Long-term Exposure to Nicotine, via Ras Pathway, Induces Cyclin D1 to Stimulate G1 Cell Cycle Transition*

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Nicotine is a major component in tobacco, has been implicated as a potential factor that promotes the development of lung cancer. However, the molecular mechanism of its action is still unclear. In this study, we have shown that, via nicotinic acetylcholine receptors, persistent exposure of mouse epithelial cells to nicotine elicits Ras signaling and subsequent Raf/MAP kinase activity, accompanied by a significant increase in cyclin D1 promoter activity and its protein expression. AP-1 is required for activation of the cyclin D1 promoter. The induction of cyclin D1 expression and its promoter activity by nicotine is abolished by the suppression of Raf/MAP kinase signaling. Furthermore, upon nicotine treatment, the cells do not arrest in the G1 phase of the cell cycle following serum starvation. The perturbation of the G1 cell cycle checkpoint is caused by the deregulation of retinoblastoma/E2F activity. Therefore, our data indicated that by targeting the Ras pathway, long-term exposure to nicotine disrupts cell cycle restriction machinery and thus potentiates tumor development.

Nicotine is an addictive component of cigarettes and exists at high concentrations in the bloodstream of smokers (1). In the brain, nicotine mediates fast synaptic transmission in key regions controlling behavior via nicotine acetylcholine receptors (nAChRs)1 (2). Recently, it has been demonstrated that through the stimulation of nAChRs on endothelial cells, nicotine regulates angiogenesis in murine models of inflammation, ischemia, and atherosclerosis (3, 4). High affinity nAChRs are also found on the surface of lung cancer cells of all histologic types and in normal lung tissue (5–8). Growing evidence indicates that nicotine is able to activate several mitogen-related signaling pathways upon its interaction with nAChRs on the surface of bronchial epithelial cells (3, 9–11). Although this effect has been suggested to contribute directly to lung carcinogenesis, the molecular mechanism(s) involved in nicotine-mediated tumorigenesis has yet to be fully understood.

Studies show that the addition of nicotine in pharmacological concentrations (90–1000 μM) results in the activation of several important, pro-survival signaling pathways (12–14). For example, an increase in protein kinase C (PKC) activity was detected in various human or murine lung cancer cell lines in response to nicotine exposure, which antagonized opioid-induced apoptotic signaling (8, 10). The activities of MAP kinase and extracellular signal-regulated kinase 1/2 were also elicited by nicotine. The activation of these kinases is responsible for the phosphorylation of Bcl-2, which blocks apoptosis in lung cancer cells (9, 13, 15, 16). Using short-term cultures of normal human or murine bronchial epithelial cells or airway epithelial cells, studies show that nicotine is able to induce the activation of Akt. Therefore, this kinase was suggested to be involved in tobacco-related carcinogenesis (17, 18). Because these results were obtained from the transient exposure of cells to nicotine, the impact of persistent nicotine exposure on the cells is not clear.

Ras plays an essential role in cellular proliferation, cell development, and cellular differentiation (19–21). As a crucial signal transducer, Ras governs multiple downstream effector pathways, including Raf/MAP kinase, PI3K, JNK/p38, and Rho (22, 23). Growing evidence links Ras activity to cell cycle machinery (24, 25). Ras acts at different phases of the cell cycle, including early G1, the G1/S boundary, and at G2/M (26, 27). In the early G1 phase, Ras activity is required for the release of cells from quiescence (28). Using Ras effector loop mutants, one study demonstrates that Ras uses multiple downstream effectors to disrupt cell cycle control, resulting in anchorage-independent growth (26, 29). Other studies show that the introduction of Ras or Raf into mouse embryo fibroblasts causes the induction of the cyclin-dependent kinase inhibitors p16INK4A and p21WAF1/CIP1 in a p53-dependent manner, leading to senescence (30, 31).

Cyclin D1 is an important factor in the regulation of cell proliferation (32). The association of cyclin D1 with the cyclin-dependent kinases cdk4 and cdk6 results in phosphorylation of the retinoblastoma (Rb) protein, which releases the transcription factor E2F. E2F subsequently activates S-phase-specific genes (21, 27). Studies have suggested an interaction between Ras and cyclin D1 (47, 59). In cells overexpressing ras, cyclin D1 mRNA levels were increased (1). It has been shown that Ras can cause the phosphorylation and activation of transcription factors, such as c-Jun (33, 34). As one of the AP-1 proteins, c-Jun promotes G1 phase progression and further elicits the related gene inductions (32, 37). The suppression of Jun expression prevented cell cycle transition by the addition of serum to G0/G1-arrested cells (38, 39). Overall, these data indicate that AP-1 family members are involved in promoting cell cycle progression.

