Protein Kinase C Inhibition and X-Linked Inhibitor of Apoptosis Protein Degradation Contribute to the Sensitization Effect of Luteolin on Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Induced Apoptosis in Cancer Cells

Ran-Xin Shi, Choon-Nam Ong, and Han-Ming Shen

Department of Community, Occupational, and Family Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Republic of Singapore

Abstract

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is an important member of the TNF superfamily with great potential in cancer therapy. Luteolin is a dietary flavonoid commonly found in some medicinal plants. Here we found that pretreatment with a noncytotoxic concentration of luteolin significantly sensitized TRAIL-induced apoptosis in both TRAIL-sensitive (HeLa) and TRAIL-resistant cancer cells (CNE1, HT29, and HepG2). Such sensitization is achieved through enhanced caspase-8 activation and caspase-3 maturation. Further, the protein level of X-linked inhibitor of apoptosis protein (XIAP) was markedly reduced in cells treated with luteolin and TRAIL, and ectopic expression of XIAP protected against cell death induced by luteolin and TRAIL, showing that luteolin sensitizes TRAIL-induced apoptosis through down-regulation of XIAP. In search of the molecular mechanism responsible for XIAP down-regulation, we found that luteolin and TRAIL promoted XIAP ubiquitination and proteasomal degradation. Next, we showed that protein kinase C (PKC) activation prevented cell death induced by luteolin and TRAIL via suppression of XIAP down-regulation. Moreover, luteolin inhibited PKC activity, and bisindolylmaleimide I, a general PKC inhibitor, simulated luteolin in sensitizing TRAIL-induced apoptosis. Taken together, these results present a novel anticancer effect of luteolin and support its potential application in cancer therapy in combination with TRAIL. In addition, our data reveal a new function of PKC in cell death: PKC activation stabilizes XIAP and thus suppresses TRAIL-induced apoptosis. (Cancer Res 2005; 65(17): 7815-23)

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily (1). TRAIL is an ideal therapeutic agent for cancer treatment because it has been shown to be a potent apoptosis inducer in a wide variety of cancer and transformed cells without damaging most normal cells. TRAIL induces apoptosis through binding to its receptors on cell surface. To date, four types of receptors have been identified: DR4 and DR5 as death receptors and DcR1 and DcR2 as decoy receptors (1). Ligation of TRAIL to its receptors results in trimerization of receptors and clustering of intracellular death domains, which then recruit Fas-associated death domain protein and caspase-8 to form the death-inducing signaling complex. Caspase-8 activation within death-inducing signaling complex subsequently activates executor caspase-3, which in turn cleaves its substrates and eventually induces apoptosis (2, 3). On the other hand, activation of caspase-3 by caspase-8 can be greatly facilitated through the mitochondrial amplification pathway, in which activated caspase-8 cleaves the proapoptotic Bcl-2 family member Bid into truncated Bid. Translocation of truncated Bid together with other proapoptotic Bcl-family members promotes release of cytochrome *c* and SMAC/DIABLO from mitochondria, leading to caspase-3 activation and apoptosis (3, 4).

However, the potential application of TRAIL in cancer therapy is limited as many cancer cells are found to be resistant to the cytotoxicity of TRAIL. The resistance may be due to low expression of proapoptotic molecules (death receptors or caspase-8) or high expression of antiapoptotic molecules (decoy receptors, FLICE-like inhibitory protein (FLIP), inhibitor of apoptosis proteins (IAP), and Bcl-2; ref. 1). Thus, combination TRAIL with other agents has been a promising strategy to potentiate the cytotoxicity of TRAIL and its therapeutic applications (5–7).

Luteolin, 3′,4′,5,7-tetrahydroxyflavone, is a flavonoid commonly found in many types of fruits and vegetables as well as in some medicinal plants (8). There were reports about its anticancer properties including induction of cancer cell apoptosis, cell cycle arrest, inhibition of cancer cell proliferation, and antiangiogenesis activity (9–14). Recently, we have found that luteolin greatly sensitizes TNF-induced cell apoptosis via inhibition of NF-κB and sustained and augmented activation of c-jun NH_2-terminal kinase (JNK; ref. 15). We here showed that luteolin also sensitizes TRAIL-induced apoptosis in various human cancer cells. Interestingly, such sensitization is achieved via enhanced X-linked inhibitor of apoptosis protein (XIAP) ubiquitination and proteasomal degradation. Furthermore, our study shows that the enhanced XIAP ubiquitination and degradation are likely due to suppressed protein kinase C (PKC) activation by luteolin. Data from this study thus present a novel function of luteolin as a potential anticancer agent.

Materials and Methods

Reagents and plasmids. Luteolin and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO). Human recombinant TRAIL was from R&D Systems (Minneapolis, MN) and was dissolved in 1% bovine serum albumin as stock solution (50 μg/mL). The following reagents were from Calbiochem (San Diego, CA): pan caspase inhibitor z-VAD-fmk, caspase-8 inhibitor z-IETD-fmk, caspase-3 inhibitor z-DEVD-fmk,

Requests for reprints: Han-Ming Shen, Department of Community, Occupational, and Family Medicine, Yong Loo Lin School of Medicine, National University of Singapore, 16 Medical Drive, Singapore 117597, Republic of Singapore. Phone: 65-6874-4998; Fax: 65-6779-1489; E-mail: cofshm@nus.edu.sg.

doi:10.1158/0008-5472.CAN-04-3875

phorbol-12-myristate-13-acetate (PMA), general PKC inhibitor bisindolylmaleimide I (BIM), phosphatidylinositol 3-kinase (PI3K) inhibitors LY-294002 and Wortmannin, and proteasome inhibitors MG132, PSI, and PSII. The Flag-XIAP expression vector was a generous gift from Dr. Colin Duckett. Anti–caspase-8, anti–caspase-3, anti–Bcl-2, anti–Bcl-xL, anti-Bid, anti–phospho (Ser)-PKC substrate, and anti-ubiquitin antibodies were from Cell Signaling Technology (Beverly, CA). Anti-XIAP and anti–poly(ADP-ribose) polymerase antibodies were from BD Transduction Laboratories (San Diego, CA). Anti–Mcl-1 and anti-tubulin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatments. Human cervical cancer cells HeLa, human liver cancer cells HepG2, and human colorectal cancer cells HT29 were from American Type Culture Collection (Manassas, VA) and human nasopharyngeal cancer cells CNE1 were obtained from Sun Yet-sat University (Guangzhou, China). HeLa, HepG2, and CNE1 cells were maintained in DMEM medium (Sigma) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT); HT29 cells were maintained in RPMI 1640 with 10% FBS.

Apoptosis assessment with 4′,6-diamidino-2phenylindole staining. The cells undergoing apoptosis were evaluated by chromatin condensation, nuclear shrinkage, and formation of apoptotic bodies, all visualized with DAPI staining (16). After various designated treatments, medium was removed and cells were fixed with 70% ethanol at room temperature for 10 minutes. Cells were then stained with 0.3 μg/mL DAPI (in PBS) at room temperature for 10 minutes and visualized under an inverted fluorescence microscope and photographed.

Colony formation assay. Cancer cells (HT29, HeLa, and HepG2) were plated on six-well plates (5,000 cells/well) for 24 hours followed by various treatments. After 3 weeks, the survival clones were stained by 0.5% crystal violet for 1 hour and photos were taken using digital camera (17).

Transient transfection. HeLa cells were transiently transfected with either pcDNA or Flag-XIAP using the Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA). A red fluorescent protein expression vector (pDsRed; Clontech, Inc., Palo Alto, CA) was cotransfected as a transfection marker. After 24 hours of transfection, the cells were pretreated with luteolin (40 μmol/L × 2 hours) followed by TRAIL (1 ng/mL × 6 hours). Cell death was determined by morphologic changes examined under an inverted fluorescent microscope.

