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Signalling pathways involved in nicotine regulation of apoptosis of human lung cancer cells

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Although nicotine has been implicated as a potential factor in the pathogenesis of human lung cancer, its mechanism of action in the development of this cancer remains largely unknown. The present study provides evidence that nicotine (a) activates the mitogen-activated protein (MAP) kinase signalling pathway in lung cancer cells, specifically extracellular signal-regulated kinase (ERK2), resulting in increased expression of the *bcl*-2 protein and inhibition of apoptosis in these cells; and (b) blocks the inhibition of protein kinase C (PKC) and ERK2 activity in lung cancer cells by anti-cancer agents, such as therapeutic opioid drugs, and thus can adversely affect cancer therapy. Nicotine appears to have no effect on the activities of *c-jun* NH_2-terminal protein kinase (JNK) and p38 MAP kinases, which have also been shown to be involved in apoptosis. While exposure to nicotine can result in the activation of the two major signalling pathways (MAP kinase and PKC) that are known to inhibit apoptosis, nicotine regulation of MAP (ERK2) kinase activity is not dependent on PKC. These effects of nicotine occur at concentrations of 1 μM or less, that are generally found in the blood of smokers, and could lead to disruption of the critical balance between cell death and proliferation, resulting in the unregulated growth of cells. The findings suggest caution in the use of smokeless tobacco products to treat smoking addiction, as they could have a potentially deleterious effect in patients with undetectable early tumour development.

Introduction

Cigarette smoking and the resultant nicotine addiction are closely tied to the development of human lung cancer. Recent studies have shown a direct molecular link between benzo[*a*]-pyrene, a chemical carcinogen found in tobacco smoke, and the development of this cancer. The metabolites of this carcinogen were found to form adducts at mutational hotspots in the tumour suppressor gene *p53* (1). Nicotine is another major component in tobacco. However, apart from its addictive properties, a direct role for nicotine in the development of lung cancer has not yet been defined. Specific, high affinity nicotine acetylcholine receptors (nAChRs*) are found on human lung cancer cells of all histologic types and in normal lung tissue (2–4), and nicotine has been shown to increase lung cancer cell numbers (4–6), suggesting a role in pulmonary carcinogenesis. We have found that nicotine blocked opioid-induced apoptosis (programmed cell death) in a number of human lung cancer cell lines (7). A similar suppressive effect of nicotine on apoptosis induced by diverse stimuli, such as tumor necrosis factor, UV light, chemotherapeutic drugs and calcium ionophore, was observed in other tumour cells (8).

It has been suggested that a common property shared by known and suspected tumour promoters is their ability to block the process of apoptosis (9), which is an important mechanism in the regulation of cancer cell growth and in response to anti-cancer therapy (for reviews see references 10,11). Based on this definition, our previous studies (7) and those of other investigators (8) suggest that nicotine can act as a tumour promoter. However, knowledge of the intracellular mechanisms involved in nicotine suppression of apoptosis in human lung cancer cells is fragmentary. We have found that the two therapeutic opioid drugs, methadone and morphine, that are generally used in the treatment of cancer pain, are also potent inducers of apoptosis in human lung cancer cells, but their apoptotic effects appear to involve different signalling pathways. Nicotine, however, blocked apoptosis induced by both opioid drugs. In several of the lung cancer cell lines, morphine significantly decreased both basal and phorbol 12-myristate 13-acetate (PMA)-stimulated total protein kinase C (PKC) activity, while nicotine stimulated PKC activity in these cells, and reversed the inhibitory effect of morphine on PKC activity (7). Activation of PKC has been shown to be involved in the mechanism of action of tumour promoters (12), and in the desensitization of cells to programmed cell death (11). In contrast, methadone did not decrease PKC activity in lung cancer cells, suggesting involvement of a second pathway in the actions of nicotine in these cells.

The aim of the present study is to elucidate the key cellular events involved in the anti-apoptotic effects of nicotine in human lung cancer cells, in order to determine whether it has a direct role in the development of lung cancer, in addition to its inhibitory effect on apoptosis-inducing agents. This study is important because the widespread use of smokeless tobacco products, such as nicotine patches and gums to treat smoking addiction, provides another source of nicotine that could play a potentially harmful role in lung tumour development.

