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TRAIL Apoptosis Is Enhanced by Quercetin Through Akt Dephosphorylation

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Abstract TNF-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapy that preferentially induces apoptosis in cancer cells. However, many neoplasms are resistant to TRAIL by mechanisms that are poorly understood. Here we demonstrated that human prostate cancer cells, but not normal prostate cells, are dramatically sensitized to TRAIL-induced apoptosis and caspase activation by quercetin. Quercetin, a ubiquitous bioactive plant flavonoid, has been shown to inhibit the proliferation of cancer cells. We have shown that quercetin can potentiate TRAIL-induced apoptotic death. Human prostate adenocarcinoma DU-145 and LNCaP cells were treated with various concentrations of TRAIL (10–200 ng/ml) and/or quercetin (10–200 μM) for 4 h. Quercetin, which caused no cytotoxicity by itself, promoted TRAIL-induced apoptosis. The TRAIL-mediated activation of caspase, and PARP (poly(ADP-ribose) polymerase) cleavage were both enhanced by quercetin. Western blot analysis showed that combined treatment with TRAIL and quercetin did not change the levels of TRAIL receptors (death receptors DR4 and DR5, and DcR2 (decoy receptor 2)) or anti-apoptotic proteins (FLICE-inhibitory protein (FLIP), inhibitor of apoptosis (IAP), and Bcl-2). However, quercetin promoted the dephosphorylation of Akt. Quercetin-induced potent inhibition of Akt phosphorylation. Taken together, the present studies suggest that quercetin enhances TRAIL-induced cytotoxicity by activating caspases and inhibiting phosphorylation of Akt. J. Cell. Biochem. 100: 998–1009, 2007.

Key words: quercetin; TRAIL; apoptosis; caspase; Akt

Tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL), also known as Apo2L [Wiley et al., 1995; Pitti et al., 1996], 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 [Rus et al., 2005]. 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 [Griffith and Lynch, 1998a; Walczak et al., 1999; Zhang et al., 1999].

Previous studies show that chemotherapeutic agents [Griffith et al., 1998b; Keane et al., 1999; Nagane et al., 2000] and ionizing radiation [Chinnaiyan et al., 2000] can increase TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP [Griffith et al., 1998b] or increasing DR5 gene expression [Sheikh et al., 1998; Chinnaiyan et al., 2000; Nagane et al., 2000]. 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. 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 [Block et al., 1992]. Quercetin has been shown to inhibit the enzymes involved in proliferation and in the signal transduction pathway including protein

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kinase C [Agullo et al., 1997], tyrosine kinase [Hagiwara et al., 1988], cdc25 phosphatase [Aligiannis et al., 2001], PI-3 kinase [Gamet-Payrastre et al., 1999], DNA topoisomerase II [Constantinou et al., 1995], proline-directed protein kinase fatty acid in human prostate carcinoma cells [Lee et al., 1998], and c-Jun N-terminal kinase (JNK) [Yoshizumi et al., 2001]. Quercetin has a wide range of biological activities including inhibition of mutant p53 expression [Avila et al., 1994] and androgen receptor expression and function in LNCaP cells [Xing et al., 2001]. Quercetin potentiates the cytotoxic action of 1-β-D-arabinofuranosylcytosine [Teofili et al., 1992]. Quercetin also inhibits cell invasion and induces apoptosis through a pathway involving heat shock proteins [Wei et al., 1994]. These activities of quercetin make it a promising candidate for treatment and prevention of various cancers. 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 [Wang et al., 1999; Yoshizumi et al., 2001; Gupta and Panda, 2002; Ong et al., 2004]. However, the role of Akt phosphorylation in the quercetin-induced apoptosis in the TRAIL-treated cells is still not clear.

In this study described here, we investigated the effects of quercetin in combination with TRAIL on human prostate cancer cells. We hypothesized that treatment with quercetin enhances TRAIL-induced apoptosis by inhibiting the PI3K-Akt signaling pathway and subsequently promoting 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.

