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Inhibition of Cholinergic Signaling Causes Apoptosis in Human Bronchioalveolar Carcinoma

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Abstract

Recent case-controlled clinical studies show that bronchioalveolar carcinomas (BAC) are correlated with smoking. Nicotine, the addictive component of cigarettes, accelerates cell proliferation through nicotinic acetylcholine receptors (nAChR). In this study, we show that human BACs produce acetylcholine (ACh) and contain several cholinergic factors including acetylcholinesterase (AChE), choline acetyltransferase (ChAT), choline transporter 1 (CHT1, SLC5A7), vesicular acetylcholine transporter (VACHT, SLC18A3), and nACh receptors (AChRs, CHRNAS). Nicotine increased the production of ACh in human BACs, and ACh acts as a growth factor for these cells. Nicotine-induced ACh production was mediated by $\alpha 7$ -, $\alpha 3\beta 2$ -, and $\beta 3$ -nAChRs, ChAT and VACHT pathways. We observed that nicotine upregulated ChAT and VACHT. Therefore, we conjectured that VACHT antagonists, such as vesamicol, may suppress the growth

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of human BACs. Vesamicol induced potent apoptosis of human BACs in cell culture and nude mice models. Vesamicol did not have any effect on EGF or insulin-like growth factor-II–induced growth of human BACs. siRNA-mediated attenuation of VACHT reversed the apoptotic activity of vesamicol. We also observed that vesamicol inhibited Akt phosphorylation during cell death and that overexpression of constitutively active Akt reversed the apoptotic activity of vesamicol. Taken together, our results suggested that disruption of nicotine-induced cholinergic signaling by agents such as vesamicol may have applications in BAC therapy.

Introduction

Bronchioalveolar carcinoma (BAC) is a subtype of lung adenocarcinoma arising from type II pneumocytes in the lung. The World Health Organization revised its classification of BACs in 2004 to include lung adenocarcinomas, which grow in a lepidic fashion along the alveolar septa without invasion into the stroma, pleura, blood vessels, or lymphatics (1–3). The incidence of pure BACs is about 4%; however, mixed subtypes, including BACs with stromal invasion and pulmonary adenocarcinoma with BAC-like morphologic features, account for almost 20% of all non–small cell lung cancers (NSCLC; ref. 4). Cigarette smoking is the leading risk factor for the development of lung cancer. It is estimated that smoking is associated with 80% to 90% of lung cancer cases throughout the world (5). Smoking has a stronger association with small cell lung cancer (SCLC) than with adenocarcinoma (6). BAC is a relatively rare type of adenocarcinoma; therefore, only a few epidemiologic studies have investigated the relationship between BAC and smoking (7–9).

Traditionally, the role of tobacco smoking in the etiology of human BAC originates from a series of early studies involving human patients with lung cancer, most of which were nonsmokers (1). These non-smoking patients with lung cancer had higher incidence of BAC than other types of lung cancer. The resulting impression was that smoking is unimportant in the etiology of human BACs (1). However, these reports did not contain any cohort or case–control studies that formally determined the relationship of human BACs to cigarette smoking.

Epidemiologic data have shown an association between human BACs and smoking (7, 8, 10). The risk of developing BACs is greater for people who started smoking at a younger age, smoked for a longer time, or smoked more cigarettes per day. Conversely, the risk decreases in proportion to the duration of smoking cessation. Smoking habits have been correlated to both the mucinous and non-mucinous form of human BACs (11). Rolen and colleagues conducted a case–control study and compared smoking status of 198 patients with BACs with an equal number of controls. They observed that the risk of BACs is strongly related to the smoking history of patients. They also found that both current and former smokers were at risk of developing BACs (9). Recently, Boffetta and colleagues analyzed 7 case-controlled studies in the United States comprising 799 cases of BACs and 15,859 controls. They found that ever smokers are at a 2-fold greater risk of developing BACs than never smokers. They also observed a positive correlation between the duration and amount of smoking and the development of lung BACs (8).

Although cigarette smoke is composed of a mixture of many compounds, nicotine is the addictive component of cigarette smoke (12). Several convergent studies have shown that nicotine promotes the progression of human BACs and confers resistance against chemotherapy (12, 13). All of these observations suggest that nicotine-induced mitogenic and prosurvival pathways contribute to the pathophysiology of BACs.

The proliferative activity of nicotine is mediated by nicotinic acetylcholine receptors (nAChRs; ref. 12). The endogenous ligand for nAChRs is acetylcholine (ACh; ref. 14). Recent studies have shown that nAChRs are present in non-neuronal tissues, including lung cancer cells, lung epithelial cells, endothelial cells, and keratinocytes (12). SCLCs and squamous cell carcinoma of the lung (SCC-L) express all components of the ACh autocrine loop, including acetylcholinesterase (AChE), choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VACHT), choline transporter1 (CHT1), nAChRs, and muscarinic acetylcholine receptors. SCLCs and SCC-L cells secrete ACh, which promotes their proliferation (14–17). Song and colleagues found that the muscarinic receptor antagonist darifenacin displayed antitumor activity in human SCC-L in both cell culture and nude mice models (15, 18). Such observations suggest that the cholinergic network may be a viable molecular target in the therapy of human lung cancer.

The present manuscript investigates whether the cholinergic loop exists in human BACs. We show that human BAC cell lines produce ACh and express cholinergic proteins. We also show for the first time that nicotine can amplify the components of the cholinergic loop in human BACs. Nicotine increased the production of ACh in human BAC cell lines in a time- and dose-dependent manner. ACh acted as a growth factor for human BAC cells. Nicotine upregulated VACHT and ChAT expression in human BAC cells. We conjectured that nicotine-induced increase of VACHT levels may provide a viable molecular target in human BACs. The VACHT antagonist vesamicol induced robust apoptosis of human BAC cells in both cell culture and *in vivo* models. Vesamicol did not affect EGF or insulin-like growth factor (IGF)-II-induced growth pathways in human BACs. This finding suggests that the pro-apoptotic activity of vesamicol is specific to the acetylcholine signaling pathway in human BACs. The pro-apoptotic activity of vesamicol was mediated via suppression of Akt activation. The data presented in this article characterize the cholinergic system in human BACs and offer novel avenues for BAC therapy. The results of our experiments are relevant to patients with BACs who are exposed to secondhand smoke or use nicotine-based cessation devices (e.g., patches and gums) to quit smoking.

Materials and Methods

Ethical use and care of laboratory animals

Nude mice (Charles River Laboratories International, Inc.) were acclimatized for 1 week. They were housed in autoclaved cages with *ad libitum* access to food and water in HEPA-filtered racks and closely monitored by animal facility staff. All procedures involving nude mice were conducted according to the Animal Care and Use Guidelines in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and were approved by the Institutional Animal Care and Use Committee of

the Joan C. Edwards School of Medicine, Marshall University (Huntington, WV; Protocol # 418).

