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ORIGINAL ARTICLE

Pycnogenol Induces Nuclear Translocation of Apoptosis-inducing Factor and Caspase-independent Apoptosis in MC-3 Human Mucoepidermoid Carcinoma Cell Line

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Background: Pycnogenol is extracted from the pine bark of a tree known as *Pinus pinaster* that has variety biological effects. However, its anticancer activity has not yet been completely studied. The aim of this study is to investigate anticancer effect of pycnogenol in MC-3 human mucoepidermoid carcinoma (MEC) cell line.
Methods: We describe the effect of anti-cancer of pycnogenol in MC-3 human oral MEC cells using trypan blue exclusion assay, 3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay, Western blot, preparation of cytosolic and nuclear fractions, immunocytochemistry and reverse transcriptase polymerase chain reaction.
Results: Pycnogenol significantly decreased cell viability and also induced caspase-independent apoptosis. We confirmed that pycnogenol induced the translocation of apoptosis-inducing factor into nucleus and regulated apoptosis. Also, Bak protein stability was partly enhanced by pycnogenol to elevate the expression level of Bak protein.
Conclusions: Overall, pycnogenol may be a fascinating therapeutic drug candidate for the treatment of MEC.
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Key Words: Electrical stimulation, Quadriceps muscle, muscle

INTRODUCTION

Mucoepidermoid carcinoma (MEC), representing 40-52% of all major and minor salivary gland malignancies, is the most common malignancy of the salivary gland.[1] It shows morphological diversity even within a specific tumor type.[2] Although MEC sometimes exhibits slow growth resembling that of a benign lesion, this neoplasm can be highly aggressive with a poor prognosis.[3] For this reason, there is a continuing need for a finding of new therapeutic agents for MEC.

Apoptosis-inducing factor (AIF), one of the mitochondrial proteins contributed to apoptosis, is a flavoprotein with NADH oxidase activity normally contained in the mitochondrial intermembrane space or loosely associated with the inner mitochondrial membrane.[4] Initially, AIF has been discovered by exploring apoptotic processes. It is not only interestingly executed in the complete absence of caspase activation but also translocated from mitochondria into nucleus where this protein induces apoptosis.[5] The intrinsic pathway of apoptosis is regulated by the B cell lymphoma (Bcl)-2 family proteins including anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1) and pro-apoptotic proteins (Bax, Bak, Bim and Bad). Especially Bak and Bax, BH3-only proteins, are essential effectors in intrinsic pathway because either is required for perturbation of the mitochondrial outer membrane. Following an apoptotic stimulus, both of the proteins undergo significant conformational changes and form apoptotic pore to release apoptotic proteins.[6] Therefore, they are necessary for apoptotic cell death.

Pycnogenol, mixture of water-soluble bioflavonoids extracted from the bark of French maritime pine (*Pinus maritima* Aiton,

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currently known as *Pinus pinaster* Aiton), is known to a potent antioxidant. The main components of pycnogenol are monomeric phenolic compounds (catechin, epicatechin and taxifolin) and condensed flavonoids (procyanidines and proanthocyanidines). It has been shown to have excellent radical scavenger and antioxidant properties in model reactions that are superior to those of other fruit and plant extracts and other antioxidants.[7,8] In addition, it was also reported to have anti-cancer, anti-inflammatory and anti-aging activities. However, little is known about the potential apoptotic effect of pycnogenol in MC-3 cells.

In the present study, we analyzed the effect of pycnogenol on apoptotic cell death as well as its related mechanism in MC-3 cells. As shown here, pycnogenol may induce apoptosis through nuclear translocation of AIF and posttranslational modification of Bak protein.

