Tobacco carcinogen 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone initiates and enhances pancreatitis responses

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ACUTE PANCREATITIS IS AN ACUTE inflammation of the pancreas resulting in death for approximately one-third of patients who develop severe disease. Acute pancreatitis is most often caused by gallstones and alcohol abuse and is initiated by premature activation and retention of digestive zymogens (enzymes) within the pancreatic acinar cell (17, 22, 23, 37). Subsequent stages of the disease are characterized by ischemia, inflammation, and cell death.

Recently, cigarette smoking has emerged as a key risk factor for developing acute pancreatitis. Previous studies identified it as a risk factor for chronic pancreatitis (12, 19, 20, 30, 33, 36). Furthermore, cigarette smoking has been shown to increase the risk of developing alcohol-related pancreatitis (36). Recent reports have shown that cigarette smoking is an independent and dose-dependent risk factor for acute pancreatitis (33, 36). A Danish population-based study that included subjects who smoked but did not abuse alcohol demonstrated a dose-dependent association between smoking and chronic pancreatitis and identified a similar association with acute pancreatitis (33).

Although clinical advances have identified a role for cigarette smoke in the development of pancreatitis, only a few studies have investigated the effects of tobacco smoke and its components in animal models (7, 35). In a 12-wk study, rats exposed to tobacco smoke developed pancreatic damage (35). In another study, graded nicotine doses fed to rats for 3–16 wk caused cellular damage (pyknotic nuclei, karyorrhexis, vacuolization, and edema) in direct relationship to the dose of nicotine. In addition, a decreased secretory response and cellular damage were reported in isolated acinar cells treated with nicotine or isolated acinar cells harvested from nicotine-treated rodents (7, 9–11, 21). Although these studies show that cigarette smoke and nicotine can induce cellular damage in acinar cells, they do not demonstrate a mechanism or examine other major cigarette smoke toxins.

Cigarette smoke has a number of potentially toxic components; one of the most active, abundant, injurious, and best-studied is 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) (27, 28). NNK has been shown to induce pancreatic cancer in experimental models (1, 2), but its effects on pancreatitis have not been determined. In this study, we have focused on potential effects of NNK in the induction and enhancement of pancreatitis. We show that incubation of isolated rat acinar cells with increasing concentrations of NNK leads to premature activation of digestive zymogens. Furthermore, we find that the effects of NNK with supraphysiological (10–100 times physiological) concentrations of the secretagogues cerulein (100 nM, Cer), a CCK ortholog, and carbachol (1 mM, Carb), a muscarinic agonist, on zymogen activation are additive. In vivo stimulation of rats with NNK (100 mg/kg body wt ip) three times per week for 2 wk caused pancreatitis responses, including premature zymogen activation, vacuolization, pyknotic nuclei, and edema. Some of these responses increased when NNK treatment was combined with Cer. β-Adrenergic receptors (β-ARs) and α7-nicotinic ACh receptors (α7-nAChRs), both high-affinity NNK receptors, were identified in acinar cells. Furthermore, NNK-induced zymogen activation was reduced in acinar cells pretreated with the nAChR blocker mecamylamine. Our study suggests that NNK, a major com-

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ponent of tobacco, can cause and enhance pancreatitis responses, potentially through a receptor-mediated pathway.

**METHODS**

**Preparation of isolated pancreatic acini.** All experiments and procedures using animals were approved by the Veterans Affairs Institutional Animal Care and Use Committee (West Haven, CT). Pancreatic acini were isolated as described elsewhere (22). Briefly, fasted male Sprague-Dawley rats (100–125 g body wt; Charles River Laboratories, Wilmington, MA) were euthanized by CO2 inhalation. The pancreas was minced in buffer A [10 mM HEPES (pH 7.4), 95 mM NaCl, 4.7 mM KCl, 0.6 mM MgCl2, 1 mM Na2HPO4, 10 mM glucose, 2 mM glutamine, 0.1% bovine serum albumin, and 1× MEM amino acids (GIBCO-BRL, San Jose, CA)] and washed twice. Cells were then digested for 1 h at 37°C in buffer A containing 50 U/ml of type IV collagenase (Worthington, Freehold, NJ) under O2 with constant shaking. The digest was filtered through a 300- to 400-μm mesh (Sefar American, Depew, NY), and the resulting groups of acinar cells, acini, were distributed in a 24-well Falcon tissue culture plate (Becton Dickinson, Franklin Lakes, NJ). All reagents were purchased from Sigma Biochemical (St. Louis, MO), unless otherwise noted.

