Effects of chronic nicotine on the autocrine regulation of pancreatic cancer cells and pancreatic duct epithelial cells by stimulatory and inhibitory neurotransmitters

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Pancreatic ductal adenocarcinoma (PDAC) has a mortality rate near 100%. Smoking is a documented risk factor. However, the mechanisms of smoking-associated pancreatic carcinogenesis are poorly understood. We have shown that binding of nicotine to nicotinic acetylcholine receptors (nAChRs) expressing subunits $\alpha 7$, $\alpha 3$ and $\alpha 5$ in PDAC and pancreatic duct epithelial cells *in vitro* triggered the production of the neurotransmitters noradrenaline and adrenaline by these cells. In turn, this autocrine catecholamine loop significantly stimulated cell proliferation via cyclic adenosine 3',5'-monophosphate-dependent signaling downstream of beta-adrenergic receptors. However, the observed responses only represent acute cellular reactions to single doses of nicotine whereas nicotine exposure in smokers is chronic. Using the PDAC cell lines BxPC-3 and Panc-1 and immortalized pancreatic duct epithelial cell line HPDE6-C7, our current experiments reveal a significant sensitization of the nAChR-driven autocrine catecholamine regulatory loop in cells pre-exposed to nicotine for 7 days. The resulting increase in catecholamine production was associated with significant inductions in the phosphorylation of signaling proteins ERK, CREB, Src and AKT, upregulated protein expression of nAChR subunits $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$ and increased responsiveness to nicotine in 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide and cell migration assays. All three cell lines produced the inhibitory neurotransmitter γ -aminobutyric acid, an activity inhibited by gene knockdown of the $\alpha 4\beta 2$ nAChR and suppressed by chronic nicotine via receptor desensitization. All of the observed adverse effects of chronic nicotine were reversed by treatment of the cells with γ -aminobutyric acid, suggesting the potential usefulness of this agent for the improvement of PDAC intervention strategies in smokers.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) comprises over 90% of all pancreatic cancers and has a mortality rate near 100% within 2 years of diagnosis (1,2). Smoking is a documented risk factor (3–6) and smokers have a 2-fold risk to develop PDAC (7,8). However, the mechanisms of smoking-associated pancreatic carcinogenesis are poorly understood. This lack of mechanistic insight may significantly contribute to the poor clinical outcomes of currently available preventive and therapeutic strategies for pancreatic cancer (9).

Among numerous carcinogenic and toxic substances contained in cigarette smoke, nicotine has been widely studied because of its documented addictive properties (10,11). Most biological effects of nicotine are mediated by nicotinic acetylcholine receptors (nAChRs), which operate as pentameric ion channels enclosed by homomeric α subunits or heteromeric α and β subunits (12). Classic

research on the function of nAChRs has focused on the nervous system. However, discoveries that nAChRs regulate the proliferation (13) and apoptosis (14) of lung cancer cells have triggered numerous investigations on the regulatory role of this receptor family in a variety of cancers. It has thus been shown that binding of nicotine to the homomeric $\alpha 7$ nAChR stimulates the proliferation, angiogenesis, neurogenesis and metastatic potential of the most common human cancers [reviewed in (15)]. The majority of these studies have interpreted the observed cancer-stimulating effects of nicotine as direct signaling responses downstream of the $\alpha 7$ nAChR (15). By contrast, we have recently shown that binding of nicotine to nAChRs expressing subunits $\alpha 7$, $\alpha 3$ and $\alpha 5$ in PDAC and pancreatic duct epithelial cells *in vitro* triggered the synthesis and release of the stress neurotransmitters noradrenaline and adrenaline by these cells (16). In turn, this autocrine catecholamine loop significantly stimulated cell proliferation via cyclic adenosine 3',5'-monophosphate (cAMP)-dependent signaling downstream of beta-adrenergic receptors (16). However, the observed responses only represent acute cellular reactions to single doses of nicotine, whereas nicotine exposure in smokers is chronic. Our current experiments reveal significant sensitization of the nAChR-driven autocrine catecholamine regulatory loop by chronic nicotine. In addition, our data show that PDAC and pancreatic duct epithelial cells produce the inhibitory neurotransmitter γ -aminobutyric acid (GABA), an activity regulated by the $\alpha 4\beta 2$ nAChR and desensitized by chronic nicotine. Interestingly, all of these effects of chronic nicotine were reversed by treatment of the cells with GABA.

