Nicotine Stimulates PPARβ/δ Expression in Human Lung Carcinoma Cells through Activation of PI3K/mTOR and Suppression of AP-2α

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Abstract

We previously showed that nicotine stimulates non–small cell lung carcinoma (NSCLC) cell proliferation through nicotinic acetylcholine receptor (nAChR)–mediated signals. Activation of peroxisome proliferator–activated receptor β/δ (PPARβ/δ) has also been shown to induce NSCLC cell growth. Here, we explore the potential link between nicotine and PPARβ/δ and report that nicotine increases the expression of PPARβ/δ protein; this effect was blocked by an α7 nAChR antagonist (α-bungarotoxin), by α7 nAChR short interfering RNA, and by inhibitors of phosphatidylinositol 3-kinase (PI3K; wortmannin and LY294002) and mammalian target of rapamycin (mTOR; rapamycin). In contrast, this effect was enhanced by PUN282987, an α7 nAChR agonist. Silencing of PPARβ/δ attenuated the stimulatory effect of nicotine on cell growth, which was overcome by transfection of an exogenous PPARβ/δ expression vector. Of note, nicotine induced complex formation between α7 nAChR and PPARβ/δ protein and increased PPARβ/δ gene promoter activity through inhibition of AP-2α as shown by reduced AP-2α binding using electrophoretic gel mobility shift and chromatin immunoprecipitation assays. In addition, silencing of Sp1 attenuated the effect of nicotine on PPARβ/δ. Collectively, our results show that nicotine increases PPARβ/δ gene expression through α7 nAChR–mediated activation of PI3K/mTOR signals that inhibit AP-2α protein expression and DNA binding activity to the PPARβ/δ gene promoter. Sp1 seems to modulate this process. This study unveils a novel mechanism by which nicotine promotes human lung carcinoma cell growth. [Cancer Res 2009;69(16): 6445–53]

Introduction

Lung carcinoma is one of the most common malignant tumors in the world and is the leading cause of carcinoma death in the United States (1, 2). Despite recent advances in understanding the molecular biology of lung carcinoma and the introduction of multiple new chemotherapeutic agents for its treatment, its dismal 5-year survival rate (<15%) has not changed substantially (3). Tobacco use is one of the most important risk factors for the development of lung carcinoma and is associated with at least 87% of cancer deaths (4). In particular, non–small cell lung carcinoma (NSCLC) shows a strong etiologic association with smoking. Nicotine in tobacco leads to tobacco addiction and therefore represents an important target of investigation. Although nicotine does not seem to be carcinogenic by itself, its metabolism leads to the generation of potent carcinogens (5). In addition, nicotine can stimulate cancer cell proliferation and angiogenesis and suppress apoptosis induced by certain agents (6). Several lines of evidence suggest that these effects by nicotine and its derivatives are mediated by nicotinic acetylcholine receptors (nAChR) expressed on the surface of tumor cells (7, 8). However, the molecular mechanisms underlying the role that nicotine plays in promoting lung cancer progression remain incompletely elucidated.

Peroxisome proliferator–activated receptors (PPAR) are members of the nuclear hormone receptor superfamily of ligand-dependent transcription factors. The major PPAR isoforms, α, β/δ, and γ, each have distinct tissue and cellular distributions, different modes of expression, and diverse agonist binding properties (9). In contrast to PPARα and PPARγ, the consequences of PPARβ/δ activation are not well known (10). PPARβ/δ is expressed throughout the body in most tissues (11), and it is linked to cell proliferation, differentiation and survival, lipid metabolism, and development (12, 13). Activation of PPARβ/δ has also been shown to increase human cancer growth, including liver, colon, breast, prostate, and lung (14–16), although opposite results have also been observed (17, 18).

We recently showed that nicotine stimulated NSCLC cell proliferation through nAChR-mediated signals that include activation of the extracellular signal-regulated kinase and phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathways (19). Here, we explore whether the effect of nicotine on lung cancer cell growth is mediated through transcriptional activation of the *PPARβ/δ* gene. We found that nicotine increased PPARβ/δ expression through α7 nAChR–mediated PI3K/mTOR activation that reduced AP-2α and promoted tumor cell proliferation.

Materials and Methods

Culture and chemicals. The human NSCLC cell lines H1838, H1792, A549, H522, and H358 were obtained from the American Type Culture Collection and grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum as previously described (20). Polyclonal antibodies for Akt and phospho-Akt (Ser^{473}) were purchased from Cell Signaling. Polyclonal antibodies against PPARβ/δ, α7 nAChR, AP-2α, AP-2β, AP-2γ, and Sp1