Immunoprecipitation and Western blot. At the end of treatment, cells were collected by scrapping and then washed with ice-cold PBS twice. Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% Triton X-100] for 1 hour on ice. The supernatant was collected after centrifugation at 20,000 × *g* for 15 minutes. Each sample was added with 0.5 μg anti-XIAP body (BD Biosciences PharMingen, San Jose, CA) and 50 μL protein A/G agarose beads (Roche Molecular Biochemicals, Indianapolis, IN) and rotated overnight at 4°C. The beads were washed four times using ice-cold PBS buffer and then eluted with SDS sample buffer before analysis by Western blot. For Western blot, equal amounts of proteins were fractionated on SDS-polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad, Hercules, CA) and blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk in TBST [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20], the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce, Rockford, IL) using a Kodak Image Station (Kodak, Rochester, NY).

RNA extraction and reverse transcription-PCR. RNA extraction was carried out using a total RNA extraction kit Purescript (Gentra Systems, Inc., Minneapolis, MN) following the instructions from the manufacturer. Five micrograms of total RNA from each sample were subjected to reverse transcription using M-MLV reverse transcriptase (Promega, Madison, IL). For PCR, the amplification reaction was carried out with 200 pmol of each primer, 200 μmol/L each of deoxynucleotide triphosphates, and 0.5 units of Taq DNA polymerase II (Promega). The PCR conditions were optimized to achieve exponential amplification in which the PCR product formation is proportional to the starting cDNA. The primers of human XIAP (18) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH; ref. 19) were based on literature. PCR products were size-fractionated using 1.8% agarose gel and visualized by ethidium bromide staining.

Results

Luteolin sensitizes cancer cells to tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis. We have recently shown that luteolin was able to sensitize TNF-induced apoptosis in human cancer cells (15). Here we further assessed the effect of luteolin on TRAIL-induced cell death. First, we tested the cytotoxicity of TRAIL on human cancer cell lines originated from various tissues, including human live cancer cells HepG2, human colorectal cancer cells HT29, human nasopharyngeal cancer cells CNE1, and human cervical cancer cells HeLa. Some cancer cells were found to be TRAIL resistant. For instance, up to as high as 200 ng/mL TRAIL exerted no significant cytotoxicity on HepG2, HT29, or CNE1 cells even after 24 hours treatment. In contrast, HeLa cells were sensitive to as low as 5 ng/mL TRAIL (Fig. 1*A*). Luteolin (40 μmol/L) alone did not induce cell death in any of these cells. However, when the cells were pretreated with luteolin for 2 hours, followed by a noncytotoxic concentration of TRAIL for as short as 6 hours, all the four cell lines tested underwent dramatic apoptotic cell death (Fig. 1*B*). Figure 1*C* shows the chromosome condensation in HeLa cells treated with luteolin and TRAIL. These data suggest that luteolin pretreatment not only markedly sensitizes TRAIL-resistant cancer cells but also significantly expedites the cell death process. To test the long-term effect of luteolin and TRAIL on cancer cell growth, the colony formation assay was done using HT29 cells. As shown in Fig. 1*D*, luteolin (40 μmol/L) alone reduced HT29 colony size evidently, although it was not cytotoxic in the short-term apoptosis assay (Fig. 1*A* and *B*). However, a combination of luteolin and TRAIL completely suppressed cancer cell growth and colony formation. Similar results were also found in HeLa and HepG2 cells (data not shown).

Luteolin facilitates tumor necrosis factor–related apoptosis-inducing ligand–initiated caspase-3 maturation. TRAIL-induced apoptosis is mainly executed by the extrinsic cell death receptor pathway, involving caspase-8 as the initiator caspase and caspase-3 as the executor. Here we examined the effect of luteolin on TRAIL-initiated caspase cascade. As shown in Fig. 2*A*, TRAIL alone induced obvious caspase-8 cleavage, producing both p44 and p23. Whereas luteolin alone had no effect on caspase-8 activation, luteolin pretreatment greatly promoted TRAIL-induced caspase-8 activation, as evidenced by the enhanced cleavage of procaspase-8 p55 to its intermediate form p44 and further to its active form p23. We next examined the pattern of caspase-3 activation. In cells treated with TRAIL alone for 6 hours, there was only slight cleavage of caspase-3, producing its inactive fragment p21 (Fig. 2*A*). Although luteolin alone did not cause any change in caspase-3, its pretreatment followed by TRAIL led to the complete cleavage of caspase-3, resulting in formation of the active form p17. This result indicates that luteolin, in combination with TRAIL, facilitates the maturation of caspase-3. Similar caspase changes were also observed in CNE1, HT29, and HepG2 cells (data not shown). Finally, we found that only combined treatment with luteolin and TRAIL resulted in evident poly(ADP-ribose) polymerase cleavage, downstream of caspase-3 activation and a hallmark of apoptosis (Fig. 2*A*), which is consistent with cell death results shown in Fig. 1*B*.

We then used various caspase inhibitors to confirm the role of the observed caspase cascade in the cell death induced by luteolin and TRAIL. Figure 2*B* and *C* shows that z-DEVD-fmk (a caspase-3 inhibitor), z-IETD-fmk (a caspase-8 inhibitor), and z-VAD-fmk (a pan caspase inhibitor) completely blocked caspase-3 activation

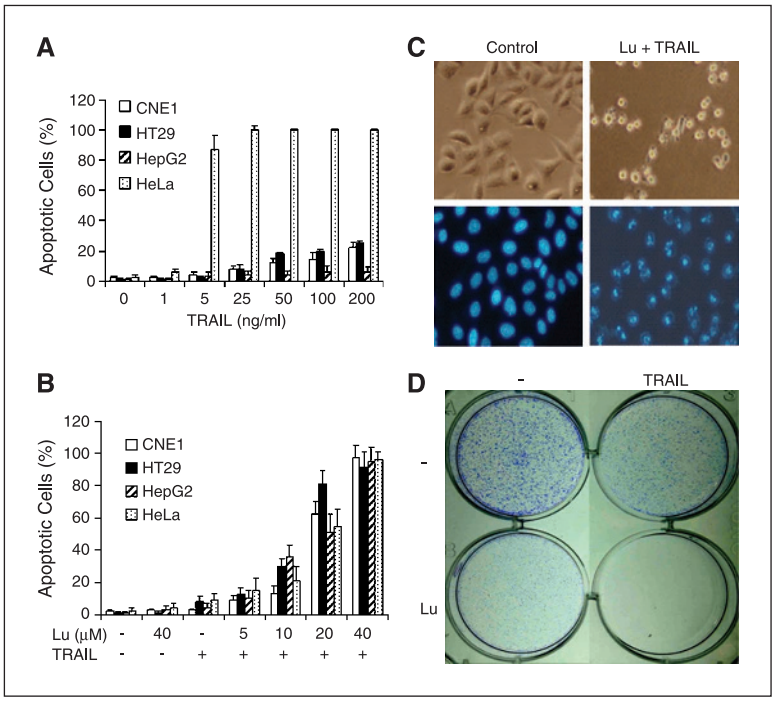


Figure 1. Luteolin sensitizes human cancer cells to TRAIL-induced apoptosis. *A,* CNE1, HT29, HeLa, and HepG2 cells were treated with various concentrations of TRAIL for 24 hours. *B,* cells were first pretreated with indicated concentrations of luteolin for 2 hours, followed by treatment with a subtoxic concentration of TRAIL for another 6 hours (1 ng/mL for HeLa and CNE1, 5 ng/mL for HT29 and HepG2). *A* and *B,* at the end of treatment, cells were stained with DAPI and examined under an inverted fluorescent microscope. *Columns,* percentage of cells with evident nuclear condensation in 200 randomly selected cells. *C,* apoptotic morphologic changes in HeLa cells with combined treatment with luteolin (40 μmol/L × 8 hours) and TRAIL (1 ng/mL × 6 hours). *Top,* cells pictured under a normal light microscope; *bottom,* cells with DAPI staining under an inverted fluorescence microscope. *D,* colony formation assay. HT29 cells were plated on six-well plates (5,000 cells/well) and treated with luteolin alone (40 μmol/L), TRAIL alone (1 ng/mL), or their combination for 3 weeks. The survival clones were stained with 0.5% crystal violet.

and cell death induced by luteolin and TRAIL. One interesting finding here is that z-DEVD-fmk, the specific inhibitor of caspase-3, also abrogated caspase-8 cleavage in cells treated with luteolin and TRAIL, indicating the presence of a caspase-8 and caspase-3 positive feedback loop (20).

