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Nicotine stimulates PPAR β/δ expression in human lung carcinoma cells through activation of PI3-K/mTOR and suppression of AP-2 α

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Abstract

We previously demonstrated that nicotine stimulates non small cell lung carcinoma (NSCLC) cell proliferation through nicotinic acetylcholine receptor (nAChR)-mediated signals. Activation of peroxisome proliferator-activated receptor beta/delta (PPAR β/δ) has also been shown to induce NSCLC cell growth. Here, we explore the potential link between nicotine and PPAR β/δ and report that nicotine increases the expression of PPAR β/δ protein; this effect was blocked by an $\alpha 7$ nAChR antagonist (α -bungarotoxin), by $\alpha 7$ nAChR siRNA, and by inhibitors of PI3-K (Wortmannin and LY294002), and mTOR (rapamycin). In contrast, this effect was enhanced by PUN282987, an $\alpha 7$ nAChR agonist. Silencing of PPAR β/δ attenuated the stimulatory effect of nicotine on cell growth which was overcome by transfection of an exogenous PPAR β/δ expression vector. Of note, nicotine induced complex formation between $\alpha 7$ nAChR and PPAR β/δ protein and increased PPAR β/δ gene promoter activity through inhibition of AP-2 α as demonstrated by reduced AP-2 α binding using electrophoretic gel mobility shift and ChIP assays. In addition, silencing of Sp1 attenuated the effect of nicotine on PPAR β/δ . Collectively, our results demonstrate that nicotine increases PPAR β/δ gene expression through $\alpha 7$ nAChR-mediated activation of PI3-K/mTOR signals that inhibit AP-2 α protein expression and DNA binding activity to the PPAR β/δ gene promoter. Sp1 appears to modulate this process. This study unveils a novel mechanism by which nicotine promotes human lung carcinoma cell growth.

Keywords

PPAR β/δ ; Nicotine; $\alpha 7$ nAChR; AP-2 α ; PI3-K/mTOR; human lung carcinoma cells

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

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chemotherapeutic agents for its treatment, its dismal five-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 cancer (NSCLC) demonstrates 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 appear to be carcinogenic by itself, its metabolism leads to the generation of potent carcinogens (5). Also, 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 (nAChRs) 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 (PPARs) 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 PPAR α and PPAR γ , the consequences of PPAR β/δ activation are not well known (10). PPAR β/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 PPAR β/δ has also been shown to increase human cancer growth including liver, colon, breast, prostate and lung, among others (14-16), although opposite results have also been observed (17,18).

We recently demonstrated that nicotine stimulated NSCLC cell proliferation through nAChR-mediated signals that include activation of the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3-K)/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 PPAR β/δ gene. We found that nicotine increased PPAR β/δ expression through $\alpha 7$ nAChR mediated PI3-K/mTOR activation that reduced AP-2 α and promoted tumor cell proliferation.

MATERIAL AND METHODS

Culture and Chemicals

The human NSCLC cell lines H1838, H1792, A549, H522, H358 were obtained from the American Type Culture Collection (Manassas, VA), grown in RPMI-1640 medium with 10% heat-inactivated as previously described (20). Polyclonal antibodies for Akt and phosphor-Akt (Ser473) were purchased from Cell Signaling (Beverly, MA). Polyclonal antibodies against PPAR β/δ , $\alpha 7$ nAChR, AP-2 α , AP-2 β , AP-2 γ , and Sp1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PI3-K inhibitors LY294002, Wortmannin, $\alpha 7$ nAChR antagonist α -bungarotoxin, protein kinase A (PKA) inhibitor H-89, and mTOR inhibitor rapamycin, were obtained from Calbiochem (San Diego, CA). The $\alpha 7$ nAChR agonist, PUN282987, was purchased from TOCRIS Bioscience (Ellisville, Missouri). 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 2x 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 three times with wash buffer (1x TBST). Blots were incubated with

primary antibodies against PPAR β/δ , $\alpha 7$ nAChR, AP-2 α , AP-2 β , AP-2 γ or Sp1 (1:1000) 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 ECL solution (Pierce, Rockford IL), exposed to X-ray film and proteins were quantified by densitometric scanning using a Bio-Rad GS-800 calibrated densitometer.

