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Role of HER-2/neu Signaling in Sensitivity to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand: Enhancement of TRAIL-Mediated Apoptosis by Amiloride

Ki M. Kim and Yong J. Lee*

Department of Surgery and Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

Abstract Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in numerous transformed cell lines but not in most normal cells. Although this selectivity offers a potential therapeutic application in cancer, not all cancers are sensitive to TRAIL-mediated apoptosis. In this study, we observed that amiloride, a current clinically used diuretic drug, which had little or no cytotoxicity, sensitized TRAIL-resistant human prostate adenocarcinoma LNCaP and human ovarian adenocarcinoma SK-OV-3 cells. The TRAIL-mediated activation of caspase, and PARP cleavage, were promoted in the presence of amiloride. Western blot analysis showed that combined treatment with TRAIL and amiloride did not change the levels of TRAIL receptors (DR4, DR5, and DcR2) and anti-apoptotic proteins (FLIP, IAP, and Bcl-2). However, amiloride dephosphorylated HER-2/neu tyrosine kinase as well as Akt, an anti-apoptotic protein. Interestingly, amiloride also dephosphorylated PI3K and PDK-1 kinases along with PP1α phosphatase. In vitro kinase assay revealed that amiloride inhibited phosphorylation of kinase as well as phosphatase by competing with ATP. Taken together, the present studies suggest that amiloride enhances TRAIL-induced cytotoxicity by inhibiting phosphorylation of the HER-2/neu-PI3K-Akt pathway-associated kinases and phosphatase. J. Cell. Biochem. 96: 376–389, 2005.

Key words: amiloride; TRAIL; apoptosis; caspase; Akt; PDK-1; HER-2/neu

Amiloride (3,5 diamino-6-chloro-*N*-(diaminomethylene) pyrazinecarboximide) is a diuretic drug and has been known to produce a low pH in the intracellular environment by blocking the intracellular pH regulatory mechanism, the Na^+/H^+ antiport, on the plasma membrane surfaces of mammalian cells [Zhuang et al., 1984; Mahnensmith and Aronson, 1985]. Amiloride is also known to inhibit tyrosine kinase activity of growth factor receptors [Davis and Czech, 1985], Na^+/Ca^{2+} exchange [Smith et al., 1982], (Na^+–K^+)-ATPase [Soltoff and Mandel, 1983], and serine kinase activity [Ralph et al., 1982]. Previous studies demonstrated that amiloride enhances thermal killing and inhibits development of thermotolerance [Miyakoshi et al., 1986; Kim et al., 1991]. Recently, we observed that amiloride enhances TRAIL-induced apoptotic death in human prostate adenocarcinoma DU-145 cells (an androgen-independent cell line expressing low levels of HER-2/neu) [Kim and Lee, 2005]. In this study, we examined whether amiloride can also sensitize TRAIL-resistant human prostate

Abbreviations used: DcR1, decoy receptor 1; DcR2, decoy receptor 2; DR4, death receptor 4; DR5, death receptor 5; DTT, dithiothreitol; FADD, Fas-associated death domain; FasL, Fas ligand; FLICE, Fas-associated death domain-like interleukin-1β-converting enzyme; FLIP, FLICE inhibitory protein; IAP, inhibitor of apoptosis; PAGE, polyacrylamide gel electrophoresis; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; PP1, protein phosphatase 1; PTEN, phosphatase and tensin homolog deleted on chromosome 10; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end-labeling.

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*Correspondence to: Dr. Yong J. Lee, Department of Surgery, University of Pittsburgh, The Hillman Cancer Center, 5117 Centre Ave., Pittsburgh, PA 15213.
E-mail: leeyj@msx.upmc.edu

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adenocarcinoma LNCaP cells (an androgen-dependent cell line expressing high levels of HER-2/neu) and human ovarian adenocarcinoma SK-OV-3 cells (a cell line expressing high levels of HER-2/neu).

HER-2/neu (also known as erbB2) is a gene in the epidermal growth factor receptor (EGFR) family. The HER-2/neu gene encodes a 185 kDa transmembrane receptor tyrosine kinase. Unlike the other epidermal growth factor receptors (EGFR), HER-2/neu has an intrinsic tyrosine kinase activity that activates PI(3)K in the absence of ligand [Fruman et al., 1998] PI(3)K consists of a regulatory subunit (p85) that binds to an activated growth factor/cytokine receptor and undergoes phosphorylation, which results in the activation of its catalytic subunit (P110) [Rodriguez-Viciana et al., 1996]. PI(3)K phosphorylates phosphoinositides at the 3′-position of the inositol ring, and its major lipid product is phosphatidylinositol 3,4,5-triphosphate (PIP_3) [Rameh and Cantley, 1999]. PIP_3 facilitates the recruitment of Akt to the plasma membrane through binding with the pleckstrin homology (PH) domain of Akt [Rameh and Cantley, 1999]. Akt is activated by phosphoinositide-dependent kinase-1 (PDK-1) through phosphorylation at threonine 308 and serine 473 [Alessi et al., 1997]. A number of pro-apoptotic proteins have been identified as direct Akt substrates, including BAD, caspsase-9, and Forkhead 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. Recent studies also show that Akt induces the degradation of IκB by promoting IKKα activity and subsequently stimulating the nuclear translocation of NF-κB [Ozes et al., 1999]. Nam et al. [2002] and Panka et al. [2001] reported that the PI(3)K-Akt-NF-κB pathway may regulate the expression of FLICE-inhibitory protein (FLIP), an anti-apoptotic molecule. Several studies reveal that TRAIL induces apoptosis in a wide variety of tumor cells, but does not cause toxicity to most normal cells. However, recent studies reveal that a polyhistidine-tagged TRAIL induces apoptosis in normal human hepatocytes in culture [Jo et al., 2000]. This is probably due to an aberrant conformation and subunit structure of TRAIL in the presence of low zinc concentrations [Lawrence et al., 2001]. In contrast, native-sequence, non-tagged recombinant TRAIL, when produced under optimized zinc concentrations, is markedly more active against tumor cells than the polyhistidine-tagged ligand, but has minimal toxicity toward human hepatocytes in vitro [Lawrence et al., 2001]. Moreover, preclinical studies in mice and primates have shown that administration of TRAIL can induce apoptosis in human tumors, but no cytotoxicity to normal organs or tissue [Walczak et al., 1999]. In addition, unlike tumor necrosis factor (TNF) and Fas ligand (FasL), TRAIL mRNA is expressed constitutively in many tissues [Wiley et al., 1995; Pitti et al., 1996].

