Apoptotic potential of sesquiterpene lactone ergolide through the inhibition of NF-κB signaling pathway

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Abstract

Treatment with ergolide, a sesquiterpene lactone from *Inula britannica* var *chinensis*, caused the induction of apoptosis in Jurkat T cells, which was confirmed by DNA fragmentation, caspase-3 activation and cleavage of poly(ADP-ribose) polymerase in response to ergolide. Furthermore, mitochondrial dysfunction appeared to be associated with ergolide-induced apoptosis, because Bax translocation and cytochrome *c* release were stimulated by ergolide. In parallel, the nuclear factor-κB (NF-κB) signaling pathway was significantly inhibited by ergolide, which was accompanied by down-regulation of cell survival molecules, such as X-chromosome-linked inhibitor of apoptosis and Bcl-2. In addition, the JNK signaling pathway was involved in ergolide-induced apoptosis. Collectively, our results identified a new mechanism for the anti-cancer property of ergolide, attributable to the induction of apoptosis through down-regulation of cell survival signal molecules resulting from inhibition of the NF-κB signaling pathway.

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Introduction

Apoptosis is closely involved in many physiological and pathological processes, such as development, maintenance of homoeostasis and eliminating damaged cells (Hengartner 2000). Many chemotherapeutic drugs induce death of their target cells by activating apoptotic pathways (Kaufmann & Earnshaw 2000). There are two major highly regulated apoptotic pathways — the death receptor pathway that begins with cell death receptors, such as TNFR1, Fas, DR-3, DR-4 and DR-5, located on cell membranes, and the mitochondrial pathway, which involves mitochondrial dysfunction, usually by pro-apoptotic Bcl-2 protein, dissipation of transmembrane potential and the release of mitochondrial proteins, including cytochrome *c* (Kaufmann & Hengartner 2001). Cytosolic cytochrome *c* binds to Apaf-1, induces autoprocessing of caspase-9 and subsequently activates caspase-3 (Li et al 1997; Cecconi 1999).

Nuclear factor-κB (NF-κB) is well known as an inducible transcription factor that plays a pivotal role in the expression of a variety of genes involved in immunity, inflammation, adhesion, cell cycle and cell survival (Baldwin 1996; Ghosh et al 1998). It has been established that NF-κB acts as a cell survival mechanism through its regulatory role over the expression of an array of anti-apoptotic genes, including anti-apoptotic Bcl-2 family proteins, inhibitors of apoptosis proteins and superoxide dismutase (Barkett & Gilmore 1999; Pahl 1999). This notion is further supported by the recent observation that tumour necrosis factor α (TNFα)-induced apoptosis is potentiated by inhibiting the NF-κB signaling pathway in human cancer cells (Zhang et al 2004a). These facts suggest that the NF-κB signaling pathway could be a plausible molecular target to induce apoptosis of cancer cells.

Ergolide is the principal component of sesquiterpene lactones contained in *Inula britannica* var *chinensis*, which has been used as a traditional remedy for inflammatory diseases in Korea (Han et al 2001; Lee et al 2002). Ergolide contains an α-methylene-γ-lactone ring, which is able to interact readily with nucleophilic sites, especially cysteine sulfhydryl groups, of biological molecules by a Michael-type addition (Han et al 2001). Previous

studies demonstrated that the NF-κB signaling molecule is the main molecular target for ergolide, and that inhibition of the NF-κB signaling pathway is the underlying mechanism responsible for its remarkable anti-inflammatory property (Han et al 2001). In comparison with its well-established anti-inflammatory activity, the anti-cancer effect of ergolide is less demonstrated. Recently, parthenolide, another sesquiterpene lactone, was found to induce apoptosis in a number of human cancer cells by depleting intracellular thiols and activating caspase cascades (Wen et al 2002; Zhang et al 2004b), and to prevent UVB-induced skin cancer in hairless mice (Won et al 2004). Therefore, we attempted to evaluate the apoptotic potential of ergolide in human T lymphocyte Jurkat cells and investigated the mechanism of ergolide-induced apoptosis by analysing the signaling pathway of apoptosis. Here, we provide evidence that ergolide induces apoptosis through inhibition of the NF-κB signaling pathway, which causes suppressed expression of anti-apoptotic proteins.

