INTRODUCTION

Lung cancer is the most common form of cancer and is the leading cause of cancer-related mortality worldwide, causing approximately 1.2 million deaths annually (Jemal et al., 2009). Tobacco smoking is known to be the most important risk factor for lung cancer. Tobacco and tobacco smoke contain more than 60 carcinogens, such as N-nitrosamines, polycyclic aromatic hydrocarbons and aromatic amines (Hecht, 1998, 1999 and 2002). N-nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK), require metabolic activation (mainly by cytochrome P450s (CYPs or P450s)) to exert their mutagenicity and carcinogenicity (Hecht, 1998). Many studies have been performed to identify the association between genetic polymorphisms of P450 genes and lung cancer risk (Le Marchand et al., 1998; Nakachi et al., 1991; Song et al., 2001).

We previously clarified that CYP2A6 is a major enzyme involved in the metabolic activation of NNK (Fujita and Kamataki, 2001; Kushida et al., 2000), and that null and reduced-function polymorphisms of CYP2A6 are significantly associated with lower risk of tobacco-related lung cancer (Ariyoshi et al., 2002; Fujieda et al., 2004; Miyamoto et al., 1999). In our hypothesis, subjects with reduced CYP2A6 capacity do not activate
N-nitrosamines to the same extent as those with higher capacity. This hypothesis was confirmed by our previous finding that 8-methoxypsoralen, a potent inhibitor of CYP2A, completely inhibited the occurrence of NNK-induced adenoma in female A/J mice (Miyazaki et al., 2005; Takeuchi et al., 2003). CYP2A13, a CYP2A isoform that has 95.4% amino acid sequence identity to CYP2A6, was found to be expressed predominantly in the human respiratory tract. CYP2A13 has a higher potency to metabolically activate NNK than CYP2A6 does (Su et al., 2000), and CYP2A13*2 allele, Arg257Cys amino acid substitution, are reported to be associated with the reduction in risk of lung adenocarcinoma (Wang et al., 2003). On the other hand, a recent study has shown that various alleles of CYP2A13 including CYP2A13*1-CYP2A13*10 might have no association with risk of lung cancer in Japanese (Tamaki et al., 2011). These results were consistent with our previous data that it was no clear relationships between CYP2A13*2 allele and tobacco-related lung cancer risk in the previous epidemiology study (Fujieda et al., 2004). On the basis of discrepancy in these results of genetic polymorphisms in CYP2A13, it is difficult to rationalize our previous epidemiological finding by only CYP2A6-mediated metabolic activation of NNK. CYP2A6 is also responsible for the metabolism of nicotine, which is a major component of tobacco, to cotinine (Nakajima et al., 1996a, 1996b), and it has been reported that CYP2A6 genotypes are associated with different smoking behaviors (Fujieda et al., 2004; Pianezza et al., 1998; Thorgerisson et al., 2010). Furthermore, the association of CYP2A6 with lung cancer risk was significant even when adjusting for smoking behavior (Fujieda et al., 2004). These lines of evidence suggest the possibility of unknown mechanism(s) related to CYP2A6.

It has been reported that nicotine and its derivatives, including NNK, activate the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway and suppress apoptosis (Heeschen et al., 2001, 2002). We hypothesized that cotinine could exhibit tumor promotion effects, just as nicotine does, and could result in synergistic effects of NNK-induced initiation and cotinine-induced promotion of the development of lung cancer. In the present study, we examined the effects of cotinine on lung tumorigenesis using human lung adenocarcinoma cell lines and in vivo mouse models.

MATERIALS AND METHODS

Materials

NNK was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Cotinine was obtained from Sigma-Aldrich (St. Louis, MO, USA), and nicotine, doxorubicin and LY294002 were from Wako (Osaka, Japan). Phospho-Akt (Ser472), Akt antibodies and rabbit anti-goat IgG were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Human lung adenocarcinoma-derived A549 cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). A549 cells were maintained in Dulbecco’s modified Eagles medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD, USA), non-essential amino acids (Invitrogen, Carlsbad, CA, USA) and 1 mM sodium pyruvate (Invitrogen) at 37°C in 5% CO₂.

Animals

Female C57BL/6 and A/J mice (Japan SLC, Shizuoka, Japan) were maintained in the Graduate School of Pharmaceutical Sciences, Hokkaido University, according to the institutional animal care guidelines. All animals were housed in polycarbonate cages with white wood chips for bedding and were given free access to drinking water and a basal diet of Oriental MF (Oriental Yeast, Tokyo, Japan).

