Glycogen synthase kinase-3β suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer

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Abstract

Prostate cancer is a major health threat for American men. Therefore, the development of effective therapeutic options is an urgent issue for prostate cancer treatment. In this study, we evaluated the effect of glycogen synthase kinase-3β (GSK-3β) suppression on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in human prostate cancer cell lines. In the presence of lithium chloride (LiCl) or SB216763, the GSK-3β inhibitors, TRAIL-induced cell death was dramatically enhanced, and the enhanced cell death was an augmented apoptotic response evidenced by increased Annexin V labeling and caspase-3 activation. GSK-3β gene silencing mediated by a small interference RNA (siRNA) duplex also sensitized the cells to TRAIL, confirming the specificity of GSK-3β suppression. Importantly, TRAIL stimulation increased GSK-3β tyrosine phosphorylation at Y216, suggesting that GSK-3β is activated by TRAIL. Furthermore, TRAIL sensitization was associated with increased proteolytic procession of caspase-8 and its downstream target BID, and z-IETD-FMK, the inhibitor specific to active caspase-8 totally blocked LiCl-induced TRAIL sensitization. Finally, Trichodion, a potent nuclear factor-κB (NF-κB) inhibitor, could not affect LiCl-induced TRAIL sensitization, although GSK-3β inhibitors significantly blocked TRAIL-reduced NF-κB activity in prostate cancer cells. These results indicate that GSK-3β suppression sensitizes prostate cancer cells to TRAIL-induced apoptosis that is dependent on caspase-8 activities but independent of NF-κB activation, and suggest that a mechanism involving GSK-3β activation may be responsible for TRAIL resistance in prostate cancer cells. (Mol Cancer Ther. 2003;2:1215–1222)

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Introduction

Prostate cancer is the second only to lung cancer in the frequency of mortality (1). Although patients with advanced prostate cancer are effectively treated with androgen ablation, the effect on disease progression is temporary. The diseases ultimately become unresponsive to androgen ablation and are then classified as having hormone-refractory prostate cancer with no means to cure (2). Therefore, the development of novel therapeutic options is an urgent issue for prostate cancer treatment.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has received significant attention as a novel anticancer agent (3, 4). Similar to tumor necrosis factor (TNF)-α or Fas, TRAIL triggers apoptosis through binding with death receptors (DR4 and DR5) and it appears less toxic based on animal safety studies (4, 5). Although it seems specifically kill transformed and cancer cells originally (3), concerns about TRAIL toxicity in humans emerged with recent reported adverse effects on primary cultures of human tissues (6) or normal prostate-derived cells (5). On the other hand, recent studies revealed that an increasing number of cancer cell lines including prostate cancer cells and fresh tumor isolates are resistant to TRAIL-induced apoptosis, while chemotherapeutic agents can reverse TRAIL resistance, indicating that combination therapy may be a potent approach against prostate cancer (8–13).

Glycogen synthase kinase-3 (GSK-3) is an old gene with homologue identified in every eukaryotic species examined. It was named because of its phosphorylation activity toward glycogen synthase. It has been implicated in fundamental processes including cell fate determination, metabolism, transcriptional control, and, in mammals, oncogenesis and neurological diseases. Two GSK-3 genes (α and β) have been cloned in mammals and these kinase homologues show strong sequence conservation within their catalytic domain (14). Recently, it has been demonstrated that GSK-3β plays a critical role in cell survival by phosphorylating nuclear factor-κB (NF-κB) p65 subunit and subsequently NF-κB transactivation in hepatocytes (15, 16).

The aim of this study is to evaluate the effect of GSK-3β suppression on TRAIL-induced apoptosis in human prostate cancer cells. We demonstrated that suppression of GSK-3β activity, through specific inhibitors or by gene silencing, dramatically sensitized prostate cancer cells to TRAIL-induced apoptosis. This occurs by enhancing caspase-8 activation and subsequent downstream cascade, but independently of NF-κB pathway.

Materials and Methods

Cells and Reagents

The human prostate cancer cell lines (LNCaP, DU145, and PC-3) and HEK293 cells were obtained from the

American Type Culture Collection (Manassas, VA). The prostate cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Recombinant human TRAIL/Apo2 and TNF-α were purchased from PeproTech Inc. (Rocky Hill, NJ). The recombinant TRAIL is a non-cross-linked protein of M_r 19,600 comprising the full length of the TNF-like extracellular domain of TRAIL. They were dissolved as stock solutions of 10 μg/ml in PBS and stored at −20°C until use. The caspase-8 specific inhibitor z-IETD-FMK and anti-human Fas antibodies (agonistic antibodies; clone DX2) were obtained from Calbiochem (San Diego, CA). The NF-κB specific inhibitor Trichodion was obtained from Alexis Biochemicals Corp. (San Diego, CA). SB216763, antibodies to GSK-3β and actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GSK-3β Y216 phospho-specific antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Lithium chloride (LiCl) and other reagents were supplied by Sigma Chemical Co. (St. Louis, MO). Antibodies against caspase-3, caspase-8, and BID were obtained from Cell Signaling (Beverly, MA).

