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Overexpression of Par-4 Enhances Thapsigargin-Induced Apoptosis Via Down-Regulation of XIAP and Inactivation of Akt in Human Renal Cancer Cells

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Abstract The prostate-apoptosis-response-gene-4 (Par-4) protein has been shown to function as an effector of cell death in response to various apoptotic stimuli that trigger mitochondria and membrane receptor-mediated cell death pathways. We found that overexpressing Par-4 by stable transfection sensitizes Caki cells to induction of apoptosis by TRAIL and drugs that induce endoplasmic reticulum (ER) stress [thapsigargin (TG), tunicamycin (TU) and etoposide]. Ectopic expression of Par-4 is associated with decreased levels of XIAP protein in TG-treated cells, caused in part by XIAP protein instability and caspase activation. Levels of phospho-Akt are decreased in Caki/Par-4 cells to a significantly greater extent than in Caki/Vector cells by treatment with TG, and this is in turn associated with decreased levels of phospho-PDK1, the kinase upstream of Akt. In conclusion, we provide evidence that ectopic expression of Par-4 sensitizes Caki cells to TG and that XIAP protein instability and inactivation of Akt are important in cellular pathways affected by Par-4. J. Cell. Biochem. 103: 358–368, 2008.

Key words: Par-4; XIAP; pAkt; ER-stress; thapsigargin (TG)

Prostate-apoptosis-response-gene-4 (Par-4) was identified by differential screening for genes up-regulated after induction of programmed cell death in prostate cancer cells [Sells et al., 1994]. Par-4 abrogates TNF-α-induced NF-κB activity, which withdraws the anti-apoptotic roadblocks and allows the caspase cascade to precede uninterrupted [Chakraborty et al., 2001]. Furthermore, Par-4 triggers apoptosis through an activation of the Fas death receptor-signaling pathway by promoting the Fas/FasL translocation to the cell membrane and by protecting the Fas apoptotic pathway from the inhibitory effects of PKCζ [Chakraborty et al., 2001; de Thonel et al., 2001]. It have been reported that Par-4-mediated apoptosis was implicated with the inactivation of anti-apoptotic protein Bcl-2 and overexpression of Par-4 down-regulated Bcl-2 expression in human prostate cancer cells [Qiu et al., 1999]. Recently, it has been reported that binding of Akt1 to Par-4 results in both Par-4 phosphorylation and inactivation of its proapoptotic potential [Goswami et al., 2005]. Although, endogenous Par-4 expressed in normal and cancer cells does not, by itself, cause apoptosis, inhibition of endogenous Par-4 result in inhibition of apoptosis induced by exogenously applied apoptotic insult such as chemotherapeutic agents, tumor necrosis factor, serum deprivation, or ionizing radiation,

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thus suggesting Par-4 function is essential for apoptosis via diverse cell death pathways such as death receptor- and mitochondria-mediated apoptosis [Sells et al., 1997; Diaz-Meco et al., 1999; Gurumurthy et al., 2001; El-Guendy and Rangnekar, 2003]. Importantly, overexpression of Par-4 in prostate cancer xenografts by injections of adenoviral Par-4 results in apoptosis and tumor growth inhibition, implying that Par-4 has therapeutic potential [Chakraborty et al., 2001; El-Guendy et al., 2003].

Endoplasmic reticulum (ER) stress is induced in many eukaryotic cells by a multitude of causes including protein misfolding, UV, viral infection, and nutritional deprivation [Wek et al., 2006]. ER stress triggers the unfolded protein response (UPR) and ER-associated protein degradation (ERAD), which help to re-establish the cell's homeostatic environment; failure to do so ultimately results in cell death through activation of apoptotic pathways [Schroder and Kaufman, 2005]. There is evidence that expression of Par-4 causes apoptosis on its own or enhances the apoptotic response to TG [Sells et al., 1997]. It was reported that TG inhibits Ca^{2+}-ATPase in the ER that blocks the sequestration of calcium by the ER and causes increase in the intracellular concentration of calcium, accumulation of unfolded or misfolded proteins, and activation of caspase-3-mediated apoptosis [Thastrup et al., 1990; Lytton et al., 1991]. However, the molecular events and the exact underlying mechanisms responsible for Par-4-mediated apoptosis against ER stress inducing agents such as TG are poorly understood. Therefore, we in present study examined functional consequences of ectopic expression of Par-4 on apoptosis of human renal cancer cells after treatment with ER stress inducing agents.

MATERIALS AND METHODS

Cells and Materials

Human renal carcinoma Caki cell was obtained from ATCC (Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. TG was directly added to cell cultures at the indicated concentrations while untreated cells contained the solvent alone. Anti-PLC-γ1 and anti-procaspase-3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against XIAP proteins was obtained from R&D systems (Minneapolis, MN), and benzyloxycarbony-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) from Biomol. TG was purchased from Calbiochem (San Diego, CA).

Par-4 Constructs and Stable Cell

The human Myc-tagged Par-4 expression vector (pcDNA3.1-Par-4) was kindly provided by Dr. Shin J (School of Medicine, Sunkyunkwan University, Korea). The Caki cells were transfected in a stable manner with the pcDNA3.1-Par-4 and control plasmid pcDNA 3.1(+) Neo vector using LipofectAMINE as prescribed by the manufacturer (Invitrogen). After 48 h of incubation, transfected cells were selected in cell culture medium containing 700 μg/mL G418 (Invitrogen). After 2 or 3 weeks, single independent clones were randomly isolated, and each individual clone was plated separately. After clonal expansion, cells from each independent clone were tested for Par-4 expression by immunoblotting.

Cell Proliferation Assay

CCK-8 assay is nearly proportional to the cell number and thus can be used to determine proliferation. Cells were plated at a concentration of 10,000 cells in 0.2 ml of 10% FBS-DMEM per well in 96-well plates. After 24-h incubation, the cells were incubated with 10% FBS-DMEM for 24, 48, and 72 h. The CCK-8 assay was carried out using the manufacturer's instructions (Dojindo Molecular Technologies, Japan).