To understand the molecular mechanisms of nicotine-induced lung cancer development, our study investigated the impact of long-term nicotine exposure on mouse epithelial cells and the intracellular signaling pathways that are activated by
such treatment. We have demonstrated that chronic exposure of mouse epithelial LA4 cells to nicotine activates Raf/MAP kinase pathway through Ras activation. Upon treatment, the expression of cyclin D1 and its promoter activity was augmented in cells. Cells treated with nicotine for 8 weeks were no longer able to arrest in the G1 phase of the cell cycle in response to serum starvation, displaying perturbation of Rb/E2F signaling. Overall, the results suggested that persistent exposure to nicotine mitigates cell growth control through activation of the Ras pathway, which potentiates the development of lung cancer.

**EXPERIMENTAL PROCEDURES**

**Cells and Materials**—Mouse lung epithelial cells, LA4, were obtained from the American Type Culture Collection (Rockville, MD), propagated for two passages, and immediately stored in liquid nitrogen. For each experiment, the cells were freshly thawed and cultured at 1 × 10^5 cells/ml density in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For the long-term treatment, cells were split every 3 days with the medium supplemented with 0.5 μM nicotine to keep the drug at a constant concentration. For the controls, cells were cultured in parallel. Untreated cells and any 3 days post nicotine treatment, the drug was added into cell culture at a concentration of 0.5 μM. Cells were transiently infected with retroviral vector containing cyclin D1 or dn-Raf on the last 2 days of the 8-week nicotine treatment. The expression of the proteins was confirmed by Western analysis. PKC inhibitor G06976 was from LC Laboratories. Nicotine was obtained from Sigma. The sequence of cyclin D1 antisense oligo is 5'–AGGACCTGTGTTCTACAGGCG–3'.

**Immunoblotting Analysis**—After the nicotine treatment, cells were treated with various inhibitors for 60 min and washed with ice-cold phosphate-buffered saline and lysed in detergent buffer. The samples were incubated with various inhibitors for 60 min and washed with ice-cold PBS twice and fixed with fixation solution containing 65% ethanol and 35% Dulbecco’s modified Eagle’s medium. The samples were stained with staining solution (1 μM phospho-specific antibody, 8 mg/ml RNase, and 18 mg/ml propidium iodide) and incubated in the dark for over 30 min at room temperature. A BD Biosciences FACScan was used to analyze the samples. For the suppression of Raf, after 8 weeks of nicotine treatment cells were transiently transfected with a dn-Raf construct using DMRIE-C reagent (Invitrogen) and were cultured in the growth medium supplemented with nicotine for 48 h. Subsequently, the cells were subjected to 48 h of serum starvation. PKC activity was measured using a commercial assay kit (Upstate Biotechnology). After the treatments, cells were lysed in lysis buffer containing 25 mM Tris (pH 7.5), 1% Triton X-114, 20 mM MgCl₂, 150 mM NaCl, and 100 μg of aprotinin and leupeptin/ml. Equal amounts of cell lysates were immunoprecipitated with anti-Ras Ab (Oncogene Science, Uniondale, NY), followed by the addition of a second Ab conjugated with protein A-Sepharose beads. Precipitates were washed with lysis buffer, and bound nucleotides were eluted with elution buffer containing 20 mM EDTA and 25 μM each of cold GDP and GTP at 65 °C. The eluted products were separated on polyethyleneimine-cellulose plates and exposed on the films.

**Assay of Mitogen-activated Protein Kinase (MAP Kinase) Activity**—Cells were treated with nicotine for various times and then lysed in lysis buffer containing 20 mM Tris (pH 7.5), 2 mM EGTA, 5 mM NaF, 0.5% Triton X-100, 1 mM Na₃VO₄, 1 mM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride, and 0.1 mM benzamidine. Supernatant was collected. Fifty micrograms of cell extracts were incubated for 20 min at 37 °C in buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl₂, 100 μM ATP containing 2 μCi of [γ-32P]ATP, 2 mM EGTA, 1 mM dithiothreitol, 100 μM okadaic acid, 100 μM Na₃VO₄, and 1 μg of myelin basic protein (Sigma). Reaction mixes were then spotted onto p81 filters. Filters were washed in 50 mM phosphoric acid and dried with ethanol. Radioactively labeled myelin basic protein bound to the washed filters was determined by scintillation counting (40). For the inhibition of Ras or nAChRs, following the 8-week nicotine exposure cells were treated with 1.5 mM sodium selenite (SeTT), 10 μM Beattle, 37 °C in buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl₂, 100 μM ATP containing 2 μCi of [γ-32P]ATP, 2 mM EGTA, 1 mM dithiothreitol, 100 μM okadaic acid, 100 μM Na₃VO₄, and 1 μg of myelin basic protein (Sigma). Reaction mixes were then spotted onto p81 filters. Filters were washed in 50 mM phosphoric acid and dried with ethanol. Radioactively labeled myelin basic protein bound to the washed filters was determined by scintillation counting (40).