Recent studies also revealed that TRAIL, which is constitutively expressed on murine natural killer cells in the liver, plays an important role in surveillance of tumor metastasis [Takeda et al., 2001]. The apoptotic signal of TRAIL is transduced by binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are members of the TNF receptor superfamily. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. TRAIL also binds to TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which act as decoy receptors by inhibiting TRAIL signaling [Degli-Esposti et al., 1997a,b; Marsters et al., 1997; Pan et al., 1997a,b; Sheridan et al., 1997; Walczak et al., 1997]. Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif [Pan et al., 1997b]. Differential sensitivity between normal and tumor cells to TRAIL has been explained by the presence of a high concentration of the decoy receptors in normal cells [Gura, 1997; Ashkenazi and Dixit, 1999]. Recently, this hypothesis has been challenged based on the results showing poor correlations between DR4, DR5, and DcR1 expression and sensitivity to TRAIL-induced apoptosis in normal and cancerous breast cell lines [Keane et al., 1999]. This discrepancy indicates that other factors such as death inhibitors (FLIP, FAP-1, or IAP) are also involved in the differential sensitivity of TRAIL. Previous studies demonstrated that the Fas-associated death domain (FADD) is also required for TRAIL-induced apoptosis [Kischkel et al., 2000]. TRAIL triggers apoptosis by recruiting the apoptosis initiator procaspase-8 through the adaptor FADD

[Bodmer et al., 2000]. Caspase-8 can directly activate downstream effector caspases including procaspase-3, -6, and -7 [Cohen, 1997]. Caspase-8 also cleaves Bid and triggers mitochondrial damage that in turn leads to cytochrome *c* release [Li et al., 1997]. Cytochrome *c* in the cytoplasm binds to Apaf-1, which then permits recruitment of procaspase-9. Caspase-9 cleaves and activates procaspase-3 [Slee et al., 1999].

The activation of caspases is counteracted by anti-apoptotic molecules of the Bcl-2 and IAP families [Tamm et al., 2000]. The Bcl-2 family proteins (Bcl-2, Bcl-X_L) heterodimerize with pro-apoptotic members of the family (Bax, Bak) and interfere with release of cytochrome *c* by pore-forming proteins (Bid, Bik) [Fruman et al., 1998]. Members of the inhibitor of apoptosis (IAP) family protein (c-IAP1, c-IAP2, XIAP) can directly bind and inhibit activation of caspases including caspase-3, -7 and -9 [Roy et al., 2001]. The expression of these Bcl-2 family and IAP family anti-apoptotic proteins is promoted by NF-κB, a family of dimeric transcription factors [Chen et al., 2000]. The NF-κB family of proteins, including NF-κB1, NF-κB2, RelA, RelB, and c-Rel, can form homo- and heterodimers in vitro, except for RelB. In mammals, the most widely distributed NF-κB is a heterodimer composed of p50 and p65 (also called RelA) subunits [Baeuerle and Baltimore, 1989]. NF-κB activity is regulated by the IκB family of proteins that interacts with and sequesters the transcription factor in the cytoplasm. IκB proteins become phosphorylated by the multisubunit IκB kinase (IKK) complex, which subsequently targets IκB for ubiquitination and degradation by the 26S proteasome [Zandi and Karin, 1999].

In this study, we postulate that blockage of HER-2/neu-mediated survival signals can sensitize HER-2/neu overexpressing cancers to TRAIL-induced cell death. Our data revealed that amiloride, which is known to inhibit tyrosine kinase activity of growth factor receptors [Davis and Czech, 1985], inhibits the HER-2/neu associated PI(3)K-Akt signal transduction pathway and thus renders the cell more sensitive to TRAIL-induced apoptotic death.

MATERIALS AND METHODS

Cell Culture and Survival Assay

Human prostate adenocarcinoma LNCaP cell line and two human ovarian adenocarcinoma cell lines, OVCAR-3 and SK-OV-3, were obtained from the American Tissue Type Culture Collection (Manassas, VA). LNCaP and SK-OV-3 cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (HyClone, Logan, Utah), 1 mM L-glutamine, and 26 mM sodium bicarbonate for monolayer cell culture. OVCAR-3 cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum, 10 mM HEPES buffer (Sigma Chemical Co., St. Louis, MO), 1 mM sodium pyruvate (Gibco BRL), 25 mM glucose, 17.9 mM sodium bicarbonate, and 0.01 mg/ml human recombinant insulin (Sigma). The dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. One 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

Wortmanin, sodium orthovanadate, 3-amino-6-chloro-5-(1-homopiperidyl)-*N*-(diaminomethylene) pyraxinecarboxamide (HMA), and 3,5-diamino-6-chloro-*N*-(diaminomethylene) pyrazinecarboximide (amiloride) were obtained from Sigma Chemical Co. LY294002 was purchased from Cell Signaling (Beverly, MA), and okadaic acid from Calbiochem (San Diego, CA). A stock solution was prepared in dimethylsulfoxide (DMSO).

Production of Recombinant TRAIL

A human TRAIL cDNA fragment (amino acids 114–281) obtained by RT-PCR was cloned into pET-23d (Novagen, Madison, WI) plasmid, and His-tagged TRAIL protein was purified using the Qiaexpress 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%

Triton X-100, 0.1% sodium citrate, and then washed twice 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 0.5M EDTA. Cells were observed under a fluorescence microscope.

Morphological Evaluation

Morphological changes in the nuclear chromatin in cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome 4′,6-diamidino-2-phenylindole (DAPI). Cells were grown on glass coverslips at a density of 2×10^5 cells/well. Cells treated with TRAIL, amiloride, or TRAIL in combination with amiloride for 4 h. Cells were washed twice with PBS and fixed by incubation in 70% ethanol for 30 min. Following washes with PBS, cells were incubated in 1 μg/ml DAPI solution for 30 min in the dark. Coverslips were then washed with PBS and analyzed by fluorescence microscopy.

UV Irradiation

For UV irradiation experiments, cells were exposed to UV-C at an intensity of 100 J/m^2 in a UV cross-linker (Spectronics Corporation, Westbury, New York).

Antibodies

Polyclonal anti-phospho(Tyr508)-PI3K, anti-Bcl-X_L, anti-Bad, 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-HER-2/neu from Dako-Cytomation (Carpinteria, CA), anti-cIAP-1 and anti-cIAP-2 from R&D Systems (Minneapolis, MN), anti-phospho(Tyr1248)-HER-2/neu, anti-phospho(Ser473)-Akt, anti-Akt, anti-phospho-(Ser241)-PDK-1, anti-PDK-1, anti-phospho-(Thr320)-PP1, and anti-PP1 from Cell Signaling, and anti-FLIP from Calbiochem. Monoclonal antibodies were purchased from the following companies: anti-PI3K from Santa Cruz, anti-caspase-8 from Upstate Biotechnology, anti-cytochrome *c* from PharMingen (San Diego, CA), anti-PARP from Biomol Research Laboratory (Plymouth Meeting, PA), and anti-Bcl-2 and anti-actin from ICN (Costa Mesa, CA).