In certain cells, TRAIL has been shown to induce apoptosis via the intrinsic mitochondrial pathway via caspase-8–mediated Bid cleavage (20). However, in this study we found that a caspase-9 inhibitor did not offer significant protection against luteolin- and TRAIL-induced apoptosis (data not shown). Therefore, it is believed that luteolin enhances TRAIL-induced apoptosis mainly by utilizing the cell death receptor pathway.

It has been reported that modulation of surface expression of death receptors could sensitize cells to TRAIL-induced apoptosis (21, 22). We then tested the changes in various TRAIL death receptors after luteolin treatment by using immunofluorescence staining for the cell surface protein level and reverse transcription-PCR (RT-PCR) for the mRNA level. However, it was found that luteolin treatment did not alter the surface expression of death receptors (DR4, DR5, DcR1, or DcR2; data not shown), suggesting that luteolin promotes caspase activation via other mechanisms. On the other hand, NF-κB is a potent antiapoptotic factor in TNF-induced apoptosis (23, 24). Previously, we have reported that luteolin sensitized TNF-induced cell death through inhibition of NF-κB (15). Although TRAIL-induced NF-κB activation has been observed in certain cells (25), in this study NF-κB is unlikely to be important in the sensitization activity of luteolin on TRAIL-induced apoptosis, based on the finding that either TRAIL or luteolin did not change NF-κB luciferase activity (data not shown). Such a finding is consistent with an earlier report that sensitivity to TRAIL-induced apoptosis is not significantly modulated by transfection of dominant-negative mutants of IκB kinase β or IκBα (26).

X-linked inhibitor of apoptosis protein down-regulation contributes to sensitized cell death. It has been well documented that a number of cellular proteins are important regulators in apoptosis via inhibition of the caspase cascade. These proteins include FLIP, c-IAP, Bcl-2, Bcl-xL, and XIAP, which are known to be regulated by NF-κB at the transcriptional level (24, 27, 28). In search of the molecular mechanisms which may be involved in the sensitization activity of luteolin, we tested the changes in these proteins in cells treated with TRAIL with or without luteolin pretreatment. The protein levels of FLIP, c-IAP1, c-IAP2, Bcl-2, and Bcl-xL remained constant among various treated groups (data not shown). This finding is basically consistent with the earlier observation that TRAIL or luteolin is unable to affect NF-κB transcriptional activity in HeLa cells (data not shown). Interestingly, the protein levels of two antiapoptotic proteins, Mcl-1 and XIAP, significantly decreased in cells undergoing apoptosis (Fig. 3*A*). However, the decrease of Mcl-1, but not of XIAP, was reversed in the presence of z-VAD-fmk, a pan caspase inhibitor, indicating that the reduction of Mcl-1 protein level is the result of caspase activation (29), whereas XIAP down-regulation is caspase independent. Figure 3*B* shows the dose-dependent pattern of XIAP down-regulation in cells treated with luteolin and TRAIL, which is consistent with the dose-dependent pattern of cell death observed above (Fig. 1*B*). Luteolin-dependent reduction of XIAP protein level was also observed in two other TRAIL-resistant cell lines (HT29 and HepG2; data not shown).

To further confirm the role of XIAP in cell death induced by luteolin and TRAIL, we examined whether XIAP overexpression will provide protection against cell death. HeLa cells were transiently transfected with either wild-type XIAP plasmid (Flag-XIAP) or an empty vector (pcDNA). Red fluorescence protein plasmid (pDsRed) was used as a transfection marker. In pcDNA-transfected cells, almost all cells died after luteolin and TRAIL treatment based on morphologic changes. In contrast, most XIAP-overexpressing cells remained alive whereas nontransfected cells underwent cell death (Fig. 3*C* and *D*). The above results thus strengthen our argument that XIAP down-regulation plays a critical role in luteolin- and TRAIL-induced cell death.

X-linked inhibitor of apoptosis protein down-regulation is mediated by ubiquitination and proteasomal degradation. The down-regulation of XIAP could be due to modulation at either the transcriptional or posttranscriptional level. To elucidate the molecular mechanism involved, we first measured the XIAP mRNA level using RT-PCR. As shown in Fig. 4*A*, either luteolin, TRAIL, or their combined treatment did not alter the XIAP mRNA level up to 4 hours, suggesting that the XIAP is mainly regulated posttranscriptionally.

To determine whether the decreased XIAP level is due to proteasomal degradation, here we tested the effects of proteasome inhibitors on XIAP protein level. As shown in Fig. 4*B*, MG132 (1 μmol/L), PSI (5 μmol/L), or PSII (5 μmol/L) completely abolished the XIAP down-regulation induced by luteolin and TRAIL. The effect of MG132 was also found to be dose dependent; a low concentration of MG132 (0.1 μmol/L) only partially prevented XIAP degradation (data not shown). Because XIAP is probably the most potent apoptosis inhibitor, the stabilization of XIAP would render cells resistant to apoptosis induced by luteolin and TRAIL. Such a hypothesis was supported by the results shown in Fig. 4*C* that the three proteasome inhibitors were able to completely prevent cell death induced by luteolin and TRAIL. It is thus believed that the down-regulation of XIAP protein through proteasomal degradation is the underlying mechanism in the sensitization effect of luteolin on TRAIL-induced apoptosis. Similar results were also found in other cell lines such as HT29 and HepG2 (data not shown).

It is known that XIAP has ubiquitin protease ligase (E3) activity and the autoubiquitination and degradation is an important