MATERIALS AND METHODS

1. Chemical and reagents

Pycnogenol was purchased from Carbosynth Ltd. (Compton, Berkshire, UK). Dulbecco's modified essential medium, fetal bovine serum, Trypsin and Dulbecco's phosphate buffered saline (PBS) were supplied from WelGENE Inc. (Daegu, Korea). The 3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulph ophenyl)-2H-tetrazolium (MTS) Assay Kit was obtained from Promega (Madison, WI, USA). 4',6-diamidino-2-phenylindole (DAPI) was purchased from Sigma (St. Louis, MO, USA). Annexin V-fluorescein 5 isothiocyanate (FITC) Apoptosis Detection Kit was supplied from BD Biosciences (San Diego, CA, USA). Cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase 3, Bak and Bax antibodies were supplied from Cell Signaling Technology, Inc. (Charlottesville, VA, USA). AIF, β-actin, α-tubulin and lamin B antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A pan caspase inhibitor was from R&D system (Minneapolis, MN, USA). Cycloheximide (CHX) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2. Cell culture and chemical treatment

MC3 human MEC cells were provided by Professor Wu Junzheng (Forth Military Medical University, Xi'an, China). MC-3 cells were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum and 100 U/mL each of penicillin and streptomycin antibiotics at 37°C in a 5% CO_2 incubator. Cells were treated with vehicle (dimethyl sulfoxide [DMSO]) or pycnogenol (5, 10, 20 and 40 μg/mL) for 24 hours.

3. Trypan blue exclusion assay

Trypan blue exclusion assay was used to determine cell proliferation by pycnogenol. MC-3 cells were treated with DMSO or pycnogenol for 24 hours. The cells were stained with 0.4% trypan blue dye and then counted using a hematocytometer. Each experiment was conducted in triplicate and the results were expressed as the mean ± SD for each treatment group.

4. 3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium assay

Cell viability was determined by CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega) in accord with the manufacturer's instructions for MTS assay. MC-3 cells were seeded in 96-well plates and incubated with DMSO or various dose of pycnogenol (5, 10, 20 and 40 μg/mL) for 24 hours. After the treatment, MTS solution was added to each well and the plates were incubated for 2 hours at 37°C. Then the cell viability was determined by measuring the absorbance at 482 nm (background) using Plate CHAMELEONTMV (HIDEX, Turku, Finland). The data were expressed as the percentage of cell viability compared to the vehicle control.

5. Annexin V-fluorescein 5 isothiocyanate and propidium iodide staining

The induction of apoptosis was determined using an Annexin V-FITC/propidium iodide (PI) double staining. Briefly, MC-3 cells treated with pycnogenol (5, 10, 20 and 40 μg/mL) or 0.1% DMSO were harvested by trypsinization and washed twice with PBS and transferred to a 5 mL polystyrene round-bottom tube. Then 5 μL Annexin V-FITC and 5 μL PI were added and incubated for 15 minutes at 37°C. The results were performed using a FACSCaliber (BD Biosciences).

6. DAPI staining

DAPI staining was executed to determine the morphology of cell nuclei following treatment with pycnogenol. Shortly, MC-3 cells treated with pycnogenol (5, 10, 20 and 40 μg/mL) or 0.1% DMSO were harvested by trypsinization and fixed in 100% ethanol overnight at −20°C. The cells were re-suspended in PBS, then deposited on slides, and stained with DAPI solution (2 μg/mL). Cell morphology was observed under a fluorescence microscope.

7. Western blotting

Whole cell lysates were extracted by lysis buffer and protein

concentration of these lysates was quantified using the DC Protein Assay (BioRad Laboratories, Hercules, CA, USA). Samples containing equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to ImmunoBlot polyvinylidene fluoride membranes (BioRad Laboratories). Membranes were blocked with 5% skimmed milk in trimethyl benzene sulfonyl tetrazole at room temperature (RT) for 2 hours and incubated overnight at 4°C with primary antibodies against cleaved PARP, cleaved caspase 3, Bak, Bax, AIF, β-actin, α-tubulin and lamin B, followed by incubation with horseradish peroxidaseconjugated secondary antibodies. After 2 hours, the membranes were washed and detected using the enhanced chemiluminescence Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc.).

