Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL)-Troglitazone-induced Apoptosis in Prostate Cancer Cells Involve AMPK-activated Kinase

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Prostate cancer (PCa) is one of the most frequently diagnosed cancers in men with limited treatment options for the hormone-resistant forms. Development of novel therapeutic options is critically needed to target advanced forms. Here we demonstrate that combinatorial treatment with the thiazolidinedione troglitazone (TZD) and TNF-related apoptosis-inducing ligand (TRAIL) can induce significant apoptosis in various PCa cells independent of androgen receptor status. Because TZD is known to activate AMP-activated protein kinase (AMPK), we determined whether AMPK is a molecular target mediating this apoptotic cascade by utilizing PCa cell lines stably overexpressing AMPKα1 dominant negative (C4-2-DN) or empty vector (C4-2-EV). Our results indicated a significantly higher degree of apoptosis with TRAIL-TZD combination in C4-2-EV cells compared with C4-2-DN cells. Similarly, results from a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed a larger reduction of viability of C4-2-EV compared with C4-2-DN cells when treated with TRAIL-TZD, thus suggesting that C4-2-DN cells were more apoptosis-resistant. Additionally, siRNA-mediated knockdown of endogenous AMPKα1 expression showed a reduction of TRAIL-TZD-induced apoptosis, further confirming the participation of AMPK in mediating this apoptosis. Apoptosis induction by this combinatorial treatment was also associated with a cleavage of β-catenin that was inhibited in both C4-2-DN cells and those cells in which AMPKα1 was knocked down. In addition, time course studies showed an increase in pACC579 (AMPK target) levels coinciding with the time of apoptosis. These studies indicate the involvement of AMPK in TRAIL-TZD-mediated apoptosis and β-catenin cleavage and suggest the possibility of utilizing AMPK as a therapeutic target in apoptosis-resistant prostate cancer.

Prostate cancer (PCa) is the most commonly diagnosed malignancy in the male population and remains the second leading cause of cancer-related deaths in the western world (1). Androgen deprivation therapy is the currently used therapeutic option for treating advanced PCa. Although the tumors initially respond to this therapeutic regimen, they eventually become androgen-independent and thus castration-resistant (2). The median survival rate of men with metastatic castration-resistant PCa (CRPC) is less than 2 years (3) with no effective drugs currently available to treat these resistant forms. Thus the development of alternate therapeutic agents that can target CRPCs is critically needed. Because apoptosis resistance is one of the major hallmarks and a critical pathophysiological factor contributing to therapeutic resistance in cancer, agents that can sensitize resistant tumor cells toward apoptosis might be helpful for treating CRPC. Despite a significant amount of studies directed toward developing apoptosis-sensitizing agents, our understanding of the mechanism that leads to therapeutic resistance in prostate cancer is still limiting (4). More insight toward this mechanism is critically needed to serve as a basis for future drug development to specifically target apoptosis-resistant forms as found in advanced prostate cancer. This strategy will help in the identification of specific candidates, targeting of which alone or as combination therapy might ameliorate advanced CRPC.

TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) (5) has recently gained significant attention as a candidate cancer

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2 The abbreviations used are: PCa, prostate cancer; ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; CRPC, castration-resistant prostate cancer; DN, dominant negative; EV, empty vector; FL, full-length; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PPARγ, peroxisome proliferator-activated receptor γ; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TZD, troglitazone.
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drug, because of its unique capability of inducing apoptosis in cancer cells, with minimal off target effects on the normal or nontransformed cells (5, 6). Signaling by TRAIL is activated following binding of TRAIL to the death receptors DR4 and DR5, leading to the assembly of a death-inducing signaling complex (7) and recruitment of adaptor protein FAS-associated death domain and resulting in caspase activation (8, 9). Although TRAIL receptors are expressed in both androgen-dependent and -independent prostate cancer cells, the majority of them develop resistance to TRAIL-induced apoptosis (10). Identification of drugs or agents that can overcome TRAIL resistance and sensitize prostate cancer cells toward TRAIL-induced apoptosis might be an important first step towards developing TRAIL-based combination therapies for CRPC. In fact, several studies have demonstrated that TRAIL combination therapy is effective in sensitizing resistant prostate cancer cells (11–15). Recent studies by us (16) as well as by others (17–21) have demonstrated that a combination of TRAIL with ligands for PPARγ (the thiazolidinediones) can ameliorate TRAIL resistance in various cancer cells and sensitize them toward apoptosis. Studies by various groups have also shown that AMP-activated protein kinase (AMPK) activation can sensitize cancer cells to TRAIL-induced apoptosis (22, 23). These interesting observations, despite providing potential avenues of combination approaches to overcome TRAIL resistance, do not provide the detailed mechanism involved in TRAIL resistance, which needs to be critically elucidated.