Cytotoxicity Assays and Flow Cytometry

Cells were seeded at 5×10^4 cells/well in a 12-well plate. The next day, cells were stimulated with various reagents as indicated in the figure legend. In case of small interference RNA (siRNA)-mediated gene silencing, the siRNA duplexes were transfected for 3 days before adding TRAIL. Typically, cell viability was assessed with a trypan blue exclusion assay (17). Confirmation of apoptosis was conducted by using Annexin V-FITC Apoptosis Detection Kit (BD PharMingen, San Diego, CA) according to manufacturer's manual. Briefly, after treatment, cells were collected and washed with ice-cold PBS and then suspended in Annexin V binding buffer. Then, cells were stained for 15 min at room temperature in the dark and analyzed on a FACS Calibur flow cytometer using CELLQuest software.

Isolation of Cell Extracts and Western Blotting

After incubation and treatment, cells were washed in PBS and lysed in a radioimmunoprecipitation assay (RIPA) buffer supplied with protease inhibitors (CytoSignal, Irvine, CA) and lysates were cleared by centrifugation. An equal amount of protein was subjected to SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and blocked with 5% dry milk in Tris-buffered saline containing 0.1% Tween 20. Western blot analysis was performed as described previously (18) to assess the protein expression level of GSK-3β, Actin, caspase-3, caspase-8, and BID. Blots were developed with a SuperSignal West Dura Substrate kit (Pierce Biotech, Rockford, IL).

siRNA Synthesis and Transfection

Sequence information regarding human *GSK-3β* gene (GenBank accession NM_002093) and *AR* gene (GenBank accession NM_000044) were extracted from the NCBI Entrez nucleotide database. Several siRNAs with different targeting sequence for each gene were selected and each targeting segment was searched with NCBI BlastN to confirm specificity only to the targeted gene. The 29-mer sense and antisense DNA oligonucleotide templates (21 nucleotides specific to the targets and 8 nucleotides specific to T7 promoter primer sequence 5′-CCTGTCTC-3′) were synthesized by IDT (Coralville, IA). The siRNAs were prepared by a transcription-based method using the *Silencer* siRNA construction kit (Ambion, Austin, TX) according to manufacturer's instructions. The quality of the synthesized siRNA was estimated by agarose gel analysis and found to be very clean. RNAs were quantified by using RiboGreen fluorescence (Molecular Probes, Eugene, OR). To determine the efficiency of the siRNAs, preliminary experiments were conducted in HEK293 cells by transfection of the siRNAs at various concentrations (0.1–10 nM) in the culture with the Oligofectamine reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Two days later, the protein levels of the targeted genes were verified by Western blot. The siRNAs targeting the human *GSK-3β* gene (5′-AAG AAT CGA GAG CTC CAG ATC-3′, designated as #4 and 5′-AAG TAA TCC ACC TCT GGC TAC-3′, designated as #16), dramatically reduced GSK-3β protein expression at 10 nM concentration; however, the AR siRNA (5′-AAG AAG GCC AGT TGT ATG GAC-3′) had no effect on GSK-3β expression.

NF-κB Reporter Gene Assay

A NF-κB-luciferase reporter construct (pNFκB-Luc) was obtained from Clontech (Palo Alto, CA). The reporter vector pCMV-SEAP was described previously (19). The cells were plated in six-well tissue culture plates and transfected the following day with 1.0 μg of the pNFκB-Luc and 0.5 μg of the pCMV-SEAP construct by using the Cytofectene reagent (Bio-Rad, Hercules, CA) according to manufacturer's protocol in serum-free media. After 24 h, the cells were treated with various agents as indicated in the figure legends. After 24 h, culture supernatants were collected and assayed for SEAP activity (19), and cells were harvested for luciferase activity. Protein concentration in the cell lysates was measured by a Protein Assay Kit (Bio-Rad). An equal amount of protein from each cell lysate was assayed in triplicate for luciferase enzyme activity by using the Promega's Luciferase Assay System (Madison, WI) and Berthold Lumat LB9501 reader (Oak Ridge, TN) as described previously (19). The luciferase activity of each sample was normalized against the corresponding SEAP activity before the fold induction value relative to control cells was calculated.

Statistical Analysis

All experiments were repeated two or three times. Western blot results are presented from a representative experiment. The mean and SD from two experiments for cell viability and luciferase assay are shown. The number of viable cells before treatment is assigned a relative value of 100%. The significant differences between groups were analyzed using the SPSS computer software (SPSS Inc., Chicago, IL).

Results

LiCl Sensitizes Prostate Cancer Cells to TRAIL-Induced Apoptosis

Previous reports have demonstrated that LiCl enhances TNF-α-mediated anti-tumor activity *in vitro* and *in vivo* (20, 21). With the aim of preclinical investigation of lithium's effect on TRAIL sensitization, three commonly used prostate cancer cell lines PC-3, LNCaP, DU145 were tested. Cell viability was determined by trypan blue exclusion assay. In PC-3 cells, TRAIL alone at 10 ng/ml did not cause significant cell death after 24 h treatment. However, LiCl addition dramatically enhanced TRAIL-induced cell death despite LiCl alone did not cause significant cytotoxicity (Fig. 1A). To rule out the eventual intervention of the anion (Cl^-) and the osmotic disturbance, the effect of LiCl was compared to those of equimolecular concentrations of potassium chloride (KCl) or sodium chloride (NaCl), which were also added to the culture medium individually. As shown in Fig. 1B, when exposing PC-3 cells to KCl or NaCl at the same concentration as LiCl, TRAIL-induced cell death remained unchanged, suggesting the selectivity of Li^+ on TRAIL sensitization.

