Nicotine Enhances the Antiapoptotic Function of Mcl-1 through Phosphorylation

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Introduction

Lung cancer has a strong etiologic association with cigarette smoking. Nicotine, a major component in tobacco smoke, functions as a survival agonist that inhibits apoptosis following various stresses. However, the mechanism of action remains elusive. Mcl-1, a major antiapoptotic protein of the Bcl2 family, is extensively expressed in both small cell and non–small cell lung cancer cells, suggesting that Mcl-1 may be a therapeutic target of patients with lung cancer. Here, we found that nicotine induces Mcl-1 phosphorylation through activation of extracellular signal-regulated kinase (ERK)1/2 in association with increased chemoresistance of human lung cancer cells. Since nicotine stimulates Mcl-1 phosphorylation and survival in cells expressing wild-type but has no such effects in cells expressing T163A Mcl-1 mutant, this indicates that nicotine induces Mcl-1 phosphorylation exclusively at the T163 site and that phosphorylation of Mcl-1 at T163 is required for nicotine-induced survival. Mechanistically, nicotine-induced Mcl-1 phosphorylation significantly enhances the half-life of Mcl-1, which renders Mcl-1 a long-term survival activity. Specific depletion of Mcl-1 by RNA interference blocks nicotine-stimulated survival and enhances apoptotic cell death. Thus, nicotine-enhanced survival of lung cancer cells may occur through activation of Mcl-1 by phosphorylation at T163 site, which may contribute to development of human lung cancer and/or chemoresistance. (Mol Cancer Res 2009;7(12):OF1–8)

Abstract

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Introduction

Lung cancer is the main cause of cancer deaths in both sexes with an annual mortality rate of 91% (1). Cigarette smoking is by far the most important risk factor in the development of lung cancer. For example, cigarette smokers have a 20-fold higher relative risk of developing lung cancer compared with non-smokers (1). Ninety percent of all lung cancers are caused by cigarette smoke including second hand smoke (2). Cigarette smoke contains about 4,000 chemicals, 55 of which have been evaluated as carcinogens (3). Nicotine is a major component in tobacco that exists at high concentrations (~90-1,000 nmol/L) in the blood of smokers (4). Nicotine functions as a survival agonist to inhibit apoptosis induced by diverse stimuli, including chemotherapeutic drugs (5). However, the intracellular signal transduction mechanism(s) involved in nicotine suppression of apoptosis remains enigmatic.

Bcl-2 family members are key regulators of apoptotic cell death and deregulation of these proteins could be oncogenic (6, 7). There are at least 20 members in the Bcl2 family, all of which share at least one BH (Bcl-2 homology) domain (8). Recent studies suggest that prognosis of lung cancer is closely linked to the Bcl-2 family members (9-11). Our previous studies have shown that nicotine induces Bcl2 phosphorylation at serine (S) 70 in association with prolonged cell survival (12). We recently discovered that nicotine can also stimulate phosphorylation and inactivation of the proapoptotic proteins (i.e., Bad and Bax; refs. 13, 14). However, whether other Bcl-2 family member(s), for example, Mcl-1, is involved in nicotine signal transduction pathways remains unclear. Mcl-1 is a major antiapoptotic member of the Bcl2 family, which is extensively expressed in various human lung cancer cells (15). Mcl-1 is one unique member of the Bcl2 family because of its short half-life (30 minutes-3 hours in various cell types) and short-term prosurvival function, which probably relates to the presence of a long proline-, glutamic acid-, serine-, and threonine-rich (PEST) region upstream of the BH domain (16-19). Thus, the mechanism(s) to prolong the half-life of Mcl-1 protein is critical for its long-term survival function. Mcl-1 protein can be phosphorylated at multiple sites that distinctly regulate Mcl-1 protein turnover. For example, extracellular signal-regulated kinase (ERK)1/2–mediated T163 site phosphorylation enhances the half-life and antiapoptotic function of Mcl-1 (20, 21). In contrast, S159 phosphorylation by GSK-3β facilitates Mcl-1 ubiquitination and degradation to reduce its survival activity (22). Additionally, the Cdk1/2–mediated S64 site phosphorylation increases the antiapoptotic function but has no effect on its half-life (16). Since nicotine can activate ERK1/2 (1, 12), which is a physiologic T163 site kinase of Mcl-1 (20), in this study, we have shown that nicotine induces Mcl-1 phosphorylation at T163 through activation of ERK1/2, which leads to prolonged half-life of Mcl-1 with enhanced antiapoptotic function in human lung cancer cells.
Results
Nicotine Induces Mcl-1 Phosphorylation in Association with Prolonged Survival of Human Lung Cancer Cells