**Treatment of pancreatic acini with NNK and/or Cer.** Acini were treated as follows after recovery: 1) 1 mM PBS for 2 h (unstimulated control), 2) 1 mM PBS for 1 h followed by 100 nM Cer for 1 h, 3) 1–100 nM NNK (Toronto Research Chemicals, Toronto, ON, Canada) for 2 h, or 4) 1–100 nM NNK for 1 h followed by 100 nM Cer for 1 h. In some experiments, acini were treated with Carb (1 mM), instead of Cer, to induce pancreatitis responses. At the conclusion of the experiment, cells were centrifuged at 30 g, and a 50-μl sample of cell-free medium was removed for determination of amylase content. The remaining sample was frozen at –80°C until it was assayed.

**Enzymatic activity assays.** Enzymatic activity assays were carried out as a marker of zymogen activation (32). Briefly, samples from in vitro studies were thawed, homogenized, and centrifuged. To each well of a 24-well plate (Greiner Bio-one Cellstar TC-Plate), 100 μl of postnuclear supernatant and 350 μl of zymogen assay buffer [50 mM Tris (pH 8.1), 150 mM NaCl, 1 mM CaCl2, and 0.01% BSA] were added. The assay was initiated by addition of 50 μl of 400 μM enzyme substrate [fluorometric trypsin substrate (Boc-Gln-Ala-Arg-MCA; catalog no. 3135-v, Peptides International, Louisville, KY) and fluorometric chymotrypsin substrate (Suc-Ala-Ala-Pro-Phe-AMC; catalog no. 230914, Calbiochem, San Diego, CA)] diluted in zymogen assay buffer (40 M final concentration). The plate was read with a fluorometric microtiter plate reader (model HTS 7000, Perkin-Elmer) using an Axiophot microscope (Carl Zeiss, Thornwood, NY) at ×40 and ×60 magnification, and images were collected with a Spot Digital camera (Diagnostic Instruments, Sterling Heights, MI). Samples were assessed using a histological scoring system (34).

**Treatment of pancreatic acini with CCK-JMV-180, atropine, propranolol, and mecamylamine.** To determine if NNK was acting through the CCK receptor, isolated acini were treated with CCK-JMV-180 (100 nM), an antagonist for the low-affinity CCK binding site (the site through which pancreatitis responses are initiated) for 30 min, and then with NNK (100 nM) for 2 h. To determine if action of NNK was via a muscarinic receptor, acini were treated for 30 min with the muscarinic blocker atropine (1 μM) and then with NNK, as described above. Given that NNK is a high-affinity agonist for β-ARs and nonneuronal α1-nAChRs, acini were treated with the β-AR blocker propranolol (10 μM) or the nAChR blocker mecamylamine (500 μM) for 30 min and then with NNK (100 nM) for 2 h. At the conclusion of the experiment, acini were processed as described above.

**PCR.** Total RNA was isolated from rat pancreatic acini and testis using the RNeasy Midi kit (Qiagen, Valencia, CA), and cDNA was prepared using the SuperScript II kit (Invitrogen, Carlsbad, CA). PCR was carried out using 1 μl of first-strand cDNA in a 50-μl reaction volume containing 20 mM Tris (pH 8.4), 50 mM KCl, 2 mM MgCl2, deoxynucleotide triphosphates at 200 mM each, 1.25 U of Taq DNA polymerase (Invitrogen), and primers at 250 nM each (Table 1; W. M Keck Foundation Biotechnology Resource Laboratory, Yale University (14, 31). Amplification conditions were as follows: initial denaturation for 3 min at 94°C followed by 40 cycles of denaturation at 94°C, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