Protein Extracts and Polyacrylamide Gel Electrophoresis (PAGE)

Cells were lysed with 1× Laemmli lysis buffer (2.4M glycerol, 0.14M Tris, pH 6.8, 0.21M sodium dodecyl sulfate, 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 1.28M β-mercaptoethanol, and equal amounts of protein were loaded on 8%–12% sodium dodecyl sulfate (SDS)–polyacrylamide gels. SDS–PAGE analysis was performed according to Laemmli [1970] using a Hoefer gel apparatus.

Immunoblot Analysis

Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS-Tween-20 (0.1%, v/v) 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).

In Vitro Enzyme Assays

For in vitro PP1 phosphatase assays, 10 ml of active PP1a proteins (1 U/ml; Upstate Biotechnology) were incubated with 3 ml of active Akt (0.5 mg; Upstate Biotechnology) for 30 min at 30°C in 50 ml of buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM ATP, 150 mM KCl, 5 mM b-glycerolphosphate, 0.1 mM sodium orthovanadate, 2 mM DTT). The reaction tubes contained either 1 mM amiloride or the same volume of DMSO. The reaction was stopped by adding 2× SDS sample buffer and heating at 100°C for 5 min. Thereafter, the sample was subjected to SDS–PAGE and the phosphorylation of Akt was analyzed by anti-phospho Akt antibody (Cell Signaling).

For in vitro PDK-1 kinase assays, LNCaP cells were transiently transfected with PDK-1 constructs (pcDNA3myc-PDK-1; 2 μg of DNA/dish) using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA). One day after transfection, cells were lysed with 500 μl of buffer A (20 mM Tris-HCl, pH 7.5, 1% Trition X-100, 0.5% deoxycholate, 5 mM EGTA, 150 mM NaCl,

10 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor cocktail solution (Sigma)). Cell lysates were immunoprecipitated by incubation with mouse anti-myc antibody (9E10; Roche, Indianapolis, IN) and protein G-Plus agarose (Gibco BRL) for 2 h at 4°C. Immune complexes were washed twice with buffer B (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.5 mM DTT, 1 mM Na_3VO_4, and 1 mM PMSF) at 4°C and then incubated with 2 μg of purified inactive Akt protein (Upstate Biotechnology) in a volume of 50 μl of a kinase buffer (100 μM ATP, 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 mM EDTA) for 30 min at 30°C. Thereafter, the sample was subjected to SDS–PAGE and the phosphorylation of Akt was analyzed by rabbit anti-phospho-Akt antibody (Cell Signaling).

RESULTS

TRAIL in Combination With Amiloride Induces Cytotoxicity

To investigate the effect of amiloride on TRAIL-induced cytotoxicity, human prostatic adenocarcinoma LNCaP cells were treated with TRAIL in the presence or absence of amiloride. Little or no cytotoxicity was observed with TRAIL (1–200 ng/ml) alone (Fig. 1A) or amiloride (0.1–1 mM) alone (Fig. 1B). In contrast, TRAIL in combination with amiloride significantly induced cytotoxicity (Fig. 1C). TdT-mediated dUTP Nick end labeling (TUNEL) assay showed that apoptotic death occurred during combined treatment with TRAIL and amiloride (Fig. 1D). Similar results were observed by DAPI staining (Fig. 1E). DAPI staining of cells treated TRAIL in combination with amiloride showed the presence of many cells with condensed nuclei, a morphological change that is associated with apoptosis.

Combined Treatment With TRAIL and Amiloride Induces Apoptosis

Additional studies were designed to examine whether the combination of amiloride and TRAIL treatment in LNCaP cells enhances poly (ADP-ribose) polymerase (PARP) cleavage, the hallmark feature of apoptosis. Previous studies show that PARP (116 kDa) is cleaved yielding a characteristic 85 kDa fragment in the presence of TRAIL in human prostate adenocarcinoma DU-145 cells [Lee et al., 2004]. Figure 2A,B shows that the cleavage of PARP was not

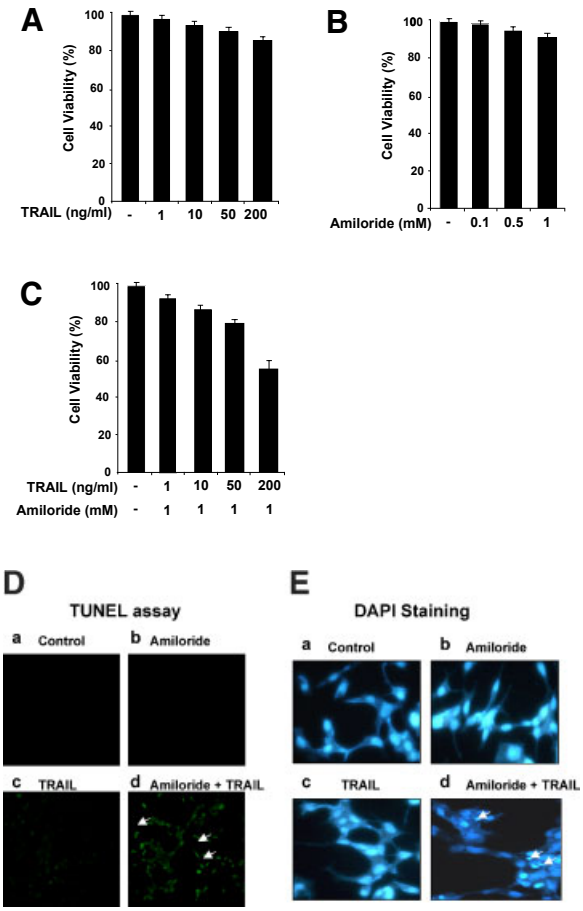

Fig. 1. Effect of amiloride on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity in human prostate adenocarcinoma LNCaP cells. **A**: Cells were treated for 4 h with various concentrations of TRAIL (1–200 ng/ml). **B**: Cells were treated with various concentrations of amiloride (0.1–1 mM). **C**: Cells were treated with various concentrations of TRAIL (1–200 ng/ml) in the presence of 1 mM amiloride. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. **D**: Cells were treated for 4 h with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride. After treatment, apoptosis was detected by the TUNEL assay. Apoptotic cells are indicated by arrows. **a**: Untreated control; (**b**) amiloride; (**c**) TRAIL; (**d**) amiloride + TRAIL. **E**: Cells were treated for 4 h with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride. After treatment, cells were stained with DAPI (1 μg/ml), and morphological features were analyzed with a fluorescence microscope.

observed by treatment with amiloride (0.1–1 mM) alone or TRAIL (1–1,000 ng/ml) alone in LNCaP cells. Interestingly, PARP was cleaved by combined treatment with TRAIL (50–1,000 ng/ml) and amiloride (0.5–1 mM). Similar results were observed with HMA, an analogue of amiloride (Fig. 2C). Figure 2D demonstrates that TRAIL in combination with amiloride, but

not TRAIL alone, activated caspases. Amiloride alone did not activate caspases (data not shown). However, Western blot analysis shows that procaspase-8 (54/55 kDa) was cleaved to the intermediates forms (41 and 43 kDa) and active form (18 kDa) by treatment with TRAIL in the presence of amiloride. The combined treatment of TRAIL and amiloride also resulted in caspase-9 activation (Fig. 2D). TRAIL in combination with amiloride induced proteolytic processing of procaspase-9 (48 kDa) into its active form (37 kDa). The combined treatment with TRAIL and amiloride also induced caspase-3 activation. Western blot analysis shows that procaspase-3 (32 kDa), the precursor form of caspase-3, was cleaved to active form (17 kDa) in the presence of TRAIL and amiloride.