Authentication of cell lines

The human BAC cell lines, A549, NCI-H358, and NCI-H650 (hereinafter referred to as A549, H358, and H650), and the human SCC-L cell line H520 were obtained from American Type Culture Collection (ATCC). The A549, H358, and H520 cells were authenticated by the ATCC Cell Authentication Service in October 2012. They used short tandem repeat (STR) profiling for authentication of these cells, and the results are summarized in Supplementary Fig. S1. The human BAC cell line H650 was passaged for less than 6 months and therefore did not require authentication. This cell line was obtained from ATCC, which used STR profiling for its characterization. Primary human pulmonary alveolar epithelial cells (HPAEPiC) were obtained from ScienCell. These cells were characterized by ScienCell using immunostaining for specific markers. A certificate of analysis was provided.

Cell culture

A549 and H358 were cultured in RPMI-1640, supplemented with 2.0 mmol/L glutamine, 100 units/mL penicillin, 50.0 µg/mL streptomycin, 1.0 mg/mL bovine serum albumin (BSA), 1× insulin, transferrin, sodium selenite (ITS) supplement (Invitrogen Corp), 50 nmol/L hydrocortisone, and 1.0 µg/mL human EGF (15, 16). This medium will be referred to hereafter as serum-free RPMI (SF-RPMI). For a few experiments, the cells were rendered quiescent by incubating them in SF-RPMI containing $\frac{1}{4}$ ITS supplement, 12.5 nmol/L hydrocortisone and 0.25 µg/mL EGF. This media, reduced serum-free RPMI, will be referred to hereafter as SF-RPMI-R. H650 was grown in a 1:1 mixture of Dulbecco's Modified Eagles' Media (DMEM) and Ham/F-12K, supplemented with 2.0 mmol/L L-glutamine, 100 units/mL penicillin, 50.0 µg/mL streptomycin, 0.02 mg/mL bovine insulin, 1× ITS, 50 nmol/L hydrocortisone, 100 mmol/L ethanolamine, 100 mmol/L O-phosphorylethanolamine, 100 mmol/L 3,3',5-triiodo-L-thyronine, 5% (w/v) BSA, 0.5 mmol/L sodium pyruvate, 10 mmol/L HEPES, and 100 µg/mL EGF. The culture conditions for H520 and HPAEPiCs are described in Supplementary Methods.

Measurement of ACh production

A549 cells were grown in SF-RPMI. On the day of the assay, 100 µmol/L neostigmine (an acetylcholinesterase inhibitor) was added to each plate (16). Four hours after the addition of neostigmine, the indicated doses of nicotine were added and the cells were incubated at 37°C for 36 hours. The supernatant was collected and spun at 800 × g. Subsequently, the supernatant was lyophilized, reconstituted with one-fifth volume autoclaved water, and stored at -80°C until further analysis. The amount of ACh in the sample was measured using the Choline/Acetylcholine Quantification Kit (Biovision; refs. 15–17). Each sample was assayed in triplicate, and the whole experiment was carried out 2 independent times for each cell line.

Immunohistochemical staining of VACHT, ChAT in human BAC tissue microarray and normal lung TMA

Human BAC tissue microarray (TMA) slides (Abnova) were deparaffinized and rehydrated as described previously (19, 20). The immunostaining was conducted using Vectastain ABC Kit (Vector Laboratories) following the manufacturer's protocol. The dilutions of primary antibodies used were 1:50 (polyclonal VACHT antibody) and 1:25 (monoclonal ChAT antibody). The images were captured by phase contrast microscopy (Leica Microsystems) at a magnification of $\times 400$. The normal lung TMA (US Biomax Inc.) was stained for VACHT and ChAT as described above.

VACHT, ChAT, and nAChR ELISA assays

The concentration of VACHT and ChAT was measured using the VACHT ELISA Kit (Antibodies Online Inc.) and the ChAT ELISA Kit (NovaTein Biosciences), according to manufacturer's instructions. The expression of nAChR subunits in human BAC cell lines was analyzed by using $\alpha 7$ -, $\alpha 3$ -, $\beta 2$ -, and $\beta 3$ - nAChR ELISA Kits (Antibodies Online Inc.). Each of these assays was completed in duplicate, and the whole experiment was carried out 2 independent times for each cell line.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assays

A549 or H358 cells (10,000 cells per well) were seeded into an 8-well chamber slide in SF-RPMI and incubated overnight at 37°C (13, 19). Subsequently, the medium was replaced with SF-RPMI-R for 24 hours. After 24 hours, cells were treated with 100 nmol/L nicotine in the presence or absence of 50 $\mu\text{mol/L}$ vesamicol. Cells were incubated for 48 hours at 37°C. Apoptosis was measured by the colorimetric TUNEL Assay (Promega Corporation), according to manufacturer's protocol. The magnitude of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells in the untreated control wells was considered to be equal to 1, and the TUNEL-positive cells in the remaining wells were calculated as fold increase relative to the control. The experiment was carried out 2 independent times with 2 replicates in each experiment.

Caspase-3 activity assay

Human BAC cells were incubated in SF-RPMI-R for 24 hours. Subsequently, cells were treated with 100 nmol/L nicotine in the presence or absence of 50 $\mu\text{mol/L}$ vesamicol for 48 hours at 37°C. Lysates were made using the Caspase-3 Activity Kit (EMD Millipore Corporation). Caspase-3 activity in untreated lysates was considered to be equal to 1, and the activity observed in treated lysates was calculated as fold increase relative to the control cells. The experiment was carried out 2 independent times with 2 replicates in each experiment.

Antitumor studies in nude mice

Four-week-old male nude mice were acclimatized for 1 week and housed in autoclaved cages with *ad libitum* access to food and water in HEPA-filtered racks. A549 cells were resuspended in a 1:1 (v/v) solution of serum-free media and Matrigel matrix (BD Biosciences). One million cells were injected subcutaneously between the scapulae of

each mouse (15, 21). After the tumors reached approximately 100 mm³, the mice were randomized into 2 groups, a control ($n = 8$) and a treatment ($n = 8$) group. The control group was fed an AIN-76A–based diet containing 10% corn oil. The treatment group was fed a diet containing 50 mg vesamicol/kg food (~10 mg vesamicol/kg body weight) per day. Both groups were administered nicotine in their drinking water (200 µg/mL in 2% saccharin sodium; refs. 20, 21). The drug treatment was continued until tumors of the control group reached approximately 1,500 mm³. Mice were weighed once per week. Their food consumption and water consumption were monitored daily. Tumor lengths (l), widths (w), and height (h) were measured daily (6 d/wk) for each mouse. Tumor volumes were calculated as $(l \times w \times h)/2$ (22, 23). After euthanizing the mice, the tumors were excised. Half of the tumor was snap-frozen in liquid nitrogen. Tumor lysates were prepared using T-Per lysis buffer (Pierce Biotechnology), according to manufacturer’s protocol (24). The other half of the tumor was fixed in 10% formalin-buffered saline and used for immunohistochemistry.

Statistical analysis

All data were plotted using GraphPad Prism 5 Software, Inc. and were represented as the mean \pm SEM. Results from the control and treated samples were compared using ANOVA followed by a Neumann–Keuls multiple comparison test. All analyses were completed using a 95% confidence interval (CI). Data were considered significant when $P < 0.05$.

