Up-regulation of c-Jun-NH2-kinase pathway contributes to the induction of mitochondria-mediated apoptosis by α-tocopheryl succinate in human prostate cancer cells

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Abstract
Previously, α-tocopheryl succinate (α-TOS) has been reported to induce caspase-mediated apoptosis in PC-3 human prostate cancer cells. Caspase-9 was among several initiator caspases activated by α-TOS, suggesting a potential contribution of the intrinsic apoptotic pathway in mediating the response to α-TOS. Gene expression microarray was carried out as a screen to identify novel signaling molecules modulated by α-TOS, with a special focus on those known to play a role in mitochondria-mediated apoptosis. We discovered that Ask1, GADD45β, and Sek1, three key components of the stress-activated mitogen-activated protein kinase pathway, are novel targets of α-TOS. Western blot analysis showed increased levels of phospho-Sek1 and phospho-c-Jun-NH2-kinase (JNK) in addition to total Ask1, GADD45β, and Sek1. α-TOS also altered JNK-specific phosphorylation of Bcl-2 and Bim in a manner consistent with enhanced mitochondrial translocation of Bax and Bim. Because the expression level of most Bcl-2 family members remained unchanged, the posttranslational modification of Bcl-2 and Bim by JNK is likely to be a driving force in α-TOS activation of the intrinsic apoptotic pathway. Based on our findings, we propose a working model to capture the salient features of the apoptotic signaling circuitry of α-TOS. [Mol Cancer Ther 2005;4(1):43–50]

Introduction
Vitamin E is a generic term for two distinct, but structurally related, groups of compounds: tocopherols and tocotrienols. Numerous studies have convincingly shown the growth-inhibitory activities of vitamin E compounds in a variety of cancer cell lines (1–5). Among the various naturally occurring vitamin E compounds and their synthetic derivatives, α-tocopheryl succinate (α-TOS), a redox-silent analogue of α-tocopherol, is the most commonly used form in in vitro studies of cancer research (6, 7). The chemotherapeutic effects of α-TOS include repressed cell proliferation, cell cycle block, reduced DNA synthesis, and induction of apoptosis (3, 8–15). Of these different mechanisms, the last one has been studied most extensively.

It is generally accepted that there are two principal apoptotic cascades: the receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway. In the presence of external stimuli such as inflammatory cytokines or withdrawal of growth factors, specific ligands bind to death receptors such as Fas, leading to the assembly of a death-inducing signal complex, and the recruitment and activation of initiator caspase-8. Independent of the above mechanism, the mitochondria-mediated pathway is activated by internal death stimuli such as reactive oxygen species, culminating in the release of cytochrome c from the mitochondria into the cytoplasm. Following the binding of cytochrome c to Apaf-1, procaspase-9 is recruited to the complex and activated by the cofactor Apaf-1 through the caspase recruitment domain. Once activated, initiator caspase-8 and caspase-9 cleave and activate downstream effector caspase-3, -6, and -7 to complete the escalation of the caspase cascade. The whole process is geared ultimately to the execution of apoptotic cell death (16, 17).

The Bcl-2 family proteins are critical regulators of the mitochondrial pathway of apoptosis (16–19). They are believed to modulate the permeability of the mitochondrial membrane directly and thereby control the release of cytochrome c. Based on the Bcl-2 homology domain structures and protein functions, the Bcl-2 family can be classified into three groups: the antiapoptotic members (containing all four BH domains) such as Bcl-2 and Bcl-xL, the proapoptotic members (containing only BH1, BH2, and BH3 domains) such as Bax and Bak, and the BH3 domain–only members such as Bad, Bim, and Bid. Apoptotic signaling triggers Bax translocation from the cytosol to the outer mitochondrial membrane. In contrast to Bax, Bak is localized primarily in the outer mitochondrial membrane. When cued by apoptotic signals, Bax and Bak...
oligomerize and form heterotetrameric channels for the exit of cytochrome c. Bcl-2 and Bcl-xL, on the other hand, prevent apoptosis by inhibiting the formation of the Bax/Bak heterotetrameric channels. Furthermore, the phosphorylation of the Bcl-2 protein is involved in the regulation of its function. The BH3 domain—only proteins are usually localized in the cytosol and function as death ligands to the multidomain Bcl-2 family members. Upon activation by apoptotic stimuli, the BH3 domain—only proteins translocate to the mitochondria; the above process is regulated by different mechanisms. For example, Bim is sequestered in the cytosol by binding to the dynein motor complexes. Phosphorylation by c-Jun-NH2-kinase (JNK) disrupts the binding motif of Bim and facilitates the release of Bim, thus making it available to antagonize Bcl-2 in the mitochondria.