To confirm that the enhanced cell death by LiCl was due to augmented apoptotic response, two hallmark markers for apoptosis, externalization of the membrane phospholipids phosphatidylserine (Annexin V-FITC assay) and procaspase-3 reduction (due to cleavage of procaspase-3 after activation), were utilized. To detect the early apoptotic response after TRAIL and LiCl addition, PC-3 cells were treated with TRAIL plus or minus LiCl, and then subjected to Annexin V-FITC assay. As shown in Fig. 1C, apoptotic cells at earlier stage (Annexin V-FITC positive/PI negative) were dramatically increased as early as 6 h after addition of TRAIL plus LiCl compared to TRAIL or LiCl alone. To detect the later stage of apoptotic events, procaspase-3 reduction was determined by Western blot. Procaspase-3 reduction was not noted when TRAIL was used alone at low dose of 10 ng/ml; however, significant reduction of procaspase-3 was observed when TRAIL was used at 10 ng/ml together with LiCl (Fig. 1D).

Next, we tested LiCl's effect on TRAIL sensitization in DU145 and LNCaP cells, in which LNCaP cells were reported to be less sensitive to TRAIL-induced apoptosis compared to other cell lines. As shown in Fig. 2, A and B, TRAIL-induced cell death was greatly enhanced in both cell lines when LiCl was added.

LiCl-Induced TRAIL Sensitization Is Due to GSK-3β Suppression

It has been demonstrated that LiCl has multiple non-competitive targets in addition to GSK-3β (22–24). To evaluate if LiCl's effect on TRAIL sensitivity is due to GSK-3β suppression, we examined the effect of a synthetic GSK-3β specific inhibitor, SB216763 (25), on TRAIL sensitization. Similar to the effect of LiCl, SB216763 also caused a significant increase of TRAIL-mediated cell death in PC-3 cells (Fig. 2C). Furthermore, SB216763 enhanced TRAIL-induced procaspase-3 reduction in PC-3 cells

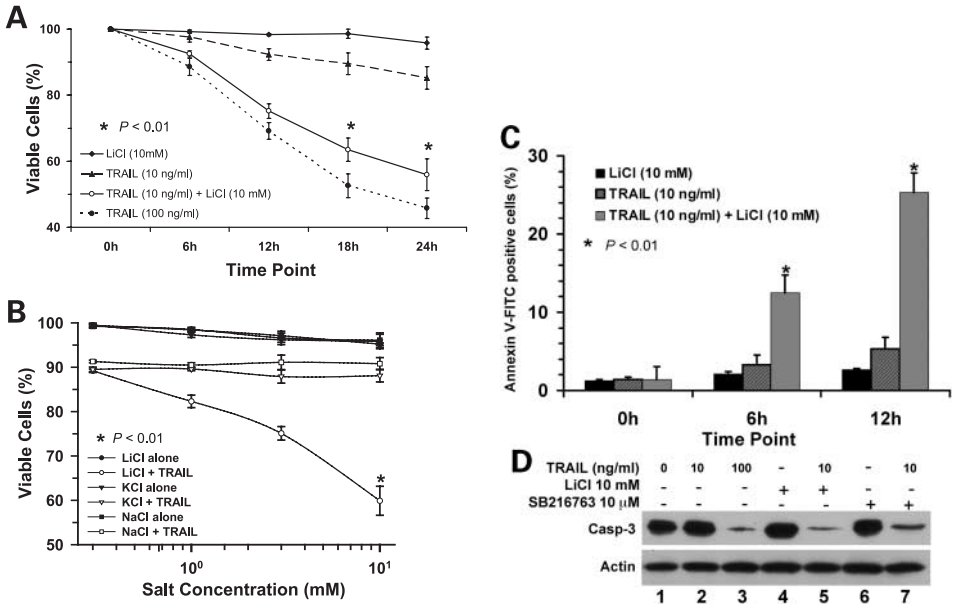

Figure 1. LiCl enhances TRAIL-induced cell death. **A,** PC-3 cells were seeded in 12-well plates overnight and then treated with TRAIL together with or without LiCl at indicated doses. Cells were harvested at indicated time points, stained in 0.4% trypan blue solution. Viable cells were counted using a hemacytometer under an inverted microscope. **B,** PC-3 cells were treated with TRAIL (10 ng/ml) together with or without the indicated salt and harvested 24 h later. Viable cells were counted as described above. **C,** PC-3 cells were seeded in six-well plates overnight and then treated with TRAIL together with or without LiCl at indicated doses. At indicated time points, cells were harvested and subjected to fluorescence-activated cell sorting analysis after staining with Annexin V-FITC kit as mentioned in the text. The *asterisk* indicates a significant difference between TRAIL alone *versus* TRAIL plus LiCl. **D,** PC-3 cells were seeded in 35-mm dish overnight and then treated with TRAIL together with or without LiCl at indicated doses for 24 h. Procaspase-3 were assessed as described in the text. Actin blot served as loading control. Data are from three independent experiments.

