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Akt Kinase Mediates the Pro-Survival Effect of Smoking Compounds in Pancreatic Ductal Cells

Chang-Hwan Park, MD^{1,2}, In-Seok Lee, MD^{1,3}, Paul Grippo, PhD⁴, Stephen J. Pandol, MD¹, Anna S. Gukovskaya, PhD¹, and Mouad Edderkaoui, PhD¹

¹Veterans Affairs Greater Los Angeles Healthcare System and University of California at Los Angeles, Los Angeles, California

²Division of Gastroenterology, Department of Internal Medicine, Chonnam National University Medical School, Gwangju, Korea

³Division of Gastroenterology, Department of Internal Medicine, The Catholic University of Korea, Seoul, Korea

⁴Department of Surgery, Feinberg School of Medicine, Northwestern University, Chicago, IL

Abstract

Objectives—Cigarette smoking is a major risk factor for pancreatic cancer (PaCa). However, the mechanisms of smoking-induced PaCa remain unknown. Here we investigated the effect of smoking compounds on cell death pathways in pancreatic ductal cells, precursors of PaCa.

Methods—Human pancreatic ductal cells (HPDE6-c7) were cultured with cigarette smoking extract (CSE) or smoking compound 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Apoptosis and autophagy were assessed by DNA fragmentation and immunofluorescence, respectively.

Results—Exposure to CSE or NNK decreased DNA fragmentation and up-regulated Bcl_{xL}. Akt kinase was activated by smoking compounds through ROS-dependent mechanism. Specifically, Akt activation was prevented by inhibition of NAD(P)H oxidase. Molecular or pharmacologic inhibitions of Akt prevented anti-apoptotic effect of smoking compounds. Smoking compounds stimulated rapid (1h) and transient activation of AMPK and formation of autophagic vacuoles indicating stimulation of autophagy. Repeated exposure to CSE/NNK (48h or longer) abolished the early activation of autophagic markers. Inhibition of Akt prevented the anti-autophagic effect of long exposure to smoking compounds indicating that smoking-induced late activation of Akt prevents autophagy.

Address correspondence to: Mouad Edderkaoui, PhD, 11301 Wilshire Blvd., Bldg. 258, Rm. 340, Los Angeles, CA 90073. Tel: 310-478-3711 ext.48545. Fax: 310-268-4578 medderkaoui@mednet.ucla.edu.

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Conclusions—long exposure of pancreatic ductal cells to smoking compounds inhibited apoptosis and autophagy. The results revealed a central role for Akt kinase in mediating key pro-carcinogenic effects of smoking compounds.

Keywords

Pancreatic cancer; smoking; apoptosis and autophagy

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths among both men and women in the United States (1). Resistance of pancreatic cancer cells to current treatment regimens contributes to the dismal prognosis of this malignancy and presents a major challenge in oncology (2). Therefore, understanding the mechanisms mediating initiation of the disease is critical in order to develop preventive strategies.

Cigarette smoking is an established risk factor for PDAC. The risk of pancreatic cancer increases with increase in the duration and the intensity of smoking (3). The molecular mechanisms of PDAC induction by smoking are unknown.

NNK (4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone) is one of the most abundant carcinogens in tobacco smoke (4). It is found in the pancreatic juice of smokers at a level 7 times higher than in non-smokers (5). NNK was associated with activating mutation in the K-ras gene (6). Cigarette smoke extract (CSE) is a combination of most of tobacco smoke components including NNK. CSE and NNK are widely used to investigate the pathologic carcinogenic effects of smoking.

Here we studied the effect of smoking compounds NNK and CSE on pancreatic ductal cell death pathways. At the concentrations used in this study the content of nicotine in CSE overlaps with the content of nicotine in the plasma of smokers (7).

Disabled apoptosis is a key “hallmark” of cancer cells. Akt kinase is the major inhibitor of apoptosis in cancer cells. Akt is over-expressed both in PDAC and pancreatic cancer cells, and was shown to mediate the resistance of pancreatic cancer cells to death (8-13). Important regulator of Akt kinase is NADPH oxidase (14). NADPH oxidase mediates the anti-apoptotic effect of both growth factors and extra-cellular matrix proteins in pancreatic cancer cells (15;16) suggesting it as a key mediator of PDAC development.

