Induction of apoptosis in human lung carcinoma cells by the water extract of *Panax notoginseng* is associated with the activation of caspase-3 through downregulation of Akt

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Abstract. The root of *Panax notoginseng* is highly valued and commonly used in Oriental medicine. Although recent experimental data have revealed the proapoptotic potency of *P. notoginseng* extracts, the underlying molecular mechanisms of this apoptotic activity have not yet been studied in detail. In the present study, the effects of the water extract of *P. notoginseng* (WEPN) on the growth of human lung carcinoma cells were investigated. It was found that the exposure of A549 and NIC-H460 cells to WEPN resulted in growth inhibition and the induction of apoptosis in a dose-dependent manner. The WEPN treatment induced the upregulation of pro-apoptotic Bax, downregulation of anti-apoptotic Bcl-2 expression and loss of mitochondrial membrane potential (MMP), which was associated with the proteolytic activation of caspases and the concomitant degradation of poly(ADP ribose) polymerase (PARP) protein. However, the caspase-3-specific inhibitor z-DEVD-fmk blocked PARP degradation and increased the survival rate of WEPN-treated cells. Moreover, the activity of Akt was downregulated in WEPN-treated cells and the phosphatidyl-inositol-3 kinase (PI3K)/Akt inhibitor LY294002 sensitized the cells to WEPN-induced apoptosis through enhancing the activation of caspase-3 and loss of MMP. The results indicated that the major regulators of WEPN-induced apoptosis in human lung carcinoma cells are the Bcl-2 family and caspase-3, which are associated with mitochondrial dysfunction and dephosphorylation of the Akt signaling pathway.

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Introduction

Ginseng is a medicinal herb consumed worldwide. Over thirteen different species of ginseng have been identified (1,2). Within the *Panax* genus, *P. notoginseng* Burk. F.H. Chen (Araliaceae) (*Sanqi* or *Tianqi* in Chinese) is a highly valuable and important herb in Oriental traditional medicine for its therapeutic abilities to stop hemorrhaging and to influence blood circulation; it is also the most common drug used to treat chronic liver disease in Korea (2-7). The main root of *P. notoginseng*, called notoginseng, is also known to have many pharmacological activities such as anti-inflammatory and anti-tumor effects and it is also known to alter the functional balance of the immune system (8,9). Recently, many studies have indicated that *P. notoginseng* extracts inhibit the growth and metastasis of various cancer cells *in vitro* and *in vivo* that are associated with cell cycle arrest and the stimulation of apoptotic cell death (10-17). However, the precise mechanisms of cell death are largely unknown in human cancer cells.

The regulation of apoptosis, which is a programmed cell death, has become an area of extensive study in cancer research and has been considered an ideal way of eliminating precancerous and/or cancerous cells (18,19). Apoptosis is characterized by a series of distinct morphological and biochemical alterations to cells such as DNA fragmentation, chromatin condensation, cell shrinkage and plasma membrane blebbing. However, most cancer cells can block apoptosis, which allows them to survive despite the genetic and morphological transformations. Therefore, the induction of

apoptotic cell death is an important mechanism in many anti-cancer drugs (18,20,21). Accumulating data have shown that many chemopreventive and/or chemotherapeutic agents that induce apoptosis target the mitochondria and promote the activation of caspases, which are important proteolytic enzymes responsible for the execution of apoptosis. The caspase-dependent initiation of apoptosis is controlled by a number of regulatory proteins, including members of the Bcl-2 family (22-24). Previous evidence has also shown that phosphatidylinositol-3 kinase (PI3K)/Akt is modulated in response to a variety of stimuli in particular the activation of Akt signaling promotes survival (25,26).

The present study aimed at elucidating the effect of the water extract of *P. notoginseng* (WEPN) in human lung carcinoma cells and the underlying intracellular signal transduction pathways involved in regulating apoptosis. The results of this study demonstrate that WEPN induces caspase-3-dependent apoptosis in human lung carcinoma cells through mitochondrial dysfunction and the Akt signaling pathway.

Materials and methods

Plant material. P. notoginseng was supplied by Daejeon University Oriental Hospital, Daejeon, Korea. WEPN was prepared in the following manner. Distilled water at 90˚C was added to dry root (5 ml/g) and the temperature was maintained for 10 h. The mixture was allowed to cool to room temperature and was then filtered and lyophilized. The yield of the lyophilized residue corresponded to 4.5% of the original dry root weight and the extract powder was dissolved directly in distilled water.

