Nicotine Stimulates a Serotonergic Autocrine Loop in Human Small-Cell Lung Carcinoma

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ABSTRACT

Small-cell lung carcinoma cells express different plasma membrane nicotinic acetylcholine receptor subtypes. We have now found that interacting with these receptors (−)-nicotine induces a dose-dependent and stereoselective release of [3H]serotonin which is dependent on external calcium and blocked by the specific ganglionic nicotinic antagonist mecamylamine. With the same potency (−)-nicotine stimulates tumor cell proliferation, an effect also blocked by mecamylamine. Serotonin itself stimulates cell proliferation in a dose-dependent manner, an effect blocked by the selective serotonergic receptor antagonists methysergide and metergoline. These data suggest that nicotine might affect proliferation of small-cell lung carcinoma cells by inducing the release of hormones (such as serotonin) with autocrine capabilities and place both the nicotinic and the serotonergic receptors at key positions in the biological and, possibly, pharmacological approach to this human lung cancer.

INTRODUCTION

Although nicotine is a major component of tobacco smoke, few studies have addressed the issue of the interaction between nicotine and lung cancer at the molecular level. There is evidence that exposure to tobacco smoke induces hormone secretion in the lung and in particular from the diffuse neuroendocrine cells of the bronchial mucosa (1, 2); associated with this, an increased proliferation of pulmonary neuroendocrine cells has been shown after exposure to tobacco smoke (2).

SCLC is a very aggressive neuroendocrine lung tumor associated with tobacco abuse; it is supposed to arise from the diffuse neuroendocrine cells of the bronchial mucosa, and, characteristically, SCLC cells synthesize and release a large number of polypeptide hormones and neurotransmitters (3).

These secreted products are responsible for well-known peripheral symptoms such as a carcinoid syndrome due to serotonin release, a Cushing syndrome due to ACTH release, and a peculiar syndrome related to inappropriate release of antidiuretic hormone (4). Furthermore, for some of these secreted products either positive (5–7) or negative (7–9) autocrine effects on cell proliferation have been demonstrated.

Muscarinic acetylcholine receptors have been described in SCLC (10), but different subtypes of nicotinic AchRs have also been shown only recently to be expressed by these cells (7, 11, 12). In particular, binding sites for different nicotinic ligands, such as [3H]nicotine (7), [125I]-α-bungarotoxin (7, 11), [3H]methylthiophenyl phosphonium bromide (12), and [125I]-neuronal bungarotoxin, have been found in SCLC cells. Furthermore, some AChR subunits, such as α3 and β4, typically expressed in the peripheral nervous system are also expressed by SCLC cell lines (12).

Nicotine, interacting with neuronal type nicotinic acetylcholine receptors, stimulates neurotransmitter release (13). We have suggested that these neuronal type AchRs could be involved in the control of hormone release from SCLC cells and possibly in cell proliferation as well (12). We have focused our studies on the control of 5-HT release from SCLC because this "classical" neurotransmitter is synthesized by SCLC in vivo (3), but no information is available concerning either the mechanisms controlling its release or its direct biological effects on SCLC. Our present data provide evidence that (a) nicotine stimulates serotonin release from SCLC cells as well as cell proliferation; (b) serotonin, acting on 5-HT receptors, is also a mitogen for SCLC cells; (c) both nicotinic and serotonergic antagonists are able to inhibit in vitro drug-induced SCLC cell proliferation.

MATERIALS AND METHODS

Cell Culture. SCLC cell lines (kindly provided by G. Gaudino, University of Turin) were grown as previously described (11) in RPMI 1640 medium, supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, in an humidified incubator and in an atmosphere of 10% CO2 in air at 37°C. The cells were plated at a concentration of 1 × 10^5 dishes of 10-cm diameter Petri dishes and used 1 week later.