<table>
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<th>Table 1. Primers for PCR</th>
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<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Nicotinic α1-nAChR Forward</td>
<td>5'-ATCTGGGATGCGAGAATGATC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCCCATAGACCCATTGCTC-3'</td>
</tr>
<tr>
<td>β2-AR Forward</td>
<td>5'-CATGCTATGCGACGCTTGGCA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AAATGCGACGCTTGGGTC-3'</td>
</tr>
<tr>
<td>β2-AR Forward</td>
<td>5'-AGCTGCCATTTCTGGGATC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TAGTTTGGAGAAGGACCC-3'</td>
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α1-nAChR, α1-neuronal ACh receptor; β2-AR, β2- and β2-adrenergic receptors.
94°C for 45 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. For the β1-AR, an additional round of 40 cycles using 1 μl of starting material from the previous round was followed by a single extension step for 10 min at 72°C. PCR products were analyzed on agarose gels that contained ethidium bromide.

**Statistical analysis.** Values are means ± SE of at least three individual experiments, with each experiment performed at least twice. Statistical significance was determined by one-way ANOVA with a posttest analysis (Newman-Keuls). *P < 0.05* designated significance.

**RESULTS**

**NNK induces zymogen activation in isolated rat pancreatic acinar cells.** Isolated pancreatic acinar cells were incubated with 0.1–100 nM NNK for a total of 2 h and then assayed for trypsin and chymotrypsin activity as a marker for zymogen activation. We observed increasing enzyme activity as concentrations of NNK increased, resulting in a three- and fivefold increase in trypsin and chymotrypsin activity, respectively, over control, at 100 nM NNK (Fig. 1, A and B). Higher levels (1 and 10 μM) of NNK did not cause increases in trypsin and chymotrypsin activity beyond that seen with 100 nM NNK (data not shown). NNK (10 and 100 nM) also increased amylase secretion (from control value of 5–10%), although this increase was not statistically significant (Fig. 1C). Since maximal effects were seen with 2 h of treatment with 100 nM NNK, these conditions were used for subsequent in vitro experiments.

**NNK enhances secretagogue-induced zymogen activation in isolated rat pancreatic acinar cells.** The effects of NNK on zymogen activation were determined in secretagogue-stimulated models of acute pancreatitis. Isolated pancreatic acinar cells were incubated with 100 nM NNK and, in the final hour of the experiment, treated with 100 nM Cer or 1 mM Carb. NNK alone caused levels of zymogen activation comparable to those caused by Cer (Fig. 2, A and B) or Carb (Fig. 2, C and D) alone. In addition, in preparations treated with NNK and then with secretagogue, zymogen activation was increased (Fig. 2, A–D). NNK did not affect secretagogue-mediated amylase secretion (Fig. 2E).

**NNK alone does not affect acinar cell LDH release or enhance the effects of Cer.** The effects of NNK on cellular injury were evaluated by LDH release as an index of cellular damage (Fig. 3). Acini were treated with Cer in the presence or absence of 100 nM NNK for 120 min, and percent LDH release into the cell culture medium was determined. After treatment with 100 nM NNK, LDH release was comparable to that of an unstimulated control. After Cer (100 nM) treatment, LDH release rose from a control level of 7% to 13%; with NNK + Cer, LDH release remained at 13% with no further increase. Thus NNK did not cause or enhance secretagogue-induced cellular damage as determined by LDH release from isolated acini.

**NNK mediates zymogen activation in vivo.** Trypsin and chymotrypsin activity increased in pancreatic homogenates from NNK- compared with PBS-treated animals (Fig. 4). Furthermore, Cer-induced zymogen activation was enhanced by the addition of NNK. The data suggest that in vivo treatment with NNK alone induces zymogen activation and increases secretagogue-induced zymogen activation.

**NNK causes edema in vivo.** In rats treated with NNK (100 mg/kg body wt) for 2 wk, edema increased 21.2% compared with rats treated with PBS (Fig. 5). In rats treated with PBS for 2 wk and then hyperstimulated for 1 h with Cer, edema increased 15.6% compared with PBS-treated controls. Finally, in rats treated with NNK and then stimulated for 1 h with Cer, edema increased 16.0%. Comparisons between each treatment group and the PBS control were statistically significant (*P < 0.05*). Comparisons between the treatment groups were not significant. This indicates that treatment with NNK alone can cause edema comparable to that caused by treatment with Cer alone, although NNK + Cer does not cause additional edema.