Cell culture and cell viability study. The human lung carcinoma cell lines A549 and NCI-H460 were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 37˚C incubator with 5% CO_2. Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. For the morphological study, the cells were treated with WEPN for 48 h and photographed directly using an inverted microscope (Carl Zeiss, Germany).

Nuclear staining with DAPI. After treating the cells with WEPN for 48 h, the cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The cells were washed twice more with PBS and analyzed via a fluorescence microscope (Carl Zeiss).

Protein extraction and Western blot analysis. Cell lysates were lysed in extraction buffer, as previously described (27). The protein concentrations were measured using a Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA, USA) according to the manufacturer's instructions. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gels and transferred by electroblotting to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody and visualized by enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp., Arlington Heights, IL, USA). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp.

Measurement of the loss of mitochondrial membrane potential (MMP, $\Delta\Psi_m$). To measure the MMP, the dual-emission potential-sensitive probe 5,5 V, 6,6 V-tetrachloro-1,1 V,3,3 V-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma), was used. After treatment with various concentrations of WEPN for 48 h, the cells were scraped from the bottom of the wells and aliquots of $5x10^5$ cells were placed into FACS tubes. Cells were then stained with 2 mg/l JC-1 at 37˚C for 20 min and analyzed using flow cytometry (Becton-Dickinson, Heidelberg, Germany).

In vitro caspase activity assay. The caspase activity was determined by a colorimetric assay using caspase-3, -8 and -9 activation kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Briefly, the cells were lysed in a lysis buffer for 30 min in an ice bath. The supernatants were collected and incubated at 37˚C with the supplied reaction buffer, which contained dithiothreitol and the substrates Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) for caspase-3, Ile-Glu-Thr-Asp (IETD)-pNA for caspase-8 and Leu-Glu-His-Asp (LEHD)-pNA for caspase-9. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

Statistical analysis. The data are expressed as mean ± SD. A statistical comparison was performed using one-way ANOVA followed by a Fisher test. Significant differences between the groups were determined using an unpaired Student's t-test. A P-value <0.05 was considered significant.

Results

Inhibition of cell viability and induction of apoptosis by WEPN. In order to determine if WEPN decreases lung cancer cell viability, the A549 and NCI-H460 cells were stimulated with various concentrations of WEPN for 48 h and the cell viability was measured using the MTT assay. As shown in Fig. 1, the WEPN treatment significantly inhibited the cell viability of both cell lines in a concentration-dependent manner (after 48 h treatment at 3.2 mg/ml, WEPN decreased the NCI-H460 and A549 cell viability by ~74 and 89%, respectively, compared with the controls). Direct observation using an inverted microscope demonstrated that the cells treated with WEPN showed many morphological changes compared with the control cells (Fig. 2A). In particular, cell

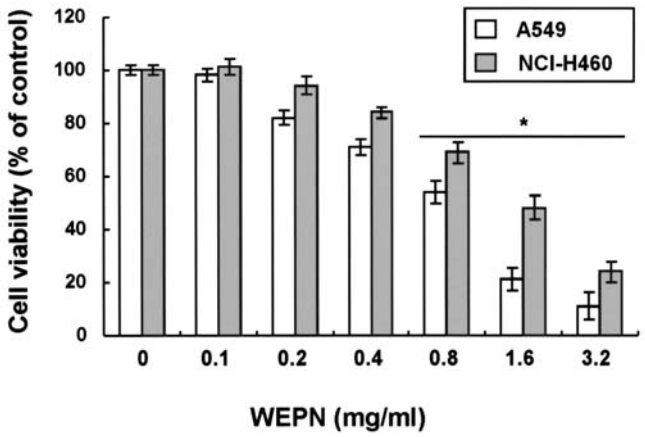

Figure 1. Inhibition of cell viability by the water extract of *P. notoginseng* (WEPN) in A549 and NCI-H460 human lung cancer cells. The cells were seeded at an initial density of $2.5x10^5$ cells per 60-mm plate, incubated for 24 h and treated with various concentrations of WEPN for 48 h. The cell viability was measured using an MTT assay. Each point represents the mean ± SD of three independent experiments. Significance was determined using a Student's t-test (*P<0.05 vs. untreated control).