Immunoprecipitation assays (IP)

Protein from NSCLC cells treated with or without nicotine for 24 h were harvested in RIPA buffer (Santa Cruz Biotechnology), sonicated, centrifuged at $12,000 \times g$ for 15 min at 4°C and the supernatant removed for IP. Protein (200 μ g) was pre-cleared for 30 min with 30 μ l of Protein A/G Plus-agarose (sc-2003, Santa Cruz Biotechnology) and incubated for 1 h at 4°C with appropriate antibodies (anti-PPAR β/δ and anti- $\alpha 7$ 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 (siRNA) treatment

The PPAR β/δ siRNA (sc-36305), $\alpha 7$ nAChR siRNA (sc-42532), AP-2 α siRNA (sc-29200), Sp1 siRNA (sc-29487), control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. Cells (70% confluence) were transfected with PPAR β/δ , $\alpha 7$ nAChR, AP-2 α , Sp1 or control siRNAs using Lipofectamine 2000 reagent (Invitrogen). Briefly, Lipofectamine was incubated with serum-free medium for 10 min., mixed with siRNA (100 nM), incubated for 15 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 media, treated with or without nicotine for an additional 24 h and analyzed by Western Blot and Cell viability assay.

Transient transfection assays

Human PPAR β/δ promoter deletion and mutation constructs ligated to a luciferase reporter gene (pGL-PPAR β/δ -1880 luc, -587 luc, -455 luc, -227 luc) have been reported previously (22). Briefly, NSCLC cells (5×10^5 cells/well, 50-60% confluence) were transfected with PPAR β/δ plasmid DNA (2 μ g/well) or internal control phRL-TK Renilla Luciferase Reporter DNA (0.1 μ g/well) using Lipofectamine reagent as previously described (23). In expression experiments, cells were transfected with control pBABE puro or pBABEpuro PPAR β/δ plasmid (2 μ g/ μ l each), or with AP-2 expression reporter construct SP (RSV) (12100) (Addgene, Inc.; Cambridge, MA) (24,25) for 24 h, treated with or without nicotine (0.1 μ M) 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 PPAR β/δ siRNAs for 40 h were exposed to nicotine (100 μ M) for an additional 72 h in 96-well plates. Afterwards, cell viability was measured using The CellTiter-Glo Luminescent Cell Viability Assay kit (Promega).

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from NSCLC cells treated with or without nicotine were prepared for EMSA as described (23). Double-stranded oligonucleotides for AP-2, Sp1 were as follows: Wild type AP-2 (5'-TCCTCCCCGCCTCCGC), Mutant AP-2 (TCCTCtttGCCTCCGC); Wild type Sp1 (5'-GGCCCCACGGGCGGG), Mutant Sp1 (5'- GGCCCCACGGtGGG). Nuclear protein (5 μ g) was incubated with 32 P-labeled oligonucleotide probe with or without AP-2 α or Sp1 antibodies (2 μ g/ μ l). 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 non-mutated oligonucleotide was used as another control.

Chromatin immunoprecipitation assay (ChIP)

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), 0.5 mmol/L phenylmethyl-sulfonyl fluoride and sonicated. Fragmented chromatin was pre-cleared 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 Sciences, MA) 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 means ± SD. The data presented in some figures are from a representative experiment which 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 h in the presence of 10^{-7} M nicotine in H1838 cells (Fig. 1A). Similar results were also observed in other NSCLC cells (Fig. 1B).

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 non-cytotoxic (19,27), inhibited, while PUN282987 enhanced the effect of nicotine on PPARβ/δ protein expression (Fig. 1C, upper panel). Note that PUN282987 alone slightly induced PPARβ/δ protein expression (Fig. 1C, upper panel) although not as efficiently as nicotine. Silencing α7 nAChR by siRNA also blocked the stimulatory effect of nicotine on PPARβ/δ expression (Fig. 1C, lower panel). 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. 1C). Similar results were also observed in H1792 NSCLC cells (Supplementary Fig. 1A, C).