Western Blotting

Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Flow Cytometry Analysis

Approximately 1×10^6 cells were suspended in 100 μl PBS, and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

Asp-Glu-Val-Asp-Ase (DEVDase) Activity Assay

To evaluate DEVDase activity, cell lysates were prepared after their respective treatment with TG. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μl reaction buffer (1% NP-40, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing the caspases substrate [Asp-Glu-Val-Asp-chromophore-p-nitroanilide (DVAD-pNA)] at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Cell Death Assessment by DNA Fragmentation Assays

The cell death detection ELISAplus kit (Boerhringer Mannheim, Indianapolis, IN) was used for assessing apoptotic activity by detecting fragmented DNA within the nucleus in TG-treated cells. Briefly, each culture plate was centrifuged for 10 min at 200*g*, the supernatant was removed, and the pellet was lysed for 30 min. After centrifuging the plate again at 200*g* for 10 min, the collected supernatant containing cytoplasmic histone-associated DNA fragments was incubated with an immobilized anti-histone antibody, and the reaction products were determined by spectrophotometry. Finally, absorbance at 405 and 490 nm (reference wavelength), upon incubating with a peroxidase substrate for 5 min, was determined with a microplate reader. Signals in the wells containing the substrate only were subtracted as background.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted by using the TRI reagent. Single-strand cDNA was synthesized from 2 μg of total RNA using M-MLV (Moloney–Murine leukemia virus) reverse transcriptase. The cDNA for XIAP was PCR amplified using the following specific primers: XIAP (sense) 5′-CTTGAGGAGTGTC-TGGTAA-3′ and (antisense) 5-GTGACTAGAT GTCCACAAGG-3. PCR amplification was carried out as follows: 1 × (94°C, 3 min); 30 × (94°C, 45 s; 59°C, 45 s; and 72°C, 1 min); and 1 × (72°C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Statistical Analysis

Three or more separate experiments were performed. Statistical analysis was done by Student's *t*-test. A *P*-value <0.05 was considered to have pronounced difference between experimental and control groups.

RESULTS

Overexpression of Par-4 Increased the Sensitivity to TRAIL and ER Stress-Inducing Agents

In order to evaluate the functional role played by Par-4 in enhancing apoptosis induced by the various apoptotic inducing agents, we first established Par-4 overexpressing cells. Caki cells were transfected with an expression vector containing Myc-tagged Par-4 cDNA. After 4 weeks, geneticin (G418) resistant cells were isolated and the relative expression level of Par-4 was determined by Western blot. Caki/Par-4 (Caki/Par-4 #1 and #2) cells exhibited increase in Myc-tagged Par-4 expression compared with cells containing empty-vector only (Fig. 1A).

To compare the proliferative capacities of control and Par-4 overexpressing cells, cells were cultured in complete media containing 10% FBS for 24, 48, and 72 h and analyzed CKK-8 assay to compare the cell proliferation. As shown in Figure 1B, we failed to detect the difference of growth rate between control and Par-4 overexpressing cells. It has been reported that overexpression of Par-4 increased the sensitivity to TRAIL in Jurkat T cells [Boehrer et al., 2006]. To examine the role of Par-4 in TRAIL-mediated apoptosis in Caki cells, Par-4 overexpressing cell lines were treated with 100 ng/ml TRAIL for 12 h and examined cytotoxicity using FACS analysis. As shown in Figure 1C, TRAIL treatment in Caki/Par-4 cells (Caki/Par-4 #1 and #2) resulted in a markedly