Cell viability assay and caspase-3/7 assay

One day before pre-treatment, cells were seeded at a density of 8 × 10³ cells/well onto a 96-well plate. Cells were pre-treated with various concentrations (0.01-1 μM) of cotinine, nicotine (1 μM) or an equal volume of DMSO (as a vehicle control) in the absence or presence of LY294002 (20 μM), a PI3K inhibitor in FBS-free medium for 1 hr, and then 10 μM doxorubicin was added to this incubation. After incubation for 48 hr, cell viability was measured using Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany) or caspase-3/7 activities were measured using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA).

Immunoblotting

For dose-dependent induction of Akt phosphorylation, cells were incubated in 0.5% FBS-containing medium for 2 hr prior to treatment. Cells were then treated with cotinine (0.001-1 μM) for 45 min. When the effects of kinase inhibitors were examined, cells were pre-treated with LY294002 (20 μM) for 45 min prior to cotinine treatment. Cotinine was then added to this incubation and cultured for 45 min. The cell lysates were separated by SDS-PAGE and then transferred to polyvinylidene
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fluoride membrane. After treatment with phospho-Akt (Ser473) or Akt antibodies (1:1,000 dilution), the protein-antibody complexes were detected by an ECL enhanced chemiluminescent detection system (Amersham, Arlington heights, IL, USA).

**In situ TUNEL staining**

Cells were seeded onto 100-mm² tissue culture plates in 10% FBS-containing medium. After 24 hr, the cells were pre-treated with cotinine (1 μM), nicotine (1 μM) or an equal volume of DMSO in FBS-free medium for 1 hr, and then treated with 10 μM doxorubicin for 24 hr. In situ DNA cleavage was assessed by the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) reaction using a DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA). Stained cells were observed using confocal microscopy (Olympus, Tokyo, Japan).

**Lewis lung cancer model**

Lewis lung carcinoma cells (1 × 10⁶ cells/mouse) were subcutaneously injected into each flank of 10-week-old C57BL6 mice. Mice received cotinine (100 μg/ml in 2% saccharine), nicotine (100 μg/ml in 2% saccharine) or vehicle in the drinking water. There was no difference in water consumption between these test and control groups. At day 14, tumors were dissected from the mice. The tumor volume was calculated as the product of length × width × 0.5 cm³.

**In vivo tumorigenicity test**

When female A/J mice were 7 weeks old, they were pre-treated with NNK (2 mg/ 0.1 ml saline/mouse, i.p.) or an equal volume of saline as a vehicle control. The next day, each group was given drinking water containing nicotine (100 μg/ml in 2% saccharine) (group 3), cotinine (100 and 300 μg/ml in 2% saccharine) (groups 4 and 5) or 2% saccharine alone as a vehicle control (group 2). The experiment was terminated 16 weeks after the first NNK treatment, and the surviving mice were killed under ether anesthesia. At autopsy, their lungs were excised and weighed, infused with 10% neutral buffered formalin and underwent careful gross inspection. All macroscopically detected lung nodules were counted, and each lung lobe was examined histopathologically. Lung lesions, hyperplasias and adenomas were diagnosed according to the criteria of “Tumors of the mouse” (Rehm et al., 1994). The number of adenomas was counted under a microscope.

**Statistical analysis**

One-way ANOVA followed by Dunnett’s multiple comparison test was performed to examine the significance of differences in cell viability and caspase-3/7 activity. To evaluate the effects of LY294002, Student’s t test was used. The data for multiplicity in A/J mice were also analyzed by Student’s t test. All P values are two tailed, and the significance level was set at P < 0.05.

**RESULTS**

**Effects of cotinine on doxorubicin-induced death of A549 cells**

We investigated whether cotinine suppressed cell death induced by doxorubicin, a representative apoptosis inducer, using human lung adenocarcinoma-derived A549 cells. Survival of A549 cells decreased to 18.5% on treatment with 10 μM doxorubicin, whereas survival was reduced to 24.4%-40.0% and 45.0% on co-treatment with cotinine (0.01-1 μM) and doxorubicin (Fig. 1), and nicotine (1 μM) and doxorubicin, respectively, indicating that cotinine dose-dependently inhibited doxorubicin-induced cell death at a level almost equivalent to that of nicotine. The addition of LY294002, a PI3K-specific inhibitor, reduced the cotinine-elevated cell survival to 27.0%. In addition, we examined the effects of cotinine on the caspase-3/7 activation induced by doxorubicin (Fig. 1B). The caspase-3/7 activity in cells treated with doxorubicin was 4.4-fold higher than that of control. Cotinine repressed the doxorubicin-induced caspase-3/7 activation to 1.3- to 1.9-fold of control in a dose-dependent manner. To assess whether cotinine inhibits apoptosis, we then investigated the effects of cotinine on genomic DNA fragmentation, which is a hallmark of apoptosis. Although TUNEL-stained cells were not observed in control (Fig. 2A), treatment with doxorubicin gave rise to TUNEL-positive cells (arrowhead) and apoptotic bodies (arrow) (Fig. 2B). The number of TUNEL-positive cells was reduced in cells treated with doxorubicin and cotinine or doxorubicin and nicotine (Figs. 2C and 2D). Furthermore, cotinine blocked the genomic DNA fragmentation induced by doxorubicin (supplementary Fig. 1). These results suggest that cotinine suppresses apoptosis through the PI3K/Akt pathway just as nicotine does.