Inhibiting autophagy is emerging as a new “hallmark” of cancer cells. The main function of autophagy is to regulate the normal turnover of proteins and organelles, and to remove those with compromised function (17). This effect is anti-cancer as it prevents cells from conditions that lead to accumulation of damaged organelles and oxidative stress. In cancer cells autophagic flux is often retarded; and tumorigenesis is associated with loss of autophagy mediators. Furthermore, autophagy inhibition promotes tumorigenesis through accumulation of damaged organelles, oxidative stress and DNA alterations (18;19).

The effects of smoking compounds on apoptosis and autophagy, and the role of Akt in these processes are poorly studied. The effects are cell and smoking compound specific. The effects of smoking compounds on apoptosis and autophagy in pancreatic ductal cells, the potential precursors of PDAC, have not been studied. The role of Akt kinase in smoking induced PDAC remains unknown.

The goal in this study is to determine the effect of smoking compounds on apoptosis and autophagy and their mediating signaling pathways as well as demonstrate potential strategies for preventing the pro-carcinogenic effects of the smoking compounds. We found that long exposure to smoking compounds decreased apoptosis and prevented autophagy stimulation in human and mouse pancreatic ductal cells. This effect was mediated by NAD(P)H oxidases, Akt and AMP kinases suggesting their role in smoking-induced PDAC development.

Experimental Procedures

Reagents

Antibodies against Akt1 phosphorylated at Ser473, AMPK phosphorylated at Thr-172, Bcl_{xL}, and LC3 were from Cell Signaling (Beverly, MA). Cigarette smoke extract (CSE) dissolved in DMSO (40 mg/ml total particulate matter, nicotine content of 6%) was from Murty Pharmaceuticals (Lexington, KY). The tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was purchased from ChemSyn Science Laboratories (Lenexa, KS, USA). Akt inhibitor VIII Isozyme selective Akti-1/2 inhibitor and diphenylene iodonium (DPI) were from Calbiochem (Gibbstown, NJ). All other antibodies and chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell culture

The immortalized human pancreatic duct epithelial cell line, HPDE6-c7, was a gift from Dr. M-S Tsao (Ontario, Canada) (20;21). The HPDE6-c7 cells were grown in keratinocyte serum free (KSF) medium supplemented with bovine pituitary extract (25 µg/500 ml), epidermal growth factor (2.5 µg/500 ml), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and used between passages 2 and 8. For the experiments, HPDE6-c7 cells were cultured between 10 minutes and 1 week. When cells were exposed to CSE or NNK for longer than 24 hours the smoking compounds were added every 24 hours.

Primary mouse pancreatic ductal cells isolation and culture

Mouse pancreatic ductal cells were isolated according to the protocol developed by Schreiber *et al.* (22). Briefly, the pancreata of 3–4-month-old mice were dissected, minced, and digested at 37°C in a Hank's balanced salt solution (Hank's balanced salt solution + 5 mmol/L glucose + 0.05 mmol/L CaCl₂) containing 2 mg/mL type V collagenase (Sigma) with agitation by a magnetic stir bar. After 20 minutes, the digested material was filtered through a 105-µm nylon mesh (Small Parts, Inc., Miami Lakes, FL). Fragments trapped on the mesh were digested further in 0.05% trypsin-0.53 mmol/L ethylenediaminetetraacetic acid (Life Technologies, Grand Island, NY) for 2 minutes, and then proteases were

inactivated by the addition of Dulbecco's modified Eagle medium/F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. The tissue was washed 3 times in Hank's balanced salt solution. The ductal fragments were plated on 2.31 mg/mL rat tail collagen type I (BD Biosciences, Palo Alto, CA) pre-coated plastic dishes.

Mouse pancreatic ductal cells were grown at 37°C and 5% CO₂ in Dulbeccos' modified Eagle medium/F12 medium supplemented with 5 mg/mL D-glucose (Sigma), 0.1 mg/mL soybean trypsin inhibitor type I (Sigma), 5 mL/L insulin-transferrin-selenium (BD Biosciences), 25 µg/mL bovine pituitary extract (BD Biosciences), 20 ng/mL epidermal growth factor (BD Biosciences), 5 nmol/L 3,3',5-triiodo-L-thyronine (Sigma), 1 µmol/L dexamethasone (Sigma), 100 ng/mL cholera toxin (Sigma), 10 mmol/L nicotinamide (Sigma), 5% Nu-serum IV culture supplement (Collaborative Biomedical Products), and antibiotics (penicillin G 100 U/mL, streptomycin 100 µg/mL, amphotericin B 0.25 µg/mL (Gibco-BRL, Grand Island, NY).