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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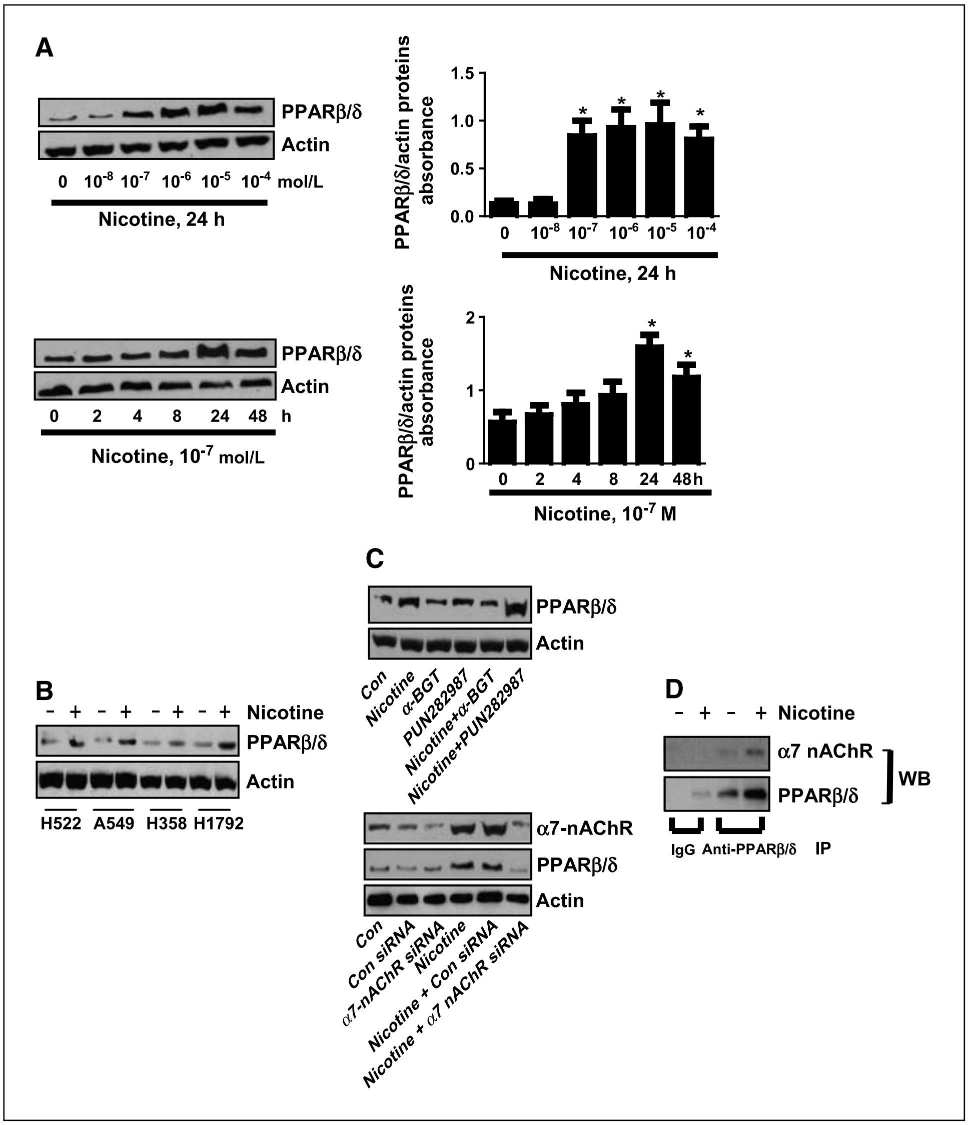

Figure 1. Nicotine increases PPARβ/δ protein expression through α7 nAChR in NSCLC cells. *A,* protein was isolated from H1838 cells treated with increasing concentrations of nicotine for 24 h (*top*) for the indicated period of time (*bottom*) and PPARβ/δ protein was analyzed by Western blot. Actin was used for loading control. *Columns,* mean of PPARβ/δ/actin of three independent experiments; *bars,* SD. *, significant difference from untreated control. *B,* protein was isolated from NSCLC cell lines cultured with nicotine for up to 24 h followed by Western blot for PPARβ/δ protein. *C,* protein was isolated from H1838 cells cultured for 1 h in the presence or absence of α-bungarotoxin (1 μmol/L) and PUN282987 (0.1 μmol/L; *top*) or transfected with control or α7 nAChR siRNAs (*bottom*) and exposed to nicotine (0.1 μmol/L) for an additional 48 h followed by Western blot for PPARβ/δ protein. *Con,* untreated control cells. *D,* lysates from cells treated with nicotine for 24 h, immunoprecipitated with IgG or anti-PPARβ/δ antibodies, and analyzed by Western blot using anti-PPARβ/δ and anti–α7 nAChR antibodies.

were purchased from Santa Cruz Biotechnology. The PI3K inhibitors LY294002 and wortmannin, α7 nAChR antagonist α-bungarotoxin, protein kinase A (PKA) inhibitor H-89, and mTOR inhibitor rapamycin were obtained from Calbiochem. The α7 nAChR agonist PUN282987 was purchased from TOCRIS Bioscience. Nicotine, Sp1 inhibitor mithramycin A, and other chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Western blot analysis. The procedure was performed as previously described (21). Briefly, cells were washed, lysed in 0.2 mL cell extraction buffer, and sonicated. Equal amounts of protein were solubilized in 2× SDS sample buffer, separated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose, blocked with 5% nonfat dry milk containing 0.1% Tween 20 for 1 h at room temperature, and washed thrice with wash buffer (1× TBST). Blots were incubated with primary antibodies against PPARβ/δ, α7 nAChR, AP-2α, AP-2β, AP-2γ, or Sp1 (1:1,000) overnight at 4°C, washed, and incubated with secondary anti-rabbit IgG conjugated to horseradish peroxidase (1:2,000 dilution; Cell Signaling) for 1 h at room temperature. Blots were transferred to enhanced chemiluminescence solution (Pierce) and exposed to X-ray film, and proteins were quantified by densitometric scanning using a Bio-Rad GS-800 calibrated densitometer.

Immunoprecipitation assays. Proteins from NSCLC cells treated with or without nicotine for 24 h were harvested in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology), sonicated, and centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatant was removed for immunoprecipitation. Protein (200 μg) was precleared for 30 min with 30 μL of protein A/G Plus-agarose (Santa Cruz Biotechnology) and incubated for 1 h at 4°C with appropriate antibodies (anti-PPARβ/δ and anti–α7 nAChR) or normal IgG preabsorbed to protein A/G Plus-agarose. Immune complexes were washed, mixed with SDS sample buffer, and analyzed by Western blot.

Short interfering RNA treatment. The PPARβ/δ short interfering RNA (siRNA), α7 nAChR siRNA, AP-2α siRNA, Sp1 siRNA, and control siRNA were purchased from Santa Cruz Biotechnology. Cells (70% confluence) were transfected with PPARβ/δ, α7 nAChR, AP-2α, Sp1, or control siRNAs using Lipofectamine 2000 reagent (Invitrogen). Briefly, Lipofectamine was incubated with serum-free medium for 5 min, mixed with siRNA (100 nmol/L), incubated for 20 min at room temperature before the mixture was diluted with medium, and added to cells. After culturing for 40 h, cells were washed, resuspended in fresh medium, treated with or without nicotine for an additional 24 h, and analyzed by Western blot and cell viability assay.

Transient transfection assays. Human PPARβ/δ promoter deletion and mutation constructs ligated to a luciferase reporter gene (pGL-PPARβ/δ −1880 luc, −587 luc, −455 luc, −227 luc) have been reported previously (22). Briefly, NSCLC cells (5×10^5 per well, 50–60% confluence) were transfected with PPARβ/δ plasmid DNA (2 μg/well) and internal control phRL-TK *Renilla* luciferase reporter DNA (0.1 μg/well) using Lipofectamine reagent as previously described (23). In expression experiments, cells were transfected with control pBABE puro or pBABEpuro PPARβ/δ plasmid (2 μg/well each) or with AP-2 expression reporter construct SP (RSV; Addgene, Inc.; refs. 24, 25) for 24 h and treated with or without nicotine (0.1 μmol/L) for an additional 24 h before luciferase activity was determined using the Dual-Luciferase Reporter kit (Promega). Firefly luciferase activity was normalized with *Renilla* luciferase activity within each sample.