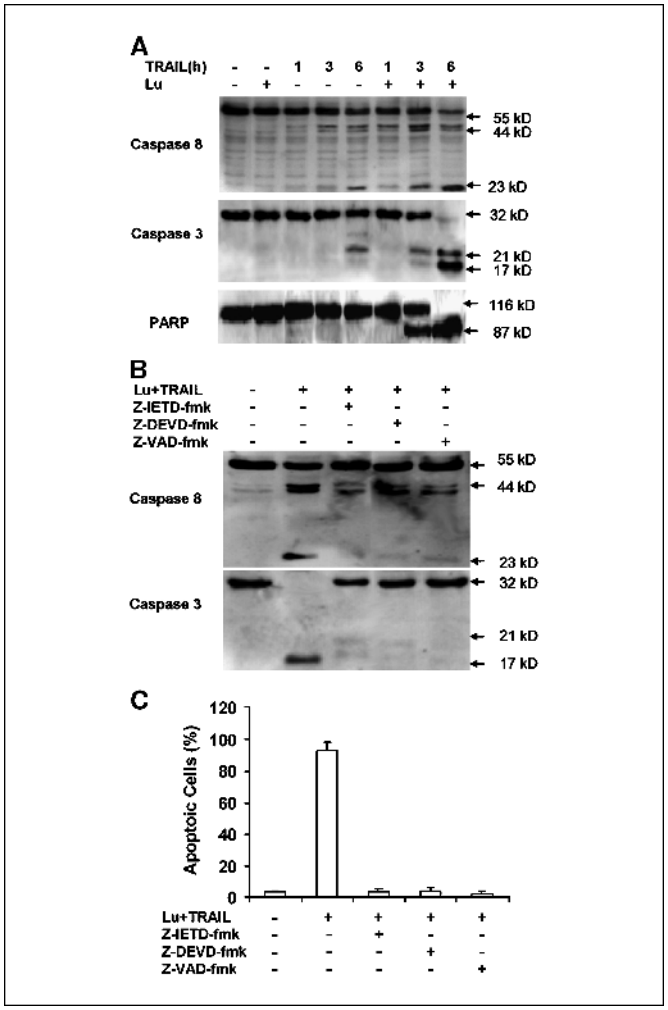


Figure 2. Luteolin facilitates TRAIL-induced caspase-3 maturation. *A,* HeLa cells were treated with TRAIL (1 ng/mL) for the indicated periods with or without the presence of luteolin pretreatment (40 μmol/L × 2 hours). Cells were collected and subjected to Western blot for detection of cleavage of caspase-8, caspase-3, and poly(ADP-ribose) polymerase (*PARP*). *B,* HeLa cells were pretreated with z-IETD-fmk (25 μmol/L), z-DEVD-fmk (25 μmol/L), or z-VAD-fmk (25 μmol/L) for 30 minutes, then cells were treated with a combination of luteolin (40 μmol/L × 8 hours) and TRAIL (1 ng/mL × 6 hours). Cells were collected for measurement of caspase-3 and caspase-8 cleavage by Western blot. *C,* HeLa cells were pretreated with various caspase inhibitors, followed by luteolin and TRAIL, as described in *B*. The percentage of apoptosis was evaluated using DAPI staining as described in Fig. 1.

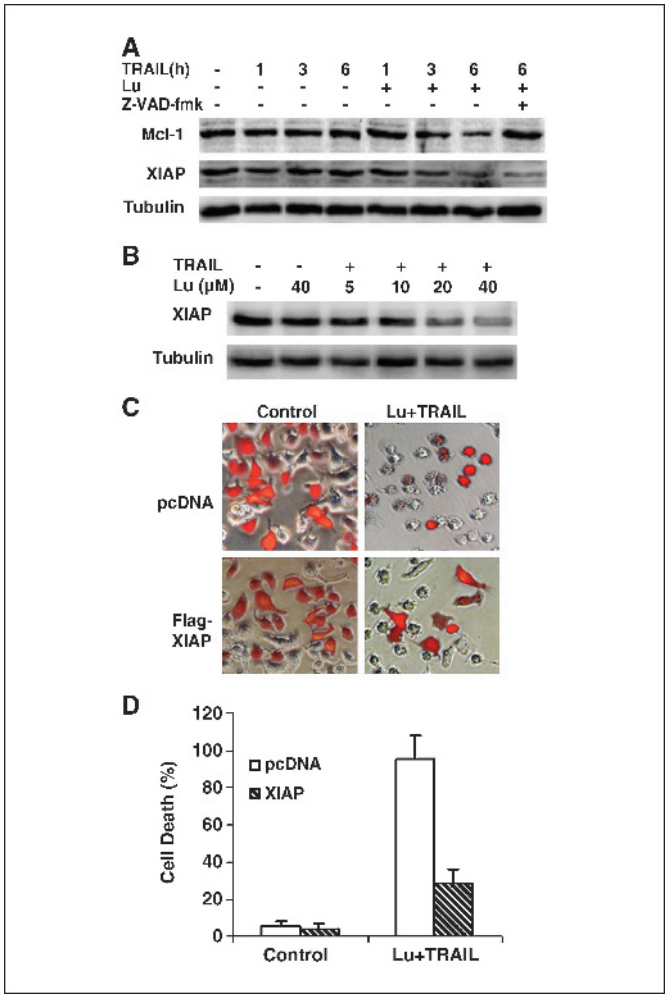


Figure 3. XIAP down-regulation contributes to apoptosis induced by luteolin and TRAIL. *A,* HeLa cells were first pretreated with z-VAD-fmk (25 μmol/L × 30 minutes), then treated with TRAIL (1 ng/mL) for indicated periods with or without luteolin pretreatment (40 μmol/L × 2 hours). Cells were collected for detection of Mcl-1 and XIAP protein level by Western blot. Tubulin was used as a loading control. *B,* HeLa cells were treated with indicated concentrations of luteolin for 2 hours, followed by TRAIL (1 ng/mL) for additional 6 hours, and then cells were collected for detection of XIAP protein level using Western blot. *C,* HeLa cells were transiently transfected with either pcDNA or Flag-XIAP-wt, together with pDsRed as a transfection marker. After 24 hours, the cells were treated with the combination of luteolin (40 μmol/L × 8 hours) and TRAIL (1 ng/mL × 6 hours). Cell death was then evaluated by morphologic changes under a fluorescent microscope; successfully transfected cells gave a bright red color. *D,* quantification of cell death by counting the percentage of dead cells among transfected cells in a total of 200 randomly selected transfected cells. *Columns,* mean of three independent transfection experiments; *bars,* SD.

mechanism for regulating the XIAP function in apoptosis (30, 31). Here we further examined whether treatment with luteolin and TRAIL promotes XIAP ubiquitination by directly measuring XIAP ubiquitination. It was found that the combined treatment with luteolin and TRAIL significantly enhanced the level of ubiquitylated XIAP in HeLa cells in the presence of proteasome inhibitor MG132 (Fig. 4*D*). Consistent with this, the level of total protein ubiquitination was also increased by luteolin and TRAIL in the presence of MG132. Similar results were also found in HT29 and HepG2 cells (data not shown). The above results thus clearly show that luteolin and TRAIL promote XIAP degradation by enhancing its ubiquitination.