Mcl-1 is a major antiapoptotic member of Bcl2 family. Intriguingly, Mcl-1 is more widely expressed in human lung cancer cells, including small cell lung cancer and non–small cell lung cancer cells, than Bcl2 (Fig. 1A). This suggests that Mcl-1 may play a more extensive role in survival and chemoresistance of human lung cancer cells, especially in those cells that express low or undetectable levels of endogenous Bcl2. To test whether nicotine stimulates Mcl-1 phosphorylation, H1299 cells that express high levels of endogenous Mcl-1 but do not express Bcl2 were metabolically labeled with 32P-orthophosphoric acid and treated with nicotine (1 μmol/L) for 30 minutes. Results indicate that nicotine potently stimulates phosphorylation of endogenous Mcl-1 in human lung cancer H1299 cells (Fig. 1B). Cisplatin and etoposide (VP-16) are currently the most useful clinical drugs for treatment of patients with lung cancer (23). To test the effect of nicotine-induced Mcl-1 phosphorylation on apoptosis, H1299 cells were treated with cisplatin or VP-16 in the absence or presence of nicotine for 96 hours. Results indicate that cisplatin or VP-16 induces >60% of cells undergoing apoptosis and nicotine significantly prolongs cell survival following treatment with cisplatin or VP-16 (Fig. 1C). These findings reveal that nicotine-prolonged cell survival is closely associated with Mcl-1 phosphorylation. Data represent the mean ± SD of three determinations. Other lung cancer cell lines (i.e., H69 or H157) were also tested and similar results were obtained (data not shown).

Nicotine Induces Activation of ERK1/2 Which Co-localizes with Mcl-1, and Active ERK1 and ERK2 Directly Phosphorylate Mcl-1 In Vitro

It has been reported that ERK-mediated phosphorylation of Mcl-1 at T163 can positively regulate its antiapoptotic activity (20). To test whether nicotine-stimulated Mcl-1 phosphorylation occurs through ERK1/2, H1299 cells were treated with increasing concentrations of nicotine for 30 minutes. Phosphorylation of ERK1/2 was analyzed by Western blot using a phosphospecific ERK antibody as previously described (12). Results reveal that nicotine induces phosphorylation and activation of ERK1/2 in a dose-dependent manner (Fig. 2A). Immunofluorescent staining using p-ERK and Mcl-1 antibodies shows that treatment of cells with nicotine significantly enhances the phosphorylated form of ERK1/2 (i.e., pERK) to colocalize with Mcl-1 (Fig. 2B). Intriguingly, nicotine not only enhances pERK (green) but also Mcl-1 (red), suggesting that pERK may phosphorylate Mcl-1 and stabilize/enhance Mcl-1 levels because the active ERK1/2 can directly phosphorylate Mcl-1 in vitro (Fig. 2B and C). Thus, nicotine-induced phosphorylation of Mcl-1 may occur through activation of ERK1/2.

Nicotine Stimulates Mcl-1 Phosphorylation at T163 Site, Which Is Required for Nicotine-Induced Survival of Lung Cancer Cells