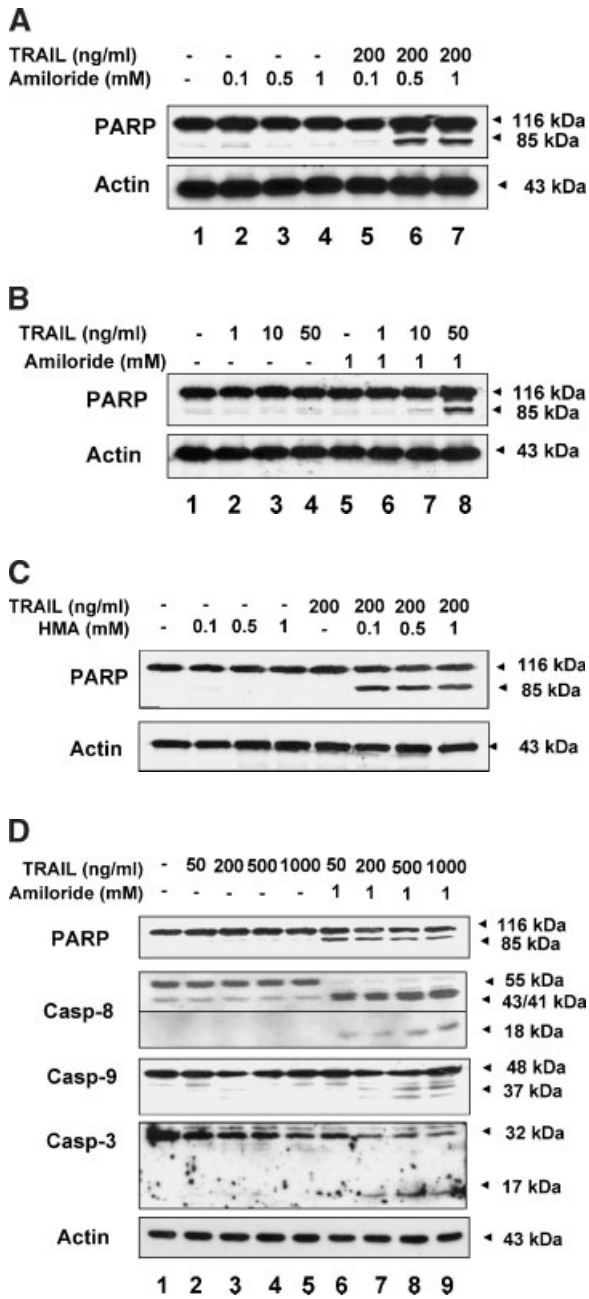

Fig. 2. Effect of amiloride or HMA on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in LNCaP cells. Cells were treated for 4 h with various concentrations of amiloride (0.1–1 mM) in the presence or absence of 200 ng/ml TRAIL (**A**), various concentrations of TRAIL (1–50 ng/ml) in the presence or absence of 1 mM amiloride (**B**), various concentrations of HMA (0.1–1 mM) in the presence or absence of 200 ng/ml TRAIL (**C**), or various concentrations of TRAIL (50–1,000 ng/ml) in the presence or absence of 1 mM amiloride (**D**) and then harvested. Cell lysates were subjected to immunoblotting for PARP, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects inactive form (55 kDa), cleaved intermediates (41, 43 kDa), and active form (18 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa), and cleaved active form (17 kDa). Immunoblots of PARP show the 116 kDa PARP and the 85 kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

Effect of Combined Treatment With TRAIL and Amiloride on the Level of TRAIL Receptor Family and Anti-Apoptotic Proteins

Previous studies demonstrate that increased DR5 levels induced by genotoxic agents [Sheikh et al., 1998; Chinnaiyan 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 amiloride. LNCaP cells were treated with TRAIL (50–1,000 ng/ml) in the presence of 1 mM amiloride. Data from western blot analysis reveal that the combined treatment did not significantly alter the levels of DR4, DR5, DcR2, $FLIP_L$, $FLIP_S$, IAP-1, IAP-2, Bcl-X_L, and Bcl-2 (Fig. 3). Amiloride alone also did not change the levels of TRAIL receptors and anti-apoptotic proteins (data not shown).

Effect of Amiloride on Akt Phosphorylation

It is well known that elevated Akt activity protects cells from TRAIL-induced apoptosis [Nesterov et al., 2001]. Since several researchers have also reported that overexpression of Na^+/H^+ exchanger promotes cell survival and Akt activity [Barriere et al., 2001; Wu et al.,

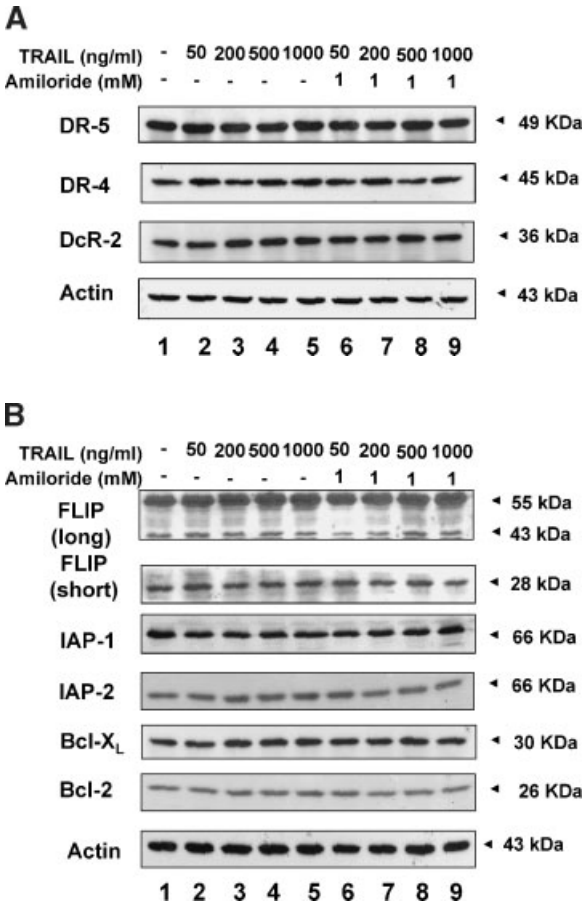

Fig. 3. Intracellular levels of TRAIL receptors (**A**) or anti-apoptotic proteins (**B**) during treatment with TRAIL in the presence or absence of amiloride. LNCaP cells were treated for 4 h with various concentrations of TRAIL (50–1,000 ng/ml) in the presence or absence of 1 mM amiloride. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted as described in ''Materials and Methods.'' Actin is shown as an internal standard.