8. Preparation of cytosolic and nuclear fractions

MC-3 cells were briefly washed with PBS, and cell pellets were resuspended in hypotonic buffer (10 mM N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.2 mM Na_3VO_4, 0.4 mM toluenesulfonyl fluoride, 1 mM dithiothreitiol, leupeptin, aprotinin and 1.5% Nonidet P-40) for 15 minutes on ice. After centrifugation at 13,000 rpm at 4°C for 5 minutes, the supernatant was used as cytoplasmic lysates for Western blotting. The pellets were resuspended in high salt extraction buffer (50 mM N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid, 50 mM KCl, 300 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol and 1 mM dithiothreitiol) for 30 minutes on ice subsequent to washed with hypotonic buffer. The supernatant containing nucleus proteins was isolated from the last centrifugation at 13,000 rpm for 30 minutes at 4°C.

9. Immunocytochemistry

Cells were seeded on 4-well culture plate and treated with DMSO or pycnogenol (20 μg/mL). After 24 hours, cells were fixed and permeabilized using the cytofix/cytoperm solution (BD Bioscience) for 1 hour at 4°C. Cells were then blocked with 1% bovine serum albumin in PBS for 1 hour at RT and incubated overnight at 4°C with Bak antibody. Subsequently, the cells were exposed to the FITC-conjugated secondary antibodies for 1 hour at RT and were visualized using a fluorescence microscope equipped with the appropriate filters for DAPI and FITC dyes.

10. Reverse transcriptase polymerase chain reaction

Total RNA was isolated using an easyBLUE Total RNA Extraction Kit (Intron, Daejeon, Korea). Subsequently, cDNA was synthesized from 1 μg total RNA using the Reverse Transcription System (Promega) and amplified using specific primers: Bak sense 5'-CTG CCC TCT GCT TCT GAG GA-3', Bak antisense 5'-CTG TCA GGA TGG GAC CAT TG-3', glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3'and GAPDH antisense 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'. The polymerase chain reaction (PCR) condition of Bak was as follows: 32 cycles of 1 minute at 94°C, 1 minute at 62°C, and 1 minute at 72°C, and the PCR condition of GAPDH was as follows: 28 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C. PCR products were separated by electrophoresis on a 2% agarose gel and made visible by ethidium bromide staining.

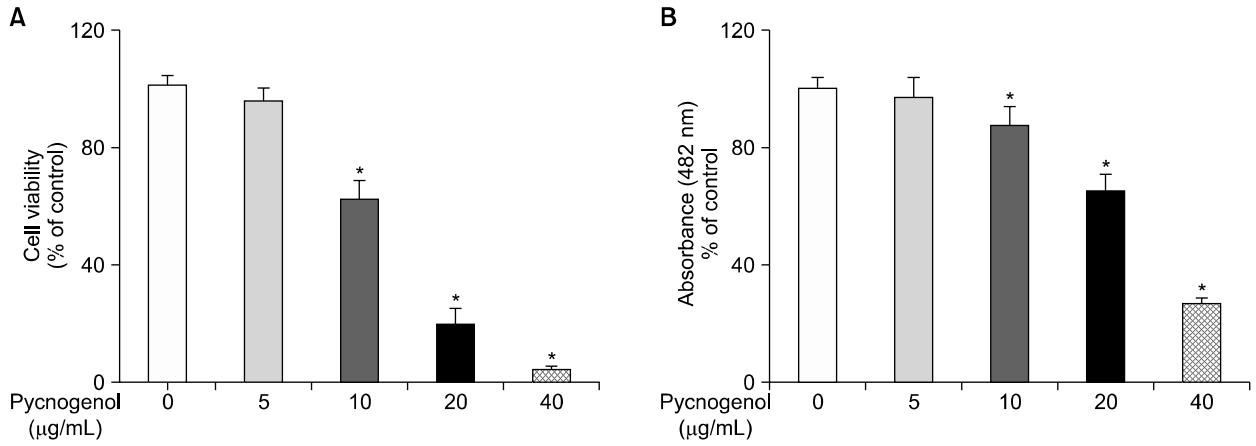

Figure 1. Effect of pycnogenol on the viability of MC-3 cells. MC-3 cells were treated with dimethyl sulfoxide (DMSO, vehicle control) or diverse doses of pycnogenol (5, 10, 20 and 40 μg/mL) for 24 hours. Cell viability was confirmed with trypan blue exclusion assay (A) or 3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (B). Bars represent the mean ± SD of triplicate experiments. $*P < 0.05$ significantly different compared with the control group.

