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Role of Annexin A5 on Mitochondria-Dependent Apoptosis Induced by Tetramethoxystilbene in Human Breast Cancer Cells

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Abstract

We have previously shown that 2,4,3',5'-tetramethoxystilbene (TMS), a *trans*-stilbene analogue, induces apoptosis in human cancer cells. However, the detailed mechanisms of mitochondria-dependent apoptosis induced by TMS are not fully understood. In the present study, the possible roles of annexin A5 in TMS-mediated apoptosis were investigated in MCF7 human breast cancer cells. Quantitative real-time PCR analysis and Western blot analysis showed that the expression of annexin A5 was strongly increased in TMS-treated cells. TMS caused a strong translocation of annexin A5 from cytosol into mitochondria. Confocal laser scanning microscopic analysis clearly showed that TMS induced translocation of annexin A5 into mitochondria. TMS increased the expression and oligomerization of voltage-dependent anion channel (VDAC) 1, which may promote mitochondria-dependent apoptosis through disruption of mitochondrial membrane potential. When cells were treated with TMS, the levels of Bax, and Bak as well as annexin A5 were strongly enhanced. Moreover, we found that the cytosolic release of apoptogenic factors such as cytochrome c, or apoptosis-inducing factor (AIF) in mitochondria was markedly increased. Annexin A5 depletion by siRNA led to decreased proapoptotic factors such as Bax , Bak, and annexin A5. Taken together, our results indicate that annexin A5 may play an important role in TMS-mediated mitochondrial apoptosis through the regulation of proapoptotic proteins and VDAC1 expression.

Key Words: Tetramethoxystilbene, Annexin A5, VDAC1, Bax

INTRODUCTION

Human cytochrome P450 1B1 (CYP1B1) is an important enzyme involved in the metabolic activation of diverse procarcinogens, such as arylamines, and polycyclic and nitro aromatic hydrocarbons (Shimada *et al*., 1996). A major interest in CYP1B1 arises from the fact that it is a major 17-estradiol (E2) 4-hydroxylase, and 4-hydroxy E2 has been suggested to be mutagenic (Hayes *et al*., 1996; Shimada *et al*., 1999). Because of the postulated significant role of CYP1B1 on carcinogenicity of E2, CYP1B1 is regarded as a target enzyme for cancer prevention, and selective inhibition of CYP1B1 may prevent E2-related tumor formation (Liehr, 1997; Shimada *et al*., 1997).

Previously, 2,4,3',5'-tetramethoxystilbene (TMS), a methoxy derivative of oxyresveratrol, was found to act as a potentially selective inhibitor of CYP1B1 (Chun *et al*., 2001; Chun and Kim, 2003). TMS is also able to promote apoptosis in MCF-7 and HL-60 cancer cells (Chun *et al*., 2005). TMS increases chromosomal DNA fragmentation and annexin V-positive cells, which are indication of apoptosis. TMS induces the level of $p27^{kip1}$ through reduction of Akt-mediated skp2 expression (Kim *et al*., 2008). However, the detailed mechanisms of mitochondrial apoptosis induced by TMS are still unclear. Recently, we found that cisplatin induces annexin A5 expression and translocation into mitochondria and mitochondrial annexin A5 may play a crucial role in changing mitochondrial membrane potential to trigger mitochondrial apoptosis in human and rat renal epithelial cells (Jeong *et al*., 2014).

In the present study, we explored the effect of TMS in mitochondrial apoptosis in MCF-7 human mammary tumor cells to elucidate whether annexin A5 may involve in mitochondrial apoptosis induced by TMS, and found that TMS induced mitochondrial apoptosis through the expression and translocation of annexin A5 into mitochondria. Annexin A5 may promote Bax, Bak and VDAC1 expression and channel formation to trigger release of apoptogenic factors including cytochrome c and AIF.

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MATERIALS AND METHODS

Reagents

FBS and DMEM medium were purchased from HyClone (Logan, UT, USA). The Neon transfection system, JC-1 assay kit and cytochrome oxidase subunit (COX)-4 antibody were from Life Technologies (Carlsbad, CA, USA). The bicinchoninic acid (BCA) protein assay kit and ECL kit were from Thermo Scientific (Rockford, IL, USA). Antibodies against VDAC1, Bax, Bak, GAPDH or goat anti-rabbit IgG-Texas Red and Ultra Cruz™ mounting medium were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cytochrome *c*, or AIF antibodies were from Millipore Co. (Bedford, MA, USA). All other chemicals were of the highest purity or molecular biology grade and were obtained from commercial sources.