Materials and methods

Chemicals, primers and antibodies

Lipofectamine 2000 Reagent, stealth-1973 for the *CHRNA4* gene, stealth RNAi Negative Control Low GC Duplex and Opti-MEM I reduced serum medium 1X were all purchased from Invitrogen Corporation (Carlsbad, CA, USA). The primer used to interfere with the $\alpha 4$ subunit mRNA was sense, GAC CGC AUC UUC CUC UGG AUG UUC A and antisense, UGA ACA UCC AGA GGA AGA UGC GGU C.

The TE Buffer 1X was purchased from Promega Corporation (Madison, WI, USA). The 2-Cat and GABA-Research ELISA Kits were purchased from Rocky Mountain Diagnostics Incorporation (Colorado Springs, CO, USA). ELISA kit for human dopamine beta-hydroxylase was purchased from MyBioSource (San Diego, CA, USA). ELISA kits for extracellular signal-regulated kinase (ERK)1/2 [pTpY185/187] and CREB [pS133] were purchased from Invitrogen Corporation (Carlsbad, CA, USA). The CytoSelect Cell Migration Assay was purchased from Cell BioLabs, Inc. (San Diego, CA, USA).

The antibodies AKT (60 kDa), p-AKT (60 kDa), Src (60 kDa), p-Src (60 kDa), antirabbit and antimouse were all purchased from Cell Signaling (Danvers, MA, USA). The primary antibody anti-nicotinic acetylcholine receptor $\alpha 4$ (55 kDa) was purchased from Millipore (Billerica, MA, USA). The nicotinic acetylcholine receptor subunits $\alpha 7$ (56 kDa), $\alpha 3$ (57 kDa), $\alpha 5$ (53 kDa), GAD65 (65 kDa), GAD67 (67 kDa) and β -actin (42 kDa) antibodies were purchased from Abcam (Cambridge, MA, USA). Nicotine ((–)-Nicotine hydrogen tartrate salt, minimum 98% TLC) and GABA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lysis buffer used to extract proteins along with Pierce ECL western-blotting substrate were purchased from Thermo Scientific (Rockford, IL, USA).

Cell culture

The human PDAC cell lines Panc-1 and BxPC-3 were purchased from the American Type Culture Collection (Manassas, VA, USA). The immortalized human pancreatic duct epithelial cell line, HPDE6-C7, was clonally established after transduction of the *HPV16-E6E7* genes into primary cultures of pancreatic duct epithelial cells and was a kind gift from Dr. Tsao (Division of Cellular and Molecular Biology, Department of Pathology, Ontario Cancer Institute/Princess Margaret Hospital, University of Toronto, Toronto, ON, Canada). All cell lines have been authenticated at the beginning of the current

Abbreviations: cAMP, cyclic adenosine 3',5'-monophosphate; CREB, cAMP response element binding; ERK, extracellular signal-regulated kinase; GABA, γ -aminobutyric acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; nAChRs, nicotinic acetylcholine receptors; PDAC, pancreatic ductal adenocarcinoma.

study by RADIL (Research Animal Diagnostic Laboratory, Columbia, MO, USA) by species-specific PCR evaluation.

The Panc-1 cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. BxPC-3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. HPDE6-C7 cells were maintained in keratinocyte serum free medium supplemented with 25 mg/500 ml bovine pituitary extract and 2.5 µg/500 ml epidermal growth factor (GIBCO Invitrogen Corporation, Grand Island, NY, USA). All cell lines were grown without antibiotics in an atmosphere of 5% CO₂, 99% relative humidity and 37°C.