**RESULTS**

**Ras Activity Is Elicited by Chronic Exposure to Nicotine**—Nicotine is one of the major components in tobacco and exists at concentrations of 90–1000 nm in the bloodstream of smokers (1). Apart from its addictive effects on the central nervous system, nicotine has been suggested to act as a tumor promoter through its ability to activate various mitogenic-related signaling pathways, such as PKC and Akt (8, 10, 17, 18). However, these observations were obtained under the experimental settings of transient exposure to nicotine; thus, the molecular mechanism of nicotine-mediated tumor promotion is still unclear. To further investigate the effect of nicotine on lung tu-
morigenesis, normal mouse lung epithelial LA4 cells were used. The cells were exposed to nicotine for a short or long period. For the long-term (8 weeks) treatment, cells were split every 3 days, and the fresh-made medium containing nicotine was then added into the culture. Untreated cells were cultured in parallel with the treated cells, and the medium was changed every 3 days. Ras activation is associated with more than 30% of human lung malignancy, and in many cases the occurrence of lung cancer is often linked to a prolonged period of smoking (1). This led us to first examine the expression and activity of Ras in response to nicotine. The cells were treated with nicotine (0.5 μM) for 1 h or 2 or 8 weeks. Immunoblotting for Ras expression was then performed (Fig. 1a, left panel). A moderate amount of Ras was detected by an anti-Ras Ab in untreated cells, and the level of the protein did not alter at the various time points of nicotine exposure. Ras is a small GTPase and functions as a molecular switch. In response to growth stimulation, Ras binds to GTP and further interacts with its downstream effectors to elicit various biologic activities in cells (22, 23). The activity of Ras, following the same treatment, was determined by GTP/GDP exchange assay (Fig. 1b, right panel). The GTP-bound form of Ras was not detected in untreated cells or cells treated with nicotine for 1 h. Two weeks after the exposure, there was a slightly increased amount of Ras bound to GTP. However, exposure to nicotine for 8 weeks resulted in a significant increase in the active form of Ras. Ras activity was also examined after 12 weeks of nicotine exposure, and the level remained at a similar level as that of 8 weeks of exposure (data not shown).

nAChRs belong to the superfamily of ligand-gated ion channels that are predominantly expressed in neural tissue as well as in other tissues (2–7). nAChRs have been reported to be found on normal lung epithelial and cancer cells with high affinity (5–8). By binding to nAChRs, nicotine can mediate many biological processes, such as controlling behavior by affecting the central nervous system as well as regulating cell growth in non-neuronal cells. To determine whether nAChRs are required for nicotine-mediated Ras activation, MCA (an nAChR antagonist) was used. Following 8 weeks of exposure to nicotine, the cells were treated with MCA (50 μM) for 60 min, and an immunoblotting assay was conducted. The level of Ras expression did not change in response to the co-treatment (Fig. 1b, right panel). However, the addition of MCA blocked nicotine-induced Ras activation (Fig. 1b, left panel), indicating that the increase of Ras activity mediated by chronic nicotine exposure is through nAChR ligand-receptor interaction. This also suggests that the activation of Ras elicited by 8 weeks of nicotine exposure is still reversible.

Effect of Nicotine on Ras Downstream Effectors—Ras controls the activity of multiple effectors (22, 23). The best characterized Ras downstream signaling is Raf/MAP pathway. Through a serine/threonine phosphorylation cascade, Ras activates MAP and extracellular signal-regulated kinase 1/2 kinases, which in turn regulate the activity of transcriptional factors (22, 23). Because the activity of Ras is dramatically increased in LA4 cells in response to chronic exposure to nicotine, it led us to examine whether Raf is activated under the same conditions. Immunoblotting for Raf expression was performed (Fig. 2a, left panel). The nicotine treatment did not alter...
the level of Raf protein expression compared with untreated control. Upon mitogenic stimulation, Raf is able to interact with Ras at the plasma membrane and transmit activation signals to downstream effectors (22, 23). We next tested whether Raf translocated from the cytosol to the plasma membrane in response to nicotine exposure. After treating the cells