MATERIALS AND METHODS

Cell Culture and Survival Assay

Human prostate adenocarcinoma LNCaP and DU-145 cell lines, and normal prostate YPEN-1 cell line were obtained from the American Type Culture Collection (Manassas, VA). LNCaP and YPEN-1 or DU-145 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) or DMEM medium (Gibco BRL, Gaithersburg, MD), respectively, with 10% fetal bovine serum (HyClone, Logan, Utah) and 26 μM sodium bicarbonate for monolayer cell culture. The dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. At 1 day prior to the experiment, cells were plated into 60-mm dishes. For trypan blue exclusion assay [Burow et al., 1998], trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min and examined under a light microscope. At least 300 cells were counted for each survival determination.

Drug Treatment

Quercetin was obtained from Sigma Chemical Co. (St. Louis, MO). LY294002 was purchased from Calbiochem (San Diego, CA). A stock solution was prepared in DMSO.

Antibodies

Polyclonal anti-phospho-P13K, anti-phospho-PDK1, anti-PDK1, anti-Bc1-X_L, anti-caspase-3, and anti-caspase-9 antibodies were purchased from Santa Cruz (Santa Cruz, CA), anti-DR5 and anti-DcR2 from StressGen (Victoria, BC, Canada), anti-DR4 from Upstate Biotechnology (Lake Placid, NY), anti-cIAP-1, anti-cIAP-2, and anti-survivin from R&D Systems (Minneapolis, MN), anti-phospho-Akt and anti-Akt from Cell Signaling (Beverly, MA), and anti-FLIP from Calbiochem. Monoclonal antibodies were purchased from the following companies: anti-P13K from Santa Cruz, anti-caspase-8 from Upstate Biotechnology, anti-PARP from Biomol Research Laboratory (Plymouth Meeting, PA), and anti-Bcl-2 and anti-actin from ICN (Costa Mesa, CA).

Production of Recombinant TRAIL

A human TRAIL cDNA fragment (amino acids 114–281) obtained by RT-PCR was cloned into a pET-23d (Novagen, Madison, WI) plasmid, and His-tagged TRAIL protein was purified using the Qiagen express protein purification system (Qiagen, Valencia, CA).

TUNEL Assay

For detection of apoptosis by the TUNEL method, cells were plated in slide chambers. After treatment, cells were fixed with 70% ethanol in PBS. Cells were washed once, permeabilized by incubating with 100 μl of 0.1% Trioton X-100 and 0.1% sodium citrate,

and then washed twice with in PBS. The TUNEL reaction was carried out at 37°C for 1 h with 0.3 nmol of fluorescein isothiocyante-12-dUTP, 3 nmol of dATP, 2 μl pf $CoCl_2$, 25 U of terminal deoxynucleotidyl transferase, and TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate) in a total reaction volume of 50 μl. The reaction was stopped with 2 μl of 0.5 M EDTA. Cells were observed under a fluorescence microscope.

Protein Extracts and PAGE

Cells were lysed with 1 × Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, and 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with 1 × lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS–polyacrylamide gels (SDS–PAGE). SDS–PAGE analysis was performed according to Laemmli [1970] using a Hoefer gel apparatus.

Infection of Adenoviruses

In brief, all recombinant adenoviruses were constructed by employing the Cre-lox recombination system [Hardy et al., 1997]. The selective cell line CRE8 had a β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells. Cells (5×10^5) were plated into a 6-well plate 1 day before transfection. For the production of recombinant adenovirus, CRE8 cells were co-transfected with shuttle vector and ψ 5 viral genomic DNA by using LipofectAMINE Reagent (Invitrogen). The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and ψ 5 viral DNA. The new virus had an intact packaging site and carried a recombinant gene. Plaques were harvested, analyzed, and purified.

Immunoblot Analysis

Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% non-fat dry milk in PBS-Tween-20 (0.1%, v/w) at 4°C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights IL). Quantitation of X-ray film was carried out by scanning densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA) using area integration.

In Vitro Kinase Assay

DU-145 cells were lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution (Sigma). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with mouse Akt antibody (Cell Signaling) and protein G-agarose (Santa Cruz Biotechnology). The beads were washed twice with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution and washed once with the kinase buffer solution, and then they were subjected to kinase assays. To examine the Akt catalytic activity, GST-tagged fusion GSK protein (Cell Signaling) was used as a substrate of Akt. One microgram of GSK was incubated with immunoprecipitated Akt in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM sodium orthovanadate, 2 mM DTT, and 20 μM ATP at 30°C for 1 h. Finally, the reaction was stopped by adding 2× Laemmli buffer. Phosphorylated proteins were resolved by SDS–PAGE and analyzed by immunoblotting using anti-phospho-GSK3α/β (Ser 21/9) antibody (Cell Signaling).