Uptake and Release of [3H]Serotonin. SCLC cells were recovered and resuspended (3 × 10^5 cells/ml) in KRH buffer containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM 4-(2-hydroxyethyl)-1-piperazinetanesulfonic acid-NaOH, 2 mM CaCl2, 6 mM glucose, 0.5 mM ascorbate, 0.5 mM pargyline, and 500 μM [3H]-5-HT (final concentration; specific activity, 14.6 Ci/mmol). Uptake was performed for 30 min at 37°C in the incubator, and then the cells were pelleted and resuspended in the same KRH buffer without [3H]-5-HT but with the 5-HT reuptake inhibitor zimelidine (1 μM). Under these conditions, GLC8 cells uptake was 7618 ± 634 dpm (mean ± SE) of [3H]-5-HT/mg of protein.

(−)-Nicotine and (−)-nicotine were added at the indicated concentrations for 5 min at 37°C; mecamylamine was preincubated with the cells for 15 min at 37°C before the addition of the agonist; basal [3H]-5-HT release was ~12% of the total amount of stored [3H]-5-HT over 5 min of incubation at 37°C. At the end of the incubation, the cells were washed three times with ice-cold buffer, and the final pellets were dissolved in 1 mL NaOH; the samples were then counted in a Beckman LS7500 scintillation counter to determine the amount of radioactivity remaining in the pellets.

[3H]Thymidine Incorporation. SCLC cells were plated in RPMI 1640 in a 96-well microtiter plate at a density of 2–5 × 10^3 cells/well and incubated for 48 h with the indicated concentrations of drugs and [3H]thymidine (5 μCi/ml). Mecamylamine did not affect cell viability as evaluated by trypan blue exclusion. Cells were then collected on filters with an automatic cell harvester (Tiltertek; Flow), and the radioactivity associated with the filters was counted in a liquid scintillation counter.

RESULTS

Nicotine Induces Serotonin Release from SCLC Cells. 5-HT is present in GLCS cells in vitro (28.5 ± 5.4 pmol/mg of protein, as determined by high-pressure liquid chromatography-electrochemical detection analysis). Exogenous [3H]-5-HT is taken up by SCLC cells.
through a specific, zimelidine-sensitive, 5-HT transporter (see "Materials and Methods").

(-)-Nicotine induces a dose-dependent release of [3H]5-HT from GLC8 SCLC cells, with a significant effect obtained at concentrations as low as 1 pM and a maximal effect at 10 nM (EC50, 10 pM; Fig. 1). (-)-Nicotine-induced [3H]5-HT release was also found in other SCLC cell lines [20 ± 3 and 23 ± 2% with a saturating (100 nM) concentration of (-)-nicotine in NCI-H-69 and NCI-N-592, respectively], all of them expressing "neuronal" AchR (12). Endogenous 5-HT levels were similarly affected by (-)-nicotine (27 ± 3% reduction after stimulation with 100 nM (-)-nicotine, n = 3).

(+)-Nicotine is much less potent than (-)-nicotine (only 50% of the effect at a concentration of 10 nM); furthermore, (-)-nicotine-induced [3H]5-HT release is antagonized in a dose-dependent manner by the selective ganglionic nicotinic antagonist mecamylamine (maximal inhibition of >95% at 10 µM mecamylamine; Fig. 1, inset), suggesting a receptor-mediated mechanism of action of nicotine. The removal of extracellular Ca2+ and the addition of ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid completely prevents (-)-nicotine-induced [3H]5-HT release from SCLC cells (Fig. 1, inset). The Ca2+-dependent release of [3H]5-HT induced by nicotine might be due to nicotine-induced cell depolarization and a subsequent opening of the voltage-gated Ca2+ channels previously shown to be expressed in these cells (14).

Nicotine Stimulates SCLC Cell Proliferation. (-)-Nicotine stimulates SCLC cell proliferation in GLC8, NCI-H-69, and NCI-N-592 cells (Table 1).

The effect of nicotine on the proliferation rate of human SCLC (7) and hamster neuroendocrine lung tumors (15) have been reported. SCLC cells had to be inhibited in their proliferation rate by the selective AchR antagonist mecamylamine (maximal inhibition of >95% at 10 µM mecamylamine; Fig. 1, inset), suggesting a receptor-mediated mechanism of action of nicotine. The removal of extracellular Ca2+ and the addition of ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid completely prevents (-)-nicotine-induced [3H]5-HT release from SCLC cells (Fig. 1, inset). The Ca2+-dependent release of [3H]5-HT induced by nicotine might be due to nicotine-induced cell depolarization and a subsequent opening of the voltage-gated Ca2+ channels previously shown to be expressed in these cells (14).