Next, we examined whether this process was associated with PPARβ/δ and α7 nAChR protein-protein interactions. Co-immunoprecipitation experiments showed that the interaction between

PPAR β/δ and $\alpha 7$ nAChR was enhanced by nicotine (Fig. 1D). Note that the control IgG had no effect (Fig. 1D).

PPAR β/δ siRNA attenuates, while overexpression of PPAR β/δ enhances the effect of nicotine on cell growth

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

Involvement of PI3-K and mTOR in the induction of PPAR β/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 PPAR β/δ expression by nicotine was mediated by these pathways. Western blot analysis revealed nicotine-induced PPAR β/δ protein expression was inhibited in the presence of the PI3-K inhibitors, Wortmannin (0.2 μ M) and LY294002 (25 μ M), and the mTOR inhibitor, rapamycin (20 nM) (Fig. 3A and 3B). These findings indicate that the stimulatory effects of nicotine on PPAR β/δ are associated with the activation of the PI3-K and mTOR signaling pathways. In contrast, the PKA inhibitor H-89 (10 μ M), had no effect (Fig. 3A). Similar results were also observed in H1792 NSCLC cells (Supplementary Fig. 3).

Nicotine increases PPAR β/δ promoter activity

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

The role of AP-2 in mediating the effect of nicotine on PPAR β/δ expression

In order to further define the effect of nicotine on PPAR β/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-2 α , while it had little effect on AP-2 β , AP-2 γ , and Sp1 protein expression (Fig. 5A, upper panel). Note that nicotine also reduced AP-2 α mRNA levels (Supplementary Fig. 5A). Silencing of AP-2 α by siRNA enhanced the effect of nicotine on PPAR β/δ protein expression (Fig. 5A, lower panel) and promoter activity (Fig. 5B, left panel). On the contrary, cells transfected with the AP-2 expression construct, SP(RSV)AP-2, had reduced PPAR β/δ promoter activity and protein expression (Fig. 5B, right panel). Note that control vector had no effect on other AP-2 family members such as AP-2 β and AP-2 γ (not shown). Next, gel mobility shift assays showed nicotine reduced AP-2 DNA binding which was blocked by Wortmannin, LY294002, and rapamycin (Fig. 5C). Furthermore, Chip assay confirmed that

nicotine reduced AP-2 α binding in the promoter of PPAR β/δ gene (Fig. 5D). However, the use of anti-PPAR β/δ or AP-1 antibodies in ChIP analysis (not shown) did not show binding, suggesting specificity of AP-2 α binding. Similar results were also observed in H1792 NSCLC cells (Supplementary Fig. 5A-D).

The role of Sp1 in modulating the effect of nicotine on PPAR β/δ expression

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

DISCUSSION

While 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 appear 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 upregulates $\alpha 7$ 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 PPAR β/δ . As a member of the nuclear hormone receptor superfamily of transcription factors, PPAR β/δ has been implicated in several processes including insulin sensitivity, terminal differentiation, and tumor growth (15,20,33). We report that silencing of PPAR β/δ inhibited, while overexpression of PPAR β/δ enhanced the mitogenic effect of nicotine, demonstrating a tumor promoting role for PPAR β/δ in mediating the effect of nicotine on cell growth. In line with this finding, one recent study showed that PPAR β/δ is strongly expressed in the majority of lung cancers, and activation of PPAR β/δ induces NSCLC cell proliferation and survival (34). It should be highlighted that results implicating PPAR β/δ activation in the upregulation 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 PPAR β/δ agonist. Note that L-165041 has also been shown to act as an agonist to PPAR γ , which is known to reduce tumor cell proliferation (36).

Our observations that PPAR β/δ and $\alpha 7$ nAChR interact and that this is enhanced by nicotine are intriguing. This suggests the possibility that some PPAR β/δ recycles to cytoplasm and interacts with $\alpha 7$ nAChR, 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 PPAR β/δ expression in NSCLC have not been elucidated. The PI3-K/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 PI3-K 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 PPAR β/δ expression. Together, our results highlight the involvement of $\alpha 7$ nAChR and PI3-K/mTOR signaling in mediating the stimulatory effect of nicotine on PPAR β/δ expression.