RESULTS

Sensitization for TRAIL-Induced Apoptosis by Quercetin

In search of novel strategies to target tumor cells, we investigated an anti-tumor 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 within 4 h. Unlike short-term

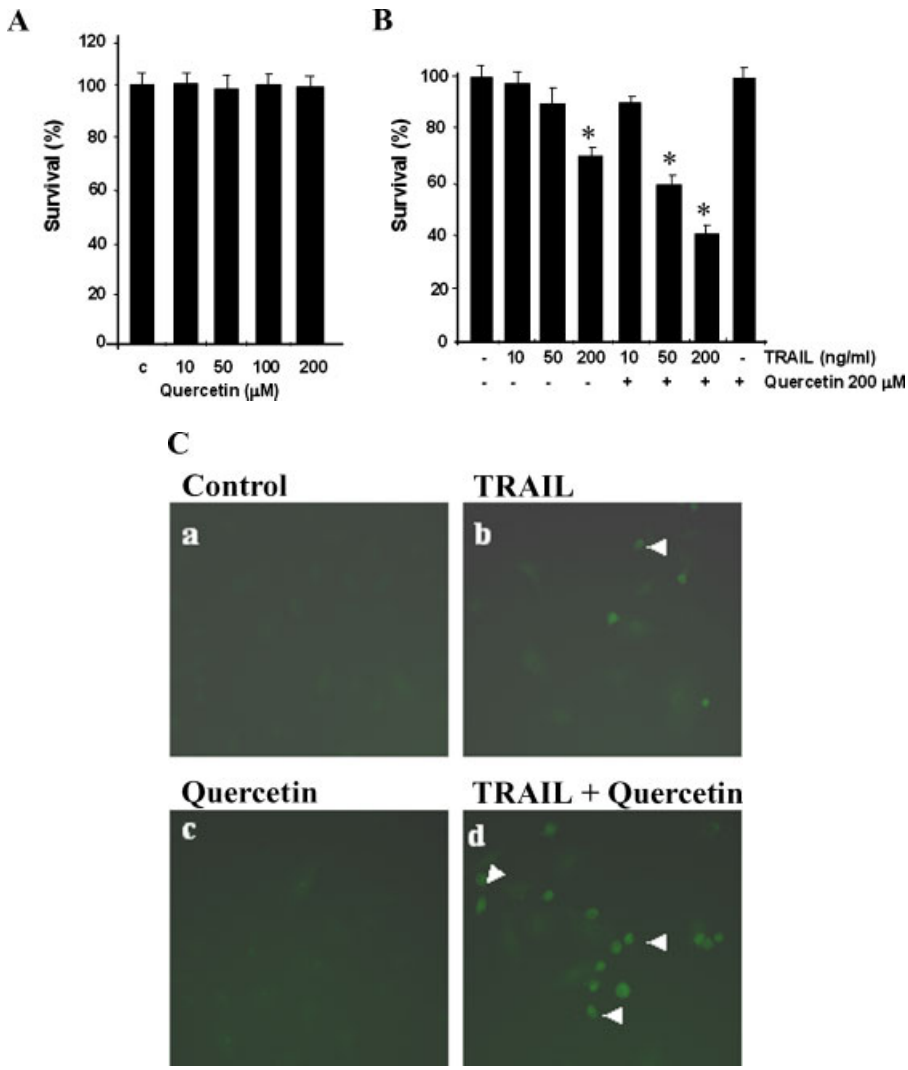

Fig. 1. Effect of quercetin on TRAIL-induced cytotoxicity in human prostate adenocarcinoma DU-145 cells. **A**: Cells were treated for 4 h with quercetin (10–200 μM). **B**: Cells were treated for 4 h with various concentrations of TRAIL (0–200 ng/ml) in the presence or absence of 200 μM quercetin. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error from the mean (SEM) for three separate experiments. Asterisks indicate values which are different from the respective control (*t*-test, $P < 0.05$). **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 cells are indicated by arrows. **a**: Untreated control; (**b**) TRAIL only; (**c**) quercetin only; (**d**) TRAIL + quercetin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treatment, long-term treatment with quercetin leads to cytotoxicity in a variety of prostate cancer cells [Huynh et al., 2003; Vijayababu et al., 2005]. Given the lack of low cytotoxic activity of quercetin as a single agent during short-term treatment, 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 4 h (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 50 ng/ml TRAIL and 200 μM quercetin (Fig. 1C).