Cell viability assay. NSCLC cells transfected with PPARβ/δ siRNAs for 40 h were exposed to nicotine (100 μmol/L) for an additional 72 h in 96-well plates. Afterwards, cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) and recorded as relative luciferase units.

Electrophoretic mobility shift assays. Nuclear extracts from NSCLC cells treated with or without nicotine were prepared for electrophoretic mobility shift assay (EMSA) as described (23). Double-stranded oligonucleotides for AP-2 and Sp1 were as follows: wild-type AP-2, 5′-TCCTCCCCGCCTCCGC; mutant AP-2, TCCTCtttGCCTCCGC; wild-type Sp1, 5′-GGCCCCCACGGGCGGG; and mutant Sp1, 5′-GGCCCCCACGGttGGG. Nuclear protein (5 μg) was incubated with ^{32}P-labeled oligonucleotide probe with or without AP-2α or Sp1 antibodies (2 μg/μL). For cold competition, 100-fold excess of the respective unlabeled oligonucleotide was added before adding probe. Mutated labeled oligonucleotide or 100-fold excess mutated or nonmutated oligonucleotide was used as another control.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (26). Briefly, cells were incubated in 1% formaldehyde for 10 min at 37°C, quenched with 125 mmol/L glycine, lysed in SDS buffer with protease inhibitors (Roche) and 0.5 mmol/L phenylmethylsulfonyl fluoride, and sonicated. Fragmented chromatin was precleared by adding salmon sperm-DNA/protein A-agarose beads. A portion of the supernatant was kept as "input" material. The remaining cleared chromatin was incubated overnight with or without 5 μg of anti–AP-2α antibody or normal human IgG (Upstate Biotechnology). DNA (10 μg) from each immunoprecipitation was reserved for input controls. DNA was purified with QIAquick PCR purification column (Qiagen) and sequences of interest were amplified by PCR using the following primers: forward (−1683 to −1669 bp), 5′-TCGGGCTCTAATATCCGCC; reverse (−1543 to −1528 bp), 5′-CCTCTCGTCGCACTGAAAC.

Statistical analysis. All experiments were repeated a minimum of three times. All data were expressed as mean ± SD. The data presented in some figures are from a representative experiment that was qualitatively similar in the replicate experiments. Statistical significance was determined with Student's *t* test (two tailed) comparison between two groups of data sets. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition ($P < 0.05$, see figure legends).

Results

Nicotine increases PPARβ/δ protein expression through α7 nAChR. Because of data implicating PPARβ/δ in NSCLC proliferation, we explored the role of this nuclear transcription factor in mediating the effect of nicotine. We began by evaluating the effect of nicotine on PPARβ/δ expression in NSCLC cells. Western blot analysis revealed a time- and dose-dependent induction of PPARβ/δ protein by nicotine with a significant increase at 24 hours in the presence of 10^{-7} mol/L nicotine in H1838 cells (Fig. 1*A*). Similar results were also observed in other NSCLC cells (Fig. 1*B*).

Having established that nicotine induces PPARβ/δ expression, we set out to investigate the mechanisms responsible for nicotine-induced PPARβ/δ expression. We and others have reported nicotine induction of α7 nAChR–dependent signals in several cancer cell types, including lung cancer (6, 19). Thus, we speculated that nicotine induces PPARβ/δ expression by activating α7 nAChRs. To test this, α-bungarotoxin, an inhibitor of α7 nAChR, and PUN282987, a selective α7 nAChR agonist, were used. We found that α-bungarotoxin, used at doses previously shown to be noncytotoxic (19, 27), inhibited, whereas PUN282987 enhanced, the effect of nicotine on PPARβ/δ protein expression (Fig. 1*C, top*). Note that PUN282987 alone slightly induced PPARβ/δ protein expression (Fig. 1*C, top*), although not as efficiently as nicotine. Silencing α7 nAChR by siRNA also blocked the stimulatory effect of

