Activation of p-ERK1/2 by nicotine in pancreatic tumor cell line AR42J: effects on proliferation and secretion

Chhanda Bose,1,3 Hailing Zhang,2 Kodetthoor B. Udupa,1,2,3 and Parimal Chowdhury2
1Donald W. Reynolds Department of Geriatrics, 2 Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, College of Medicine; and 3Medical Research, Central Arkansas Veterans Health Care System, Little Rock, Arkansas

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Bose, Chhanda, Hailing Zhang, Kodetthoor B. Udupa, and Parimal Chowdhury. Activation of p-ERK1/2 by nicotine in pancreatic tumor cell line AR42J: effects on proliferation and secretion. Am J Physiol Gastrointest Liver Physiol 289: G926–G934, 2005. First published July 28, 2005; doi:10.1152/ajpgi.00138.2005.—The objectives of the present study were to determine the effect of nicotine on MAPK signaling and on the proliferation of AR42J cells as well as to assess the relationship between MAPK activation and exocrine secretion in these cells. AR42J cells were incubated with nicotine and analyzed for the activation of MAPK by Western blot analysis using their respective antibodies and confirmed by immunohistochemistry. The effect of nicotine on cell proliferation was determined by the spectrophotometric method, and cell function was assessed by cholecystokinin (CCK)-stimulated amylase release into the culture medium. Nicotine at a dose of 100 μM induced phospho-ERK1/2 activation maximally in 3 min compared with untreated cells. Furthermore, immunofluorescence study confirmed the nicotine-induced increase in translocation of phospho-ERK1/2 to the nucleus. Activation of phospho-ERK1/2 was inhibited by an ERK1/2 pathway inhibitor but not by a nicotine receptor antagonist. At the same dose, there was a significantly enhanced proliferation of AR42J cells until 72 h without toxic effect, as the percentage of lactate dehydrogenase release remained unchanged. Other MAPKs, c-Jun NH2-terminal kinase 1/2 and p38 MAPK, were not affected by nicotine treatment. At a nicotine dose of 100 μM, the CCK-stimulated release of amylase was maximal at 6 min, and, although a nicotinic receptor antagonist inhibited this response, it was not inhibited by the ERK1/2 pathway inhibitor. We conclude that nicotine treatment induced activation of ERK1/2 and increased the proliferation of AR42J cells. The data further indicate that MAPK signaling by nicotine is independent of the secretory response.

cell signaling; stimulus-secretion coupling; nicotine effect

SEVERAL REPORTS have suggested that smokers have a higher risk for developing various types of cancers than nonsmokers (41a, 53, 59, 69). It has, however, been shown that chronic pancreatitis is associated with smoking, and it is also a possible risk factor for the induction of pancreatic cancer (6, 33, 45). Earlier studies conducted in rats with nicotine have shown that nicotine, a component derived from cigarette smoking, induces metabolic, pathological, and functional changes in the pancreas (8, 10) that are identical to those reported in patients dying with chronic pancreatitis (35, 11), experimental animal models of pancreatitis (54), and in vitro cell culture studies (7, 17, 29). The mechanism by which nicotine alters the stimulus-secretion coupling in exocrine pancreatic acinar cells is as yet unknown.

In this study, we report on the mitogenic effects of nicotine in AR42J cells, a stable rat pancreatic tumor cell line derived from the hyperplastic pancreatic nodules of male rats after the administration of azaserine (32). Because of their unique properties of stability, secretory capacity, and growth potential, these cells have been extensively used as an in vitro model for the biological investigation of isolated pancreatic acinar cells, in terms of their secretory processes and associated signal transduction pathways (13, 21, 66, 67). Because regulatory peptides can stimulate AR42J cells to secrete enzymes and induce proliferation (13, 29, 58), we wondered whether or not nicotine would influence AR42J cells in a similar manner and thus might play an important role in the pathogenesis of pancreatic disorders.

Nicotine is known to activate several MAPK signaling pathways in a variety of tissue and cell types (25, 40, 41, 50, 56, 60). MAPK enzymes play a critical role in the regulation of cell proliferation, differentiation, and apoptosis and are comprised of a ubiquitous family of tyrosine/threonine kinases that include ERK1/2, c-Jun NH2-terminal kinases (JNK)1/2, and p38 MAPK. Cytokines and mitogens trigger a signaling cascade that leads to the activation of MAPK, which has been well documented in studies with different cell lines (3, 14, 30, 39, 43). The induction of MAPKs, however, leads to the phosphorylation and activation of a variety of proteins, including a number of transcription factors involved in regulating the expression of genes controlling cellular proliferation (4, 18, 65). ERK1/2 is found in most tumors and is involved in the carcinogenesis of gastric cancer (52).