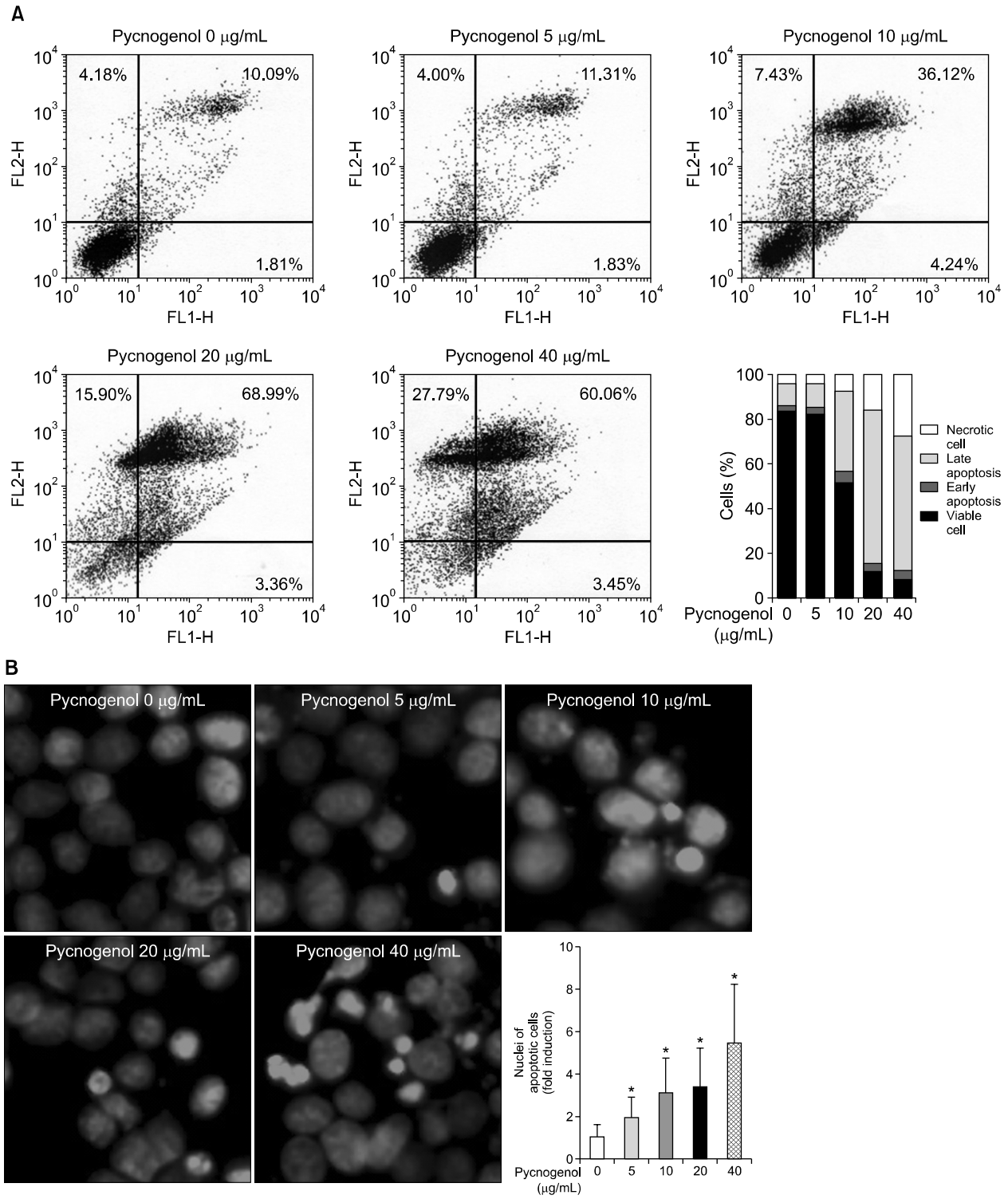

Figure 2. Apoptotic effect of pycnogenol on MC-3 cells. (A) Induction of apoptosis was determined by Annexin V-fluorescein 5 isothiocyanate (FITC)/propidium iodide (PI) double staining (low left: viable cells, low right: early apoptosis, upper left: necrosis, upper right: late apoptosis). (B) Chromatin condensation was detected by fluorescence microscopy (magnification, ×400). $*P < 0.05$ significantly different compared with the control group.