Analysis of intracellular and secreted GABA in response to acute nicotine treatments

All three cell lines were maintained in their respective complete medium until reaching 65% confluence, at which time they were switched to basal medium for 24 h starvation. Cells were then switched into fresh basal media and were divided into two groups. The first group of cells was either untreated or treated with 1 µM nicotine for 1, 5, 15 or 30 min. The second group of cells was either untreated or treated with 10 pM, 500 pM, 1 nM, 500 nM, 1 µM or 10 µM nicotine for 30 min. The culture media, containing secreted GABA, were then collected in 15 ml test tubes. The cells, which contained synthesized intracellular GABA, were lysed and harvested into 1.5 ml Eppendorf tubes after a one-time wash with warm 1× phosphate-buffered saline (PBS). Quantitative analyses of intracellular and secreted GABA of five samples per treatment group were conducted using GABA-Research ELISA kit following the vendor's recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Analysis of total GABA, adrenaline and noradrenaline in response to chronic nicotine

Unpretreated cells from each cell line or cells pretreated for 7 days with nicotine (1 µM) were exposed to a range of nicotine concentrations (10 pM, 500 pM, 1 nM, 500 nM, 1 µM or 10 µM) for 30 min prior to harvesting. For the 7-day treatment group, media and treatment were replaced every 24 h. The culture media, containing secreted catecholamines and GABA, were then collected in 15 ml test tubes. The cells that contained synthesized intracellular catecholamines and GABA were lysed and harvested into 1.5 ml Eppendorf tubes after a one time wash with warm 1× PBS. Total (secreted plus intracellular) catecholamines and GABA of five samples per treatment group was analyzed using 2-Cat and GABA-Research ELISA kits, respectively, following the vendor's recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Gene knockdown of the α4nAChR

Cells from all three cell lines were grown for 24 h in their respective complete media. Cells were then switched to Opti-MEM I media and were divided into several groups. Groups 1 and 2 from each cell line were left untreated in Opti-MEM I media for 24 h. Group 3 was transfected for 24 h using stealth RNAi Negative Control Low GC Duplex. Groups 4 and 5 were transfected with stealth-1973 for the *CHRNA4* gene for 24 h in Opti-MEM I media. Once the 24-h transfection was complete, all cells were switched into their respective basal media. Groups 1, 3 and 4 were left untreated for 30 min in basal media, whereas Groups 2 and 5 were treated with 1 µM nicotine for 30 min in basal media. All transfections were done using Lipofectamine 2000 reagent following the instructions of the manufacturer. Cell lysates were then harvested and collected in 1.5 ml Eppendorf tubes after one time wash with warm 1× PBS for GABA analysis by immunoassays as described above. The transfection efficiency was assessed by western blots using α4-nAChR as the primary antibody and actin as the loading control following the procedure that is outlined below. Following background subtraction, mean densities of two rectangular areas of standard size per band from three independent westerns were determined for the calculation of mean values and standard deviations ($n = 6$) of protein expression.

Assessment of dopamine beta-hydroxylase levels

Cells from the three cell lines were divided into four groups. Group 1 was left untreated. Group 2 was treated with 1 µM nicotine for 30 min. Group 3 was treated with 1 µM nicotine for 7 days. Group 4 was treated with 1 µM nicotine for 7 days followed by 1 µM nicotine 30 min before harvesting. Media and treatments were replaced every 24 h for Groups 3 and 4. The cells were then lysed and harvested into 1.5 ml Eppendorf tubes after a one-time wash with warm 1× PBS. Quantitative analyses of dopamine beta-hydroxylase activity of five samples per treatment group were conducted using human dopamine beta-hydroxylase ELISA kit following the vendor's recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument

ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Protein analyses by western blotting and ELISA

The levels of phosphorylated signaling proteins ERK and cAMP response element binding (CREB) were assessed by ELISAs, using ERK1/2 [pTpY185/187] and CREB [pS133] ELISA kits from Invitrogen. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Protein expression of nAChR subunits α3, α4, α5 and α7, as well as GAD65, GAD67 and the phosphorylated and unphosphorylated forms of signaling proteins AKT and Src was determined using western blotting.