Transfections

Transient transfections of HPDE6-c7 cells were performed using the electroporation Amaxa System Nucleofector™ (Amaxa Inc, Gaithersburg, MD) according to the manufacturer protocol. To knockdown Akt 1 or p22^{phox}, Akt1 and p22^{phox} siRNAs (MWG biotech, High Point, NC) were applied to the cells using electroporation according to the Amaxa kit protocol. Control cells were transfected with the Silencer Negative Control siRNA #1 (Ambion, Foster City, CA).

Measurement of apoptosis

Internucleosomal DNA fragmentation was measured by using Cell Death Detection ELISA^{Plus} kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions (14;15;23).

Staining and immunofluorescence

HPDE6-c7 Cells were fixed for 15 min at -20°C in methanol/acetone 1:1, unspecific binding was blocked with 5 % normal goat or donkey serum (Abcam, Cambridge, MA) and 1% aurion BSA in PBS with 0.2 % triton. After incubation with primary antibody and fluorescent-labeled secondary antibody, cells were counterstained with DAPI mounted with antifade (Invitrogen, Carlsbad, CA) and analyzed with a Nikon Eclipse 2000S fluorescent microscope. Sections of paraffin embedded mouse normal pancreas were deparaffinized by xylol for 30 min and then rehydrated with an ethanol gradient. Antigen retrieval was done by boiling in 10mM citrate buffer for two times 5 minutes. Unspecific binding was blocked with 5% aurion BSA. Sections were incubated with primary antibody followed by secondary Alexa Fluor coupled antibody for immunofluorescence and developed with the Vecta stain ABC kit (Vector Laboratories, Burlingame, CA) or dehydrated and permanently mounted. The samples were then analyzed by fluorescence or conventional microscopy.

Western blot analysis

Cells were re-suspended in RIPA phosphorylation buffer (50 mM NaCl, 50 mM Tris/HCl pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 10 mM Na₂HPO₄ + NaH₂PO₄,

100 mM NaF, 2 mM Na₃VO₄, 80 μM glycerophosphate, 20% glycerol, 1 mM PMSF, 5 μg/ml each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin), sonicated and centrifuged for 15 min at 16,000 × g at 4 °C. Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose or PVDF membranes. Non-specific binding was blocked for 1 h with 5% bovine serum albumin or non-fat dry milk in Tris-buffered saline (4 mM Tris base, 100 mM NaCl, pH 7.5) containing 0.05% Tween 20. Membranes were incubated with primary antibody overnight at 4°C, and then for 1 h with peroxidase-conjugated secondary antibody. Blots were developed using Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL).

Measurement of NADPH oxidase activity

Superoxide production was measured in total cell homogenates by using lucigenin-derived chemiluminescence according to (24;25) as we described in (15). Briefly, cells were collected, washed in PBS, then lysed in the buffer containing 50 μM phosphate buffer, 1 mM EGTA and 150 mM sucrose and homogenized in a dounce homogenizer with 100 strokes. Homogenates were centrifuged and pellets (membrane fractions) were stored at -80°C. For superoxide measurements, 50 μg of protein was diluted in 500 μl of the same lysis buffer. Dark-adapted lucigenin (15 μM) was added to the sample, and chemiluminescence measurement immediately started. Chemiluminescence (in arbitrary units) was measured at 15-sec intervals for 1 min in a Turner 20/20 luminometer (Turner Designs, Sunnyvale, CA). NADPH was used as a substrate. In this and other assays, protein concentration was measured by the Bradford assay (BioRad Laboratories, Hercules, CA).

Statistical analysis

Results are expressed as means ± SEM and represent data from at least 3 independent experiments. Differences between 2 groups were analyzed using Student's *t* test. *P* < 0.05 was considered statistically significant.

Results

CSE and NNK dose and time -dependently inhibit apoptosis in pancreatic ductal cells

First, we cultured human HPDE6-c7 and mouse pancreatic ductal cells (passages 2 to 4) in the presence of CSE or NNK for 48 hours. CSE and NNK were added every 24h. Exposure to CSE and NNK caused dose-dependent decrease in apoptosis assessed as the rate of DNA fragmentation in both human and mouse pancreatic ductal cells (Fig. 1A, B). Correspondingly, prolong culture with CSE or NNK for up to 1 week increased the level of anti-apoptotic Bcl_{xL} protein after 24 hours (Fig. 1C). The effect was sustained for up to 1 week. Of note, CSE and NNK were not toxic to cells and did not affect necrosis (not shown).