Mammalian AMPK acts as a metabolic sensor to monitor cellular AMP and ATP levels and is activated by an increase in AMP/ATP ratio to regulate various cellular processes (24). AMPK can be activated in response to hypoxia, ischemia, long term starvation, and increased reactive oxygen species (25, 26). These processes cause an increase in cellular AMP/ATP ratio leading to its allosteric activation by AMP. AMP binding also reduces the dephosphorylation of Thr172 within AMPK catalytic α subunit by protein phosphatase 2Cα (PP2Cα), thus maintaining higher phosphorylation by upstream kinase LKB1 (27–29). In a separate pathway that does not require increased AMP levels, an increase in cellular Ca2+ can also phosphorylate and activate AMPK Thr172 via activating Ca2+/calmodulin-dependent protein kinase kinase β (29). A variety of drugs and xenobiotics have been reported to activate AMPK, including 5-aminoimidazole-4-carboxamide riboside (24), the antidiabetic biguanides, metformin (30) and phenformin (31), phenobarbital (32), resveratrol (33), berberine (34), and more. In addition, there are reports that the thiazolidinedione group of antidiabetic drugs can also activate AMPK (35, 36) and mediate growth suppression (37). Because in our earlier studies, a combination of the thiazolidinedione troglitazone (TZD) and TRAIL significantly promoted apoptosis of cancer cells (16), in this study we determined the efficacy of this combination toward PCa cells. In addition, we determined the possible involvement of AMPK in this combinatorial apoptosis. Our studies demonstrate that PCa cell lines overexpressing AMPKα1 dominant negative (C4-2-DN cells) were resistant to TRAIL and TZD combination-induced apoptosis compared with the empty vector expressing (C4-2-EV) cells. Knockdown of endogenous AMPKα expression in the PCa cells by siRNA showed a similar reduction of apoptosis following knockdown of AMPKα. In addition, the caspase-induced cleavage of β-catenin protein observed following TRAIL-TZD treatment was significantly attenuated in the C4-2-DN cells or following AMPKα knockdown. These studies indicate an involvement of AMPK in TRAIL and TZD combination-induced apoptosis and β-catenin cleavage in PCa cells.

Experimental Procedures

Reagents—RPMI tissue culture media, Lipofectamine 2000, and a β-galactosidase assay kit were purchased from Invitrogen. The luciferase assay reagent was purchased from Promega (Madison, WI); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma; In Situ cell death detection kit (fluorescein), was from Roche Diagnostics; troglitazone and TRAIL were purchased from EMD Biosciences (Gibbstown, NJ). The antibodies were obtained from the following sources: poly(ADP-ribose) polymerase (PARP), caspase-3, cleaved caspase-8, caspase 9, PPARγ, AMPKα1, AMPKα2, pAMPKα1/2, pAMPKα1/2-Thr172, ACC, and pACC-Thr79 were from Cell Signaling Technologies (Danvers, MA); GAPDH was from Ambion Inc. (Austin, TX); β-catenin was from BD Biosciences (San Jose, CA); and FLAG was from Sigma-Aldrich. The tk-PPRE3-luc reporter construct was obtained from Dr. Ron Evans (38).

Cell Culture—LNCaP and DU 145 cells were purchased from ATCC, C4-2, C4-2B, C4-2-DN, and C4-2-EV cells were used as described earlier (39–41). Cells were maintained in RPMI medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. In TRAIL and TZD experiments, confluent populations of cells were treated with DMSO (as vehicle) or 100 ng/ml TRAIL or 50 μM TZD (unless indicated otherwise) alone or in combination for various lengths of time followed by Western blot analyses.