Mitogen-activated protein (MAPK) ERK1 and ERK2 are the proline (Pro)-directed kinases that can phosphorylate substrate (s) at serine (S) or threonine (T) residues immediately followed by Pro (20). Interestingly, T163 site in the PEST region of Mcl-1 represents a complete consensus MAPK phosphorylation sequence (PXT163P). Previous report showed that ERK1/2-mediated Mcl-1 phosphorylation occurs at T163 site, which enhances Mcl-1 stability and antiapoptotic activity (20). Because ERK1/2 functions as nicotine-activated Mcl-1 kinase (Figs. 1 and 2), nicotine-induced Mcl-1 phosphorylation may occur at T163 site (a proline-directed phosphorylation site). To test this, we have created the nonphosphorylatable T163A and the phosphomimetic T163E Mcl-1 mutants. Wild-type (WT) and each of the Mcl-1 mutants were overexpressed in H82 cells that express relatively low levels of endogenous Mcl-1. Because nicotine can induce phosphorylation of WT but not T163A Mcl-1 mutant, this indicates that nicotine stimulates Mcl-1 phosphorylation exclusively at T163 (Fig. 3A). Intriguingly, nicotine enhances survival of cells expressing WT but not the cells expressing the T163A Mcl-1 mutant (Fig. 3B). Nicotine has no additional survival activity in cells expressing T163E

FIGURE 1. Nicotine induces Mcl-1 phosphorylation in association with increased chemoresistance of lung cancer cells. A, Expression of Mcl-1 or Bcl2 in various lung cancer cell lines was analyzed by Western blot. B, H1299 cells were metabolically labeled with 32P-orthophosphoric acid for 90 min and treated with nicotine for 30 min. Phosphorylation of Mcl-1 was determined by autoradiography. C, H1299 cells were treated with cisplatin (40 μmol/L) or VP-16 (40 μmol/L) in the presence or absence of nicotine (1 μmol/L) for 96 h. Cell viability was determined by analyzing annexin V binding on fluorescence-activated cell sorting (FACS). Columns, mean of three separate determinations; bars, SD.
These findings provide genetic evidence that phosphorylation of Mcl-1 at T163 is required for nicotine-induced survival.

The MAPK/ERK Kinase/ERK Inhibitor PD98059 Suppresses Nicotine-Induced Mcl-1 Phosphorylation and Survival

Our data strongly suggest that ERK1 and ERK2 function as nicotine-activated Mcl-1 kinase to induce Mcl-1 phosphorylation at T163 (Figs. 1 and 2). To further test whether inhibition of MAPK/ERK1/2 (MEK) affects nicotine-induced Mcl-1 phosphorylation, H1299 cells expressing high levels of endogenous Mcl-1 were metabolically labeled with 32P-orthophosphoric acid and treated with nicotine in the absence or presence of increasing concentrations of propranolol or α-bungarotoxin. Interestingly, propranolol but not α-bungarotoxin can inhibit nicotine-induced activation of ERK1/2 as well as Mcl-1 phosphorylation in a dose-dependent manner (data not shown; Fig. 5A and B). Functionally, propranolol also blocks nicotine-induced cell survival and promotes apoptosis following treatment with cisplatin (Fig. 5C). These results reveal that nicotine-induced Mcl-1 phosphorylation may occur in a mechanism involving the upstream β-adrenergic receptor in human lung cancer cells.

Nicotine-Induced Mcl-1 Phosphorylation Enhances Mcl-1 Expression and Its Half-Life

To test whether nicotine affects Mcl-1 expression, H82 cells expressing low levels of endogenous Mcl-1 or H157 and H1299 cells expressing high levels of endogenous Mcl-1 were treated with increasing concentrations of nicotine for 3 hours. Results indicate that nicotine induces a dose-dependent increase of Mcl-1 expression and its half-life (Fig. 3B).
Mcl-1 expression in various lung cancer cells (Fig. 6A). To further determine whether nicotine regulates the turnover rate of Mcl-1, the half-life of Mcl-1 was measured using the classic $^{35}$S-methionine pulse-chase method as described (26). H157 or H1299 cells were metabolically labeled with $^{35}$S-methionine for 60 minutes. The $^{35}$S-labeled cells were washed and incubated in fresh methionine-replete RPMI 1640 in the absence or presence of nicotine (1 μmol/L) for various time points up to 12 hours. $^{35}$S-labeled Mcl-1 was immunoprecipitated using Mcl-1 antibody. The samples were subjected to SDS-PAGE. The half-life of Mcl-1 was determined by electronic autoradiography. Intriguingly, exposure of cells to nicotine significantly prolongs the half-life of Mcl-1 (i.e., from 2.2-6 hours in H157 cells or from 1.5-5 hours in H1299 cells, respectively; Fig. 6B). These data suggest that nicotine-enhanced Mcl-1 expression may occur, at least in part, through prolonging the half-life of Mcl-1, which may contribute to its long-term survival activity. Mule has been identified as the E3 ubiquitin ligase for Mcl-1 (27). Nicotine-induced T163 phosphorylation of Mcl-1 may render Mcl-1 more resistant to Mule-induced polyubiquitination and degradation. This may be a potential mechanism by which nicotine-induced Mcl-1 phosphorylation increases the stability of Mcl-1. Further work is required to show this hypothesis.