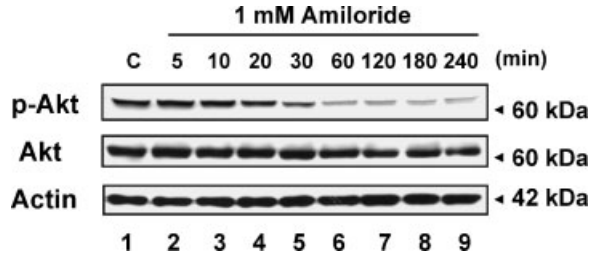

Fig. 4. Effect of amiloride on the level of phosphorylated Akt in LNCaP cells. Cells were treated with 1 mM amiloride for various times (5–240 min) and then harvested. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-phospho-Akt, or anti-Akt antibody. Actin is shown as an internal standard.

2004], we postulated that amiloride inhibits Akt activity and consequently enhances TRAIL-induced cytotoxicity. To examine whether amiloride inhibits Akt activity by dephosphorylating Akt, LNCaP cells were treated with 1 mM amiloride for various periods (5–240 min) and the level of phosphorylated Akt was measured. Figure 4 shows that Akt was rapidly dephosphorylated within 30 min of amiloride addition without changing the Akt protein level. TRAIL treatment did not alter amiloride-induced dephosphorylation of Akt (data not shown). Since amiloride is acting as a Na^+/H^+ exchanger inhibitor, promoting intracellular acidification may inhibit Akt activity. However, low extracellular pH does not dephosphorylate Akt (data not shown). To examine whether dephosphorylation of Akt during amiloride treatment is mediated through activation of phosphatase, we treated LNCaP cells with okadaic acid, a serine phosphatase inhibitor. Figure 5A shows that amiloride-induced Akt dephosphorylation (lane 5) was suppressed by pretreatment with 0.1–5 μM okadaic acid (lanes 6–8), but not with low concentrations (<0.1 μM) of okadaic acid (data not shown). Since okadaic acid inhibits both protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1), (both serine/threonine phosphatases), at higher concentrations ($IC_{50} = 150$ nM), but it inhibits only PP2A at low concentrations ($IC_{50} < 0.1$ nM), PP1 rather than PP2A may play an important role in the regulation of Akt phosphorylation during treatment with amiloride. Recent studies have also demonstrated that dephosphorylation of Akt is regulated by PP1, but not by PP2A [Xu et al., 2003]. Figure 5B shows that reduced phosphorylation of PP1 in the presence of amiloride (activation of PP1; lane 2) was partially antagonized by pretreatment with okadaic acid (lanes 5 and 6). Figure 5B also shows that amiloride in combination with TRAIL led to greater dephosphorylation of PP1 than amiloride alone and that this was not easily blocked by okadaic acid. These results indicate that reduction of phosphorylation is more responsible than stimulation of dephosphorylation during treatment with amiloride. We further examined whether okadaic acid prevents the combined treatment with TRAIL and amiloride-induced PARP cleavage (apoptosis). Amiloride-promoted TRAIL-induced PARP cleavage (lanes 4 and 7 in Fig. 5C) was significantly inhibited by treatment with 1 μM okadaic acid (lanes 5 and 8 in Fig. 5C).

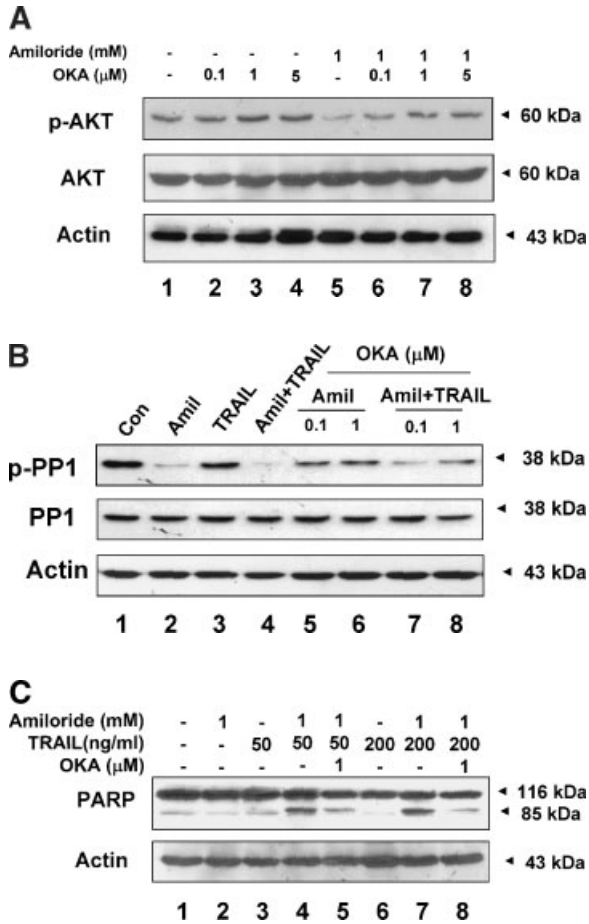

Fig. 5. Effect of okadaic acid on amiloride-induced PP1 dephosphorylation (**A**), Akt dephosphorylation (**B**), and TRAIL cytotoxicity (**C**). A: LNCaP Cells were pretreated with okadaic acid (OKA: 0.1–5 μM) for 30 min and then treated or untreated with 1 mM amiloride. B: Cells were pretreated with okadaic acid (0.1–1 μM) for 30 min and treated with TRAIL in the presence or absence of 1 mM amiloride (Amil). Con, untreated control cells. C: Cells were pretreated with 1 μM okadaic acid for 30 min and treated with TRAIL (50–200 ng/ml) in the presence or absence of 1 mM amiloride. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-phospho-Akt, anti-Akt, anti-phospho-PP1, anti-PP1, or anti-PARP antibody. Actin is shown as an internal standard.