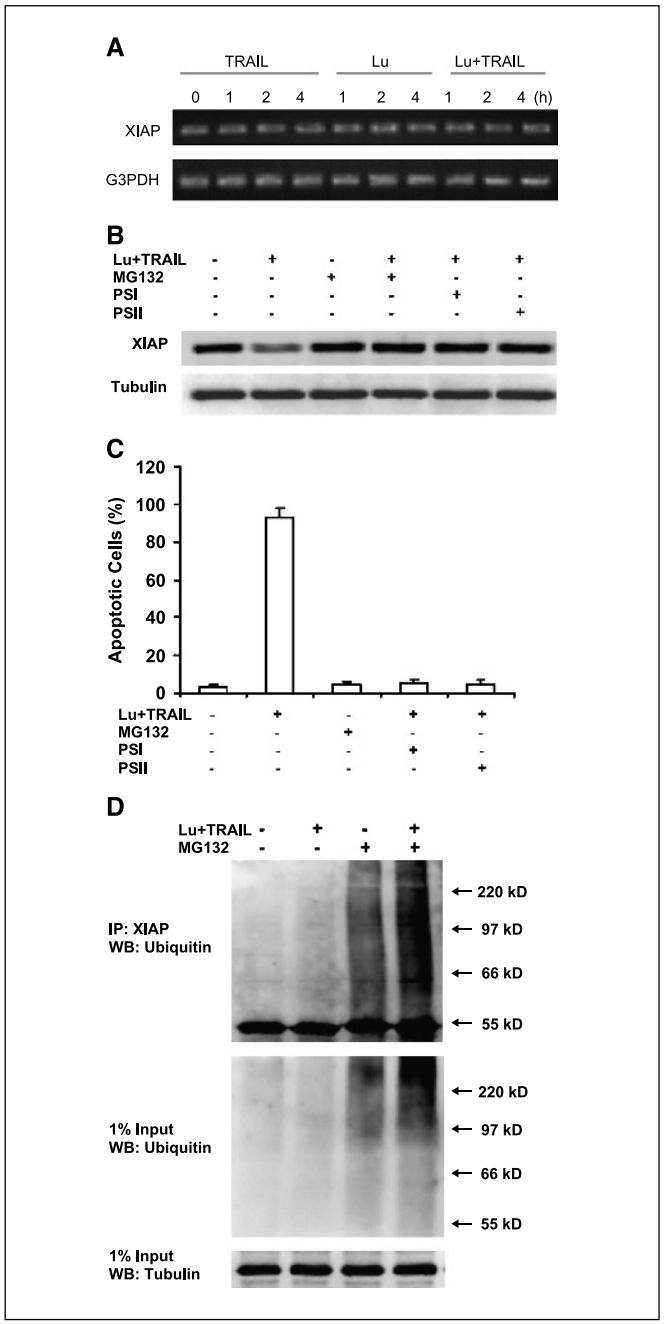


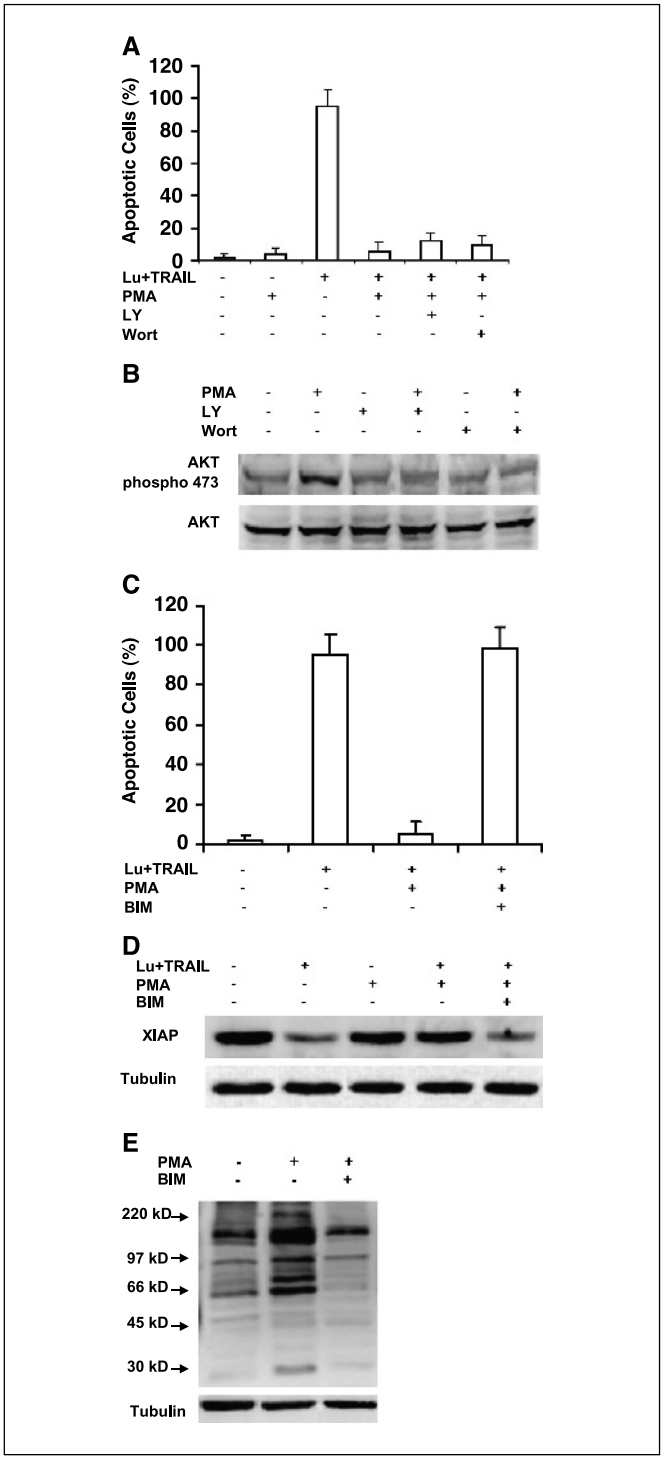
Figure 4. XIAP down-regulation was through ubiquitination and proteasomal degradation. *A,* HeLa cells were treated with luteolin (40 μmol/L) or TRAIL (1 ng/mL) or a combination of both for indicated periods. Cells were collected for detection of XIAP mRNA level using RT-PCR. G3PDH was used as a loading control. *B,* HeLa cells were pretreated with proteasome inhibitor MG132 (1 μmol/L), PSI (5 μmol/L), or PSII (5 μmol/L) for 1 hour, followed by combined treatment with luteolin (40 μmol/L × 8 hours) and TRAIL (1 ng/mL × 6 hours). XIAP protein level was determined by Western blotting. *C,* HeLa cells were treated as described in *B* and the percentage of apoptotic cell death was evaluated by DAPI staining. *D,* HeLa cells were pretreated with proteasome inhibitor MG 132 (1 μmol/L) for 2 hours before combined treatment with luteolin (40 μmol/L) and TRAIL (1 ng/mL) for another 2 hours. Cell lysate was used for immunoprecipitation with anti-XIAP antibody, followed by Western blot with anti-ubiqutin antibody.

Protein kinase C activation blocks X-linked inhibitor of apoptosis protein degradation and prevents cell death induced by luteolin and tumor necrosis factor–related apoptosis-inducing ligand. Previous studies have shown that the PI3K-AKT pathway plays a protective role in TRAIL-induced apoptosis (32) and one of the mechanisms is phosphorylation and stabilization of XIAP by AKT through inhibition of its ubiquitination (33). On the other hand, it is known that PMA is capable of protecting cells from TRAIL-induced apoptosis (34). In our study, PMA pretreatment also completely prevented luteolin- and TRAIL-induced cell death (Fig. 5*A*). It has been well established that PMA stimulates a series of downstream signals including PI3K-AKT, mitogen-activated protein kinase (MAPK), and PKC (34, 35). We examined the involvement of each signaling pathway in the protective activity of PMA using various specific inhibitors. The two PI3K inhibitors (LY-294002 and Wortmannin) failed to reverse the protective effect of PMA (Fig. 5*A*). Similar negative results were also found with a JNK inhibitor (SP600125), a p38 inhibitor (SB203580), or an extracellular signal-regulated kinase inhibitor (PD98059; data not shown). The effectiveness of these two inhibitors on the PI3K-AKT pathway was confirmed in PMA-stimulated cells (Fig. 5*B*). We also found that either TRAIL or luteolin alone or their combination has no effect on AKT activation (data not shown). Therefore, the above data indicate that neither the PI3K-AKT nor the MAPK pathway plays a critical role in the protective effect of PMA against apoptosis induced by luteolin and TRAIL.

It has been reported that PKC activation plays a protective role against TRAIL-induced apoptosis (34). Here we attempted to explore the possible role of PKC in luteolin- and TRAIL-induced apoptotic cell death. First, BIM, a general PKC inhibitor, is capable of abolishing the protective effect of PMA on luteolin- and TRAIL-induced cell death (Fig. 5*C*), suggesting that the protective effect of PMA is mediated via PKC activation. Second, we asked whether PKC activation is associated with changes in XIAP protein level. As shown in Fig. 5*D*, PMA pretreatment completely prevented XIAP degradation in cells treated with luteolin and TRAIL. Moreover, such an effect by PMA on XIAP was completely abolished by BIM, thus suggesting that PMA-mediated PKC activation is able to stabilize XIAP and subsequently prevent apoptosis. The effectiveness of BIM in PKC activation was confirmed by the overall PKC activity, which was assessed by Western blot with anti–phospho (Ser)-PKC substrate antibody (35). As expected, PMA readily activated PKC and this activation was completely blocked by BIM (Fig. 5*E*).