**Mcl-1 Is a Required Target in Nicotine-Induced Survival of Human Lung Cancer Cells**

To test whether Mcl-1 is a required target for nicotine-induced survival of human lung cancer cells, an RNAi approach was used. Recent studies have shown that transfection of cells with siRNA concentrations of >100 nmol/L frequently produces nonspecific off-target effects, and a concentration of 20 to 100 nmol/L only occasionally produces effects (28). One group has reported that siRNA concentrations of 10 to 20 nmol/L generally do not exert nonspecific effects (29). To minimize the nonspecific effect, a low concentration (i.e., 15 nmol/L) of Mcl-1 siRNA was used in the experiment. H1299 cells were transfected with Mcl-1 siRNA or control siRNA. Cells expressing Mcl-1 siRNA displayed a >95% reduction of Mcl-1 protein expression (Fig. 7A). Importantly, specific knockdown of Mcl-1 expression by RNAi significantly enhances apoptotic cell death following treatment with the chemotherapeutic drug cisplatin in the absence or presence of nicotine (Fig. 7B). Nicotine had no significant survival effect on cells expressing Mcl-1 siRNA, suggesting that Mcl-1 is a required target in nicotine-induced survival and/or chemoresistance of H1299 lung cancer cells.

**FIGURE 4.** The MEK/ERK-specific inhibitor PD98059 blocks nicotine-induced Mcl-1 phosphorylation and enhances apoptosis of human lung cancer cells. A. H1299 cells were metabolically labeled with $^{32}$P-orthophosphoric acid for 90 min and treated with nicotine in the absence or presence of increasing concentrations of PD98059 for 30 min. Phosphorylation of Mcl-1 was determined by autoradiography. B. H1299 cells were treated with cisplatin (40 μmol/L) and nicotine (1 μmol/L) in the presence or absence of increasing concentrations of PD98059 for 48 h. Cell viability was determined by analyzing annexin V binding on FACS. Columns, mean of three separate determinations; bars, SD.

**FIGURE 5.** The β-adrenergic receptor–specific inhibitor propranolol suppressed nicotine-induced Mcl-1 phosphorylation and enhances chemosensitivity of human lung cancer cells. A. H1299 cells were treated with nicotine in the absence or presence of increasing concentrations of propranolol for 30 min. Western blot analysis was done to detect the p-ERK1/2 using a phospho-specific ERK antibody. B. H1299 cells were metabolically labeled with $^{32}$P-orthophosphoric acid for 90 min and treated with nicotine in the absence or presence of increasing concentrations of propranolol for 30 min. Phosphorylation of Mcl-1 was determined by autoradiography. C. H1299 cells were treated with cisplatin (40 μmol/L) and nicotine (1 μmol/L) in the presence or absence of increasing concentrations of propranolol for 48 h. Cell viability was determined by analyzing annexin V binding on FACS. Columns, mean of three separate determinations; bars, SD.
The antiapoptotic Bcl2 family member Mcl-1 has been originally identified in the ML-1 human myeloid leukemia cell line which undergoes phorbol ester–induced differentiation (29). Mcl-1 can be stimulated by multiple growth factors including interleukin (IL)-3, IL-5, IL-6, IL-7, granulocyte-macrophage colony-stimulating factor, vascular endothelial growth factor, αIFN, and epidermal growth factor (30). A recent report and our findings indicate that Mcl-1 is extensively expressed in both small cell lung cancer and non–small cell lung cancer cells (Fig. 1A; ref. 15). Importantly, nicotine, a major component of cigarette smoke, can mimic growth factor(s) to simulate Mcl-1 phosphorylation, which is associated with enhanced chemoresistance of human lung cancer cells (Fig. 1). ERK1 and ERK2 are physiologic Mcl-1 kinases that can phosphorylate Mcl-1 at T163 site in the PEST region (amino acid 104-176; refs. 20, 31). Intriguingly, nicotine not only stimulates phosphorylation and activation of ERK1/2 but also facilitates the phosphorylated, active ERK1/2 to colocalize with Mcl-1 in cytoplasm (Fig. 2A and B). Because active ERK1 and ERK2 can directly phosphorylate the Mcl-1 protein isolated from human lung cancer H1299 cells (Fig. 2C) and the specific MEK/ERK inhibitor PD98059 blocks nicotine-stimulated Mcl-1 phosphorylation (Fig. 4), we propose that ERK1 and ERK2 function as nicotine-activated Mcl-1 kinases to phosphorylate Mcl-1 and regulate its activity in human lung cancer cells.