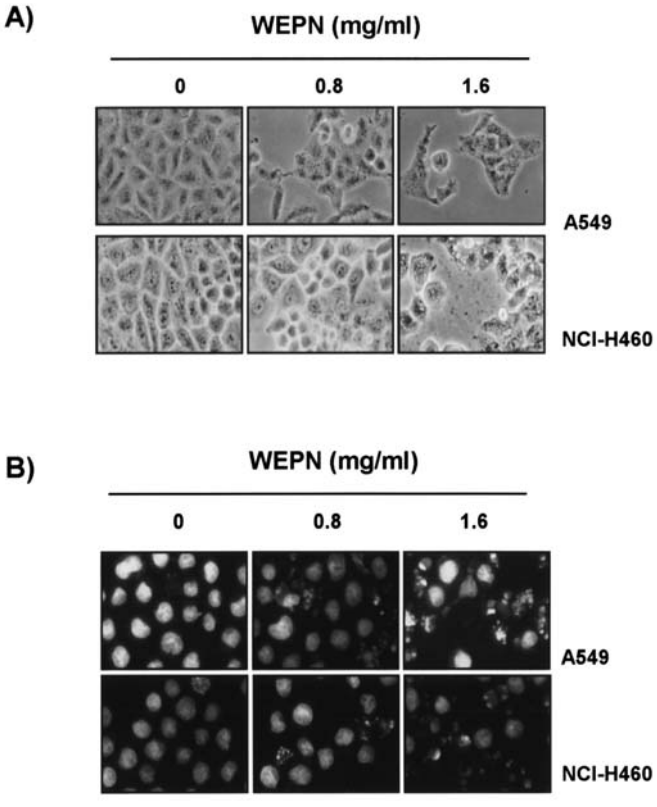

Figure 2. Morphological changes of A549 and NCI-H460 human lung cancer cells due to WEPN treatment. (A) The cells were treated with various concentrations of WEPN for 48 h. The cells were sampled and examined using an inverted microscope (magnification, x200). (B) The cells grown under the same conditions as (A) were sampled, fixed and stained with DAPI. The stained nuclei were then observed under a fluorescent microscope using a blue filter (magnification, x400).

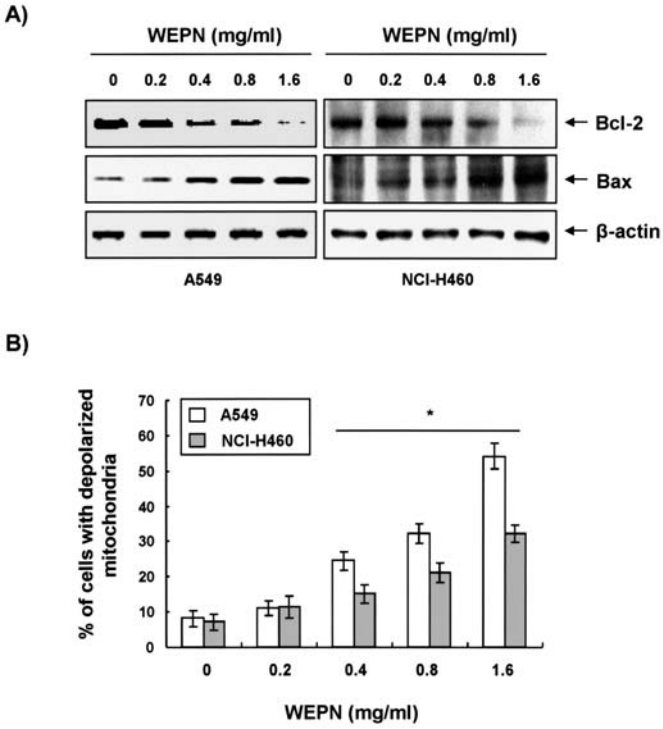

Figure 3. Modulation of anti-apoptotic Bcl-2 and pro-apoptotic Bax protein expression and loss of MMP by WEPN in A549 and NCI-H460 human lung cancer cells. (A) The cells were treated with the indicated concentrations of WEPN for 48 h. Equal amounts of the cell lysates (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose and probed with the anti-Bcl-2 and anti-Bax antibodies. Proteins were visualized using the ECL detection system. Actin was used as the internal control. (B) The cells were treated with the indicated concentrations of WEPN for 48 h, stained with JC-1 and incubated at 37˚C for 20 min. The mean JC-1 fluorescence intensity was detected using a flow cytometer. The results are expressed as the mean ± SD of three independent experiments. The significance was determined by using the Student's t-test (*P<0.05 vs. untreated control).

shrinkage, cytoplasm condensation and formation of cytoplasmic filaments appeared after 0.8 mg/ml WEPN treatment for 48 h. Further experiments using fluorescence microscope analyses were carried out to determine if the anti-proliferative effect of WEPN is the result of apoptotic cell death. Morphological analysis with DAPI staining showed nuclei with chromatin condensation and the formation of apoptotic bodies in the cells cultured with WEPN in a concentration-dependent manner, in contrast, very few were observed in the control culture (Fig. 2B). These results indicate that the cytotoxic effects observed in response to WEPN are associated with the induction of apoptosis.