**Pancreatic histology after NNK treatment and Cer stimulation in vivo.** Morphological markers of pancreatitis, including cytoplasmic vacuole and lobular separation, an indicator of pancreatic edema, were evaluated in a blinded manner. The control (PBS alone) group did not show evidence of pancre-
atitis (Fig. 6A). PBS + Cer-treated animals displayed increased vacuolization and some edema (Fig. 6B). Furthermore, these markers were prominent in tissues isolated from NNK-treated animals (Fig. 6C). Increased edema, vacuolization, and pyknotic nuclei were also observed in the NNK + Cer-treated group (Fig. 6D). Histological evaluation showed a score of 2.0 for PBS + Cer-treated animals compared with 0.1 for PBS-treated animals (Fig. 6E) and scores of 6.0 and 5.3 for NNK- and NNK + Cer-treated animals, respectively. The histological scores for each treatment group were significantly different from the score for the PBS control group. Furthermore, scores for the NNK and NNK + Cer groups were significantly greater than the score for the PBS + Cer group but were not different from each other. This indicates that NNK causes a significant increase in the morphological severity of pancreatitis, but this effect was not further increased when NNK was combined with Cer. In Fig. 6F, the individual scoring is given for each marker of pancreatitis: edema, vacuolization, and pyknotic nuclei. Each parameter increased significantly in NNK-treated animals; however, a lack of leukocyte infiltration was observed following NNK treatment.

NNK does not mediate zymogen activation through CCK or muscarinic receptors. An in vitro approach was used to determine if NNK was mediating zymogen activation nonspecifically through the CCK receptor. Isolated acini were treated...
with CCK-JMV-180 (100 nM), an antagonist for the low-affinity CCK binding site (the site through which pancreatitis responses are initiated) for 30 min and then with 100 nM NNK for 2 h. As shown in Fig. 7, NNK-induced zymogen activation was unaffected by treatment with CCK-JMV-180. In Fig. 2, we also show that zymogen activation can be induced by a supraphysiological concentration of the muscarinic agonist Carb. To determine if NNK was nonspecifically acting through a muscarinic receptor, acini were treated for 30 min with the muscarinic blocker atropine (1 μM) and then with NNK, as previously described. NNK-induced zymogen activation was unaffected by atropine treatment (Fig. 7). Thus NNK does not appear to be acting on CCK or muscarinic acinar cell receptors.

Potential NNK target receptors (β-ARs and nonneuronal α7-nAChRs) are present in pancreatic acinar cells. NNK is a high-affinity agonist for β-ARs and nonneuronal α7-nAChRs, which could mediate NNK effects in the exocrine pancreas (2, 28). The presence of β1- and β2-ARs and the nonneuronal α7-nAChR in isolated pancreatic acinar cells was confirmed by PCR analysis (Fig. 8).

NNK-mediated zymogen activation is unaffected by propranolol but is reduced by mecamylamine. Pharmacological inhibitors were used to determine if NNK could mediate zymogen activation through β1- or β2-ARs or the nonneuronal α7-nAChR. Isolated acinar cells were treated with the β-blocker propranolol (10 μM) or the nAChR inhibitor mecamylamine (500 μM) for 30 min and then with 100 nM NNK for 2 h and assayed for zymogen activation. As shown in Fig. 9A, propranolol did not affect NNK- or Cer-mediated zymogen activation. However, mecamylamine significantly reduced NNK-induced zymogen activation but did not affect Cer-induced zymogen activation (Fig. 9B). These data imply that NNK is affecting zymogen activation potentially through a nonneuronal α7-nAChR.

**DISCUSSION**

Recent clinical studies directly link cigarette smoking to the development of acute pancreatitis. The smoking effects are independent of risks imparted by gallstones and alcohol abuse (26, 33). The risk of cigarette smoke-induced disease is directly related to the levels and duration of smoking. However, little experimental evidence has been available regarding the underlying cellular mechanisms associated with smoking-related pancreatitis.