Several transcription factor binding sites within regions of the PPAR β/δ promoter have been characterized including regulatory elements for AP-2, C/EBP, and Sp1, among others (22, 30). Our findings demonstrate a critical role for AP-2 α in mediating the effect of nicotine on the expression of PPAR β/δ . AP-2 α proteins are known to be essential biological factors during development, cell growth, differentiation, and apoptosis (39,40). Loss of AP-2 α expression has been associated with several invasion and metastasis promoting events (39,40). Conversely, overexpression of AP-2 α has been associated with survival in colon cancer cells (41). Our results demonstrated that a reduction in AP-2 α gene expression is needed for nicotine to stimulate PPAR β/δ . Supershift and ChIP assays highlight the key role of AP-2 α transactivation in the regulation of PPAR β/δ promoter activity by nicotine. Additional studies using sitedirected mutagenesis of key AP-2 sites are required to confirm their role in nicotine-induced PPAR β/δ expression. However, this is commitment 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 PPAR β/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), appeared to block the inhibitory effect of nicotine on AP-2 α protein expression via inhibition of Sp1 activity. Of note, while the PPAR β/δ -227 promoter construct showed induction by nicotine, the PPAR β/δ -587 and -445 promoter constructs did not, suggesting the presence of co-repressors. Moreover, since nicotine induced the interaction between Sp1 and the AP-2 cis-acting element, additional mechanisms that enhance the effects of nicotine may exist as demonstrated in previous studies where both 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-2 α influence the stimulatory effect of nicotine on expression of PPAR β/δ .

In summary, we have demonstrated that nicotine increases PPAR β/δ expression through $\alpha 7$ nAChR-mediated activation of PI3-K and mTOR pathways and inhibition of AP-2 α expression and DNA binding activity in the PPAR β/δ gene promoter. Sp1 appears to modulate these processes. Nicotine also enhances the formation of the $\alpha 7$ nAChR- PPAR β/δ protein complex (Fig. 6D). To our knowledge, this represents the first link between nicotine and the PPAR β/δ gene thereby unveiling a novel mechanism by which nicotine stimulates NSCLC cell growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Felip E, Rosell R. Testing for excision repair cross-complementing 1 in patients with non-small-cell lung cancer for chemotherapy response. *Expert Rev Mol Diagn* 2007;7:261–8. [PubMed: 17489733]
2. Socinski MA. Combined modality trials in unresectable stage III non-small cell lung cancer: the Cancer and Leukemia Group B experience. *Semin Oncol* 2005;32:S114–8. [PubMed: 16015547]
3. Sun S, Schiller JH, Spinola M, Minna JD. New molecularly targeted therapies for lung cancer. *J Clin Invest* 2007;117:2740–50. [PubMed: 17909619]
4. Meuwissen R, Berns A. Mouse models for human lung cancer. *Genes Dev* 2005;19:643–64. [PubMed: 15769940]
5. Li MD. The genetics of nicotine dependence. *Curr Psychiatry Rep* 2006;8:158–64. [PubMed: 16539894]
6. Tsurutani J, Castillo SS, Brognard J, et al. Tobacco components stimulate Akt-dependent proliferation and NFkappaB-dependent survival in lung cancer cells. *Carcinogenesis* 2005;26:1182–95. [PubMed: 15790591]
7. Dasgupta P, Chellappan SP. Nicotine-mediated cell proliferation and angiogenesis: new twists to an old story. *Cell Cycle* 2006;5:2324–8. [PubMed: 17102610]
8. Dasgupta P, Rastogi S, Pillai S, et al. Nicotine induces cell proliferation by beta-arrestin-mediated activation of Src and Rb-Raf-1 pathways. *J Clin Invest* 2006;116:2208–17. [PubMed: 16862215]
9. Semple RK, Chatterjee VK, O'Rahilly S. PPAR gamma and human metabolic disease. *J Clin Invest* 2006;116:581–9. [PubMed: 16511590]
10. Petrashevskaya NN, Schwarz A. Peroxisome proliferator-activated receptor beta/delta: a new antihypertrophic drug target? *Cardiovasc Res* 2005;65:770–1. [PubMed: 15721856]
11. Braissant O, Wahli W. Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. *Endocrinology* 1998;139:2748–54. [PubMed: 9607781]
12. Piqueras L, Reynolds AR, Hodivala-Dilke KM, et al. Activation of PPARbeta/delta induces endothelial cell proliferation and angiogenesis. *Arterioscler Thromb Vasc Biol* 2007;27:63–9. [PubMed: 17068288]
13. Kim DJ, Bility MT, Billin AN, Willson TM, Gonzalez FJ, Peters JM. PPARbeta/delta selectively induces differentiation and inhibits cell proliferation. *Cell Death Differ* 2006;13:53–60. [PubMed: 16021179]
14. Aung CS, Faddy HM, Lister EJ, Monteith GR, Roberts-Thomson SJ. Isoform specific changes in PPAR alpha and beta in colon and breast cancer with differentiation. *Biochem Biophys Res Commun* 2006;340:656–60. [PubMed: 16378595]
15. Stephen RL, Gustafsson MC, Jarvis M, et al. Activation of peroxisome proliferator-activated receptor delta stimulates the proliferation of human breast and prostate cancer cell lines. *Cancer Res* 2004;64:3162–70. [PubMed: 15126355]
16. Xu L, Han C, Lim K, Wu T. Cross-talk between peroxisome proliferator-activated receptor delta and cytosolic phospholipase A²alpha/cyclooxygenase-2/prostaglandin E² signaling pathways in human hepatocellular carcinoma cells. *Cancer Res* 2006;66:11859–68. [PubMed: 17178883]
17. Girroir EE, Hollingshead HE, Billin AN, et al. Peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta) ligands inhibit growth of UACC903 and MCF7 human cancer cell lines. *Toxicology* 2008;243:236–43. [PubMed: 18054822]
18. Marin HE, Peraza MA, Billin AN, et al. Ligand activation of peroxisome proliferator-activated receptor beta inhibits colon carcinogenesis. *Cancer Res* 2006;66:4394–401. [PubMed: 16618765]
19. Zheng Y, Ritzenthaler JD, Roman J, Han S. Nicotine stimulates human lung cancer cell growth by inducing fibronectin expression. *Am J Respir Cell Mol Biol* 2007;37:681–90. [PubMed: 17600315]
20. Han S, Ritzenthaler JD, Wingerd B, Roman J. Activation of peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) increases the expression of prostaglandin E2 receptor subtype