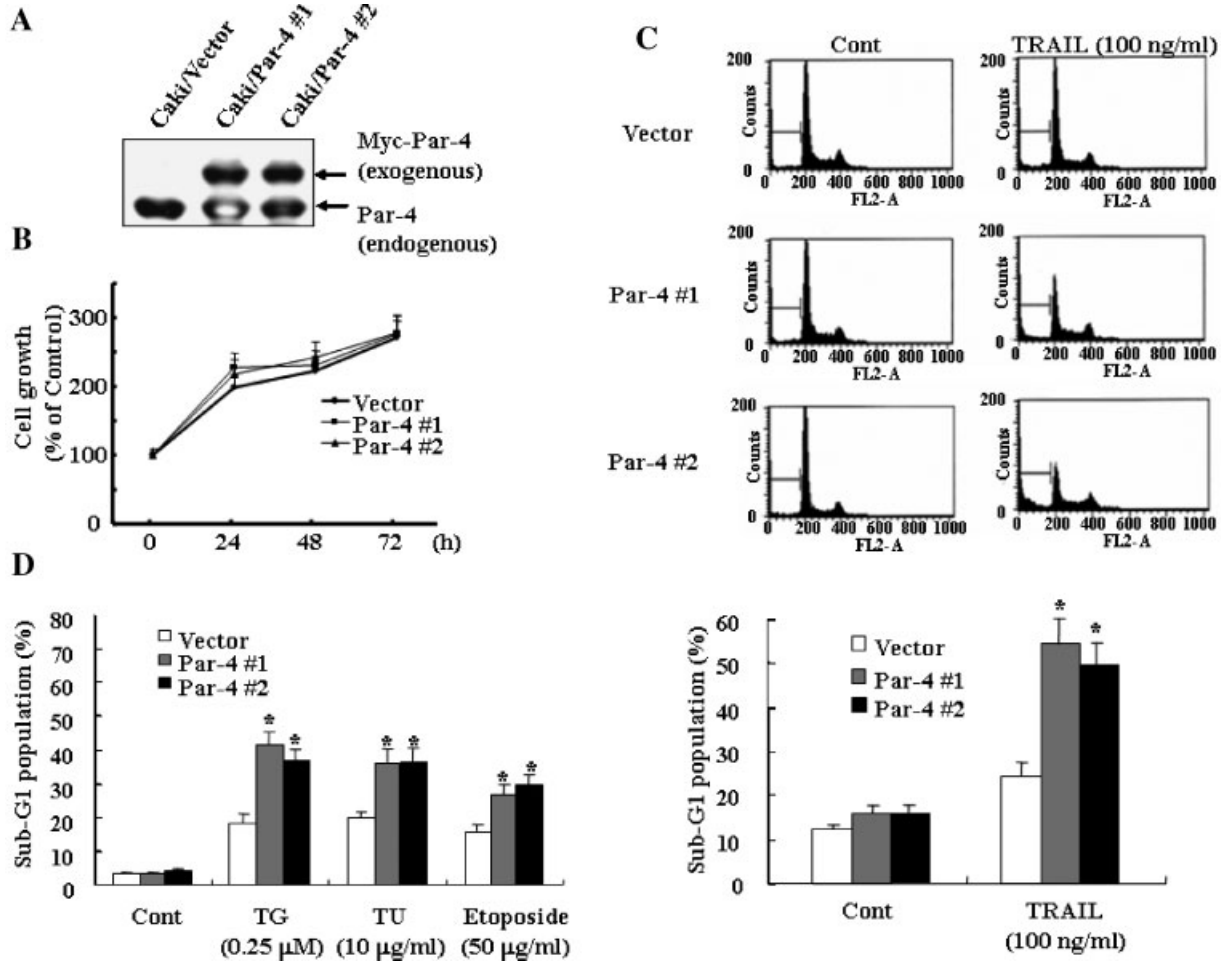

Fig. 1. Overexpression of Par-4 increased the sensitivity to TRAIL and ER stress-inducing agents in Caki cells. **A**: Analysis of Par-4 expression in the stably transfected cell lines. Western blotting using an anti-Par-4 antibody was performed to confirm the overexpression of Par-4 in the selected cells (Clone No. 1; Caki/Par-4-#1, Clone No. 2; Caki/Par-4-#2). **B**: Comparison of proliferative rates between control and Par-4 overexpressing cells. Caki/Vector and Caki/Par-4 were seeded in 96-well plates. The cell proliferation assay was performed using CCK-8 assay kit. **C**: Overexpression of Par-4 enhanced TRAIL-induced apoptosis in Caki cells. Caki/Vector and Caki/Par-4 were treated for 12 h with the indicated concentration of TRAIL and their DNA content was measured after propidium iodide staining. The FACS data were shown in **upper panel**. The proportion of apoptotic cells is indicated. Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus TRAIL-treated Vector cells. **D**: Overexpression of Par-4 potentiated ER stress-induced apoptosis in Caki cells. Caki/Vector and Caki/Par-4 were treated for 24 h with the indicated concentrations of TG, TU, and etoposide and their DNA content was measured after propidium iodide staining. The proportion of apoptotic cells is indicated. Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus indicated drugs-treated Vector cells.

increased accumulation of sub-G1 phase compared with Caki/Vector cells (top panel). Although sub-G1 population looked like to increase in untreated Caki/Par-4 cell, statistical analyses showed no significant differences between control and Par-4 overexpressing cells. In addition, we also tested whether overexpression of Par-4 could increase the sensitivity to ER-stress-inducing drugs such as thapsigargin (TG) and tunicamycin (TU). We found that treatment with 0.25 μM TG or 10 μg/ml TU increased the sub-G1 populations in Par-4 overexpressing cells, suggesting that overexpression of Par-4 potentiated ER-stress-induced apoptosis in Caki cells (Fig. 1D). Also, ectopic expression of Par-4 increased the sensitivity to etoposide (50 μg/ml) in Caki cells (Fig. 1D).

Overexpression of Par-4 Enhanced TG-Induced Apoptosis

To investigate the effect of overexpression of Par-4 on ER-stress-mediated apoptosis, Caki/Par-4 #1 and Caki/Vector cells were treated

with TG (0.1–0.5 μM). We first determined apoptosis in both cells using flow cytometric analysis to detect hypodiploid cell populations. After the Caki/Vector cells were incubated with the indicated concentrations of TG, the percentage of apoptotic cells was slightly increased (Fig. 2A). However, the percentage of TG-induced apoptosis in Par-4 overexpressing Caki cells was markedly increased in a dose-dependent manner (Fig. 2A). We next analyzed whether treatment with TG gave rise to the more activation of caspases in Caki/Par-4 #1 cells (Fig. 2B). Exposure of Caki/Par-4 #1 cells to TG strongly stimulated DEVDase activity and led to a reduction of the protein levels of XIAP and 32-kDa caspase-3 precursor together with a concomitant cleavage of phospholipase C-γ1 (PLC-γ1), a substrate protein of caspases (Fig. 2C). Furthermore, exposure of Caki/Par-4 #1 cells to TG strongly increased cytoplasmic histone-associated DNA fragments as determined with the DNA fragmentation detection kit compared with Caki/Vector cells (Fig. 2D).