Transient Transfection and Luciferase Assays—Subconfluent populations of DU 145 cells were transiently transfected using Lipofectamine 2000 with tk-PPRE3-luc reporter construct (38) and a β-galactosidase vector as described earlier (42) as per the manufacturer’s instructions. After 48 h of transfection, the cells were treated with increasing concentrations of TZD alone or in combination with TRAIL (100 ng/ml) for 6 h. Each transfection was performed in triplicate, and each experiment was repeated at least twice. Luciferase and β-gal assays were performed using a luminometer (Berthold Technologies, Centro XS3 LB 960) and a plate reader (Power Wave XS, Biotek), respectively. The results obtained were calculated as the ratio of relative light units to β-gal values and expressed as the percentage of increase compared with controls.

Small Interference RNA—ON-TARGETplus smart pool human PPARγ siRNA, human PRKAA1 siRNA (AMPKα1), and human PRKAA2 siRNA (AMPKα2) were purchased from Dharmacon (Lafayette, CO). A negative control siRNA from Ambion Inc. (Austin, TX) was used as control siRNA. siRNA transfection was performed using Lipofectamine 2000 as per the manufacturer’s instructions and as described earlier (16). Subconfluent populations of cells were transfected with either 50 nm control siRNA or the target protein siRNA for 24 h followed by recovery in serum containing medium. After ~72 h of
transfection with siRNA, cells were treated with either DMSO or a combination of TRAIL and TZD for an additional 4–16 h followed by Western blot analysis.

**MTT Assay**—Cell viability was determined by MTT assay as described (43). Briefly, C4-2-EV and C4-2-DN cells were plated at a density of 10,000 cells/well in 96-well plate and allowed to attach overnight. The next day, the cells were treated with medium containing DMSO alone (as vehicle) or with a combination of TRAIL (100 ng/ml) and TZD (50 μM) for 16 h. At the end of each treatment, the cells were incubated with 50 μl of freshly diluted 0.5 mg/ml MTT solution (dilution made in medium from an MTT stock solution of 5 mg/ml) for 4 h at 37 °C. Thereafter, 150 μl of DMSO was added to each well, followed by incubation for another 30 min at 37 °C and measurement of absorbance at 570 nm in a microtiter plate reader.

**TUNEL Assay**—In Situ cell death detection kit (fluorescein), was used to detect apoptosis based on labeling of DNA strand breaks in TRAIL-TZD treated cells as per the manufacturer’s protocol. Cells treated with either DMSO or TRAIL (100 ng/ml) and TZD (25 μM) combination for 4 h in chamber slides were fixed with freshly prepared 4% paraformaldehyde in PBS. They were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, labeled with TUNEL reaction mixture, and analyzed by fluorescence microscopy. The images were captured using Olympus microscope (model IX81) equipped with QIMAGING QI Click cooled camera.

Western Blot Analysis—Western blot analysis was performed utilizing procedures described previously (44). Briefly, equal amounts of total cell extracts were fractionated by SDS-PAGE, transferred to PVDF membranes, and subjected to Western blot analysis utilizing various antibodies.

**Results**

Combination of TRAIL with Troglitazone Induces Apoptosis in Various Prostate Cancer Cells—Our earlier studies have shown that combinatorial treatment with TRAIL and PPARγ ligand TZD can induce potent apoptosis in various cancer cells (16). To determine the efficacy of this combination in PCa cells, a more detailed analysis was carried out using androgen-dependent, androgen-independent, and castration-resistant PCa cells. Western blot analysis showed increased cleavage of caspases 3, 9, and 8 and PARP with TRAIL-TZD combination compared with TRAIL alone within an 8-h time frame in the androgen-independent DU 145 cells (Fig. 1A). Similar apoptosis induction with TRAIL-TZD combination was also observed in the androgen-dependent LNCaP cells (Fig. 1B) and castration-resistant C4-2 (Fig. 1C) and C4-2B (Fig. 1D) cells. Induction of caspase cleavage with TRAIL-TZD combination peaked at ~8 h and was reduced by 24 h, likely because the cells were mostly dead by that time. These suggested that combinatorial treatment with TRAIL and TZD can successfully sensitize both androgen-dependent and -independent PCa cells toward apoptosis.