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 (Fig. 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. Data from Western blot analysis (Fig. 2A) and its densitometer analysis (Fig. 2B) clearly show that procaspase-8 (57 kDa) was cleaved to the

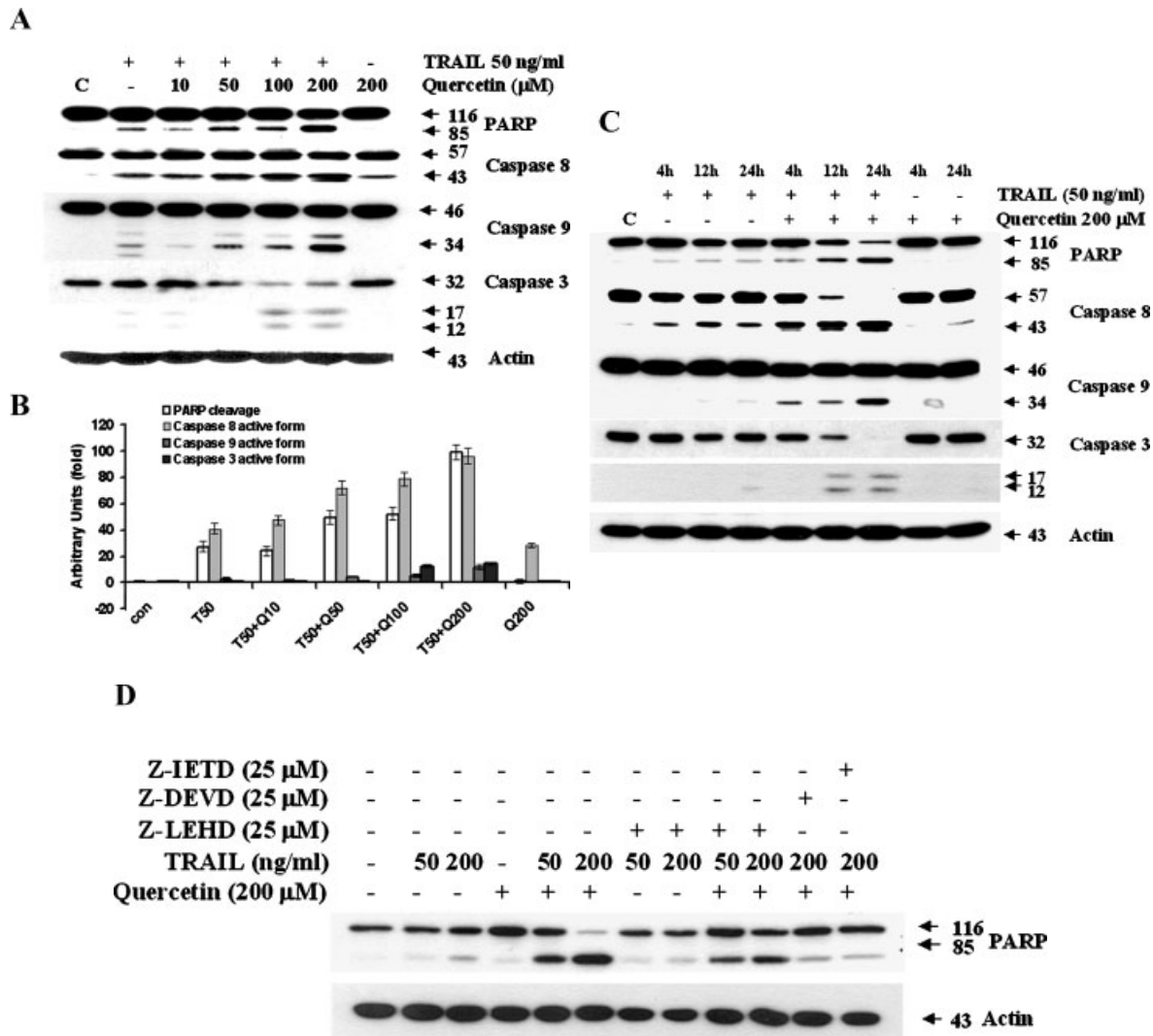

Fig. 2. Effect of quercetin on TRAIL-induced proteolytic cleavage of PARP and activation of caspases and caspase inhibitors in DU-145 cells. **A**: Cells were treated for 4 h with various concentrations of quercetin in the presence or absence of 50 ng/ml TRAIL and then harvested. Cell lysates were subjected to immunoblotting for caspases-8, -9, -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**: The immunoblot was analyzed with a densitometer. **C**: 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 caspases-8, -9, -3, or PARP. **D**: 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 50–200 ng/ml. Cell lysates were subjected to immunoblotting for PARP. Actin was used to confirm the amount of proteins loaded in each lane.

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, was cleaved to its active form (17 and 12 kDa) by treatment with TRAIL and quercetin. After treatment of DU-145 cells with TRAIL (50 ng/ml) for various times (4, 12, 24 h), 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 cleavage (Fig. 2C). Although treatment with quercetin (200 μM) alone for 4 h 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), and Z-DEVD-fmk (caspase-3 inhibitor) peptides capable of inhibiting caspase activity. The inhibitors are included in Figure 8, a schematic diagram of the effect of quercetin on the TRAIL-induced apoptotic pathway. 