In pulmonary neuroendocrine cells, nicotine binds to nicotinic receptors, resulting in the phosphorylation of ERK and stimulation of DNA synthesis (1, 25). Furthermore, nicotine induces Ca2+ influx and stimulates the Ras/ERK cascade, which promotes cell survival in neuronal cells (28). Thus ERK1/2 is thought to be one of the possible signaling pathways involved in nicotine-induced cell proliferation. It was, therefore, of interest to us to investigate the involvement of ERK1/2 in the AR42J cell proliferation response to nicotine and to simultaneously examine the changes in the secretory function of these cells.

Our results demonstrate that nicotine can induce the proliferation of AR42J cells by activating ERK1/2 and that this process is independent of its secretory response.

Address for reprint requests and other correspondence: P. Chowdhury, Dept. of Physiology and Biophysics, Univ. of Arkansas for Medical Sciences, College of Medicine, 4301 W. Markham St., Little Rock, AR 72205 (e-mail: Pchowdhury@uams.edu).

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MATERIALS AND METHODS

Cell culture. The AR42J cell line, derived from rat pancreatic tumors, was obtained from American Type Culture Collection (Rockville, MD). Cells were grown in 75-cm² flasks containing 12 ml medium, consisting of Ham's F-12 nutrient medium (F12K) with 2 mM L-glutamine, 1% antibiotic, 1.5% sodium bicarbonate, and 10% FBS. Flasks were placed in an incubator maintained at 37°C and in a 5% CO₂-95% air atmosphere.

Nicotine treatment and inhibitor studies. For experiments with MAPK, confluent cells were trypsinized with 0.25% trypsin and 1 mM EDTA. On day 0, 1.6 × 10⁵ cells were plated into 100-mm culture dishes containing 10 ml F-12 nutrient medium, which included 10% FBS and 1% antibiotic solution. On day 1, when the cells were attached to the culture dishes, the medium was changed to serum-free nutrient medium. On day 2, cells were treated with 10 μM-2 mM nicotine (Sigma; St. Louis, MO) in 6.0 ml of serum-free medium for periods of 30 s to 10 min. Two specific MAPK inhibitors, PD-98059 (Calbiochem Biosciences; La Jolla, CA) or U0126 (Cell Signaling Technology; Beverly, MA), were used in the MAPK inhibitor studies. Cells were pretreated with PD-98059 (50 or 10 μM) or U0126 (5 or 10 μM) for 30 min before the addition of nicotine. For function studies, cells were grown in F-12 nutrient medium containing 10% FBS until 70–80% confluence. Cells (4–6 × 10⁵) were treated with HEPES-Ringer (HR) buffer and buffer with nicotine concentrations ranging from 50 μM to 1 mM with periods of exposure from 30 s to 10 min. Studies were performed with or without cholecystokinin-8 (CCK; Bachem; Philadelphia, PA).

Preparation of whole cell lysate. After treatment with nicotine, cells were washed with ice-cold 1× PBS and lysed with RIPA buffer on ice (1× PBS, 1% Nonidet P-40, 0.5% sodiumdeoxycholate, and 0.1% SDS plus freshly added protease and phosphate inhibitors). Cells were collected, homogenized in a hand homogenizer for 5 s, passed through a syringe fitted with a 21-gauge needle, and incubated on ice for 40 min. Cell lysates were then microcentrifuged at 10,000 g at 4°C for 10 min, the supernatant was collected, and protein was estimated using the method described by Bradford (5).