Next, we carried out time kinetics studies of the apoptotic effects of TG on Caki/Par-4 #1 and Caki/Vector cells. Since 0.25 μM TG was enough to inhibit the viability of Caki/Par-4 #1 cells, this single concentration was utilized for further assessment of apoptosis. Treatment with TG induced markedly increase in sub-G1

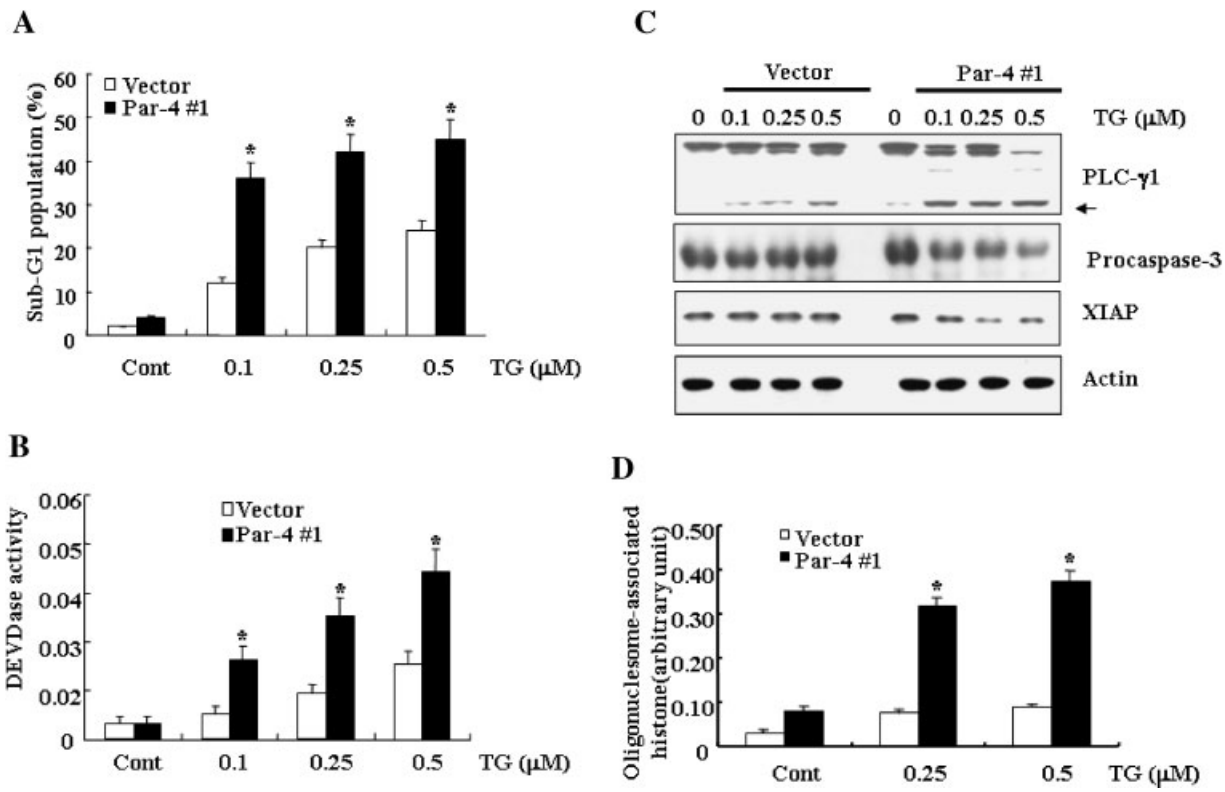

Fig. 2. Overexpression of Par-4 enhances TG-induced apoptosis. **A**: Overexpression of Par-4 enhanced TG-induced apoptosis in Caki cells. Caki/Vector and Caki/Par-4 #1 were treated for 24 h with the indicated concentrations of TG and their DNA content was measured after propidium iodide staining. The proportion of apoptotic cells is indicated. Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus TG-treated Vector cells. **B**: Overexpression of Par-4 enhanced DEVDase activities in TG-treated cells. Caki/Vector and Caki/Par-4 #1 were treated for 24 h with the indicated concentrations of TG and DEVDase activities was measured. DEVDase activities were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (DEVD-pNA) in a 100 μl assay buffer for 2 h at 37°C. The release of chromophore p-nitroanilide (pNA) was monitored spectrophotometrically (405 nm). Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus TG-treated Vector cells. **C**: Overexpression of Par-4 enhanced cleavage of PLC-γ1, procaspase-3, and XIAP proteins in TG-treated cells. Cells treated as above were harvested in lysis buffer and equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for pro-caspase 3, PLC-γ1 and XIAP. The proteolytic cleavage of PLC- γ1 was indicated by arrow. **D**: Overexpression of Par-4 potentiated DNA fragmentation in TG-treated Caki cells. Cells were treated with the indicated concentrations of TG for 24 h and cytoplasmic histone associated DNA fragments were quantified using a commercially available ELISA kit as described in Materials and Methods Section. Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus TG-treated Vector cells.

population and DEVDase activity after 18 h treatment in Par-4 overexpressing cells (Fig. 3A,B). The levels of pro-caspase 3 and XIAP were progressively degraded from 12 to 24 h after TG treatment in Caki/Par-4 #1 cells (Fig. 3C).

TG-Induced Apoptosis Was Partly Mediated by Caspase-Dependent Pathway in Caki/Par-4 #1 Cells

To investigate whether activation of caspase is directly associated with TG-induced apoptosis in Caki/Par-4 #1 cells, we determined FACS analysis to quantitate sub-G1 population in Caki/Vector and Caki/Par-4 #1 cells after pretreatment with a pan-caspase inhibitor, z-VAD-fmk, followed by TG treatment. As shown in Figure 4A, pretreatment with z-VAD-fmk did not completely inhibit TG-induced apoptosis. However, z-VAD-fmk prevented caspase-related events such as decrease of procaspase-3 and cleavage of PLC-γ1 (Fig. 4B). These results suggest that TG-induced cell death was partly mediated by caspase-dependent and caspase-independent cell death pathways.

