Rapid Activation of Stat3 and ERK1/2 by Nicotine Modulates Cell Proliferation in Human Bladder Cancer Cells

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Cigarette smoke is a major risk factor for bladder cancer. The main component in cigarette smoke, nicotine, can be detected in the urine of smokers. Nicotine has been implicated as a co-carcinogen that promotes lung cancer development through prosurvival pathways. Although the mechanisms of nicotine-induced cell proliferation have been well studied in lung epithelial cells, the molecular mechanism of its action in bladder epithelial cells is still unclear. The aims of this study were to investigate whether there is nicotine-induced bladder epithelial cell proliferation and to identify the signaling transduction pathway regulated by nicotine. We found that nicotine simultaneously activates Stat3 and extracellular signal regulated kinase 1/2 (ERK1/2) in T24 cells. Stat3 activation via nicotinic acetylcholine receptor (nAChR)/protein kinase C signaling pathway was closely linked to Stat3 induction and nuclear factor-kB DNA binding activity, which is associated with Cyclin D1 expression and cell proliferation. ERK1/2 activation through nAChR and β-adrenoceptors plays a dual role in cell proliferation; it phosphorylates Stat3 at Ser727 and regulates cell proliferation. We conclude that through nAChR and β-adrenoceptors, nicotine activates ERK1/2 and Stat3 signaling pathways, leading to Cyclin D1 expression and cell proliferation. This is the first study to investigate signaling effects of nicotine in bladder cells. The current findings suggest that people exposed to nicotine could be at risk for potential deleterious effects, including bladder cancer development.

Key Words: nicotine; bladder cancer; nicotinic acetylcholine receptor; Stat3; ERK1/2.

Nicotine has been linked to a variety of cell types, including bladder epithelial cells (Beckel et al., 2006). Growing evidence indicates that nicotine activates several mitogen-related signaling pathways upon its interaction with nAChR on the surface of several epithelial cells (Nakayama et al., 2002; West et al., 2003; Ye et al., 2004). Nicotine has also been reported to promote the growth of cancer cells through transactivation of β-adrenoceptors (Wong et al., 2007) and epidermal growth factor (EGF) receptor (Laag et al., 2006). Although nicotine-induced carcinogenesis has led to the activation of survival pathways in some cancer cells, the molecular mechanism(s) involved in nicotine-mediated bladder tumorigenesis remain unclear.

Previous studies demonstrate that nicotine can increase the cell numbers of certain cell lines (West et al., 2003; Ye et al., 2004). This suggests that nicotine exposure can lead to the disruption of the dynamic balance between cell death and proliferation, which is required for normal cellular functioning. In response to mitogenic stimuli, three major types of signal transduction pathways are activated in a cell, such as mitogen-activated protein kinase (MAPK) pathways (including extracellular signal regulated kinase 1/2 [ERK1/2], c-Jun NH2-terminal kinases 1/2 and p38MAPK), protein kinase C (PKC) pathways, and JAK/STAT pathways (JAK: the Janus family of tyrosine kinases; STAT: signal transducers and activators of transcription). Among these survival pathways, ERK1/2 is thought to be the major signaling pathway involved in nicotine-induced cell proliferation, which is also considered a precursor event in tumorigenesis (Shin et al., 2004). Apart from ERK1/2, Stat3, a member of the STAT family, is a key signal transduction protein that mediates signaling by numerous cytokines, growth factors and oncoproteins (Nadiminty et al., 2006). Upon stimulation, Stat3 is activated by tyrosine and serine phosphorylation, dimerizes and translocates to the nucleus, which it binds specific gene-promoter sequences and induces gene expression. Genes that are regulated by activated Stat3 include antiapoptotic and proliferation-associated genes such as Bcl-2, Bcl-2, Fas, Cyclin D1, Survivin, and c-Myc (Bromberg, 2002). A large body of evidence has further demonstrated that elevated Stat3 activity is frequently observed in a variety of human...
malignancies, including ovarian, prostate cancer, and bladder cancer (Bromberg, 2002; Campbell et al., 2001; Itoh et al., 2006). Although abnormal Stat3 activation has been proved to be an early event and a critical mediator of tumorigenesis, none of the studies has examined the effects of nicotine on Stat3 activation. Thus, one of the aims of the current study was to test whether nicotine could induce bladder epithelial cell proliferation through ERK1/2 and Stat3 signaling pathways.