Western blot analysis. SDS-PAGE was carried out in 4–12% bis-Tris NuPage separating gel (Invitrogen Life Technologies; Carlsbad, CA). Equal amounts of protein were loaded into each lane, and the fractionated protein was electroblotted onto nitrocellulose membranes at 30 V for 1 h at room temperature. Membranes were blocked in casein blocker (Pierce; Rockford, IL) for 1 h at room temperature and then probed with primary antibodies (anti-p44/42ERK1/2, anti-p46/44MAPK, anti-p38MAPK diluted to 1:2,000 in casein blocker) and incubated with gentle shaking at room temperature for 1 h. Phospho-specific antibodies of the same were diluted 1:1,000 and incubated with membranes overnight at 4°C using gentle shaking. Membranes were washed five times (5 min each) with Tris-buffered saline-Tween 20 [TBST; 20 mM Tris·HCl (pH 7.6), 137 mM NaCl, and 0.2% (vol/vol) Tween 20] and incubated with horseradish peroxidase-coupled anti-IgG (secondary antibody, dilution 1:3,000) for 1 h. All antibodies were purchased from Cell Signaling Technology. Enhanced chemiluminescence (ECL+; Amersham Biosciences; Piscataway, NJ) and fluorescence detection steps were followed per the manufacturers' instructions for visualization of the bands, and bands were quantified using a Storm 860 Imager (Molecular Dynamics; Sunnyvale, CA).

Cell proliferation and cell toxicity assay. The effect of nicotine on the proliferation of AR42J cells was assessed utilizing commercially available Cell Counting Kit-8 (Dojindo Molecular Technologies; Gaithersburg, MD), which uses a highly sensitive spectrophotometric assay as described by Itano et al. (28). AR42J cells were seeded in 96-well microplates at 20 × 10⁴ cells/well, allowed to attach, and then incubated overnight in F-12 nutrient media containing 10% FBS. In these studies, before the treatment, cells were transferred to nutrient media containing 0.05% serum for 10–12 h. In a preliminary study, we determined that AR42J cells were viable only for 40 h in serum-free condition, whereas in 0.05% serum-containing medium, cells were viable beyond 96 h.) Cells were treated with 100 μM of nicotine for up to 96 h. Proliferation was measured from 24 to 96 h using Cell Counting Kit-8 according to the manufacturer’s instructions. Briefly, after treatment with nicotine for a fixed period, the dye solution was added to the wells, and incubation continued at 37°C for another 2–3 h. The amount of reduced 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt produced by the viable cells was assessed by the absorbance difference between treated and control (untreated) samples measured at a wavelength of 450 nm.

To determine the cellular toxicity induced by different doses of nicotine, media from control and nicotine added cultures were analyzed for the percentage of lactate dehydrogenase (LDH) released using a commercially available colorimetric kit, the Cytoscan-LDH Cytotoxicity Assay Kit, from Genotech (Geno Technology; St. Louis, MO) according to the manufacturer’s instructions.

Immunofluorescence studies. Immunofluorescence studies were performed as described by Sharma et al. (51) with minor modifications. AR42J cells were plated in 4-well Lab-Tek chamber slides (Becton-Dickinson Labware; Franklin Lakes, NJ) at a density of 40 × 10⁴ cells. Cells were serum starved overnight in basal F-12 nutrient media (without any growth factor) and then treated with 100 μM of nicotine for 3 min. After being briefly washed with cold PBS, cells were fixed with 2% paraformaldehyde for 20 min at room temperature, permeabilized with 1% Triton X-100 in PBS for 5 min, and then washed extensively with PBS. Cells were first incubated at room temperature for 30 min with 1% BSA and 5% goat serum in PBS and subsequently with primary antibodies against ERK1/2 (1:200) or phospho-ERK1/2 (p-ERK1/2; 1:100) at room temperature for 1 h. After three washes with PBS, cells were finally incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:50, Sigma) at room temperature for 45 min. Cells were washed extensively with PBS, mounted in an aqueous mounting medium (Molecular Probes; Eugene, OR), and observed under a fluorescence microscope (Olympus America; Melville, NY). The negative controls for immunostaining were those cells that were incubated with rabbit IgG without the specific primary antibody (51) with minor modifications.

Amylase secretion from AR42J cells after nicotine treatment. AR42J cells were washed free of media after being washed two times with HR buffer (pH 7.4) and incubated in the same buffer with or without nicotine (50–1,000 μM) for periods of 0 to 10 min and then washed twice with HR buffer to make the cells nicotine free. Cells were then dispersed in fresh HR buffer and incubated with or without CCK (10⁻⁹ M) for 30 min at 37°C. The selection of the CCK dose (10⁻⁹ M) for stimulation was based on our initial study, which showed a maximal stimulated response of amylase release in the CCK dose-response curve (7). After the incubation period, the media were removed by centrifugation and analyzed for amylase activity by the method of Jung (27) with Procion yellow starch as a substrate (PRO Chemical & Dye; Somerset, MA). Cell pellets were washed with ice-cold PBS, lysed with water by sonication, and centrifuged. The cell lysate was analyzed for both amylase and protein content. The release of amylase was expressed as the percent initial from the total.