SCLC cells are mitogenic and releasable by (-)-nicotine but that it is able to act as a mitogen for these cells.

Table 1 (-)-Nicotine-stimulated [3H]thymidine incorporation in NCI-H-69, NCI-N-592, and GLC8 SCLC cell lines

<table>
<thead>
<tr>
<th></th>
<th>NCI-H-69</th>
<th>NCI-N-592</th>
<th>GLC8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>3156 ± 288</td>
<td>5833 ± 111</td>
<td>1453 ± 35</td>
</tr>
<tr>
<td>Nicotine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 pm</td>
<td>4895 ± 542</td>
<td>6858 ± 86</td>
<td>1693 ± 53</td>
</tr>
<tr>
<td>100 pm</td>
<td>5761 ± 897*</td>
<td>8300 ± 132*</td>
<td>2032 ± 91*</td>
</tr>
<tr>
<td>10 nM</td>
<td>6223 ± 489*</td>
<td>8215 ± 408*</td>
<td>2731 ± 178*</td>
</tr>
</tbody>
</table>

*P < 0.01 with Sheffé's test after a significant overall analysis of variance.

DISCUSSION

Nicotinic acetylcholine receptors of the neuronal type have recently been characterized in SCLC cells (7, 11, 12). We now report the first evidence for a functional role of these receptors: activation of SCLC AchRs by nicotine stimulates serotonin release from these cells, a process strictly related to SCLC cell proliferation.

Our data are in agreement with the evidence that nicotine or some of its metabolites might act directly on AchRs of lung neuroendocrine cells, thus inducing hormone release (1, 2, 16) and cell proliferation (2, 17). The role of tobacco smoking on lung cancer development should, therefore, also be considered in the light of the possible presence and function of AchRs in epithelial neuroendocrine lung cells.

These results show for the first time that 5-HT is not only present in SCLC cells and releasable by (-)-nicotine but that it is able to act as a mitogen for these cells.
Mitogenic effects of both nicotine and serotonin have been recently described in a non-SCLC (carcinoid) human lung cancer cell line (18). However, this is the first report of serotonin stimulatory effects on SCLC cells. Furthermore, both nicotine and serotonin were effective on SCLC at concentrations much lower than those effective on the carcinoid cell lines.

Serotonin should, therefore, be added to the list of autocrine growth factors produced and released by SCLC cells. It is worth noting that a similar autocrine role of 5-HT has been recently demonstrated in pancreatic carcinoid tumors (19).

Previous reports on the mitogenic properties of 5-HT on transfected fibroblasts, Chinese Hamster Lung fibroblasts, and jejunal epithelial, renal mesangial, and smooth muscle cells (for a review, see Ref. 20) have suggested a possible involvement of either 5-HT, 5-HT1B, or 5-HT2 receptor subtypes. Our pharmacological data suggest an involvement of 5-HT1 receptor subtypes in mediating the mitogenic effects of 5-HT on SCLC; a more detailed molecular analysis of the serotonergic receptor subtypes present in SCLC is in progress.

The above data suggest that nicotine might affect SCLC cell proliferation specifically by the activation of a serotonergic autocrine loop. The concentrations of endogenous 5-HT achieved in the medium as a consequence of nicotinic receptor activation (30–50 nM) are compatible with this mechanism.

This suggests that both the nicotinic and the serotonergic receptors should be evaluated as potential targets of a new pharmacological approach to the control of SCLC proliferation. We have already found that ganglionic nicotinic antagonists (such as mecamylamine) and serotonergic antagonists (such as methiothepine and metergoline) do inhibit nicotine- and serotonin-induced in vitro SCLC proliferation, respectively. Furthermore, we have preliminary evidence that methiothepine also might partially inhibit nicotine-induced cell proliferation (without interacting directly with the nicotinic receptor), while nicotinic agents have no effect on 5-HT-induced cell proliferation (not shown), further suggesting that one major effect of nicotine is to stimulate a serotonergic autocrine loop.

\* A. Codignola and Marco Lanza, unpublished observation.
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