Smoking compounds activate Akt through ROS dependent mechanism

Akt kinase is an established regulator of apoptosis. In order to determine the mechanism through which CSE and NNK regulate apoptosis we measured time-dependent changes in Akt phosphorylation (a measure of their activation) in cells cultured with smoking compounds CSE and NNK for up to 1 week. In these experiments we applied the

concentrations of CSE and NNK which cause maximal effect on apoptosis as shown in Figure 1.

CSE and NNK did not significantly affect Akt phosphorylation at early times (10 min and 1h), but increased it at 24h. The increase in Akt phosphorylation was sustained for up to 1 week (Fig. 2A).

We recently showed correlation between Akt activation level and NADPH oxidase activity in pancreatic cancer cells (26,27). To test whether normal pancreatic ductal cells utilize the same ROS-dependent mechanism we measured the effects of CSE and NNK on the activity of NADPH oxidase. The results indicate that both smoking compounds stimulated NADPH oxidase activity at 48 hours (Fig. 2B). Importantly, NADPH oxidase inhibition using pharmacological inhibitor DPI (Fig. 2C) or molecular NADPH oxidase-p22*phox* siRNA (Fig. 2D) prevented the up-regulation of Akt kinase phosphorylation induced by smoking compounds. However, molecular inhibition of NADPH oxidase using p22*phox* siRNA did not completely inhibit the effect of NNK on Akt phosphorylation as DPI did suggesting that other oxidases (other than the p22*phox*-dependent NADPH oxidase) are involved in this late effect. Indeed, DPI inhibits the mitochondrial NADH dehydrogenase as well.

Akt kinase mediates the down-regulation of apoptosis by CSE and NNK

To determine the effect of Akt inhibition in mediating the anti-apoptotic effect of CSE and NNK we measured the level of DNA fragmentation and anti-apoptotic Bcl_{xL} protein level in human and mouse pancreatic ductal cells. Inhibiting Akt with both pharmacological and molecular approaches prevented apoptosis suppression induced by CSE and NNK (Fig. 3A, B). In addition, transfection with Akt1 siRNA prevented the increase in anti-apoptotic Bcl_{xL} protein level induced by NNK (Fig. 3C).

CSE and NNK transiently stimulate autophagy, this effect is abolished after repeated exposure to smoking compounds

Next; we measured the effect of smoking compounds on autophagy. We measured the level of autophagic vacuoles by using their marker LC3-II. We first quantified LC3-II labeled structures (punctate “LC3 dots” visualized by immunofluorescence (Fig. 4A, B). Then we measured by immunoblot another characteristic of autophagic flux, i.e., the conversion of cytosolic LC3-I to the autophagosomal LC3-II form and the level of p62 (Fig. 2C). CSE and NNK markedly increased LC3-II dots as well as LC3-I/LC3-II conversion. The increase was maximal at 1h, and much less pronounced at 48h. Importantly, CSE and NNK decreased p62 at 1h but had no effect at 48h. This indicates that smoking compounds induce transient (1h) stimulation of autophagy, which disappears after long and repeated exposure (48h) to the smoking compounds.

Akt negatively regulates AMP kinase and autophagy

Data in figure 4 indicate that short exposure (1h) to smoking compounds stimulates autophagy; whereas, long exposure down-regulates autophagy back to basal level. Thus, it is important to determine the cell signaling molecules that mediate the effect of smoking compounds. Literature data showed before that AMPK is an ATP sensor that up-regulates

autophagy when cells need energy (28). Therefore, we measured the effect of smoking compounds on AMPK activation.

CSE and NNK have a biphasic effect on AMPK phosphorylation. Increase in phospho-AMPK was evident at 10min, reached maximum at 1h, and then decreased later on (24h and later) (Fig. 5A). Data in figures 5A and 2A indicate that the decrease in phospho-AMPK and increase in phospho-Akt occur at the same time interval suggesting a possible inter-regulation between the two kinases. Therefore, we checked the effect of Akt inhibition on the effect of smoking compounds on AMPK. Indeed, Akt inhibition with a pharmacologic inhibitor (Fig. 5B) or with Akt1 siRNA (Fig. 5C) both prevented the decrease in AMPK phosphorylation induced by repeated exposure to smoking CSE and NNK. The results demonstrate that Akt kinase negatively regulates AMPK. In addition, Akt inhibition induced stimulation of autophagy as indicated by increase in the level of LC3-II in both human and mouse pancreatic ductal cells (Fig. 5D).