Results

Cholinergic proteins are expressed on BACs

The cholinergic pathway proteins have been traditionally found in neuromuscular junctions and in neuronal cells (14). However, studies have shown that genes for these proteins are found in SCC-L and SCLC cells (14). ELISA experiments were carried out to examine whether cholinergic proteins were expressed in human BAC cell lines and in HPAEpiCs. Figure 1A shows that multiple nAChR subunits are expressed on A549, H358, and H650 human BAC cells. Similarly, HPAEpiCs also expressed a diverse array of nAChR subunits. Immunoblotting experiments were carried out to examine the presence of AChE, ChAT, CHT1, and VACHT (Fig. 1B) in human BAC cell lines and in HPAEpiCs. H520 human SCC-L cells were used as the positive control for both of the experiments. We observed that human BAC cell lines, as well HPAEpiCs normal lung cells, express VACHT, ChAT, AChE, and CHT1. The antibodies to VACHT, ChAT, CHT1, and AChE were found to be specific and showed only a single band at the correct molecular weight in full-screen Western blots (Supplementary Fig. S2A–S2D). The expression of ChAT and VACHT was examined in human BAC tumors isolated from patients using BAC TMA. The specific VACHT and ChAT antibodies described above were used for the immunostaining. Each TMA contained 81 samples of human BAC tumors from patients. Human BAC tumors displayed robust expression of both ChAT (Fig. 1C, left) and VACHT (Fig. 1D, right). Both VACHT and ChAT were found to be expressed in the cytoplasm. This observation is in agreement with previous data from several research groups showing that VACHT and ChAT are localized in the cytoplasm of cells (25–28). We also analyzed the expression of VACHT and ChAT in normal lung tissues using normal lung TMA (Supplementary Fig. S3A and S3B). This TMA

contained 20 samples of normal lung tissue. We found that normal lung tissues also express VAChT and ChAT, and these proteins are localized in the cytoplasm.

Nicotine induces acetylcholine production in BACs

Next we wanted to assess the effect of nicotine on acetylcholine production in human BACs. We observed that nicotine increased ACh production from A549 cells in a concentration-dependent manner (Fig. 2A). The maximum ACh production (2 $\mu\text{mol/L}$) was observed at 100 nmol/L nicotine and remained constant thereafter. A similar pattern was observed in H358 and H650 human BAC cells (Fig. 2A). We also carried out a time kinetics experiment with nicotine on A549 cells and found that the maximal ACh production (2 $\mu\text{mol/L}$) occurred at 36 hours and remained relatively constant thereafter (Fig. 2B). Therefore, we selected a concentration of 100 nmol/L nicotine and a 36-hour time point for all of our ACh production experiments. A point to note here is that 100 nmol/L nicotine is within the concentration range found in the plasma of an average smoker (1 nmol/L–1 $\mu\text{mol/L}$; ref. 29).

Studies by Song and colleagues have shown that ACh acts as an autocrine growth factor for SCLCs (16, 17). We analyzed the mitogenic activity of ACh in human A549 BAC cells and found that ACh stimulated the proliferation of A549 cells in a concentration-dependent manner; the maximal proliferation observed at 2 $\mu\text{mol/L}$ ACh (Fig. 2C, white bars). This finding is significant because the maximal mitogenic activity of ACh is similar to the amount of ACh secreted by human BACs. We repeated the bromodeoxyuridine (BrdUrd) cell proliferation assays in H358 human BAC cells and obtained similar results (Fig. 2C, black bars). Our observations raise the possibility that nicotine induces the production of ACh in human BACs, which, in turn, promotes the growth of human BACs in an autocrine manner.

Next, we wanted to determine the role of nAChRs in nicotine-induced ACh production. The treatment of A549 cells with the generalized nAChR antagonist mecamylamine (MCA) suppressed nicotinic-induced ACh production, whereas atropine, an antagonist to the closely related muscarinic receptor, had little to no effect on nicotine-induced ACh levels. We obtained similar results in another human BAC cell line H358. Our results show that nicotine promotes ACh levels in human BACs in an nAChR-dependent manner (Supplementary Fig. S4A).

The next series of experiments aimed to investigate which specific nAChR subunits were responsible for nicotine-induced ACh production. Our data showed that the treatment of A549 and H358 human BAC cells with $\alpha 7$ -nAChR subunit antagonists, methyllycaconitine (MLA) and α -bungarotoxin (α -BT), ablated nicotine-induced ACh production. In addition, 1 $\mu\text{mol/L}$ of α -conotoxin MII (α -CT; $\alpha 3\beta 2$ and $\beta 3$ subunit antagonist) reversed the pro-secretory effect of nicotine, whereas DH β E ($\alpha 3\beta 2$ and $\alpha 4\beta 2$ nAChR antagonist) had little to no effect (Supplementary Fig. S4B). Taken together, these results suggest that nicotine-induced ACh secretion is mediated via nAChRs, specifically through the $\alpha 7$ -, $\alpha 3\beta 2$ -, and $\beta 3$ -containing nAChR subunits.

Finally, we wanted to examine the effect of choline/acetylcholine transporters on nicotine-induced ACh production. The treatment of A549 and H358 cells with hemicholinium (an

antagonist of CHT1) suppressed nicotine-induced ACh secretion. Similarly, vesamicol (an antagonist of VAcHT) potently abrogated nicotine-induced ACh production (Supplementary Fig. S4C). Taken together, this shows that CHT1 and VAcHT function are vital for nicotine-induced ACh production.

Nicotine increases VAcHT and ChAT levels in human BAC cells

We wanted to investigate whether nicotine promoted ACh secretion via the VAcHT/ChAT pathway in BAC cells. We used an ELISA kit to measure VAcHT and ChAT levels, which allowed us to quantitate these levels in an accurate and sensitive manner. We observed that the VAcHT ELISA Kit detected VAcHT levels in asynchronous A549, H358, and H650 BAC cells and HPAEpic normal lung cells (Supplementary Fig. S5). The relative pattern of VAcHT expression in the ELISA correlated well with the results obtained from western blotting (Fig. 1B, fourth panel). ELISA The treatment of A549 cells with 100 nmol/L nicotine caused a 4-fold increase in VAcHT levels (Fig. 3A) and a 1.5-fold increase in ChAT levels (Fig. 3B). Similarly, nicotine increased VAcHT levels in H358 by 5.5-fold and ChAT levels by 1.4-fold (Fig. 3A and B). We also tested the effect of nicotine on AChE and CHT1 in human BACs. We found that nicotine decreased AChE levels in H358 and A549 human BACs (Fig. 3C). The levels of CHT1 were relatively unaffected by nicotine in both the cell lines (Fig. 3C).