Apoptosis Induction by TRAIL-TZD Is Attenuated by AMPKα Dominant Negative Overexpression—To obtain a mechanistic insight toward the mediators involved and to elucidate the overall mechanism behind this apoptotic pathway, we focused on AMPK, because the drugs in the thiazolidinedione group are known to activate AMPK pathway and mediate its biological effects (35–37). To determine the involvement of AMPK in mediating apoptosis following combinatorial treatment with TRAIL and TZD, we utilized stable PCa cells overexpressing either dominant negative AMPKα (C4-2-DN cells) or the empty vector (C4-2-EV cells) (41). Treatment of C4-2-EV cells with TRAIL-TZD showed a time-dependent increase in apoptosis in these cells as indicated by cleavage of caspases 3, 9, and 8 and PARP (Fig. 2A), which seemed to be maximal within 4–8 h. This apoptosis induction was, however, significantly attenuated in the C4-2-DN cells as indicated by reduced cleavage of caspases 3, 9, and 8 and PARP particularly at 4 h (Fig. 2B, compare lanes 6 and 8). In addition, MTT assays carried out indicated a higher degree of cell survival in the C4-2-DN cells compared with the EV cells (Fig. 2C). To establish this further, TUNEL assays were performed following combinatorial treatment, which showed a significant induction of TUNEL-positive cells indicative of apoptosis in the C4-2-EV cells compared with the DN cells (Fig. 2D).

Knockdown of Endogenous AMPKα Attenuates TRAIL and TZD-induced Apoptosis—To establish the role of AMPK in TRAIL-TZD combination-induced apoptosis cascade more conclusively, we designed siRNA studies to knock down endogenous AMPKα1 expression in the DU 145 cells. Transfection with AMPKα1-siRNA resulted in a decrease in endogenous AMPKα1 expression in these cells (Fig. 4, A and B). Treatment with TRAIL and TZD combination showed a significant attenuation of cleavage of caspases 3, 9, and 8 and PARP following knockdown of AMPKα1 expression (Figs. 4, A and B). In addition, because these cells also expressed the AMPKα2 isoform, we determined whether knocking down both AMPKα1 and α2 isoforms produced any synergistic effects in antagonizing this apoptosis pathway. These studies showed that knocking down either AMPKα1 or α2 antagonized TRAIL-TZD-induced apoptosis with AMPKα1 playing a more predominant role (Fig. 4B). These studies confirmed that induction of apoptosis following combinatorial treatment with TRAIL and TZD in PCa cells involves AMPK.

Role of PPARγ in TRAIL and TZD-induced PCa Cell Apoptosis—Because TZD is an agonist of PPARγ, we also determined the role of PPARγ in this apoptotic cascade. To determine the involvement of PPARγ, we first estimated changes in luciferase activity of PPARγ-responsive luciferase reporter (PPRE-luc) following treatment with this apoptotic combination. Because the expression of PPARγ was higher in the DU 145 compared with the LNCaP cells (data not shown), PPRE-luc assays were carried out in the DU 145 cells. Treatment of
these cells with a combination of TRAIL (100 ng/ml) and increasing concentrations of TZD (5–100 μM) for 6 h did not show any induction of PPRE-luc activity and rather showed a gradual reduction in luciferase activity as shown in Fig. 5A. However, similar combination treatment was able to induce apoptosis in the DU 145 cells as indicated by cleavage of caspases 3, 9, and 8 and PARP (Fig. 5C). To rule out any effect of apoptosis on PPRE-luc activity, we performed the PPRE-luc assays with increasing concentrations of TZD alone (without TRAIL), which also showed no significant induction of PPRE-luc activity (Fig. 5B). To conclusively determine any involvement of PPARγ in TRAIL-TZD-induced apoptosis, we knocked down endogenous PPARγ expression in DU 145 cells using PPARγ-siRNA. These results in Fig. 5D showed a significant reduction of PPARγ expression with PPARγ-siRNA. Knockdown of PPARγ expression showed a reduction in the cleavage of caspase 3 and of caspase 8 (p43/41 and p18 KD forms), during the early stages of apoptosis but not at the later stages of apoptosis, whereas the cleavages of caspase 9 and PARP were unaffected. These results suggested a partial involvement of PPARγ in mediating the early stages of this apoptosis cascade, which does not seem to be mediated through PPRE. In addition, knockdown of PPARγ expression also seemed to reduce endogenous AMPKα1 and α2 expressions and thus might mediate apoptosis indirectly via regulating AMPKα expression.