It has been demonstrated that the Cyclin D1 expression and its promoter activity are augmented in cells in response to nicotine stimulation (Chu et al., 2005). Cyclin D1 is an important factor in regulating cell cycle progression, in which Cyclin D1 associated with cdk4 and cdk6, resulting in retinoblastoma protein (Rb) phosphorylation to promote Rb dissociate from E2F and permit progression from G1 to the S phase of the cell cycle (Fu et al., 2004). Cyclin D1 overexpression is believed to play an important role in the progression of bladder cancer (Yang et al., 2002). Induction of Cyclin D1 gene expression is regulated by different signaling pathways such as ERK1/2 or Stat3 (Sinibaldi et al., 2000) and requires several different transcriptional factors such as the nuclear factor-kB (NF-kB) (Ho et al., 2005). In the current study, we also examined the possible role of ERK1/2, Stat3 and NF-kB in Cyclin D1 expression induced by nicotine.

Although numerous reports suggest that nicotine promotes tumorigenesis through phosphatidylinositol-3-kinase (PI3K)/Akt, Raf/mitogen extracellular signal-regulated kinase kinase (MEKK)/ERK1/2 or NF-kB signaling pathways in various cancer cells (Bose et al., 2005; Chowdhury et al., 2007; West et al., 2004), the detailed molecular mechanisms of survival signaling pathways between nicotine and bladder epithelial cells have apparently never been reported. To characterize the precise roles of ERK1/2 and Stat3 activation in the signaling network in cell survival after nicotine stimulation, we tested the hypothesis that ERK1/2 and Stat3 cooperate in NF-kB activation, Cyclin D1 expression, and cell proliferation in response to nicotine. Our results indicated that inhibition of ERK1/2 or Stat3 by pretreatment with specific inhibitors block the activation of NF-kB, Stat3, and Cyclin D1 expression in a loop-like connection. The present study revealed a novel connection of cell proliferation with activation of ERK1/2, Stat3, and NF-kB in bladder epithelial cells after nicotine stimulation.

**MATERIALS AND METHODS:**

**Materials.** Nicotine, staurospmine (STP), nonspecific nicotinic receptor inhibitor hexemethonium bromide (Hexa), α7 subunit inhibitor methylcyclo-nitine (MLA), 24/β2 subunit inhibitor α7-lobeline (Lob), U0126, AG490, and nonselective beta-adrenergic receptors antagonist, propanolol (Pro) were purchased from Sigma-Aldrich, Inc. (St Louis, MO). Bay 11-7082 was purchased from Calbiochem (EMD Bioscience, Inc., Darmstadt, Germany). Polyclonal anti-α4, α7, α9, and β2-nAChR antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against Cyclin D1, Cyclin A, Bel-2, α-tubulin, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were obtained from Transduction Laboratories (Lexington, KY). Anti-ERK1/2, Stat3, phospho-ERK1/2, phospho-Stat3 (Serine 727), phospho-Stat3 (Tyrosine 705), and horseradish peroxidase–conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Cell Signaling (Beverly, MA).

**Cell culture and pharmacological treatments.** The T24 bladder epithelial cancer cell line was purchased from ATCC. T24 cells were maintained in 10-cm² dishes in McCoy 5A medium (Sigma-Aldrich, Inc.) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc., Gaithersburg, MD), and 10% heat-inactivated fetal calf serum (HyClone, South Logan, UT). Human urinary bladder cancer cell line U874 was a kind gift of Dr. Hsiao-Sheng Liu (National Cheng Kung Medical College, Institute of Molecular Medicine, Tainan, Taiwan). U874 cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum and 100 μg/ml penicillin, 100 μg/ml streptomycin. Both cell lines were incubated at 37°C in a 5% CO₂ atmosphere incubator. For the time-dependent induction of signaling pathways, cells were maintained in a serum-free medium exposed to nicotine.