***Abbreviations:** MAP, mitogen-activated protein; ERK2, extracellular signal-regulated kinase; PKC, protein kinase C; JNK, *c-jun* NH_2-terminal protein kinase; nAChRs, nicotine acetylcholine receptors; PMA, phorbol 12-myristate 13-acetate; PVDF, immobilon-P polyvinylidene difluoride; DAMGO, [D-Ala^2, N MePhe, Gly^5-ol]-enkephalin; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HRP, horseradish peroxidase; ERK, extracellular signal-regulated kinase; SCLC, small cell lung carcinoma cells; GRP, gastrin-releasing peptide.

Materials and methods

Cell lines and reagents

Previously characterized human lung cancer cell lines NCI-H345, NCI-N417 and NCI-H157 (a gift from Drs John D.Minna and Adi Gazdar, UT Southwestern Medical Centre at Dallas) were grown in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum, as described (13), and were found to be free of mycoplasma contamination. The cells were maintained in an atmosphere of 5% CO_2 in air at 37°C.

Commercial sources of reagents: (–)nicotine ditartarate, (±)methadone hydrochloride, PMA, D-sphingosine, Immobilon-P polyvinylidene difluoride (PVDF) transfer membranes (Sigma, St Louis, MO); morphine sulphate (Mallinckrodt, Phillipsburg, NJ); [D-Ala^2, N MePhe, Gly^5-ol] (DAMGO)-enkephalin (Peninsula Laboratories, Belmont, CA); BIOTRAK^{TM} MAP kinase assay kit (Amersham, Arlington Heights, IL); phospho-specific MAP Kinase polyclonal antibody, PhosphoPlus^{TM} p38 MAP Kinase (Tyr182) and SAPK/JNK (Thr183/Tyr185) antibody kits, MAP kinase inhibitor PD98059 (New England Biolabs, Beverly, MA); polyclonal antibodies to the phosphatases PAC1 (A-19) and MKP-1 (C-19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); human *bcl*-2 (Ab-1) antibody (Oncogene Science, Cambridge, MA). PROTRAN nitrocellulose transfer membranes (Schleicher & Schuell, Keene, NH). Protein concentrations were measured using the Bio-Rad protein assay kit.

MAP kinase assay

Human lung cancer cells were grown to confluency in 10 cm tissue-culture dishes. Prior to drug treatment, the cells were cultured overnight in serum-free medium. Varying concentrations of the different drugs (in sterile distilled water) were added directly to the medium and the cells incubated for 5 min at 37°C. The time course of MAP kinase activation shows that nicotine rapidly stimulates enzyme activity, reaching a maximum by 5 min and gradually declining thereafter (65% from peak values after 30 min of stimulation). Control cells were treated with the same volume of sterile distilled water used to dissolve the drugs. Following incubation with the drugs, cells were collected either by scraping with a rubber policeman or by centrifugation, depending upon the cell type. NCI-H157 cells grow as a monolayer attached to the dish, while the NCI-N417 and NCI-H345 cells are floating aggregrates. The culture medium was then removed by centrifugation, and the cells washed with 3×10 ml of ice-cold Ca^{2+}-free phosphate-buffered saline (PBS). Cells were lysed by sonication in 10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, containing a 2 mM concentration of the phosphatase inhibitor, sodium orthovanadate. Cellular debris was precipitated at 25 000 *g* for 20 min and the supernatant retained to obtain MAP kinase activity. Protein concentrations of the supernatants were measured, and 15 μl of the supernatants were assayed immediately for MAP kinase activity with the BIOTRAK^{TM} MAP kinase assay kit (Amersham), according to the manufacturer's instructions. The assay system is based upon the p42/44 MAP kinase catalyzed transfer of the γ-phosphate group of adenosine-5′-triphosphate to a peptide that is highly selective for the p42/44 MAP kinase. The reaction was initiated by the addition of [γ-^{32}P]ATP (Amersham). Incubation proceeded for 30 min and the peptide was separated from the unincorporated activity using a binding paper separation step. The linear range of the assay was between 0.9–27 pmol Pi transferred per min. MAP kinase activity detected in the samples was normalized for protein concentration and expressed as the percentage ratio of each activity to the initial activity.