The VAcHT antagonist vesamicol causes apoptosis in human BACs

Our results showed that nicotine-induced ACh production was blocked by the VAcHT inhibitor vesamicol (Supplementary Fig. S4C). It may be envisaged that vesamicol will suppress nicotine-induced ACh production and thereby block Ach-induced growth of human BACs. In addition, we conjectured that nicotine-induced upregulation of VAcHT should provide a viable molecular target for vesamicol therapy in human BACs. MTT assays (Supplementary Methods online) showed that vesamicol decreased the viability of nicotine-treated A549 and H358 human BAC lines in a concentration-dependent manner. The maximum reduction in cell viability was observed at 50 $\mu\text{mol/L}$ vesamicol (Fig. 4A). Therefore, we used 50 $\mu\text{mol/L}$ vesamicol for all further experiments.

Next, we wanted to examine whether vesamicol induced apoptosis in human BAC cell lines. Quiescent A549 and H358 cells were treated with 100 nmol/L nicotine in the presence or absence of 50 $\mu\text{mol/L}$ vesamicol. Apoptosis was measured by TUNEL assays. Figure 4B shows that vesamicol caused robust apoptosis in both A549 and H358 human BACs. The results of the TUNEL assays were verified by caspase-3 activity assay. We observed that vesamicol caused 2.5- to 3-fold increase in apoptosis relative to nicotine-treated human BAC cells (Fig. 4C).

We also wanted to assess the effect of vesamicol on other mitogenic signaling pathways in human BACs (30–35). Several convergent studies indicate that EGF and IGF-II are potent growth factors for human BAC cells (30–35). We conducted a BrdUrd assay (Supplementary Methods online) to test the effect of vesamicol on EGF-induced proliferation of human BAC cell lines. We found that vesamicol has no effect on EGF-induced proliferation of A549 and H358 human BAC cell lines (Fig. 4D). Similarly, vesamicol did not affect IGF-II-induced

proliferation of human A549 and H358 cells (Fig. 4E). Our findings seem to suggest that vesamicol specifically inhibits the acetylcholine-proliferative pathway in human BACs.

Vesamicol induces apoptosis in human BAC cells by specifically targeting VACHT

Vesamicol is a well-characterized antagonist of VACHT (36). However, previous studies indicate that vesamicol also binds to the sigma-receptor in several types of human cancer cells (37, 38). We wanted to examine whether vesamicol induced apoptosis in human BAC cells by specifically targeting VACHT. For this purpose, we used siRNA methodology to suppress the expression of VACHT or sigma receptor in A549 cells. A549 cells were transfected with VACHT-siRNA or sigma-receptor siRNA (Supplementary Methods Online). Eighteen hours posttransfection, the cells were rendered quiescent by incubating them in SF-RPMI-R medium for 24 hours (19). Subsequently, the cells were treated with 100 nmol/L nicotine in the presence or absence of 50 μ mol/L vesamicol for 48 hours at 37°C. A549 cells transfected with a nontargeting control siRNA were used as the negative control for the experiment. After 48 hours, cell lysates were made and vesamicol-induced apoptosis was measured by the caspase-3 activity assay.

We observed that vesamicol-induced apoptosis was decreased upon transfection of VACHT-siRNA and unaffected by sigma-receptor siRNA. The apoptotic activity of vesamicol was also unaffected by the control nontargeting siRNA (Fig. 5A). We repeated these experiments in H358 human BAC cells and obtained similar results (Fig. 5B). These experiments were also repeated with a second independent VACHT-siRNA (Ambion Inc.), and similar results were obtained (Fig. 5C and D).

Parallel transfection experiments were carried out to test the efficacy of VACHT-siRNA and sigma-receptor siRNA in A549 cells. ELISA assays show that the levels of VACHT are robustly suppressed upon transfection with both sets of VACHT-siRNA (Supplementary Fig. S6A and S6B) in A549 and H358 human BAC cells. Western blotting experiments show that the levels of sigma-receptor are ablated upon transfection of sigma-receptor siRNA (Supplementary Fig. S6C and S6D) in both A549 and H358 cells.

We also examined whether vesamicol was targeting the VACHT pathway at concentrations lower than 50 μ mol/L. We chose 10 and 25 μ mol/L vesamicol for our experiments. Caspase-3 activity assays show that the cellular apoptosis induced by 10 μ mol/L vesamicol was suppressed by 2 independent sets of VACHT-siRNA in both A549 and H358 cells (Supplementary Fig. S7A and S7B). Similarly, the transfection of VACHT-siRNA efficiently abrogated the apoptotic activity of 25 μ mol/L vesamicol in nicotine-treated A549 and H358 cells (Supplementary Fig. S7C and S7D). ELISA assays indicate that both the VACHT-siRNA decreased the expression of VACHT in A549 and H358 cells (Supplementary Fig. S7E). Taken together, our data show that vesamicol caused apoptosis in human BAC cells by specifically targeting the VACHT pathway.

Vesamicol-induced apoptosis is mediated by the Akt pathway in human BACs

The Akt signaling pathway plays a vital role in nAChR signaling in normal lung cells and lung cancer cells (12). We observed that the treatment of A549 and H358 human BAC cells with nicotine and vesamicol caused potent decreases in phosphorylated Akt levels (Fig. 6A

and B). The levels of both phosphorylated Akt (Thr308) and phosphorylated Akt (Ser473) are robustly suppressed upon vesamicol treatment, whereas total Akt levels remain constant (Fig. 6A and B, respectively). Western blotting also showed that vesamicol had little to no effect on the expression of phosphorylated Akt in untreated A549 (or H358) cells (Fig. 6C and D). The transfection of constitutively active Akt (pcDNA3-HA-Akt-CA; Supplementary Methods Online) reversed the apoptotic effect of vesamicol, showing that vesamicol induces cell death by suppressing Akt activation (Fig. 6E and F). Western blotting confirms the overexpression of hemagglutinin (HA)-tagged Akt in A549 and H358 human BAC lines upon transfection (Supplementary Fig. S8A and S8B).

Vesamicol inhibited the growth of human A549 cells in vivo

The antitumor activity of vesamicol was examined *in vivo* using a nude mice model (15, 21). A549 human BAC cells were injected between the scapulae of nude mice. The tumors were allowed to grow until approximately 100 mm³, after which they were randomized into 2 groups. The control group was administered nicotine in the drinking water. The vesamicol group was administered 50 mg vesamicol/kg food along with nicotine. Our results showed that the administration of vesamicol decreased the tumor growth rate of A549 human BAC tumors (Fig. 7A). The administration of 50 mg vesamicol/kg food in the diet was well tolerated and caused no discomfort or weight loss in mice (mean control = 25.6 ± 0.6 g; mean vesamicol treated = 25.2 ± 0.5 g). In addition, food intake (mean control = 6.2 ± 0.04 g/d; mean vesamicol-treated = 6.4 ± 0.04 g/d) and water consumption (mean control = 11.0 ± 0.4 mL/d; mean vesamicol-treated = 11.2 ± 0.3 mL/d) were similar between groups.