Previously, we found that caspase-9, the key initiator caspase of mitochondria-mediated apoptosis, is activated in PC-3 human prostate cancer cells treated with α-TOS (15). The significance of the mitochondrial pathway in α-TOS-induced apoptosis has similarly been implicated by Kline et al. and Neuzil et al. in different cell models (20–22). In the present study, we used gene expression microarray as a first step to identify novel signaling molecules modulated by α-TOS, with a special focus on those that are known to play a role in mitochondria-mediated apoptosis. Based on further research aimed at validating the discovery, a working model is proposed to delineate the molecular mechanism that may underlie the proapoptotic activity of α-TOS.

Materials and Methods

Cell Culture Condition

The PC-3 human prostate cancer cell line was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine, and maintained in an atmosphere of 5% CO2 in a 37°C humidified incubator. Ethanol was used to dissolve α-TOS (Sigma, St. Louis, MO); the final concentration of ethanol in the culture medium was kept at 0.2% (v/v).

MTT Cell Growth Assay

This assay is based on the conversion of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan crystals by metabolically active cells. It provides a quantitative determination of cell viability. Cells were seeded in 24-well plates at a density of 8,000 cells/mL. At 72 hours after seeding, cells were treated with α-TOS at different concentrations as specified in Results. The experiment was done in triplicate. After 24, 48, or 72 hours of treatment, 200 μL of MTT was added to each well of cells, and the plate was incubated for 4 hours at 37°C. The MTT crystals from both attached and floating cells were solubilized in isopropanol, and the solution was centrifuged to pellet the cellular debris. Spectrophotometric absorbance of each sample was measured at 570 nm using a Spectra Microplate Reader (SLT, Australia).

Quantification of Apoptosis by Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling

PC-3 cells were plated at a density of 4,000 cells/cm² in T175 culture flasks. At 72 hours after seeding, cells were exposed to 20 or 40 μmol/L α-TOS for 24 hours. Adherent cells harvested by mild trypsinization were pooled together with detached cells. The cell pellets were then fixed in 1% paraformaldehyde in PBS (pH 7.4), washed in PBS, and stored in 70% ethanol at 4°C overnight. The ethanol solution was subsequently removed following centrifugation, and cells were treated with the enzyme terminal deoxynucleotidyl transferase-mediated nick end labeling, labeled with bromodeoxyuridine, and stained with fluorescein isothiocyanate and propidium iodide using the apo-bromodeoxyuridine kit from Phoenix Flow Systems (San Diego, CA) as per the manufacturer's protocol. Apoptotic cells were counted by flow cytometry, and the data were analyzed with the WinList software.

Oligonucleotide Array Analysis

PC-3 cells were plated at a density of 4,000 cells/cm² in 15-cm culture dishes. Synchronization of cells was achieved by starving in serum-free medium for 48 hours. After returning to regular growth medium for 6 hours, cells were exposed to regular growth medium or 20 μmol/L α-TOS for 24 hours. Total RNA and protein were then isolated using TRIZol (Life Technologies, Inc., Gaithersburg, MD). The experiment was repeated twice more, and total RNA collected from the three replicates were pooled and submitted to microarray analysis using the U95A chip from Affymetrix (Santa Clara, CA). Biotinylated cRNA probe generation, array hybridization, and other procedures were carried out according to the standard Affymetrix GeneChip protocol. Fluorescence intensity for each chip was captured with a Hewlett-Packard laser confocal scanner. Three data analysis parameters from the Affymetrix Microarray Suite software were used to determine a change in gene expression between the control and treatment samples: detection (a qualitative measure of the presence or absence of a particular transcript), change (a quantitative measure of the increase or decrease of a particular transcript), and signal log ratio (a quantitative measure of the increase or decrease of a particular transcript). A detailed description of the analysis of the microarray data was published previously (23). For a single comparison between two groups, a log₂-transformed treatment/control signal ratio of ≥1 or ≤−1 was chosen as the criterion for induction or repression, respectively. These values are recommended by guidelines described in the Affymetrix Data Analysis Fundamentals Manual.