**Cell proliferation assay.** Cell proliferation was induced by nicotine, and was quantified using two approaches: trypsin blue exclusion assay and BrdU (5-bromo-2-deoxyuridine, Sigma-Aldrich, Inc.) incorporation assay. For trypsin blue exclusion assay, T24 or U874 cells were cultured in 96-well plates at a density of 2 × 10⁴ cells/well for 24 h. Cells were cultured in serum rich media and treated daily with nicotine alone, or with a combination of nicotine and inhibitors. Cell numbers were counted at 1, 2, 3, 4, and 5 days using a cell counting chamber. Cell proliferation was also determined by a BrdU assay to measure the incorporation of the pyrimidine analog during DNA synthesis. Cells were plated in a 10 ml dish with a density of 1.5 × 10⁶ cells/dish, and cultured for 24 h. Next, cells were serum starved for 24 h, then released with medium containing 10% fetal bovine serum (FBS). Cells were treated with nicotine at the time of release. After 6, 12, 18, and 24 h of nicotine incubation, cells were incubated with 10μg/ml BrdU for 15 min, and then harvested. Cells were trypsinized, washed with phosphate buffered saline (PBS), and resuspended in 75% ethanol for 24 h. After fixation, cells were denatured with 2N HCl for 30 min. Subsequently, a fluorescein isothiocyanate (FITC)- conjugated anti-BrdU antibody (Becton Dickinson, San Jose, CA) was added, and cells were incubated at room temperature for 30 min. After resuspension in PI (propidium iodide, 50 μg/ml) solution, a fluorescence-activated cell sorter (Becton Dickinson) was used to determine BrdU incorporation and cell cycle progression. Samples were analyzed by WinMDI software programs.

**Immunoblotting and immunoprecipitation.** The isolation of total cellular lysates, immunoprecipitation, gel electrophoresis, and immunoblotting were performed according to the methods described previously (Lee et al., 2003). Immunoreactive proteins were visualized with the enhanced chemoluminescent detection system (PerkinElmer Life Science, Inc., MA) and BioMax Light Film (Eastman Kodak Company, New Heaven, CT), according to the manufacturer’s instructions.

**Determination of adrenalin and EGF levels.** T24 cells were plated in a 12 well plate at a density of 2 × 10⁶ cells per well. Cells were treated daily with 1μM nicotine, and supernatants were collected for determination of adrenalin and EGF concentrations. Adrenalin levels were detected using an adrenalin ELISA (enzyme-linked immunosorbent assay) kit (Immuno-Biological Laboratories, Hamburg, Germany). EGF levels were detected using an EGF ELISA kit according to the manufacturer’s instructions (R&D systems, Inc., Minneapolis, MN).

**Electrophoretic mobility shift assay.** Nuclear extracts were performed according to Trombino et al., (2004). DNA probes were biotin-labeled using a biotin 3’ end labeling kit (Pierce Biotechnology, Rockford, IL). Double stranded labeled DNA oligonucleotides (AGTTAGGGAGCTTTTTCCAGG) encompassing the κB binding site and Stat3 consensus oligonucleotides (GATCTCTTTGGATTTCTTAGATC) (Protech Technology Enterprise Co., Ltd. TaoYuan, Taiwan) were used for a binding reaction. The binding reaction contained 1× biotin binding buffer, 2.5% glycerol, 5mM MgCl₂, 0.05% NP-40, 50 ng/μl poly[dI-dC], 1mM ethylenediaminetetraacetic acid, 500mM KCl, 6 μg of nuclear extract, and 20 fmol of biotin end-labeled target DNA. The contents were incubated at room temperature for 20 min and stopped with 5 μl

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of 5× DNA loading buffer. The mixture was loaded onto 4% native polyacrylamide gel, electrophoresed, transferred to nitrocellulose, and probed with antibodies directed against α4, α7, α9, β2-nAChR, and G3PDH. (B) T24 and UB47 cells were plated in 96-well culture plates and cell proliferation was measured after nicotine (N) or (C) cotinine (Cot) treatment using trypan blue exclusion assay. Cells were trypsinized and cell numbers were measured at 1, 2, 3, 4, and 5 days after treatment. Each point represents the mean ± SD of at least two independent experiments performed in triplicate. *p < 0.05 by Student’s t-test for nicotine treatment.