Amylase secretion by AR42J cells with ERK inhibitors and the nicotine receptor antagonist mecamylamine. For amylase secretion in the presence of an ERK inhibitor, cells were pretreated with PD-98059 (50 μM) or U0126 (10 μM) for 30 min. After being washed, cells were incubated with 100 μM nicotine for 6 min. The method used for CCK stimulation was similar to that described in Amylase secretion from AR42J cells after nicotine treatment. For studies of AR42J cells with the nicotine antagonist mecamylamine, cells were pretreated with 500 μM mecamylamine for 30 min before the addition of nicotine and incubated further with nicotine for an additional 6 min. At the end of the incubation period, cells were washed with HR buffer and incubated with either HR buffer or buffer containing 10⁻⁹ M...
CCK for 30 min. Amylase secretion and protein concentration were determined as outlined in "Amylase secretion from AR42J cells after nicotine treatment."

Statistics. Results were reported as means ± standard error of the mean (SE). The results were analyzed either by students’ t-test (two groups) or analysis of variance (three or more groups), using a multiple statistics software package. Differences were considered significant at a probability of 0.05 or lower.

RESULTS

Activation of MAPK by nicotine in AR42J cells. First, we investigated nicotine-induced activation of one of the MAPKs, ERK1/2, in AR42J cells by Western blot analysis with phospho-specific antibodies to ERK1/2. Incubation of AR42J cells with varying doses of nicotine from 50 μM to 2 mM for 3 min produced higher phosphorylation of ERK1/2 compared with the control (Fig. 1). Maximal induction of p-ERK1/2 occurred at a nicotine dose of 100 μM, and the incubation time needed to achieve this maximum was 3 min (Fig. 2). Total ERK1/2 activity remained unchanged during all of these conditions (Figs. 1A and 2A). Because the highest response was observed at a nicotine dose of 100 μM and incubation time of 3 min, these conditions were employed for all subsequent ERK1/2 stimulation studies. For localization of phospho-specific ERK1/2 in AR42J cells after nicotine treatment, the immunohistochemical technique was used. As depicted in Fig. 3, immunofluorescence staining showed a considerably higher quantity of p-ERK1/2 present in AR42J cells 3 min after the 100 μM nicotine treatment, as indicated by the higher fluorescence intensity, corresponding to ERK1/2 in the cytoplasm and nucleus, compared with the nicotine-free untreated control. This observation confirmed our result of activation of ERK1/2 by nicotine, noted in the Western blot analysis.

Fig. 1. Dose-dependent induction of ERK1/2 in AR42J cells. Cell lysates were loaded onto a SDS gel, separated by electrophoresis, blocked by casein, and probed with antibodies to total and phosphorylated (p)ERK1/2. Horseradish peroxidase-coupled anti-IgG was used as a secondary antibody. Bands were visualized with an enhanced chemiluminescence kit and quantified with a Storm Imager. A: representative ERK1/2 bands on Western blots. B: increased (fold) induction of ERK1/2 as means ± SE of 5 separate studies.

To evaluate whether the other two members of MAPKs, namely, JNK1/2 and p38, would follow similar patterns to ERK1/2, studies were then repeated for these two members of the MAPK family after nicotine treatment. As depicted in Fig. 4, Western blot analysis conducted with the respective phospho-specific antibodies showed that p-JNK1/2 was slightly, but not significantly, elevated at 5 min with a dose of 100 μM nicotine treatment (1.21 ± 0.12 and 1.66 ± 0.17 arbitrary units in control samples and in samples treated with 100 μM nicotine for 5 min, respectively), whereas p-p38 did not change at any time during nicotine treatment (Fig. 4). Thus these two members of MAPK did not produce a similar effect comparable to ERK1/2 after nicotine treatment.