- EP4. The roles of phosphatidylinositol 3-kinase and CCAAT/enhancer-binding protein beta. *J Biol Chem* 2005;280:33240–9. [PubMed: 16061473]
21. Han SW, Lei ZM, Rao CV. Up-regulation of cyclooxygenase-2 gene expression by chorionic gonadotropin during the differentiation of human endometrial stromal cells into decidua. *Endocrinology* 1996;137:1791–7. [PubMed: 8612516]
 22. Tan NS, Michalik L, Di-Poi N, et al. Essential role of Smad3 in the inhibition of inflammation-induced PPARbeta/delta expression. *Embo J* 2004;23:4211–21. [PubMed: 15470497]
 23. Han S, Sidell N, Roser-Page S, Roman J. Fibronectin stimulates human lung carcinoma cell growth by inducing cyclooxygenase-2 (COX-2) expression. *Int J Cancer* 2004;111:322–31. [PubMed: 15221958]
 24. Brun RP, Tontonoz P, Forman BM, Ellis R, Chen J, Evans RM, Spiegelman BM. Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev* 1996;10:974–84. [PubMed: 8608944]
 25. Williams T, Tjian R. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev* 1991;5:670–82. [PubMed: 2010091]
 26. Zheng Y, Ritzenthaler JD, Sun X, Roman J, Han S. Prostaglandin E2 Stimulates Human Lung Carcinoma Cell Growth through Induction of Integrin-Linked Kinase: The Involvement of EP4 and Sp1. *Cancer Res* 2009;69:896–904. [PubMed: 19176380]
 27. Roman J, Ritzenthaler JD, Bechara R, Brown LA, Guidot D. Ethanol stimulates the expression of fibronectin in lung fibroblasts via kinase-dependent signals that activate CREB. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L975–87. [PubMed: 15653713]
 28. Roman J, Ritzenthaler JD, Gil-Acosta A, Rivera HN, Roser-Page S. Nicotine and fibronectin expression in lung fibroblasts: implications for tobacco-related lung tissue remodeling. *Faseb J* 2004;18:1436–8. [PubMed: 15247149]
 29. Wang Y, Pereira EF, Maus AD, et al. Human bronchial epithelial and endothelial cells express alpha7 nicotinic acetylcholine receptors. *Mol Pharmacol* 2001;60:1201–9. [PubMed: 11723227]
 30. Di-Poi N, Desvergne B, Michalik L, Wahli W. Transcriptional repression of peroxisome proliferator-activated receptor beta/delta in murine keratinocytes by CCAAT/enhancer-binding proteins. *J Biol Chem* 2005;280:38700–10. [PubMed: 16166081]
 31. Schuller HM. Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. *Biochem Pharmacol* 1989;38:3439–42. [PubMed: 2573356]
 32. Cattaneo MG, Codignola A, Vicentini LM, Clementi F, Sher E. Nicotine stimulates a serotonergic autocrine loop in human small-cell lung carcinoma. *Cancer Res* 1993;53:5566–8. [PubMed: 8221698]
 33. Burdick AD, Kim DJ, Peraza MA, Gonzalez FJ, Peters JM. The role of peroxisome proliferator-activated receptor-beta/delta in epithelial cell growth and differentiation. *Cell Signal* 2006;18:9–20. [PubMed: 16109478]
 34. Pedchenko TV, Gonzalez AL, Wang D, DuBois RN, Massion PP. Peroxisome proliferator-activated receptor beta/delta expression and activation in lung cancer. *Am J Respir Cell Mol Biol* 2008;39:689–96. [PubMed: 18566335]
 35. Fukumoto K, Yano Y, Virgona N, et al. Peroxisome proliferator-activated receptor delta as a molecular target to regulate lung cancer cell growth. *FEBS Lett* 2005;579:3829–36. [PubMed: 15978581]
 36. Wurch T, Junquero D, Delhon A, Pauwels J. Pharmacological analysis of wild-type alpha, gamma and delta subtypes of the human peroxisome proliferator-activated receptor. *Naunyn Schmiedeberg Arch Pharmacol* 2002;365:133–40. [PubMed: 11819031]
 37. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002;2:489–501. [PubMed: 12094235]
 38. Carraway H, Hidalgo M. New targets for therapy in breast cancer: mammalian target of rapamycin (mTOR) antagonists. *Breast Cancer Res* 2004;6:219–24. [PubMed: 15318929]
 39. Pellikainen JM, Kosma VM. Activator protein-2 in carcinogenesis with a special reference to breast cancer—a mini review. *Int J Cancer* 2007;120:2061–7. [PubMed: 17330235]