**RESULTS**

**Expression of nAChR Subunits in Bladder Cancer Cells and Effect of Nicotine on Cell Proliferation**

Nicotine binds to nAChR and mediates the biological effects. nAChR are composed of 12 subunits (α2–α10, β2–β4). Functional nAChR are composed of homopentamers of α7–α10 subunits or heteropentamers derived from 5 α (α2–α6) and 3 β (β2–β4) subunits (Itier and Bertrand, 2001). We characterized expression of nAChR subunits in T24 and UB47 cells. Figure 1A demonstrates that both cell types express α4, α7, and β2 subunits and T24 cells selectively express α9 subunit. We also wanted to ascertain if nicotine treatment modified the expression of nAChR subunits in T24 and UB47 cells. We found that both cells incubated with nicotine 1 μM for 1, 3, and 5 days did not upregulate the expression of nAChR subunits (Supplementary Data, Fig. 1).

To examine the direct effect of nicotine on bladder epithelial cell proliferation, T24 and UB47 cells were cultured in 96-well culture plates, in which nicotine (0.1, 1, or 10 μM) was added to the medium containing 10% fetal calf serum (FCS) for 5 days. Nicotine significantly stimulated T24 and UB47 cell
proliferation in a time-dependent manner (Fig. 1B) \( p < 0.05 \), and nicotine-induced cell proliferation was more obvious in T24 cells, as compare to UB47 cells. However, cotinine, the major metabolite of nicotine did not increase cell proliferation in a dose or time-dependent manner in both cell types (Fig. 1C). To assure that nicotine-induced cell proliferation was a result of increased DNA synthesis, we determined optimal duration of nicotine treatments by time course experiments. T24 and UB47 cells were starved for 24 h, then released into medium containing 10% of FBS and 1 \( \mu \)M nicotine. Cells were double stained with PI and BrdU-FITC, and cell cycle progression and BrdU incorporation were assayed by flow cytometry. Figure 2A shows that T24 cells entered S phase at 18 h, then declined at 24 h. Nicotine increased the population of T24 and UB47 cells in the S phase (Fig. 2A). We observed maximum BrdU incorporation at 24 h in T24 cells and at 18 h in UB47 cells. Nicotine-treated T24 and UB47 cells were significantly more proliferative than control cells, as determined by BrdU incorporation assay (Fig. 2B). Our results confirm that nicotine-induced cell proliferation observed at later time point by trypan blue exclusion assay resulted from increased DNA synthesis at earlier time points.

**Effects of Nicotine on Cell Survival-Related and Cell Cycle Regulatory Proteins**

Because of the remarkable effect of nicotine on T24 cell proliferation relevant to UB47 cells, T24 cells were used in subsequent study. To gain a better understanding of the mechanism of cell growth stimulation induced by nicotine, the possible effects of nicotine on cell cycle regulatory and cell survival-related proteins were tested using Western Blot analysis. Figure 3A shows that nicotine (0.1–10 \( \mu \)M) strongly enhanced the expression of the cell cycle regulatory proteins Cyclin D1 and Cyclin A as well as the survival protein Bcl-2 at 24 h, in a dose-dependent manner. Next, we examined Cyclin D1 expression and the phosphorylation state of Rb protein in T24 cells exposed to nicotine for 2, 4, and 6 h. Figure 3B indicates that Cyclin D1 protein levels were increased at 4 and 6 h after nicotine stimulation. Meanwhile, the increased phosphorylation of Rb was observed. Proliferating cell nuclear antigen (PCNA) is a marker for cell proliferation involving DNA synthesis. Increased PCNA expression was observed at 12, 18, and 24 h after nicotine treatment (Fig. 3C), which is consistent with the increased S phase analyzed by flow cytometry (Fig. 2A).

**Effect of Nicotine on Mitogenic Signaling**

To identify the signaling mechanisms of nicotine-mediated proliferation, we examined three signaling pathways, the PI3K/AKT, ERK1/2, and JAK/Stat3 pathways, which have been reported to be involved in Cyclin D1 protein expression and cell proliferation (Ho et al., 2005; West et al., 2003). As shown in Figure 4A, 1 \( \mu \)M of nicotine rapidly increased the phosphorylation of ERK1/2 at 15 to 60 min; meanwhile, the
total ERK1/2 protein levels were not altered. Similar results were also observed for Stat3 activation. Although AKT is known to be involved in nicotine-induced proliferation by protecting cells from apoptosis (West et al., 2004), unexpectedly, we did not observe effects from nicotine on AKT phosphorylation in T24 cells (data not shown).