Western blotting for activated ERK, JNK and p38 MAP kinases

2×10^6 lung cancer cells were serum-starved overnight, treated with the different drugs, incubated for different lengths of time (indicated in figure legends), and washed as described above. In general, standard Western blotting procedures were employed, using the Phosphoplus MAP Kinase antibody kits from New England Biolabs. Cell lysates were prepared as follows: for the ERKs, the lysates were prepared by sonicating the cells on ice in the buffer described above for the MAP kinase assay. Protein concentrations were determined and 10 μg of protein were dissolved in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue). For the JNK and p38 MAP kinases, cells were lysed on ice by sonication in SDS sample buffer. 10 μg of protein were boiled for 5 min, cooled on ice, and subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred electrophoretically to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (blocking buffer) for 2 h at room temperature and then incubated overnight at 4°C with a 1:1000 dilution of phospho-specific ERK and 1:200 dilutions of JNK and p38 antibodies in PBS containing 5% bovine serum albumin (BSA) and 0.05% Tween-20. After three washes with blocking buffer, the membranes were incubated with alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:5000) for 2 h at room temperature, and signals detected using the Phototope-Star Chemiluminescent Western blot detection kit from New England Biolabs and X-ray films, according to the manufacturer's instructions.

Western blots for phosphatase expression

Cells were treated with the drugs for 30 min and lysed in 25 mM HEPES (pH 7.4), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin. Western blots were carried out with a 1:100 dilution of either PAC1(A-19), an affinity-purified goat polyclonal antibody to the protein tyrosine phosphatase PAC1, or MKP-1 (C-19), an affinity purified rabbit polyclonal antibody to MAP kinase phosphatase-1. The method used was the same as described above, except that the proteins were transferred to nitrocellulose membranes, and the appropriate IgG conjugated with horseradish peroxidase (HRP) was used as the secondary antibody at a dilution of 1:5000. The signals were detected using the ECL chemiluminescent detection system from Amersham.

Western blots for bcl-2 protein expression

Experiments were performed according to the method described by McDonnell *et al*. (14). Cells were treated with drugs for 90 min at 37°C, washed with PBS and lysed in 1% Triton X-100, 10 mM Tris (pH 7.4), 150 mM NaCl, 50 μg/ml PMSF, 10 μg/ml aprotinin at 4°C for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation at 15 000 *g* for 10 min and protein concentrations of the cleared lysates were determined. Ten μg of these lysates were boiled in SDS sample buffer (composition described above), loaded onto 12.5% SDS–PAGE gels, transferred overnight to nitrocellulose membranes, blotted with a 1:100 dilution of anti-human mouse monoclonal antibody to *bcl*-2 (Ab-1), and a 1:5000 dilution of HRP-conjugated anti-mouse secondary antibody, and the signals detected using the ECL chemiluminescent detection system.

Results

Nicotine activates ERK2 MAP kinase and blocks methadone-induced inhibition of the activity of this kinase