Cell Lysis and Fractionation

PC-3 cells were plated at a density of 4,000 cells/cm² in T175 culture flasks. At 72 hours after seeding, cells were exposed to 40 μmol/L α-TOS for 12 or 24 hours. Whole cell lysate was prepared using 1× cell lysis buffer (Cell Signaling Technology, Beverly, MA), and protein concentration determined by using the bicinchoninic acid protein
assay kit (Pierce Biotechnology, Rockford, IL). The cytosol and mitochondria-enriched fractions were collected using the cytochrome c release apoptosis assay kit from Oncogene Research Products (San Diego, CA) as per the manufacturer’s protocol. Briefly, adherent cells harvested by mild trypsinization were pooled together with detached cells. Approximately $5 \times 10^7$ cells were collected for each sample. After washing with ice-cold PBS, cell pellets were resuspended in 1 mL of 1× cytosol extraction buffer containing 1 mol/L DTT and protease inhibitors. The cell suspension was then incubated on ice for 10 minutes, and the cells were homogenized in a tissue grinder on ice for 30 to 50 passes. The homogenate was centrifuged at $700 \times g$ for 10 minutes at $4^\circ C$, and the supernatant was centrifuged at $10,000 \times g$ for 30 minutes at $4^\circ C$. The supernatant from the high-speed centrifuge was collected as the cytosol fraction. The cell pellets from the high-speed centrifuge were resuspended in 0.1 mL of mitochondrial extraction buffer containing 1 mol/L DTT and protease inhibitors, and saved as the mitochondrial fraction. Western blot was used to analyze for cytochrome c release, and for Bcl-2 family protein translocation in the mitochondrial fraction.

**Western Blot Analysis**

Briefly, ~50 µg of protein was resolved over 10% to 15% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The blot was blocked in blocking buffer [5% nonfat dry milk, 10 mmol Tris (pH 7.5), 10 mmol NaCl, and 0.1% Tween 20] at 37°C for 1 hour, incubated with the primary antibody overnight at 4°C, followed by incubation with an anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA) at room temperature for 30 minutes. Individual proteins were visualized by an enhanced chemiluminescence kit obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Immunoreactive bands were quantitated by volume densitometry using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and normalized to loading controls.

The following polyclonal antibodies (source) were used in this study: anti-PARP, phospho-Bcl-2 (Ser70), Bcl-xL, Bax, Bak, Ask1, Sek1, phospho-Sek1, JNK, and phospho-JNK (Cell Signaling Technology), anti-Bim (BD PharMingen, San Diego, CA), and anti-phospho-Bim EL (Upstate Cell Signaling Solutions, Charloettesville, VA).

The following monoclonal antibodies (source) were used in this study: anti-cytochrome c (Oncogene Research Products), anti-Bcl-2 (BD PharMingen), anti-GADD45 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-β-actin (Sigma), anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA), and anti-heat shock protein 70 (StressGen Biotechnologies Corp., Victoria, BC, Canada).

**Statistical Analysis**

The Student’s two-tailed $t$ test was used to determine statistical significance between treatment and control values, and $P < 0.05$ was considered significant.

**Results**

**Sensitivity of PC-3 Cells to α-TOS**

The effect of α-TOS on the viability of PC-3 cells was assessed by the MTT assay (Fig. 1). Treatment with α-TOS at a concentration of 10 µmol/L did not produce any change in cell number even after 72 hours. Raising the concentration to 20 µmol/L inhibited cell viability by ~10% and ~40% at 48 hours and 72 hours, respectively. Further increases of α-TOS in the culture medium resulted in a greater magnitude of inhibition with a shorter exposure time. Thus, at a concentration of 40 µmol/L, α-TOS was able to reduce cell number by ~50% only after 24 hours. Based on the above data, it is evident that α-TOS inhibited the accumulation of PC-3 cells in a time-dependent and dose-dependent manner.