**TRAIL and TZD-induced β-Catenin Cleavage Is Mediated by AMPK**—Our earlier studies showed that induction of apoptosis with TRAIL and TZD combination was also associated with an increase in cleavage of β-catenin protein (16) mediated by caspases. Once we established in the preceding studies the involvement of AMPK in mediating caspase activation in TRAIL-TZD-induced apoptosis, the next set of studies were designed to determine any role of AMPK in mediating β-catenin cleavage. To address this, endogenous AMPKα1 was knocked down from DU 145 cells using AMPKα1-siRNA.
which showed a significant reduction of AMPK levels. Knocking down AMPK levels also resulted in a reduction of β-catenin cleavage by TRAIL-TZD (Fig. 6A, compare cl-β-catenin in lanes 2 and 4 with lanes 6 and 8) and restoration of full-length β-catenin (compare FL-β-catenin in lane 4 with lane 8). Similar antagonism on β-catenin cleavage was also observed in the LNCaP cells following knockdown of AMPK (Fig. 6B). To conclusively demonstrate the involvement of AMPK in mediating β-catenin cleavage, TRAIL-TZD studies were also carried out in the C4-2-EV and C4-2-DN cells. Although an increase in β-catenin cleavage was detected by 8 h in the C4-2-EV, it was significantly attenuated in the C4-2-DN cells (Fig. 6C). It is important to note that the partially cleaved β-catenin fragment (indicated by an asterisk) that appears in the C4-2-DN cells (lanes 5–8) is most likely a non-apoptosis-related band, because this is also observed in the vehicle-treated samples. These results suggest that TRAIL-TZD treatment leads to the cleavage of β-catenin protein via AMPK pathway.

**TRAIL and TZD Modulates AMPK Pathway**—The information provided above showed an involvement of AMPK in mediating the effects of TRAIL-TZD combination treatment toward induction of apoptosis and β-catenin cleavage. To determine whether this combination also modulated AMPK pathway, we determined the status of phosphorylation of ACC at Ser79, which is a downstream target of AMPK (45). Data shown in Fig.
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**FIGURE 3.** Effect of increasing concentrations of TRAIL and TZD on C4-2-EV and C4-2-DN cell death. C4-2-EV and C4-2-DN cells were treated with DMSO or a combination of TZD (50 μM) and increasing concentrations of TRAIL (25–100 ng/ml) (A) or a combination of TRAIL (100 ng/ml) and increasing concentrations of TZD (10–100 μM) (B). The cells were harvested after 4 h of treatment and analyzed by Western blots.

**FIGURE 4.** Effect of knockdown of endogenous AMPKα1 or α2 on TRAIL and TZD-induced apoptosis. A, DU 145 cells were transiently transfected with 50 nM of either control siRNA (lanes 1 and 2) or AMPKα1 siRNA (lanes 3 and 4) followed by treatment with DMSO or TRAIL-TZD combination for 8 h. At the end of the incubation, Western blot analysis was performed utilizing the antibodies indicated. B, DU 145 cells were transiently transfected with either control siRNA (lanes 1 and 2), AMPKα1 siRNA (lanes 3 and 4), AMPKα2 siRNA (lanes 5 and 6), or a combination of AMPKα1 and α2 siRNA (lanes 7 and 8) followed by treatment with TRAIL-TZD and Western blot analysis as in A.

7A (WCE panel) indicated an increase in pACC Ser79 phosphorylation as early as 1 h in LNCaP cells, which preceded apoptosis induction as shown by cleavage of caspase 3. A similar induction of pACC Ser79 with TRAIL-TZD was also observed in C4-2-EV cells with a peak induction at 4–8 h. (Fig. 7B, WCE panel) associated with increased apoptosis (Fig. 2A). These suggest an activation of AMPK pathway following TRAIL-TZD treatment, which then mediates the induction of apoptosis. Since total AMPKα expression shows some reductions with TRAIL-TZD treatment, we estimated the effect of this treatment on AMPKα phosphorylation at Thr172 following normalization of total AMPKα levels. These showed a modest increase in pAMPKα Thr172 levels in both cell types with TRAIL-TZD treatment (Fig. 7, A and B, Normalized panels). However, the detailed mechanism by which AMPK is activated by this combination is still unclear and might involve novel pathways.