To examine the contribution of a specific signaling pathway to cell proliferation-induced by nicotine, T24 cells were treated with nicotine in combination with ERK1/2 inhibitor, U0126, or JAK/Stat3 inhibitor, AG490 for 24 h. Figure 4B shows that increased BrdU incorporation was significantly reduced in the combined treatment (*p < 0.05). These results indicate that ERK1/2 and Stat3 signaling pathway activation could be necessary for increased cell proliferation after exposure to nicotine.

Blockade of β-Adrenoceptors and nAChRs Abrogated Mitogenic Signals Activated by Nicotine

In order to elucidate which receptor was involved in nicotine-induced ERK1/2 and Stat3 activation, we used the following inhibitors: Hexa, a nonspecific nAChR antagonist (Beckel et al., 2006), MLA, a selective α7 selective antagonist...
Cross-Talk between ERK1/2 and Stat3 Signaling Pathways in Response to Nicotine

Previous studies have implied that nicotine binding to nAChR opens voltage-gated ion channels, resulting in Ca\(^{2+}\) influx and PKC activation (Tomizawa and Casida, 2002). Next, we examined whether PKC was involved in ERK1/2 or Stat3 activation. nAChR/PKC signaling inhibited by PKC inhibitor STP at 1, 5, and 10\(\mu\)M attenuated the phosphorylation of Stat3 Ser727. However, nicotine-induced ERK1/2 activation was not affected (Fig. 5C). These results imply that Stat3 activation could be mediated by the nAChR/PKC pathway, whereas the regulation of the nAChR on ERK1/2 activation may not through PKC signaling.

It has been shown that Stat3 activity could be mediated by ERK1/2, indicating a point of interaction between the MEK/ERK1/2 and Jak/Stat pathways (Steelman et al., 2004). Next, we tested whether cross-talk between ERK1/2 and Stat3 could exist in response to nicotine by using an ERK1/2 inhibitor U0126. As indicated in Figure 5D, U0126 strongly inhibited Stat3 Ser727 phosphorylation in a dose-dependent manner. At a concentration of 5\(\mu\)M U0126, nicotine-induced Stat3 Ser727 was completely inhibited at 24 h. We conclude that nicotine-induced Stat3 phosphorylation was mediated by PKC and ERK1/2 signaling cascades, and we demonstrate that an interaction between ERK1/2 and PKC/Stat3 pathways occurred in T24 cells exposed to nicotine.

Proliferation of T24 Cells by Nicotine Stimulation is Mediated by nAChR and \(\beta\)-Adrenoceptors

The importance of nAChR/PKC signaling on cell proliferation was examined by BrdU incorporation assay from cells pretreated with Hexa, MLA, Lob, or STP. As shown in Figure 5E, cells treated with nicotine showed a significant increase in the rate of BrdU incorporation when compared with controls (\(p < 0.05\)). Cells treated with nicotine combined with inhibitors significantly reduced the rate of BrdU incorporation, with a \(-15\%\) decrease in nAChR antagonist treatment groups and a \(-20\%\) decrease in STP treatment groups compared with nicotine treatment groups (\(p < 0.05\)). The current results confirm that nAChR, PKC, and their downstream signaling pathways play important roles in nicotine-induced cell proliferation.

Previous reports showed that nAChR and \(\beta\)-adrenoceptors might promote nicotine-induced cancer growth (Park et al., 1995; Shin et al., 2007). The involvement of these receptors in nicotine-stimulated T24 cell proliferation remains undefined. To this end, we pretreated cells with Pro and found that Pro significantly suppressed nicotine-induced T24 cell proliferation.
in a dose-dependent manner (20, 50, and 100µM) (Fig. 5F). Our results suggest that the regulatory effects of β-adrenoceptors may also play an important role in mediating the proliferative action stimulated by nicotine.