40. Orso F, Fassetta M, Penna E, et al. The AP-2alpha transcription factor regulates tumor cell migration and apoptosis. *Adv Exp Med Biol* 2007;604:87–95. [PubMed: 17695722]
41. Schwartz B, Melnikova VO, Tellez C, et al. Loss of AP-2alpha results in deregulation of E-cadherin and MMP-9 and an increase in tumorigenicity of colon cancer cells in vivo. *Oncogene* 2007;26:4049–58. [PubMed: 17224907]
42. Zannetti A, Del Vecchio S, Romanelli A, et al. Inhibition of Sp1 activity by a decoy PNA-DNA chimera prevents urokinase receptor expression and migration of breast cancer cells. *Biochem Pharmacol* 2005;70:1277–87. [PubMed: 16143315]
43. Ray R, Snyder RC, Thomas S, Koller CA, Miller DM. Mithramycin blocks protein binding and function of the SV40 early promoter. *J Clin Invest* 1989;83:2003–7. [PubMed: 2542379]
44. Zhang G, Veldhuis JD. Requirement for proximal putative Sp1 and AP-2 cisdeoxyribonucleic acid elements in mediating basal and luteinizing hormone- and insulin-dependent in vitro transcriptional activation of the CYP17 gene in porcine theca cells. *Endocrinology* 2004;145:2760–6. [PubMed: 15001547]
45. Xu Y, Porntadavity S, St Clair DK. Transcriptional regulation of the human manganese superoxide dismutase gene: the role of specificity protein 1 (Sp1) and activating protein-2 (AP-2). *Biochem J* 2002;362:401–12. [PubMed: 11853549]
46. Hirano F, Tanaka H, Hirano Y, et al. Functional interference of Sp1 and NF-kappaB through the same DNA binding site. *Mol Cell Biol* 1998;18:1266–74. [PubMed: 9488441]
47. Choi WI, Jeon BN, Park H, et al. Proto-oncogene FBI-1 (Pokemon) and SREBP-1 synergistically activate transcription of fatty-acid synthase gene (FASN). *J Biol Chem* 2008;283:29341–54. [PubMed: 18682402]