**FIG. 2.** Activation of Ras downstream effectors in response to persistent nicotine exposure. *a,* after treating the cells with nicotine for various time points, immunoblotting for Raf expression was conducted. Equal protein loading was determined by anti-actin Ab (left panel). After the treatment, the plasma membrane fraction was isolated and immunoblotted for Raf expression. Equal protein loading and relative purity of subcellular fractions was determined by anti-EGF receptor Ab (right panel). *b,* after exposing cells to nicotine in the presence or absence of different genetic or chemical inhibitors, MAP kinase activity was measured. Equal protein loading was determined by anti-actin Ab. c–e, cells were treated with nicotine for various times, and PI3K, JNK, or p38 kinase was immunoblotted with the corresponding Ab. Immunoblotting was also performed to detect the phosphorylated forms of either the downstream effector (Akt) or the kinases (JNK or p38 kinase) with the phosho-specific Abs. Equal protein loading was determined by anti-actin Ab.
with nicotine for various periods, the plasma membrane fraction was isolated for immunoblotting (Fig. 2a, top panel at the right). A moderate amount of Raf protein was detected in the plasma membrane fraction from the cells treated with nicotine for 2 weeks. Eight weeks of nicotine exposure caused a significant increase in the amount of Raf translocated to the membrane fraction. The protein could not be detected in this subcellular fraction from untreated cells or cells treated with nicotine for 1 h. The relative purity of the plasma membrane from the cells, with or without nicotine exposure, was confirmed by the expression of epidermal growth factor receptor in the plasma membrane fraction (Fig. 2a, middle panel at the right) and compared with the cytosol fraction (Fig. 2b, bottom panel at the right). The data indicate that Raf activity, but not expression, is augmented by persistent nicotine treatment.

MAP kinase is one of the Raf downstream effectors (22, 23). To further determine the activation of Raf induced by nicotine treatment, we examined the status of MAP kinase using a MAP kinase assay (Fig. 2b). Exposure of the cells to nicotine for 2 weeks moderately increased MAP kinase activity. The kinase activity was significantly augmented by exposure to nicotine for 8 weeks. The kinetics of MAP kinase activation is consistent with that of Ras or Raf activation. Exposure to nicotine for 1 h had no effect on MAP kinase activity. FTI is a potent competitive inhibitor of methyltransferase and is able to block methylation of the carboxyl-terminal S-prenylcysteine of Ras, leading to the suppression of Ras activity (42). In the presence of FTI or the introduction of dn-Raf, the increased MAP kinase activity of 8-week nicotine-treated cells was suppressed. This indicated that MAP kinase acts downstream of Ras and Raf in the signaling elicited by nicotine. The requirement of nAChRs for the activation of MAP kinase was also tested. The addition of MCA blocked the induction of MAP kinase activity, which again suggests the linear relationship between the ligand/receptor and Ras/Raf/MAP signaling. The activation of MAP kinase was confirmed by its ability to phosphorylate extracellular signal-regulated kinase 1/2 (data not shown).

Phosphoinositide-3-OH kinase (PI3K) is another Ras downstream effector that is able to phosphorylate Akt at its Ser-473 domain, subsequently activating this serine/threonine kinase (18). Therefore, the level of PI3K was tested by immunoblotting (Fig. 2c, upper panel). After exposing the cells to nicotine for different periods, the level of PI3K expression did not change. To test whether PI3K activity is increased under such conditions, immunoblotting to detect the phosphorylation status of the Ser-473 residue of Akt was conducted (Fig. 2c, lower panel). Exposure to nicotine for 1 h caused Akt phosphorylation at the Ser-473 domain. However, addition of wortmannin (a PI3K inhibitor) suppressed this phosphorylation, which indicates that the activation of Akt by nicotine is through PI3K. The phosphorylation of this enzyme could not be detected after 8 weeks of nicotine exposure. The data suggest that PI3K is transiently activated by nicotine. The involvement of other Ras downstream effectors, JNK and p38 kinase, in response to nicotine treatment was also examined. After exposing cells to nicotine for various periods, immunoblotting for the expression of JNK and p38 kinase was conducted (Fig. 2, d and e, upper panels). Nicotine exposure had no effect on the expression of these kinases. Because the activation of JNK or p38 kinase requires phosphorylation, antibodies against their phosphorylated forms were used to perform immunoblotting analysis (Fig. 2, d and e, lower panels). A basal phosphorylation of JNK or p38 was revealed by the phosphospecific Ab to each kinase under normal growth conditions or in response to nicotine treatment. Overall, the data indicate that these kinases play no role in the action elicited by nicotine.

Nicotine Stimulates Transient Activation of PKC—PKC is a family of phospholipid-dependent serine/threonine protein kinases consisting of multiple members (43, 44). Changes in PKC activity/expression have been demonstrated to affect the biological responses in neoplastic cells (43, 44). In lung cancer cells, nicotine has been demonstrated to stimulate PKC and further block apoptosis (10, 15). Although the role of PKC in Ras signaling is not yet clear, the existence and the interaction between these two molecules have been suggested (45–47).