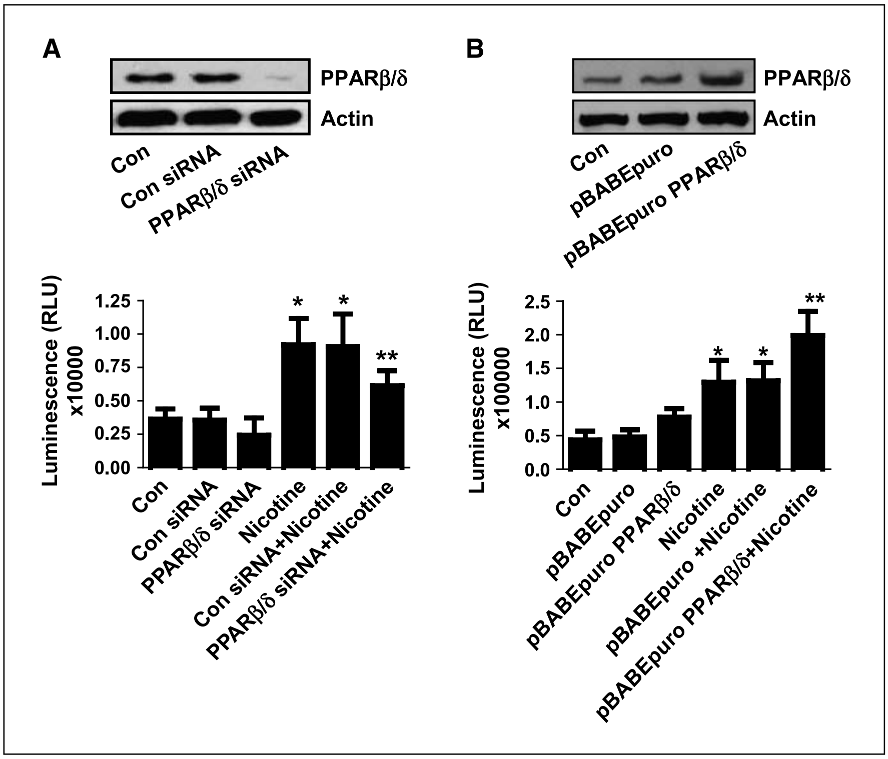

Figure 2. The role of PPARβ/δ in mediating the effect of nicotine on cell growth. *A,* H1838 cells were transfected with control or PPARβ/δ siRNA (100 nmol/L each) for 40 h before exposure to 100 μmol/L nicotine for up to 5 d. Afterwards, viable cells were detected using CellTiter-Glo Luminescent Cell Viability Assay kit. *Inset,* Western blot results for PPARβ/δ. *B,* H1838 cells were transfected with pBABE puro or pBABEpuro PPARβ/δ plasmid (2 μg/well each) using Lipofectamine 2000 for 24 h and treated with 100 μmol/L nicotine for up to 5 d, and viable cells were detected using CellTiter-Glo Luminescent Cell Viability Assay kit. *Inset,* Western blot results for PPARβ/δ protein. *Columns,* mean; *bars,* SD. *, significant difference from untreated control condition; **, significance of combination treatment compared with nicotine alone.

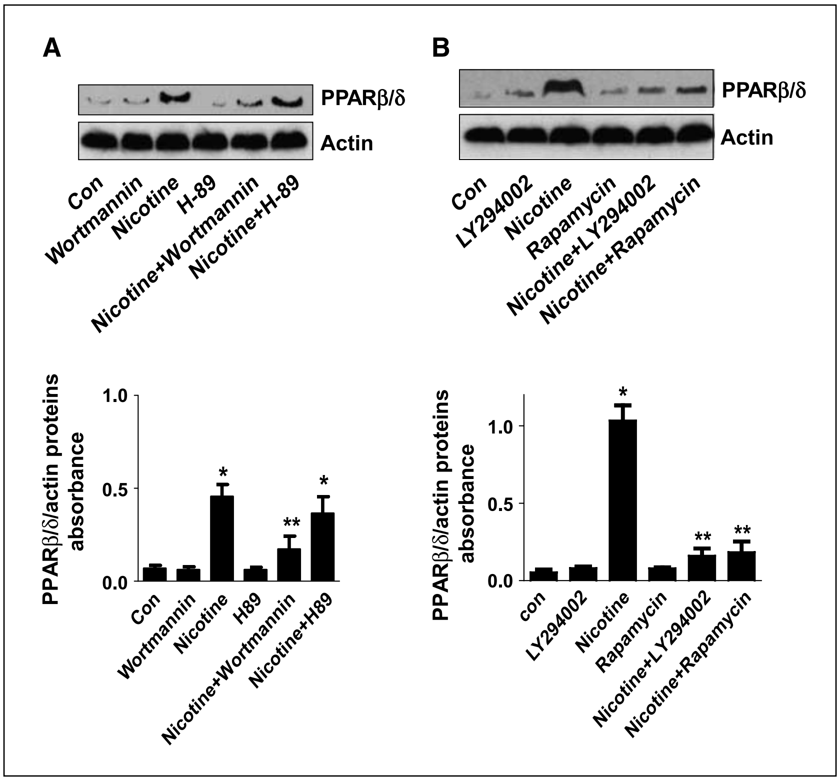

Figure 3. Involvement of Akt and mTOR in the induction of PPARβ/δ expression by nicotine. *A,* protein isolated from H1838 cells treated with wortmannin (0.2 μmol/L) or H-89 (10 μmol/L) for 2 h before exposure to nicotine for an additional 24 h was assayed by Western blot using anti-PPARβ/δ antibodies. *B,* protein was isolated from H1838 cells treated with LY294002 (25 μmol/L) or rapamycin (20 nmol/L) for 2 h before exposure to nicotine for an additional 24 h. Afterwards, Western blot analysis was performed using anti-PPARβ/δ antibodies. *Columns,* mean of PPARβ/δ/actin of at least three independent experiments; *bars,* SD. *, significant difference from untreated control; **, significance of combination treatment compared with nicotine alone.

nicotine on PPARβ/δ expression (Fig. 1*C, bottom*). Note that control siRNA had no effect, and in line with other reports in lung fibroblasts and human bronchial epithelial cells (28, 29), nicotine stimulated the expression of α7 nAChR (Fig. 1*C*). Similar results were also observed in H1792 NSCLC cells (Supplementary Fig. S1*A* and *C*).

Next, we examined whether this process was associated with PPARβ/δ and α7 nAChR protein-protein interactions. Coimmunoprecipitation experiments showed that the interaction between PPARβ/δ and α7 nAChR was enhanced by nicotine (Fig. 1*D*). Note that the control IgG had no effect (Fig. 1*D*).

PPARβ/δ siRNA attenuates, whereas overexpression of PPARβ/δ enhances, the effect of nicotine on cell growth. Because nicotine stimulates NSCLC cell proliferation, we set out to examine the role of PPARβ/δ in this process. We found that the stimulatory effect of nicotine on cell proliferation was significantly reduced in cells silenced for the *PPARβ/δ* gene using PPARβ/δ siRNA, whereas control siRNA had no effect (Fig. 2*A*). Note that PPARβ/δ siRNA slightly reduced cell proliferation at baseline (Fig. 2*A*). The PPARβ/δ expression vector induced proliferation slightly but significantly enhanced the stimulatory effect of nicotine (Fig. 2*B*). The control vector (p-BABEpuro) had no effect. Similar results were also observed in H1792 NSCLC cells (Supplementary Fig. S2*A* and *B*).

Involvement of PI3K and mTOR in the induction of PPARβ/δ expression by nicotine. Because of the role of multiple kinase signals in mediating the effect of nicotine on lung carcinoma cell growth, we tested whether the regulation of PPARβ/δ expression by nicotine was mediated by these pathways. Western blot analysis revealed that nicotine-induced PPARβ/δ protein expression was inhibited in the presence of the PI3K inhibitors wortmannin (0.2 μmol/L) and LY294002 (25 μmol/L) and the mTOR inhibitor rapamycin (20 nmol/L; Fig. 3*A* and *B*). These findings indicate that the stimulatory effects of nicotine on PPARβ/δ are associated with the activation of the PI3K and mTOR signaling pathways. In contrast, the PKA inhibitor H-89 (10 μmol/L) had no effect (Fig. 3*A*). Similar results were also observed in H1792 NSCLC cells (Supplementary Fig. S3).