**Effects of cotinine on phosphorylation of Akt**

To confirm that cotinine activates the PI3K/Akt signaling pathway, we analyzed the phosphorylation level of Akt at Ser473. Cotinine enhanced Akt phosphorylation in a dose-dependent manner (Fig. 3A), whereas LY294002 inhibited the phosphorylation of Akt induced by cotinine.
These results indicate that cotinine induces Akt phosphorylation and suppresses apoptosis via the PI3K/Akt pathway.

**Effects of cotinine on tumor growth in mice**

On the basis of the above in vitro analyses, we evaluated whether cotinine enhanced tumor growth using the in vivo Lewis lung cancer model (Fig. 4). Two weeks after implantation of Lewis lung carcinoma cells and treatment with cotinine, tumor growth in the cotinine group significantly exceeded that in the vehicle-treated group by 2.3-fold ($P = 0.020$); nicotine-treated mice also showed significantly higher tumor growth than control ($P = 0.013$). We further examined the in vivo effects of cotinine on lung tumorigenesis induced by NNK in A/J mice (Table 1). Lung adenocarcinoma was not seen in any of the animals. The number of microscopically observed adenomas induced by NNK per mouse increased from 2.3 (NNK alone) to 2.7, 3.6 and 4.0 on treatment with 100 mg/l of nicotine, 100 mg/l of cotinine and 300 mg/l of cotinine, respectively; the increase in the group treated with 300 mg/l of cotinine compared to control group was statistically significant ($P < 0.05$). These results suggest that cotinine accelerates NNK-induced lung tumorigenesis.

**DISCUSSION**

In previous studies, we clearly demonstrated that reduced-function or null polymorphisms of CYP2A6 were significantly associated with lower risk of tobacco-related lung cancer (Ariyoshi et al., 2002; Fujieda et al., 2004; Miyamoto et al., 1999). The following two hypotheses to explain these associations were raised from the findings of several reports, including ours: first, differences in CYP2A6 activity caused by genetic polymorphisms result in different exposures to metabolically activated NNK,

*Fig. 1. Effects of cotinine on doxorubicin (Dox)-induced apoptotic death in A549 cells. (A) Cell viability. A549 cells were pre-treated with cotinine (0.01-1 μM) or nicotine (1 μM) in the presence or absence of LY294002 (20 μM), a PI3K inhibitor for 1 hr, and then treated with 10 μM doxorubicin for a further 48 hr. Cell viabilities were assessed by XTT assay. The percentage of viable cells relative to the vehicle control is given as the mean ± S.D. from three independent experiments. **Significantly different compared to doxorubicin alone at $P < 0.01$. †Significantly different compared to doxorubicin plus cotinine at $P < 0.05$. (B) Caspase 3/7 activity. A549 cells were pre-treated with cotinine (0.01-1 μM) or nicotine (1 μM) for 1 hr and then treated with 10 μM doxorubicin for a further 48 hr. Caspase-3/7 activities were measured using the Caspase-Glo 3/7 Assay Kit. Caspase-3/7 activities relative to vehicle control are given as means ± S.D. from three independent experiments. **Significantly different compared to doxorubicin alone at $P < 0.01$.***
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Fig. 2. Inhibitory effects of cotinine on genomic DNA fragmentation of A549 cells induced by doxorubicin. *In situ* TUNEL staining of control (A), and cells treated with doxorubicin alone (B), cotinine and doxorubicin (C), and nicotine and doxorubicin (D). A549 cells were pre-treated with cotinine (1 μM) or nicotine (1 μM) for 1 hr and then treated with doxorubicin (10 μM) for a further 24 hr. The arrowhead and arrows indicate TUNEL-positive nuclei (brown) and apoptotic bodies (dark brown), respectively. All panels display cells at 150 × magnification. Results are typical data found in three independent experiments.