The activity of PKC in cells exposed to a short or long period of nicotine treatment was tested (Fig. 3). A PKC-specific enzymatic assay for detecting total PKC activity was used to measure the phosphotransferase activity of the kinase in nicotine-treated cells at different time points. Adding nicotine for 1 h dramatically increased PKC activity (~7-fold) in comparison with the control, and this activity was suppressed by GO6976 (a PKC inhibitor). In comparison, PKC activity was sustained at a moderate level after 4 or 8 weeks of nicotine treatment (only ~2-fold). The addition of MCA blocked the transient PKC activation elicited by nicotine, which is consistent with findings by others that the up-regulation of the kinase by nicotine has been demonstrated to affect the biological responses in neoplastic cells (43, 44). In lung cancer cells, nicotine has been demonstrated to stimulate PKC and further block apoptosis (10, 15). Although the role of PKC in Ras signaling is not yet clear, the existence and the interaction between these two molecules have been suggested (45–47).

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FIG. 3. Transient increase in PKC activity in response to nicotine exposure. After exposure to nicotine for various time points in the presence or absence of different inhibitors, relative [32P] incorporation into PKC-specific substrate peptide was assayed. Error bars represent the S.D. over five independent experiments.

Persistent Exposure to Nicotine Blocks Serum Starvation-mediated G1 Arrest—In response to serum withdrawal, cells are able to enter the G1/G0 phase of the cell cycle. Activated Ras in murine quiescent fibroblasts promotes entry into the S-phase (25). Studies using Ras mutants or inhibitors suggest that Ras activity is required throughout the G1 phase of the cell cycle, leading us to test the effect of nicotine on cell cycle progression (24). After exposure to nicotine for 8 weeks, LA4 cells were cultured in the medium containing 10 or 0.5% serum
for 48 h. After harvesting the cells, DNA profile analysis was performed using a flow cytometer (Fig. 4). LA4 cells arrested in the G1 phase of the cell cycle in response to serum starvation. In contrast, after nicotine treatment more than 22% of the cells were still progressing through the cell cycle even during serum starvation. Various genetic or chemical inhibitors were used to determine whether Ras is involved in the deregulation of cell growth control and which downstream effectors participate in this process. The addition of FTI (a Ras inhibitor) re-established this negative cell cycle control. After blocking Raf/MAP kinase signaling, but not PI3K or PKC, cells also regained control of the cell cycle. The data suggest that, via the Raf/MAP kinase pathway, the nicotine-induced increase in Ras activity disrupts serum withdrawal-mediated growth arrest.

Activation of Ras/Raf Signaling by Nicotine Stimulates Cyclin D1 Expression and Its Promoter Activity—Cyclin D permits cells to transit from G1 to S-phase by influencing the cyclin-associated kinases, especially cdk4 or 6, and affecting the phosphorylation of the Rb tumor suppressor. Activation of Ras causes the increase of cyclin D expression and further affects the G1 phase of the cell cycle (48–51). To further investigate the mechanism by which chronic exposure to nicotine perturbs cell cycle control, the expression of cyclin D1 and its promoter activity were examined (Fig. 5). A moderate level of cyclin D1 was detected in untreated cells, and the protein expression was increased in response to 8 weeks of nicotine treatment. Serum starvation dramatically reduced this G1 cyclin expression to an undetectable level and had a minimal effect on the nicotine-treated cells (Fig. 5a). In the presence of FTI or introduction of dn-Raf into the cells, persistent exposure to nicotine did not maintain the increased cyclin D1 expression; thus the expression level was reduced to that seen in untreated cells. Because PKC activity was moderately increased upon persistent exposure to nicotine, the effect of this kinase on cyclin D1 was then tested. In nicotine-treated cells, the addition of GO6976 to suppress PKC activity did not affect the overexpression of cyclin D1.

