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Introduction

Tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL), also known as Apo2L (1, 2), is synthesized, similar to other tumor necrosis factor (TNF) superfamily members, as a membrane-bound apo-protein that can be cleaved to generate soluble TRAIL (3). TRAIL is being actively investigated as a cancer therapeutic agent, because different types of tumor cells are vulnerable to apoptotic death by soluble TRAIL, whereas normal cells are relatively insensitive to this effect (4 – 6).

Previous studies show that chemotherapeutic agents (7 – 9) and ionizing radiation (10) can increase TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP (7) or increasing DR 5 gene expression (9 – 11). We hypothesize that quercetin promotes TRAIL-induced apoptotic death by modulating the levels of TRAIL receptors and anti-apoptotic molecules.

In this study, we investigated whether quercetin can promote TRAIL-induced apoptotic death in prostate cancer. Epidemiological studies have shown that the consumption of vegetables, fruits and tea, of which quercetin is frequently a component, is associated with a low risk of cancer (12). Quercetin has been shown to inhibit the enzymes involved in proliferation and signal transduction pathway including protein kinase C (13), tyrosine kinase(14), cdc25 phosphatase (15), PI-3 kinase (16), DNA topoisomerase II (17), proline-directed protein kinase fatty acid in human prostate carcinoma cells (18), and c-Jun N-terminal kinase (JNK) (19). Quercetin has a wide range of biological activities including inhibition of mutant p53 expression (20), and androgen receptor expression and function in LNCaP cells (21). Quercetin potentiates the cytotoxic action of 1-β-D-arabinofuranosylcytosine (22). Quercetin also inhibits cell invasion and induces apoptosis through a pathway involving heat shock proteins (23). These activities of quercetin make it a promising candidate for treatment and prevention of various cancers including prostate cancer. Moreover, quercetin-mediated apoptosis may result from the induction of stress proteins, disruption of microtubules and mitochondrial, release of cytochrome c, and activation of caspases (19, 24 – 26). However, the role of Akt phosphorylation in the quercetin-induced apoptosis in the TRAIL treated cells are still not clear.

In this study described here, we investigated the effects of quercetin in combination with TRAIL-resistant human prostate cancer cells. We hypothesized that treatment with quercetin enhances TRAIL-induced apoptosis by activation of the PI3K-Akt signaling pathway and the caspase cascade. Our studies demonstrate that quercetin augments TRAIL-induced apoptosis by the dephosphorylation of Akt which subsequently leads to an increase in caspase activation.

Body :

The long-term goal of our research project is to develop a novel therapy for HER-2/neu overexpressing prostate cancer. Previous studies have shown that the HER-2/neu homodimer constitutively activates the PI(3)K-Akt-NF-κB signal transduction pathway. In this budget period, we examined whether quercetin, a PI3K inhibitor, promotes TRAIL cytotoxicity by inhibiting Akt activity. As DU-145 cells were treated with quercetin, we observed that TRAIL-induced cytotoxicity was promoted. Our observations are illustrated below:

Sensitization for TRAIL-induced apoptosis by Quercetin

In search of novel strategies to target tumor cells, we investigated an antitumor effect of the chemopreventive, natural compound quercetin on human tumor cell line. To investigate the effect of quercetin on TRAIL-induced cytotoxicity, human prostatic adenocarcinoma DU-145 cells were treated with TRAIL in the presence or absence of quercetin. As shown in Figure 1A, no cytotoxicity was observed with quercetin (10-200 μM) alone. Given the lack of low cytotoxic activity of quercetin as a single agent, we then tested quercetin in combination treatments. Surprisingly, we observed that quercetin acted in synergy with the death ligand TRAIL to increase apoptosis in DU-145 cells in a dose-dependent manner. TRAIL plus quercetin significantly induced cell death in a concentration-dependent manner; 90, 60, and 40% of the cells survived after exposure to 10, 50, and 200 μM quercetin, respectively, for 4h (Fig. 1B). Similar results were observed by TdT-mediated dUTP Nick end labeling (TUNEL) assay. TUNEL assay showed that apoptotic death was promoted during combined treatment with TRAIL and quercetin (Fig. 1C).