11. Statistical analysis

Student's *t*-test was used to determine the significance of differences between the control and treatment groups; Values of $P < 0.05$ were considered significant.

RESULTS

1. Pycnogenol inhibited cell viability and induced caspase-independent apoptosis in MC-3 human mucoepidermoid carcinoma cells

To clarify whether pycnogenol was an effective inhibitor of cell viability in MC-3 cells, trypan blue exclusion and MTS assays were initially performed. The viability of MC-3 cells was shown to be significantly decreased by pycnogenol in a dose-dependent manner (Fig. 1). In order to demonstrate whether pycnogenol inhibits cell viability through inducing apoptosis, we performed Annexin V and DAPI staining. The results showed that pycnogenol significantly increased Annexin V-positive cells and nuclear condensation and fragmentation in a dose-dependent manner (Fig. 2). To evaluate if caspase 3 is involved in pyconogenol-induced apoptosis, Western blot analysis using cleaved PARP and cleaved caspase 3 antibodies was carried out. Pycnogenol increased expression of cleaved PARP protein level, but expression of cleaved caspase 3 was rarely detected in MC-3 cells whereas 1 μM of Withaferin A as positive control clearly increased its expression level (Fig. 3A). To confirm the effect of pycnogenol on caspase-independent apoptosis, we used z-VAD, a pancaspase inhibitor. The results showed that z-VAD did not block pycnogenol-induced apoptosis (Fig. 3B). These results suggest that pycnogenol significantly decreased cell viability and induced caspase-independent apoptotic cell death.

2. Nuclear translocation of apotosis-inducing factor may play an important role in regulating pycnogenol-induced apoptosis

A previous study has reported that AIF leads to caspase-independent apoptosis by its translocation into nucleus from mitochondria.[5] Thus, cytosolic and nuclear fraction was prepared in MC-3 cells to determine the effect of pycnogenol on nuclear translocation of AIF. As shown in Figure 4A, expression of AIF in nucleus was significantly increased in a dose-dependent manner. Immunostaining also confirmed that AIF was clearly observed in nucleus of cells treated with pycnogenol whereas it was not observed in control group (Fig. 4B). These results indicate that translocation of AIF into nucleus may be related to the apoptotic effect of pycnogenol in MC-3 cells.

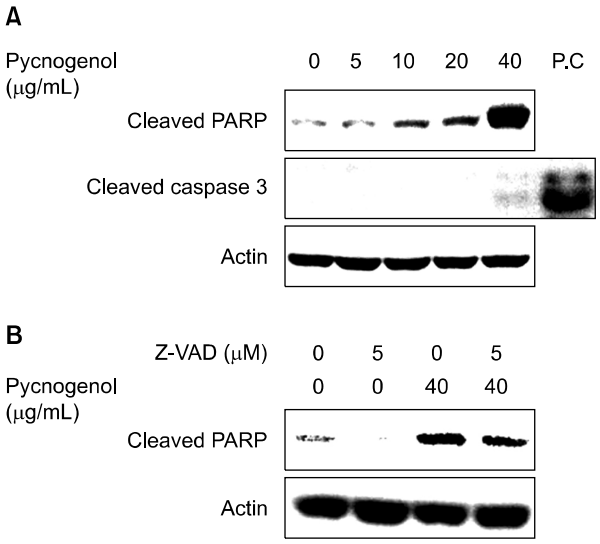

Figure 3. Caspase 3-independent apoptosis in pycnogenol-treated MC-3 cells. (A) Expression levels of cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase 3 were investigated by Western blotting and actin was used as loading control. 1 μM Withaferin A-treated human oral squamous carcinoma (HSC-3) cells were used as a positive control (P.C). (B) benzyloxycarbonylvalyl-alanyl-aspartyl fluoromethyl ketone (Z-VAD), pan caspase inhibitor) was used to estimate the connection of caspase 3 in pycnogenol-induced apoptosis.