To test whether persistent exposure to nicotine regulates the transcription of cyclin D1, the activity of its promoter was measured using the cyclin D1-luciferase reporter construct that corresponds to the fragment of cyclin D1 5'-sequence (−1745)/Fig. 5b). The luciferase activity could not be detected under serum starvation condition and was strongly induced after adding back the growth medium. The nicotine treatment moderately increased this serum-induced reporter activity. However, the reporter was still responsive after 8 weeks of nicotine treatment following serum starvation, and FTI blocked this nicotine-mediated activation of the cyclin D1 promoter.
Persistent exposure to nicotine up-regulates cyclin D1 expression and its promoter activity. After the treatments, cell lysates were isolated and immunoblotted with anti-cyclin D1 Ab. Equal protein loading was verified with anti-actin Ab. a, b, and c, after the treatments, LA4 cells were co-transiently transfected with a full-length cyclin D1 promoter luciferase (-1745) and β-galactosidase (internal control) constructs. Forty-eight hours posttransfection, cells were harvested and luciferase activity was analyzed. Error bars represent the S.D. over five independent experiments. d, LA4 cells were transiently infected with a cyclin D1 vector or treated with nicotine for 8 weeks. Subsequently, the transfectants were grown in medium containing 0.5% serum for 48 h. The nicotine-treated cells were cultured in the same medium in the presence of cyclin D1 antisense oligos for 48 h. Immunoblotting for the suppressed expression of cyclin D1 (cln D1) was shown by exposing to antisense oligos (as) for 48 h, and even protein loading was verified with anti-actin Ab (upper panels). DNA profiles of the cells in response to the treatments as described above (lower panel).
Because long-term exposure to nicotine via Ras signaling activates the cyclin D1 promoter, it led us to further investigate which Ras downstream effector was utilized to stimulate cyclin D transcription and the role of PKC in this nicotine-induced action. To address these questions, genetic or chemical inhibitors that suppress Ras downstream effectors and PKC were used to assay the luciferase activity (Fig. 5c). The addition of Raf antisense oligos significantly inhibited the reporter gene activity induced by persistent nicotine treatment. In contrast, the suppression of JNK, PI3K, p38, as well as PKC, by the inhibitors had no negative effect on the induction of the cyclin D1 promoter activity. These results suggest that Ras, via the Raf pathway, participates in the nicotine-mediated up-regulation of cyclin D1 promoter activity.

The effect of cyclin D1 on cell cycle progression in nicotine-treated or untreated cells in response to serum starvation was also examined (Fig. 5d). LA4 cells were transiently infected with cyclin D1 or exposed to nicotine for 8 weeks. Subsequently, the cells overexpressing cyclin D1 were subjected to serum starvation for 48 h or the nicotine-treated cells were grown in serum-free medium containing cyclin D1 antisense oligos for 2 days. The DNA profiles of these cells were then analyzed (Fig. 5d, lower panel). More than 30% of LA4 cells overexpressing cyclin D1 were actively progressing in the cell cycle under the normal growth conditions. After serum withdrawal, a similar portion of the cells still remained in the S and G2 + M phases. The data indicate that the overexpression of cyclin D1 abolishes the negative growth control rendered by serum starvation. However, the nicotine-treated cells that express an increased amount of cyclin D1 (shown in Fig. 5a) regained this negative control in the presence of cyclin D1 antisense oligos. The elimination of cyclin D1 expression by the antisense oligos was confirmed by immunoblotting (Fig. 5d, upper panels). The data further suggest that cyclin D1 plays a role in nicotine-mediated cell proliferation promotion.

**AP-1 Binding Site Is Required for Activation of the Cyclin D1 Promoter in Response to Chronic Nicotine Treatment**—Distinguishable regions of the cyclin D1 promoter were targeted by different signaling pathways (35). To further determine whether nicotine elicits general transcription machinery or regulates a specific transcription factor binding site of the cyclin D1 promoter, various cyclin D1 promoter constructs were used (Fig. 6a, upper panel). The –964 promoter fragment has an AP-1 binding site that is necessary for Ras responsiveness (35). The reporter constructs carrying AP-1 and Ets (−163) deletions, or just 22 bp of the cyclin D1 promoter, were also employed. Chronic nicotine treatment strongly transactivated the full-length as well as the –964 fragment of the cyclin D1 promoter (Fig. 6a, lower panel). Serum starvation had no effect on the promoter activity. Once the AP-1 site was deleted, the promoter activity was almost completely abolished, which indicates that long-term exposure to nicotine affects the cyclin D1 promoter specifically through the AP-1 site. The Ets (−163) and –22-bp reporter genes were unresponsive under the same conditions. We next tested the requirement of nAChRs in the nicotine-induced cyclin D1 transcription activity (Fig. 6b). The full-length (−1745) and minimally responsive 5’-sequence (−964) of the cyclin D1 promoter constructs were introduced into the cells. Addition of MCA completely suppressed both promoter activities conferred by chronic nicotine exposure.

To determine whether AP-1 proteins were able to bind to the cyclin D1 AP-1 site, gel mobility assays were performed (Fig. 6c). After treatment with nicotine for 8 weeks, with or without FTI, nuclear extracts were prepared for gel mobility assay using the cyclin D1 AP-1 site. The AP-1 protein complex was strongly induced and bound to the site by the activated ras (V12). In comparison, nicotine elicited a moderate binding of AP-1 to the binding site. The AP-1 binding activity induced by nicotine treatment was inhibited by FTI or dn-Raf, which again indicates the participation of Ras in this nicotine-mediated action. The addition of cold AP-1 oligos suppressed the formation of the hot AP-1 complex, which highlights the specificity of AP-1 binding activity. Overall, these data suggest that the AP-1 site is a target for activation of the cyclin D1 promoter as a result of long-term nicotine exposure in LA4 cells.