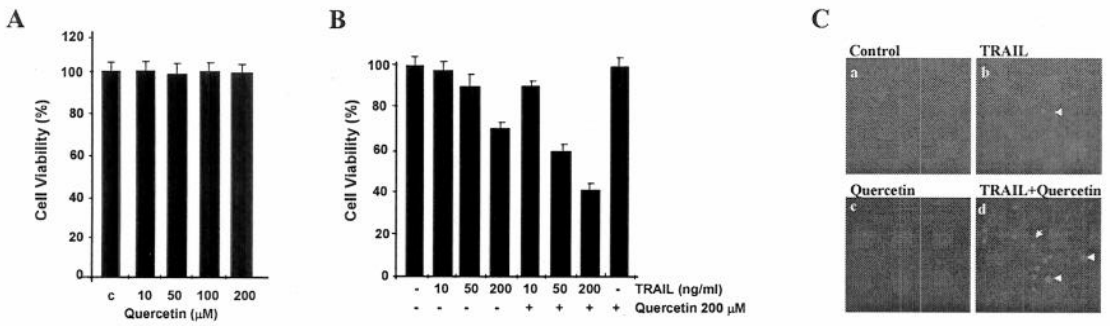
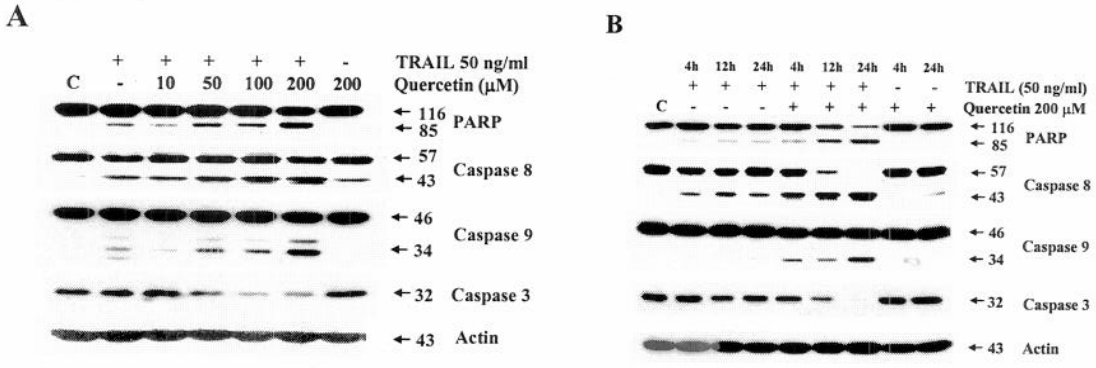


Figure 1. ***Effect of quercetin on TRAIL-induced cytotoxicity in human prostate adenocarcinoma DU-145 cells.*** (A) Cells were treated for 4h with quercetin (10-200 μM). (B) Cells were treated for 4 h with various concentrations of TRAIL (0-200 ng/ml) in the presence or absence of 200 μM quercetin. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error from the mean (SEM) for three separate experiments. (C) Cells were treated for 4 h with TRAIL (50 ng/ml) in the presence or absence of 200 μM quercetin. After treatment, apoptosis was detected by the TUNEL assay. Apoptotic cell are indicated by arrows. (a) untreated control; (b) TRAIL only; (c) quercetin only; (d) TRAIL + quercetin.