Protein kinase C inhibition promotes down-regulation of X-linked inhibitor of apoptosis protein and apoptosis in tumor necrosis factor–related apoptosis-inducing ligand–treated cells. As the above data on PKC were all obtained from cells stimulated with PMA, we next examined whether the sensitization activity of luteolin also involves PKC without the presence of PMA. An earlier report showed that TRAIL activates PKC in one pancreatic adenocarcinoma cell line (PancTuI) but

only marginally in another (Colo357 cells; ref. 36), suggesting the effect of TRAIL on PKC is cell type specific. In this study, there was a considerable degree of basal PKC activation in the control HeLa cells and a marginal PKC activation by TRAIL. In contrast, luteolin pretreatment markedly reduced both basal PKC and TRAIL-induced PKC activations, which is similar to the effect of BIM (Fig. 6*A*). These data thus support the hypothesis that luteolin sensitizes TRAIL-induced apoptosis through PKC inhibition.



To confirm the role of PKC in XIAP stability and the possible mechanisms involved, we further tested the effect of BIM on XIAP protein level in cells treated with TRAIL. Combined treatment with BIM and TRAIL significantly down-regulated XIAP level, a process not affected by z-VAD-fmk (data not shown), but prevented by MG132 (Fig. 6*B*), which is similar to the effect of luteolin on TRAIL-induced XIAP down-regulation as shown earlier (Figs. 3*A* and 4*B*). Furthermore, similar to the sensitization activity of luteolin, BIM also significantly enhanced TRAIL-induced cell apoptosis (Fig. 6*C*). Similar results were found in TRAIL-resistant cells (HT29 and HepG2; data not shown). Therefore, data from this part of our study show that PKC activation plays a protective role in TRAIL-induced apoptosis via stabilization of XIAP and that luteolin may act as a PKC inhibitor to sensitize cancer cells to TRAIL-mediated apoptotic cell death.

Discussion

TRAIL is a potent therapeutic agent due to its unique property of killing cancer cells by apoptosis but sparing normal cells. However, many cancer cells are found to be resistant to TRAIL, thus limiting its clinical application. A number of factors may be involved in the resistance, including modified expression of surface death receptors or changes in antiapoptotic proteins (1). One effective strategy to overcome TRAIL resistance is to combine TRAIL with other anticancer agents (5–7, 21, 22). We showed in an earlier study that luteolin, a natural flavonoid commonly found in vegetables and fruits, sensitized TNF-induced apoptosis in cancer cells (15). We here reported that luteolin also sensitizes TRAIL-induced apoptosis in a number of cancer cells. Such sensitization effect of luteolin is probably achieved via inhibition of PKC activation and promotion of XIAP ubiquitination and proteasomal degradation, which then removes the blockage on caspase and enhances apoptosis.

Data from this study have shown that the sensitization activity of luteolin on TRAIL-induced apoptosis is mainly executed through the cell death receptor pathway and luteolin sensitizes TRAIL-induced apoptosis via enhanced caspase-3 maturation (Fig. 2). Because luteolin treatment did not alter the expression level of TRAIL death receptors (DR4 and DR5) or its decoy receptors (DcR1 and DcR2; data not shown), we went on to screen the changes in other apoptosis regulatory proteins after luteolin and TRAIL treatment. Among many antiapoptotic proteins tested, we found that XIAP was significantly down-regulated by combined treatment with luteolin and TRAIL, but not by their individual treatment (Fig. 3*A* and *B*). More importantly, the XIAP down-regulation was caspase independent, indicating the down-regulated XIAP level is

Figure 5. PKC activation protects against cell death and XIAP down-regulation induced by luteolin and TRAIL *A,* HeLa cells were pretreated with either 10 μmol/L LY-294002 or 0.5 μmol/L Wortmannin for 30 minutes, followed by treatment with PMA (80 ng/mL × 30 minutes), and finally with a combination of luteolin (40 μmol/L × 8 hours) and TRAIL (1 ng/mL × 6 hours). Cell death was evaluated by DAPI staining. *B,* HeLa cells were pretreated with luteolin for 2 hours followed by TRAIL for 1 hour; or HeLa cells were pretreated with LY-294002 (10 μmol/L) or Wortmannin (1 nmol/L) for 60 minutes followed by PMA (80 nmol/L) treatment for 1 hour. Cells were collected for detection of AKT activation using anti–phospho Ser473-AKT antibody. Total AKT level was used as loading control. *C,* HeLa cells were first pretreated with 10 μmol/L BIM for 30 minutes, followed by treatment with PMA (80 ng/mL × 30 minutes), and finally with combined treatment with luteolin (40 μmol/L × 8 hours) and TRAIL (1 ng/mL × 6 hours). Cell death was evaluated by DAPI staining. *D,* HeLa cells were treated as in *C* and XIAP protein level was detected using Western blot. *E,* HeLa cells were pretreated with 10 μmol/L BIM for 30 minutes, followed by treatment with PMA (80 ng/mL × 30 minutes). Cells were collected for detection of PKC activation by Western blot using anti–phospho (Ser)-PKC substrate antibody.

upstream of caspase-3 activation. XIAP, a member of IAP family, is probably the most potent apoptosis inhibitory protein and plays important roles in cell survival. XIAP is characterized by baculoviral IAP repeat domains, which can inhibit caspase-3 and caspase-9 activity by direct binding (27, 37, 38). In this study, it seems that luteolin sensitizes TRAIL-induced apoptosis by targeting XIAP to remove the blockage on caspase-3 activation and cell death. Such a hypothesis was further supported by the fact that overexpression of XIAP protein offers complete protection against luteolin- and TRAIL-induced apoptosis (Fig. 3*C*).

We further examined the possible mechanisms contributing to the reduced XIAP protein level in cells treated with luteolin and TRAIL. Because luteolin, TRAIL, or their combined treatment had no effect on the XIAP mRNA level (Fig. 4*A*), the reduced XIAP protein level is most probably the result of enhanced posttranscriptional degradation. The effect of luteolin is apparently different from that of flavopiridol, which acts synergistically with TRAIL by suppression of XIAP gene transcription (5). It is known that the RING finger domain of XIAP has ubiquitin protease ligase (E3) activity and is responsible for its autoubiquitination and degradation after an apoptosis stimulus (30). Here we tested whether luteolin and TRAIL promote XIAP ubiquitination and subsequent proteasomal degradation; the three proteasome inhibitors offered complete protection against both XIAP degradation (Fig. 4*B*) and cell death (Fig. 4*C*) in cells treated with luteolin and TRAIL. By performing XIAP immunoprecipitation and ubiqutin Western blot analysis, we then provided direct evidence showing that the level of ubiquitylated XIAP was enhanced in cells treated with luteolin and TRAIL. It is thus believed that the decreased XIAP protein level is mediated via ubiquitination and proteasomal degradation, a process crucial for deciding the susceptibility to apoptosis induced by luteolin and TRAIL in cancer cells.

One interesting finding in this study is that PMA pretreatment completely blocked XIAP down-regulation and apoptotic cell death induced by luteolin and TRAIL (Fig. 5*A*). PMA is a potent inducer for a number of important cell signaling pathways, including the PI3K-AKT pathway (39). It has been reported that AKT activation stabilizes XIAP through enhanced phosphorylation and suppressed ubiquitination and proteasomal degradation (33). It has also been noted that overexpression of active AKT renders TRAIL-sensitive cells to be TRAIL resistant (40, 41). In this study, pretreatment with the two PI3K inhibitors (LY-294002 or Wortmannin) did not alter the cell sensitivity to TRAIL-induced apoptosis (Fig. 5*A*). No evident change in AKT activation was found in cells treated with luteolin alone, TRAIL alone, or their combination (data not shown). Moreover, LY-294002 or Wortmannin failed to abrogate the protection effect of PMA against apoptosis induced by luteolin and TRAIL (Fig. 5*A*). Therefore, it seems that the PI3K-AKT pathway is not an important factor in luteolin- and TRAIL-mediated XIAP down-regulation and apoptosis.