(Fig. 1D). We next evaluated the effect of *GSK-3β* gene silencing mediated by siRNA duplex (26) on TRAIL sensitivity. Two siRNA duplexes against human GSK-3β mRNA at two different regions were synthesized *in vitro* and transfected into PC-3 cells. Another siRNA duplex against human AR mRNA was used as negative control because PC-3 cells lack AR expression (27). Three days after transfection, cells were treated with TRAIL for another 24 h. As shown in Fig. 3A, the protein level of GSK-3β was knocked down dramatically by transfection of the GSK-3β siRNA duplexes compared to the controls. In parallel, transfection of the GSK-3β siRNA duplexes caused a significant increase of TRAIL-induced cell death, whereas the AR siRNA duplex did not affect TRAIL sensitivity (Fig. 3B). These results confirmed that GSK-3β suppression leads to TRAIL sensitization in prostate cancer cells.

It was believed that GSK-3 is a constitutively active enzyme, the activity of which is decreased in response to cell stimulation, such as insulin and Wnt pathways (14). However, recent evidences demonstrated that GSK-3β can be activated in response to certain cellular stimuli, and its activity is associated with the phosphorylation status of the tyrosine residue Y216 (28). Because GSK-3β suppression enhances TRAIL-mediated cell death, we asked if GSK-3β is activated by TRAIL stimulation. To address this issue, we evaluated tyrosine phosphorylation of GSK-3β using an Y216 phospho-specific antibody. DU145 cells were serum-starved for 24 h and then treated with TRAIL in serum-free media. As shown in Fig. 3C, TRAIL stimulation considerably increased tyrosine phosphorylation of GSK-3β at the Y216 site in a time-dependent manner. These data strongly suggest that GSK-3β is activated after TRAIL stimulation.

GSK-3β Inhibitor-Induced TRAIL Sensitization Depends on Caspase-8 Activity

Caspase-8 is a critical mediator for death receptor-induced apoptosis (reviewed in Ref. 29). Thus, we asked whether TRAIL sensitization of prostate cancer cells by GSK-3β inhibition is acting through the caspase-8 pathway or by other novel caspase-independent mechanism that in turn contributes to TRAIL-induced apoptosis. To this end, we tested if LiCl enhances TRAIL-mediated caspase-8 activation via proteolytic procession of the pro-form caspase. DU145 cells were treated with TRAIL in the presence or absence of LiCl for 18 h, and the cleavage of procaspase-8 was determined by Western blot. As shown in Fig. 4A, in the presence of LiCl, TRAIL-induced cleavage of procaspase-8 was significantly enhanced compared to LiCl or TRAIL alone. Consistent to this, the amount of BID proform, the immediate downstream target of active caspase-8 (30), was also significantly reduced. These data indicate that LiCl enhances TRAIL-induced caspase-8 activation and subsequently BID cleavage.

Next, we used a caspase-8 specific inhibitor z-IETD-FMK (31) to establish the involvement of caspase-8 in GSK-3β inhibitor-induced TRAIL sensitization. DU145 cells were pretreated with z-IETD-FMK followed by addition of TRAIL together with or without LiCl or SB216763. As shown in Fig. 4B, when TRAIL was added together with