Materials and Methods

Cell culture and reagents

The human T lymphocyte Jurkat cells were grown in suspension in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 1% penicillin/streptomycin and 1% L-glutamine (Gibco, Grand Island, NY). Ergolide was isolated from the dried flowers of *Inula britannica* var *chinensis* and structural identity of ergolide was determined spectroscopically (^{1}H and ^{13}C NMR, IR, MS) as described previously (Han et al 2001; Lee et al 2002).

Cell viability

Cells (5×10^4) were cultured in a 96-well plate in the presence of ergolide. Finally, 10 μL of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution (10 mg mL^{-1} in phosphate-buffered saline, pH 7.4) was added to each well 4 h before stopping. Optical density (OD) was measured with a microplate reader at 570 nm (OD570-630). The mean value of OD content of four wells was used for calculating the viability (% of control).

Apoptosis assays

After treatment of cells with ergolide under indicated conditions, the cells were harvested and the extent of apoptosis was determined by quantitation of nucleosomes released into the cytoplasm using the Cell Death Detection ELISA Plus kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's directions.

Preparation of mitochondria and cytosolic fractions

Jurkat T cells treated with ergolide were harvested and rinsed with ice-cold phosphate-buffered saline (PBS) and cell pellets were resuspended in 300 μL of buffer A (in mM: 20 Hepes-KOH, (pH 7.5), 10 MgCl$_2$, 1 EDTA, 1 EGTA, 1 DTT) containing 250 mM sucrose and proteinase inhibitors. After homogenization, unbroken cells, pieces of large plasma membrane and nuclei were removed by centrifugation at 1000 g for 10 min at 4°C. The supernatant was centrifuged at 10 000 g for 20 min at 4°C. The pellet fraction containing the mitochondria was dissolved in 50 μL of TNC buffer (10 mM Tris-acetate, (pH 8.0), 0.5% Nonidet P-40, 5 mM CaCl$_2$).

Preparation of cell extracts and western blot

Jurkat T cells were rinsed twice with ice-cold wash buffer solution containing (in mM) 50 Tris-HCl (pH 7.5), 120 NaCl, 20 NaF, 1 EDTA, 5 EGTA, 15 sodium pyrophosphate, 30 p-nitrophenyl phosphate, 1 benzamidine and 0.1 phenylmethylsulfonyl fluoride, and then extracted with the same buffer containing 1% Nonidet P-40. Cells were collected by pipetting and were homogenized and centrifuged at 15 000 g for 15 min at 4°C. The extracts were boiled in Laemmli sample buffer for 3 min and 30 μg of each total protein were subjected to SDS-polyacrylamide gel electrophoresis on 12 or 15% slab gels. Proteins were transferred to a polyvinylidene difluoride membrane and the membrane was blocked for 30 min in TBS containing 0.1% Tween 20 and 5% (w/v) dry skimmed milk powder, and incubated overnight with primary antibodies (poly(ADP-ribose), polymerase (PARP; Santa Cruz Biotechnology), cleaved caspase-3 (Cell Signaling), BAX (Santa Cruz Biotechnology), cytochrome c (Transduction Laboratory), IκBα (Santa Cruz Biotechnology), XIAP (Cell Signaling), Bcl-2 (Santa Cruz Biotechnology), phospho-JNK (Cell Signaling) and JNK (Cell Signaling)). The membrane was then washed with TBS-T and incubated for 1 h with a secondary antibody. Bound antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, IL).

RT-PCR

Total RNA was extracted, using TRI$_{ZOL}$ reagent (Invitrogen, Carlsbad, CA). The integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT-PCR) by using RNA PCR kit (Perkin Elmer).

Luciferase reporter gene assay

HeLa cells were seeded into 24-well plates and cultured for 24 h in DMEM–10% FBS before transfection. NF-κB-luciferase reporter plasmid DNA (0.5 μg; Stratagene) was transfected with expression plasmids encoding various genes, using ProFection transfection reagent (Promega, Madison, WI). Twenty-four hours after the transfection, the media were replaced by media with or without indicated stimuli and cell lysates were collected for the luciferase assay 24 h later. The luciferase activity of the cell lysates was measured according to the

manufacturer's recommendations. Relative luciferase activity was normalized by cotransfection of renilla luciferase reporter plasmids.