Genetic studies indicate that nicotine stimulates Mcl-1 phosphorylation exclusively at T163 site because only WT but not the T163A Mcl-1 mutant can be phosphorylated when cells were exposed to nicotine (Fig. 3A). The nonphosphorylatable T163A Mcl-1 mutant displays less antiapoptotic activity than WT Mcl-1. Because nicotine only prolongs survival of cells expressing WT but not the T163A Mcl-1 mutant, this suggests that phosphorylation of Mcl-1 at T163 is essential for nicotine-induced Mcl-1 phosphorylation and survival. Moreover, a substitution of T163 with glutamate (E), which mimics T163 site phosphorylation in the PEST region, also enhances the antiapoptotic activity of Mcl-1 (Fig. 3). These findings indicate that either the phosphomimetic mutation at T163 or nicotine-induced T163 site phosphorylation can enhance the survival activity of Mcl-1. Thus, the T163 site should be a critical target for nicotine to positively regulate the antiapoptotic function of Mcl-1.

In addition to the nicotine acetylcholine receptors, high levels of β-adrenergic receptor have been found to be expressed in human lung cancer cells (32). Mounting evidence now indicates that nicotine can function as a β-adrenergic receptor agonist and its effect is abrogated by propranolol (a β-adrenergic receptor inhibitor; refs. 25, 33). It has been reported that ERK1 and ERK2 are the signal downstream components in nicotine/β-adrenergic receptor signaling pathway (34-36). Importantly, ERK1 and ERK2 are physiologic Mcl-1 kinases (20). Because treatment of cells with β-adrenergic receptor inhibitor (propranolol) not only suppresses nicotine-stimulated ERK1/2 activation (Fig. 5A) but...
also inhibits Mcl-1 phosphorylation (Fig. 5B), the β-adrenergic receptor should be linked to nicotine-stimulated Mcl-1 phosphorylation through ERK activation. Thus, the β-adrenergic receptor may function as the major upstream receptor in nicotine-stimulated ERK/Mcl-1 survival signal pathway. Importantly, inhibition of nicotine-induced ERK activation and Mcl-1 phosphorylation by propranolol suppresses nicotine-stimulated survival and enhances apoptotic cell death (Fig. 5). Thus, propranolol may have the potential to be developed as a clinically useful drug that specifically targets the β-adrenergic receptor to block nicotine-stimulated survival signal pathway in patients with lung cancer expressing high levels of β-adrenergic receptor and Mcl-1.

Mcl-1 protein is rapidly degraded in response to cell death signals and is immediately reinduced by survival stimuli (30). Mcl-1 has a very short half-life (i.e., 30 minutes-3 hours) in various cells (16-19). Because nicotine can induce Mcl-1 phosphorylation at T163 site through activation of ERK1/2 (Figs. 1-3), this may positively regulate Mcl-1 stability and expression level. As expected, nicotine not only markedly prolongs the half-life of Mcl-1 from 1.5 to 2.2 hours to 5 to 6 hours but also upregulates its expression level in various human lung cancer cells (Fig. 6). This supports the notion that nicotine-stimulated Mcl-1 phosphorylation at T163 may prevent Mcl-1 from Mule-induced polyubiquitination and degradation, which could render Mcl-1 a longer half-life and long-term survival activity. H82 cells express very low levels of endogenous Mcl-1 (Fig. 1A) and exposure of H82 cells to nicotine upregulates Mcl-1 expression (Fig. 6A). However, nicotine failed to protect the vector-only H82 cells from cisplatin treatment (Fig. 3B). This consequence may result from the ability of cisplatin to block nicotine-enhanced Mcl-1 expression in H82 cells (data not shown). In support of this, cisplatin has also been reported to facilitate enhanced Mcl-1 expression in H82 cells (data not shown). In H82 cells from cisplatin treatment (Fig. 3B). This consequence is expected, nicotine not only markedly prolongs the half-life of Mcl-1 from 1.5 to 2.2 hours to 5 to 6 hours but also upregulates its expression level in various human lung cancer cells (Fig. 6).