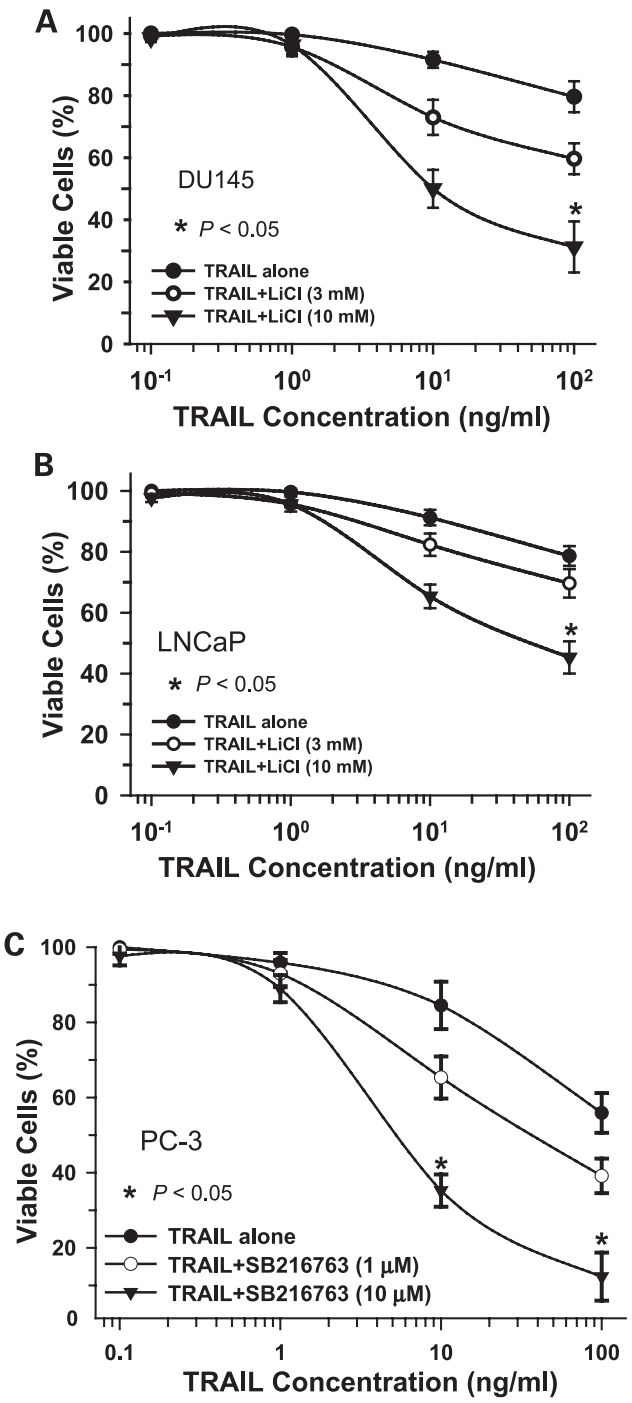

Figure 2. Effects of LiCl on TRAIL-induced cell death in DU145 (**A**) and LNCaP (**B**) cells. Cells were seeded overnight and then treated with TRAIL or TRAIL plus LiCl at the indicated doses. Cells were harvested for counting the survival rate as described earlier. The *asterisk* indicates a significant difference between TRAIL alone *versus* TRAIL plus 10 mM LiCl. **C,** effect of SB216763 on TRAIL-induced cell death. PC-3 cells were treated with TRAIL with or without SB216763 at the indicated doses for 24 h. Cells were harvested 24 h later for counting the survival rate as described earlier. The *asterisk* indicates a significant difference between TRAIL alone *versus* TRAIL plus 10 μM SB216763. Data are from three independent experiments.

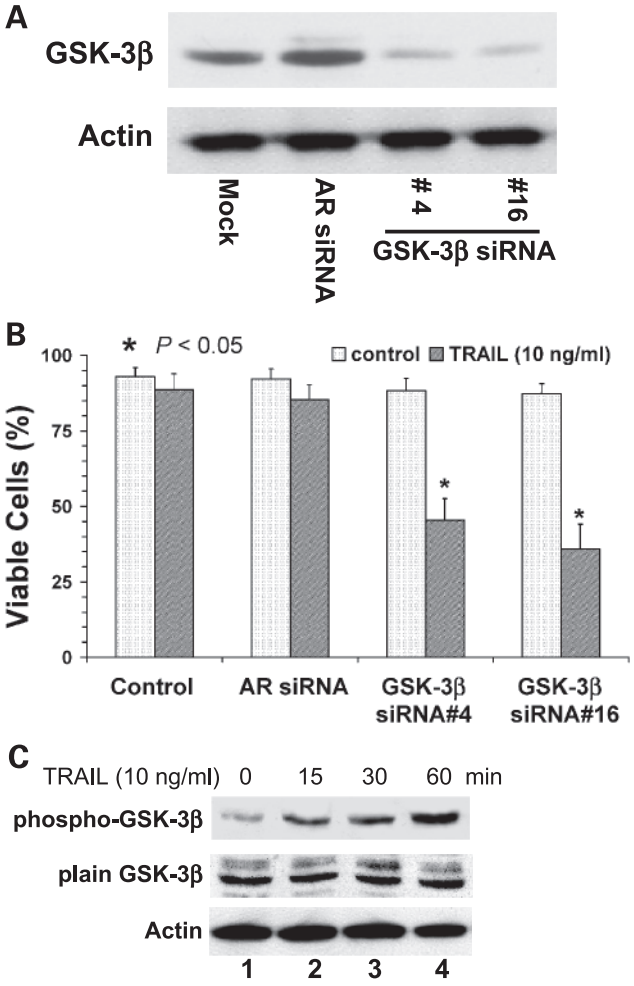

Figure 3. Effect of GSK-3β gene silencing mediated by a siRNA duplex on TRAIL-induced cell death. **A,** PC-3 cells were transfected with the siRNA duplexes (10 nM) as indicated in the figure by Oligofectamine transfection reagent. Mock control cells received transfection reagent only. Cells were harvested 3 days later and the protein level of GSK-3β was determined by Western blot as described in the text. Actin blot served as loading control. **B,** PC-3 cells were transfected with the siRNAs as described above for 3 days followed by treatment with TRAIL or solvent control at the indicated doses for 24 h. Viable cells were counted as mentioned earlier. The *asterisk* indicates a significant difference between cells treated with TRAIL *versus* the solvent. Data are from two independent experiments. **C,** TRAIL stimulation increases GSK-3β phosphorylation at Y216. DU145 cells were serum-starved for 24 h followed by addition of TRAIL in fresh serum-free media. Cells were harvested at indicated time points and GSK-3β phosphorylation at Y216 was evaluated as described in the text. Plain GSK-3β and Actin blots served as loading control. Data are from two independent experiments.

either LiCl or SB216763, significant cell death was induced; however, pretreatment with Z-IETD-FMK totally blocked cell death. These data indicate that the sensitization of TRAIL-mediated cell death by GSK-3β inhibitor depends on caspase-8 activity.