Cell culture

Human breast cancer MCF7 cells were obtained from Korean Society Cell Bank (KCLB). Cells were grown in DMEM medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the cells were harvested by scrapping and solubilized in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Cells were centrifuged at 1,000 x *g* for 4 min at 4°C and the pellets were resuspended and stored in -70°C.

Subcellular fractionation

After treatment, cells were harvested and washed with ice-cold PBS. Subcellular fractionation was performed using the Mitochondria Isolation kit for Cultured Cells (Thermo Scientific) according to the manufacturer's protocol. Western blotting was carried out using antibodies against the following control marker proteins: GAPDH for the cytosolic fraction, and COX-4 for the mitochondrial fraction.

Measurement of mitochondrial membrane potential (Δψm)

Mitochondrial membrane potential (Δψm) was also measured in cells with TMRM using a flow cytometer. After cell treated with TMS for 48 h, cells were loaded with 100 nM TMRM for 20 min in culture medium at 37°C. After washing with PBS, cells were resuspended in PBS and analyzed immediately using the BD FACScan flow cytometer. Red fluorescence was measured in the FL2 mode. For each sample, 10,000 events were acquired.

Immunofluorescence

Cells grown on poly D-lysine-coated coverslips were treated for 48 h with TMS in growth medium, rapidly washed with PBS, and then treated with growth medium including 100 nM MitoTracker® probes (Invitrogen). After 1 h, the cells were fixed with 3.7% (w/v) paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature. After washing with PBS, the cells were blocked for 15 min in PBS containing 5% goat serum and 0.2% Triton X-100, then incubated with primary antibody (1:1000) for 1 h, washed extensively, and stained for 1 h with goat anti-rabbit IgG-Texas Red (1:500). After further washes, the coverslips were mounted on glass slides using Ultra Cruz™ mounting medium. Fluorescence signals were analyzed by using a LSM 510 META Confocal Laser Scanning Microscope (Carl Zeiss, Germany).

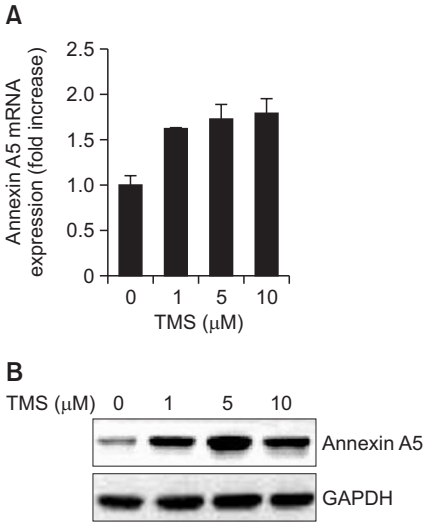

Fig. 1. TMS induces annexin A5 mRNA and protein expression in MCF-7 cells. MCF-7 cells were treated with various concentrations of TMS (0, 1, 5, or 10 μM) for 48 h. The mRNA and protein levels of annexin A5 were determined using quantitative real-time PCR and Western blot analysis. Total RNA was isolated and human annexin A5 genes were amplified with specific primers. Expression of GAPDH mRNA was determined as a RNA control. Total cellular lysates were prepared for Western blot analysis using annexin A5 antibody. GAPDH was used as a loading control. (A) Quantitative real-time PCR, (B) Western blot analysis. The data shown are representative of three independent experiments.

VDAC cross-linking

Following chemical treatment, cells were washed twice with PBS. Sulfo-EGS in DMSO was added to a final concentration of 250 μM. After 25-min incubation at 30°C, the crosslinker was quenched by the addition of 1 M Tris-HCl (pH 7.5) to a final concentration of 20 mM. Samples were then solubilized in 1% NP-40 and sonicated five times for 7 s with a 30% pulse using a Vibra-Cell sonicator (Sonics and Materials, Newtown, CT, USA). VDAC1 was detected by Western blotting using an anti-VDAC1 antibody.