Modulation of the expression of Bcl-2 family proteins and loss of MMP by WEPN. To elucidate the mechanisms underlying WEPN-induced apoptosis, the levels of Bcl-2 family proteins were examined using Western blotting. When A549 and NCI-H460 cells were treated with WEPN, a clear decrease in anti-apoptotic Bcl-2 protein expression was observed in a concentration-dependent manner (Fig. 3A). In the case of the pro-apoptotic protein Bax, there was a concentration-dependent upregulation observed in both A549 and NCI-H460 cells treated with WEPN. Next, the MMP was measured in WEPN-treated cells using a fluorescent cationic dye, JC-1, in order to determine whether WEPN-induced apoptosis is associated with the loss of MMP. As shown in Fig. 3B, the exposure of cells to various concentrations of WEPN led to a significant reduction in the level of MMP, indicating that WEPN-induced apoptosis is associated with mitochondrial dysfunction.

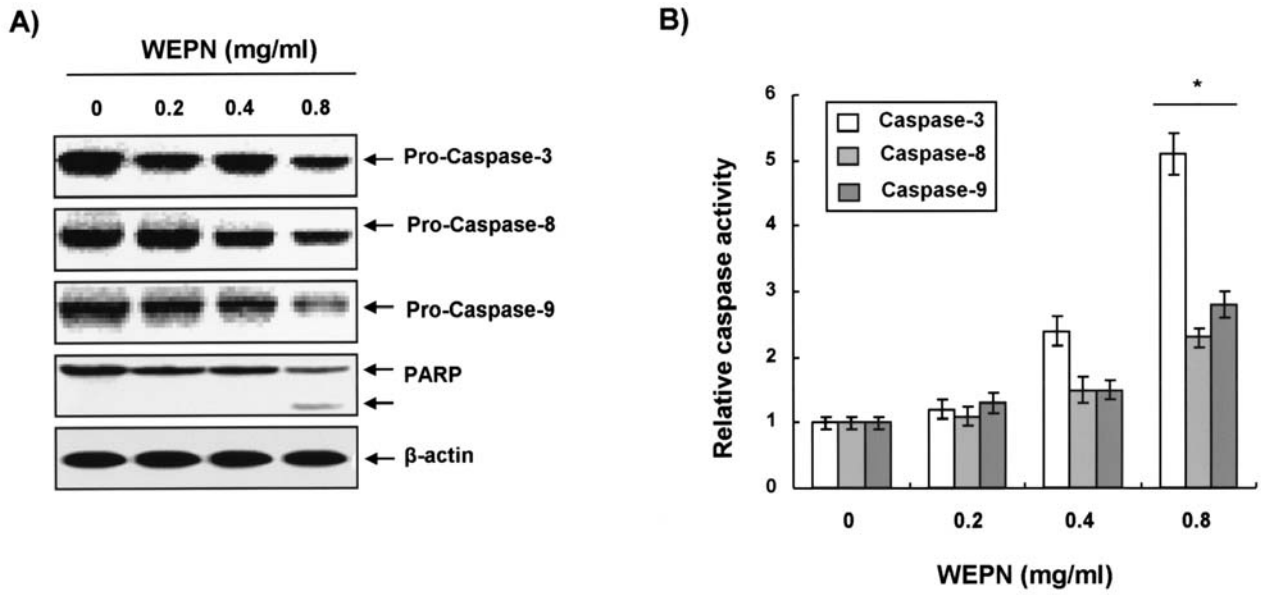

Figure 4. Activation of caspases and degradation of PARP protein due to the WEPN treatment in A549 cells. (A) After 48 h incubation with WEPN, the cells were lysed. The cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-caspase-3, anti-caspase-8, anti-caspase-9 and anti-PARP antibodies. The proteins were visualized using an ECL detection system. Actin was used as the internal control. (B) The cell lysates from the cells treated with WEPN for 48 h were assayed for the *in vitro* caspase-3, -8 and -9 activity using DEVD-pNA, IETD-pNA and LEHD-pNA, respectively, as substrates. The concentrations of the fluorescent products released were measured. The results are expressed as the mean ± SD of three independent experiments. The significance was determined using the Student's t-test (*P<0.05 vs. untreated control).