Effect of Amiloride on Kinases and Phosphatases Associated With the HER-2/neu-PI3K-Akt Pathway

Since previous studies have revealed that HER-2/neu activates the PI3K-Akt pathway, we further examined whether amiloride nonspecifically affects the HER-2/neu-PI3K-Akt pathway-associated kinases or phosphatases or specifically activates PP1 activity. As shown previously, amiloride induced dephosphorylation (activation) of PP1 (Fig. 6A). Interestingly,

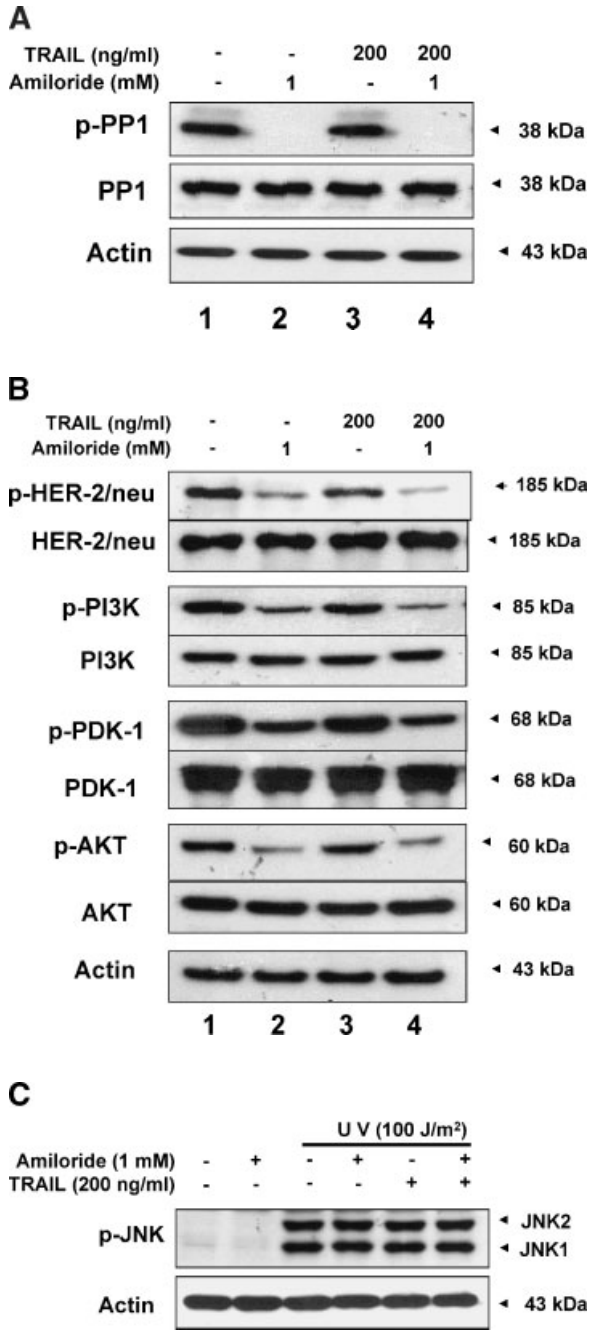

Fig. 6. Effect of amiloride on phosphatase (**A**), kinases (**B**), or JNK (**C**) in the presence or absence of TRAIL. A, B: LNCaP cells were treated for 2 h with 1 mM amiloride in the presence or absence of 200 ng/ml TRAIL. C: LNCaP cells were pretreated with 1 mM amiloride for 30 min, irradiated with UV (100 J/m^2), incubated for 1 h in the presence or absence of TRAIL (200 ng/ml)/amiloride (1 mM), and then harvested. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted as described in ''Materials and Methods.'' Actin is shown as an internal standard.

amiloride also induced dephosphorylation (inactivation) of the HER-2/neu-PI3K-Akt pathway-associated kinases such as HER-2/neu, PI3K, and PDK-1 (Fig. 6B). These results suggest that amiloride nonspecifically induced dephosphorylation of the HER-2/neu-PI3K-Akt pathway-associated kinases as well as phosphatase. We further examined whether amiloride has an affect on phosphorylation of a substrate that is not in the HER-2/neu-PI3K-Akt pathway. Figure 6C shows that UV exposure activated JNK (phosphorylation of JNK1 and JNK2) and its phosphorylation was not inhibited by treatment with amiloride.

The effect of amiloride on Akt was compared with the effects of LY294002 and wortmannin, inhibitors of PI3K. Figure 7A shows that all these drugs induced dephosphorylation of Akt. However, unlike amiloride, LY294002 and wortmannin did not induce dephosphorylation of PI3K, PDK-1, and PTEN. We postulated that if amiloride-induced dephosphorylation of Akt is responsible for promoting TRAIL cytotoxicity, amiloride should enhance the effect of these kinase inhibitors. Indeed, amiloride in combination with LY294002 or wortmannin promoted TRAIL-induced cytotoxicity (Fig. 7B) and apoptosis (Fig. 7C). These results indicate that Akt inactivation (dephosphorylation) is responsible for the amiloride-induced enhancement of TRAIL cytotoxicity.

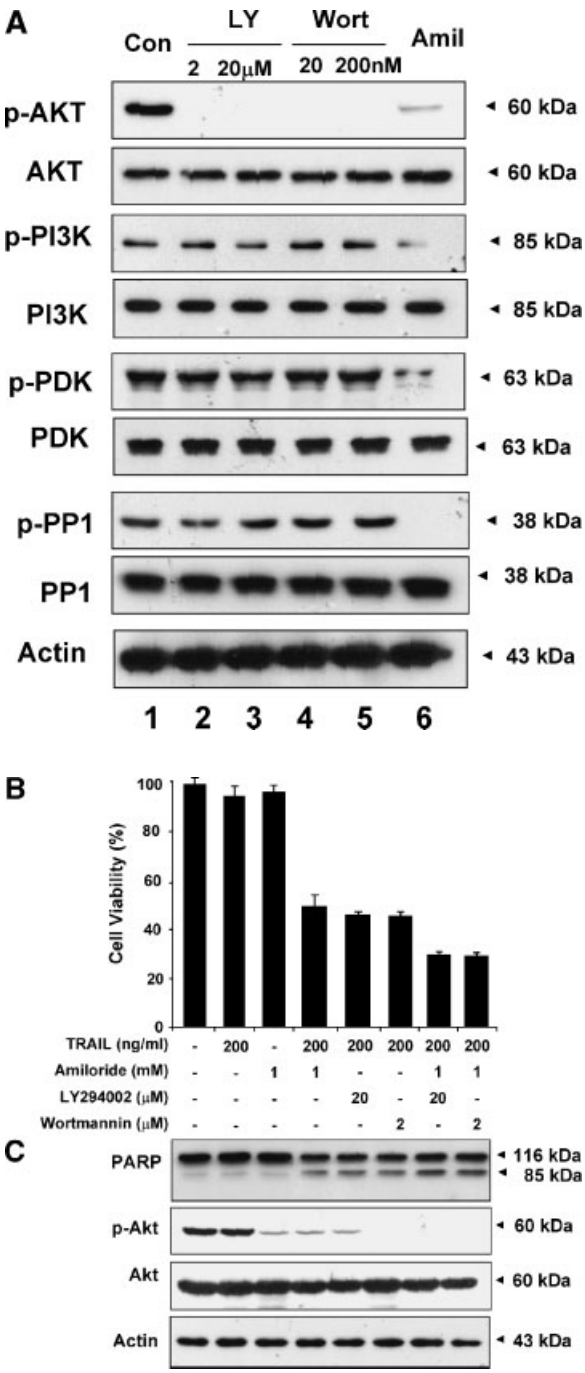

Fig. 7. Effect of LY294002 or wortmannin on kinases/phosphatases (**A**), TRAIL-induced cytotoxicity (**B**), and TRAIL-induced PARP cleavage (**C**) in LNCaP cells. A: Cells were treated for 1 h with LY294002 (LY; 2–20 μM), wortmannin (Wort; 20–200 nM), or 1 mM amiloride (Amil). Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted as described in ''Materials and Methods.'' Actin is shown as an internal standard. Con, untreated control cells. B, C: Cells were pretreated with 20 μM LY294002 or 200 nM wortmannin for 20 min and then treated with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride for 4 h. 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, or anti-Akt antibody. Actin is shown as an internal standard.