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 (**upper panel**) for the indicated period of time (**lower panel**) and PPARβ/δ protein was analyzed by Western blot. Actin was used for loading control. The bar graphs (**right panels**) represent the mean ± SD PPARβ/δ/actin of three independent experiments. * indicates 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 isolated from H1838 cells was cultured for 1 h in the presence or absence of α-bungarotoxin (1 μM), PUN282987 (0.1 μM) (**upper panel**), or transfected with control or α7 nAChR siRNAs (**lower panel**), exposure to nicotine (0.1 μM) for an additional 48 hr 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.

Figure 2. The role of PPAR β/δ in mediating the effect of nicotine on cell growth

A, H1838 cells were transfected with control or PPAR β/δ siRNA (100 nM each) for 40 h before exposure to 100 μ M nicotine for up to 5 days. Afterwards, viable cells were detected using Cell Titer-Glo Luminescent Cell Viability Assay Kit. Insert; Western Blot results for PPAR β/δ . *Con*, indicates untreated control cells. **B**, H1838 cells were transfected with pBABE puro or pBABEpuro PPAR β/δ plasmid (2 μ g/ μ l each) using Lipofectamine 2000 (Invitrogen) for 24 h, treated with 100 μ M nicotine for up to 5 days and viable cells were detected using Cell Titer-Glo Luminescent Cell Viability Assay Kit. Insert; Western Blot results for PPAR β/δ protein. *Con*, untreated control cells. All data are depicted as mean $\pm$ SD. * denotes significant difference from untreated control condition. ** indicates significance of combination treatment as compared to nicotine alone.

Figure 3. Involvement of Akt and mTOR in the induction of PPAR β/δ expression by nicotine
A, Protein was isolated from H1838 cells treated with LY294002 (25 μ M) or rapamycin (20 nM) for 2 h before exposure to nicotine for an additional 24 h. Afterwards, Western blot analysis was performed using anti-PPAR β/δ antibodies. **B**, Protein isolated from H1838 cells treated with Wortmannin (0.2 μ M) or H89 (10 μ M) for 2 h before exposure to nicotine for an additional 24 h was assayed by Western blot using anti-PPAR β/δ antibodies. Each bar (**lower panel**) represents the mean $\pm$ SD of PPAR β/δ /actin of at least three independent experiments. * indicates significant difference from untreated control. ** indicates significance of combination treatment as compared with nicotine alone. *Con*, untreated control cells.

Figure 4. Nicotine increases PPARβ/δ promoter activity

A, Schematic of human PPARβ/δ promoter constructs. **B**, H1838 cells transfected with wild type or deletion PPARβ/δ promoter luciferase constructs along with an internal control Renilla Reporter Vector were treated with or without nicotine for 24 h or **C**, treated with α-bungarotoxin (1 μM), Wortmannin (0.2 μM), or rapamycin (20 nM) for 2 h before exposure to nicotine. *Con*, untreated control cells. Bars represent the mean ± SD of four independent experiments. * indicates significant increase of activity as compared to controls. ** indicates significance of combination treatment as compared to the 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 μ M) for 24 h (**upper panel**), or transfected with AP-2 α siRNA or control siRNA for 40 h followed by exposure to nicotine for 24 h (**lower panel**) was assayed by Western Blot using antibodies against AP-2 α , AP-2 β , AP-2 γ , Sp1, and PPAR β/δ protein. **B**, H1838 cells transfected with AP-2 α or control siRNA and exposed to nicotine for 24 h (**left panel**), were re-transfected with control or AP-2 expression constructs (SP(RSV)AP-2) along with an internal Renilla control Reporter Vector then treated with vehicle or nicotine for an additional 24 h (**right panel**). Insert; upper panel represents Western blot results for PPAR β/δ and AP-2 α protein. Bars below represent the mean $\pm$ SD of at least four independent experiments. Firefly/renilla luciferase activity was quantified