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. 2D). These results provide further evidence that the quercetin-enhanced TRAIL cytotoxicity involves a caspase-8- and caspase-3-dependent pathway.

Effect of Quercetin on the Level of the TRAIL Receptor Family and Anti-Apoptotic 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 anti-apoptotic 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 anti-apoptotic 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 anti-apoptotic proteins.

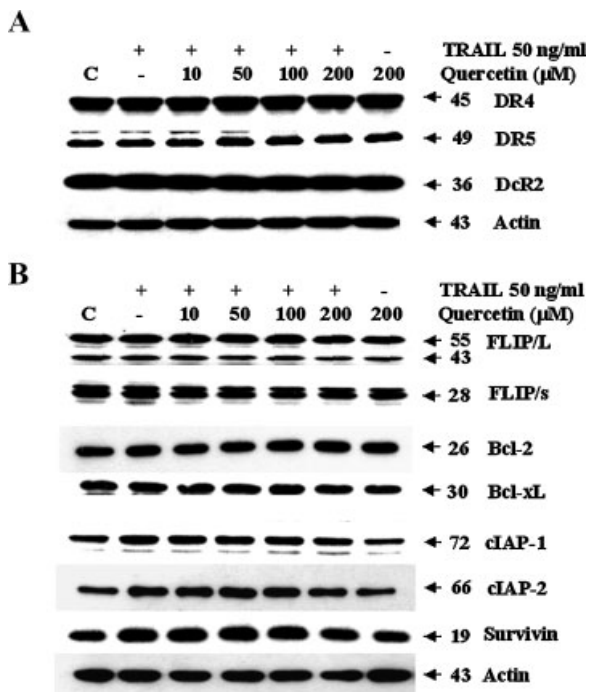

Fig. 3. Intracellular levels of TRAIL receptors (**A**) or anti-apoptotic proteins (**B**) during treatment with quercetin in the presence or absence of TRAIL. DU-145 cells were treated for 4 h with various concentrations of quercetin (10–200 μM) in the presence or absence of 50 ng/ml TRAIL. Equal amounts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

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) and measured the level of phophorylated Akt (Fig. 4B) and its enzyme activity with in vitro kinase assay (Fig. 4C). We observed that Akt was rapidly dephosphorylated within 10 min of quercetin addition without changing the Akt protein level and its activity was markedly inhibited by treatment with quercetin (Fig. 4B,C). 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.