Mechanism of Amiloride-Induced Alterations of Phosphatase and Kinase Activities

Davis and Czech [1985] reported that amiloride acts as an ATP analogue which causes the formation of nonproductive enzyme–substrate complexes. We hypothesized that amiloride competes with ATP, thereby inhibiting protein phosphorylation. To test this hypothesis, we examined whether amiloride blocks the kinase-mediated phosphorylation process. Figure 8A shows that purified unphosphorylated Akt protein (Upstate Biotechnology; data not shown) was phosphorylated by active PDK-1 in vitro. Amiloride, but not DMSO, inhibited phosphorylation of Akt. Figure 8B shows that

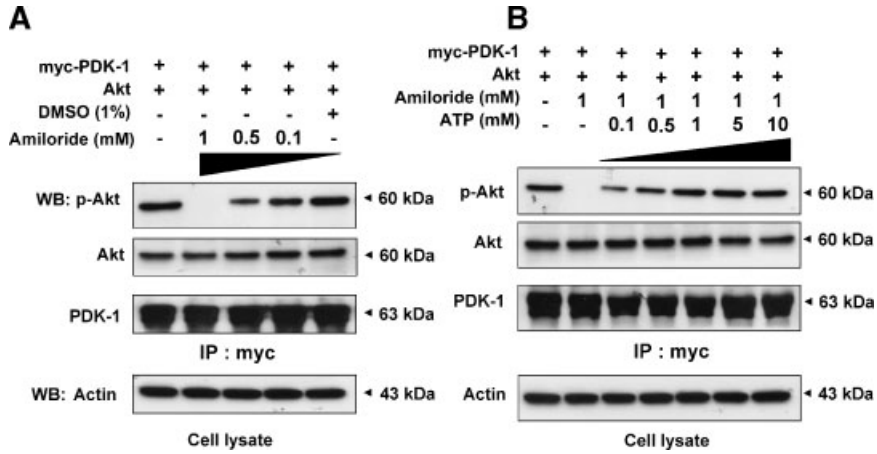

Fig. 8. Effect of amiloride on PDK-1 kinase activity. LNCaP cells were transfected with pcDNA3myc-PDK-1. One day after transfection, myc-PDK-1 was immunoprecipitated with anti-myc antibody. For in vitro PDK-1 kinase assays, the immune complex was incubated with purified inactive Akt protein in the presence or absence of various concentrations of amiloride (**A**) or various concentrations of ATP (**B**). The sample was separated by SDS–PAGE and immunoblotted with anti-phospho-Akt, anti-Akt, or anti-PDK-1 antibody. Actin is shown as an internal standard.

amiloride blocks the kinase-mediated phosphorylation process by competing with ATP.

Ability of Amiloride-Induced TRAIL-Sensitivity Is not Specific to LNCaP Cells

To examine whether our findings can be generalized for more than one cell line, human ovarian adenocarcinoma SK-OV-3 and OVCAR-3 cell lines were employed. Figure 9 shows that SK-OV-3 cells containing a relatively high level of Akt activity and HER-2/neu are resistant to TRAIL. In contrast, OVCAR-3 cells containing a relatively low level of Akt activity and HER-2/neu are sensitive to TRAIL. When SK-OV-3 or

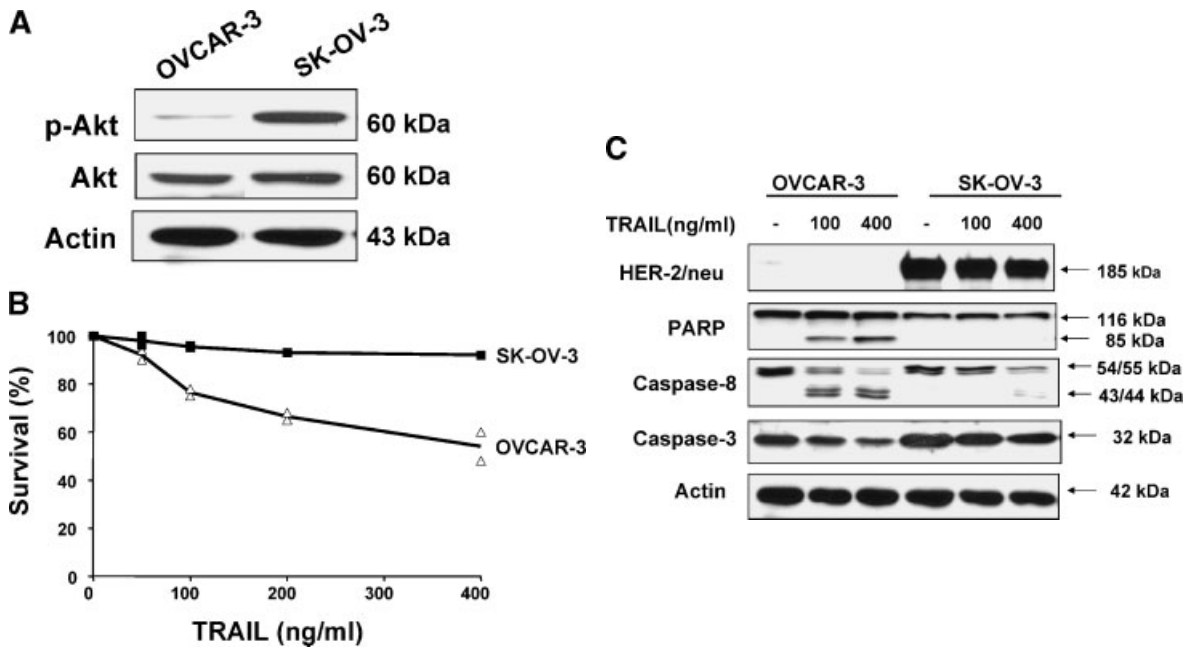

Fig. 9. Expression of HER-2/neu, basal Akt activity, and TRAIL sensitivity in ovarian adenocarcinoma SK-OV-3 and OVCAR-3 cells. **A**: Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-Akt antibody (Akt) or anti-phospho-S473 Akt antibody (p-Akt). Actin is shown as an internal standard. **B**: Cells were treated with TRAIL (0–400 ng/ml) for 4 h. Survival was analyzed by the trypan blue exclusion assay. Data from two separate experiments are compiled. **C**: Cells were treated with TRAIL (0–400 ng/ml) for 4 h. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-HER-2/neu, anti-PARP, anti-caspase-8, anti-caspase-3, or anti-actin antibody.