for normalization purposes. * indicates significant increase of activity as compared to controls. ** indicates significance of combination treatment as compared to the nicotine alone. **C**, AP-2 oligonucleotides were end-labeled with [γ - 32 P]-ATP and incubated with nuclear extracts (5 μ g) from H1838 cells treated with Wortmannin (0.2 μ M), LY294002 (25 μ M) or rapamycin (20 nM) for 2 h before exposure to 1 μ M nicotine for 24 h in the absence or presence of AP-2 α antibody (2 μ g/ μ l each). For competition assays, a molar excess (x100) 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 for 24 h were isolated, sonicated and chromatin was immunoprecipitated using antibodies against AP-2 α protein (anti-AP-2 α) or preimmune serum (pre-immune). PCR analysis indicates that AP-2 α protein binds to the endogenous AP-2 site in this region of the PPAR β/δ promoter (-1683/-1528 bp). Non-AP-2 sequence was used as control. Input indicates aliquot of the chromatin analyzed before immunoprecipitation. *Con*, untreated control cells.

Figure 6. The role of Sp1 in mediating the effect of nicotine on PPAR β/δ expression

A, Protein from H1838 cells treated with mithramycin A (100 nM) for 2 h before exposure to nicotine for 24 h was analyzed by Western Blot for PPAR β/δ and AP-2 α (**upper panel**). H1838 cells were transfected with wild type PPAR β/δ promoter construct (PPAR β/δ -1880 bp luc) and treated with or without mithramycin A (100 nM) for 24 h, followed by nicotine exposure for 24 h. Firefly/renilla luciferase activity was quantified. Bars represent the mean $\pm$ SD of at least four independent experiments (**lower panel**). **B**, **Upper panel**, H1838 cells transfected with control or Sp1 siRNA (100 nM) for 30 h were analyzed by Western blot for Sp1 protein. **Lower panel**, H1838 cells transfected with control or Sp1 siRNA for 30 h were re-transfected with the wild type PPAR β/δ promoter construct (PPAR β/δ -1880 bp luc) along with an internal

control Renilla Vector and treated with or without nicotine for an additional 24 h. The ratio of firefly/renilla luciferase activity was quantified; bars represent mean $\pm$ SD of at least four independent experiments. * indicates significant increase of activity as compared to controls. ** indicates significance of combination treatment as compared to the nicotine alone. *Con*, untreated control cells. **C, Upper panel**, AP-2 oligonucleotides were endlabeled with [γ - 32 P]-ATP and incubated with nuclear extracts (5 μ g) from H1838 cells treated with nicotine for 24 h in the absence or presence of Sp1 antibody (2 μ g/ μ l each). **Lower panel**, Sp1 oligonucleotides were end-labeled with [γ - 32 P]-ATP and incubated with nuclear extracts (5 μ g) from H1838 cells treated with 0.1 μ M nicotine for 24 h. For competition assays, a molar excess (x100) of Sp1 (Cold Sp1) oligonucleotide was added to the binding reaction. Mutated Sp1 (Mut Sp1) oligonucleotides end-labeled with γ - 32 PATP were used to confirm binding specificity. *Con*, indicates untreated control cells. **D**, Diagram demonstrates that nicotine increases PPAR β/δ expression through α 7 nAChR-mediated activation of PI3-K and mTOR pathways and inhibition of AP-2 α expression and DNA binding activity in the PPAR β/δ gene promoter. Sp1 modulates these processes. Nicotine also enhances the formation of α 7 nAChR and PPAR β/δ protein complex. In turn, this may further stimulate NSCLC cell proliferation.