Effect of quercetin on TRAIL-induced apoptosis by caspase activation

It has been demonstrated that the proteolytic cleavage of PARP, which synthesizes poly (ADP-ribo) from β-nicotinamide adenine dinucleotide (NDA) in response to DNA strand breaks, is a biochemical event during apoptosis. As PARP cleavage is a hallmark of caspase activation, we determined whether the apoptosis machinery was activated by quercetin and TRAIL treatment, using an anti-PARP antibody. As shown in Figure 2A, PARP (116 kDa) was cleaved yielding a characteristic 85 kDa fragment in the presence of TRAIL. This cleavage was enhanced by treatment with quercetin (Figure 2A). We extended our studies to investigate whether quercetin enhances TRAIL-induced cytotoxicity by increasing the activation of caspases. Figure 2A demonstrates that quercetin promoted TRAIL-induced caspase-8 activation. Western blot analysis shows that procaspase-8 (57 kDa) was cleaved to the intermediates (43 kDa) in the presence of TRAIL, and the cleavage of procaspase-8 was promoted by treatment with quercetin. The combined treatment of TRAIL and quercetin also resulted in an increase in caspase-9 activation. TRAIL induced proteolytic processing of procaspase-9 (46 kDa) into its active form (34 kDa), and the activation of caspase-9 induced by TRAIL was enhanced by quercetin. Quercetin also increased TRAIL-induced caspase-3 activation. Western blot analysis shows that procaspase-3 (32 kDa), the precursor form of caspase-3, decreased the level of the procaspase-3 form in the presence of TRAIL. After treatment of DU-145 cells with TRAIL (50 ng/ml) for various times (4, 12, 24h), immunoblot analysis of cell lysates demonstrated processed polypeptides for both initiator caspases, caspase-8 and caspase-9, and processed forms of caspase-3, an effector caspase, and PARP cleaveage (Fig. 2B). Although quercetin (200 μM) alone did not activate this signaling cascade, quercetin enhanced TRAIL-induced activation of the caspases-8, -9, -3 and the associated cleavage of PARP. Having shown that sensitization of TRAIL-induced cytotoxicity by treatment with quercetin is associated with an increase in the activation of caspases-8, -9, and -3, we next wanted to see if this sensitization was blocked using Z-IETD-fmk (caspase-8 inhibitor), Z-LEHD-fmk (caspase-9 inhibitor), Z-DEVD-fmk (caspase-3 inhibitor) peptides capable of inhibiting caspase activity. The presence of Z-IETD-fmk and Z-DEVD-fmk significantly reduced the ability of quercetin to sensitize cells to TRAIL in DU-145 cells, but Z-LEHD-fmk only partially inhibited the sensitization (Fig 2C). These results provide further evidence that the quercetin-enhanced TRAIL cytotoxicty involves a caspase-8 and caspase-3-dependent pathway.



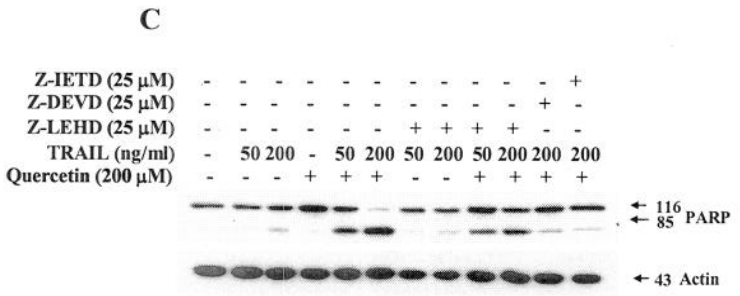


Figure 2. ***Effect of quercetin on TRAIL-induced proteolytic cleavage of PARP and activation of caspases and caspase inhibitors in DU-145 cells.*** (A) Cells were treated for 4 h with various concentrations of quercetin in the presence or absence of 50 ng/ml TRAIL and then harvested. Cell lysates were subjected to immunoblotting for casapse-8, caspase-9, caspase-3, or PARP. Antibody against caspase-8 detected the inactive form (57 kDa) and cleaved intermediate (43 kDa). Anti-caspase-9 antibody detected both the inactive form (46 kDa) and cleaved intermediate (34 kDa). Anti-caspase-3 antibody detected the inactive form (32 kDa). (B) Cells were treated for various times with quercetin in the presence or absence of TRAIL and then harvested. Cell lysates were subjected to immunoblotting for caspase-8, -9, -3, or PARP. (C) DU-145 cells were pretreated with caspase inhibitors (caspase-8 inhibitor: Z-IETD-fmk 25 μM, caspase-9 inhibitor: Z-LEHD-fmk 25 μM, caspase-3 inhibitor: Z-DEVD-fmk 25 μM,) for 30 min, then cells were treated with a combination of quercetin (200 μM) and TRAIL 50 to 200 ng/ml. Cell lysates were subjected to immunoblotting for PARP. Actin was used to confirm the amount of proteins loaded in each lane.