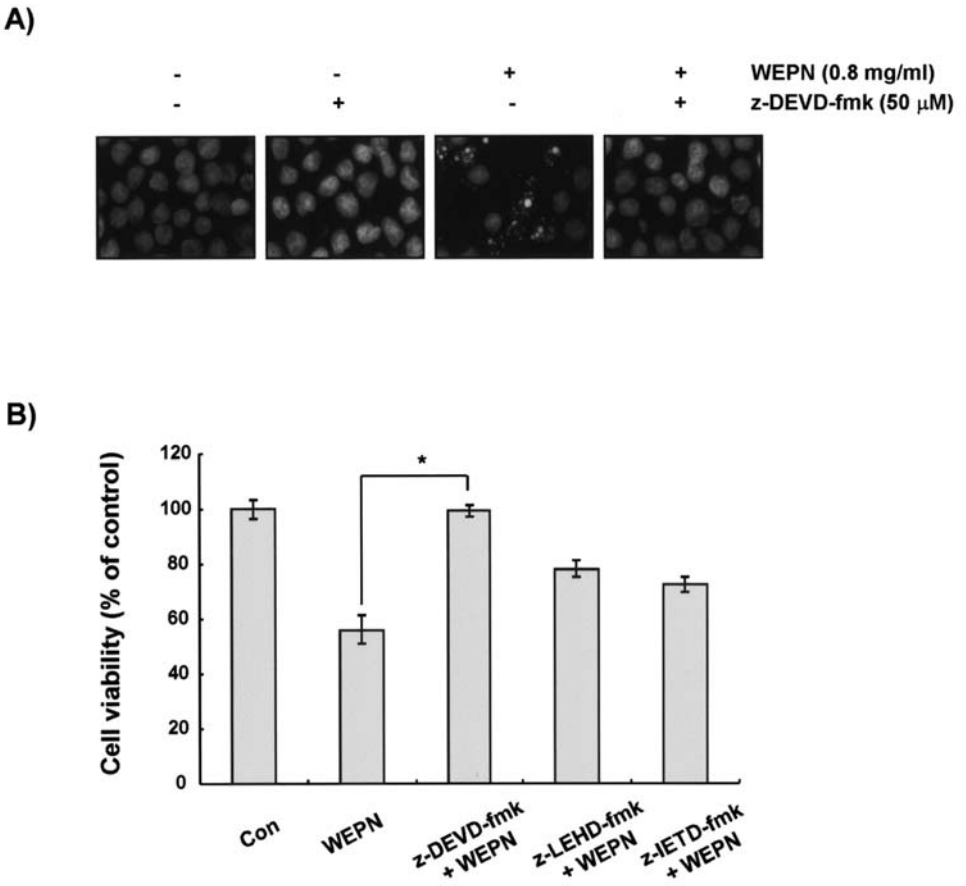

Figure 5. Inhibition of WEPN-induced chromatin condensation and growth inhibition by the caspase-3 inhibitor in A549 cells. (A) The cells were treated with z-DEVD-fmk (50 μM) for 2 h before being challenged with 0.8 mg/ml WEPN for 48 h. The cells were sampled and stained with DAPI for 10 min and photographed with a fluorescence microscope using a blue filter (magnification, x400). (B) The cells were grown under the same conditions as (A) and the cell viability was measured using an MTT assay. Each point represents the mean ± SD of three independent experiments. The significance was determined using the Student's t-test (*P<0.05 vs. untreated control).