To further understand the mechanism involved in the effect of nicotine on MAPK and the role of an upstream ERK1/2 activator, MEK1/2, two specific inhibitors of MEK1/2 activity, PD-98059 and UO126, were used. These two agents block MEK1/2 phosphorylation and subsequent activation of ERK1/2. Both of these inhibitors significantly blocked the increase in ERK1/2 phosphorylation induced by nicotine (Fig. 5), although UO126 was more potent than PD-98059. As before, with the addition of 100 μM nicotine, ERK1/2 activation was significantly increased from 1.21 ± 0.09 to 2.09 ± 0.21 arbitrary units in 3 min (P < 0.05). Incubation with 10 μM UO124 before the addition of nicotine of the same dose abolished such an increase, with the value being 0.49 ± 0.10 arbitrary units, which was lower than the control (Fig. 5B). There was no effect of inhibitors on the amount of total ERK1/2 (Fig. 5A). These studies demonstrated that nicotine activation of ERK1/2 was dependent on MEK1/2. Pretreatment of cells with MEK1/2 inhibitors had no effect on nicotine-induced JNK or p38 activity (data not shown).

Activation of ERKs is mediated through the mitogenic response of nicotine. To evaluate the role of nicotine-induced ERK1/2 activation in the growth of AR42J cells, we investi-
gated the proliferation potential of these cells after treatment with nicotine by the spectrophotometric method. Cells were maintained in 0.05% serum, and their proliferation was evaluated in response to 100 μM nicotine, as described in MATERIALS AND METHODS. The addition of 100 μM nicotine caused an increase in proliferation at 24–72 h, with significant increases observed at 24, 48, and 72 h compared with untreated cells (Fig. 6A). Proliferation of cells with both nicotine treatment and control were maximum at 72 h, and, at that time interval, the proliferation profile measured in control wells by absorption was 0.780 ± 0.006 arbitrary units compared with the absorption noted in nicotine-added wells of 0.982 ± 0.008 arbitrary units (P ≤ 0.01). In both instances, proliferation of cells decreased at 96 h.

Nicotine-mediated proliferation was inhibited by the MEK inhibitor UO126. To determine the inhibitory effect of the MEK-ERK1/2 pathway on the proliferation of AR42J cells, the specific MEK1/2 pathway inhibitor UO126 was used. Cells were treated with either 10 μM UO126 or medium alone for 30 min, and, after this interval, 100 μM nicotine was added to both. Cell proliferation was monitored at 48 h using the spectrophotometric proliferation assay kit, as described earlier. The addition of UO126 alone to the AR42J cells did not exert any significant effect on proliferation of the AR42J cells, with the values being 0.731 ± 0.010 and 0.726 ± 0.009 absorption units for control and UO126-added samples, respectively. As shown earlier, with the addition of 100 μM nicotine, the proliferation of AR42J cells was significantly elevated at 48 h to 1.042 ± 0.013 absorption units (P ≤ 0.01). With the addition of 10 μM UO126 and 100 μM nicotine, the proliferation did not increase, with the value of 0.743 ± 0.011 absorption units being similar to the control without nicotine. Thus this study indicated a direct correlation between the increase in ERK1/2 and the proliferation of AR42J cells.

Evaluation of cytotoxicity of nicotine. AR42J cells were exposed to different doses of nicotine for various time intervals to determine the time-dependent increase in cytotoxicity of these cells as measured by the percentage of LDH released to the medium. Results showed that the nicotine dose range used in the study (from 0.05–0.1 mM) had no toxic effect on AR42J cells. As shown in Fig. 6B, the percentage of LDH released with 0.1 mM nicotine was 7.80 ± 0.43% compared with the percentage of LDH released, which was 8.96 ± 0.91% in control, untreated cells (P ≥ 0.05). With the increase of nicotine concentration to 0.2 mM, the percentage of LDH released was significantly elevated to 14.04 ± 0.54% compared with control (P ≤ 0.01). The percentages of LDH released in the presence 100 μM nicotine from 24 to 96 h were
of ERK1/2 and CCK-stimulated amylase secretion at a maximal rate. To identify whether these two processes were inter-related, we measured nicotine-induced amylase secretion in AR42J cells after incubation with inhibitors of the ERK1/2 pathway, namely, PD-98059 and UO126. As shown in Fig. 8A, pretreatment of cells with 50 μM PD-98059 or 10 μM UO126 for 30 min had no effect on amylase secretion, both in the absence or presence of CCK. Hence, this study demonstrated that ERK1/2 activation by nicotine is independent of stimulus-secretion coupling mediating amylase secretion, even though both the MAPK activation and secretory phenomena were increased at the same dose of nicotine.