Statistical analysis

Results are expressed means ± s.d., and a one-way analysis of variance with Tukey's test was used for the statistical analysis of multiple comparisons of data. $P < 0.05$ was considered statistically significant.

Results and Discussion

Ergolide, a sesquiterpene lactone isolated from dried flowers of *Inula britannica* var. *chinensis*, has been shown to exhibit anti-inflammatory activity by inhibiting the NF-κB signaling pathway (Han et al 2001; Lee et al 2002). Recent studies demonstrated that NF-κB has an important role in blocking apoptosis through its upregulation of several anti-apoptotic molecules, including X-chromosome-linked inhibitor of apoptosis (XIAP) and antiapoptotic Bcl-2 family members (Barkett & Gilmore 1999; Pahl 1999; Tang et al 2001). These observations prompted us to investigate the possibility of apoptotic potential of ergolide. Therefore, we first examined the effect of ergolide on the growth of Jurkat T cells using the MTT assay. Treatment of Jurkat T cells with up to 1 μM of ergolide for 48 h caused only a slight decrease in viable cells (Figure 1), although increasing concentrations of ergolide (3 and 10 μM) resulted in a sharp decline in viable cell number (IC50 (concentration reducing viability by 50%) = 3.56 μM), indicating extensive cell death within 48 h. In addition, ergolide also significantly decreased the viability of a human acute promyelocytic leukaemia cell line, HL60 cells, with an IC50 value of 4.21 μM (data not shown). Next, we examined whether the effect of ergolide on cell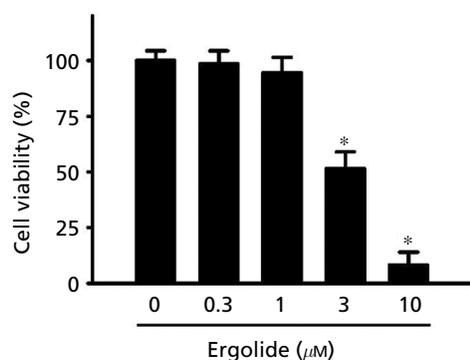 death was attributable to the induction of apoptosis. Since cells undergoing apoptosis show a characteristic cleavage of DNA into oligonucleosome fragments, the cytoplasmic histone-bound DNA fragment was measured by using a cell death detection ELISA kit as a hallmark of apoptosis (Mayo et al 2003). We exposed Jurkat T cells to different concentrations of ergolide for 24 h and determined apoptosis levels. While a concentration of 1 μM of ergolide induced little, if any, DNA fragmentation, increasing the concentration of ergolide to 3 and 10 μM resulted in an appearance of DNA fragmentation up to approximately 3.6 fold (Figure 2A). This result suggests that ergolide triggers an apoptotic pathway in Jurkat T cells.

A family of aspartate-specific cysteinyl proteases (caspases) plays a pivotal role in the execution of apoptosis (Grutter 2000). Thus, to gain a molecular insight into the mechanism by which ergolide induces apoptosis, we investigated the effects of ergolide on caspases. We examined the in-vivo effect of ergolide on caspase-3, which is activated by a number of apoptotic signals. We found that incubation of Jurkat T cells with ergolide resulted in activation of capsase-3, evidenced by the appearance of the catalytically active effector protease (p17) resulting from cleavage of the proenzyme form of caspase-3 (p32) (Figure 2B). Activation of caspase-3 protease during ergolide-induced apoptosis was also confirmed by examining
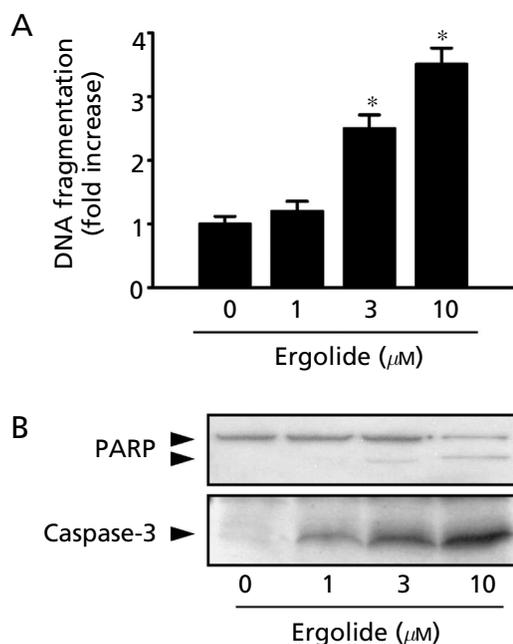