Effect of quercetin on the level of the TRAIL receptor family and antiapoptotic proteins

Previous studies demonstrated that increased DR5 levels induced by genotoxic agents (Sheikh *et al.*, 1998; Chinnaiyan *et al.*, 2000; Nagane *et al.*, 2000) or decreased FLIP expression induced by glucose deprivation (Nam *et al.*, 2002) is responsible for increasing TRAIL cytotoxicity. Thus, we examined whether changes in the amounts of TRAIL receptors and antiapoptotic proteins are associated with the promotion of apoptosis by TRAIL in combination with quercetin. DU-145 cells were treated with 50 ng/ml TRAIL in the presence of quercetin (10-200 μM). Data from Western blot analysis reveal that the combined treatment did not significantly alter the levels of DR4, DR5, or DcR2 protein expression (Fig. 3A). As antiapoptotic proteins such as $FLIP_L$, $FLIP_S$, cIAP-1, cIAP-2, survivin, Bcl-X_L, and Bcl-2 have also been implicated in the regulation of TRAIL-induced apoptosis, expression of these proteins was next assessed in the presence of quercetin (10-200 μM). Neither $FLIP_L$, $FLIP_S$, cIAP-1, cIAP-2, survivin, Bcl-X_L, nor Bcl-2 cellular protein level was altered by quercetin treatment of DU-145 cells (Fig. 3B). Quercetin alone also did not change the levels of TRAIL receptors and antiapoptotic proteins.

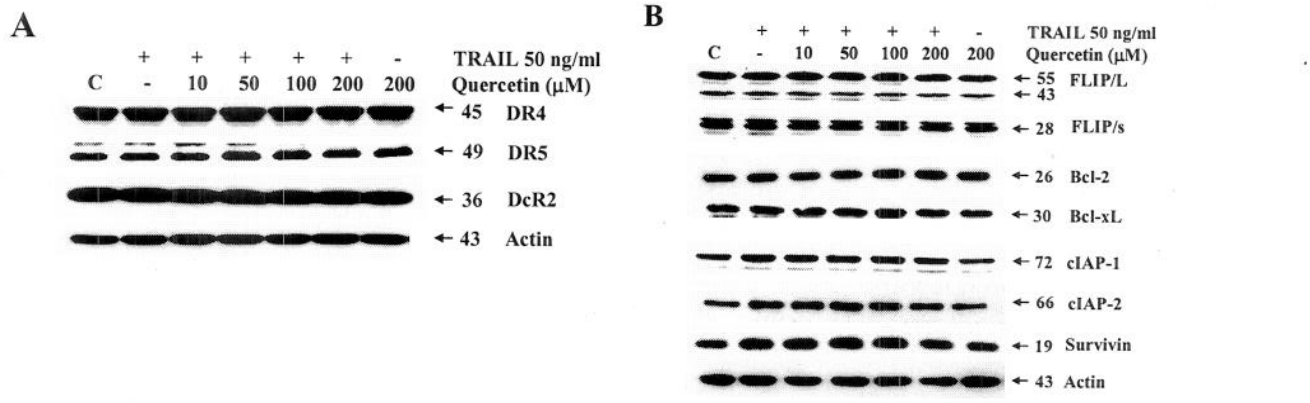


Figure 3. ***Intracellular levels of TRAIL receptors (A) or antiapoptotic proteins (B) during treatment with quercetin in the presence or absence of TRAIL.*** DU-145 cells were treated for 4 h with various concetrations of quercetin (10-200 μM) in the presence or absence of 50 ng/ml TRAIL. Equal amounts of protein (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as described in Materials and Methods.