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. Figure 5A,B shows that all these drugs induced dephosphorylation of Akt. These results indicate that Akt inactivation (dephosphorylation) is responsible

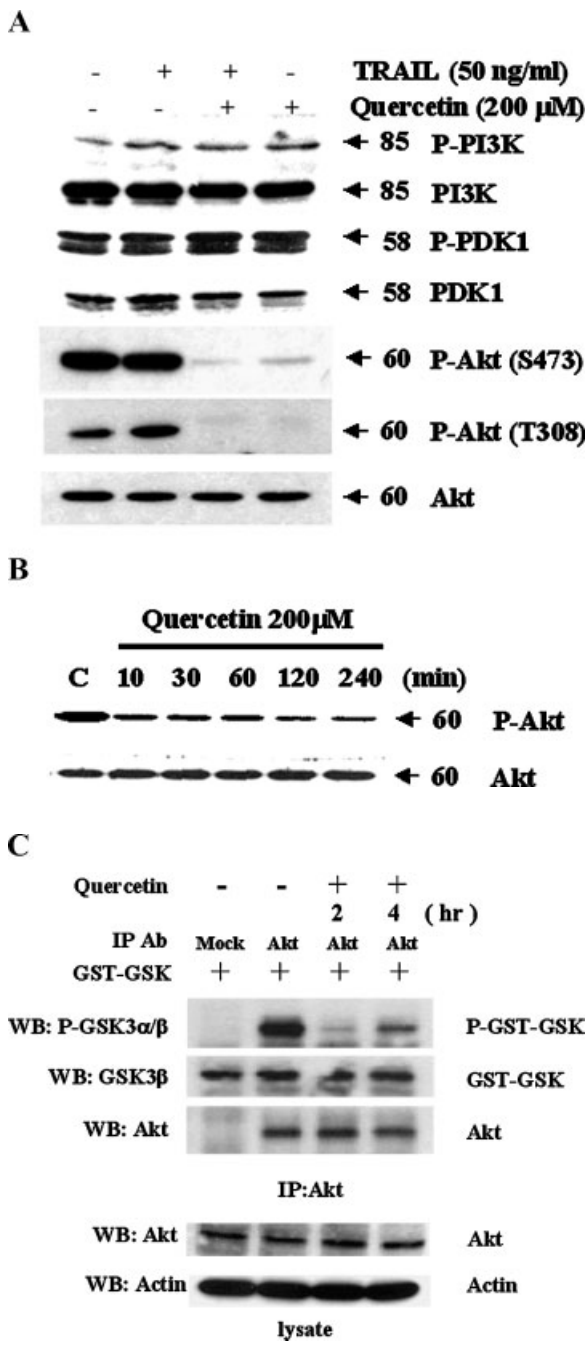

Fig. 4. Effect of quercetin on P13K-Akt pathway-associated kinases in the presence or absence of TRAIL in DU-145 cells. **A**: Cells were treated for 4 h with 200 μM quercetin in the presence or absence of 50 ng/ml TRAIL. Cells were treated with quercetin (200 μM) for 4 h and then harvested. **B**: Cells were treated for various times with 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-PDK1, anti-PDK1, anti-phospho-Akt (S473, T308), and anti-Akt antibody. Actin was shown as an internal standard. **C**: Cells were treated with 200 μM quercetin for 2 or 4 h. Proteins were immunoprecipitated with either mouse IgG (mock IgG) or anti-Akt antibody. The immune complex was incubated with GST-GSK3β. The presence of Akt, GST-GSK3β, or phosphorylated GST-GSK3β was detected with anti-Akt, anti-GSK3β, or anti-phospho-GSK3β antibody, respectively (**upper panels**). Endogenous level of Akt or actin was examined by Western blot analysis with anti-Akt or anti-actin antibody (**bottom panels**).