Hematoxylin and eosin (H&E) staining of the tumors revealed the presence of apoptotic bodies in the tumors belonging to the vesamicol-treated mice (Fig. 7B). Caspase-3 activity assays indicate that tumor lysates from vesamicol-treated mice displayed about 2.5-fold greater apoptosis relative to control nicotine-treated mice (Fig. 7C). Our data from cell culture suggested that vesamicol induced apoptosis by suppression of Akt activation. We wanted to investigate whether vesamicol-treated tumors had lower levels of phosphorylated Akt. Western blotting revealed that vesamicol-treated tumors had substantially lower levels of phosphorylated Akt (Thr308 and Ser 473) relative to nicotine-treated A549 tumors (Fig. 7D, top 2 panels). The total Akt levels were similar in all 4 pairs of tumors (Fig. 7D, third panel from the top). Taken together, these data indicate that vesamicol decreases tumor growth rates of A549 human BAC tumors *in vivo* by inducing robust apoptosis via an Akt-dependent pathway.

Discussion

Our study shows for the first time that human BACs produce ACh and contain a functional acetylcholine signaling system. A functional cholinergic loop has also been detected in SCLCs, SCC-L, and normal bronchial epithelial cells (14). Most importantly, nicotine regulates the cholinergic machinery and increases ACh levels by about 10-fold in human BACs. Nicotine upregulates the levels of cholinergic proteins, namely VACHT and ChAT, and concomitantly downregulates AChE in human BACs. Nicotine-induced increases of VACHT and ChAT promote ACh content and its transport into the extracellular

environment. On the other hand, nicotine decreases AChE levels, which, in turn, suppress ACh degradation. Thus, the enhanced levels of ACh provide proliferative stimuli to human BACs. The amplification of the cholinergic network by nicotine offers novel therapeutic strategies for BAC therapy. These phenomena are highly significant as about 30% of patients with lung cancer continue to smoke, use nicotine-based cessation patches or gum, or are exposed to environmental tobacco smoke after their diagnosis (39–41).

We observed that nicotine caused a 3-fold increase in VACHT and ChAT levels in human BAC cells. This led us to hypothesize that antagonists of VACHT should attenuate nicotine-induced ACh production and thereby suppress the growth of human BACs. As a proof-of-principle, we decided to use the well-characterized VACHT antagonist, vesamicol, for our experiments. Our hypothesis was supported by the results of Song and colleagues who showed that vesamicol suppressed the growth of H82 human SCLC cells *in vitro*. We believe that our results are the first to report characterizing the anticancer activity of VACHT antagonists in human BACs using both cell culture and *in vivo* model systems. We found that vesamicol induced robust apoptosis in human BAC cells in both cell culture and *in vivo* systems.

A survey of literature shows that only very few studies have evaluated the antitumor activity of vesamicol. Ogawa and colleagues evaluated radioiodinated vesamicol analogs for tumor imaging and antitumor activity (42, 43). They found that radioiodinated vesamicol analogues suppressed the growth of human prostate cancer cells in mice model. However, their results showed that vesamicol exerted its antitumor activity by binding to the sigma-receptor on DU145 prostate cancer cells (42). Several convergent studies have shown that vesamicol is also a ligand for the sigma-receptor. However, our experiments involving VACHT-siRNA and sigma-receptor siRNA showed that the anticancer activity of vesamicol was specifically mediated by VACHT. It is possible that sigma-receptors do not play a vital role in nicotine-induced proliferative signaling in human BAC cells.

The present manuscript shows that apoptotic effects of vesamicol are mediated via suppression of the Akt pathway. Clinical studies have shown that the activation of Akt is highly prevalent in human BAC tumors. Nicotine causes rapid activation of Akt and its downstream substrates. Studies by West and colleagues have speculated that inhibition of the Akt pathway may be a viable strategy for treatment of tobacco-related lung cancers (44, 45). The EGF receptor (EGFR) inhibitors gefitinib and erlotinib have been shown to suppress Akt phosphorylation in human lung cancer cells (46, 47). Similarly, phosphoinositide 3-kinase (PI3K)/Akt inhibitors, that is, LY294002 and deregulin, suppress proliferation of human BAC cells *in vitro* and in mouse models of tobacco carcinogenesis (48). Our results suggest that VACHT antagonists such as vesamicol are another class of therapeutic agents capable of inhibiting activated Akt in human BACs.

The acetylcholine signaling pathway has been found to regulate multiple cellular functions, such as proliferation, cell-to-cell contact, differentiation, and cytoskeletal integrity (14). Apart from lung cancer cells, endothelial cells, mesothelial cells, immune cells, and keratinocytes have been found to synthesize, transport, and degrade acetylcholine (14). The pharmacologic manipulation of this non-neuronal cholinergic system by agents, such

as vesamicol, could lead to the development of novel therapies for multiple tobacco-related diseases including lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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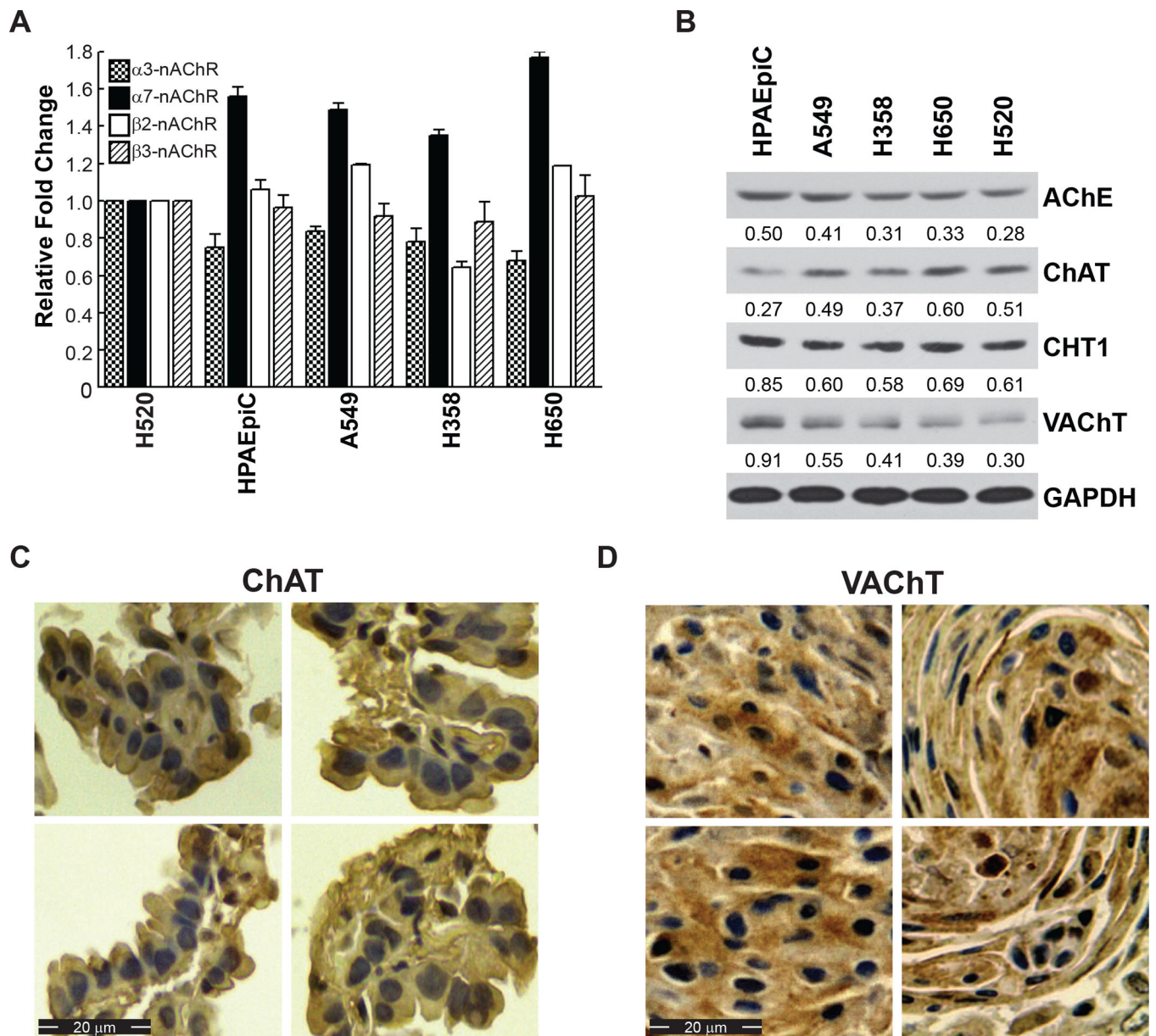