As mentioned in the Introduction, PKC appears to be involved in the actions of morphine, but not of methadone, in human lung cancer cells (7). To determine whether a common pathway is involved in the actions of nicotine, methadone and morphine in lung cancer cells, we examined the role of the other major signalling pathway, the MAP kinase cascade, that has been shown to be involved in apoptosis of other cells, such as the rat PC-12 pheochromocytoma cells (15). This cascade consists of a series of serine/threonine kinases that are activated by sequential phosphorylation, and they play a crucial role in the transmission of growth factor signals to the nucleus (for review see reference 16). The MAP kinase family includes extracellular signal-regulated kinases (ERK), *c-jun* NH_2-terminal protein kinase (JNK) and p38. The pathways involved in the activation of these three MAP kinases are functionally distinct. Lung cancer cells are classified into small cell lung carcinoma cells (SCLC) representing ~25% of all cases, and non-SCLCs representing the remaining 75%. In addition, SCLC cell lines have been subgrouped into two major classes—'classic' and 'variant'—based on biochemical, morphological and growth properties (17). In the present study, human lung cancer cells representing the three major histologic types, NCI-H345 ('classic' type SCLC), NCI-N417 ('variant' type SCLC) and NCI-H157 (non-SCLC) were treated with nicotine and opioids, and the effects of these drugs on ERK activity analyzed on Western blots with a phospho-specific antibody to MAP kinase which detects only the active phoshorylated isoforms, p42 and p44. This antibody revealed selective involvement of the p42 (ERK2) isoform in the actions of nicotine and methadone in these cells. The 42 kDa band could be detected in the control (no drug) and nicotine-treated, but not the methadone-treated lysates of the NCI-N417 and NCI-H157 cells, within 5 min of treatment, indicating that methadone induces an inactive non-phosphorylated state of ERK2 in these cells (Figure 1). However, methadone did not inhibit basal activity of this kinase in the NCI-H345 cells, although we could detect the 42 kDa band in the nicotine-treated cells (data not shown). When 1 μM nicotine was added to the culture medium together with methadone, it reversed the inhibitory effect of methadone on ERK2 activity in the NCI-N417 and NCI-H157 cells, as indicated by the reappearance of the 42 kDa band (Figure 1). These effects of nicotine and

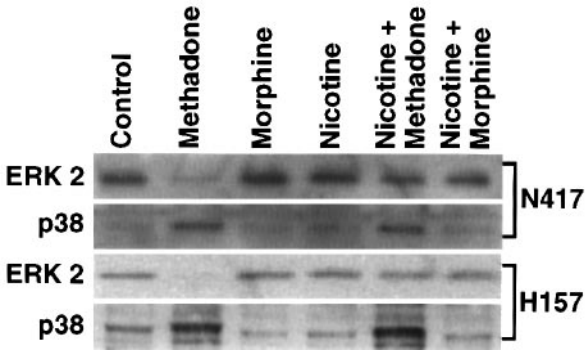

Fig. 1. Western blot analysis demonstrating the effects of nicotine and opioids on ERK2 and p38 MAP kinase activities in NCI-N417 and NCI-H157 cells, using rabbit polyclonal phospho-specific antibodies to ERK (1:1000) and p38 (1:200) kinases. The ERK MAP kinase antibody detects ERK1 and ERK2 (p42/44) only when catalytically activated by phosphorylation at Tyr 204, and the p38 antibody detects p38 only when activated by phosphorylation at Tyr 182 and has no appreciable cross-reactivity with either ERK or JNK MAP kinases. The cells were cultured overnight in serum-free medium and then treated with 1 μM concentrations of the different drugs for 5 min (for ERK activity) and 30 min (for p38 activity), and the samples prepared and analyzed, as described in Materials and methods. The experiment was repeated at least three times.

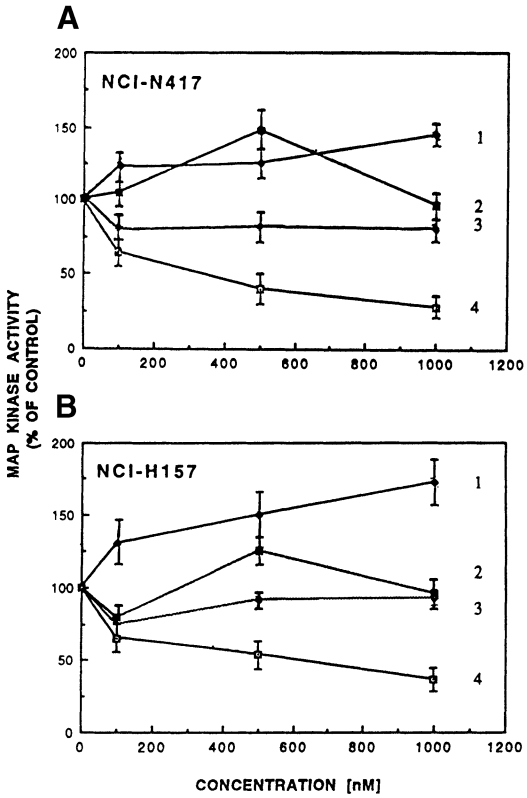

Fig. 2. Effects of nicotine, methadone and morphine on MAP kinase activity in cell lysates derived from (**A**) NCI-N417 and (**B**) NCI-H157 cells, as measured by the BIOTRAK^{TM} MAP kinase assay kit (Amersham) that uses a synthetic peptide highly selective for the p42/p44 (ERK1 and ERK2) MAP kinase. Serum-starved cells were treated with varying concentrations of the different drugs for 5 min, and the samples prepared and assayed, as described in Materials and methods. Results are the average of three different experiments (± SEM). 1 = nicotine; 2 = morphine; 3 = nicotine + methadone; 4 = methadone.