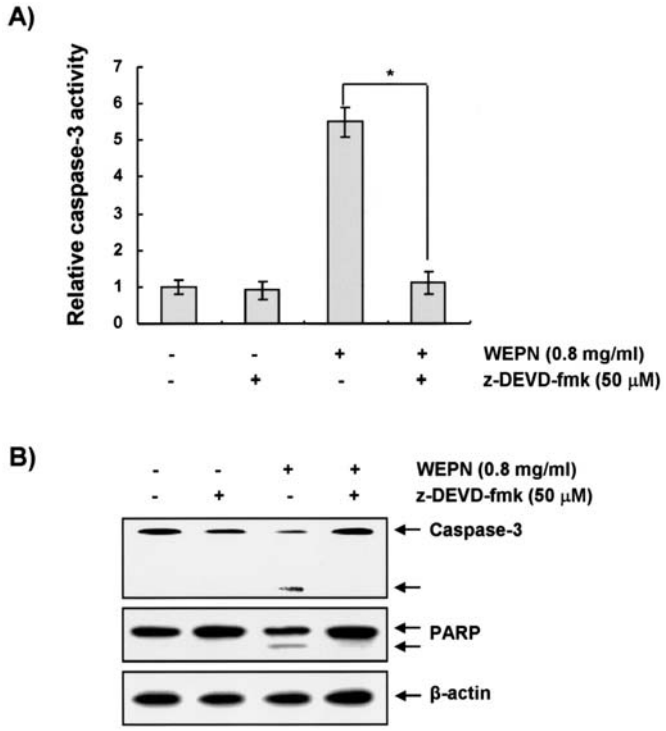

Figure 6. Inhibition of caspase-3 activation and PARP degradation by z-DEVD-fmk, a caspase-3 inhibitor, in WEPN-treated A549 cells. (A) The cell lysates from the cells treated with z-DEVD-fmk (50 μM) for 2 h before being challenged with 0.8 mg/ml WEPN for 48 h were assayed for the *in vitro* caspase-3 activity using DEVD-pNA. The concentrations of the fluorescent products released were measured. The results are expressed as the mean ± SD of three independent experiments. The significance was determined using the Student's t-test ($^*P<0.05$ vs. untreated control). (B) The cells grown under the same conditions as (A) and equal amounts of the cell lysates (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with the anti-caspase-3 and anti-PARP antibodies. The proteins were visualized using the ECL detection system. Actin was used as an internal control.

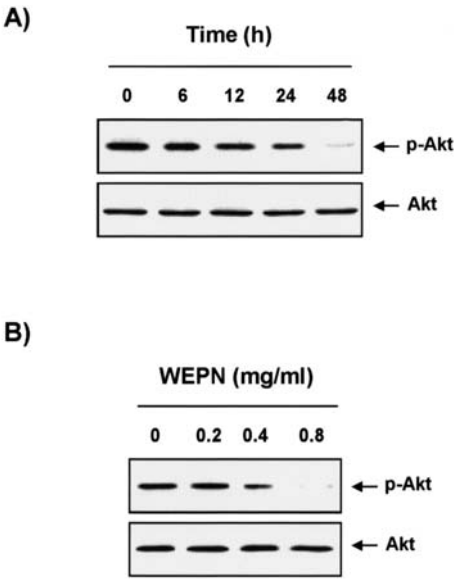

Figure 7. Inhibition of phosphorylation of Akt by WEPN treatment in A549 cells. The cells were treated with 0.8 mg/ml WEPN for the indicated times (A) or with the indicated concentrations of WEPN for 48 h (B). Equal amounts of the cell lysates (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose and probed with the anti-Akt and anti-p-Akt antibodies. Proteins were visualized using the ECL detection system.

Activation of caspases and degradation of PARP by WEPN. The expression levels and activities of caspase-3, -8 and -9 in cells that had been exposed to WEPN were measured in order to determine if WEPN-induced apoptosis is associated with the activation of caspases. As seen in Fig. 4A, Western blot data showed that the levels of pro-caspase-3, -8 and -9 proteins were decreased in a concentration-dependent manner in WEPN-treated A549 cells. In order to further quantify the proteolytic activation of caspases in A549 cells, the lysates equalized for protein from the cells treated with WEPN were assayed for their activities using colorimetric assay kits and it was found that WEPN caused a dose-dependent increase in the proteolytic activities of caspases, especially caspase-3 (Fig. 4B). Subsequent Western blot analyses showed the progressive proteolytic cleavage of poly (ADP ribose) polymerase (PARP) in A549 cells after WEPN treatment (Fig. 4A), suggesting that the activation of caspase is involved in the WEPN-induced apoptotic pathway.

Inhibition of WEPN-induced apoptosis by a caspase-3 inhibitor. In order to show that the activation of caspases is a key step in the WEPN-induced apoptotic pathway, cells were pretreated with z-DEVD-fmk, z-LEHD-fmk and z-IETD-fmk for 2 h, followed by treatment with 0.8 mg/ml WEPN for 48 h. The blocking of the caspase-3 activity by pretreatment of the cells with z-DEVD-fmk prevented the WEPN-induced chromatin condensation and a decrease of cell viability (Fig. 5). Furthermore, z-DEVD-fmk significantly blocked not only caspase-3 activation, but also PARP degradation (Fig. 6). However, the cytotoxic effects of WEPN treatment were not markedly blocked by pretreatment with z-LEHD-fmk and z-IETD-fmk, caspase-8 and -9 inhibitor, respectively. These results clearly show that WEPN-induced apoptosis is associated with the activation of caspase-3.