In summary, our studies show that nicotine promotes survival of human lung cancer cells in a novel mechanism by activating the antiapoptotic function of Mcl-1 via its phosphorylation. Nicotine activates ERK1/2 through the upstream β-adrenergic receptor, which can induce Mcl-1 phosphorylation at T163 site in the PEST region. Nicotine-induced Mcl-1 phosphorylation at T163 enhances the half-life of Mcl-1, which leads to its long-term survival function and/or chemoresistance of human lung cancer cells. Thus, disruption of the antiapoptotic function of Mcl-1 by blocking its T163 site phosphorylation may represent a new strategy for the treatment of tobacco-related cancer especially lung cancer and other malignancies that express Mcl-1.

**Materials and Methods**

**Materials**

Nicotine, propranolol, cisplatin, and VP-16 were purchased from Sigma. Purified ERK1 and ERK2 were obtained from Calbiochem. ERK1, ERK2, phospho-specific ERK, Mcl-1, and actin antibodies as well as Mcl-1 siRNA were purchased from Santa Cruz Biotechnology. Alexa Fluor 594–conjugated goat anti-mouse IgG antibody and Alexa Fluor 488–conjugated goat anti-rabbit IgG antibody were obtained from Invitrogen. WT Mcl-1 cDNA in the pcDNA3.1/V5-His-TOPO was kindly provided by Dr. Ulrich Maurer (University of Ulm, Ulm, Germany). [32P]Orthophosphate and [32P]γ-ATP were purchased from MP Biomedica. All reagents used were obtained from commercial sources unless otherwise stated.

**Cell Lines, Plasmids, cDNA, Mutagenesis, and Transfections**

H23, H69, H82, H157, H358, H460, and H1299 cells were maintained in RPMI 1640 with 10% fetal bovine serum. A549 cells were maintained in F-12K medium with 10% fetal bovine serum. WT Mcl-1 cDNA was cut from Mcl-1 cDNA/pcDNA3.1/ V5-His-TOPO construct using BamHI and XbaI restriction enzymes. The Mcl-1 cDNA insert was cloned in pUC19. Nucleotides corresponding to threonine (T) 163 residue was substituted to create a conservative alteration to alanine (A) or glutamic acid (E). The 5′ phosphorylated mutagenic primers for Mcl-1 mutants were synthesized as follow: T163A, 5′-GTC ACT ACC TTC GGC GCC GCC GCC AGC-3′; T163E, 5′-GTC ACT ACC TTC GGA GCC GCC GCC AGC-3′. The WT-Mcl-1/pUC19 construct was used as the target plasmid, which contains a unique NdeI restriction site for selection against the unmutated plasmid. The NdeI selection primer is 5′-GAG TGC ACC ATG GCC GGT GTG AAA-3′. The nonphosphorylatable (T163A) and the phosphomimetic (T163E) Mcl-1 mutants were created using a site-directed mutagenesis kit (Clontech) according to the manufacturer’s instructions. Each single mutant was confirmed by sequencing of the cDNA and was then cloned into the pcDNA (Promega) mammalian expression vector. The pcDNA plasmids containing WT, T163A, or T163E were transfected into H82 cells using Lipofectamine 2000 (Invitrogen). Clones stably expressing WT, T163A, or T163E were selected in medium containing G418 (0.8 mg/mL). The expression levels of exogenous Mcl-1 were compared by Western blot analysis using a Mcl-1 antibody. Three separate clones for each mutant expressing similar amounts of exogenous Mcl-1 were selected for analysis.