For both types of analysis, cells from HPDE6-C7, BxPC-3 and Panc-1 were either untreated or treated with 1 µM nicotine, 30 µM GABA or GABA plus nicotine for 7 days in complete media. Media and treatments were changed every 24 h for all groups. Protein samples were prepared using lysis buffer (50 mmol/L Tris-HCl, 1% NP-40, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₂VO₄, 1 mmol/L NaF and 1 µg/ml of aprotinin, leupeptin and pepstatin). After heat denaturation, protein samples were electrophoresed using 12% sodium dodecyl sulfate gels (Invitrogen) and blotted onto membranes. The membranes for western blots were blocked (5% nonfat dry milk solution) for 1 h at room temperature. Membranes of Group 1 were then incubated overnight at 4°C with the following primary antibodies: nicotinic acetylcholine receptor subunit α3, α4, α5 and α7, GAD65, GAD67, p-AKT and p-Src determine expression or phosphorylation levels of these signaling proteins or receptors. The primary antibodies Src, AKT and β-actin were used as a loading control to ensure equal loading of proteins. All membranes were then washed (0.5% Tween 20/tris-buffered saline) and incubated with their respective fluorescent secondary antibodies for 2 h. Protein bands were then visualized with enhanced chemiluminescence reagent (Pierce ECL Western Blotting Detection Substrate). Following background subtraction, mean densities of two rectangular areas of standard size per band from three independent westerns were determined and mean values and standard deviation ($n = 6$) of protein expression were calculated.

Cell proliferation by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cells from the three cell lines were seeded in six-well plates at a density of 20 000 cells per well in their respective complete media. Cells were then divided into five groups ($n = 5$). The first group of cells was left untreated for 7 days and harvested 24 h later. The second group was left untreated for 7 days, then treated with a single dose of 1 µM nicotine that was removed 30 min later and harvested 24 h later. The third group was treated with 30 µM GABA for 7 days and harvested 24 h later. The fourth group was treated with 1 µM nicotine for 7 days followed by a single dose of nicotine at the EC₅₀ concentration of nicotine established in the ELISAs for adrenaline production after chronic nicotine for each cell line (Figure 2d-f: 1.184 nM for HPDE6-C7, 13.96 nM for BxPC-3 and 14.14 nM for Panc-1) that was removed 30 min later and harvested 24 h later. The fifth group was similar to the fourth one except that these cells were additionally pretreated with 30 µM GABA for 7 days. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) colorimetric assay (Sigma-Aldrich St Louis, MO, USA) was used to assess cell proliferation following instructions by the vendor. This assay is based on the cleavage of the tetrazolium salt MTT to formazan in metabolically active cells. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at a primary and reference wavelengths of 570 and 650 nm, respectively.

Cell migration assay

Using six-well plates that contain polycarbonate membrane filter inserts (8 µm pore size) provided by a cell migration assay kit (Cell BioLabs, San Diego, CA, USA), cells from each cell line were seeded onto the top chamber above the filter insert at a density of 20 000 cells per well in their respective complete media. Cells were then divided into identical treatment groups ($n = 5$) as described for the MTT assays (above). Migration ability of the cells was assessed following the instructions provided in the kit. Optical density of samples was read at 560 nm using an uQuant Bio-Tek Instrument ELISA reader.

Statistical analysis of data

GraphPad Instat 3 software (GraphPad Instant biostatistics, San Diego, CA, USA) was used for the transformation of data to fold increase and normalization and to test significant differences among treatment groups. Statistical tests used included parametric one way analysis of variance and Tukey-Kramer multiple comparison test when the data followed a normal distribution. When data failed to pass the normality test of Kolmogorov and Smirnov provided by the GraphPad software, they were instead analyzed by nonparametric Kruskal-Wallis analysis of variance followed by the nonparametric Dunn's

Fig. 1. Secreted (a) and intracellular (b) GABA levels in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine from 1 to 30 min. (c) ELISAs showing intracellular GABA levels in cells in the presence and absence of gene knockdown of the $\alpha 4$ -nAChR in the presence and absence of 1 μ M nicotine for 30 min. Data points are mean $\pm$ SD from five samples per treatment group. (d) Western blots showing the effects of gene knockdown on protein expression of the $\beta 4$ -nAChR in the presence and absence of nicotine (1 μ M for 30 min). The house keeping protein β -actin was used as a control to ensure equal loading of proteins. The columns in the graph represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in expression of $\beta 4$ -nAChR.

multiple comparison test. In addition, ImageJ from NIH was used for mean density determination of bands from three independent western blots. Data of the immunoassays are expressed as mean and $\pm$ standard deviation of five samples per treatment group. Densitometry data of western blots are expressed as mean values and standard deviations of two density determinations per band from three independent westerns per antibody ($n = 6$). Nonlinear regression analysis of sigmoidal dose-response curves for GABA, noradrenaline and adrenaline was used to determine EC_{50} values of dose-response curves for nicotine in untreated and 7-day pretreated cells.