RNA isolation, reverse transcription and RT-PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Crawley, UK). Total RNA (500 ng) was transcribed at 37°C for 1 h in a volume of 20 μl containing 5× RT buffer, 10 mM dNTPs, 40 units of RNase inhibitor, 200 units of M-MLV reverse transcriptase, and 100 pmol oligo-dT primer. Subsequently, 0.8 μL of the reaction mixture from each sample was amplified with 10 pmol of each oligonucleotide primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1.25 units of *Taq* DNA polymerase in a final volume of 25 μL. PCR was performed as follows: one cycle of 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 62°C for 30 sec, and extension at 72°C for 15 sec. Human annexin A5 cDNA was amplified using a sense primer (5'-CAGTCTAGGTGCAGCTGCCG-3') and an antisense primer (5'-GGTGAAGCAGGACCAGACTGT-3'). Human GAPDH cDNA was amplified using a sense primer (5'-TGAACGGGAAGCTCACTGG-3') and an antisense primer (5'-TCCACCACCCTGTTGCTGTA-3'). The number of amplification cycles was optimized in preliminary experiments to ensure that the PCR did not reach a plateau. PCR products

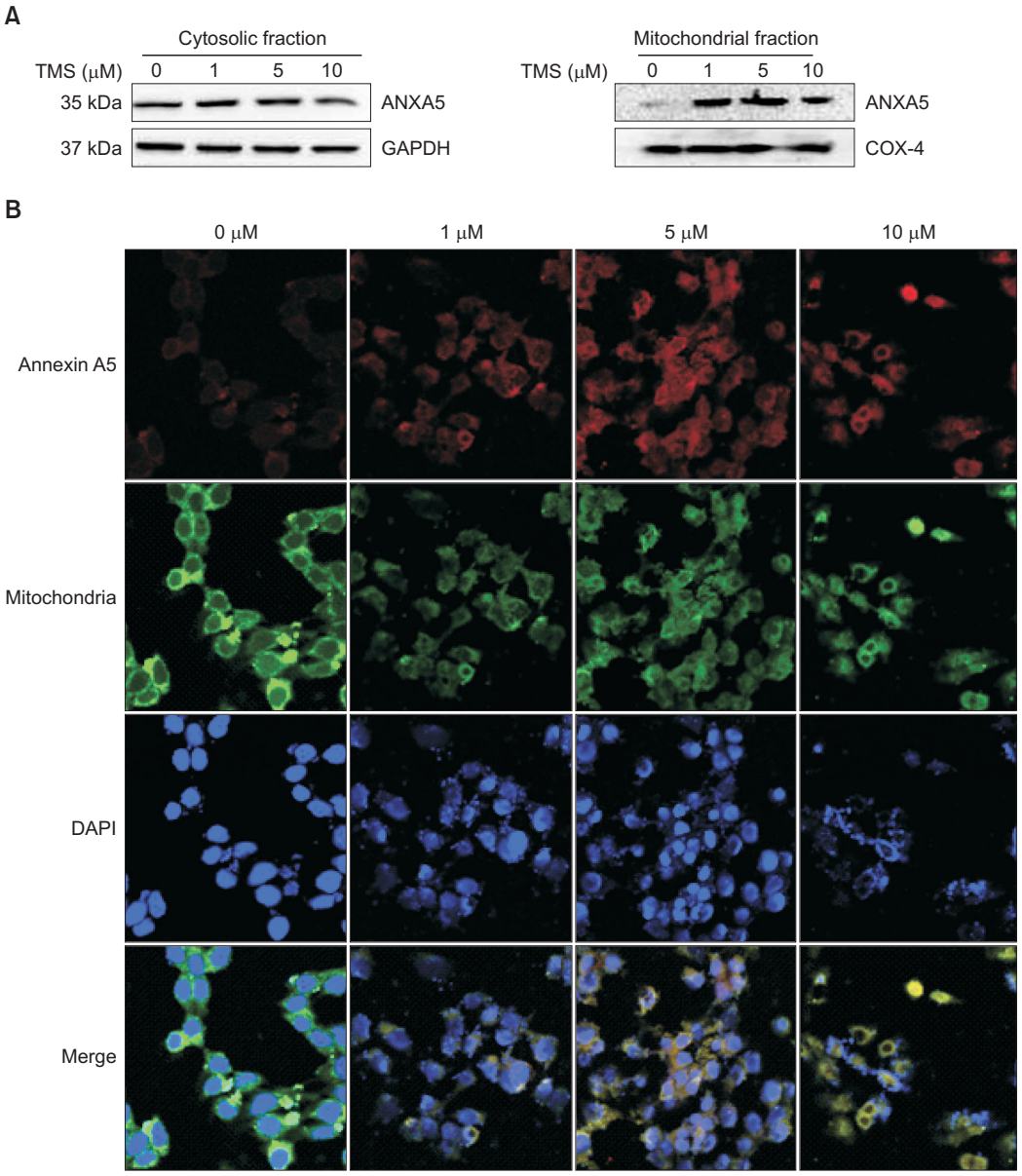

Fig. 2. Translocation of annexin A5 into mitochondria induced by TMS. (A) Western blot analysis. MCF-7 cells were treated with various concentrations of TMS (0, 1, 5, or 10 μM) for 48 h. After incubation, cells were harvested and the cytosolic and mitochondrial fractions were isolated. Extracted proteins were resolved by SDS-PAGE (10%) and