In this study, we have focused on a potent tobacco carcinogen and nicotine metabolite, NNK, and its potential effects in development of acute pancreatitis. We have used two pancreatitis models published by our laboratory and others. 1) In the in vitro model, isolated pancreatic acini recapitulate the initial responses (i.e., inappropriate intracellular activation of zymogens) in acute pancreatitis when treated with supraphysiological concentrations of Cer or muscarinic agonists (Carb), without the influence of inflammation, nerves, or decreased blood flow. 2) In the in vivo model, Cer hyperstimulation consistently causes mild-to-moderate pancreatitis responses, including inflammation and vascular injury (6, 32, 38).

We found that pretreatment of pancreatic acinar cells for 1 h with NNK (100 nM; the level commonly used in in vitro cancer studies (4)) caused pathological intracellular activation of the zymogens trypsinogen and chymotrypsinogen (Fig. 1). A high concentration of Cer for 1 h also caused zymogen activation; the effects of NNK + Cer were additive (Fig. 2). The response elicited by Carb was similar to that seen with both NNK and Cer (Fig. 2). These findings show that NNK can initiate zymogen activation and increase secretagogue responses. Furthermore, the effects of NNK occur regardless of which secretagogue is used to induce zymogen activation.

NNK-mediated increases in zymogen activation would likely result in cellular injury, although this was found not to be the case when LDH release was measured as a marker for cellular injury (Fig. 3). It is possible that the short duration of NNK treatment in vitro studies is sufficient to cause zymogen activation but no appreciable injury. Furthermore, we saw in previous studies that changes in zymogen activation do not always correlate with some markers of cellular injury (18, 32).
To better assess effects of NNK in terms of cellular damage and altered morphology, we carried out longer treatments with NNK in vivo.

In the in vivo studies, intraperitoneal injection of NNK over a 2-wk period also induced zymogen activation and enhanced secretagogue-induced zymogen activation (Fig. 4). Additional parameters of pancreatitis, including tissue swelling or edema (Fig. 5), development of pyknotic nuclei, and generation of cytoplasmic vacuoles (Fig. 6), were observed. The responses in animals treated with NNK were at least as severe, if not more so, than those in animals treated with Cer alone (Fig. 6). Severity of the responses was not greater in animals treated with NNK and then with Cer than in animals treated with either agent alone (Fig. 6). This could suggest that NNK-mediated responses result in a maximal amount of tissue damage that cannot be exceeded, even when NNK and Cer are given in combination.

No significant leukocyte infiltration was observed in animals treated with NNK, Cer, or NNK + Cer. Our findings suggest that NNK causes pancreatic damage without inflammation, although it is possible that an even longer duration of NNK treatment may be required to induce the latter response. Potential effects of NNK on inflammation need to be studied in greater detail, but such an investigation is beyond the scope of the present study.
The NNK concentrations used in our studies (0.1–100 nM) were selected because they reflect levels of NNK to which a smoker can be exposed. The level of NNK can be as high as 2 g/cigarette; if a smoker uses 20 cigarettes/day, exposure will be 40 g in a 24-h period (3, 13). Given that the body weight of an average smoker is 75 kg, NNK concentration as a function of wet weight will be 4 nM. Levels of NNK in human pancreatic juice from cigarette smokers have been reported to be 1.4–600 ng/ml (7 nM–3 M) (24), which is similar to the concentration range used in our studies (0.1–100 nM). Furthermore, the NNK concentrations used in our in vitro and in vivo studies also correspond to those used in cancer studies (1, 2). Another issue in administering NNK for this study was choosing the duration of treatment. In some cancer studies, animals were treated with NNK for up to 10 wk, and NNK-induced carcinogenesis occurred in pancreas and lung (1). To preclude potentially carcinogenic effects in our study, we selected a 2-wk treatment regimen. We observed pancreatic damage after 2 wk of NNK but found no histological evidence of neoplasia.

Since the effects of NNK in these studies were rapid, we suspected that they were receptor-mediated. To exclude NNK effects on CCK or muscarinic receptors, we pretreated acini with the CCK receptor antagonist CCK-JMV-180 (JMV, 100 nM) and then treated with NNK (100 nM). NNK-stimulated trypsin activity was significant vs. control, although the inhibitors did not reduce this activity. Values are means ± SE; n = 4. *P < 0.05 vs. CTL.