Figure 1 Ergolide treatment promotes cell death of Jurkat T cells. Jurkat T cells were seeded at a density of 5×10^4/well in 96-well plates and treated with various concentrations of ergolide for 48 h. Cell viability was determined using the MTT assay as described in Materials and Methods and expressed as percentage of the control without ergolide treatment. Data are expressed as mean ± s.d. from four separate experiments in duplicate. *$P < 0.05$, ergolide-treated groups vs control.

Figure 2 Ergolide treatment induces apoptosis of Jurkat T cells. Jurkat T cells were harvested 24 h following the addition of ergolide and analysed for DNA fragmentation levels, caspase-3 activation and proteolytic cleavage of PARP. A. Histone-associated DNA fragments were detected using the Cell Death Detection ELISA Plus kit as described in Materials and Methods. Data are expressed as mean ± s.d. from four separate experiments in duplicate. *$P < 0.05$, ergolide-treated groups vs control. B. The activation of caspase-3 and the cleavage of PARP were examined by Western blot analysis using specific antibody against cleaved caspase-3 (p17) or PARP. Results show a representative blot from three separate experiments.

poly(ADP-ribose) polymerase (PARP) cleavage, a known endogenous substrate for caspase-3 (Tian et al 1999). Caspase-3 protease activation was accompanied by cleavage of PARP (116 kDa) into an 85 kDa C-terminal fragment and a 28 kDa N-terminal fragment (Figure 2B). This suggested that caspase-3 was involved in the induction of apoptosis by ergolide. To further confirm the involvement of caspase-3 in ergolide-induced apoptosis, we examined the effect of a cell-permeable caspase inhibitor, z-DEVD-fmk, on ergolide-induced apoptosis. As shown in Figure 3, pretreatment with z-DEVD-fmk significantly inhibited ergolide-induced DNA fragmentation. Consistent with these results, cleavage of PARP induced by ergolide was also abrogated by z-DEVD-fmk pretreatment (Figure 3). Taken together, these results indicate that ergolide-induced apoptosis appears to be mediated by activation of caspase-3.

Previous studies with several models of apoptosis have shown that Bax translocates from the cytosol to the mitochondria when over expressed or in response to certain cell death stimuli. Moreover, translocation of Bax to the mitochondria in a number of systems has been suggested to be responsible for the release of cytochrome c from the mitochondria to the cytosol and the activation of apoptosis (Murphy et al 2000; Wood & Newcomb 2000). To examine whether this pathway was activated by ergolide, we investigated the effect of ergolide on Bax translocation and cytochrome c release. Ergolide-treated Jurkat T cells were collected and fractionated into cytosolic and mitochondrial fractions, and the distribution of Bax and cytochrome c in these fractions was examined by Western blot analysis. Increasing amounts of Bax were detected in the
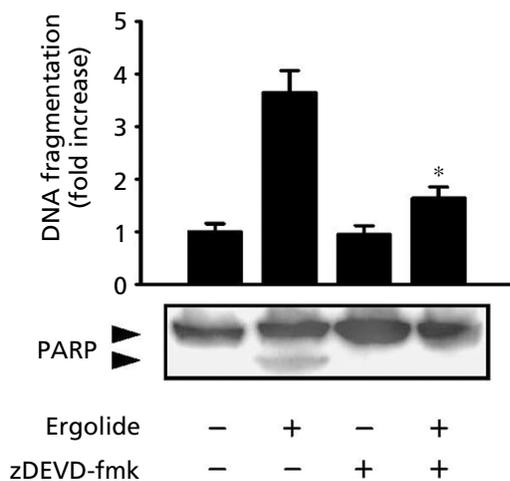
Figure 4 Effect of ergolide on Bax translocation and cytochrome c release. Jurkat T cells were treated with ergolide (10 μM) for the time intervals indicated. Cytosolic and mitochondrial fractions were prepared as described in Materials and Methods. Translocation of Bax from the cytosol to the mitochondria was analysed by Western blotting with anti-Bax antibody (A) and cytochrome c release from the mitochondria to the cytosol was analysed with anti-cytochrome c antibody (B). The levels of Bax and cytochrome c in whole cell lysates were analysed as control. Results show a representative blot from three separate experiments.