Western blot analysis was conducted. GAPDH level was determined as loading controls for cytosolic fraction and COX-4 level was determined as a loading control for mitochondrial fraction. (B) Confocal microscopic analysis. Cells were seeded on cover slip for 48 h and treated with TMS for 48 h. Cells were stained with annexin A5 antibody and fluorescence was determined using confocal microscopy. The data shown are representative of three independent experiments.

were analyzed using 2% (w/v) agarose-gel electrophoresis and a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA).

Quantitative real-time PCR

To quantify mRNA expression, quantitative real-time PCR was performed using a Rotor-Gene Q (Qiagen) system with the SYBR green I PCR kit (TaKaRa) according to the manufacturers' recommendations. Each reaction contained 10 μL of the 2× SYBR green Premix Ex Taq, 10 pmol of each oligonucleotide primer, and 2 μL of cDNA in a final volume of 20 μL. qPCR was performed as follows: one cycle of 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 62°C for 30 sec, and extension at 72°C for 15 sec. Melting-curve analysis was performed to verify amplification specificity. For data analysis, the comparative threshold cycle (C_T) method was used to calculate the relative changes in gene expression.

siRNA transfection

Cells were transfected with annexin A5 siRNA using the Neon™ transfection system (Invitrogen) according to the manufacturer's recommendations. Briefly, one day prior to