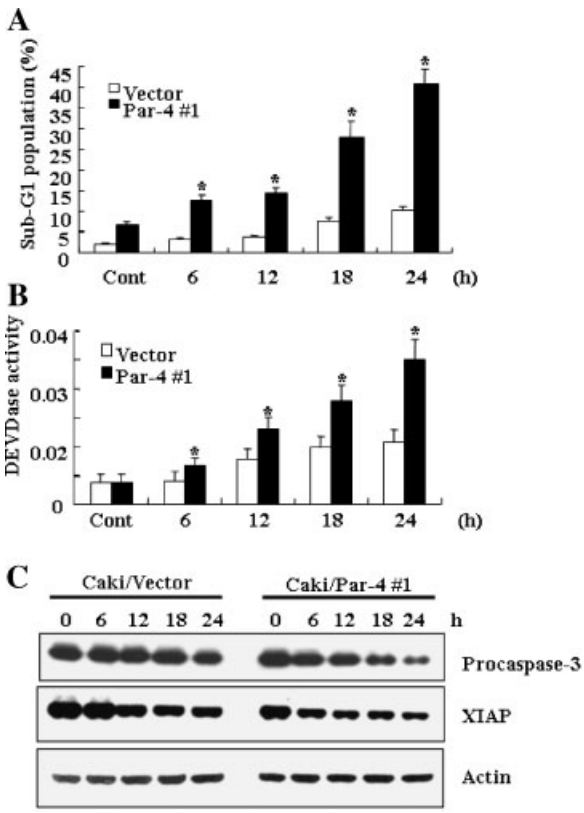

Fig. 3. TG-induced cell death was mediated in a time-dependent manner. **A**: The accumulation of Sub-G1 population was increased in time dependent manner and greater in Caki/Par-4 #1 than Caki/Vector cells. Caki/Vector and Caki/Par-4 #1 cells were treated with 0.25 μM TG for the indicated time points and their DNA content was measured after propidium iodide staining. Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus TG-treated Vector cells. **B**: The DEVDase activities were increased in time dependent manner and greater in Caki/Par-4 #1 than Caki/Vector cells. DEVDase activities were determined as above in Figure 2. Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus TG-treated Vector cells. **C**: The cleavage of PLC-γ1, procaspase-3, and XIAP proteins in time dependent manner and greater in Caki/Par-4 #1 than Caki/Vector cells. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with procaspase-3 and XIAP.

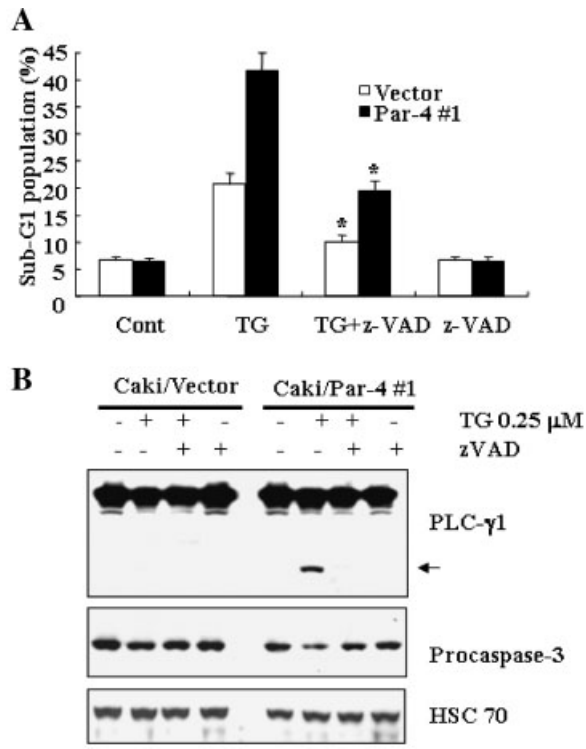

Fig. 4. TG-induced apoptosis was partly mediated by caspase-dependent pathway in Caki/Par-4 #1 cells. **A**: Pretreatment with z-VAD did not completely prevent TG-induced apoptosis in Caki/Par-4 #1 cells. Caki/Vector and Caki/Par-4 #1 cells were pretreated with 50 μM z-VAD for 30 min, and then incubated with TG for 24 h. Sub-G1 population was determined by FACS analysis. Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus TG-treated Vector/or TG-treated Par-4 #1 cells. **B**: Pretreatment with z-VAD completely prevented TG-induced cleavage of PLC-γ1 and procaspase-3. Caki/Vector and Caki/Par-4 #1 cells were pretreated with 50 μM z-VAD for 30 min, and then incubated with TG for 24 h. Cells were harvested in lysis buffer and equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for pro-caspase 3 and PLC-γ1. The proteolytic cleavage of PLC- γ1 was indicated by arrow.

Decreased Expression of XIAP Was Caused by Activation of Caspase and XIAP Protein Instability in Par-4-Overexpressing Cells

To examine whether the decrease of XIAP proteins was related with down-regulation of mRNA expression in TG-treated Caki/Par-4 #1 cells, we measured the mRNA expression level of XIAP by RT-PCR. As shown in Figure 5A, XIAP mRNA levels remain constant through the TG treatment at different times in Caki/Par-4 #1 cells, suggesting that TG-mediated degradation of total XIAP proteins are regulated by the post-transcriptional levels in Par-4 overexpressing cells. Since XIAP has been previously reported to be a substrate of caspases during apoptosis [Deveraux et al., 1997], we tested whether the decrease in the protein levels of XIAP that was caused by TG treatment was correlated with the enhanced caspase activities in Caki/Par-4 #1 cells. As shown in Figure 5B, XIAP degradation was partly prevented by pretreatment with 50 μM z-VAD-fmk in the presence of TG in Caki/Par-4 #1 cells, indicating that the decrease of XIAP protein was partly mediated by caspase-dependent pathways. To further clarify the underlying mechanisms of decreased XIAP protein level caused by caspase-independent pathway(s) in TG-treated Par-4 overexpressing cells, we analyzed XIAP protein stability test. Caki/Vector and Caki/Par-4 #1 cells were pretreated with cycloheximide for 1 h and then treated with TG for different periods of time. We found that the degradation of XIAP protein was facilitated by TG treatment in Caki/Par-4 #1 cell (Fig. 5C); implying that ectopic expression of Par-4 caused an increase of XIAP protein instability in TG-treated cells. Taken together, TG-induced decrease of total XIAP proteins were mediated through at least two different mechanisms, by activation of caspase-dependent pathway and by the enhancement of protein instability, in Par-4 overexpressing Caki cells.