Results

Effects of acute and chronic nicotine on GABA levels

Studies by our laboratory have shown that treatment of PDAC cells with GABA *in vitro* (17) and in mouse xenografts (18) inhibits tumor growth. Contrary to widely held belief that the production and release of pancreatic GABA is restricted to the endocrine cells of pancreatic islets (19), our data show that both PDAC and the pancreatic duct epithelial cells synthesize and release GABA *in vitro* (Figures 1a–b and 2a–c). Exposure of the cells to single doses of nicotine (1 μ M) over time revealed a time-dependent decrease ($P < 0.0001$) in synthesis and release of GABA (Figure 1a–b). Gene knockdown of the $\alpha 4$ nAChR subunit significantly ($P < 0.001$) reduced GABA levels in untreated cells while additionally decreasing the nicotine-induced

suppression of GABA (Figure 1c). Moreover, gene knockdown of the $\alpha 4$ -nAChR showed a significant ($P < 0.0001$) reduction in its protein expression and significantly reduced nicotine-induced expression of the receptor (Figure 1d). These findings identify nAChRs expressing the $\alpha 4$ subunit as important regulators of GABA production in the three investigated cell lines.

Acute exposure of cells for 30 min to single doses of nicotine at concentrations from 10 pM through 10 μ M revealed concentration-dependent decrease in total (intracellular plus secreted) GABA in all three cell lines (Figure 2a–c). The suppression of total GABA by identical concentrations of nicotine was enhanced even further in cells pretreated for 7 days with nicotine (Figure 2a–c) and the EC_{50} values of nicotine that caused these responses were significantly ($P < 0.0001$) lower after chronic nicotine than in the untreated cells.

Effects of chronic nicotine on production of noradrenaline and adrenaline

We have previously shown that exposure of the three investigated cell lines to a single dose of nicotine causes synthesis and release of the catecholamine neurotransmitters noradrenaline and adrenaline, both of which stimulated cell proliferation (16). In accord with these findings, our current results show a significant and concentration-dependent increase ($P < 0.0001$) in total adrenaline (Figure 2d–f) and

Fig. 2. Total (secreted plus intracellular) GABA levels (a–c) and total (secreted plus intracellular) adrenaline levels (d–f) in HPDE6-C7, BxPC-3 and Panc-1 cells treated with single doses of nicotine from 10 pM through 10 μ M for 30 min or pretreated with 1 μ M nicotine for 7 days and then exposed to identical concentrations of nicotine. Data points are mean and $\pm$ SD from five samples per treatment group.

noradrenaline (Figure 3a–c) in unpretreated cells exposed to concentrations of nicotine from 10 pM to 10 μ M for 30 min. The production of both catecholamines was markedly enhanced ($P < 0.0001$) and EC_{50} values were significantly reduced when the cells were pretreated for 7 days with nicotine and then exposed for 30 min to identical concentrations of nicotine (Figures 2d–f and 3a–c). In accord with these findings, the levels of the enzyme dopamine beta-hydroxylase, which catalyzes the formation of noradrenaline from dopamine, were significantly ($P < 0.0001$) increased after 30 min of exposure to 1 μ M nicotine in all three cell lines (Figure 3d). In turn, chronic exposure to nicotine for 7 days further upregulated the expression of this enzyme (Figure 3d).

Effects of chronic nicotine in the presence and absence of GABA treatment on protein expression of nAChRs

We have previously shown that nAChRs with subunits $\alpha 3$, $\alpha 5$ and $\alpha 7$ nAChRs jointly regulated the production of noradrenaline and adrenaline in the three investigated cell lines (16) while our current data identified the $\alpha 4$ nAChR as the regulator of GABA production. We therefore assessed the effects of chronic nicotine in the presence and absence of GABA on the protein expression of these receptors using western blots. As shown in Figure 4a and 4b, chronic nicotine significantly induced ($P < 0.0001$), the protein expression of all four receptors in each of the three cell lines. GABA treatment alone did not significantly change the expression of these receptors as compared with the control groups (Figure 4b). However, the protein induction of all four receptors in response to chronic nicotine was significantly ($P < 0.001$) reduced by simultaneous chronic exposure of each cell line to GABA (Figure 4b).