![Fig. 2](image)

Fig. 3. Effects of cotinine on phosphorylation of Akt in A549 cells. (A) A549 cells were treated with cotinine (0.001-1 μM) in 0.5% FBS-containing DMEM for 45 min. (B) A549 cells were pre-treated with LY294002 (20 μM), a PI3K inhibitor for 45 min, and then treated with cotinine (1 μM) for a further 45 min. Phosphorylation of Akt was analyzed by immunoblotting using phosphospecific Ser473 antibody.

![Fig. 3](image)

Fig. 4. Effects of cotinine on tumor growth in the Lewis lung cancer model. C57BL6 mice, subcutaneously injected with Lewis lung carcinoma cells (1 × 10^6 cells/mouse), received cotinine (100 μg/ml in 2% saccharine), nicotine (100 μg/ml in 2% saccharine) or vehicle in the drinking water. At day 14, tumors were dissected from the mice and the tumor volume was measured. *Significantly different compared to vehicle control at P < 0.05.*

![Fig. 4](image)
and second, differences in CYP2A6 activity affects individual metabolism of nicotine leading to different smoking behaviors. However, the underlying mechanisms have not been fully clarified. In the present study, we propose a novel mechanism, namely that cotinine suppresses apoptosis and promotes tumor proliferation in vitro and in vivo. To our knowledge, this is the first study providing evidence that anti-apoptotic effects of cotinine play an important role in the development of lung cancer induced by tobacco smoking.

In humans, CYP2A6 is the predominant enzyme responsible for nicotine metabolism: nicotine is metabolized mainly (70%-80%) to cotinine by CYP2A6 (Nakajima et al., 1996a, 1996b), and is also metabolized to nornicotine by CYP2A6 and CYP2B6 via N-demethylation, which contributes to about 5% of nicotine metabolism (Yamanaka et al., 2005; Yamazaki et al., 1999). Therefore, genetic polymorphisms of CYP2A6 can cause large individual variations in nicotine metabolism. Xu et al. (2002) also reported that the plasma AUC of nicotine in subjects with lowered CYP2A6 activity was > 3-fold higher than that in those with normal CYP2A6 activity, suggesting that tumor promotion effects of nicotine in subjects of reduced CYP2A6 activity were greater than those in subjects of normal CYP2A6 activity. It appears to be disagreement with our hypothesis accounting for previous epidemiological results. However, some reports suggest that the pharmacokinetics of cotinine, a main metabolite of nicotine, was closely related with the genetic polymorphisms of CYP2A6. First, terminal half-life of cotinine (19 hr) was approximately 10-fold longer than that of nicotine (2 hr) in smokers (Benowitz et al., 1983). Second, the plasma AUC of cotinine in subjects with lowered CYP2A6 activity was 10-fold lower than that in those with normal CYP2A6 activity. The plasma AUC of both cotinine and nicotine in CYP2A6 extensive metabolizer was at least twice higher than that in CYP2A6 poor metabolizer (Xu et al., 2002). Third, urinary cumulative concentrations of cotinine in CYP2A6 extensive metabolizer were approximately 7-fold higher those in CYP2A6 poor metabolizer (Kitagawa et al., 1999). Finally, it has been reported that the mean plasma concentrations of nicotine and cotinine in smokers are 0.12 and 0.85 μM, respectively (Nagano et al., 2010). These lines of evidences clarified that the disposition of cotinine in smokers was greater than that of nicotine, suggesting that cotinine stimulates anti-apoptotic effects and tumor proliferative effects more than nicotine. In this study, we clarified that cotinine can suppress apoptosis at a level similar to that of nicotine. When the much higher expose to cotinine than to nicotine in smokers is taken into consideration, it is clear that cotinine may play an important role in lung tumorigenesis in vivo.

The PI3K/Akt pathway is a key mediator in regulating anti-apoptosis, cell survival and cell proliferation (Jimenez et al., 1998; Klippel et al., 1998). Active Akt has been detected in human lung cancer precursor lesions and in established lung cancers (Tsao et al., 2003). Non-small-cell lung cancer cells, including A549 cells, have constitutively active Akt that promotes cellular survival and resistance to chemotherapy or radiation (Brognard et al., 2001). This study demonstrated that cotinine exhibits anti-apoptotic and cellular proliferative effects through the PI3K/Akt-mediated signaling pathway. Nicotine induces phosphorylation of Bcl2 and Bad through mitogen-activated protein kinases (MAPKs) and the extracellular signal-regulated kinase (ERK1/2)-mediated signaling pathway as well as through the PI3K/Akt-mediated signaling pathway (Jin et al., 2004; Mai et al., 2003). In preliminary experiments, PD98059 (MAPK/ERK kinase-specific inhibitor) tended to block the cotinine-induced suppression of apoptotic cell death (data not shown) and phosphorylation of MAPK (supplementary Fig. 2), suggesting that cotinine is also likely to be involved in these