Nicotine increases PPARβ/δ promoter activity. We next examined whether the effects of nicotine on PPARβ/δ expression occur at the transcriptional level. The PPARβ/δ gene promoter contains multiple transcription factor binding sites, including AP-2, AP-1, C/EBP, and Sp1 (Fig. 4*A*), and is differentially responsive to various stimuli (22, 30). We found that H1838 cells transfected with wild-type PPARβ/δ promoter (PPARβ/δ −1880) showed increased reporter activity in response to nicotine; this was not observed with the PPARβ/δ deletion promoter constructs (PPARβ/δ −587 and −445) lacking several distal AP-2 sites, suggesting a role for these AP-2 sites (Fig. 4*B*). Interestingly, we found a significant induction in PPARβ/δ promoter activity in cells transfected with the PPARβ/δ −227 promoter construct, suggesting a role for Sp1 (Fig. 4*B*). This increase in PPARβ/δ promoter activity was reduced by α-bungarotoxin, wortmannin, and rapamycin (Fig. 4*C*). Similar results were also observed in H1792 NSCLC cells (Supplementary Fig. S4*B*).

The role of AP-2 in mediating the effect of nicotine on PPARβ/δ expression. To further define the effect of nicotine on PPARβ/δ gene transcription, we tested the role of transcription factors AP-2 and Sp1 in mediating the effect of nicotine. We found that nicotine reduced AP-2α, whereas it had little effect on AP-2β, AP-2γ, and Sp1 protein expression (Fig. 5*A, top*). Note that nicotine also reduced AP-2α mRNA levels (Supplementary Fig. S5*A*). Silencing of AP-2α by siRNA enhanced the effect of nicotine on PPARβ/δ protein expression (Fig. 5*A, bottom*) and promoter activity (Fig. 5*B, left*). On the contrary, cells transfected with the AP-2 expression construct SP(RSV)AP-2 had reduced PPARβ/δ promoter activity and protein expression (Fig. 5*B, right*). Note that control vector had no effect on other AP-2 family members, such as AP-2β and AP-2γ (data not shown). Next, gel mobility shift assays showed that nicotine reduced AP-2 DNA binding, which was blocked by wortmannin, LY294002, and rapamycin (Fig. 5*C*). Furthermore, ChIP assay confirmed that nicotine reduced AP-2α binding in the promoter of *PPARβ/δ* gene (Fig. 5*D*). However, the use of anti-PPARβ/δ or AP-1 antibodies in ChIP analysis (data not shown) did not show binding, suggesting specificity of AP-2α binding. Similar results were also observed in H1792 NSCLC cells (Supplementary Fig. S5*A–D*).

The role of Sp1 in modulating the effect of nicotine on PPARβ/δ expression. We also assessed the role of Sp1 in the induction of PPARβ/δ expression by nicotine. Interestingly, we showed that the Sp1 inhibitor mithramycin A diminished the effect of nicotine on PPARβ/δ and AP-2α protein expression (Fig. 6*A, top*) and PPARβ/δ promoter activity (Fig. 6*A, bottom*). Similarly, silencing of Sp1 by siRNA also abrogated the effect of nicotine on PPARβ/δ promoter activity (Fig. 6*B*). This suggested that Sp1 is required for mediating the full effect of nicotine on PPARβ/δ and AP-2α. Furthermore, EMSA analysis showed increased Sp1 binding to AP-2 promoter sequences (Fig. 6*C, top*) but not to Sp1 sequences with nicotine treatment (Fig. 6*C, bottom*). Similar results were also observed in H1792 NSCLC cell line (Supplementary Fig. S6*A–C*).

Discussion

Although nicotine is not a carcinogen by itself, it has been shown to induce tumor cell proliferation and differentiation (31, 32). The mitogenic effects of nicotine in NSCLC are analogous to those of growth factors and involve activation of multiple signaling pathways (7, 8). nAChRs seem to play an important role in mediating the effects of nicotine on cell proliferation and survival. Consistent with reports in lung fibroblasts and human bronchial epithelial cells (28, 29), nicotine up-regulates α7 nAChR expression in NSCLC cells, which could amplify the effects of nicotine. We have reported that nicotine also stimulates NSCLC proliferation through the induction of fibronectin, a matrix glycoprotein highly expressed in acute and chronic forms of lung disease that has been implicated in the biology of lung cancer (19).

Herein, we show that nicotine induces NSCLC cell proliferation by stimulating the expression of PPARβ/δ. As a member of the