**Persistent Nicotine Exposure Induces Rb Phosphorylation and Augments E2F Activity**—Cyclin D1 drives cells to progress through the G1 phase of the cell cycle by regulating cyclin-dependent kinases, resulting in Rb phosphorylation and E2F activation. Therefore, it was important to explore whether nicotine may inhibit growth factor withdrawal-induced arrest by perturbing these events. To examine the effect of long-term exposure to nicotine on Rb, immunoblotting analysis of Rb protein from whole cell lysates of nicotine-treated or untreated LA4 cells was performed in the presence or absence of serum starvation (Fig. 7a). As expected, serum depletion resulted in an increase of fast migrating, hypophosphorylated Rb. Upon serum starvation, Rb in nicotine-treated LA4 cells was in the hyperphosphorylated form.

During late G1, Rb phosphorylation releases E2F from repression, triggering the activity of E2F family transcription factors (52). Activation of E2F-regulated genes is necessary for the G1/S-phase transition. A construct containing a minimal E2F-responsive promoter linked to a luciferase reporter sequence was used to test the effect of persistent exposure to nicotine on E2F activity (Fig. 7b). Under normal growth conditions, a relatively high E2F-dependent luciferase activity was detected in LA4 cells. Upon serum starvation, the reporter activity was almost completely abolished. However, E2F-dependent luciferase activity in the extract from nicotine-treated cells was higher than that of untreated, control cells. After serum starvation, the promoter activity was only slightly reduced. These experiments suggested that, by activating Rb/E2F signaling, chronic nicotine exposure perturbs the growth control induced by serum withdrawal.

**DISCUSSION**

Nicotine is being used widely to aid smoking cessation (1, 2), yet nicotine use has been associated with lung tumorigenesis (12–14, 16). Studies have demonstrated that transient nicotine exposure promotes lung epithelial cell survival by activating various intracellular growth factors, such as PKC, or by up-regulating Bel-2 activity to antagonize apoptotic signals (16). Other studies show, in vitro and in vivo, that nicotine stimulates angiogenesis in the settings of inflammation, ischemia, atherosclerosis, and tumor growth (3, 4). However, the molecular mechanisms of nicotine, especially as a result of long-term exposure, in the development of cancer are not clear. To fully understand the potential oncogenic properties of this tobacco component, we have thoroughly analyzed various intracellular signaling pathways in response to persistent nicotine exposure and the subsequent effect on cell cycle regulatory events in mouse lung epithelial LA4 cells. The data reveal that nicotine exposure for 8 weeks strongly activates Ras activity. The activation of Ras by nicotine depends upon the nACh receptor. Through the Ras/Raf/MAP kinase pathway, long-term nicotine exposure perturbs growth factor withdrawal-induced restriction events, leading to an increase of cyclin D1 expression/activity, Rb phosphorylation, and E2F activation. The experiments presented here also demonstrated that short- or long-term exposure to nicotine modulates different intracellular pathways. For example, P38K is transiently activated after 1 h of nicotine exposure. PKC activity is strongly elicited by short-
FIG. 6. Activation of cyclin D1 promoter by persistent nicotine exposure is through the AP-1 site. a, schematic representation of cyclin D1 luciferase reporter constructs and mutants (upper panel). Following nicotine treatment, various constructs were transiently introduced into LA4 cells. In the presence or absence of serum starvation, luciferase activity was analyzed. Error bars represent the S.D. over five independent experiments (lower panel). b, cyclin D1 promoter activity was assayed as described above in the presence of MCA. c, after the treatments, gel mobility assay was performed with a 32P-labeled oligo containing the cyclin D1 AP-1 site.
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term exposure to nicotine and is sustained at a moderate level after prolonged period of nicotine exposure. Interestingly, the suppression of these kinases does not block the nicotine-mediated deregulation of cell cycle events. Our data show that long-term exposure to nicotine mobilizes Ras signaling, resulting in the alteration of several important growth regulators and subsequent disruption of cell growth restriction.