**Effect of NF-κB and Stat3 Transcriptional Activity on Cyclin D1 Expression after Nicotine Treatment**

To further identify which transcriptional factors downstream of ERK1/2 and Stat3 are responsible for Cyclin D1 expression in T24 cells, nuclear extracts were prepared from nicotine-treated cells, and the NF-κB or Stat3 transcriptional activity was detected by electrophoretic mobility shift assay (EMSA). The results show an increase of NF-κB and Stat3 transcriptional activity in T24 cells treated with nicotine for 24 h (1.4- and 1.8-fold, respectively) (Figs. 6A, 6B). Next, we tested whether NF-κB, as well as Stat3 activity, was necessary in nicotine-induced cell proliferation and Cyclin D1 expression. For this purpose, an IκB phosphorylation inhibitor, Bay 11-7082, was used. Our results show that Bay 11-7082 inhibited nicotine-induced NF-κB activity, as well as Cyclin D1 expression (Fig. 6C) in a dose-dependent manner (2 and 5µM). In addition, reduced Stat3 transcriptional activity and Cyclin D1 expression by ERK1/2 inhibitor, U0126, could be observed (Fig. 6D).

Based on reports suggesting that the ERK1/2 pathway acts upstream of NF-κB activation (Kurland et al., 2003), we determine whether ERK1/2 inhibition could decrease NF-κB activity. The results revealed that treatment with the ERK1/2 inhibitor U0126 did not change the NF-κB activity (data not shown), implying that there is no direct interaction between ERK1/2 and NF-κB activation. Yoshida et al., reported that Stat3 could interact physically with NFκB subunit p65 and that the interaction appears to be bound to a specific type of NFκB motif. Their study suggested novel transcription factor cross-talk mechanism that regulate the acute phase response (Yoshida et al., 2004). Thus, we further examined the possibility that ERK1/2 may mediate NF-κB activity through Stat3 regulation. Interaction of NF-κB subunit p65 and Ser727 phospho-Stat3 was detected by immunoprecipitation in cells treated with nicotine combined with U0126 or AG490. Figures 6E and 6F show that Stat3 could be cointmunoprecipitated with p65, and the nicotine-induced Stat3 Ser727/p65 interaction could be inhibited partially by AG490, U0126 treatment alone and completely inhibited by AG490 combined with U0126. These findings suggest that nicotine stimulates a physical interaction between Stat3 and p65. Stat3 Ser727 phosphorylation by ERK1/2 is necessary for optimal interaction with p65 and may potentiate NF-κB activity in response to nicotine.

**DISCUSSION**

Numerous epidemiological studies have reported that tobacco smoking is a major risk factor for bladder cancer, but little is known about the effect of nicotine or its metabolite on bladder cells. Nicotine is rapidly absorbed, metabolized and eliminated in the urine. The major metabolic pathway of nicotine is oxidation to cotinine, which is catalyzed by CYP2A6 in livers (Nakajima and Yokoi, 2005). According to a German environmental survey, the average urine nicotine and...
cotinine concentration in smokers is 367 μg/l (2μM) and 856 μg/l (4μM) respectively (Heinrich et al., 2004). In Carty’s study, both nicotine and its metabolite cotinine at concentration of 10⁻⁷ and 10⁻⁸ mol/l were mitogenic for smooth muscle cell in vitro (Carty et al., 1997). They suggested that nicotine and cotinine either up regulates production of growth factors for cell proliferation, or enhances expression of growth factors receptors in the cell membrane. In our study, exposure of bladder epithelial cells to nicotine and cotinine was similar to the urine concentration of cigarette smokers. However, we found that nicotine, but not cotinine, induced proliferation of bladder epithelial cancer cells.