Figure 3 Activation of caspase-3 is required for ergolide-induced apoptosis. After pretreatment of Jurkat T cells with caspase-3 inhibitor z-DEVD-fmk (100 μM), the cells were harvested 24 h following the addition of ergolide (10 μM). Histone-associated DNA fragments were detected using the Cell Death Detection ELISA Plus kit as described in Materials and Methods. Data are expressed as mean $\pm$ s.d. from four separate experiments in duplicate. $*P < 0.05$, ergolide-treated control vs z-DEVD-fmk/ergolide-treated group. The PARP cleavage was examined by Western blot analysis. Result shows a representative blot from three separate experiments.
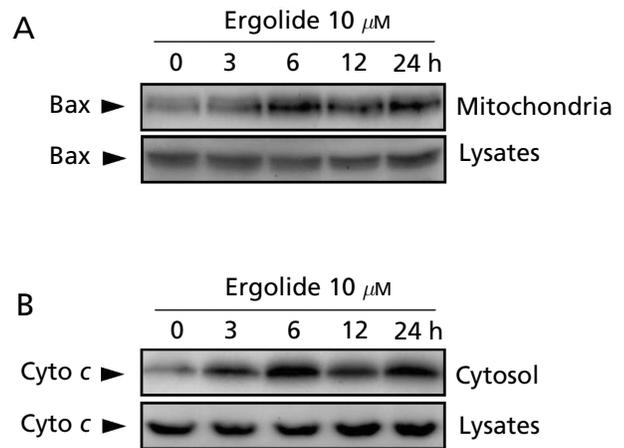
mitochondria in a time-dependent manner after the addition of ergolide to Jurkat T cells (Figure 4A). However, the amount of Bax in whole cell lysates was not changed by ergolide treatment. In parallel, cytochrome c in the cytosol fraction markedly increased in a time-dependent manner without any alteration of the level of cytochrome c in whole cell lysates (Figure 4B). These results suggest that ergolide induces translocation of Bax to the mitochondria, subsequently releasing cytochrome c preferentially into the cytosol of Jurkat T cells to induce apoptosis.

Recent studies have established that NF-κB provides an anti-apoptotic function in many different cancer cells through upregulation of several anti-apoptotic molecules (Tang et al 2001). The ability of NF-κB to suppress apoptosis is also considered to confer resistance to chemotheraphy (Mayo & Baldwin 2000). In addition, it has been demonstrated that ergolide could inhibit NF-κB signaling pathways, resulting in suppression of inducible nitric oxide synthase and cyclo-oxygenase-2 expression (Han et al 2001). Thus, we assumed that the apoptotic potential of ergolide might be attributed to the disruption of the NF-κB signaling pathway. To test this possibility, HeLa cells were transiently transfected with an NF-κB-responsive reporter gene and were then treated with phorbol-dibutyrate (PDBu), a protein kinase C activator, in the presence or absence of ergolide. As shown in Figure 5A, treatment with PDBu caused an approximately 5-fold increase in NF-κB reporter activity, whereas pretreatment with ergolide led to a significant decrease in PDBu activation of NF-κB reporter activity. In addition, basal activity of NF-κB was also significantly attenuated by ergolide treatment (Figure 5A). These results suggest that the
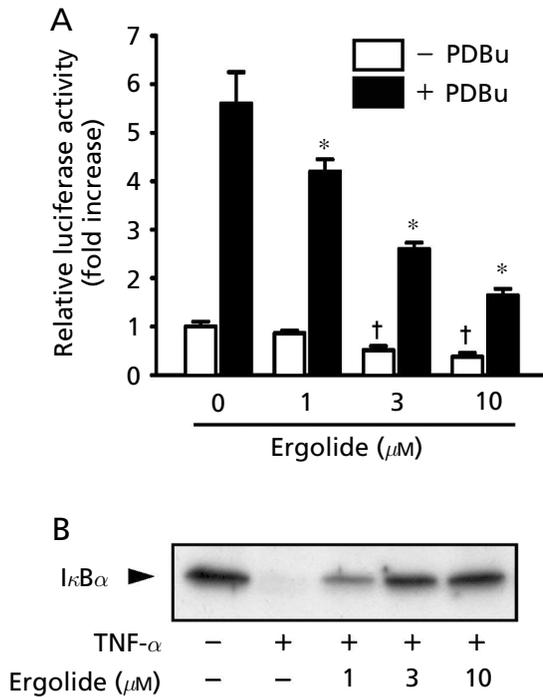
Figure 5 NF-κB signaling pathway is attenuated by treatment with ergolide. A. HeLa cells were transfected with the NF-κB luciferase reporter plasmid DNA (0.5 μg) for 24 h. After pretreatment with ergolide for 1 h, the cells were incubated for additional 24 h, following treatment with 50 nM PDBu. Cells were harvested, and luciferase activity was determined. Data are expressed as mean ± s.d. from four separate experiments in duplicate. †$P < 0.05$, ergolide-treated groups vs control; *$P < 0.05$, PDBu-control vs PDBu/ergolide-treated groups. B. After pretreatment of Jurkat T cells with ergolide, the cells were further incubated for 30 min in the presence or absence of TNFα (10 ng mL^{-1}). The cellular level of IκBα was examined by Western blot analysis. Results show a representative blot from three separate experiments.