We next turned our attention to the possible involvement of PKC in luteolin- and TRAIL-mediated XIAP down-regulation and apoptosis because PMA is also known to be a potent stimulus for both classic and novel PKC activation (35). In this study, we first confirmed that PKC activation contributes to the protective effect of PMA against luteolin- and TRAIL-induced apoptosis by the following observations: (*a*) PMA activates PKC (Fig. 5*E*) and (*b*) a general PKC inhibitor (BIM) prevents PKC activation and abolishes the protective effect of PMA (Fig. 5*C* and *D*). It has been reported that PKC activation plays a protective role in TRAIL-induced apoptosis (34). The protective effects of PKC against TRAIL-induced apoptosis could be achieved by interfering with death-inducing signaling complex formation (34), disrupting proteolytic cleavage of procaspase-8 (42), or affecting caspase-8–mediated Bid cleavage (43). Here, we propose a novel mechanism to illustrate the antiapoptotic function of PKC: PKC activation is associated with decreased XIAP proteasomal degradation and increased stability. Such a hypothesis is supported by the findings that PMA

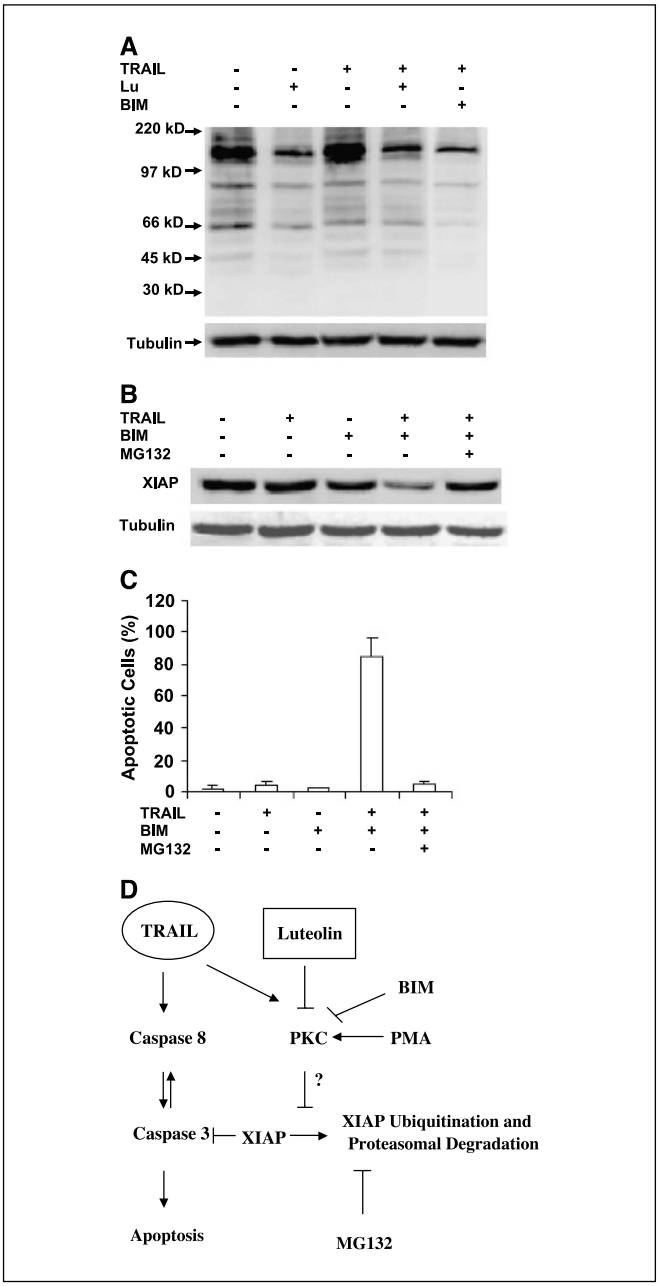


Figure 6. PKC inhibition promotes XIAP down-regulation and apoptosis in TRAIL-treated cells. *A,* HeLa cells were first pretreated with luteolin (40 μmol/L) or BIM (10 μmol/L) for 30 minutes, followed by TRAIL (1 ng/mL for 1 hour). Cells were collected for detection of PKC activation using anti–phospho (Ser)-PKC substrate antibody. *B,* HeLa cells were first pretreated with BIM (10 μmol/L) and/or MG132 (1 μmol/L) for 30 minutes, followed by TRAIL (1 ng/mL × 6 hours). XIAP protein level was detected using Western blot. *C,* cells were treated as described in *B* and cell death was evaluated by DAPI staining. *D,* illustration of the pathways involved in the sensitization activity of luteolin on TRAIL-induced apoptosis in cancer cells.

pretreatment prevents XIAP down-regulation whereas BIM reverses the effect of PMA on XIAP protein level in cells treated with luteolin and TRAIL (Fig. 5*D*). The suggested close link between PKC activation and XIAP level indicates that there might be a positive correlation between the basal PKC level and XIAP level among cells with different sensitivities. However, we did not find any correlation after comparing the basal PKC and XIAP levels among both TRAIL-sensitive cells and TRAIL-resistant cells (data not shown). It is possible that the basal level PKC activity and XIAP are not the only determining factors in the cellular response to TRAIL. It is known that phosphorylation of XIAP by some other protein kinases such as AKT protects XIAP from ubiquitination and proteasomal degradation (33). It is thus possible that PKC acts through a similar mechanism to stabilize XIAP. Thus, a number of important questions remain to be further investigated. For instance, does XIAP serve as the direct substrate for PKC? If so, which specific PKC subunit is involved? Further studies on these topics will certainly shed light on the underlying mechanisms controlling TRAIL resistance and sensitivity in cancer cells.

It was reported that certain flavonoids, including luteolin, inhibited PKC activity in some *in vitro* cell-free systems (44, 45). In this study, luteolin was found to significantly block both the basal PKC activation in control cells and the enhanced PKC activation in TRAIL-treated cells (Fig. 6*A*). Furthermore, the general PKC inhibitor (BIM) mimics the effect of luteolin: pretreatment with BIM down-regulated XIAP protein level in the presence of TRAIL (Fig. 6*B*) and greatly caused apoptosis (Fig. 6*C*). Therefore, it is likely that luteolin acts as a PKC inhibitor to facilitate XIAP degradation and to promote TRAIL-mediated apoptosis. However, it is still not clear how luteolin inhibits PKC activity. Through the structure analysis, it has been hypothesized that flavonoids might interfere with the binding of PKC to calcium or diacylglycerol, but not directly interacting with the functional domain of PKC protein (44). Interestingly, although either luteolin or BIM could effectively suppress the basal PKC activation in control cells, they failed to cause any evident reduction of basal XIAP protein level. It thus suggests that PKC-induced modification of XIAP has no effect on its stability or expression, but likely acts on the recognition process by the proteasomal degradation pathway. Further study on PKC activation and XIAP ubiquitination and degradation is obviously required to address this issue.

Taken together, we discovered a novel anticancer function of luteolin. Luteolin sensitizes TRAIL-induced apoptosis in human cancer cells via inhibition of PKC activation and promotion of XIAP degradation (Fig. 6*D*). Understanding of such an effect of luteolin supports its potential therapeutic application in overcoming TRAIL resistance, especially in cancers with elevated level of PKC activation. In addition, we also reveal a novel function of PKC in TRAIL-mediated apoptosis: PKC may protect against cell death by blocking XIAP ubiquitination and degradation although a firm biochemical link between PKC and XIAP ubiquitination and degradation remains to be further established.