Using several small cell lung cancer cell lines, several studies showed that nicotine, through binding to nAChRs, acts as a mitogen (5–8). Upon transient interaction between nicotine and the receptor, MAP kinase activity is up-regulated via a tyrosine kinase pathway (13). One study has also demonstrated that Akt in primary human airway epithelial cells is activated upon short-term exposure to nicotine, which antagonizes apoptosis. This effect has been suggested to contribute to tobacco-related carcinogenesis (18). However, these observations were obtained from the experimental settings of transient exposure to nicotine. The present study, using long-term nicotine exposure, not only supports the tumor promoter property of nicotine but also provides an insight into the molecular mechanism of its action. By activating Ras-related signaling, persistent exposure to nicotine stimulates cyclin D1 expression and perturbs cell cycle checkpoints, such as mitogen withdrawal-regulated growth control. An increase in Ras activity/expression is frequently seen in cancer. Thus, it is reasonable to hypothesize that long-term exposure to nicotine potentiates the lung carcinogenesis process, mainly by up-regulating Ras activity.

Several lines of evidence demonstrate that different signaling pathways target distinguishable regions of the cyclin D1 promoter (35). Upon Ras activation, the transcription factor c-Jun is phosphorylated by JNK, which stimulates cyclin D1 transcription activity and promotes cell cycle progression, especially the G1 transition. The AP-1 site of the cyclin D1 promoter is necessary for this activity (36). Overexpression of MAP kinase induces re-entry into the cell cycle by eliciting cyclin D1 promoter activity. It has been suggested that Ets-2 plays a role in this MAP kinase-induced action (36). Our data demonstrated that, after persistent nicotine exposure, Raf/MAP kinase signaling pathway, but not JNK, is mobilized. Using various promoter mutant constructs, we also showed that the AP-1 site, but not Ets sites, is important for nicotine-induced cyclin D1 promoter activation. The molecular mechanisms of signaling pathways bridging mitogenic stimulation and the cyclin D1 promoter activation are not fully understood yet. It is possible that different cell types use different mechanisms to activate the cis or trans activity of cyclin D1.

Our study showed that PKC activity is transiently augmented by short-term nicotine treatment and sustained at a moderate level during prolonged exposure to nicotine. In comparison, the activation of Ras by nicotine is rather a late event in the nicotine-mediated action. It is possible that PKC is not involved in the perturbation of negative growth control induced by persistent nicotine exposure; rather, PKC might participate in another, as yet unknown, pro-survival process. Numerous studies suggest the existence of the interaction between PKC and Ras signaling pathways (45–47). Upon growth stimulation, the binding sites of PKC for the SH2 domain of Grb-2 are phosphorylated, which recruits the Grb-2-Sos complex and activates Ras signaling in T lymphocytes. In lung cancer cells, PKC activity is often augmented and able to phosphorylate Bcl-2 to promote anti-apoptotic activity. This action of PKC has been linked to tumorigenesis (8, 10). Activated ras is observed in more than 30% of human lung cancer (53). Our previous studies also demonstrated a cooperation between oncogenic Ras and PKC. For instance, once endogenous PKC activity is suppressed, the increase in Ras activity is capable of inducing apoptosis in various types of cells, including lung cancer cells (27, 54). In this study, we showed that PKC activation is detected in both short- and long-term nicotine exposure. Although the activation of PKC has been suggested to be associated with tumor development, further experiments will elucidate the existence and the significance of the cooperative relationship between Ras and PKC in nicotine-mediated action.

P38K, as a Ras downstream effector, regulates cell survival signals (22, 23). Recently, many studies have shown that this serine/threonine kinase is able to suppress cell death pathways by activating Akt, which then directly phosphorylates and inactivates proteins involved in apoptosis, such as Bad and caspase 9 (55–57). The anti-apoptotic action of these kinases has also been shown to contribute to the resistance of Ras-transformed cells to antagonize apoptotic agents (55). In this study, we demonstrated that P38K pathway is transiently activated in response to short-term exposure to nicotine, and the inhibition of this Ras downstream effector pathway does not regain the growth control induced by the serum withdrawal that is perturbed by nicotine treatment. Thus, it is possible that transient activation of P38K and Akt serine/threonine pathway, in concert with other signal pathways induced by nicotine, prevents or suppresses the activation of the cell death program.

Nicotine is well known to be an addictive component of cigarettes. Nicotine-based products, such as gums, inhalers, nasal sprays, and patches, are widely used to aid smoking cessation. The chronic use of nicotine is even being studied for beneficial effects in Alzheimer’s disease and chronic pain. Our present data showing that long-term nicotine exposure dereg-
ulates cell growth restriction offer a cautions stance for the therapeutic use of this compound. However, elucidating the molecular mechanism of nicotine action can lead to the development of clinically relevant strategies that improve upon and modify the current usage of nicotine. Furthermore, our data suggest that Ras activity can be used as a biomarker for monitoring nicotine treatment, because the activation status of this protein is closely associated with early events of tumor development, such as the loss of cell cycle checkpoints.

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