-medium (data not

shown) and was attenuated when 5 mM EDTA or EGTA was added to the reaction mixture. Furthermore, the optimal pH for activity was around 7.5 because DNA ladders were obviously detected in the reaction mixture with pH 7.5, but was clearly reduced at pH 6.5 or 5.5.

A Caspase 3-like Protease Inhibitor, Ac-DEVD-CHO, Attenuates Wogonin and Fisetin induced Apoptotic Responses Associated with Decreased Endonuclease Activity

The above results clearly indicate that caspase 3-like proteases and endonuclease are activated in response to wogonin and fisetin in HL-60 cells. To determine if the activation of caspase 3-like protease is necessary for the induction of apoptosis by wogonin and fisetin, caspase inhibitors including the caspase 3-like protease inhibitor, Ac-DEVD-CHO, and the caspase 1-like protease inhibitor, Ac-YVAD-CHO, were used to block intracellular protease, and wogonin- and fisetin-induced DNA ladders and endonuclease activity were analyzed by agarose electrophoresis and in vitro chromatin digestion assay. Results show that the caspase 3-like inhibitor, Ac-DEVD-CHO (200 μM), was able to inhibit wogonin- and fisetin-induced DNA fragmentation accompanied by suppressing PARP cleavage and decreasing pro-caspase 3 protein. However, Ac-YVAD-CHO, an inhibitor of caspase-1 like activity, had no effect at a similar concentration (200 μM). In order to illustrate if activation of caspase 3-like activity participated in wogonin- and fisetin-induced endonuclease activities, in vitro chromatin digestion using cell lysates extracted from HL-60 cells under different treatments was performed. The results in Figure 7C show that wogonin- and fisetin-induced endonuclease activities were inhibited by Ac-DEVD-CHO (200 μM), but not by Ac-YVAD-CHO (200 μM). These results indicate that activation of caspase 3 and endonuclease activities is involved in wogonin- and fisetin-induced apoptosis, and that endonuclease activation might be located downstream of caspase 3 activation.

N-acetyl-cysteine (NAC) and Catalase have no Effect on Wogonin and Fisetin induced Apoptosis, but Inhibited H_2O_2-induced Cell Death

ROS producing and scavenging activities play important roles in the biological effects of flavanoids. In order to demonstrate the role that ROS play in wogonin- and fisetin-induced apoptosis, free radical-scavenging agents, NAC (a: 20 mM; b: 40 mM) and catalase (c: 200 U/ml; d: 400 U/ml), were used in this study. Results in Figure 8A show that neither NAC no catalase has any obvious inhibitory effect on wogonin- and fisetin-induced caspase 3 procession, PARP cleavage, decrease in Mcl- 1 protein, and increase in bax protein. Results of analysis of DNA integrity by agarose electrophoresis show NAC and catalase are unable to block wogonin- and fisetin-induced DNA ladders. However, both NAC and catalase show significant preventive effects on ROS (H_2O_2)-induced apoptosis. In this study, we also found that both NAC and catalase show a slight but obvious increase in wogonin- or fisetin-induced DNA ladder intensity and caspase 3 procession. We propose that the apoptosis-inducing effects of wogonin and fisetin might parallel the actions of NAC and catalase, and that proxidant activities might not be involved.

Decrease of Intracellular ROS by Wogonin and Fisetin

In order to further demonstrate the role that ROS play in wogonin- and fisetin-induced apoptosis, the intracellular peroxide level was determined by fluorescence intensity of DCF. Changes in intracellular peroxide levels were determined by flow cytometry analysis. The results show that both wogonin and fisetin treatments significantly decreased the amount of intracellular peroxide levels from 496.3±12.1 (control) to 265.9±14.3 (wogonin) and 55.2±9.5 (fisetin), respectively ($p < 0.01$, compared with control). Adding H_2O_2 (200 μM) into the medium with DCHF-DA elevated the intracellular fluorescence intensity to 1808.6±21.7 as is described as a positive control here.

成果自評

本計劃成果符合原計劃所提之研究目的, 成果具體且已被雜誌 Biochemical Pharmacology **所接受** (2001/10/9)。

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