In this study, we demonstrated for the first time that nicotine can directly affect the growth of bladder epithelial cells. Treatment of nAChR antagonist and β-adrenoceptors antagonist abolished nicotine-stimulated cell proliferation, suggesting that nAChR and β-adrenoceptors both play important roles in mediating the proliferative action stimulated by nicotine. Based on the information obtained in this study, we hypothesized a signaling model of nicotine-induced bladder epithelial cell proliferation could act through four main pathways (Fig. 7): (1) ERK1/2 activation via nAChR and β-adrenoceptor, leading to increased Cyclin D1 expression and cell proliferation; (2) nAChR/PKC and β-adrenoceptor activation followed by Stat3 Ser727 phosphorylation as well as increased DNA binding activity, leading to Cyclin D1 expression; (3) The cross-talk between ERK1/2 and PKC/Stat3, in which activated ERK1/2 serves as a kinase to phosphorylate Stat3; inhibition of ERK1/2 by U0126 decreases Stat3 Ser727 phosphorylation and transcriptional activity; (4) An interaction between Ser phospho-Stat3 and NF-κB subunit p65, which results in increasing NF-κB transcriptional activity. Using specific inhibitors, we found that all these signaling pathways contribute to increased NF-κB and Stat3 transcriptional activity and consequently induce Cyclin D1 overexpression, finally leading to an increased proliferative effect in T24 bladder epithelial cells after nicotine stimulation.
et al. (2007). Therefore, we first examined the subunits of nAChR expressed in bladder cancer cells by Western Blot Analysis and demonstrated that T24 and UB47 cells expressed nAChR, including α4, α7, and β2 subunits, and T24 cells selectively expressed αβ9 subunit. We found that nicotine-induced ERK1/2 and Stat3 activation was effectively inhibited by Hexa (nonspecific nAChR inhibitors), MLA (selective α7 antagonist) and Lob (selective α4/β2 antagonist). Furthermore, MLA and Lob abolished nicotine-induced cell proliferation (Fig. 5E). These results indicated that the effects of nicotine on ERK1/2, Stat3 activation, and cell proliferation were mediated via α7 and α4/β2 nAChR. Our findings agree with previous studies in which α7 nAChR triggered the Ras/Raf/MEK/ERK signaling pathway in epithelial cells (Jull et al., 2001; Schuller and Orloff, 1998; Schuller et al., 2003), promoting cell proliferation and cell survival (Chang and Karin, 2001). In a recent report, the effect of nicotine on the α7 nAChR receptor in a non-neuronal cell line was linked to the JAK-2/Stat3 signaling pathway (de Jonge et al., 2005). In addition, α4/β2 and α7 nAChR are the predominant nAChR subtypes in the brain. Nicotinoids activate the ERK1/2 cascade triggered by α4/β2 nAChR, induce intracellular Ca2+ mobilization, and activate sequential pathways from PKC to ERK (Tomizawa and Casida, 2002).

It has been reported that nicotine promotes colon cancer cell growth through an α7 nAChR-dependent pathway. Activation of α7 nAChR transactivates the β-adrenoceptor, and stimulates production of catecholamines such as adrenaline. Increased adrenaline production has been shown to stimulate β-adrenoceptors to produce proliferative responses in colon cancer HT-29 cells (Wong et al., 2007). It has also been shown that tobacco nitrosamine NNK cases β-adrenoceptor-mediated transactivation of EGFR, followed by ERK1/2 phosphorylation. This leads to an increased proliferative response in pancreatic cancer cells (Askari et al., 2005). These studies implied that the nicotine could activate various receptors including nAChR, β-adrenoceptors, or EGFR by transactivation. Therefore, we examined the production of adrenaline and EGF in T24 cells treated with nicotine for 1, 3, and 5 days. We found that the adrenaline (Supplementary Data, Fig. 2A) and EGF (Supplementary Data, Fig. 2B) production increased slightly when treated with nicotine for 3 days. However, this increase was not significantly different compared with controls. In addition, we also examined whether nicotine treatment could activate EGFR in T24 cells via phosphorylation of its Tyr845 residue. Immunoblotting of EGFR and EGFR Tyr845 proteins revealed that nicotine could not activate EGFR signaling after incubation with nicotine for 1, 3, and 5 days (Supplementary Data, Fig. 2C). Our results indicate that nicotine-induced T24 cell proliferation was not mediated by direct adrenaline or EGFR signaling.

The regulatory mechanisms of Stat3 activation are rather complex; we demonstrated that Stat3 Ser727 phosphorylation was completely abolished by ERK1/2 specific inhibitor U0126 (Fig. 5D). These results implied that ERK1/2 activated by nicotine could phosphorylate Stat3 Ser727. It has also been reported that Ser727 residue of Stat3 is a recognition site for ERK1/2 (Shen et al., 2004), suggesting a link between activation of PKC/Stat3 and ERK1/2 pathways. We suggested that the activation of Stat3 Ser727 after nicotine stimulation is modulated via two parallel pathways: (1) activation PKC activation and (2) activation of ERK1/2 cascade, leading to Stat3 Ser727 phosphorylation. Stat3 activation has been found to induce angiogenesis, increased tumor cell migration and invasion, and inhibition of innate and adaptive antitumor immunity gave rise to immune tolerance of tumor cells (Yu et al., 2007). These reports implicate the important role of Stat3 on tumor biology. The present study is the first to study bladder cancer with reference to the mechanism of Stat3 activation in response to nicotine. It is important that nicotine may alter the normal bladder epithelial cells growth properties through activation of Stat3 signaling.