**Induction of Apoptosis by α-TOS**

In an experiment aimed at determining the proapoptotic effect of α-TOS, exponentially growing PC-3 cells were incubated for 24 hours in the presence of 20 or 40 µmol/L α-TOS. Apoptosis was measured by flow cytometric analysis of cells labeled with bromodeoxyuridine. DNA fragmentation by cellular nucleases is a signature event of apoptosis. This process results in a substantial appearance of free 3’-hydroxyl ends in genomic DNA, which can be earmarked by labeling with bromodeoxyuridine in the presence of terminal deoxynucleotidyl transferase. As shown in Fig. 2A, cells in the right region of each cytogram are bromodeoxyuridine-positive. The percentage of apoptotic cells was quantitated from three independent experiments and shown in Fig. 2B. A concentration of 20 mol/L α-TOS caused only a marginal increase in apoptosis. Raising the concentration to 40 mol/L led to a 5-fold increase compared with the control. Thus in agreement with the cell number data, the apoptotic response to α-TOS was rather steep in the dose range between 20 and 40 mol/L.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Sensitivity of PC-3 cells to α-TOS. Cells were exposed to 10, 20, 30, or 40 µmol/L α-TOS for 24, 48, or 72 h. Results are expressed as percentages of the control. Values of $< 100\%$ represent growth inhibition. **Columns,** mean ($n = 5$); bars, SE. *, $P < 0.05$, statistically different compared with the untreated control.
Identification of Novel α-TOS Responsive Apoptosis-Related Genes

In order to avoid the confounding effect of cell death but still in a position to capture the early response genes which are exquisitely sensitive to α-TOS, we exposed PC-3 cells to 10 μmol/L α-TOS for only 24 hours for the microarray experiment. As noted in Methods, genes with a log2-transformed treatment/control ratio of ≥1 or ≤−1 are considered to be up-regulated or down-regulated by α-TOS, respectively. Based on the above analysis, we identified more than 600 genes responsive to α-TOS. With the resources provided by the NetAffx Analysis Center on the Affymetrix web site, we narrowed the roster to those genes that are implicated in the signal transduction of apoptosis. The information is summarized in Table 1. One thing caught our attention immediately from this list of 21 genes. Three upstream components of the stress-activated kinase cascade, ASK1, GADD45β, and SEK1 (boldface in Table 1), were all induced by α-TOS with a respective signal log ratio of 2.8, 2.0, and 1.4. Because the JNK pathway is known to mediate the apoptotic response of cancer cells, we thought it would be reasonable to do additional experiments with these three genes.

To confirm the array data, we used Western blot analysis to measure the changes in protein level of Ask1, GADD45β, and Sek1. The results are shown in Fig. 3. We quantified the intensity of the protein bands by densitometry and expressed the results as fold of change induced by α-TOS (Table 2). The protein levels of all three molecules were markedly increased. In the other half of Table 2, we presented the array data, but this time, calculated as fold of the power of the signal log ratio. The exercise is meant to illustrate that there is a reasonably close correlation between the changes in transcript and protein levels.