NF-κB Activity Is Not Involved in TRAIL Sensitization Induced by GSK-3β Inhibitors

It has been shown that NF-κB is activated in several cell lines after death receptor engagement, which is responsible at least in part, if not all, for TRAIL resistance (32, 33). In response to TNF-α stimulation, GSK-3β activity is required for NF-κB activation in hepatocyte (16). In the other hand, LiCl-induced NF-κB activation in human intestinal epithelial cells was also reported (34). Thus, the role of GSK-3β in NF-κB activation remains to be elucidated. To determine if TRAIL sensitization induced by GSK-3β inhibitor is due to preventing NF-κB activation in prostate cancer cells, we evaluated the effect of LiCl or SB216763 on TRAIL-stimulated NF-κB-controlled luciferase reporter activity (pNFκB-Luc). In HEK293 cells, TNF-α strongly activated NF-κB-Luc reporter while TRAIL weakly induced NF-κB activity (Fig. 5A), which is consistent with previous report (35). LiCl or SB216763 significantly enhanced either TNF-α- or TRAIL-stimulated NF-κB-Luc reporter activity although no TRAIL sensitization was induced. In prostate cancer cells, however, TRAIL alone did not induce but slightly reduced NF-κB activity in a dose-dependent manner (Fig. 5, B–D). Nevertheless, PMA, another known NF-κB activator (36), strongly stimulated NF-κB-Luc reporter in these cells (Fig. 5C and data not shown). Interestingly, TRAIL-induced reduction of NF-κB activity was significantly

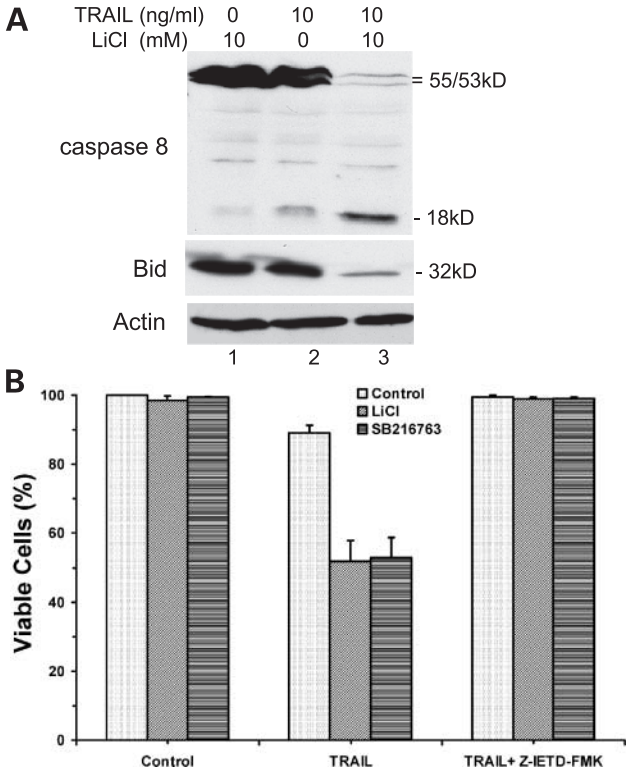

Figure 4. **A,** LiCl enhances TRAIL-induced caspase-8 activation and BID cleavage. DU145 cells were treated with TRAIL at the indicated doses together with or without LiCl for 18 h, and the cleavage of procaspase-8 or BID was assessed by Western blot. Actin blot was served as loading control. **B,** caspase-8 inhibitor Z-IETD-FMK blocks GSK-3β inhibitor-induced TRAIL sensitization. Du145 cells were pretreated with z-IETD-FMK (100 μM) for 30 min followed by addition of TRAIL (10 ng/ml) plus or minus LiCl (10 mM) or SB216763 (10 μM) for another 18 h. Cells were harvested for counting the survival rate as described earlier. Data are from three independent experiments.