Figure 1. Human BACs and normal HPAEpiCs express cholinergic proteins. A, ELISA assays show that human BAC cell lines and HPAEpiCs express multiple nAChR subunits. The assay was completed in duplicate, and the whole experiment was carried out 2 independent times. Results indicated by a different letter are significantly different ($P < 0.05$). B, HPAEpiCs and human BACs express AChE, ChAT, CHT1, and VAcHT. H520 human SCC-L cells were used as the positive control for the experiments outlined in A and B. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control, and the results were quantitated by densitometric analysis. The experiment was repeated twice, and the representative data are shown. C and D, immunohistochemistry of the human BAC microarray showed that BAC tumors (isolated from patients) express ChAT and VAcHT in the cytoplasm, adjacent to the hematoxylin-counterstained dark nuclei in the tissue samples.

A tumor microarray containing 81 cores of human BACs was used for these experiments and 4 panels of representative photos are shown. Scale bar, 20 μm .

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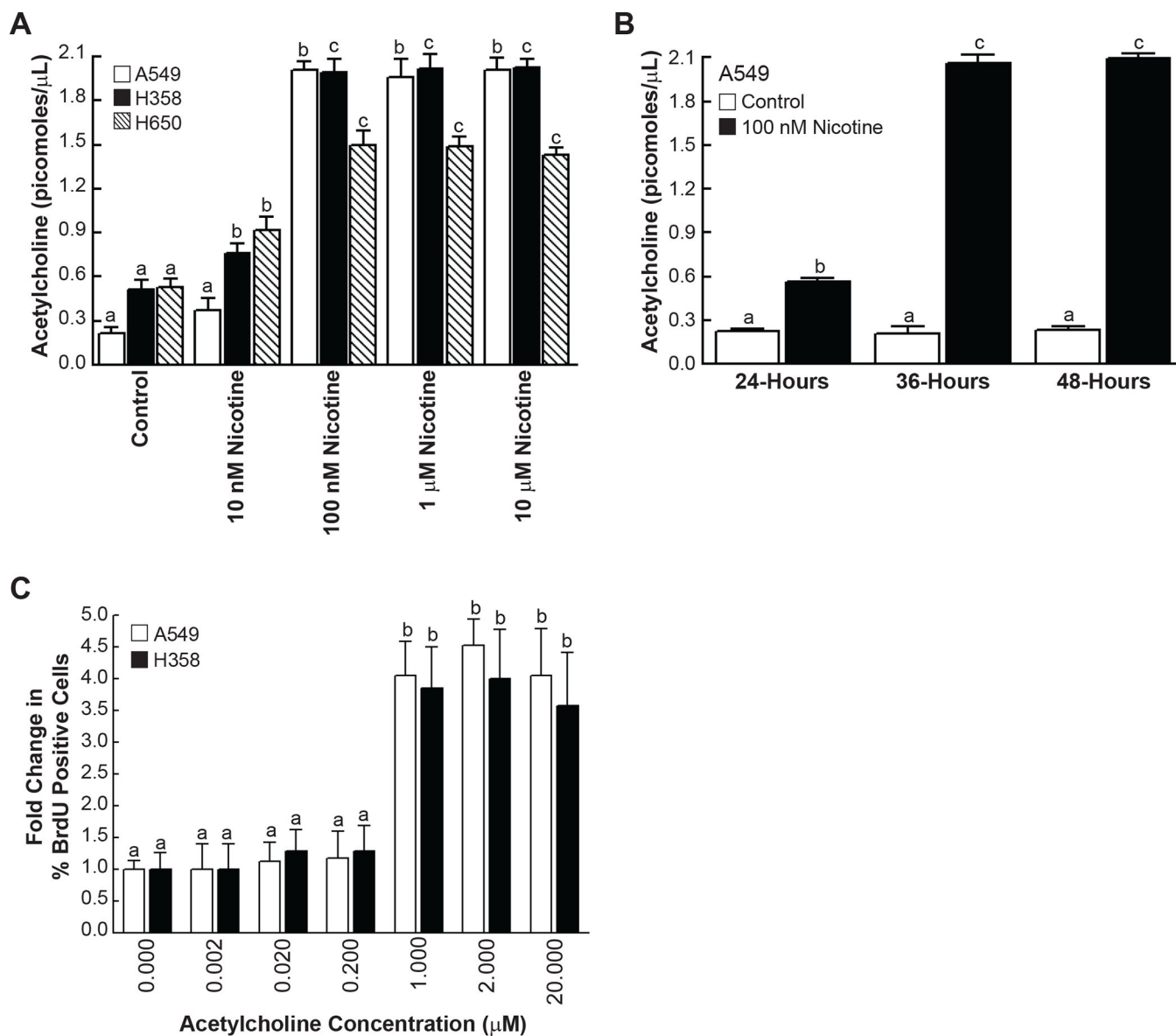


Figure 2. Nicotine induces the production of the growth factor ACh in human BACs. A, nicotine caused a concentration-dependent increase in ACh production in A549, H358, and H650 human BAC cell lines. B, time kinetics of nicotine-induced ACh production in A549 human BAC cells. The maximal ACh production was observed at 100 nmol/L nicotine at 36 hours. C, ACh stimulated proliferation of A549 and H358 cells. BrdUrd assays show that 2 μ mol/L of ACh (which is approximately the amount of ACh produced in nicotine-treated BAC cells) caused a 4- to 4.5-fold increase in proliferation of A549 and H358 human BACs. Each sample was analyzed in triplicate. Data represent mean \pm SEM from 2 independent experiments. Results indicated by a different letter are significantly different ($P < 0.05$).