Effect of quercetin on kinases associated with the PI3K-Akt pathway

It is well known that elevated Akt activity protects cells from TRAIL-induced apoptosis (Nesterov *et al.*, 2001). We postulated that quercetin inhibits Akt activity and consequently enhances TRAIL-induced cytotoxicity. Previous studies demonstrated that Akt activation is regulated through the PI3K-Akt pathway. We examined whether quercetin specifically affects the PI3K-Akt pathway-associated kinases. We observed that quercetin induced dephosphorylation of Akt but not PI3K or PDK-1 (Fig. 4A). To examine whether quercetin inhibits Akt activity by dephosphorylating Akt, We treated DU-145 cells with 200 μM quercetin for various times (10-240 min) measured the level of phophorylated Akt (Fig. 4B). We observed that Akt was rapidly dephosphorylated within 10 min of quercetin addition without changing the Akt protein level. Previous studies have demonstrated that quercetin interacts in the PI3K ATP binding site (Walker *et al.*, 2000). Our results indicate that quercetin enhanced TRAIL-induced apoptosis is coupled with PI3K-Akt pathway activity without changes in the level of PI3K. It is therefore possible that quercetin may inactivate PI3K to induce cell death in a TRAIL-induced apoptosis.

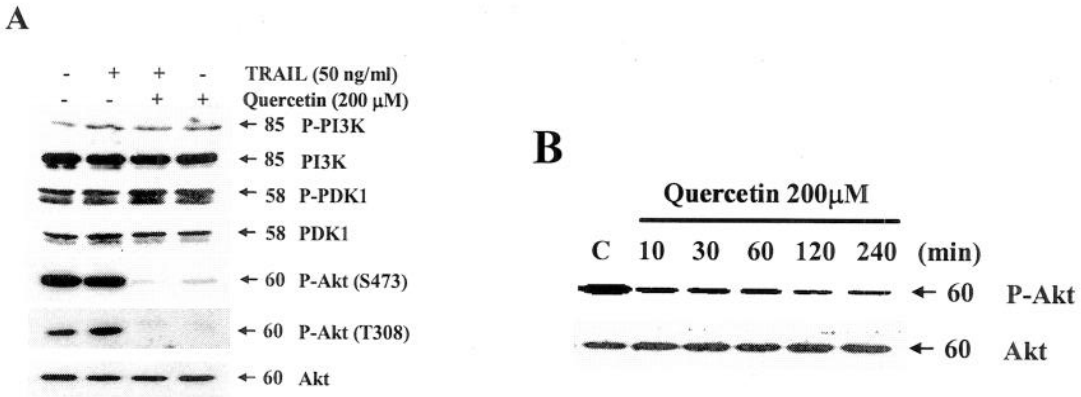


Figure 4. ***Effect of quercetin on P13K-Akt pathway-associated kinases in the presence or absence of TRAIL.*** (A) DU-145 cells were treated for 4 h with 200 μM quercetin in the presence or absence of 50 ng/ml TRAIL. Cells were treated with quercetin (200 μM) for 4 h and then harvested. (B) DU-145 cells were treated for various times with 200 μM quercetin. (C) DU-145 cells were treated for 4h with various concentrations of quercetin in the presence or absence of 50 ng/ml TRAIL. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-phospho-PI3K, anti-PI3K, anti-phospho-PDK1, anti-PDK1, anti-phospho-Akt (S473, T308), and anti-Akt antibody. Actin was shown as an internal standard.

Effect of PI3K inhibitor, LY294002 on TRAIL-induced apoptosis

TRAIL treatment did not alter quercetin-induced dephosphorylation of Akt (Fig. 5A). The effect of quercetin on Akt was compared with LY294002, an inhibitor of PI3K. Figures 5A and 5B shows that all these drugs induced dephosphorylation of Akt. These results indicate that Akt inactivation (dephosphorylation) is responsible for the quercetin-induced enhancement of TRAIL cytotoxicity.