opioids on ERK activity were measured quantitatively with the BIOTRAK^{TM} MAP kinase assay kit, which uses a synthethic peptide substrate that is highly selective for the p42/p44 MAP kinase. Nicotine stimulated total MAP (ERK) kinase activity over basal levels, within 5 min of treatment, while methadone inhibited activity of this kinase in the NCI-N417 and NCI-H157 cells in a dose–dependent manner (Figure 2A and B). In the presence of nicotine, the inhibitory effect of methadone on ERK activity was reversed, and the activity returned to basal levels. In contrast to the effect of methadone, other agonists specific for μ opioid receptor type, such as morphine (Figures 1 and 2) and DAMGO did not inhibit MAP (ERK) kinase activity in these cells. At a 1 μM concentration, DAMGO caused a 30% increase ($P < 0.05$) in ERK activity over basal levels (data not shown). These results demonstrate that nicotine activates ERK2 in the three histologic types of lung cancer cells used in this study, and reverses the inhibitory effect of methadone on ERK2 activity in the 'variant' SCLC (NCI-N417) and non-SCLC (NCI-H157). The differential effects of methadone and morphine on MAP kinase (described in the present study) and PKC activity (7) strongly suggest that these opioids utilize distinct signalling pathways for the induction of apoptosis in lung cancer cells. However, nicotine is able to reverse the inhibitory effects of both agonists on the different pathways.

Nicotine cannot reverse methadone-induced p38 MAP kinase activation

The involvement of JNK and p38 MAP kinases in the actions of nicotine and opioids in lung cancer cells was also examined. Western blot analysis of nicotine and opioid-treated cell lysates, using a phospho-specific antibody to p38, revealed specific activation of this kinase over basal levels in the NCI-N417 and NCI-H157 cells, following treatment with methadone (Figure 1). In contrast, morphine or nicotine did not significantly change p38 activity from the basal levels, and nicotine had no effect on methadone-induced activation of p38 (Figure 1). Treatment of these cells with nicotine or opioids did not have an effect on JNK activity, under the conditions used in our assay (data not shown). Thus, there appears to be a concurrent activation of p38 and inhibition of ERK2 activity in lung cancer cells in response to treatment with methadone. However, the effect of nicotine appears to be restricted to the regulation of ERK2.

Effects of nicotine and methadone on MAP kinase phosphatases

MAP kinase activation in cultured cells is a transient event that is regulated by dual specificity protein phosphatases (16). A number of MAP kinase phosphatases have been identified, and are synthesized *de novo* after initiation of the signalling cascade (16). We have observed an increase in the levels of expression of the dual-specific threonine/tyrosine phosphatase PAC1 (a 36 kDa protein that has stringent substrate specificity for ERK MAP kinase; reference 18); in the NCI-N417 and NCI-H157 cells, following a 30 min treatment with methadone (W.Heusch and R.Maneckjee, in preparation). In the present study, we find that nicotine does not appear to have a direct regulatory effect on phosphatase activity in these cells. The levels of PAC1 and MKP-1, another phosphatase commonly expressed in several human tumours, remained the same as the basal levels in the nicotine-treated cells, and nicotine did not affect the increase in phosphatase activity following treatment of the cells with methadone (data not shown). These results suggest that nicotine activation of ERK2 kinase is not through inhibition of MAP kinase phosphatase activity.