NF-κB signaling pathway in cellular systems could be effectively down-regulated in the presence of ergolide. We next determined the effect of ergolide on TNFα-mediated degradation of IκBα in Jurkat T cells by Western blots analysis. Treatment with TNFα led to rapid disappearance of the immunoreactive IκBα band within 30 min, which was returned to basal levels within 90 min (data not shown). However, this degradation of IκBα was dramatically attenuated by ergolide pretreatment (Figure 5B), indicating that ergolide treatment led to an impairment of the NF-κB signaling pathway in Jurkat T cells. These results were in good agreement with the previous observation that ergolide inhibited nuclear translocation and DNA binding activity of NF-κB resulting from blockade of the degradation of IκBα in lipopolysaccharide/interferon-γ-stimulated RAW 264.7 cells (Han et al 2001). Recent reports show that NF-κB has an important role in blocking apoptosis through its upregulation of several anti-apoptotic molecules, including XIAP and the anti-apoptotic Bcl-2 family (Barkett &
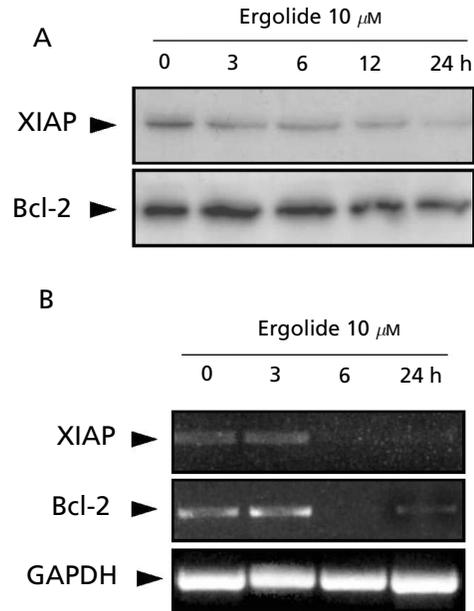

Figure 6 Ergolide treatment inhibits the expressions of XIAP and Bcl-2. Jurkat T cells were treated with ergolide (10 μM) for the time intervals indicated. Expression levels of XIAP and Bcl-2 protein were analysed by Western blot analysis (A) and the total RNA from each group was isolated and mRNA levels of XIAP and Bcl-2 were analysed by RT-PCR (B). Results show a representative blot from three separate experiments.

Gilmore 1999; Karin & Lin 2002). Thus, we examined the effect of ergolide on the expression of anti-apoptotic protein XIAP and Bcl-2 in Jurkat T cells. As shown in Figure 6A, ergolide treatment suppressed the expression of both XIAP and Bcl-2 protein in a time-dependent manner. In addition, mRNA levels of these molecules were decreased by ergolide treatment, indicating that ergolide regulates the expression level of XIAP and Bcl-2 at transcription level (Figure 6B). Taken together, these results suggest that the ability of ergolide to induce apoptosis might be due to the inhibition of NF-κB transcription factor, which results in down-regulation of cell survival signals.