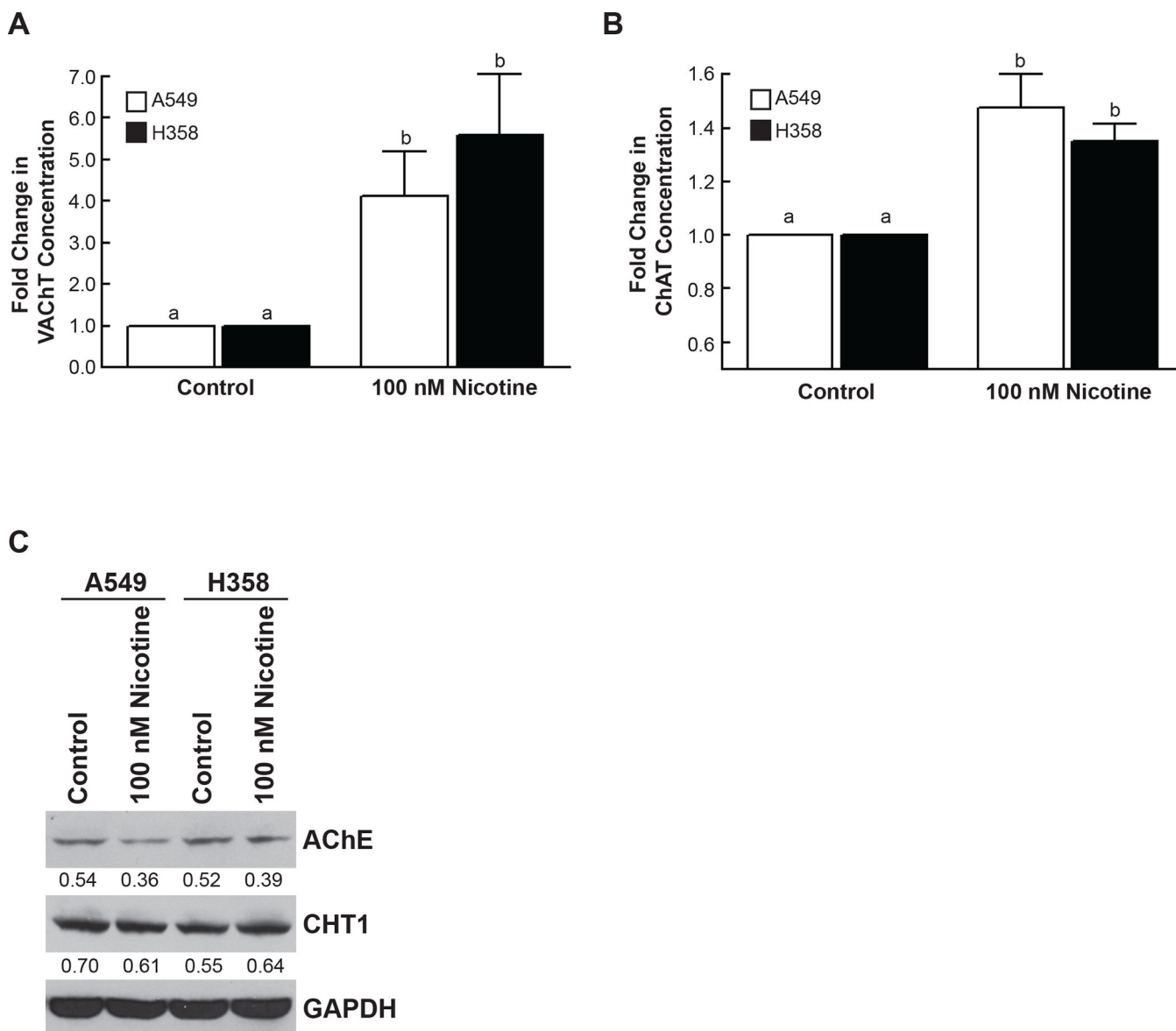
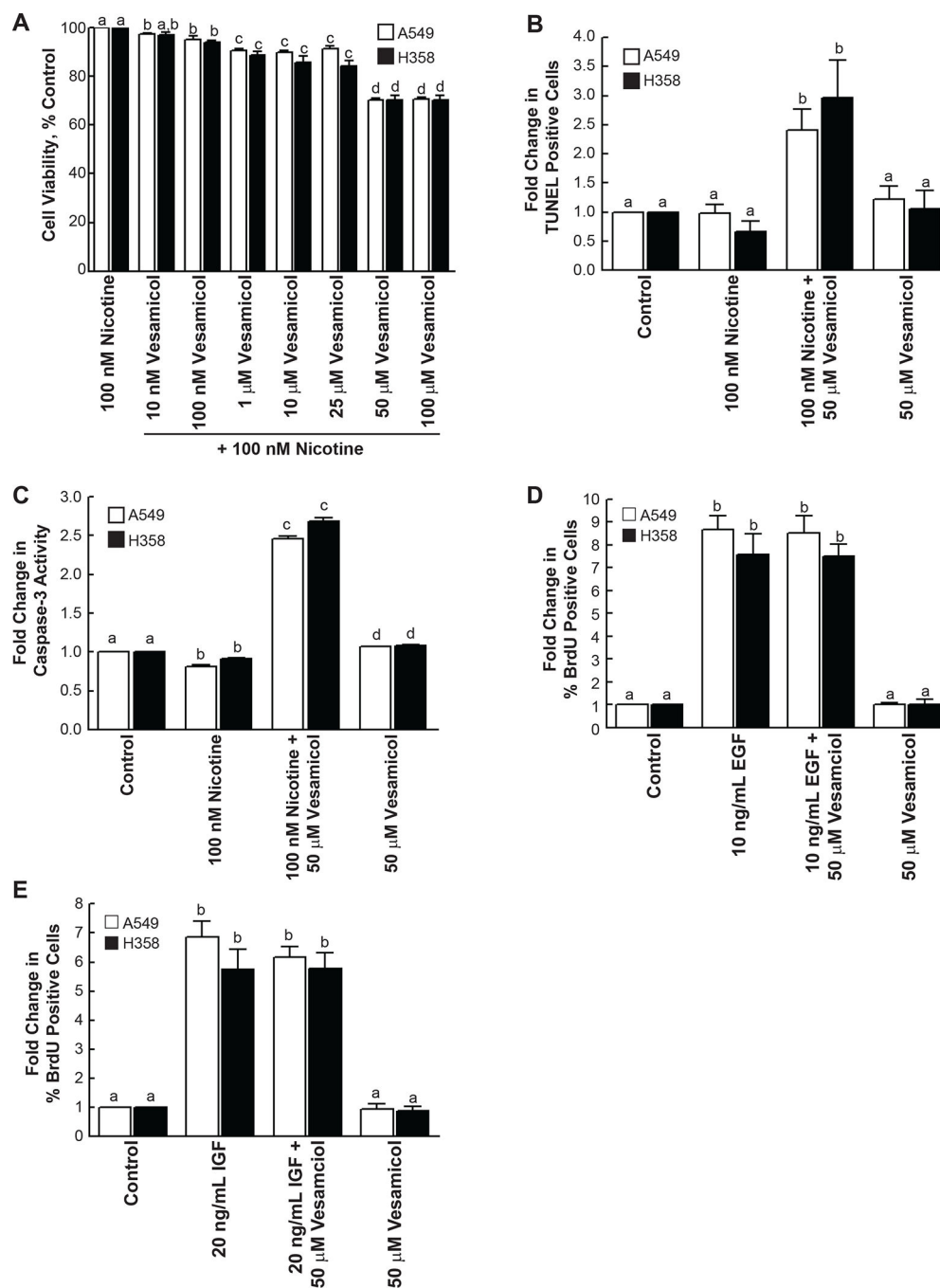