Acknowledgments

Received 10/28/2004; revised 6/20/2005; accepted 6/24/2005.

Grant support: Biomedical Research Council, Singapore (BMRC 03/1/21/19/268), the University Academic Research Fund (H.M. Shen), and the National University of Singapore Research Scholarship (Ranxin Shi).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. C. Duckett (University of Michigan, Ann Arbor, MI) for providing the Flag-XIAP plasmid and Dr. K.L. Lim from the National Neuroscience Institute, Singapore for his expert advice on the ubiquitin assay.

References

1. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 2003;22:8628–33.
2. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer 2002;2:420–30.
3. Green DR. Apoptotic pathways: paper wraps stone blunts scissors. Cell 2000;102:1–4.
4. Jiang X, Wang X. Cytochrome C-mediated apoptosis. Annu Rev Biochem 2004;73:87–106.
5. Rosato RR, Dai Y, Almenara JA, Maggio SC, Grant S. Potent antileukemic interactions between flavopiridol and TRAIL/Apo2L involve flavopiridol-mediated XIAP down-regulation. Leukemia 2004;18:1780–8.
6. von Haefen C, Gillissen B, Hemmati PG, et al. Multidomain Bcl-2 homolog Bax but not Bak mediates synergistic induction of apoptosis by TRAIL and 5-FU through the mitochondrial apoptosis pathway. Oncogene 2004;23:8320–32.
7. Huerta-Yepez S, Vega M, Jazirehi A, et al. Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-κB and inhibition of Bcl-xl expression. Oncogene 2004;23:4993–5003.
8. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr 2002;22:19–34.
9. Huang YT, Hwang JJ, Lee PP, et al. Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on cell growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. Br J Pharmacol 1999;128:999–1010.
10. Ko WG, Kang TH, Lee SJ, Kim YC, Lee BH. Effects of luteolin on the inhibition of proliferation and induction of apoptosis in human myeloid leukaemia cells. Phytother Res 2002;16:295–8.
11. Kim JH, Jin YR, Park BS, et al. Luteolin prevents PDGF-BB-induced proliferation of vascular smooth muscle cells by inhibition of PDGF β-receptor phosphorylation. Biochem Pharmacol 2005;69:1715–21.
12. Lee HJ, Wang CJ, Kuo HC, et al. Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. Toxicol Appl Pharmacol 2005;203:124–31.
13. Leung HW, Wu CH, Lin CH, Lee HZ. Luteolin induced DNA damage leading to human lung squamous carcinoma CH27 cell apoptosis. Eur J Pharmacol 2005;508:77–83.
14. Bagli E, Stefaniotou M, Morbidelli L, et al. Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3′-kinase activity. Cancer Res 2004;64:7936–46.
15. Shi RX, Ong CN, Shen HM. Luteolin sensitizes tumor necrosis factor-α-induced apoptosis in human tumor cells. Oncogene 2004;23:7712–21.
16. Fuentes L, Perez R, Nieto ML, Balsinde J, Balboa MA. Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase A2. J Biol Chem 2003;278:44683–90.
17. Mangan JK, Rane SG, Kang AD, et al. Mechanisms associated with IL-6-induced up-regulation of Jak3 and its role in monocytic differentiation. Blood 2004;103:4093–101.
18. Asselin E, Mills GB, Tsang BK. XIAP Regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. Cancer Res 2001;61:1862–8.
19. Mafune K, Tanaka Y, Mimori K, et al. Increased expression of ornithine decarboxylase messenger RNA in human esophageal carcinoma. Clin Cancer Res 1999;5:4073–8.
20. Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 2002;9:459–70.
21. Nagane M, Pan G, Weddle JJ, et al. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand *in vitro* and *in vivo*. Cancer Res 2000;60:847–53.
22. Gibson SB, Oyer R, Spalding AC, Anderson SM, Johnson GL. Increased expression of death receptors 4 and 5 synergizes the apoptosis response to combined treatment with etoposide and TRAIL. Mol Cell Biol 2000;20:205–12.
23. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998;281:1680–3.
24. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J Clin Invest 2001;107:135–42.
25. Zauli G, Sancilio S, Cataldi A, et al. PI-3K/Akt and NF-κB/IκBα pathways are activated in Jurkat T cells in response to TRAIL treatment. J Cell Physiol 2005;202:900–11.
26. Leverkus M, Sprick MR, Wachter T, et al. Proteasome inhibition results in TRAIL sensitization of primary keratinocytes by removing the resistance-mediating block of effector caspase maturation. Mol Cell Biol 2003;23:777–90.
27. Deveraux QL, Reed JC. IAP family proteins-suppressors of apoptosis. Genes Dev 1999;13:239–52.
28. Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF-κB signals induce the expression of c-FLIP. Mol Cell Biol 2001;21:5299–305.

29. Herrant M, Jacquel A, Marchetti S, et al. Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. Oncogene 2004; 23:7863–73.

30. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 2000;288:874–7.

31. Zhang HG, Wang J, Yang X, Hsu HC, Mountz JD. Regulation of apoptosis proteins in cancer cells by ubiquitin. Oncogene 2004;23:2009–15.

32. Thakkar H, Chen X, Tyan F, et al. Pro-survival function of Akt/protein kinase B in prostate cancer cells. Relationship with TRAIL resistance. J Biol Chem 2001; 276:38361–9.

33. Dan HC, Sun M, Kaneko S, et al. Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). J Biol Chem 2004;279:5405–12.

34. Harper N, Hughes MA, Farrow SN, Cohen GM, MacFarlane M. Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. J Biol Chem 2003;278:44338–47.

35. Tanaka Y, Gavrielides MV, Mitsuuchi Y, Fujii T, Kazanietz MG. Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J Biol Chem 2003;278:33753–62.

36. Trauzold A, Wermann H, Arlt A, et al. CD95 and TRAIL receptor-mediated activation of protein kinase C and NF-κB contributes to apoptosis resistance in ductal pancreatic adenocarcinoma cells. Oncogene 2001;20:4258–69.

37. Riedl SJ, Renatus M, Schwarzenbacher R, et al. Structural basis for the inhibition of caspase-3 by XIAP. Cell 2001;104:791–800.

38. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. Nature 1997;388:300–4.

39. Hah JO, Lee KA, Choi YJ, et al. Inactive caspase 3 activates Akt in human leukemia cells susceptible or resistant to apoptosis induced by phorbol ester. Int J Oncol 2003;22:1111–6.

40. Nesterov A, Lu X, Johnson M, et al. Elevated AKT activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. J Biol Chem 2001;276: 10767–74.

41. Chen X, Thakkar H, Tyan F, et al. Constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer. Oncogene 2001;20: 6073–83.

42. Meng XW, Heldebrant MP, Kaufmann SH. Phorbol 12-myristate 13-acetate inhibits death receptor-mediated apoptosis in Jurkat cells by disrupting recruitment of Fas-associated polypeptide with death domain. J Biol Chem 2002;277:3776–83.

43. Sarker M, Ruiz-Ruiz C, Robledo G, Lopez-Rivas A. Stimulation of the mitogen-activated protein kinase pathway antagonizes TRAIL-induced apoptosis downstream of BID cleavage in human breast cancer MCF-7 cells. Oncogene 2002;21:4323–7.

44. Ferriola PC, Cody V, Middleton E Jr. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. Biochem Pharmacol 1989;38:1617–24.

45. Agullo G, Gamet-Payrastre L, Manenti S, et al. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem Pharmacol 1997;53:1649–57.