Amylase secretion with nicotine is receptor mediated in AR42J cells. To evaluate whether the nicotine-mediated increase in amylase secretion was via a receptor-mediated phenomenon, we incubated AR42J cells with the specific nicotine receptor antagonist mecamylamine before treatment with nicotine. As shown in Fig. 8B, when cells were incubated with 100 μM nicotine after incubation with 500 μM mecamylamine, amylase secretion was significantly lower than for those cells incubated with nicotine alone or in the presence or absence of CCK (Fig. 8B). These results indicate that the nicotine-mediated amylase secretion by AR42J cells is receptor mediated.

The next study was performed to see whether activation of ERK1/2 by nicotine was receptor mediated. For this study, AR42J cells were pretreated first with 500 μM mecamylamine and then stimulated with 100 μM nicotine, after which cell lysates were tested with Western blot analysis to determine activation of p-ERK1/2. As shown in Fig. 9, activation of ERK1/2 by nicotine was similar in the presence or absence of mecamylamine (1.93 ± 0.03 and 1.85 ± 0.02 arbitrary units with the addition of nicotine alone or added after mecamylamine treatment, respectively). These results indicated that activation of ERK1/2 after nicotine treatment was not receptor mediated. Thus nicotine treatment induced an increase in ERK1/2 phosphorylation as well as amylase secretion in AR42J cells by two independent pathways.

DISCUSSION

The studies described here demonstrate a complex pattern of changes occurring at the cellular level of AR42J cells when they are exposed to nicotine. Nicotine is capable of modulating two major intracellular signaling pathways resulting in cell proliferation (MAPK) as well as exocytotic events resulting in increased enzyme secretion (the physiological process). Our study also presents a plausible mechanism by which nicotine can induce the growth of AR42J cells, through activation of the MAPK signaling system. The proliferation of AR42J cells by nicotine, however, appears to be independent of the stimulus-secretion coupling response of amylase secretion. At the dose used, nicotine-induced proliferation of AR42J cells is MAPK pathway specific, particularly by ERK1/2 activation, as the proliferation is suppressed by a specific inhibitor of the MEK-ERK pathway. We also found that this particular inhibitor has no effect on the proliferation of control cells in the absence of nicotine. Earlier studies have also shown that nicotine can increase the cell numbers of certain cancer cell lines (16, 46, 49), suggesting that nicotine exposure can lead to the disruption of the dynamic balance between cell death and prolifera-
tion, which is required for normal functioning of cells. We have based our argument that nicotine exposure enhances the proliferation of AR42J cells on a mechanism mediated via the early activation of ERK1/2 signaling pathways. However, the sustained increase of cell numbers at 24 h and beyond after nicotine exposure indicates that nicotine might also directly induce the release of autocrine growth factors, as reported by proliferation studies of small lung cancer cells (1, 16).

The selection of the nicotine dose in the present study was based on published literature in both in vivo and other cell culture studies (2, 20, 23, 26, 31, 37, 40, 41). Earlier studies from this laboratory have shown that between 5% and 6% of nicotine is accumulated in pancreatic tissue after either a bolus or constant infusion (9). This means that circulating levels of nicotine could be very high before it is metabolized and excreted in urine. Besides, it has been shown that peak plasma nicotine concentration in cigarette smokers can range from 10 to 15 mM within 20 min of cigarette smoking (48). In addition, in studies from other laboratories, nicotine doses from 0.75 to 25 mM have been used with isolated rat pancreatic acini (36).

As mentioned earlier, our studies have indicated that ERK1/2 is the only MAPK activated by nicotine, whereas the other two components of MAPKs, JNK and p38, are not affected significantly. Nakayama et al. (40, 41) have shown...
that nicotine treatment induces ERK activation in PC12 cells. It has been further shown that chronic exposure to nicotine via cigarette smoking results in a sustained activation of MAPK (ERK1/2) and overexpression of bcl-2 protein in human lung cancer cells (25), without affecting the activities of JNK and (ERK1/2) and overexpression of bcl-2 protein in human lung cancer cells (25). The results of our studies also demonstrate some parallelism in the time-dependent activation of ERK by CCK and nicotine, although these two secretagogues are completely different from one another in terms of their biological actions on the pancreas (7, 19, 66). Furthermore, our results show that activation of ERK1/2 preceded the activation of JNK1/2, a finding that is identical to that observed in freshly isolated pancreatic acini cells (17).