**Discussion**

Earlier studies by our group as well as others have demonstrated that combination of pro-apoptotic TRAIL with ligands of PPARγ can induce potent apoptosis in various cancer cells (16–21). These observations have important clinical implica-
tions, because they seem to be effective in ameliorating TRAIL resistance that is prevalent in various cancer cells including PCa (10). To determine the efficacy of this combination treatment in PCa cells, we utilized various PCa cells including LNCaP (androgen-dependent), DU 145 (androgen-independent), and C4-2 and C4-2B (Castration-resistant) (46, 47) cells in the current studies. Treatment with TRAIL and TZD combination seemed to induce a higher degree of apoptosis compared with TRAIL alone in all the cells types tested (Fig. 1, A–D), suggesting that this combination treatment can target both androgen-dependent and -independent PCa cells toward apoptosis. Because apoptosis resistance is one of the major hallmarks of cancer progression including castration-resistant PCa, combination of TRAIL with troglitazone might be an effective means of sensitizing these resistant forms toward cell death.

Despite observations by various groups that a combination of TRAIL with PPARγ ligands can increase the apoptotic potential of TRAIL-resistant cells, the detailed signaling mechanism and the mediators involved in this pathway are not clearly defined. Understanding of the mechanisms that ameliorates TRAIL resistance is critically required to design novel TRAIL-based combination therapeutic approaches. To gain a mechanistic insight toward this, we designed studies to determine whether AMPK might be a potential target of TRAIL-TZD-induced apoptosis. This was based on the rationale that AMPK can be activated by the thiazolidinedione group of antidiabetic drugs (35, 36), which also mediate growth suppression (37). To address the role of AMPK, we utilized PCa cells that expresses either an empty vector (C4-2-EV cells) or dominant negative AMPK (C4-2-DN cells) (41). Treatment of C4-2-EV cells with
TRAIL-TZD combination induced potent apoptosis within a 4-h time frame that was significantly attenuated in the C4-2-DN cells (Fig. 2, B–D), suggesting a potential involvement of AMPK in mediating these events. Additional studies carried out with higher concentrations of TRAIL or TZD were also unable to sensitize the C4-2-DN cells (Fig. 3, A and B). The participation of AMPK was further confirmed by AMPKα1-siRNA studies. They showed a significant reduction in TRAIL-TZD-induced apoptosis following knockdown of endogenous AMPKα (Fig. 4, A and B), thus confirming that an active AMPK signaling axis is needed for effective apoptosis induction following TRAIL combinatorial treatment. Several recent reports have indicated a potential beneficial effect of AMPK activation in PCa (48), which has led to the development of a novel AMPK activator that can antagonize cell growth in androgen-sensitive and castration-resistant PCa (49). The studies described here revealed a novel mechanism of targeting AMPK pathway to sensitize PCa cells toward apoptosis. To determine the mechanism by which this combination might be modulating AMPK pathway, we also estimated changes in the levels of phospho AMPKα1T172, which showed a modest increase during the peak time of apoptosis (Fig. 7, A and B, Normalized panels). However, estimation of AMPK downstream target ACC phosphorylation showed distinctive early induction of pACCS79 levels following TRAIL-TZD treatment (Fig. 7), indicative of AMPK pathway activation (49). In the presence of this modest increase in AMPKα1T172 phosphorylation, the detailed mechanism by which AMP is activated by this combination treatment is unclear. Studies are currently underway to investigate in depth the mechanism involved. Interest-