Table 1. Changes of α-TOS-responsive apoptosis-related genes

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Change</th>
<th>Signal log2 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1</td>
<td>v-akt murine thymoma viral oncogene homologue 1</td>
<td>Decrease</td>
<td>−2.7</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid β (A4) precursor protein</td>
<td>Increase</td>
<td>1.9</td>
</tr>
<tr>
<td>ASK1</td>
<td>MAPK kinase kinase 5</td>
<td>Increase</td>
<td>2.8</td>
</tr>
<tr>
<td>CGB</td>
<td>Chorionic gonadotropin, β polypeptide</td>
<td>Decrease</td>
<td>−2.1</td>
</tr>
<tr>
<td>CLDN3</td>
<td>Claudin 3</td>
<td>Decrease</td>
<td>−1.6</td>
</tr>
<tr>
<td>C-MYC</td>
<td>v-myc myelocytomatosis viral oncogene homologue</td>
<td>Decrease</td>
<td>−1.8</td>
</tr>
<tr>
<td>FXR1</td>
<td>Fragile X mental retardation, autosomal homologue 1</td>
<td>Increase</td>
<td>2.2</td>
</tr>
<tr>
<td>GADD45B</td>
<td>Growth arrest and DNA-damage inducible, beta</td>
<td>Increase</td>
<td>2.0</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone deacetylase 1</td>
<td>Decrease</td>
<td>−2.7</td>
</tr>
<tr>
<td>HSPB2</td>
<td>Heat shock 27 kDa protein 2</td>
<td>Decrease</td>
<td>−2.5</td>
</tr>
<tr>
<td>NFKB3</td>
<td>v-rel reticuloendotheliosis viral oncogene homologue A</td>
<td>Decrease</td>
<td>−1.5</td>
</tr>
<tr>
<td>PAR1</td>
<td>Coagulation factor II (thrombin) receptor</td>
<td>Decrease</td>
<td>−1.8</td>
</tr>
<tr>
<td>PAR4</td>
<td>PRKC, apoptosis, WT1, regulator</td>
<td>Increase</td>
<td>1.4</td>
</tr>
<tr>
<td>PRKCZ</td>
<td>Protein kinase C,</td>
<td>Decrease</td>
<td>−2.1</td>
</tr>
<tr>
<td>PSEN1</td>
<td>Presenilin 1</td>
<td>Decrease</td>
<td>−3.3</td>
</tr>
<tr>
<td>RAF1</td>
<td>v-raf-1 murine leukemia viral oncogene homologue 1</td>
<td>Decrease</td>
<td>−3.4</td>
</tr>
<tr>
<td>SEK1</td>
<td>MAPK kinase 4</td>
<td>Increase</td>
<td>1.4</td>
</tr>
<tr>
<td>SOCS2</td>
<td>Suppressor of cytokine signaling 2</td>
<td>Increase</td>
<td>2.0</td>
</tr>
<tr>
<td>STK3</td>
<td>Serine/threonine kinase 3</td>
<td>Increase</td>
<td>2.4</td>
</tr>
<tr>
<td>SURVIVIN</td>
<td>Baculoviral IAP repeat-containing 5 (survivin)</td>
<td>Decrease</td>
<td>−21.6</td>
</tr>
<tr>
<td>TNFRSF25</td>
<td>Tumor necrosis factor receptor super family, member 25</td>
<td>Decrease</td>
<td>−1.7</td>
</tr>
</tbody>
</table>

Figure 2. Quantitation of apoptosis by terminal deoxynucleotidyl transferase-mediated nick end labeling in PC-3 cells treated with 20 or 40 μmol/L α-TOS for 24 h. A, cytograms from flow cytometric analysis. Right of each cytogram, apoptotic cells. B, percentages of apoptotic cells. Columns, mean (n = 3); bars, SE. *, P < 0.05, statistically different compared with the untreated control.

Activation of JNK Pathway by α-TOS

To further investigate the effect of α-TOS on the activity of the JNK pathway components, PC-3 cells were exposed to 40 μmol/L α-TOS for 12 or 24 hours and whole cell lysates were analyzed by Western blot. The higher dose and shorter time protocol were designed to see how quickly cells respond to α-TOS induction of the stress-activated signaling pathway. Because this is a kinase cascade,
were naturally interested in the phosphorylation status of the proteins, especially when microarray data do not furnish information on posttranslational changes. As shown in Fig. 4, α-TOS markedly increased the expression level of Ask1, GADD45, Sek1, and phospho-Sek1 as early as 12 hours; the magnitude of the increase seemed to increase with longer exposure. Consistent with the above findings, phospho-JNK was also noticeably increased, although the expression level of total JNK was not affected. Given the above observations, the following question came to mind: is JNK activation by α-TOS associated with phosphorylation of some members of the Bcl-2 family?