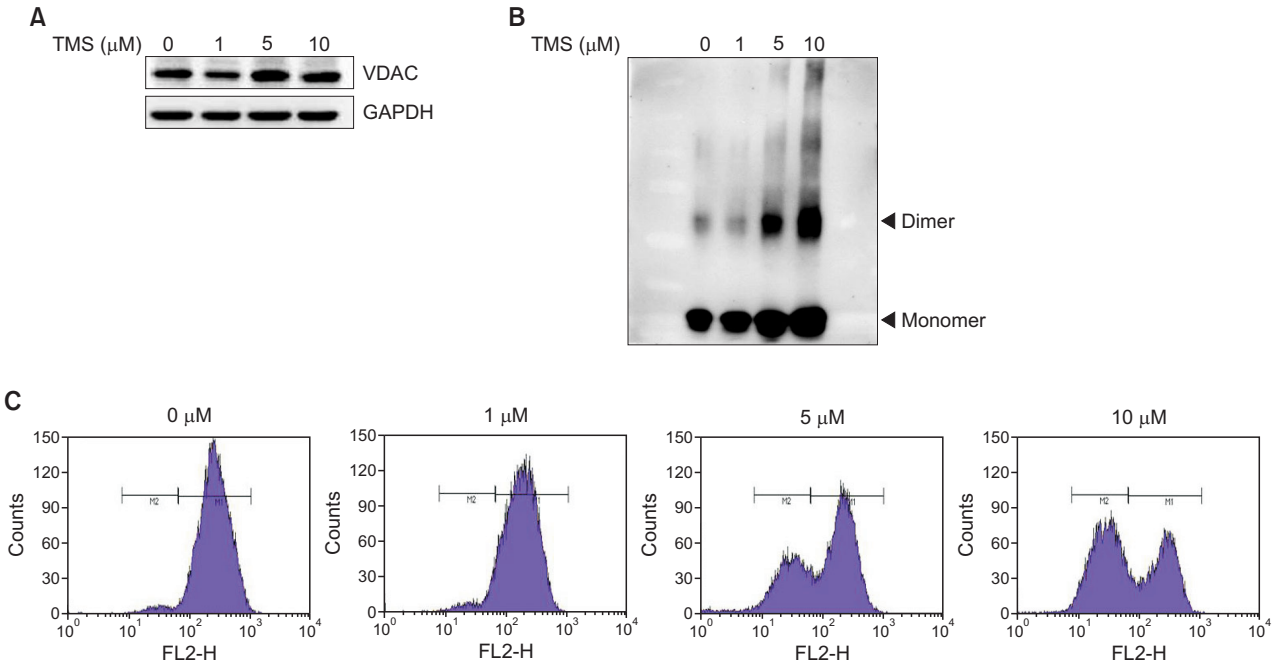

Fig. 3. TMS induces VDAC1 expression and oligomerization. (A) Western blot analysis. MCF-7 cells were treated with various concentrations of TMS (0, 1, 5, or 10 μM) for 48 h. After incubation, cells were harvested and extracted proteins were resolved by SDS-PAGE (10%) and Western blot analysis was conducted. GAPDH level was determined as loading controls. (B) VDAC oligomerization. Cells were treated with TMS (0, 1, 5, or 10 μM) for 48 h. Cells were then harvested and incubated with sulfo-EGS (250 μM) for 20 min at 30°C. After proteins were resolved by SDS-PAGE (8%), VDAC1 proteins were measured using Western blot analysis. A 33-kDa band represents VDAC monomers, while a 65-kDa band represents VDAC dimers. (C) Mitochondrial membrane potential. After cells were incubated with TMS for 48 h, cells were labeled with 100 nM TMRM for 20 min. After washing, cells were analyzed by flow cytometry. TMRM was monitored as log FL2-H (*x axis*, 574 nM) versus relative cell number (*y axis*) in the histogram. The data shown are representative of three independent experiments.

transfection, approximately 5×10^5 cells per 60 mm-plate were seeded in DMEM medium containing 10% FBS. Cells were washed in PBS and then resuspended in Opti DMEM serum-free medium. Transfection was carried out with 38 nM of annexin A5 siRNA for 5 h at 37°C. After transfection, cells were maintained in DMEM medium containing 10% FBS for 48 h. The target sequence of annexin A5 siRNA was GUAAUGGGAUCUAUAAAGG.

Western blot analysis

Following transfection, whole cell lysates were prepared and protein concentration was determined using BCA Protein Assay Reagents. Cellular extracts (20 μg) were separated on 10% SDS-PAGE at 100 V and transferred onto 0.45 μm PVDF membrane. Nonspecific binding was blocked with 5% nonfat milk in TBS-T for 1 h at room temperature. Primary antibody was used at a 1:1000 dilution. Secondary antibody was used in a 1:5000 dilution. The incubation of primary antibodies was done at 4°C for overnight incubation. Secondary antibodies were done at 4°C for 2 h. Proteins were visualized by an ECL method and the band intensity was analyzed by Chemidoc XRS densitometer system and quantified by Quantity One software (Bio-Rad).

Statistical analysis

Statistical analysis was performed by using one-way analysis of variance, followed by Dunnett's Multiple Comparison *t*-test using Graph-Pad Prism Software (GraphPad Software Inc., San Diego, CA, USA) when appropriate. The difference was considered statistically significant at $p<0.05$.

RESULTS

TMS induces expression of annexin A5

To elucidate whether TMS is able to induce expression of annexin A5 in MCF-7 cells, the mRNA and protein expression of annexin A5 were measured by quantitative real-time PCR and Western blot, respectively. As shown in Fig. 1A, when cells were treated with TMS (1, 5, or 10 μM) for 48 h, annexin A5 mRNA expression was significantly increased in a concentration-dependent manner. In agreement with increasing mRNA levels, annexin A5 protein level were also strongly enhanced by TMS (Fig. 1B).

Translocation of annexin A5 into mitochondria by TMS

To examine whether induction of annexin A5 expression by TMS also causes a translocation of annexin A5 into mitochondria, cytosolic and mitochondrial fractions were isolated and the levels of annexin A5 were determined by Western blot analysis. As shown in Fig. 2A, TMS markedly promoted translocation of annexin A5 into mitochondria. Confocal laser scanning microscopic analysis of annexin A5 confirmed that TMS strongly induced annexin A5 expression and translocation into mitochondria, indicating that annexin A5 may play an important role in mitochondria-dependent apoptosis (Fig. 2B).