The Decreased Expression of XIAP Might be Involved in TG-Induced Apoptosis in Par-4 Overexpression Cells

To determine whether the decreased expression of XIAP is related with sensitizing effect of

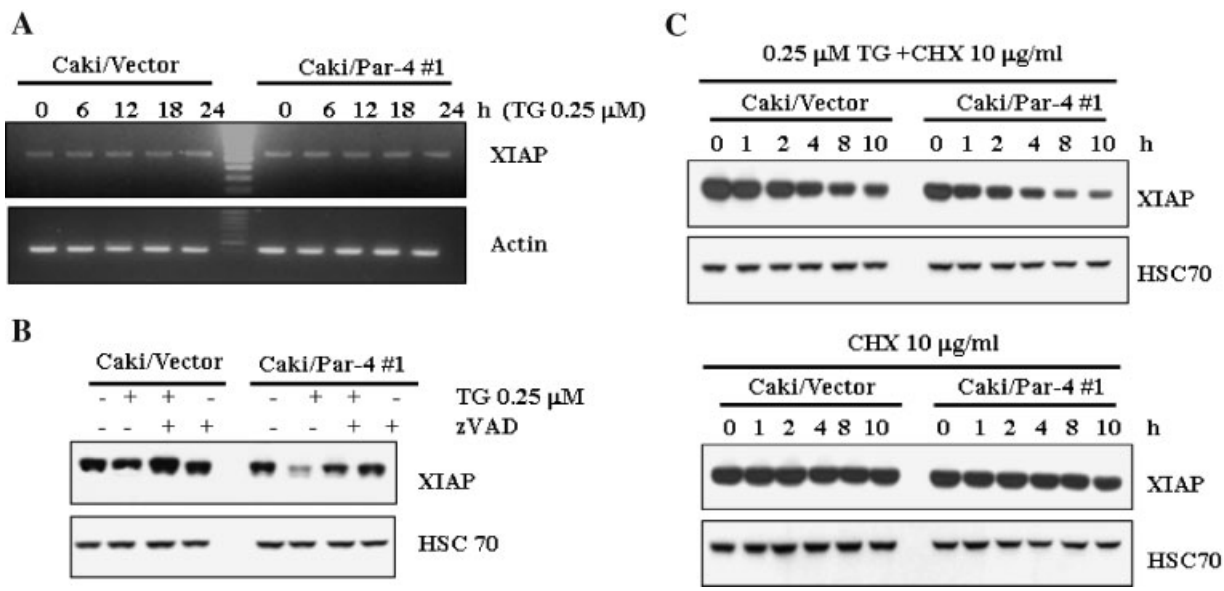

Fig. 5. Decreased expression of XIAP was caused by XIAP protein instability in Par-4 overexpressing cells. **A**: Decreased expression of XIAP was not related to down-regulation of mRNA expression in Caki/Par-4 cells. Caki/Vector and Caki/Par-4 cells were treated with 0.25 μM TG for the indicated times. Total RNA was isolated and RT-PCR analysis was performed as described in Materials and Methods Section. A representative study is shown; two additional experiments yielded similar results. **B**: Pretreatment with z-VAD did not completely prevented TG-induced decrease of XIAP protein in Caki/Par-4 #1 cells. Caki/Vector and Caki/Par-4 #1 cells were pretreated with 50 μM z-VAD for 30 min, and then incubated with TG for 24 h. Cells were harvested in lysis buffer and equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for XIAP. **C**: Decreased expression of XIAP was caused by XIAP protein instability in Par-4-overexpressing cells. Caki/Vector and Caki/Par-4 #1 cells were treated with 0.25 μM TG in the presence or absence of CHX for the indicated times. Western blotting was performed using anti-XIAP and HCS70 antibody to serve as control for the loading of protein level.

Par-4 in TG-treated cells, Caki/Par-4 #1 cells were transiently transfected with XIAP and empty Vector. Treatment with TG in Caki/Par-4 #1 cells that were transfected with Vector led to increase of sub-G1 populations, which was markedly blocked by ectopic expression of XIAP (Fig. 6, upper panel). This result indicated that restoration of XIAP expression prevented TG-mediated apoptosis in Caki/Par-4 #1 cells. XIAP-transfected Caki/Par-4 #1 cells exhibited approximately two to threefolds increase in XIAP expression compared with cells containing empty-vector only (Fig. 6, bottom panel). The introduced XIAP protein was slightly decreased by TG treatment, implying XIAP protein was decreased by TG treatment in Caki cells (Fig. 6, bottom panel).

Overexpression of Par-4 Led to Reduction of Phosphorylation of Akt That Were Caused by the Decreased Phospho-PDK1 Level

It has been known that ER stress induced by TG or TU led to a gradual reduction of phosphorylation of Akt [Srinivasan et al., 2005; Hyoda et al., 2006; Yung et al., 2007]. To investigate whether TG treatment could alter Akt activation, we determined the expression and phosphorylation levels of Akt in Caki/Vector and Caki/Par-4 #1 cells after treatment for the indicated times. As shown in Figure 7A, the levels of phosphorylated Akt were markedly decreased in response to TG in time-dependent manner in Caki/Par-4 #1 cells. In contrast, the level of phosphorylated Akt was declined by 4 h and then recovered into basal level after 16 h in TG-treated Caki/Vector cells. Total Akt protein levels remained constant during treatment with TG in both cells. These result suggested that overexpression of Par-4 appeared to inhibit phosphorylation of Akt in TG-treated cell. It is well known that Akt is phosphorylated and activated by phosphoinositide-dependent kianse (PDK) [Nicholson and Anderson, 2002]. To evaluate the contribution of upstream kinase