PI3K/Akt inhibitor sensitizes WEPN-induced apoptosis. The phosphorylation state of the Akt protein in cells after WEPN treatment was examined to determine if WEPN-induced apoptosis is closely related to the Akt signal, which is a downstream effector of PI3K for survival signaling (25,26). As shown in Fig. 7, the levels of the total Akt protein remained unchanged by WEPN treatment; however, its phosphorylation levels were markedly decreased in a time- and dose-dependent manner. Thus, the involvement of Akt signal pathways in WEPN-induced apoptosis was examined using the PI3K/Akt inhibitor LY294002 to determine if the inhibition of Akt phosphorylation was responsible for the induction of apoptosis. As shown in Fig. 8, the combined treatment with WEPN and LY294002 significantly increased the loss of MMP and activation of caspase-3 and decreased the cell viability resulting from the WEPN treatment. This indicates that WEPN-induced apoptosis is associated with the downregulation of the PI3K/Akt signaling pathway.

Discussion

Recent studies have reported that the extracts of *P. notoginseng* treatment can cause the accumulation of cells in the G1 or S phase of the cell cycle and apoptosis (10,12,13), which suggests that the growth inhibitory effect of the extracts occur

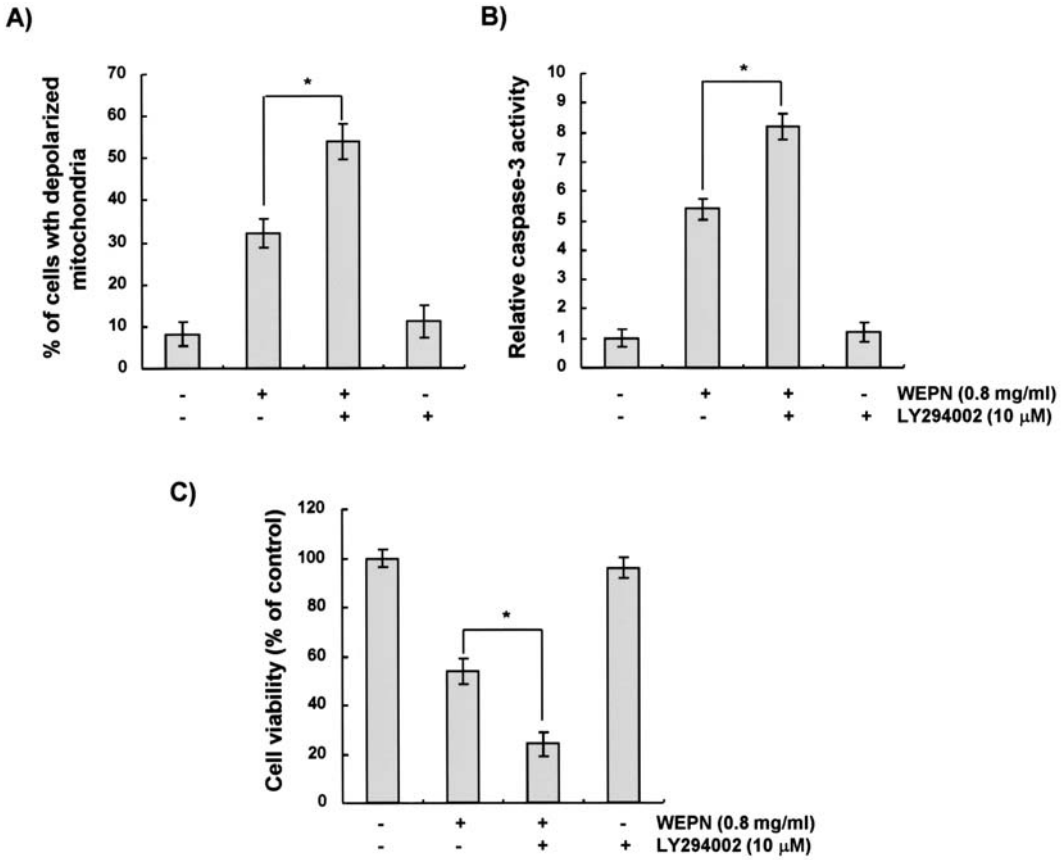

Figure 8. Increase in WEPN-induced cytotoxic effects by the inhibition of the PI3K/Akt signal pathway in A549 cells. (A) The A549 cells were treated with LY294002 (10 μM) for 2 h before being challenged with 0.8 mg/ml WEPN for 48 h. The cells were collected, stained with JC-1 and incubated at 37˚C for 20 min. The mean JC-1 fluorescence intensity for MMP was detected using a flow cytometer (B and C). The cells grown under the same conditions as (A) were collected and the caspase-3 activity (B) and the cell viability (C) were measured. Each point represents the mean ± SD of three independent experiments. The significance was determined using the Student's t-test ($^*P<0.05$ vs. untreated control).