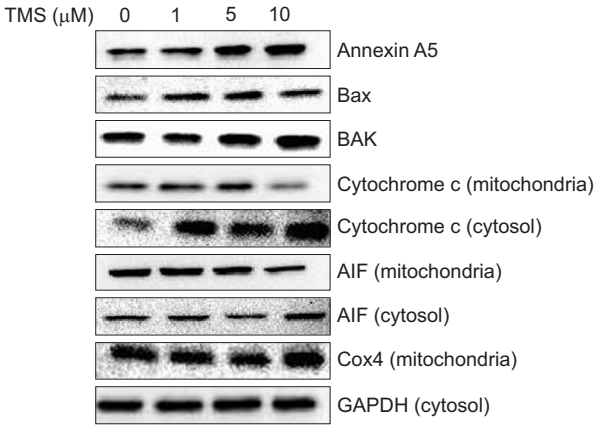

Fig. 4. TMS regulates mitochondrial apoptotic proteins. MCF-7 cells were treated with various concentrations of TMS (0, 1, 5, or 10 μM) for 48 h. After incubation, cells were harvested and the cytosolic and mitochondrial fractions were isolated. Extracted proteins were resolved by SDS-PAGE (10%) and Western blot analysis was conducted. GAPDH level was determined as loading controls for cytosolic fraction and COX-4 level was determined as a loading control for mitochondrial fraction. The data shown are representative of three independent experiments.

VDAC1 is necessary for TMS-mediated apoptosis

To elucidate the mechanism of TMS-induced apoptosis, we focused on the role of VDAC1 in mitochondria. MCF-7 cells were treated with TMS (1, 5, or 10 μM) for 48 h and VDAC1 protein expression and formation of VDAC dimer and oligomers were determined. TMS clearly increased the expression of VDAC in a concentration-dependent manner. Moreover, the formation of VDAC dimer and oligomers was significantly enhanced in TMS-treated cells (Fig. 3A, B). To determine the effect of VDAC1 on mitochondrial depolarization, cells were treated with TMS (1, 5, or 10 μM) for measuring mitochondrial membrane potential. TMS caused a significant decrease in FL2-H fluorescence, indicating TMS may induce disruption of mitochondrial membrane potential through inducing VDAC1 expression and oligomerization.

Role of annexin A5 in mitochondrial apoptosis by TMS

To elucidate how TMS causes mitochondria-dependent apoptosis, cells were treated with TMS (1, 5, or 10 μM) for 48 h and then the levels of Bax, Bak, cytochrome c, and AIF were measured. TMS increased mitochondrial Bax and Bak levels as well as translocation of annexin A5 into mitochondria. In addition, cytochrome c and AIF release from mitochondria to cytosol was significantly enhanced by TMS in a concentration-dependent manner (Fig. 4). These data indicated that Bax and Bak may involve in TMS-induced apoptosis through induction of cytochrome c and AIF release.

To investigate the role of annexin A5 in mitochondria-dependent apoptosis, annexin A5 siRNA was used. As shown in Fig. 5A, treatment with annexin A5 siRNA (40 nM) for 48 h strongly repressed annexin A5 expression. Knockdown of annexin A5 by siRNA also decreased Bax and Bak expression and TMS recovered the inhibition of Bax and Bak expression by annexin A5 siRNA. (Fig. 5B). These results demonstrated that annexin A5 is able to regulate mitochondria-dependent apoptosis through controlling Bax and Bak expression.