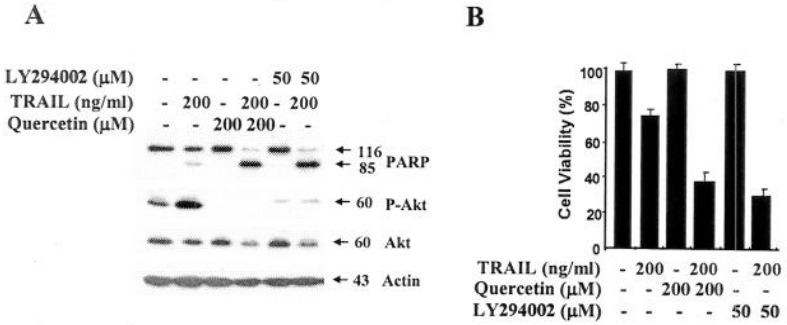


Figure 5. ***Effect of LY294002 on TRAIL-induced cytotoxicity in DU-145 cells.*** Cells were treated with 25 μM LY294002 for 30 min and then treated with TRAIL in the presence or absence of 200 μM quercetin for 4 h. (A) Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted as described in "Materials and Methods". (B) Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean

(SEM) from three separate experiments. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP, anti-phospho-Akt, and anti-Akt antibody. Actin is shown as an internal standard.

Quercetin enhances TRAIL-induced apoptosis in LNCaP, but not in YPEN-1 cells

We also examined whether quercetin promotes TRAIL-induced apoptosis in other prostate cancer cells, LNCaP and normal prostate YPEN-1 cells. Our results indicate that for LNCaP cells, like DU-145 cells, TRAIL and quercetin induce apoptosis as indicated by PARP cleavage but for YPEN-1 cells, unlike DU-145 cells, neither TRAIL nor quercetin induce PARP cleavage (Fig. 6).

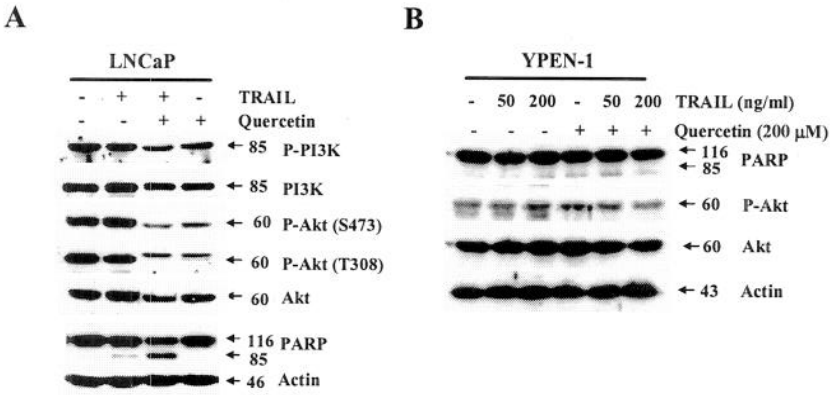


Figure 6. *Effect* ***of quercetin on TRAIL-induced proteolytic cleavage of PARP in LNCaP and YPEN-1 cells.*** (A) LNCaP cells were treated for 4 h with TRAIL (50 ng/ml) in the presence or absence of 200 μM quercetin and then harvested. (B) YPEN-1 cells were treated for 4 h with various concentrations of TRAIL (50, 200 ng/ml) in the presence or absence of 200 μM quercetin and then harvested. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt, anti-Akt and anti-PARP antibody. Actin was shown as an internal standard.

Activated Akt rescues quercetin-induced dephosphorylation of Akt as well as quercetin-enhanced TRAIL cytotoxicity

To further explore Akt function, we expressed a constitutively active form of Akt, constructed by fusing Akt to the myristoylation signal of Src protein (myr-Akt) was introduced into DU-145 cells by adenovirus-mediated gene transfer. Upon infection with the Akt adenovirals, an increase in the expression of the corresponding proteins was detected, as judged by Western blot using an anti-Akt antibody (Fig. 7). As expected, high phospho-Akt levels were observed in DU-145 cells infected with the constitutively active myr-Akt. Interestingly, expression of myr-Akt significantly reduced the apoptotic effect of quercetin plus TRAIL. The effect was proportional to the expression levels of myr-Akt. On the other hand, infection with a control vector (Ad.vector) had no effect. Taken together, these data suggest that inhibition of Akt activity is critical for quercetin plus TRAIL-mediated apoptosis in DU-145 prostate cancer cells.