**Effect of α-TOS on Bcl-2 Family Members**

The effect of α-TOS on the antiapoptotic proteins Bcl-2 and Bcl-xL was examined first by Western blot. As shown in Fig. 5, the expression level of both proteins remained unchanged with α-TOS treatment. However, phosphorylation of Bcl-2 at Ser70, a phosphorylation site specific to JNK (24), was found to increase more than 100-fold by α-TOS at 12 hours based on densitometric analysis. This robust enhancement was sustained for at least up to 24 hours. No change was detected with Bcl-xL.

The next experiment was designed to determine the effect of α-TOS on the proapoptotic Bcl-2 family proteins, including Bax, Bak, and Bim. Whole cell lysate and mitochondria-enriched fraction were used to analyze for protein level and translocation changes, respectively. Because α-TOS did not alter the protein level of Bcl-xL, we used this mitochondria-resident protein as a loading control for mitochondrial fraction Western blot. As shown in Fig. 6, α-TOS significantly increased the amount of Bax protein in whole cell lysate at 24 hours. Interestingly, an increase of Bax protein in the mitochondria was observed as early as 12 hours, suggesting that the induction of Bax translocation to the mitochondria by α-TOS was independent of the increase in expression level. In contrast to Bax, the protein level of Bak in either whole cell lysate or mitochondria was not affected by α-TOS.

The protein level of Bim EL, a generally expressed isoform of Bim, was not changed in whole cell lysate of α-TOS-treated cells. However, the amount of Bim EL protein was significantly increased in the mitochondria. The phosphorylation status of Bim EL at Ser65, a JNK-specific site (25), was also evaluated. Phospho-Bim EL was not cleanly resolved in whole cell lysate due to the presence of too many nonspecific signals. However, an increase in phospho-Bim EL was observed in the mitochondria of α-TOS-treated cells.

**Cytochrome c Release and PARP Cleavage**

To confirm the biological significance of α-TOS activation of the intrinsic apoptotic pathway, Western blot analysis was done to study cytochrome c release and PARP cleavage. Cytochrome c release is a known outcome of an activated mitochondrial pathway, whereas PARP cleavage is a sensitive marker for caspase-mediated apoptosis. As shown in Fig. 7, α-TOS induced cytochrome c release

<table>
<thead>
<tr>
<th>Gene</th>
<th>Array analysis</th>
<th>Western analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Difference call</td>
<td>Fold of change*</td>
</tr>
<tr>
<td>ASK1</td>
<td>Increase</td>
<td>7.0</td>
</tr>
<tr>
<td>GADD45β</td>
<td>Increase</td>
<td>4.0</td>
</tr>
<tr>
<td>SEK1</td>
<td>Increase</td>
<td>2.6</td>
</tr>
</tbody>
</table>

*The value is obtained by raising 2 to the power of signal log ratio (Table 1).

The value is the treatment/control signal ratio normalized by actin from the densitometric analysis.
and PARP cleavage in a time-dependent manner. Furthermore, the fact that cytochrome c release happened before PARP cleavage supports the role of the mitochondrial pathway in apoptosis signaling by α-TOS.

**Discussion**

α-TOS is considered an anticancer agent with chemotherapeutic and chemopreventive potential because of its high proapoptotic activity in malignant cells. Several studies have shown that the mitochondria pathway is central to α-TOS-induced apoptosis, but the underlying mechanism has not been fully delineated (15, 20–22). In this study, we successfully used microarray analysis to identify potential molecular triggers that might be critical to α-TOS mitochondria-mediated apoptosis signaling. We discovered that Ask1, GADD45β, and Sek1, three key components of the stress-activated mitogen-activated protein kinase (MAPK) pathway, are novel targets of α-TOS. Additional Western blot analysis showed that an increased level of phospho-Sek1 could be responsible for the activation of JNK. We also found that α-TOS altered JNK-specific phosphorylation of Bcl-2 and Bim in such a way as to hasten the mitochondrial translocation of Bax and Bim. Because the expression level of most of the Bcl-2 family proteins remain unchanged, the posttranslational modification of Bcl-2 and Bim by activated JNK is likely to play a key role in mediating the apoptotic response to α-TOS.