3. Expression of Bak protein was enhanced by pycnogenol though post-translational modification

Next, we investigated expression of Bax and Bak because earlier studies have shown that Bax activation or Bak conformational changes lead to the formation of a mitochondrial pore that facilitates the release of mitochondrial pro-apoptotic proteins including AIF.[9,10] Thus, we investigated whether pycnogenol affects Bak or Bax protein and the results showed[7] that only Bak protein was increased (Fig. 5A). Then we investigated Bak mRNA levels and the results showed that Bak mRNA levels were not augmented by pycnogenol (Fig. 5B). Because Bak was not transcriptionally regulated based on Figures 5A and 5B, we evaluated the stability of Bak protein using a protein synthesis inhibitor, CHX. The results demonstrated that co-treatment of pycnogenol and CHX slightly enhanced Bak protein levels even after new protein synthesis was totally blocked by CHX in MC-3 cells suggesting that pycnogenol partly enhanced expression of Bak by modulating protein stability (Fig. 5C).

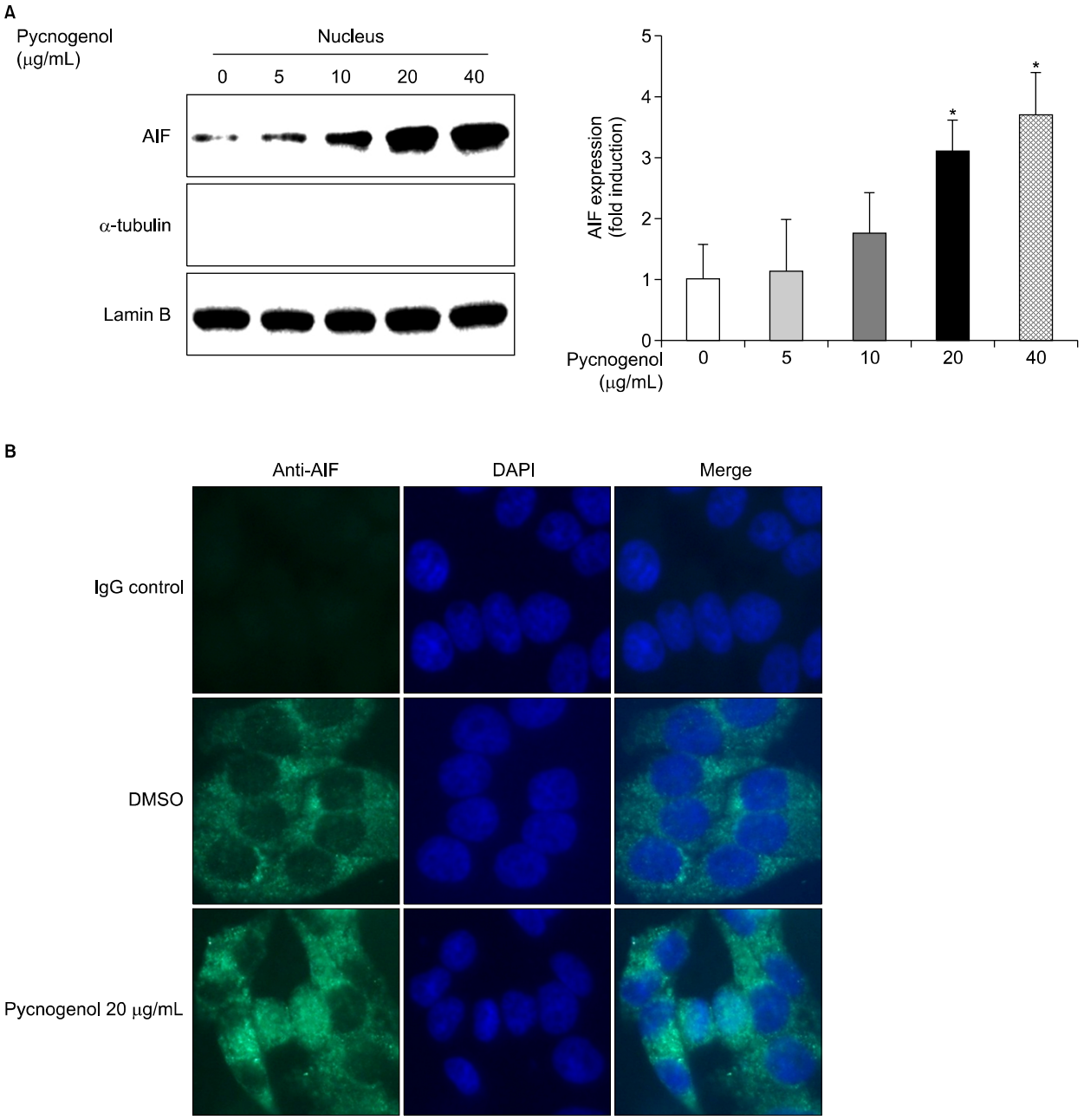

Figure 4. Effect of pycnogenol on nuclear translocation of apoptosis-inducing factor (AIF). (A) AIF protein expression from the nuclear protein extracts was detected using Western blotting. Lamin B and α-tubulin were used to normalize the nuclear and cytosolic protein level, respectively; bars represent the mean ± SD of triplicate experiments. $*P < 0.05$ significantly different compared with the control group. (B) MC-3 cells were treated with dimethyl sulfoxide (DMSO) or 20 μg/mL pycnogenol for 24 hours and immunostained with immunoglobulin G (IgG) or AIF antibodies. DAPI, 4',6-diamidino-2-phenylindole.

DISCUSSION

Our previous studies have reported that natural compounds exhibited inhibition of cell growth and apoptotic effects in various cancer cells.[11-13] Similarly, pycnogenol also has induced cell differentiation and apoptosis in human mammary cancer cells and promyeloid leukemia cells.[14,15] However, anticancer effect of pycnogenol in human MEC cell line has not been explored. In the present study, we investigated the effect of pycnogenol on apoptosis and the results showed that pycnogenol