Discussion

In the present study we investigated the effect of smoking compounds CSE and NNK on apoptotic and autophagic cell death pathways in human and mouse pancreatic ductal cells, the possible precursors of pancreatic cancer cells. Smoking compounds interact directly with pancreatic cells. Indeed, previously published data showed that blockade of β -adrenergic receptors (29) and nicotinic acetylcholine receptors (nAChR), especially $\alpha 7$ -AChR prevents the pro-survival effect of NNK in pancreatic cells (30;31).

Our results indicate that active smoking compounds CSE and NNK stimulate activation of Akt and inhibit apoptosis in pancreatic ductal cells. Both Akt activation and apoptosis inhibition are pronounced with daily repeated cell exposure (48h-1week) to the smoking compounds. Both pharmacological and molecular inhibitions of Akt prevented the anti-apoptotic effect of CSE and NNK. These data indicate that Akt mediates suppression of apoptosis by smoking compounds. Recently published data showed that Akt mediates nicotine and NNK -induced proliferation in pancreatic ductal cells and in cancer cells (32) indicating that Akt kinase is a key mediator of two important pro-survival pathways in these cells.

The study shows that smoking compounds activate Akt in pancreatic ductal cells through NAD(P)H oxidase-dependent mechanisms. Our data indicate that CSE and NNK stimulate NAD(P)H oxidase in pancreatic ductal cells. This is in accord with the publications on other cells demonstrating stimulation of NAD(P)H oxidase by smoking compounds (33;34). We showed before that ROS produced by NADPH oxidase stimulate Akt phosphorylation resulting in suppression of cell death in pancreatic cancer cells (27;35). Regulation of Akt kinase phosphorylation by NAD(P)H oxidase is likely through inhibition of PH domain and Leucine rich repeat Protein Phosphatases by ROS (35). The combined results of this and previous studies indicate a central anti-apoptotic role of ROS in both pancreatic ductal normal and cancer cells. Of note, non-NAD(P)H oxidase ROS are likely to be involved in the pro-survival effect of smoking compounds on pancreatic ductal cells as DPI is an inhibitor of NAD(P)H oxidases including the mitochondrial NADH dehydrogenase.

Our data further suggest that Akt-induced inhibition of apoptosis is mediated by anti-apoptotic protein Bcl_{xL}. Indeed, smoking compounds up-regulated Bcl_{xL} level and Akt kinase inhibition induced apoptosis and decreased Bcl_{xL} protein level.

An important finding of the study is the effect of smoking compounds on autophagy. We found that single exposure to CSE and NNK induced rapid (minutes) activation of autophagy. Autophagy protects cells from the consequences of damaged organelles accumulation such as oxidative stress and DNA alteration, and therefore tumorigenesis (18;19). However, this anti-cancer effect was transient and was followed by the return of autophagy to the basal level after repeated exposure to smoking compounds. Correspondingly, smoking compounds stimulated rapid and transient phosphorylation/activation of AMPK, a known mediator of autophagy (28), which returned to the basal level after repeated treatments with smoking compounds. This later effect is mediated by Akt. The data indicate that Akt inhibits autophagy through preventing AMPK phosphorylation. Interestingly, the phosphorylation levels of Akt and AMP kinases correlate inversely. This goes along with previously published data showing the interaction between Akt and AMP kinases in other cells (36).

Besides the rapid activation of autophagy, long and repeated exposure to smoking compounds prevents the early and transient stimulation of autophagy. Akt activation as a result of repeated and prolonged exposure to CSE or NNK induces inhibition of AMPK and limits the early stimulation in autophagy. Therefore, repeated exposure to smoking prevents the beneficial effect of autophagy through a mechanism that involves Akt activation and AMPK inhibition.

A possible mechanism through which Akt inhibits AMPK activity is by inhibition of creatine kinase. In fact, oxidative stress induced by CSE and NNK (33;34) has been shown to inhibit creatine kinase in different cells leading to a decrease in the ATP/AMP ratio and therefore, leading to the activation of AMPK (37;38).