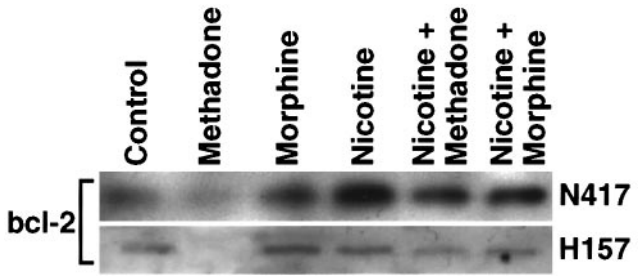

Fig. 3. Western blot analysis of the regulation of expression of *bcl*-2, a 25–26 kDa membrane protein, in NCI-N417 and NCI-H157 cells by 1 μM concentrations of methadone, morphine and nicotine. Cells were incubated with the drugs for 90 min at 37°C. Cell lysates were prepared, and assessed with anti-human mouse monoclonal antibodies to *bcl*-2 (Ab-1), as described in Materials and methods.

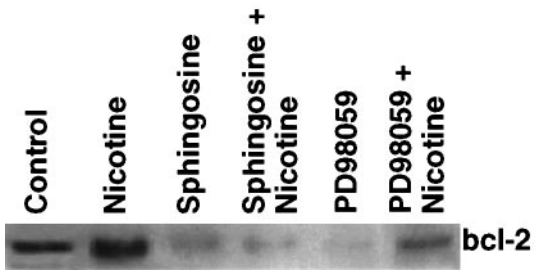

Fig. 4. Western blot analysis of the effects of sphingosine (PKC inhibitor) and PD98059 (MAP kinase inhibitor) on nicotine regulation of *bcl*-2 protein expression in NCI-N417 cells. Nicotine = 1 μM; sphingosine = 50 μM; PD98059 = 50 μM.

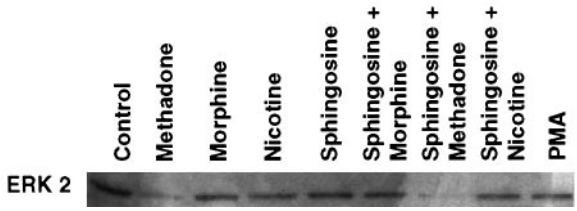

Fig. 5. Western blot analysis demonstrating the effects of sphingosine (PKC inhibitor) and PMA (PKC activator) on ERK2 MAP kinase activity in nicotine, morphine and methadone treated NCI-N417 cells. Sphingosine = 50 μM; PMA = 10 ng/ml.

Nicotine blocks the inhibitory effect of methadone on bcl-2 expression

Over-expression of the inner mitochondrial membrane protein *bcl*-2 has been shown to block the process of apoptosis in certain cell lines (for reviews see references 10,11). High levels of *bcl*-2 are expressed in human lung cancer cells, while the levels appear to be low in normal lung (19,20). Thus, experiments were carried out to determine whether nicotine and opioids could regulate the expression of this protein in human lung cancer cells. Western blot analysis, with a commercially available monoclonal antibody to this protein, shows that morphine and methadone appear to differ in their regulation of the levels of expression of the *bcl-2* protein in these cells (Figure 3). Treatment of the NCI-N417 and NCI-H157 cells with 1 μM methadone for 90 min resulted in the loss of the 25 kDa band corresponding to the *bcl*-2 protein. However, in the presence of 1 μM nicotine, this effect was blocked. In contrast, at the same concentration, morphine did not block the expression of this protein. The levels of expression of *bcl*-2 in NCI-N417 cells could also be decreased by PD98059, a highly selective inhibitor of MAP kinase activity, both in the absence and presence of 1 μM nicotine (Figure 4).

Role of PKC in nicotine-induced activation of MAP (ERK2) kinase and bcl-2 protein expression