OVCAR-3 cells were treated with 400 ng/ml TRAIL for 4 h, the survival was 92% or 60%, respectively. Similar results were observed when PARP cleavage and caspase (-8 and -3) activation were examined. TRAIL-induced activation of caspase-8 and caspase-3 as well as PARP cleavage were observed in OVCAR-3. Unlike OVCAR-3 cells, SK-OV-3 cells are resistant to TRAIL-induced PARP cleavage and caspase activation. However, the combined treatment of TRAIL and amilroide resulted in an increase in caspase (-8, -9, and -3) activation along with PARP cleavage in SK-OV-3 cells (Fig. 10). Amiloride dephosphorylated Akt regardless of the presence of TRAIL.

DISCUSSION

LNCaP and SK-OV-3 cells, which express high levels of HER-2/neu protein, are relatively insensitive to TRAIL and have a high level of Akt activation [Nesterov et al., 2001; Li et al., 2004; Fig. 9]. Previous studies demonstrated that down-regulation of the HER-2/neu protein and inhibition of Akt activity by treatment with trastuzumab (Herceptin) enhance TRAIL-mediated apoptosis [Cuello et al., 2001]. Similar results were observed by treatment with amiloride. Inactivation of HER-2/neu and Akt through treatment with amiloride results in

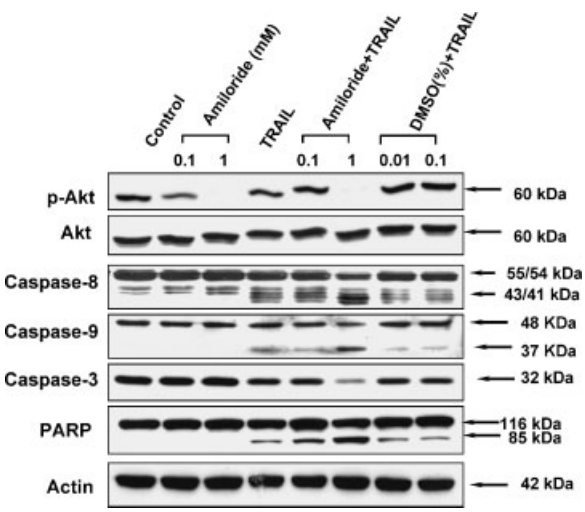

Fig. 10. Effect of amiloride on Akt activity and TRAIL-induced activation of caspases and cleavage of PARP in SK-OV-3 cells. Cells were treated with amiloride (0.1–1 mM) in the presence or absence of TRAIL (400 ng/ml) for 4 h. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-Akt, anti-phospho-S473 Akt, anti-caspase-8, anti-caspase-9, anti-caspase-3, anti-PARP, or anti-actin antibody.

cytotoxicity by TRAIL (Figs. 1, 4, and 6). Our studies also reveal that amiloride causes a decrease in the phosphorylation state of Akt by inhibiting (dephosphorylation of) PI3K and PDK-1 kinases as well as activating (dephosphorylation of) PP1 (Fig. 6). The inhibition of kinases by amiloride can be overcome at high ATP concentrations which indicates that the inhibition of kinase activity is competitive with ATP (41; Fig. 8). Thus, amiloride may act as an ATP analogue and directly inhibit kinase activity and/or activate phosphatase activity by decreasing protein phosphorylation [Davis and Czech, 1985]. Indeed, our results from in vitro kinase assays show that amiloride directly inhibits PDK-1 activity (Fig. 8).

LNCaP cells which are PTEN-null due to mutations contain a high level of Bcl-2 [Huang et al., 2001]. This is probably because Bcl-2 expression is down-regulated by PTEN [Huang et al., 2001; Rosser et al., 2004]. Previous studies revealed that transient transfection of PTEN into the PTEN-null cells results in decreased levels of Bcl-2 [Huang et al., 2001] and sensitizes cells to apoptotic agents [Huang et al., 2001; Yuan and Whang, 2002]. In this study, we observed that combined treatment with TRAIL and amiloride does not alter the level of Bcl-2 (Fig. 3B). These results suggest that amiloride-promoted TRAIL cytotoxicity is not mediated through Bcl-2. Previous studies show that amiloride inhibits tyrosine kinase activity of growth factor receptors [Davis and Czech, 1985], Na^+/Ca^{2+} exchange [Smith et al., 1982], (Na^+–K^+)-ATPase [Soltoff and Mandel, 1983], and serine kinase activity [Ralph et al., 1982]. It has previously been suggested that a possible mechanism of biochemical action of amiloride is the inhibition of kinase activity [Ralph et al., 1982; Davis and Czech, 1985]. In addition to the inhibition of kinase activity, our data indicate that amiloride activates PP1 phosphatases (Fig. 6A).

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]. However, pharmacokinetic studies reveal that PP1 rather than PP2A is responsible for the regulation of Akt phosphorylation [Xu et al., 2003]. It is well known that the activity of PP1 is regulated by phosphorylation of its catalytic subunit. Upon phosphorylation of Thr320, the COOH terminus of PP1 folds back to mask its catalytic center [Goldberg et al.,

1995]. Our data clearly demonstrate that amiloride dephosphorylates (activates) PP1 (Figs. 5B and 6A) as well as Akt (Figs. 4 and 5A). The amiloride-mediated dephosphorylation of PP1 and Akt is inhibited by okadaic acid (Fig. 5). Previous pharmacokinetics studies demonstrate that okadaic acid inhibits only PP2A at low concentrations ($IC_{50} < 0.1$ nM), but both PP1 and PP2A at higher concentrations ($IC_{50} = 150$ nM). Thus, we need to further investigate whether PP2A is also involved in the regulation of Akt phosphorylation.

Previous studies have shown that constitutively active Akt blocks TRAIL cytoxicity [Chen et al., 2001; Thakkar et al., 2001]. Down-regulation of Akt activity by PI3K inhibitors, wortmannin and LY294002, promotes TRAIL cytotoxicity [Thakkar et al., 2001; Fig. 7]. This is probably due to inhibition of Akt-mediated antiapototic effects. A number of pro-apoptotic proteins have been identified as direct Akt substrates, including BAD, caspasase-9, and Forkhead 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 proapoptotic function of these molecules is suppressed upon phosphorylation by Akt. Akt also induces the degradation of IκB by promoting IKKα activity and subsequently stimulating the nuclear translocation of NF-κB [Ozes et al., 1999]. Recently we [Nam et al., 2002] and Panka et al. [2001] reported that the PI(3)K-Akt-NF-κB pathway may regulate the expression of FLICE-inhibitory protein (FLIP), an anti-apoptotic molecule. We postulate that amiloride-mediated dephosphorylation of Akt leads to activation of proapoptotic molecules and results in enhancement of TRAIL-induced apoptotic death. Overall, our model may provide important insights into how amiloride promotes TRAIL-induced apoptotic death. We believe that this model provides a framework for future studies.

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