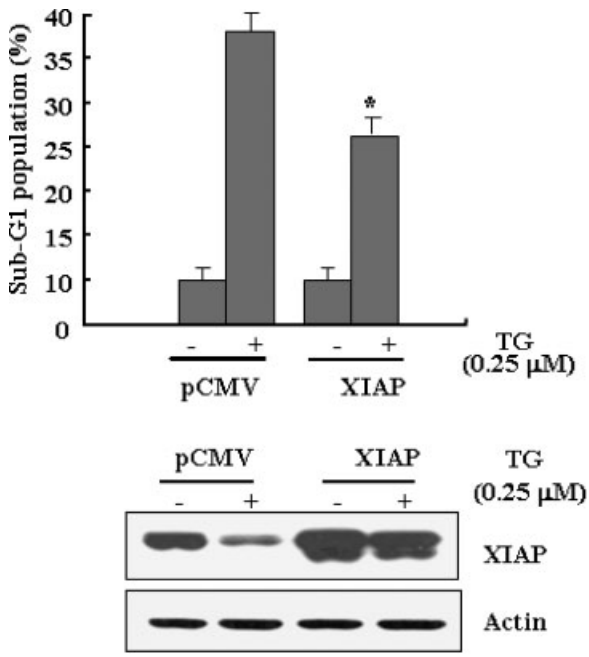

Fig. 6. The decreased expression of XIAP might be involved in TG-induced apoptosis in Par-4 overexpression cells. Ectopic expression of XIAP attenuated TG-induced apoptosis in Caki/Par-4 #1 cells (**upper panel**). Caki/Par-4 #1 cells were transiently transfected with XIAP and empty vector and treated with 0.25 μM TG for 24 h. Cells were harvested and analyzed by FACS and Western blotting. Western blotting was performed using anti-XIAP and actin antibody to serve as control for the loading of protein level (**bottom panel**). Data shown are means ± S.D. (n = 3). Statistics, Student's *t*-test for unpaired values. $^*P < 0.05$ versus TG-treated Par-4 cells that are transfected with pCMV.

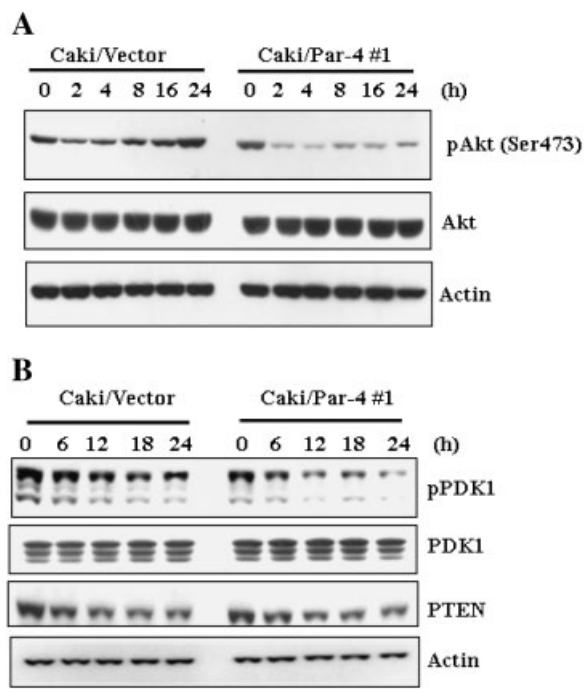

Fig. 7. Effect of Par-4 overexpression on TG-induced phosphorylation of Akt and PDK1 in Caki/Vector and Caki/Par-4 cells. **A**: Overexpression of Par-4 led to reduction of phosphorylation of Akt. Caki/Vector and Caki/Par-4 cells #1 were treated with 0.25 μM TG for the indicated time points. Phospho-Akt, Akt, and actin were detected by Western blotting. **B**: The decreased expression levels in phosphorylated Akt might be related to reduction of phosphorylation of PDK1. Caki/Vector and Caki/Par-4 cells were treated with 0.25 μM TG for the indicated time points. Phospho-PDK1, PDK1, PTEN, and actin were detected by Western blotting.

PDK1 to the phosphorylation of Akt, time course experiment for phosphorylated PDK1 was performed using the TG-treated cell lysates. TG treatment also led to reduced phospho-PDK1 level in Caki/Par-4 #1 cells compared with control cells (Fig. 7B). However, we failed to detect any differences in protein levels of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) between Caki/Vector and Caki/Par-4 #1 cells after TG treatment (Fig. 7B). Thus, these results suggested that TG-mediated inactivation of Akt was derived from decreased phospho-PDK1 level in Par-4-overexpressing cell.

The Decreased Expression of XIAP and Phosphorylated Akt Level Were Related to the Enhanced Induction of Apoptosis in Caki/Par-4

To rule out the possibility of clonal differences between the generated stable cell lines, Caki/Vector, Caki/Par-4 # 1, and Caki/Par-4 #2 cells were treated with 0.25 μM TG for 24 h and examined Western blotting analysis. Exposure of both Caki/Par-4 cells to TG strongly led to reduction of XIAP protein levels and 32-kDa caspase-3 precursor as well as phosphorylated Akt level (Fig. 8). These results suggested that the enhanced induction of apoptosis caused by Par-4 overexpression might be associated with degradation of XIAP protein and decrease in phosphorylated Akt protein level in Caki cells. TG is well known pharmacological ER stress agents that caused induction of CHOP protein in cells [Muruganandan and Cribb, 2006; Kim et al., 2007]. We attempted to test whether Par-4 overexpression increased the induction of CHOP protein against TG-induced apoptotic signals. As shown in Figure 7B, the induction levels of CHOP protein in Caki/Par-4 cells were almost similar to Caki/Vector cells, implying that the enhanced apoptotic cell death caused by Par-4 overexpression was not related to CHOP induction in TG-treated Caki cells.