Because nicotine activates both PKC and MAP kinase in these cells, we examined the possibility of intracellular cross-talk between the two pathways. We have previously found that sphingosine, an inhibitor of PKC activity in cultured cells, caused an ~80% decrease in PKC activity from control levels ($P < 0.05$), and it significantly increased DNA fragmentation (~60%, $P < 0.05$) in several lung cancer cell lines, including NCI-N417 and NCI-H157, and these effects of sphingosine could not be blocked by nicotine (7). In addition, in the presence of sphingosine, nicotine could not reverse morphine-induced decrease in PKC activity. However, sphingosine did not inhibit the increase in PKC activity observed in these cells following treatment with methadone (7). In the present study, we have found that sphingosine inhibited the expression of *bcl-2* in the NCI-N417 cells, in the absence and presence of 1 μM nicotine, similar to the effect of the MAP kinase inhibitor, PD98059 (Figure 4). To explore the role of PKC in nicotine-induced activation of MAP kinase, NCI-N417 cells were incubated with nicotine and/or opioids, in the presence and absence of the PKC inhibitor sphingosine, and PMA, which we have shown to increase PKC activity (139% over control value, $P < 0.05$) in these cells (7). Western blot analysis with the phospho-specific ERK antibody revealed that neither PMA nor sphingosine alone had any significant effect on ERK2 activity, and they did not affect the regulation of ERK2 activity by nicotine and opioids (Figure 5). These results suggest that nicotine-induced activation of ERK2 MAP kinase is not dependent on PKC activity. However, inhibition of PKC and/or MAP kinase activity in lung cancer cells appears to be critical for the suppression of *bcl-2* protein expression and subsequent induction of apoptosis in these cells.

Discussion

These studies demonstrate a complex pattern of changes induced by nicotine at the cellular level in lung cancer cells. It has the capacity to modulate the two major intracellular signalling pathways (PKC and MAP kinase), resulting in suppression of the physiological process of apoptosis. The present data indicate that chronic exposure to nicotine, through cigarette smoking, could result in sustained activation of MAP (ERK2) kinase and over-expression of *bcl*-2 protein in lung cancer cells. We have previously described the stimulatory effect of nicotine on PKC activity in lung cancer cells (7), and the present study shows that PKC is also involved in the increase in *bcl*-2 expression in lung cancer cells. However, there does not appear to be an interaction between the two pathways, as we did not observe a PKC-mediated activation of MAP kinase in these cells. The precise mechanism by which nicotine increases ERK2 activity is yet to be determined. These effects of nicotine could lead to disruption of the dynamic balance between cell death and proliferation, essential for the normal functioning of cells, and result in tumor development. This could explain the increase in lung cancer cell numbers observed in the presence of nicotine (4–6). In addition to its direct anti-apoptotic effect via the signal transduction pathways, continuous ingestion of nicotine would block the therapeutic effects of anti-cancer agents, such as

opioids, on lung cancer cells by reversing their inhibitory effects on either the MAP kinase or PKC pathways.

It is also possible that nicotine stimulates MAP kinase activity in lung cancer cells by inducing the release of autocrine growth factors, such as serotonin and gastrin-releasing peptide (GRP) or bombesin (5,21), which have been shown to stimulate proliferation of small cell lung cancer cells. In contrast to the NCI-N417 and NCI-H157 cells, methadone was unable to inhibit MAP kinase activity and induce apoptosis in the NCI-H345 cells. We have recently observed that the apoptosis-inducing effects of methadone were dependent on the concentration of bioactive GRP secreted by lung cancer cells. In a subset of 'classic' SCLC cells, such as the NCI-H345 cells, that secrete supramaximal concentrations of this neuropeptide (6 pmol as compared to < 0.01 pmol/mg protein for the NCI-N417 and NCI-H157 cells; reference 22), methadone's effects appear to be blocked by the autocrine effect of GRP. The activation of ERK2 and increased expression of *bcl*-2 observed in control lung cancer cells (no drug treatment) could be due to the autocrine effect of bombesin on these cells (W.Heusch and R.Maneckjee, in preparation). The highly malignant SCLCs are generally more common in smokers than non-smokers (23), and increased levels of pulmonary bombesin-like peptides have been found in the urine of smokers, as compared to non-smokers (21). Thus, it is possible that there are endogenous systems coupling nicotine and bombesin, resulting in the inactivation of the opioid 'tumour suppressor' system in lung cancer cells (2), and these interactions are presently being investigated.