In addition to mitogenesis, we also focused on the involvement of nicotine in the basal and CCK-stimulated secretory response in this cell line. As shown previously, AR42J rat pancreatic acinar cells retain the potential to secrete digestive enzymes in addition to their ability to proliferate upon stimulation with CCK and other growth factors (17, 21, 29, 58, 66, 67). CCK can also activate MAPK in pancreatic acinar cells in a concentration- and time-dependent manner by a mechanism involving protein kinase C and tyrosine kinase activity (21), via activation of MEK and Ras (17, 22) and p125FAK (29). The dose of CCK that maximally stimulates enzyme secretion in freshly isolated normal pancreatic acini is 10 nM. Whereas Kiehne et al. (29) have demonstrated that CCK induces maximal secretion and p42ERK2 activation in AR42J cells at a dose of 10 nM, different regulatory peptides, such as bombesin, cause only p42ERK2 activation but not significant enzyme secretion, suggesting that kinase activation and receptor-mediated signal transduction pathways leading to enzyme secretion might involve separate mechanisms. In our present study, nicotine maximally increased basal and CCK-stimulated enzyme secretions at 6 min at the same dose, whereas kinase activation was maximal at 3 min and declined steadily thereafter. Although ERK1/2 activation by nicotine is completely inhibited with specific kinase inhibitors, these inhibitors have no influence on the basal and stimulated secretory responses induced by nicotine. Together, these results suggest that nicotine-induced proliferation of AR42J cells is correlated with ERK1/2 activation as demonstrated by Western blot analysis and immunohistochemistry, which intimates that the ERK1/2 pathway is responsible for the stimulation of

![Graph A](image)

![Graph B](image)
AR42J cell proliferation. This is further supported by the data showing that inhibition of ERK1/2 activation by UO126 attenuates the nicotine-induced proliferative response of AR42J cells.

Stimulation of enzyme secretion by secretagogues in pancreatic acini has been shown to involve several second messenger pathways that are rapidly activated by G protein-coupled receptors (44, 61, 63) and changes in intracellular calcium concentration (51). Earlier, we have shown that nicotine at doses of 1 to 30 mM concentration, either administered in vivo or added to in vitro isolated acini, inhibits the CCK-stimulated enzyme secretion (7). The mechanism of this nicotine effect is unclear, although a cell-mediated intracellular Ca$^{2+}$ response appears to be involved (55). Although no study has been conducted to examine the effects of nicotine in this cellular system at a dose of lower than 1 mM, in our present study, the effect of nicotine at a dose of 100 μM induced maximal secretory responses in AR42J cells at 3 min, which persisted for 6 min before decreasing. The effects of nicotine on the basal and stimulated secretory response are abolished in the presence of mecamylamine, a nicotinic receptor antagonist, suggesting that the secretory response of nicotine is G protein coupled and receptor mediated. ERK1/2 activation by nicotine under similar conditions and in the presence of the nicotine receptor antagonist is unaffected, implying that the kinase and secretory responses induced by nicotine are completely independent from each other and, perhaps, involve a separate mechanism.

It has been reported that in rat sublingual mucous acini, nicotine first triggers the release of acetylcholine from presynaptic nerve terminals, which then activates muscarinic receptors (70). In addition, in freshly isolated pancreatic acini, ω-conotoxin, a potent Q-type calcium channel blocker, inhibited nicotine-induced pancreatic secretion completely (unpublished data), suggesting that the regulation of pancreatic secretion is physiologically regulated by a calcium-mediated process. The present study examined the specificity of nicotinic receptor in AR42J cells and demonstrated clearly its effect on the downstream events regulating exocrine secretion. A similar effect has also been observed in freshly isolated rat acini (unpublished observations). The major finding of our study is the identification of two independent pathways between MAPK signaling and exocytotic secretion by nicotine. ω-Conotoxin, a Q-type calcium channel blocker, also inhibited nicotine-induced secretion but not MAPK signal activation, confirming that pathways for MAPK signaling and receptor-mediated regulation of exocrine secretion are mediated via independent mechanisms (unpublished data).

In conclusion, our data demonstrate that nicotine at the employed doses activates pERK-1/2 signaling in AR42J cells. This is associated with cellular proliferation. The activation of kinase signaling appears not to be correlated with the stimulation of the receptor-mediated secretory response. The mechanisms of these two processes appear to be completely independent.

GRANTS

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