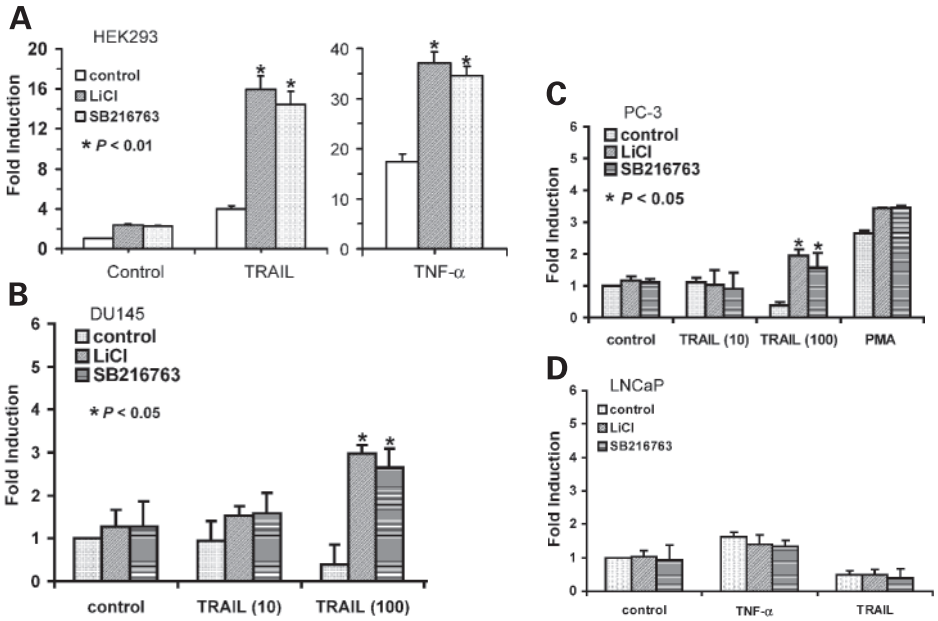

Figure 5. GSK-3β inhibitor-induced NF-κB activation is not involved in LiCl-induced TRAIL sensitization. **A–D,** cells as indicated in each panel were seeded in six-well plates overnight and then transiently co-transfected with NF-kB-Luc and pCMV-SEAP reporter constructs as described in the text. Cells were treated with the agents as indicated in serum-free media for 24 h. Control cells received the solvent only. Luciferase or SEAP activity was measured as described in the text. The luciferase activity was presented as fold induction against control sample after normalization with protein content and SEAP activity. The *asterisk* indicates a significant difference compared to the solvent control.

blocked by LiCl or SB216763 in DU145 and PC-3 (Fig. 5, B and C), while PMA-induced NF-κB activation was not affected significantly by GSK-3β inhibitors (Fig. 5C). Unexpectedly, GSK-3β inhibitors failed to block TRAIL-induced NF-κB reduction in LNCaP cells (Fig. 5D), suggesting that a cell type- or stimulus-specific distinction of NF-κB activation exists in response to GSK-3β inhibition.

It was reported recently that NF-κB possesses a pro-apoptotic effect in LNCaP cells (37). As mentioned above, we observed an increased NF-κB activation after TRAIL treatment in the presence of GSK-3β inhibitors in DU145 and PC-3 cells. One might postulate that NF-κB is playing a pro-apoptotic role in LiCl or SB216763-induced TRAIL sensitization. Thus, we used a potent NF-κB specific inhibitor Trichodion (38) in the trypan blue assay to test this possibility. As expected, Trichodion totally blocked NF-κB activation in all the cell lines we used in response to TNF-α, TRAIL, or PMA stimulation (data not shown). However, Trichodion did not sensitize the cells to TRAIL-induced cell death, while the sensitizing effect of LiCl was still observed in the presence of Trichodion (Fig. 6). These data suggest that NF-κB might not account for LiCl-induced TRAIL sensitization in prostate cancer cells.

Discussion

The principal findings of this study are: (*a*) GSK-3β suppression via its inhibitors or siRNA-triggered gene silencing sensitizes prostate cancer cells to TRAIL-induced apoptosis; (*b*) TRAIL stimulation induces GSK-3β tyrosine phosphorylation; (*c*) GSK-3β inhibitor-induced TRAIL sensitization depends on caspase-8 activities; and (*d*) NF-κB activity is not involved in TRAIL sensitization. These results provide a novel mechanism of GSK-3β modulation on TRAIL sensitivity.

The existence of TRAIL resistance was reported earlier from various cell types, including prostate cancer cells (8–13). The resistance is not solely due to differential expression of the TRAIL receptors; instead, some intracellular molecules, such as PI3K/Akt or Bcl-2 family proteins, modulate the downstream effect of TRAIL signaling (39–43). In this study, we demonstrated that LiCl, a well-known GSK-3β inhibitor, and a novel synthetic inhibitor, SB216763, at a sub-toxic concentration significantly sensitized prostate cancer cells to TRAIL-mediated cell death. The involvement of GSK-3β in TRAIL sensitization was confirmed by siRNA-mediated *GSK-3β* gene silencing. To the author's awareness, this is the first report showing GSK-3β involvement in TRAIL sensitization.