Effects of chronic nicotine in the presence and absence of GABA on phosphorylated signaling proteins

Binding of agonists to β -ARs activates adenylyl cyclase, leading to the formation of cAMP and phosphorylation of the transcription factor CREB by activated protein kinase A (20). In addition, activated protein kinase A transactivates the epidermal growth factor receptor pathway in pancreatic cancer cells and pancreatic duct epithelia, leading to the phosphorylation of the extracellular signal-regulated kinases ERK1/2 (21) and activation of the Src and AKT pathways (16). We therefore assessed the phosphorylation of AKT and Src by semiquantitative western blotting in our three cell lines after exposures to nicotine, GABA or the combination of both agents for 7 days. ELISAs were used to monitor the phosphorylation of CREB and ERK. As Figure 5a and 5b shows, chronic 1 μ M nicotine significantly ($P < 0.0001$) induced phosphorylation of AKT and Src signaling proteins in both pancreatic cancer cell lines and immortalized pancreatic duct epithelial cells. Simultaneous chronic exposure of the cells to GABA significantly ($P < 0.0001$) reduced the induction of both phosphorylated proteins by chronic nicotine while chronic GABA treatment of unpretreated cells suppressed the activation of these proteins to near background levels (Figure 5a and 5b). Analysis of p-ERK and p-CREB by ELISAs revealed a similar trend with significant ($P < 0.0001$) inductions by chronic nicotine and significant ($P < 0.001$) reversal of these effects by treatment with GABA (Figure 6a).

Effects of chronic nicotine in the presence and absence of GABA on the GABA-synthesizing enzymes GAD65 and GAD67

The isozymes GAD65 and GAD67 catalyze the formation of GABA from glutamate (22). Having shown that our investigated cell lines

Fig. 3. Total (secreted plus intracellular) noradrenaline levels (a–c) in HPDE6-C7, BxPC-3 and Panc-1 cells treated with single doses of nicotine from 10 pM through 10 μ M for 30 min or pretreated with 1 μ M nicotine for 7 days and then exposed to identical concentrations of nicotine. Levels of dopamine beta-hydroxylase (d) in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine for 30 min, pretreated with 1 μ M nicotine for 7 days, or pretreated with 1 μ M nicotine for 7 days followed by 30-min nicotine treatment prior to harvesting. Data points are mean and $\pm$ SD from five samples per treatment group.

produce GABA *in vitro*, we therefore investigated the levels of GAD65 and GAD67 by western blots in cells exposed to 1 μ M nicotine for 7 days in the presence and absence of simultaneous treatment with GABA. Our data show a significant nicotine-induced reduction ($P < 0.0001$) in the expression of both isozymes as compared with the controls. These responses were significantly ($P < 0.0001$) reversed by simultaneous chronic treatment with GABA, whereas GABA treatment alone significantly ($P < 0.001$) increased GAD levels in unpretreated cells (Figure 5a and 5c).

Effects of chronic nicotine in the presence and absence of GABA on cell proliferation and migration

We have previously shown that blocking $\alpha 7$ -nAChR by its selective antagonist, alpha-bungarotoxin, or of β -adrenergic receptors by propranolol significantly reduced cell proliferation induced by a single dose of nicotine in unpretreated PDAC cells (16). In the current study, we assessed cell proliferation as an indicator of tumor growth and cell migration as an indicator of metastatic potential. Both assays revealed a significant ($P < 0.0001$) increase in cellular responsiveness to a single dose of nicotine in the cells pretreated for 7 days with nicotine in comparison with unpretreated cells (Figure 6b and 6c). When the pretreatment with nicotine was accompanied by 7 days of pretreatment with GABA, the observed increased sensitivity to nicotine was significantly reduced ($P < 0.0001$; Figure 6b–c).