Ask1 is a MAPK kinase kinase (27–29). In the presence of stress-related stimuli, Ask1 activates Sek1, which in turn activates JNK. Overexpression of Ask1 has been shown to induce mitochondria-dependent caspase cleavage in various cell models, and the activation of Ask1 is required for stress-induced and cytokine-induced apoptosis. Taken together, Ask1 seems to function as a proapoptotic signaling molecule when cells are subjected to various kinds of stress. GADD45β is a member of a group of genes whose transcripts are induced rapidly under the conditions of growth arrest and DNA damage. Saito et al. (30, 31) have reported that GADD45-like proteins can bind to and activate MTK1/MEKK4, leading to the activation of downstream MAPK cascade molecules, including Sek1 and JNK. Sek1 is a dual-specificity protein kinase that can directly activate JNK by phosphorylation on Thr183 and Tyr185 (32). The present study is the first to report that α-TOS activates the JNK pathway through enhancing the gene expression of a string of upstream molecules. The implication of stress signaling by α-TOS opens up a new dimension in elucidating the anticancer mechanism of α-TOS.

JNK activation has been reported to be essential for stress-activated apoptosis (32). There are two possible mechanisms to support the proapoptotic action of JNK. First, JNK can activate the transcription factor c-Jun by phosphorylation, leading to c-Jun/AP-1-regulated gene expression of death receptor ligands, such as FasL (33).
Second, JNK may contribute to the initiation of mitochondria-mediated apoptosis via phosphorylation of the Bcl-2 family proteins (24, 25, 34, 35). Our present study focuses mainly on the latter. Recent evidence indicates that JNK phosphorylates Bcl-2 at Ser70 to dampen its antiapoptotic activity (36). Because the antiapoptotic activity of Bcl-2 primarily involves an inhibition of Bax mitochondrial translocation (37, 38), our findings that JNK activation by α-TOS is associated with increased phospho-Bcl-2 and Bax protein in the mitochondria lend credence to a cause and effect relationship.

JNK may also affect the proapoptotic Bcl-2 family members (25, 34, 39, 40). Kline et al. (20) recently showed that in human breast cancer cells, α-TOS induces JNK activation and Bax mitochondrial translocation. Functional knockdown of Bax significantly reverses α-TOS-induced apoptosis, suggesting a key role of Bax in this process. The presence of a chemical JNK inhibitor prevents Bax from undergoing conformational changes and interferes with its proapoptotic activity. However, the mechanism by which JNK regulates Bax mitochondrial translocation remains unclear. Structural studies of Bim and Bax implicate a direct interaction of these two proteins that leads to spontaneous activation of Bax (41). Our observation that JNK activation is accompanied by phosphorylation of Bim EL and its release from cytosolic sequestration might provide a missing link between JNK and Bax mitochondrial translocation in α-TOS treated cells.

On the basis of our findings, we propose a working model to integrate the apoptotic signaling circuitry of α-TOS (Fig. 8). Some notable vignette captured in the diagram includes (a) the activation of the Ask1/Sek1/JNK pathway, (b) phospho-Bim as a possible tether between JNK and Bax translocation, and (c) the convergence of multiple JNK targets on the mitochondrial pathway of apoptosis.

Although the model is built largely on the strength of descriptive data, it is congruent with the idea that JNK is a key player in orchestrating a favorable climate to facilitate cytochrome c release from the mitochondria and apoptosis induction by α-TOS. To confirm our working model, additional studies will be required to establish the functional significance of activating the JNK pathway. Overexpression and knockout studies of ASK1, GADD45β, and SEK1 will be appropriate in order to gain a better understanding of the biological role of up-regulation of these three genes in α-TOS apoptosis signaling. Moreover, PC-3 is an aggressive androgen-independent prostate cancer cell line. Demonstration of similar mechanisms in other prostate cancer cell lines and/or in vivo models would greatly increase the relevance of proposed mechanisms to the human disease.

Acknowledgments

We thank Dr. Yan Dong for her advice, and Cassandra Hayes and Earl Timm for their technical assistance.

References


Figure 8. Schematic illustration of mitochondria-mediated apoptosis induction by α-TOS. Highlights of the working model are described in Discussion.


Up-regulation of c-Jun-NH$_2$-kinase pathway contributes to the induction of mitochondria-mediated apoptosis by \( \alpha \)-tocopheryl succinate in human prostate cancer cells

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