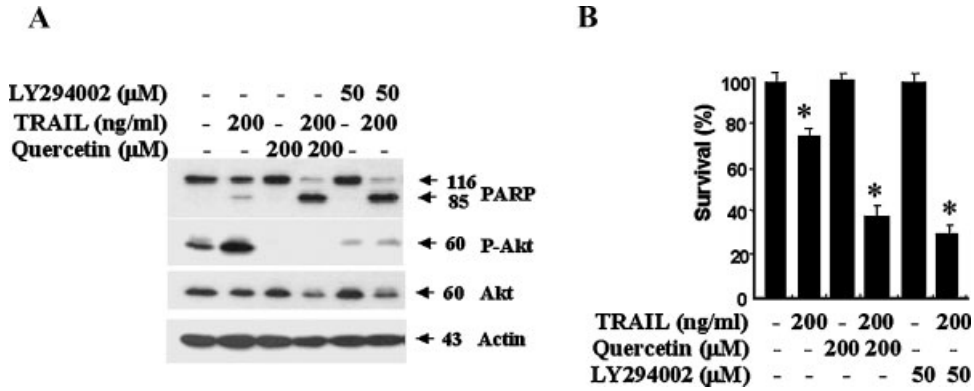

Fig. 5. Effect of LY294002 on TRAIL-induced cytotoxicity in DU-145 cells. Cells were treated with 25 μM LY294002 for 30 min and then treated with TRAIL in the presence or absence of 200 μM quercetin for 4 h. **A**: Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted as described in ''Materials and Methods Section''. **B**: Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. Asterisks indicate values which are different from the respective control (*t*-test, $P < 0.05$). 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.

for the quercetin-induced enhancement of TRAIL cytotoxicity.

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).

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). This 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

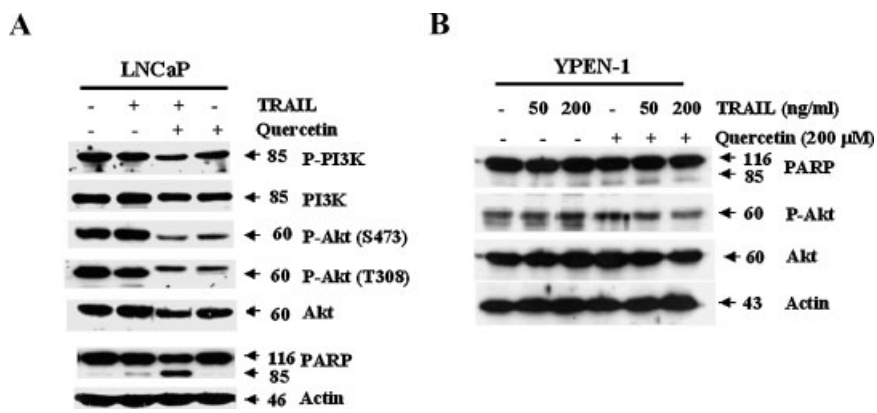

Fig. 6. Effect of quercetin on TRAIL-induced proteolytic cleavage of PARP in LNCaP and YPEN-1 cells. **A**: LNCaP cells were treated for 4 h with TRAIL (50 ng/ml) in the presence or absence of 200 μM quercetin and then harvested. **B**: YPEN-1 cells were treated for 4 h with various concentrations of 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.

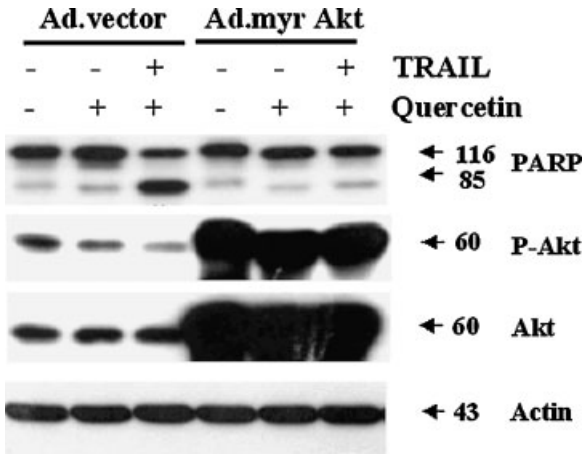

Fig. 7. Overexpression of constitutively active Akt inhibits potentiation of TRAIL-induced apoptosis by quercetin. DU-145 cells were infected with a control adenoviral vector (Ad.vector) or an adenoviral vector containing active form of Akt (Ad.myr Akt) for 24 h. Infected cells were treated with quercetin (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.

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.

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.

DISCUSSION

Because resistance of many tumors to established treatment regimens still constitutes a major concern in oncology, attempts to improve the survival of cancer patients depend largely on strategies to target tumor cell resistance [Peto, 2001]. Induction of apoptosis in cancer cells is a key mechanism for most anti-tumor therapies including chemotherapy, γ-radiation, immunotherapy, or cytokines, and thus, defects in apoptosis programs may cause resistance [Hengartner, 2000; Lowe and Lin, 2000; Herr and Debatin, 2001; Debatin et al., 2002; Johnstone et al., 2002]. One potential strategy to overcome resistance is direct induction of cell death by death receptors, for example, by using TRAIL [Ashkenazi, 2002]. However, many tumors remain resistant towards treatment with TRAIL, which has been related to the dominance of anti-apoptotic signals [Igney and Krammer, 2002]. Other candidates to overcome resistance are naturally occurring antioxidants present in diet and beverages, such as quercetin, which have gained considerable attention