The kinetics of the effects of smoking is slow and requires a prolonged exposure to smoking compounds. Multiple exposures to smoking compounds are needed to induce resistance to apoptosis and prevent autophagy. This correlates with the *in vivo* data indicating that individuals with long periods of smoking have higher risk for developing pancreatic cancer. Figure 6 illustrates the proposed mechanism of regulation of apoptosis and autophagy in pancreatic ductal cells.

Our results indicate that long-term and repeated exposure to smoking compounds stimulates ROS production, inhibits apoptosis and limits autophagy in pancreatic ductal cells, and thus create conditions favorable for cancer initiation and promotion. Our results further indicate the inhibition of Akt kinase could be considered as potential therapeutic tool to prevent pro-carcinogenic effect of smoking compounds in pancreas.

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Fig. 1. CSE and NNK decrease apoptosis in pancreatic ductal cells

Human HPDE6-c7 and mouse pancreatic ductal cells were treated daily with indicated doses of CSE (A, C) or NNK (B, C) for 48 hours. In C, HPDE6-c7 cells were treated with CSE (400 ng/ml) or NNK (1μM) for indicated times. Internucleosomal DNA fragmentation was measured using the Cell Detection ELISA kit and normalized to those in cells without treatment (A, B). (C) Protein levels of Bcl_{XL} were measured by Western blotting. Blots were re-probed with GAPDH to confirm equal loading. (A, B) Values are means ± S.E. from at least five independent experiments. *, $p < 0.05$ versus control.

Fig. 2. Smoking compounds up-regulate NADPH oxidase and Akt kinase activities

HPDE6-c7 cells were treated daily with CSE (400 ng/ml) or NNK (1μM) for indicated times (A) or for 48h in the presence or absence of NADPH oxidase inhibitor DPI (15μM) (B-D). (D) Cells were transfected with p22phox or control siRNA. (B) Activity of NADPH oxidase was measured in total cell homogenates by using lucigenin-derived chemiluminescence and normalized to those in cells without CSE and NNK. (A, C and D) Protein levels of phosphorylated Akt (p-Akt), total Akt (t-Akt) and p22phox were measured by Western blotting. Blots were re-probed for GAPDH to confirm equal loading. The intensities of p-Akt were quantified by densitometry and their ratio normalized to those in the control. Data shown are representative of three independent experiments. In B, values are means ± S.E. from at least three independent experiments. *, $p < 0.05$ versus control.

Fig. 3. Akt kinase mediates the anti-apoptotic effect of smoking

Human HPDE6-c7 and mouse pancreatic ductal cells were treated daily with CSE (400 ng/ml) or NNK (1μM) for 48h in the presence or absence of Akt inhibitor (Akt Inhibitor VIII, Isozyme-Selective, Akti-1/2) (50μM). (B, C) HPDE6-c7 cells were transfected with Akt1 or control siRNA. (C) Protein level of Bcl_{XL} was measured by Western blotting. Blots were re-probed for GAPDH to confirm equal loading. Data are representative of three independent experiments. (A, B) *, $p < 0.05$ versus control; #, $p < 0.05$ versus cells with the same treatment except Akt inhibition.

Fig. 4. Short exposure to smoking compounds up-regulates autophagy; whereas, long exposure prevents this effect

HPDE6-c7 cells were treated daily with CSE (400 ng/ml) or NNK (1μM) for 1 hour or 48 hours. (A) Levels of autophagy measured by staining autophagy marker LC3. (B) Levels of autophagy quantified by counting the number of LC3 vacuoles per cell. (C) Levels of LC3-I (~ 18 kDa) and LC3-II (~ 16 kDa) and p62 were measured using Western blotting. Blots were re-probed with GAPDH to confirm equal loading. The cytosolic LC3 ratio (LC3-II/I) were calculated using densitometric analysis of LC3 bands and normalized to those in cells without treatment (C). In B, Values are means ± S.E. from at least three independent experiments. *, $p < 0.05$ versus control.

Fig. 5. Akt kinase activation prevents autophagy in pancreatic ductal cells
Human HPDE6-c7 (A-D) and mouse pancreatic ductal (D) cells were treated daily with CSE (400 ng/ml) or NNK (1μM) for indicated times (A) or for 48h (B-D). Protein levels were measured by Western blotting. Blots were re-probed for GAPDH to confirm equal loading. Western blots shown are representative of three independent experiments. The intensities of p-AMPK, p-Akt and LC3II/LC3I were quantified by densitometry and their ratio normalized to those in the control (n=3).

Fig. 6. Proposed pathway mediating pro-cancer effect of smoking compounds CSE and NNK