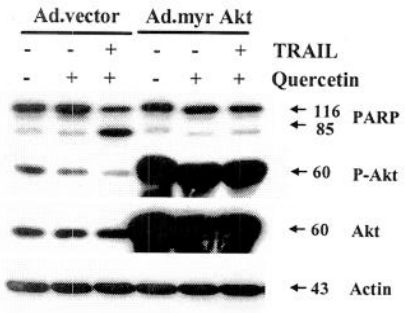


Figure 7. ***Overexpression of constitutively active Akt inhibits potentiation of TRAIL-induced apoptosis by quercetin.*** DU-145 cells were infected with a control adenoviral vector (Ad.vector) or an adenoviral vector containing active form of Akt (Ad.myr Akt) for 24 h. Infected cells were treated with quercetin (200 μM) or quercetin plus TRAIL (50 ng/ml) for 4 h. Cell lysates were subjected to immunoblotting for anti-PARP, anti-phospho-Akt, anti-Akt, or anti-actin.

A model for the effect of quercetin on the TRAIL-induced apoptotic pathway

Figure 8 shows a schematic diagram of a model which is based on the literature and our data. Quercetin blocks the PI3K-Akt survival signal pathway. The inhibition of this pathway enhances the activation of caspases through a TRAIL-induced apoptotic signal.

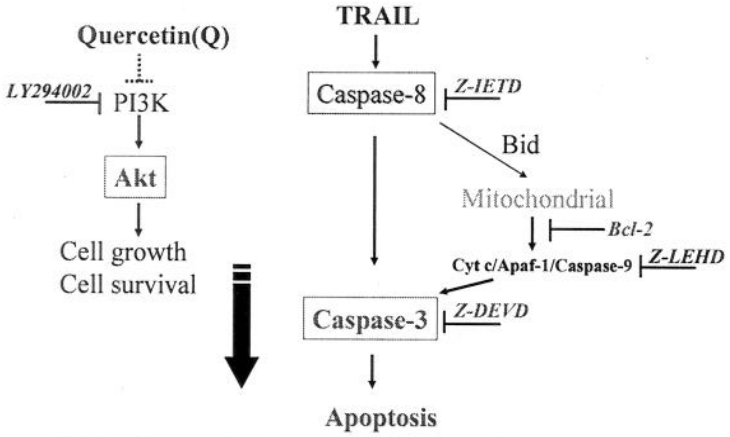


Figure 8. ***Tentative model for the mechanism of quercetin and TRAIL-induced apoptotic pathways.***

Key research accomplishments:

We previously proposed that the PI3K-Akt-NF-κB signal plays an important role in TRAIL sensitivity. In this study we observed that quercetin promotes TRAIL-induced cytotoxicity in the human prostate adenocarcinoma DU-145 cell line. The mechanism of this enhancement is shown to be almost certainly due to inhibition of Akt by treatment with quercetin. Thus, our data support our proposed hypothesis.

Reportable Outcomes

(1) Kim, Y.H. and Lee, Y.J. The Sequence and The Time Interval between TRAIL and Cisplatin Treatment Are Responsible for A Complex Pattern of Synergistic Cytotoxicity. J. Cell. Biochem., In pres.

(2) Kim, Y.H., and Lee, Y.J. TRAIL Apoptosis Is Enhanced by Quercetin through Akt Dephosphorylation. Submitted.

Conclusions

We have shown that quercetin can potentiate TRAIL-induced apoptotic death quercetin through activating caspase activity in human prostate cancer cells. Quercetin, an inhibitor of the PI3K-Akt-NF-κB signal transduction pathway by dephosphorylation of Akt, enhances TRAIL-induced cytotoxicity *in vitro*. Therefore, quercetin may also be a useful drug to promote TRAIL cytotoxicity in HER-2/neu overexpressing prostate tumor cells.

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