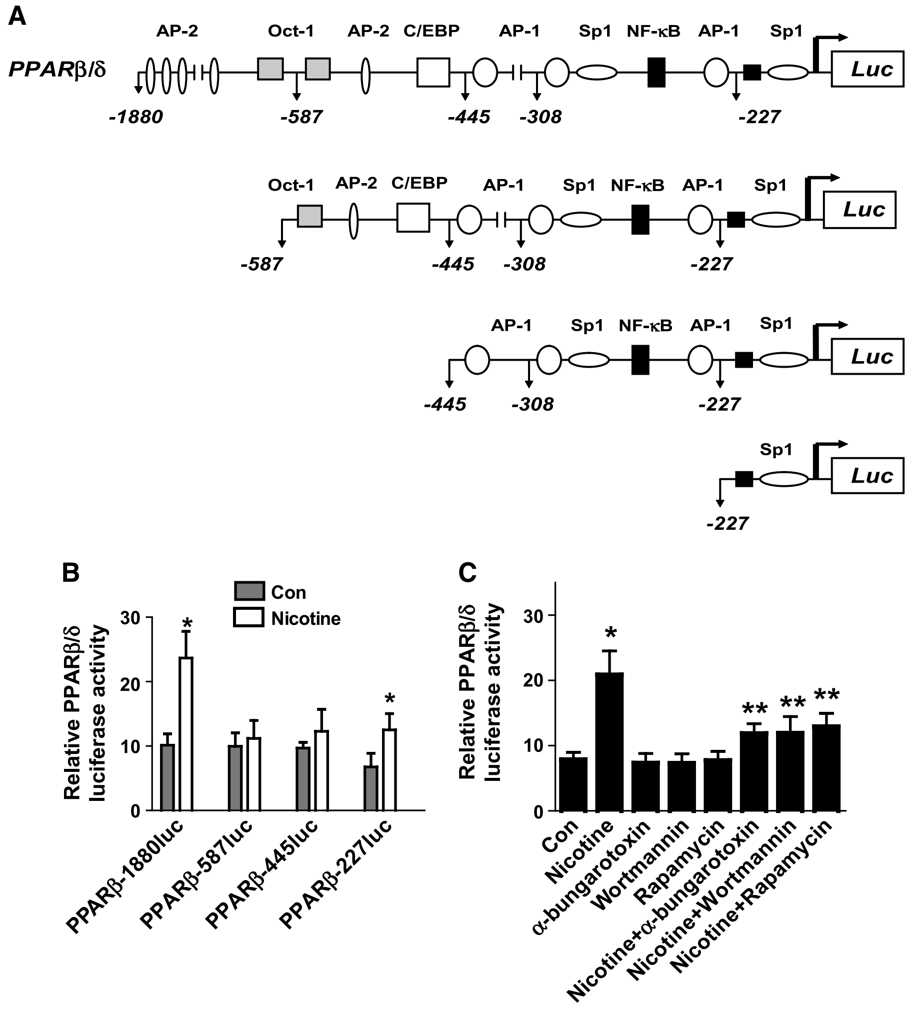

Figure 4. Nicotine increases PPARβ/δ promoter activity. *A,* schematic of human PPARβ/δ promoter constructs. H1838 cells transfected with wild-type or deletion PPARβ/δ promoter luciferase constructs along with an internal control *Renilla* reporter vector were treated (*B*) with or without nicotine for 24 h or (*C*) with α-bungarotoxin (1 μmol/L), wortmannin (0.2 μmol/L), or rapamycin (20 nmol/L) for 2 h before exposure to nicotine. *Columns,* mean of four independent experiments; *bars,* SD. *, significant increase of activity compared with controls; **, significance of combination treatment compared with nicotine alone.