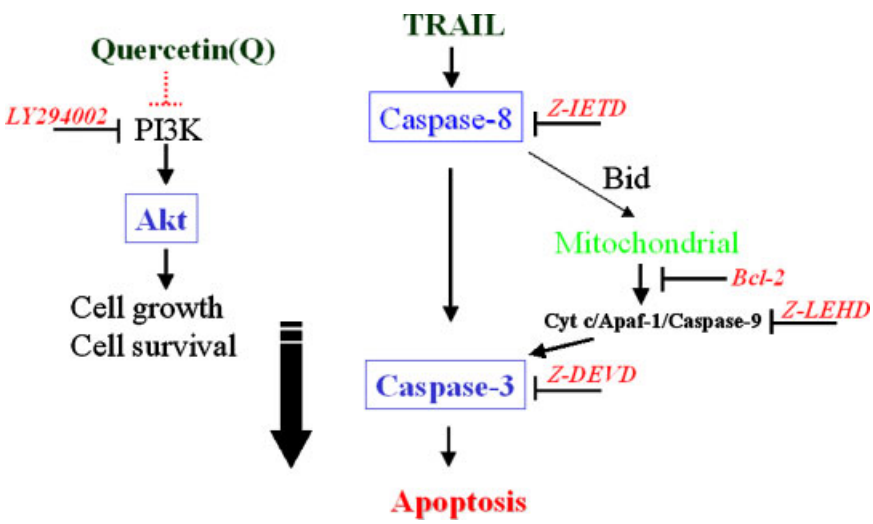

Fig. 8. Tentative model for the mechanism of quercetin and TRAIL-induced apoptotic pathways. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

because of their beneficial effects on health as cancer chemopreventive agents [Jones et al., 2004; Verschoyle et al., 2006].

Quercetin is known to inhibit various enzymes involved in proliferation and apoptosis. This suggests that quercetin experts multiple effects on cellular growth and apoptosis. The target proteins observed may differ from one another depending on experimental condition or cell type. The data presented in this report demonstrate that quercetin is a potent promoter of TRAIL-induced apoptotic death (Fig. 1B). This is probably due to the quercetin-induced dephosphorylation of Akt (Fig. 4), an anti-apoptotic protein. In the present report, we observe that quercetin inhibits Akt phosphorylation. The experimental use of PI3K inhibitors, in particular wortmannin and LY294002, has enabled investigators to link various cellular processes with the involvement of PI3K-dependent pathways. Previous studies showed that Akt activity was also regulated by Ser/Thr phosphatases such as PP2A [Resjo et al., 2002] or PP1 [Xu et al., 2003]. Thus, the possible involvement of PP2A or PP1 quercetin-induced dephosphorylation of Akt needs to be further studied.

Previous studies have shown that constitutively active Akt blocks TRAIL [Chen et al., 2001; Thakkar et al., 2001]. Downregulation of Akt activity by the PI3K inhibitors, wortmannin and LY294002, promotes TRAIL cytotoxicity [Thakkar et al., 2001; Martelli et al., 2003]. In this study, we observed that LY294002 augments TRAIL cytotoxicity in DU-145 cells (Fig. 5). This is probably due to inhibiting Akt-mediated anti-apoptotic effects. A number of pro-apoptotic proteins have been identified as direct Akt substrates, including BAD, caspase-9, and Fork-head transcription factors [Cross et al., 1995; Datta et al., 1997; del Peso et al., 1997; Cardone et al., 1998; Brunet et al., 1999; Hetman et al., 2000]. The pro-apoptotic function of these molecules is suppressed upon phosphorylation by Akt. We believe that blockade of Akt activity by quercetin leads to inhibiting phosphorylation of pro-apoptotic molecules and results in sensitization of TRAIL cytotoxicity. Obviously, further studies are necessary to understand the molecular mechanism of effect of quercetin on TRAIL-induced apoptotic death. Overall, our model in Figure 8 may provide important insights into how quercetin promotes TRAIL-induced apoptotic death. We believe that this model provides a framework for future studies.

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