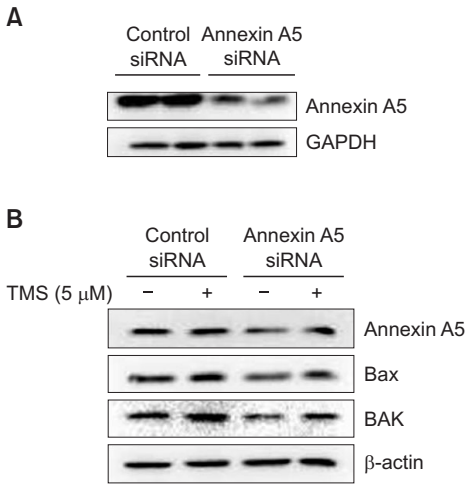

Fig. 5. Effect of annexin A5 knockdown on Bax and Bak expression induced by TMS. (A) MCF-7 cells were transfected with annexin A5 siRNA (38 nM) for 48 h. After incubation, cells were harvested and total cellular lysates were prepared. Extracted proteins were resolved by SDS-PAGE (10%) and Western blot analysis was conducted. GAPDH level was determined as loading controls. (B) Cells were transfected with annexin A5 siRNA (38 nM) for 48 h and were then treated with TMS (5 μM) for 48 h. After incubation, cells were harvested and total cellular lysates were prepared. Extracted proteins were resolved by SDS-PAGE (10%) and Western blot analysis was conducted. β-Actin level was determined as loading controls. The data shown are representative of three independent experiments.

DISCUSSION

Previously, we have demonstrated that TMS induces apoptosis in MCF-7 and HL-60 cancer cells in a concentration- and time-dependent manner (Chun *et al*., 2005). However, the detailed mechanisms of apoptosis induced by TMS are not clear. Recently, we showed that annexin A5 may play a crucial role in cisplatin-induced toxicity by mediating the mitochondrial apoptosis via the induction and oligomerization of VDAC1 in human and rat renal epithelial cells (Jeong *et al*., 2014). Thus, we investigated the role of annexin A5 in controlling mitochondria-dependent apoptosis induced by TMS.

Annexin A5 is known as a Ca^{2+}-dependent phospholipid binding protein that inhibits PKC signaling (Rothhut *et al*., 1995). Although annexin A5 has been used for the detection of apoptosis because it shows high affinity for surface exposed phosphatidylserine during apoptosis (van Engeland *et al*., 1998), annexin A5 may directly involve in apoptotic pathway (Kwon *et al*., 2013; Jeong *et al*., 2014). In this study, we found that TMS is able to activate annexin A5 mRNA and protein expression in MCF-7 cells in a concentration-dependent manner. We observed that translocation of annexin A5 into mitochondria promotes mitochondria-dependent apoptosis by TMS because annexin A5 increases mitochondrial proapoptotic proteins including Bax and Bak as well as VDAC1 in mitochondria. Bax and Bak play important roles in mitochondrial permeabilization and apoptosis and the oligomerization of Bax and Bak in the mitochondrial outer membrane is a major control point for mitochondria-dependent apoptosis. Because Bax resides predominantly in the cytosol, the translocation of Bax from cytosol into mitochondria is necessary for Bax/Bax

interaction and channel formation. Our data showed that Bax translocation caused by TMS occurs in parallel with translocation of annexin A5 into mitochondria. Moreover, when cells were treated with annexin A5 siRNA, Bax level was also markedly decreased. Thus, the present data indicated that annexin A5 expression and translocation by TMS could result in the observed alteration of Bax level, which in turn could trigger the mitochondrial pathway of apoptosis.

Mitochondrial outer membrane permeability (MOMP) is a common event of apoptotic pathways and apoptotic factors including cytochrome c or AIF normally secluded in the intermembrane space have been liberated from mitochondria through the permeabilized outer membrane (Ravagnan *et al*., 2002). Bax and Bak are the effectors of MOMP because these proteins insert into the outer mitochondrial membrane and induce the pore formation allowing for the release of apoptotic factors. It has been proposed that VDAC1 may act as a regulator of MOMP and VDAC1 is responsible for transducing Bak activation to Bax to lead MOMP (Tajeddine *et al*., 2008). Moreover, Bax increases the permeability of VDAC1 function in outer mitochondrial membrane. Our data that annexin A5 may increase Bax and Bak level in mitochondria indicate that increased mitochondrial annexin A5 by apoptosis stimuli may activate Bax and Bak to form MOMP through VDAC1 action. Because mitochondrial lipids including cardiolipin and sphingosine 1-phosphate regulates Bax/Bak oligomerization, the possibility that annexin A5 is able to change mitochondrial lipid composition in outer mitochondrial membranes needs to be determined.

In summary, we demonstrated that TMS is able to induce mitochondrial apoptosis through increasing annexin A5 expression and translocation into mitochondria. VDAC oligomerization and MOMP formation trigger release of apoptotic factors to promote caspase activation. The function of annexin A5 inducing mitochondrial apoptosis seems to be common and the detailed mechanism of annexin A5 action in cell will provide a clue for new apoptotic pathway.

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