We further demonstrated that through the activation of ERK1/2 and Stat3 signal pathways, nicotine exposure perturbs cell cycle progression, leading to increased S phase, expression of Cyclin D1, Cyclin A, PCNA, and Rb phosphorylation. The amplification of Cyclin D1 genes have been reported in many types of cancers, including bladder cancer (Wang et al., 2002). The detailed molecular signaling mechanisms bridging nicotine stimulation and Cyclin D1 expression have never been studied in bladder epithelial cells. By using pharmacological inhibitors, we confirmed that nicotine-induced Cyclin D1 expression in T24 cells is dependent on the activation of ERK1/2, NF-κB, and Stat3. Consistence with our results, nicotine-induced lung cancer cell proliferation is thought to involve AKT and NF-κB dependent pathways that are closely linked to changes in Cyclin D1 expression (Tsurutani et al., 2005). Sinibaldi et al. (2000) also indicated that the Cyclin D1 promoter was transcriptionally induced by v-Src in a Stat3 dependent manner.

ERK1/2 activation has been reported to be associated with NF-κB activation in many studies (Kurland et al., 2003; Wang et al., 2005). To ascertain whether the ERK1/2 pathway contributes to nicotine-induced NF-κB activity leading to Cyclin D1 expression in the current study, the effect of ERK1/2 inhibitor U0126 on NF-κB activity was examined by EMSA. Unexpectedly, the activity of NF-κB was not affected by U0126 (data not shown), whereas Stat3 activation was inhibited by U0126 (Fig. 7). These results implicated that ERK1/2 may indirectly regulate NF-κB activity through Stat3 activation. Consistent with this notion, Pearson et al. (2001) indicated that ERK2 indirectly regulated NF-κB activity in NIH-3T3 cells through cooperation with ERK5. Thus, we suggest that phosphorylation of Stat3 Ser727 may transactivate Cyclin D1 expression through interaction with NF-κB. Indeed, as shown in Figures 6E and 6F, we found that Stat3 Ser727...
interacted with the NF-κB p65 subunit and led to an increase in NF-κB activity. Recent studies indicate that NF-κB transcriptional regulation requires the cooperation of several proteins. The cross-talk between transcriptional factors has become a common model of gene regulation. For instance, in Yu’s study, the author reported a physical and functional interaction of Stat3 with NF-κB p65 subunit that is important for the transcriptional activity of NF-κB p65 responsive genes (Yu and Kone, 2004). Debidda et al. (2005) also indicated that Stat3 is required for Rho-induced NF-κB and cyclin D1 transcription, cell proliferation, and transformation, as well as actin stress fiber formation and migration. We concluded that the Stat3 Ser727 phosphorylation activated by ERK1/2 or PKC observed in our study is necessary for the Stat3 and NF-κB p65 interaction.

Previous reports showed that nAChR and β-adrenoceptors might be involved in the mediation of the effect of nicotine-induced cancer growth (Park et al., 1995; Tsurutani et al., 2005). The involvement of these receptors in T24 cancer cell proliferation promoted by nicotine was not understood. Here we report a unique picture of smoking-related bladder tumorigenesis. Nicotine can promote bladder cancer cell proliferation through the nAChR and β-adrenoceptors followed by activation of ERK1/2/Stat3-signaling pathway. It is also important to indicate that activation of ERK1/2 and Stat3 would further enhance NF-κB activation, Cyclin D1 expression and cell cycle progression (Fig. 7). In conclusion, our results shed new light on the mechanism of nicotine-induced bladder epithelial cell proliferation. These findings provide new perspectives on the effects of nicotine on bladder epithelial cells proliferation and may explain the role of nicotine in bladder tumorigenesis.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