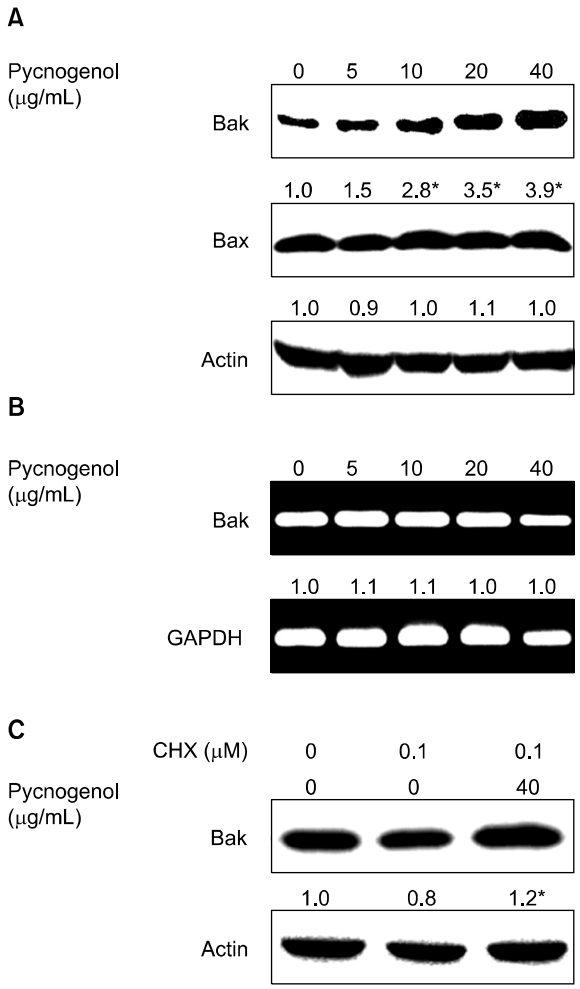

Figure 5. Effect of pycnogenol on the expression levels of Bak or Bax protein. (A) The expression levels of Bak and Bax proteins were detected by Western blotting, and actin was used as loading control. *$P < 0.05$ significantly different compared with the control group. (B) Expression level of Bak mRNA was evaluated using reverse transcriptase polymerase chain reaction. mRNA level was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C) The stability of Bak protein was investigated by Western blotting in MC-3 cells treated with 0.1 μg/mL cycloheximide (CHX) with or without 40 μg/mL pycnogenol for 24 hours. *$P < 0.05$ significantly different compared with the control group.

inhibited cell growth and induced apoptosis in MC-3 cells. Previously, Huang et al.[15] demonstrated that the activation of caspase 3 mainly mediated pycnogenol-induced apoptosis in HL-60 cells indicating that caspase activity can play an important role in its apoptotic activity. Thus, we investigated whether pycnogenol affects the expression level of caspase 3. However, there is no change of caspase 3 activity by pycnogenol and its inhibitor cannot block its apoptotic activity suggesting that that pycnogenol may be a potent caspase-independent apoptotic inducer in MC-3 cells.

A few anticancer drugs can induce apoptosis in tumor cells without caspases. It was also demonstrated that mitochondrial membrane potential and pro-apoptotic factors including caspase-independent factors are involved in programmed cell death.[16] AIF is a unique known element of the apoptotic machinery and seems to play a role in caspase-independent apoptosis as a caspase-independent factor. Previously, it was shown that AIF is released into cytosol as well as nucleus from the mitochondria during apoptosis.[17] Thus, it was undertaken to explore this possibility in the present study. The results showed that the expression of AIF into nucleus was increased by pycnogenol in a dose-dependent manner and it was confirmed by immune-staining. This results indicated that translocation of AIF into nucleus by pycnogenol may induce apoptosis.

Bak and Bax, members of the "multi domain" subset of Bcl-2 family proteins, regulate the intrinsic pathway of apoptosis through essential gateway to mitochondrial dysfunction required for cell death.[16] Besides, after the mitochondrial outer membrane permeabilization via activation of Bak and Bax, apoptotic proteins containing cytochrome *c*, AIF, endonuclease G and Smac were released from the mitochondrial intermembrane space into the cytosol.[18] Recently, Bleicken et al.[16] demonstrated that Bax and Bak formed stable protein-permeable pores of mitochondrial outer membrane during apoptosis to be responsible for the release of AIF. Thus, we investigated the involvement of Bax or Bak on nuclear translocation of AIF in MC-3 cells and the results showed that only Bak was clearly augmented by pycnogenol suggesting that the increase in Bak protein by pycnogenol may be associated with nuclear translocation of AIF. We also test how pycnogenol regulates Bak protein using CHX and the results showed that it slightly augmented the expression level of Bak protein in spite of the blockage of Bak protein synthesis suggesting that it partly modulates Bak protein through its enhancing activity of protein stability. There are several studies have reported that Bak protein can be also regulated by de novo protein synthesis.[19,20] Thus, it implied that pycnogenol may mostly affect the synthesis of Bak protein rather than its protein stability. In the previous study, it was reported that Mitochondria is the major sites for reactive oxygen species (ROS) production and excessive generation of ROS and mitochondrial membrane dysfunction are related to apoptosis.[3] Thus, further study would be necessary to evaluate the relevance between ROS and Bak.

In summary, we demonstrated that pycnogenol results in MC-3 cell death through the induction of caspase-independent apoptosis. Its related molecular mechanism may be associated

with the nuclear translocation of AIF and up-regulation of Bak protein through the increase in protein stability. Therefore, our present results conclude that pycnogenol may be a potential anti-cancer drug candidate for the treatment of human MEC.

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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