While the p38 signalling pathway appears to be involved in the actions of methadone in lung cancer cells, reversal of the apoptotic effects of methadone by nicotine does not involve this pathway. The down-regulation of ERK2 activity and the simultaneous activation of p38 MAP kinase following methadone treatment in lung cancer cells appears to involve other, as yet unknown, factors. These results suggest that the ERK pathway plays a more critical role in the induction of apoptosis in lung cancer cells, since stimulation of the p38 pathway alone is not sufficient to overcome the inhibitory effect of nicotine on methadone-induced apoptosis. Inhibition of ERK and a concurrent activation of the JNK-p38 signalling pathways were found to be critical for induction of apoptosis following NGF withdrawal in the rat PC-12 pheochromocytoma cells (15).

MAP kinases are inactivated by several dual specificity protein phosphatases (16). Thus, these phosphatases could regulate mitogenic signals, and their biological role is being actively investigated. In rat pituitary cells, dopamine inhibition of DNA synthesis has been shown to be associated with stimulation of phosphotyrosine phosphatase activity (24). The present data suggest that the two MAP kinase phosphatases, PAC1 and MKP-1, do not appear to be involved in nicotine activation of ERK2 activity in lung cancer cells. However, increased levels of phosphatase activity induced by methadone could lead to inhibition of ERK2 activity and concurrent activation of p38 resulting in apoptosis, and this sequence of events is blocked by nicotine, acting directly on ERK2.

Recently, Gallo *et al.* (25) reported a correlation between heavy cigarette smoking and increased expression of the *bcl*-2 oncogene in patients with head and neck cancer, and suggested that the *bcl*-2 gene may be the target of carcinogens in tobacco smoke. However, they did not indicate a mechanism by which tobacco smoke may influence *bcl*-2 expression in these cancers. The present studies demonstrate the critical role played by increased activation of PKC and MAP kinase in the sequence of events leading to over-expression of this protein in lung cancer cells, as specific inhibitors of these two pathways are effective in suppressing *bcl*-2 expression. Over-expression of *bcl*-2 appears to be a reversible process, and withdrawal of nicotine would restore the normal functioning of the cell.

The results presented here and in previous reports (2,7), indicate a heterogeneous effect of nicotine on the growth inhibitory effects of opioids in lung cancer cells. We have found that nicotine reversed opioid-induced growth inhibition in some, but not all (9/14) of the lines tested. This effect does not appear to be restricted to a particular histologic type of lung cancer. Since all lines expressed specific, high affinity binding for nicotine, it suggested the presence of different receptor subtypes on lung cancer cells that are 'sensitive' and 'resistant' to nicotine reversal. However, Northern blot analysis with cDNA probes specific for the different nicotinic acetylcholine receptor subunits showed no differences in subunit expression between nicotine 'sensitive' and 'resistant' cell lines (R.Maneckjee, X.Zhu and J.Minna, in preparation), suggesting that nicotine regulation of opioid effects occurs at a site downstream of the receptor, possibly as the result of a change in the post-receptor components of the signalling pathways of the nicotine 'resistant' cell lines. In lung cancer cells, such as NCI-H146 and NCI-H1299, where nicotine could not reverse opioid-induced apoptosis, nicotine was unable to stimulate PKC activity over basal levels (7), although high levels of specific nicotine binding was found in these cells (1024 fmol/mg protein, reference 2). However, further investigation is required to determine the exact cause of this heterogeneous response of lung cancer cells to nicotine, which appears to be complex, due to the involvement of several interacting growth factors in these cells.

Thus, nicotine appears to play an important role in the pathogenesis of human lung cancer, and it appears to have multiple sites of action. Chronic exposure to nicotine can lead to sustained activation of growth-promoting pathways and result in the development of new tumours, as well as decrease the efficacy of anti-cancer agents, such as methadone. These effects of nicotine occur at concentrations [~90–240 nM] that are observed in the blood of smokers (26), and indicate that the use of nicotine gums and patches to treat smoking addiction needs to be carefully evaluated. Exposure to nicotine could also result in the development of second lung primaries commonly found in lung cancer patients. We have previously shown that nicotine receptor antagonists, such as hexamethonium and decamethonium, reversed the suppressive effect of nicotine on opioid-induced apoptosis, and a combination of these antagonists with opioids or nicotine stimulated apoptosis (7), indicating that these antagonists could have potential value as chemopreventive agents for lung cancer in cigarette smokers.

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