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Incidence (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hyperplasia</th>
<th>Adenoma</th>
<th>Hyper+Adenoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline alone</td>
<td>2/11 (18.2)</td>
<td>0.1 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>0.2 ± 0.4</td>
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<tr>
<td>2</td>
<td>NNK alone</td>
<td>12/12 (100)</td>
<td>0.5 ± 0.7</td>
<td>2.3 ± 0.9</td>
<td>2.8 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>NNK + Nicotine 100 mg/l</td>
<td>12/12 (100)</td>
<td>1.3 ± 1.7</td>
<td>2.7 ± 1.6</td>
<td>4.0 ± 3.0</td>
</tr>
<tr>
<td>4</td>
<td>NNK + Cotinine 100 mg/l</td>
<td>11/11 (100)</td>
<td>0.9 ± 1.0</td>
<td>3.6 ± 1.7</td>
<td>4.5 ± 2.2</td>
</tr>
<tr>
<td>5</td>
<td>NNK + Cotinine 300 mg/l</td>
<td>12/12 (100)</td>
<td>0.9 ± 0.9</td>
<td>4.0 ± 1.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.9 ± 1.2</td>
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<sup>a</sup>Number of mice observed with lung lesions. *<sup>P</sup> < 0.05 vs Group 2.
Cotinine promotes lung tumor proliferation signaling pathways. We need to undertake further analyses to elucidate the detailed mechanisms. Because nicotine is reported to stimulate angiogenesis, we also investigated the angiogenic effects of cotinine, and clarified the cotinine-accelerated capillary formation of vascular endothelial cells as same level as those of nicotine (data not shown). A previous study reported that cotinine likely promoted the growth of vascular endothelial cells and vascular smooth muscle cells with a greater potency than that of nicotine (Carty et al., 1997), supporting the findings of our preliminary experiments. Therefore, cotinine may potentiate angiogenesis as well as suppress apoptosis, indicating that cotinine plays an important role in tobacco-related lung tumorigenesis.

In the present study, we investigated whether cotinine indicated anti-apoptotic effects and cellular proliferative effects using A549 cells, expressing nicotinic acetylcholine receptor (nAChR) and Bad (Jin et al., 2004; Plummer, 2005), but not CYP2A6/2A13 (Newland, 2011). It was reported that nicotine induced anti-apoptotic effects and angiogenesis effects via nAChR (Dwoskin et al., 1999), and improved cell survival rates by the phosphorylation of Bad through the PI3K/Akt or MAPK/extracellular signal-regulated kinase (ERK) pathway (Jin et al., 2004) (supplementary Figs. 2-4). Although it seemed to remain unsolved questions whether anti-apoptotic effects of cotinine could occur via nAChR and whether cotinine would have a higher affinity than nicotine, the affinity of cotinine to nAChR should be evaluated. We also examined the effects of cotinine and nicotine on the cell death using H82 (human small cell lung carcinoma) and H441 (human lung adenocarcinoma) in addition to A549 cells, which are pulmonary cancer cells (supplementary Fig. 4).

Cotinine and nicotine significantly suppressed the doxorubicin-induced cell death in A549 cells, whereas the cellular proliferative effects of cotinine and/or nicotine were not significant in H82 and H441 cells. The cellular proliferative effects of cotinine and/or nicotine on doxorubicin-treated cells were not significant in H82 and H442 cells under the same conditions that the significant effects were observed in A549 cells. These differences may be due to the fact that Bad and nAChR are not expressed in H82 and H442 cells, respectively (Jin et al., 2004; Plummer, 2005). Furthermore, doxorubin was the most sensitive agents for detecting the cell death in A549 cells among the apoptotic agents including cisplatin and VP-16 (supplementary Fig. 5). Together with these findings, A549 cells treated with doxorubicin are likely to be an appropriate assay condition for evaluating the anti-apoptotic effects of cotinine.

In conclusion, we clarified that cotinine promotes lung tumorigenesis via suppression of apoptosis, giving more support to the impacts of genetic polymorphism of CYP2A6 on tobacco-related lung cancer risk. The novel mechanism of lung tumor promotion mediated by cotinine emphasizes the importance of CYP2A6 as a molecular target for chemoprevention against tobacco-related cancer in the lung.

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