**FIGURE 6. Role of AMPK in mediating β-catenin cleavage following treatment with TRAIL-TZD combination.** A and B, DU 145 (A) or LNCaP (B) cells were transfected with control siRNA or AMPKα1 siRNA as in Fig. 4A followed by treatment with TRAIL and TZD combination for the indicated periods of time. Western blot analysis was performed with an antibody against β-catenin to detect both full-length (FL) and cleaved (Cl) β-catenin protein. Two different exposures were used to show the effects on full-length (low exposure) and Cl forms (high exposure). They were also blotted with an antibody against AMPKα1 to show the degree of knockdown and GAPDH (as control). C, C4-2-EV and C4-2-DN cells were treated with DMSO or TRAIL-TZD for the indicated periods of time and analyzed by Western blots. The panel labeled (FLAG) AMPK-DN indicates expression of FLAG-tagged AMPKα-DN. The asterisk next to the β-catenin panel indicates a non-apoptosis-related fragment observed under all treatment conditions.
endogenous AMPKα1 and α2 expressions. It is thus unclear whether PPARγ has a direct role in this apoptotic cascade or mediates this via regulating AMPKα expression. To understand whether TRAIL and TZD combination activates PPARγ transcriptional activity, we also estimated the luciferase activity of a PPARγ-responsive luciferase reporter tk-PPRE3-luc (38). The results showed no induction of PPARγ transcriptional activity with increasing concentrations of TZD alone (Fig. 5B) or in combination with TRAIL (Fig. 5A). The same combination of TRAIL and TZD was, however, very effective in inducing caspase cleavage (Fig. 5C) indicative of apoptosis. Thus the involvement of PPARγ seems to be PPRE-independent. In fact, PPRE-independent effect of PPARγ has been reported earlier that regulates target gene expression via a novel pathway (52).

To obtain a mechanistic insight and to identify a suitable target that mediates TRAIL-TZD-induced apoptosis, we observed a significant attenuation of β-catenin/TCF transcriptional activity associated with a reduction of β-catenin protein expression upon treatment with this combination (16). Further elucidation of this mechanism revealed that antagonism of β-catenin pathway was also associated with an increased cleavage of β-catenin protein corresponding to the time of apoptosis. To understand whether AMPK pathway was involved in mediating β-catenin cleavage following TRAIL-TZD treatment, we compared any differences in β-catenin cleavage in C4-2-EV and C4-2-DN cells. Interestingly, the cleavage of β-catenin was significantly attenuated in the C4-2-DN cells compared with the C4-2-EV cells (Fig. 6C). It is, however, important to note that we were unable to obtain a complete blockage of β-catenin cleavage in these cells as was observed in case of apoptosis induction. This was likely because β-catenin cleavage is a later event in this apoptosis cascade, which peaks at ~8–16 h, and treatment of the C4-2-DN cells for prolonged periods with TRAIL-TZD seems to inhibit ectopic AMPK-DN expression (see reduced FLAG expression in Fig. 6C, lane 8). This is also reflected in a slight increase in caspase 3 cleavage. Thus to confirm the participation of AMPK in mediating β-catenin cleavage, we also knocked down endogenous AMPKα expression in both DU 145 and LNCaP cells (Fig. 6, A and B). This showed a significant reduction in β-catenin cleavage in both cells types following knockdown of AMPKα expression. Caspase-induced cleavage of β-catenin has been reported in other cells, which was associated with a reduction of its transcriptional potential (53, 54). Cleavage of β-catenin also results in disruption of adherens junctions (54, 55), and a combination of these two events might contribute toward increased apoptosis. Although it is unclear whether cleaved β-catenin has any functional role in mediating or promoting this apoptotic cascade in prostate cancer, our results show an increased cleavage downstream of caspase activation in all cell types. We have, however, demonstrated earlier that full-length β-catenin plays an important pro-survival role in cancer cells that can be antagonized by TRAIL-TZD combination. Further studies are needed to confirm any role of cleaved β-catenin in apoptosis. Nevertheless, our results indicate a novel AMPK-mediated pathway that regulates β-catenin cleavage following induction of PCa cell apoptosis.

**FIGURE 7. Effect of TRAIL and TZD combination on AMPK pathway.** LNCaP (A) and C4-2-EV (B) cells were treated with DMSO or TRAIL-TZD combination for various lengths of time. Equal amounts of protein were then analyzed by Western blots with the indicated antibodies on the top panels labeled WCE for whole cell extract. Total and pAMPKα172 were blotted following normalization of total AMPKα levels on the bottom panels labeled Normalized.
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Author Contributions—S. S., N. V., and S. D. helped in developing the overall methodologies, performing the experiments, and acquisition and interpretation of data. S. S. and N. V. contributed to the writing, drafting, and preparation of the manuscript. A. R. helped with the analyses of the AMPK data and provided intellectual input in this collaborative study. B. R. contributed to the overall study design and interpretation of the results during all phases and drafted/edited the final manuscript, which was read and approved by all the authors.

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