![Fig. 7. NNK does not mediate zymogen activation through CCK or muscarinic receptors. Isolated acini were pretreated with the CCK receptor antagonist CCK-JMV-180 (JMV, 100 nM) or the muscarinic antagonist atropine (ATR, 1 µM) and then treated with NNK (100 nM). NNK-stimulated trypsin activity was significant vs. control, although the inhibitors did not reduce this activity. Values are means ± SE; n = 4. *P < 0.05 vs. CTL.](image)

![Fig. 8. Potential NNK target receptors [β-adrenergic receptors (β-ARs) and nonneuronal α7-nicotinic ACh receptors (α7-nAChRs)] are present in pancreatic acinar cells. PCR was carried out on unstimulated isolated acini to determine if β1- and β2-ARs and α7-nAChR were present. Amplified product was observed at the expected size for each receptor: 440 nt for β1-AR, 559 nt for β2-AR, and 178 nt for α7-nAChR (n = 3). Control tissue (CTL+) was brain; negative control (CTL−) reactions contained no cDNA.](image)

![Fig. 9. NNK-mediated zymogen activation is unaffected by propranolol but is reduced by mecamylamine. Isolated acini were pretreated with the β-AR antagonist propranolol (10 µM) or the nAChR blocker mecamylamine (500 µM) for 30 min and then treated for 2 h with NNK (100 nM). NNK-stimulated trypsin activity was unaffected by propranolol, but mecamylamine reduced trypsin activity. Values are means ± SE; n = 3. #P < 0.05.](image)
that at least its short-term effects are mediated through known receptor pathways.

Using PCR analysis of RNA from isolated acini, we found α7- nAChR and β1/β2-ARs, potential targets of NNK (Fig. 8). To determine which receptor class affected NNK responses, we pretreated acini with the β-AR blocker propranolol (10 μM) or the nicotinic receptor antagonist mecamylamine (500 μM). We found that propranolol had no effect on NNK- or Cer-induced zymogen activation, but mecamylamine significantly decreased NNK-mediated zymogen activation (Fig. 9). Thus our data would suggest that NNK could be inducing pancreatitis responses by activating a nonneuronal nicotinic receptor on the acinar cell.

These findings, while convincing, should be interpreted with caution. Our studies used pharmacological inhibitors to characterize involvement of nicotinic receptors; these are known to have nonspecific effects and could inhibit other cellular pathways. A genetic approach, such as small interfering RNA to knock down specific nAChRs, would be useful in confirming these findings. However, as it is difficult to obtain a robust knockdown or maintain the phenotype of pancreatic acinar cells in culture, such an approach is not feasible. Furthermore, there are no suitable alternative cell lines that could be substituted as a model for the pancreatic acinar cell. Alternatively, studies using appropriate genetic knockout mice could be used to determine whether they would eliminate pancreatitis responses to NNK.

Our data indicate that NNK-induced zymogen activation is mediated by a nicotinic receptor, raising the following question: Which downstream cellular pathways are involved in this response? It is widely known from the literature that sustained elevations in cytosolic calcium levels accompany zymogen activation (16, 25). Since the α7-nAChR is also a calcium entry channel, it is possible that NNK is contributing to pathological increases in calcium during development of pancreatitis. This study did not examine whether mecamylamine (or other nAChR blockers) reduces later stages of NNK-related pancreatitis, but the potential elevated levels of calcium that could arise from NNK stimulation may well contribute to pancreatitis events downstream of zymogen activation. Alternatively, these events could also be initiated through β-AR-mediated pathways; effects of β-AR blockers, if any, on later stages of NNK-induced pancreatitis have not been determined. Finally, we believe that NNK is not likely to act on nonacinar cell nicotinic receptors in our preparation of acini, but we cannot exclude effects of NNK on other cell types in our in vivo studies.

In conclusion, we have determined that a potent tobacco toxin, NNK, mediates zymogen activation and pancreatic damage, potentially through a nonneuronal nAChR on the acinar cell. This study establishes a foundation for future investigations into this important pathological mechanism. The findings presented here may ultimately lead to development of a therapy for smoking-related pancreatitis and may have implications for other diseases associated with smoking.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