**Preparation of Cell Lysates**

Cells were washed with phosphate-free RPMI and metabolically labeled with 32P-orthophosphoric acid for 90 min. After agonist or inhibitor addition, cells were washed with ice-cold PBS and lysed in detergent buffer. Mcl-1 was immunoprecipitated and subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-omat film at −80°C. Mcl-1 phosphorylation was determined by autoradiography. The same filter was then probed by Western blot analysis with a Mcl-1 antibody and developed by using an enhanced chemiluminescence kit (Amersham) as described previously (12, 14).

**Metabolic Labeling, Immunoprecipitation, and Western Blot Analysis**

Cells were washed with phosphate-free RPMI and metabolically labeled with 32P-orthophosphoric acid for 90 min. After agonist or inhibitor addition, cells were washed with ice-cold PBS and lysed in detergent buffer. Mcl-1 was immunoprecipitated and subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-omat film at −80°C. Mcl-1 phosphorylation was determined by autoradiography. The same filter was then probed by Western blot analysis with a Mcl-1 antibody and developed by using an enhanced chemiluminescence kit (Amersham) as described previously (12, 14).
were lysed by sonication and centrifuged at 14,000 × g for 10 min at 4°C. The resulting supernatant was collected as the total cell lysate.

**Immunofluorescence**

The cells were washed with 1× PBS, fixed with methanol and acetone (1:1) for 5 min, and then blocked with 10% normal mouse or rabbit serum for 20 min at room temperature. Cells were incubated with a mouse or rabbit primary antibody for 90 min. After washing, samples were incubated with Alexa 488 (green)–conjugated anti-mouse or Alexa 594 (red)–conjugated anti-rabbit secondary antibodies for 60 min. Cells were washed with 1× PBS and observed under a fluorescent microscope (Zeiss). Pictures were taken and colored with the same exposure setting for each experiment. Individual green- and red-stained images derived from the same field were merged using Openlab 3.1.5 software from Improvision, Inc.

**Mcl-1 Phosphorylation by ERK1 and ERK2 In vitro**

Mcl-1 was immunoprecipitated from lysates isolated from H1299 cells and incubated with purified, activated ERK1 or ERK2 enzyme (0.5 μg) in the assay buffer [10 mmol/L Hepes (pH 8.0), 100 μmol/L ATP, 10 mmol/L MgCl2, 1 mmol/L DTT, 0.5 mmol/L benzamidine, and 2 μCi of [γ-32P] ATP] for 30 min at 30°C as described (37). The reaction was stopped by the addition of 2× SDS sample buffer and boiling the sample for 5 min. The samples were analyzed by SDS-PAGE. Mcl-1 phosphorylation was determined by autoradiography.

**Metabolic Labeling and Pulse-Chase Analysis of Mcl-1 Half-Life**

Cells were metabolically labeled with 35S-methionine for 60 min. The 35S-methionine–labeled cells were washed and incubated in fresh methionine-replete RPMI 1640 in the absence or presence of nicotine (1 μmol/L) for various time points up to 12 h. 35S-labeled Mcl-1 was immunoprecipitated by using Mcl-1 antibody. The samples were subjected to SDS/10-20% PAGE. The half-life (t1/2) of Mcl-1 was determined by electronic autoradiography as described (26).

**RNA Interference**

Human Mcl-1 siRNA (GAAGACCAUAACAGAAATT) was purchased from Santa Cruz Biotechnology. H1299 cells were transfected with Mcl-1 siRNA using Lipofectamine 2000 (Invitrogen). A control siRNA (nonhomologous to any known gene sequence) was used as a negative control. The levels of Mcl-1 expression were analyzed by Western blot. Specific silencing of the targeted Mcl-1 gene was confirmed by at least three independent experiments.

**Cell Viability Assay**

Apoptotic and viable cells were detected using an ApoAlert Annexin-V kit (Clontech) according to the manufacturer’s instructions. The percentage of annexin Vlow (i.e., viable) or annexin Vhigh (i.e., apoptotic) cells was determined using the data obtained by fluorescence-activated cell sorter analysis as described (14). Cell viability was also confirmed using the trypan blue dye exclusion method.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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