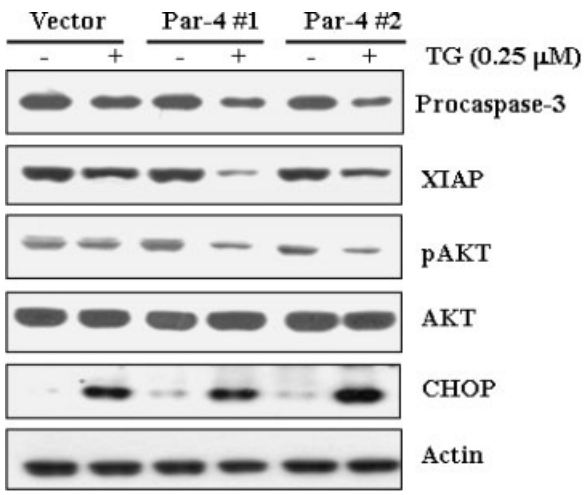

Fig. 8. The decreased expression of XIAP and phosphorylated Akt level were related to the enhanced induction of apoptosis in Caki/Par-4. Overexpression of Par-4 potentiated decrease of XIAP and phosphorylated Akt proteins levels but not CHOP protein in Caki cells. Caki/Vector and Caki/Par-4 cells (Caki/Par-4 #1 and Caki/Par-4 #2) were treated with 0.25 μM TG for 24 h and performed Western blotting analysis using anti-procaspse-3, -XIAP, Akt, -pAkt, -CHOP, and -actin antibodies.

DISCUSSION

In present study, we showed that overexpression of Par-4 significantly enhanced the sensitivity to ER stress-inducing drugs that caused increase of cell death in Caki cells. Moreover, ectopic expression of Par-4 induced decrease of XIAP protein level in TG-treated cells, which was partly caused by the increased caspase activation and XIAP protein instability. TG-induced cell death was attenuated by transient transfection of XIAP in Par-4 overexpression cells. In addition, we observed that TG treatment caused to decrease phospho-Akt levels and then subsequently recovered its levels approximately to basal level in Caki/Vector cells. However, Caki/Par-4 cells showed the decrease of its levels in a time-dependent manner.

It has been reported that Par-4 promotes apoptosis induced by chemotherapeutic drugs through downregulation of Bcl-2 expression [Boehrer et al., 2002]. We hypothesized that this proapoptotic influence of Par-4 might also extend to molecules considered to function also in ER stress-mediated cell death. In order to test this hypothesis, Caki cells containing a vector stably expressing Par-4 as well as Caki cells with control vector were incubated with ER stress-inducing drugs such as TG and TU. We demonstrated increase in the rate of apoptosis in Par-4-overexpressing cells compared to control cells treated with ER stress-inducing agents. Furthermore, we found that overexpression of Par-4 promoted activation of caspase-dependent pathway in TG-treated cells. These results indicated that overexpression of Par-4 increased the sensitivity to TG via the potentiated caspase activation.

XIAP is a member of the IAP family and plays a key role in cell survival by modulating

post-mitochondrial apoptosis signaling. XIAP is the most potent inhibitor of caspases and apoptosis among IAPs [Deveraux et al., 1997]. It has been shown that XIAP is a direct inhibitor of caspase-3 and caspase-9 and modulates the Bax/cytochrome c pathway by inhibiting caspase-9. Down-regulation of XIAP is an important mechanism for caspase activation in response to various apoptotic stimuli [Shi et al., 2005]. Furthermore, ER stress-induced programmed cell death is accompanied by a decrease in XIAP protein content [Yamaguchi and Wang, 2006]. Another reports showed that overexpression of Par-4 promoted down-regulation of XIAP by increasing caspase activity in chemotherapeutic agents- and TRAIL-treated Jurkat cells [Boehrer et al., 2002, 2006]. In these studies, they provide evidence that overexpression of Par-4-induced reduction of XIAP protein was completely blocked by z-VAD. However, we observed that pretreatment with z-VAD-fmk did not completely block down-regulation of XIAP protein levels in TG- treated Caki/Par-4 cells, indicating that reduction of XIAP protein levels during TG-induced apoptosis was partly mediated by caspase-dependent pathways. We additionally demonstrate that Par-4 overexpression results in enhanced degradation of XIAP protein via increasing protein instability after TG treatment.

PI3K/Akt pathway has been reported to be one of the most important survival pathways in a variety of cell types [Song et al., 2005]. Withdrawal of growth factors or a death signal challenge decreases its activity and promotes cell death. A recent study also showed that inactivation of PI3K/Akt is important to ER stress-induced apoptosis [Srinivasan et al., 2005; Yung et al., 2007]. The loss of Akt activity may thus be the result of suppression of protein phosphorylation, reduction of protein expression, or a combination of the two. Yung et al. [2007] reported that TU induces cell death through suppressing Akt activity through lowering its protein expression level. Alternatively, another study demonstrated that ER stress-induced apoptosis was associated with a reduction in phospho-Akt [Srinivasan et al., 2005]. In our present study, we observed that Akt was inactivated in response to ER stress via a reduction in phospho-Akt in TG-treated Caki/Par-4 cells, which might be caused by decreasing level of phospho-PDK1. However, future experiments will have to determine the contribution of the PI3K/Akt signaling pathway for the demonstrated enhancing apoptosis induced by TG treatment in Par-4-overexpressing cells. This result provides significant novel insights into the molecular mechanisms for pro-apoptotic behavior of Par-4 in ER stress. Although many questions concerning the functional role of Par-4 in Caki cells remain to be answered during ER stress, this study appears to be the first identification of endogenous Akt/XIAP signaling pathways in regulation of cell fate in Par-4 overexpressing cells.

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