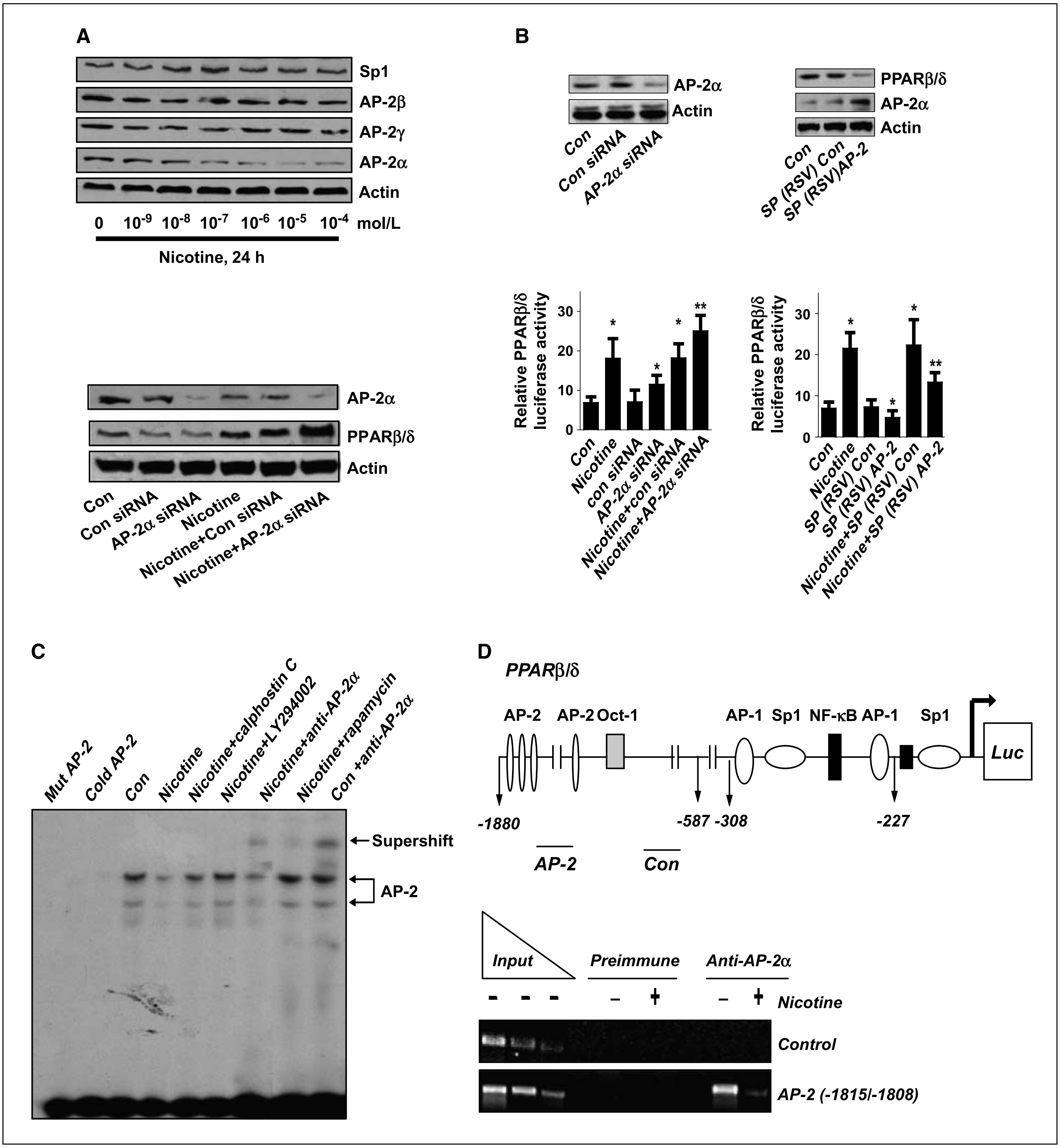

Figure 5. The role of AP-2α in mediating the effect of nicotine on PPARβ/δ expression. *A,* protein isolated from H1838 cells treated with nicotine (0.1 μmol/L) for 24 h (*top*) or transfected with AP-2α siRNA or control siRNA for 40 h followed by exposure to nicotine for 24 h (*bottom*) was assayed by Western blot using antibodies against AP-2α, AP-2β, AP-2γ, Sp1, and PPARβ/δ protein. *B,* H1838 cells transfected with AP-2α or control siRNA and exposed to nicotine for 24 h (*left*) were retransfected with control or AP-2 expression constructs [SP(RSV)AP-2] along with an internal *Renilla* control reporter vector and then treated with vehicle or nicotine for an additional 24 h (*right*). *Inset,* Western blot results for PPARβ/δ and AP-2α protein. *Columns,* mean of at least four independent experiments; *bars,* SD. Firefly/*Renilla* luciferase activity was quantified for normalization purposes. *, significant increase of activity compared with controls; **, significance of combination treatment compared with nicotine alone. *C,* AP-2 oligonucleotides were end labeled with [γ-^{32}P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with wortmannin (0.2 μmol/L), LY294002 (25 μmol/L), or rapamycin (20 nmol/L) for 2 h before exposure to 1 μmol/L nicotine for 24 h in the presence or absence of AP-2α antibody (2 μg/μL each). For competition assays, a molar excess (×100) of AP-2 (*Cold AP-2*) oligonucleotide was added to the binding reaction. Mutated AP-2-[γ-^{32}P]ATP (*Mut AP-2*) oligonucleotides were used to confirm binding specificity. *D,* nuclear protein from H1838 cells treated with nicotine (0.1 μmol/L) for 24 h was isolated and sonicated, and chromatin was immunoprecipitated using antibodies against AP-2α protein (*Anti-AP-2α*) or preimmune serum (*Preimmune*). PCR analysis indicates that AP-2α protein binds to the endogenous AP-2 site in this region of the PPARβ/δ promoter (−1683/−1528 bp). Non–AP-2 sequence was used as control. *Input,* aliquot of the chromatin analyzed before immunoprecipitation.