The mechanism underlying TRAIL sensitization mediated through GSK-3β inhibition is unknown. A recent

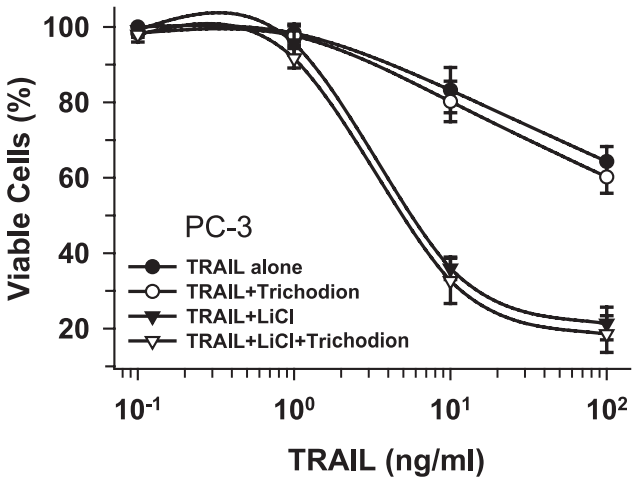

Figure 6. LiCl-induced TRAIL sensitization is not blocked by NF-κB inhibitor Trichodion. PC-3 cells were pretreated with Trichodion (10 μM) for 30 min and followed by addition of TRAIL (10 ng/ml) plus or minus LiCl (10 mM) for another 24 h. Viable cells were counted as described earlier. Data are from three separate experiments.

report demonstrated that a novel gene transcription or *de novo* protein synthesis is not required for LiCl-induced TNF-α sensitization (23). Therefore, β-catenin-mediated change in gene expression after GSK-3β suppression is unlikely involved in TRAIL sensitization. Furthermore, elevated activity of Akt kinase due to PTEN mutation is involved in TRAIL resistance in LNCaP cells (39–42), whereas activated Akt by certain stimuli leads to GSK-3β inactivation (44). In this report, we found that GSK-3β suppression sensitized LNCaP to TRAIL-mediated apoptosis, indicating that PTEN mutation or Akt activation may not be solely responsible for TRAIL resistance in LNCaP cells. In addition, we observed a sensitizing effect in DU145 cells that are lacking of Bax expression, the pro-apoptotic Bcl-2 family (45), although Bcl-2 was also reported to inhibit TRAIL-mediated cell death (43). Finally, LiCl has been shown to induce p53 stabilization (46), while we still observed an enhanced cell death in PC-3 cells that are lacking p53 expression (45). Thus, Bcl-2 family or p53 pathway may not be responsible for GSK-3β inhibitor-induced TRAIL sensitization.

Engagement of TRAIL to its receptors leads to recruit adaptor protein fas-associated death domain (FADD) [or TNF receptor-associated death domain (TRADD) in case of TNF-α stimulation] and subsequent caspase cascade. The FADD or TRADD adaptor protein plays an important role in forming the death-inducing signaling complex (DISC) that contains caspase-8 (29). In this study, we found that LiCl enhanced TRAIL-mediated caspase-8 activation and BID cleavage, and the synthetic caspase-8 specific inhibitor z-IETD-FMK totally abolished GSK-3β inhibitor-induced TRAIL sensitization. These results indicate that the sensitizing effect of GSK-3β inhibitors on TRAIL-mediated cell death is situated upstream of caspase-8. Our results are consistent with a previous report showing lithium's action on TRADD level (upstream of caspase-8) after TNF-α stimulation in L929 fibrosarcoma and KYM rhabdomyosarcoma cells (20). However, in contrast to our findings observed in prostate cancer cells, GSK-3β is not involved in LiCl-induced TNF-α sensitization observed in L929 or KYM cells (20), suggesting a cell-specific distinction.

Previous studies demonstrated that TRAIL stimulates NF-κB activation, while inhibiting NF-κB activation sensitizes cells to TRAIL-mediated apoptosis in some cell lines (32). Hepatocytes derived from GSK-3β knock-out mice showed increased sensitivity to TNF-α-induced cell death due to defect in NF-κB activation (16). In this study, however, we observed a distinct role of GSK-3β in TRAIL-induced NF-κB activation in prostate cancer cells. TRAIL or TNF-α did not stimulate but slightly reduced NF-κB activity, which was reversed by GSK-3β inhibitors in prostate cancer cells. These findings are consistent with a recent report showing that LiCl enhances NF-κB activation and IL-8 production (34). Furthermore, NF-κB specific inhibitor Trichodion did not block TRAIL sensitization induced by GSK-3β inhibitors although NF-κB activation is totally abolished by Trichodion. Therefore, our results suggest that NF-κB activation is not involved in TRAIL sensitization induced by GSK-3β inhibitors, and an alternative mechanism involving GSK-3β activity other than NF-κB activation may be responsible for TRAIL resistance in prostate cancer cells.

The therapeutic application of TRAIL in prostate cancer treatment is limited because most of the available prostate cancer cell lines are not quite sensitive to its cytotoxic action (12). Thus, secondary agent that specifically sensitizes the cells to TRAIL may overcome the TRAIL resistance and reduce the doses needed for effective treatment. Because lithium has been safely used for more than half a century in clinics to treat mental disorders (47), the combination of TRAIL plus lithium might become a hopeful therapy for prostate cancer patients.

In conclusion, our data indicate that GSK-3β suppression sensitizes prostate cancer cells to TRAIL-induced apoptosis, which is dependent on caspase-8 activity, but independent of NF-κB pathway. An understanding of the role of GSK-3β in TRAIL resistance may offer better therapeutic strategies in prostate cancers.

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