In addition, it has been demonstrated that inhibition of the NF-κB signaling pathway causes a prolonged activation of c-Jun-N-terminal kinase (JNK), leading to the induction of apoptosis (Guo et al 1998; Tobiume et al 2001). Furthermore, recent reports show that inhibition of the NF-κB signaling pathway by parthenolide leads to a sustained activation of the JNK signaling pathway, which reverses the resistance of human cancer cells to apoptosis (Nakshatri et al 2004; Zhang et al 2004a). Therefore, we examined the effect of ergolide on the JNK signaling pathway. Consistent with other observations, our data showed that the phosphorylation level of JNK was dramatically increased by ergolide treatment in Jurkat T cells (Figure 7A). In parallel, inhibition of the JNK signaling pathway using a specific JNK inhibitor, SP600125, prevented ergolide-induced apoptosis (Figure 7B). These results strongly suggest that apoptotic potential
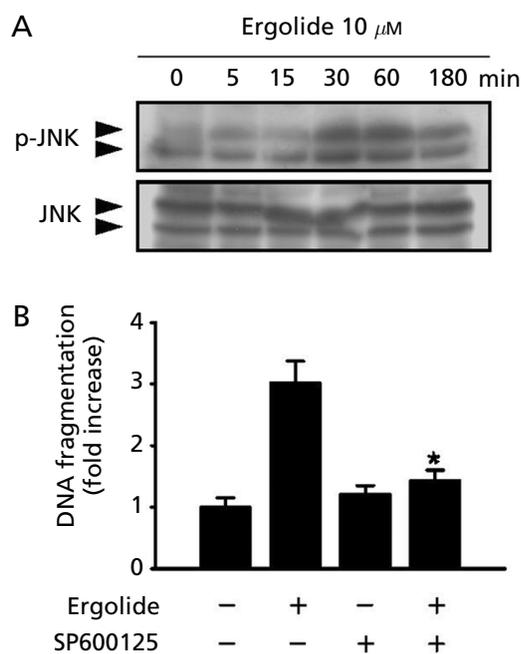
A

Ergolide 10 μM

0   5   15   30   60   180 min

p-JNK ▶

JNK ▶

B

| Ergolide | − | + | − | + |
| SP600125 | − | − | + | + |

Figure 7 Ergolide treatment activates the JNK signaling pathway. A. Jurkat T cells were treated with ergolide (10 μM) for the time intervals indicated. JNK activity was measured using phospho-specific JNK (p-JNK) and JNK antibodies. Results show a representative blot from two separate experiments. B. After pretreatment of Jurkat T cells with the JNK inhibitor SP600125 (20 μM), the cells were harvested 24 h following the addition of ergolide (10 μM). The DNA fragments were detected using the Cell Death Detection ELISA Plus kit as described in Materials and Methods. Data are expressed as mean ± s.d. from four separate experiments in duplicate. *$P < 0.05$, ergolide-treated control vs SP600125/ergolide-treated group.

of ergolide is associated with sustained activation of JNK via inhibition of the NF-κB signaling pathway.

Conclusion

In summary, we demonstrated here that ergolide, a sesquiterpene lactone, was a potent apoptotic agent in Jurkat T cells. Ergolide treatment caused cell death, probably due to the induction of apoptosis, because cell death by ergolide was accompanied by DNA fragmentation, activation of caspase-3, cleavage of PARP and cytochrome *c* release. Furthermore, the NF-κB signaling pathway was significantly inhibited by ergolide treatment to down-regulate its target gene XIAP and Bcl-2, indicating the possible involvement of the NF-κB signaling pathway in ergolide-induced apoptosis. In addition, ergolide-induced apoptosis was closely associated with activation of the JNK signaling pathway. Taken together, our results provide a molecular insight into the mechanism of apoptosis by ergolide via down-regulation of cell survival signal molecules, including XIAP and Bcl-2, resulting from the disruption of the NF-κB signaling pathway, and the usefulness of ergolide as a chemopreventive or chemotherapeutic agent for cancer treatment.

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