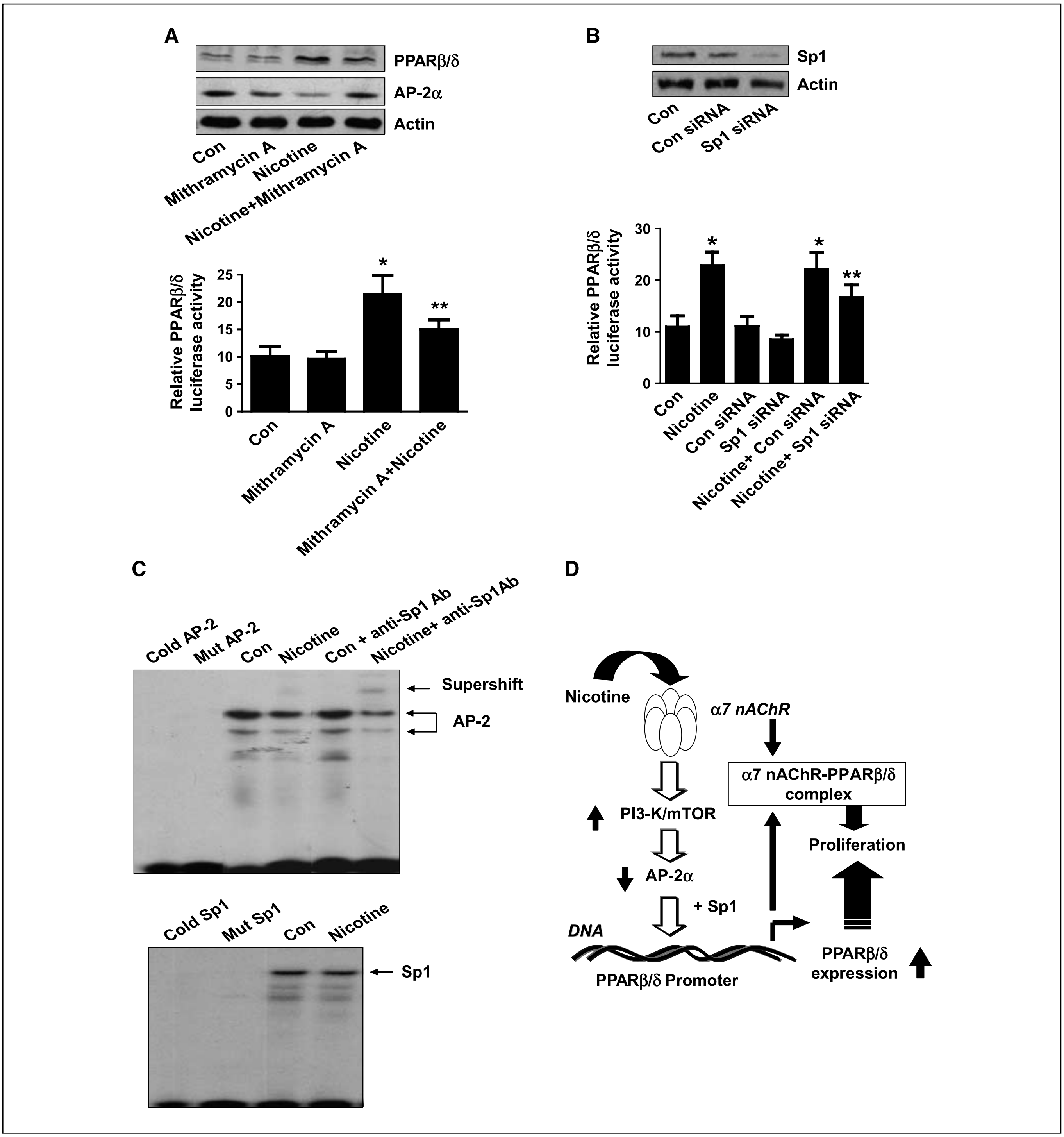

Figure 6. The role of Sp1 in mediating the effect of nicotine on PPARβ/δ expression. *A, top,* protein from H1838 cells treated with mithramycin A (100 nmol/L) for 2 h before exposure to nicotine (0.1 μmol/L) for 24 h was analyzed by Western blot for PPARβ/δ and AP-2α. H1838 cells were transfected with wild-type PPARβ/δ promoter construct (PPARβ/δ −1880 bp luc) and treated with or without mithramycin A (100 nmol/L) for 24 h followed by nicotine exposure for 24 h. Firefly/*Renilla* luciferase activity was quantified. *Columns,* mean of at least four independent experiments; *bars,* SD. *B, top,* H1838 cells transfected with control or Sp1 siRNA (100 nmol/L) for 30 h were analyzed by Western blot for Sp1 protein; *bottom,* H1838 cells transfected with control or Sp1 siRNA for 30 h were retransfected with the wild-type PPARβ/δ promoter construct (PPARβ/δ −1880 bp luc) along with an internal control *Renilla* vector and treated with or without nicotine (0.1 μmol/L) for an additional 24 h. The ratio of firefly/*Renilla* luciferase activity was quantified. *Columns,* mean of at least four independent experiments; *bars,* SD. *, significant increase of activity compared with controls; **, significance of combination treatment compared with nicotine alone. *C, top,* AP-2 oligonucleotides were end labeled with [γ-^{32}P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with nicotine (0.1 μmol/L) for 24 h in the presence or absence of Sp1 antibody (2 μg/μL each); *bottom,* Sp1 oligonucleotides were end labeled with [γ-^{32}P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with 0.1 μmol/L nicotine for 24 h. For competition assays, a molar excess (×100) of Sp1 (*Cold Sp1*) oligonucleotide was added to the binding reaction. Mutated Sp1 (*Mut Sp1*) oligonucleotides end labeled with [γ-^{32}P]ATP were used to confirm binding specificity. *D,* nicotine increases PPARβ/δ expression through α7 nAChR–mediated activation of PI3K and mTOR pathways and inhibition of AP-2α expression and DNA binding activity in the PPARβ/δ gene promoter. Sp1 modulates these processes. Nicotine also enhances the formation of α7 nAChR and PPARβ/δ protein complex. In turn, this may further stimulate NSCLC cell proliferation.

nuclear hormone receptor superfamily of transcription factors, PPARβ/δ has been implicated in several processes, including insulin sensitivity, terminal differentiation, and tumor growth (15, 20, 33). We report that silencing of PPARβ/δ inhibited, whereas overexpression of PPARβ/δ enhanced, the mitogenic effect of nicotine, showing a tumor-promoting role for PPARβ/δ in mediating the effect of nicotine on cell growth. In line with this finding, one recent study showed that PPARβ/δ is strongly expressed in the majority of lung cancers, and activation of PPARβ/δ induces NSCLC cell proliferation and survival (34). It should be highlighted that results implicating PPARβ/δ activation in the up-regulation of lung carcinoma cell growth (20, 34) contradict those reported elsewhere in which a decrease in lung cancer cell proliferation was observed (35). That particular work was performed in another lung carcinoma cell line (A549) and with the use of L-165041, a PPARβ/δ agonist. Note that L-165041 has also been shown to act as an agonist to PPARγ, which is known to reduce tumor cell proliferation (36).

Our observations that PPARβ/δ and α7 nAChR interact and that this is enhanced by nicotine are intriguing. This suggests the possibility that some PPARβ/δ recycles to cytoplasm and interacts with α7nAChR, which may be a potential mechanism for enhancing the stimulatory effect of nicotine on cell growth. This mechanism needs to be explored further.

The intracellular pathways mediating the effect of nicotine on PPARβ/δ expression in NSCLC have not been elucidated. The PI3K/Akt pathway is a critical pathway in cancer because it contributes to tumor growth, invasion, metastasis, and tumor angiogenesis (37). Therefore, targeting this pathway may represent an attractive strategy for novel anticancer therapies. Akt serves at a key point in the PI3K pathway and is likely important for the development and maintenance of lung cancer (6). mTOR also plays a central role in modulating cellular proliferation and angiogenesis in normal tissues and neoplastic processes (38). Nicotine activation of Akt increased phosphorylation of multiple downstream signals, including mTOR. Moreover, nicotine was found to stimulate Akt-dependent proliferation in lung cancer cells (6). The current report suggests that targeting these signaling pathways inhibits nicotine-induced PPARβ/δ expression. Together, our results highlight the involvement of α7 nAChR and PI3K/mTOR signaling in mediating the stimulatory effect of nicotine on PPARβ/δ expression.

Several transcription factor binding sites within regions of the PPARβ/δ promoter have been characterized, including regulatory elements for AP-2, C/EBP, and Sp1 (22, 30). Our findings show a critical role for AP-2α in mediating the effect of nicotine on the expression of PPARβ/δ. AP-2α proteins are essential biological factors during development, cell growth, differentiation, and apoptosis (39, 40). Loss of AP-2α expression has been associated with several invasion- and metastasis-promoting events (39, 40). Conversely, overexpression of AP-2α has been associated with survival in colon cancer cells (41). Our results showed that a reduction in AP-2α gene expression is needed for nicotine to stimulate PPARβ/δ. Supershift and ChIP assays highlight the key role of AP-2α transactivation in the regulation of PPARβ/δ promoter activity by nicotine. Additional studies using site-directed mutagenesis of key AP-2 sites are required to confirm their role in nicotine-induced PPARβ/δ expression. However, this is consistent with the work of others suggesting that AP-2 acts as a tumor suppressor.

Interestingly, our results also suggested a role for Sp1 in regulation of PPARβ/δ by nicotine. Sp1 regulates activation of many genes involved in tumor growth, apoptosis, and angiogenesis. Down-regulation of Sp1 activity inhibited urokinase receptor expression and reduced the migration of breast cancer cells (42). Here, mithramycin A, a Sp1 inhibitor (43), seemed to block the inhibitory effect of nicotine on AP-2α protein expression via inhibition of Sp1 activity. Of note, whereas the PPARβ/δ −227 promoter construct showed induction by nicotine, the PPARβ/δ −587 and −445 promoter constructs did not, suggesting the presence of corepressors. Moreover, because nicotine induced the interaction between Sp1 and the AP-2 *cis*-acting element, additional mechanisms that enhance the effects of nicotine may exist as shown in previous studies where Sp1 and AP-2 interaction was required for gene expression (44, 45). Competitive binding between Sp1 and other transcription factors has also been shown to be important in the control of several other genes (46, 47). Together, these studies suggest that the existence of functional Sp1 and its interaction with AP-2α influence the stimulatory effect of nicotine on expression of PPARβ/δ.

In summary, we have shown that nicotine increases PPARβ/δ expression through α7 nAChR–mediated activation of PI3K and mTOR pathways and inhibition of AP-2α expression and DNA binding activity in the PPARβ/δ gene promoter. Sp1 seems to modulate these processes. Nicotine also enhances the formation of the α7 nAChR-PPARβ/δ protein complex (Fig. 6*D*). To our knowledge, this represents the first link between nicotine and the *PPARβ/δ* gene, thereby unveiling a novel mechanism by which nicotine stimulates NSCLC cell growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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