through the blockage of the G1 or S phase and that these cells do not enter the G2 phase. While some cell killing mechanisms of these extracts have been suggested (10,13-15,28), little is known about their effects on the growth of human lung cancer cells. Therefore, this study examined whether WEPN induces apoptosis in human lung carcinoma A549 and NCI-H460 cells and the mechanisms related to cell death. In the present study, the WEPN treatment was shown to induce mitochondrial damage and apoptosis in lung carcinoma cells through modulation of the Bcl-2 family proteins and activation of caspase-3. Furthermore, a caspase-3 inhibitor significantly attenuated WEPN-induced apoptosis and the inhibition of the PI3K/Akt pathway enhanced the WEPN-induced cytotoxic and apoptotic effects.

It has previously been proposed that mitochondria are possible targets for anti-cancer drug-induced apoptosis in the death receptor (extrinsic) pathway as well as the mitochondrial (intrinsic) pathway (20,21). Since the discovery of Bcl-2, several mechanisms for the anti-apoptotic properties of this protein have been proposed (22-24). The anti-apoptotic function of Bcl-2 against pro-apoptotic Bax may be explained by its ability to control several key steps involved in death signaling. The Bcl-2 family significantly regulates apoptosis either as an activator (Bax) or as an inhibitor (Bcl-2); therefore, it has been suggested that the Bax/Bcl-2 ratio is a key factor in the regulation of the apoptotic process (22,24). The results of this study indicate that WEPN treatment results in significant inhibition of cell viability and the induction of apoptosis in A549 and NCI-H460 cells. The data also showed that WEPN-induced apoptosis was related to augmented levels of the Bax protein and downregulation of Bcl-2, as well as to the loss of MMP in WEPN-treated cells (Fig. 3), which indicates that WEPN increased the Bax/Bcl-2 ratio and induced mitochondrial dysfunction, leading to apoptosis.

The caspase family of aspartate-specific cystein proteases also plays a critical role in regulating apoptosis. Caspase signaling is initiated and propagated by proteolytic auto-catalysis and the cleavage of downstream caspases and substrate proteins (22,24). Caspase-3 is the most important executioner of apoptosis. Significant evidence indicates that caspase-3 is either partially or totally responsible for the proteolytic cleavage of many key proteins including PARP. PARP is important for cell viability, and the cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (29). In this study, it was demonstrated that WEPN induces apoptosis through the activation of caspase-3 and concomitant degradation of PARP (Fig. 4). This is confirmed by the finding that treatment with WEPN in the presence of z-DEVD-fmk, a specific caspase-3 inhibitor, inhibited the morphological changes and degradation of PARP (Figs. 5 and 6). However, neither caspase-8 (z-LEHD-fmk) nor -9 (z-IETD-fmk) inhibitors markedly blocked the inhibition

of cell viability by WEPN. These data suggest that WEPN-induced apoptosis is caused by caspase-3-dependent cell death.

Although the PI3K/Akt signal pathways play a critical role in regulating cell survival and death in many physiological and pathological settings, numerous studies have indicated that these pathways are more often associated with cell survival through activation of anti-apoptotic downstream effectors (25,26). Therefore, whether WEPN-induced apoptosis is associated with these pathways was also investigated. WEPN caused the downregulation of Akt activation (Fig. 7) and the combined exposure with LY294002, a PI3K/Akt inhibitor, made the cells more sensitive to WEPN-induced apoptosis by promoting the caspase-3 activation and mitochondrial dysfunction (Fig. 8). These results indicate that the PI3K/Akt signaling may have a survival role in response to WEPN-induced apoptosis.

In summary, these studies demonstrated that WEPN exposure induced apoptosis in human lung carcinoma cells. The apoptotic response was associated with the increase of Bax, decrease of Bcl-2, mitochondrial dysfunction, caspase-3 activation and PARP degradation. Moreover, the inactivation of Akt may play an important role in WEPN-induced apoptosis. These results provide new information on the possible mechanisms for the anti-cancer activity of WEPN. However, it is still unclear if WEPN can induce apoptosis through other pathways, such as the death receptor pathway or the endoplasmic reticulum pathway. Therefore, more research is necessary to examine the mechanisms for the phosphorylation and activation of multiple apoptosis-related proteins in WEPN-induced apoptosis.

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