Discussion

Smoking is an important risk factor for pancreatic cancer (1). However, the mechanisms of smoking-associated pancreatic carcinogenesis are far from understood. Recent studies by our laboratory have

shown that the proliferation of the three currently investigated cell lines is significantly stimulated by the activation of an autocrine catecholamine loop regulated by $\alpha 3$, $\alpha 5$ and $\alpha 7$ nAChRs upon acute exposure to single doses of nicotine (16). Single doses of nicotine thus activated multiple signaling proteins commonly overexpressed in pancreatic cancer via noradrenaline/adrenaline-induced cAMP-dependent signaling downstream of beta-adrenergic receptors (16). Our current study extends these findings to show that all three cell lines additionally synthesize and secrete GABA, a neurotransmitter identified previously by us as a potent tumor suppressor for PDAC *in vitro* (17) and in xenograft models (18). This novel finding contrasts the widely held belief that endocrine beta cells in the pancreatic islets are the sole source of pancreatic GABA that is transported to the exocrine pancreas via the pancreatic blood circulation (22). The observed significant reduction of GABA production in untreated cells and inhibition of nicotine-induced GABA suppression by gene knockdown of the $\alpha 4$ nAChR additionally identifies this nAChR as the upstream regulator of GABA in all three cell lines. Normal pancreatic duct epithelial cells, as well as PDAC cells, thus express the complete machinery for the regulation of their own growth stimulation and inhibition by neurotransmitters.

The observed chronic nicotine-induced increase in receptor protein of nAChRs $\alpha 3$, $\alpha 5$ and $\alpha 7$ in conjunction with significantly reduced EC₅₀ values yielding significantly higher levels of catecholamine production indicate sensitization of these receptors by chronic nicotine. By contrast, the significantly reduced EC₅₀ values for nicotine-induced GABA production in conjunction with significant suppression of GAD and GABA levels in cells chronically exposed to nicotine suggest that the observed increase in protein of the GABA-regulating $\alpha 4$ nAChR was a reaction to desensitization of the receptor. This

Fig. 4. Western blots assessing protein expression of nAChR subunits α 3, α 4, α 5 and α 7 in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine, 30 μ M GABA or GABA + nicotine for 7 days. The house keeping protein β -actin was used as a control to ensure equal loading of proteins (a). The columns in graph (b) represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in expression of α 3, α 4, α 5 and α 7-nAChRs.

interpretation gains strong support from the significant increases in sensitivity to nicotine in 7-day nicotine-pretreated cells observed in cell proliferation and cell migration assays. Our findings are in accord with changes of these receptors in the nicotine-addicted brain (23) and imply a shift of neurotransmitter-mediated autocrine regulation of pancreatic duct epithelial cells and PDAC cells from a balanced state to selective prevalence of cancer-stimulating catecholamine neurotransmitters accompanied by suppression of the inhibitory GABA system by chronic nicotine. This effect is further exacerbated by the observed significant increase in dopamine beta-hydroxylase levels by chronic nicotine as the induction of this enzyme leads to increased synthesis of noradrenaline.

The observed participation of three different nAChRs (α 3, α 5, α 7) in the regulation of catecholamine production is in accord with the reported redundancy of these cancer-stimulating receptors in other epithelial cells (24). In fact sequential investigations have shown that chronic exposure of oral keratinocytes to nicotine initially induce the protein expression of nAChRs containing subunit complexes comprised of α 3, α 5, α 2 and α 4 followed by induction of the homomeric α 7nAChR protein (25). In turn, this sequence in nAChR upregulation may be a reflection of the differences in the magnitude of Ca^{2+} influx in response to agonist among nAChRs, with the α 7nAChR demonstrating the greatest permeability to Ca^{2+} . The regulatory role of nAChRs expressing the α 4 subunit for GABA synthesis and release observed in our current experiments is in accord with similar findings in human small airway epithelial cells and lung adenocarcinomas

with phenotypic features of these cells (26). However, contrary to the α 7nAChR whose stimulatory role on numerous cancers has been widely published (15), current knowledge on the role of the α 4nAChR in the regulation of cancer is rudimentary at best. The desensitization of this receptor and associated suppression of GABA by chronic nicotine in the brain is considered a major cause of nicotine addiction (23). Our current data imply that analogous events in pancreatic duct epithelial and PDAC cells significantly increase their propensity for cancerous growth and metastasis.

The signaling proteins ERK, Src and AKT are commonly over-expressed in PDAC (27–29) and are current targets of pancreatic cancer therapy albeit with little success (9). Our recently published investigations on the cellular responses of PDAC cells to a single dose of nicotine have shown that all three of these signaling proteins, as well as CREB, are phosphorylated when the nAChR triggered increase in noradrenaline/adrenaline activates beta-adrenergic receptor signaling (16). Our current data show additional significant increases over single-dose acute nicotine treatments in the activated forms of these proteins in cells chronically exposed to nicotine. In conjunction with the observed changes in nAChRs, catecholamine and GABA production, chronic nicotine thus has deleterious effects at several levels on the complex network that governs the growth regulation of PDAC and pancreatic duct epithelial cells.

The significant reduction of all investigated effects of chronic nicotine in the current experiments by simultaneous chronic treatment of

Fig. 5. Western blots assessing phosphorylation of AKT, Src, GAD65 and GAD67 in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine, 30 μ M GABA and GABA + nicotine for 7 days (a). The columns in graph (b) and (c) represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in protein phosphorylation. The unphosphorylated proteins AKT, Src and β -actin were used as controls to ensure equal loading of proteins.

our three cell lines with GABA provides a mechanistic explanation for the reported reversal of nicotine-induced progression of PDAC xenografts by GABA treatment (18). We have previously established that the nicotine-induced phosphorylation of CREB, ERK, Src and AKT are cAMP-dependent events in the signaling pathway downstream of beta-adrenergic receptors (21,30) and that GABA inhibits the formation of cAMP via the G α i-coupled GABA-B receptor (17). The strong reversal by GABA treatment of increased levels in these phosphorylated signaling proteins, as well as cellular responsiveness in proliferation and migration assays in response to chronic nicotine, was therefore an expected outcome of the current study. However, it is noteworthy that GABA also significantly reduced the chronic nicotine-induced modulations in the expression and function of all investigated nAChRs, as well as the induction of dopamine beta-hydroxylase and suppression of GAD. These findings are in accord with observations in the nervous system that some of the post-transcriptional mechanisms that cause the nicotine-induced upregulation of nAChR protein are caused by increases in cAMP (31). On the other hand, mechanisms involved in the observed effects of GABA on nicotine-induced changes in dopamine beta-hydroxylase and GAD expression need yet to be elucidated.

The beneficial effects of GABA on chronic nicotine-induced changes in the autoregulation of PDAC cells and pancreatic duct epithelial cells of our current experiments are in accord with previous reports that GABA inhibits the norepinephrine-induced migration of

cell lines from colon cancer (32) and mammary gland (33) and has strong tumor-suppressor function in lung adenocarcinoma *in vitro* (34) and in PDAC xenografts (17). However, in PDAC cell lines that overexpressed the π subunit of GABA-A receptors, GABA stimulated cell growth, an effect believed to involve reversal of receptor function from hyperpolarizing to depolarizing, leading to an increase in intracellular calcium levels (35).

Nicotine is generally classified as a noncarcinogenic agent because it does not cause cancer in healthy experimental animals. However, it has been shown that sensitization of lung nAChRs by pathologically increased CO₂ levels that increase Ca²⁺ influx rendered nicotine a lung carcinogen in hamsters (36). Our current data provide strong evidence for a key role of nicotine in the development and progression of pancreatic cancer of individuals with modulated nAChR functions due to chronic nicotine exposure. Although blocking upstream regulatory receptors such as the α 3, α 4, α 5 and α 7nAChRs and β -adrenergic receptors may inhibit nicotine-induced cell proliferation, angiogenesis and migration of pancreatic cells (16), the use of such agents for cancer intervention is problematic due to the important functions of these receptors in the regulation of the nervous system, cardiovascular functions and immune responses. In addition, the presence of beta-adrenergic agonists and stimulators of cAMP in numerous widely used over-the-counter drugs would effectively nullify any beneficial effects of nAChR blockage. GABA, on the other hand, offers the promise to significantly improve clinical outcomes of pancreatic

Fig. 6. Phosphorylation of CREB and ERK (a) assessed by ELISA in HPDE6-C7, BxPC-3 and Panc-1 cells showing inhibition of nicotine-induced phosphorylation, cell proliferation and migration by GABA. Cell proliferation (b) assessed by MTT assays and metastatic potential (c) assessed by cell migration assays: data from all three cell lines were significantly ($P < 0.0001$) different from controls (*), from nicotine 30' (**), and from nicotine 7 days + 30' nicotine (***). The columns represent means and $\pm$ SD of five samples per treatment group.

cancers that do not overexpress the GABA-A receptor $\alpha 1$ subunit and may be suitable for the prevention of this malignancy in smokers.

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