Figure 3. Effect of nicotine on cholinergic proteins in human BACs. A, the treatment of A549 and H358 cells with 100 nmol/L nicotine caused 4- to 5-fold increase in VAcHT levels. B, nicotine produces a modest increase in ChAT levels. C, nicotine decreases AChE levels in both A549 and H358 cells. The levels of CHT1 are relatively unaffected by nicotine in human BAC cells. Data represent mean \pm SEM from 2 independent experiments. Each sample was analyzed in triplicate. Results indicated by a different letter are significantly different ($P < 0.05$).

**Figure 4.**

Vesamicol caused apoptosis in human BAC cells. A, MTT assays showed that vesamicol decreases the viability of A549 and H358 cells in a concentration-dependent manner. Each sample was analyzed in triplicate. B, the treatment of human BAC cell lines with 50 μ mol/L vesamicol caused 2.5- to 3-fold apoptosis in nicotine-treated A549 and H358 cells. C, the apoptotic activity of vesamicol was confirmed with caspase-3 activity assay, and similar results were obtained. D, vesamicol had no effect on EGF-induced proliferation of A549 and H358 cells. BrdUrd assays show that the treatment of A549 and H358 human BAC cells

with 10 ng/mL EGF causes robust proliferation. The addition of 50 $\mu\text{mol/L}$ vesamicol along with EGF produced no effect on EGF-induced proliferation of human BACs. E, BrdUrd assays show that vesamicol does not suppress IGF-II-induced proliferation of human BAC cell lines. Each sample was analyzed in duplicate. Data represent mean \pm SEM from 2 independent experiments. Results indicated by a different letter are significantly different ($P < 0.05$).

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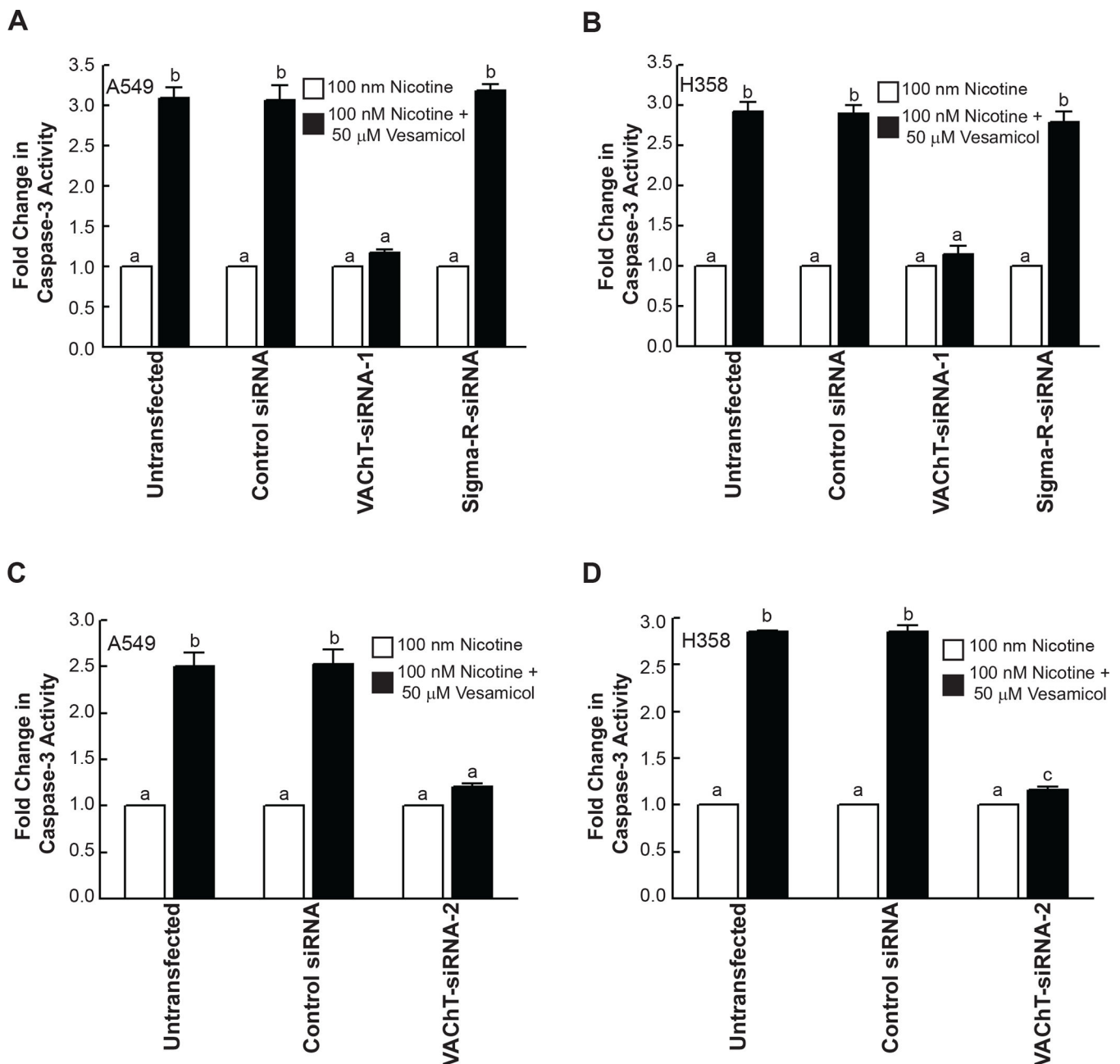


Figure 5. Vesamicol-induced apoptosis is specifically mediated by VACHT in human BAC cells. A, caspase-3 activity assays showed that the transfection of VACHT-siRNA reversed vesamicol-induced apoptosis in nicotine-treated A549 cells, whereas transfection of sigma-receptor siRNA (SigmaR-siRNA) or control-siRNA has no effect. B, the experiment was repeated in H358 human BAC cells and similar results were obtained. C, the suppression of VACHT, with another independent VACHT-siRNA (VACHT-siRNA-2), abrogated vesamicol-induced apoptosis in A549 human BAC cells. D, the experiment was repeated in H358 cells and similar results were obtained. Each sample was analyzed in duplicate. Data represent

mean \pm SEM from 2 independent experiments. Results indicated by a different letter are significantly different ($P < 0.05$).

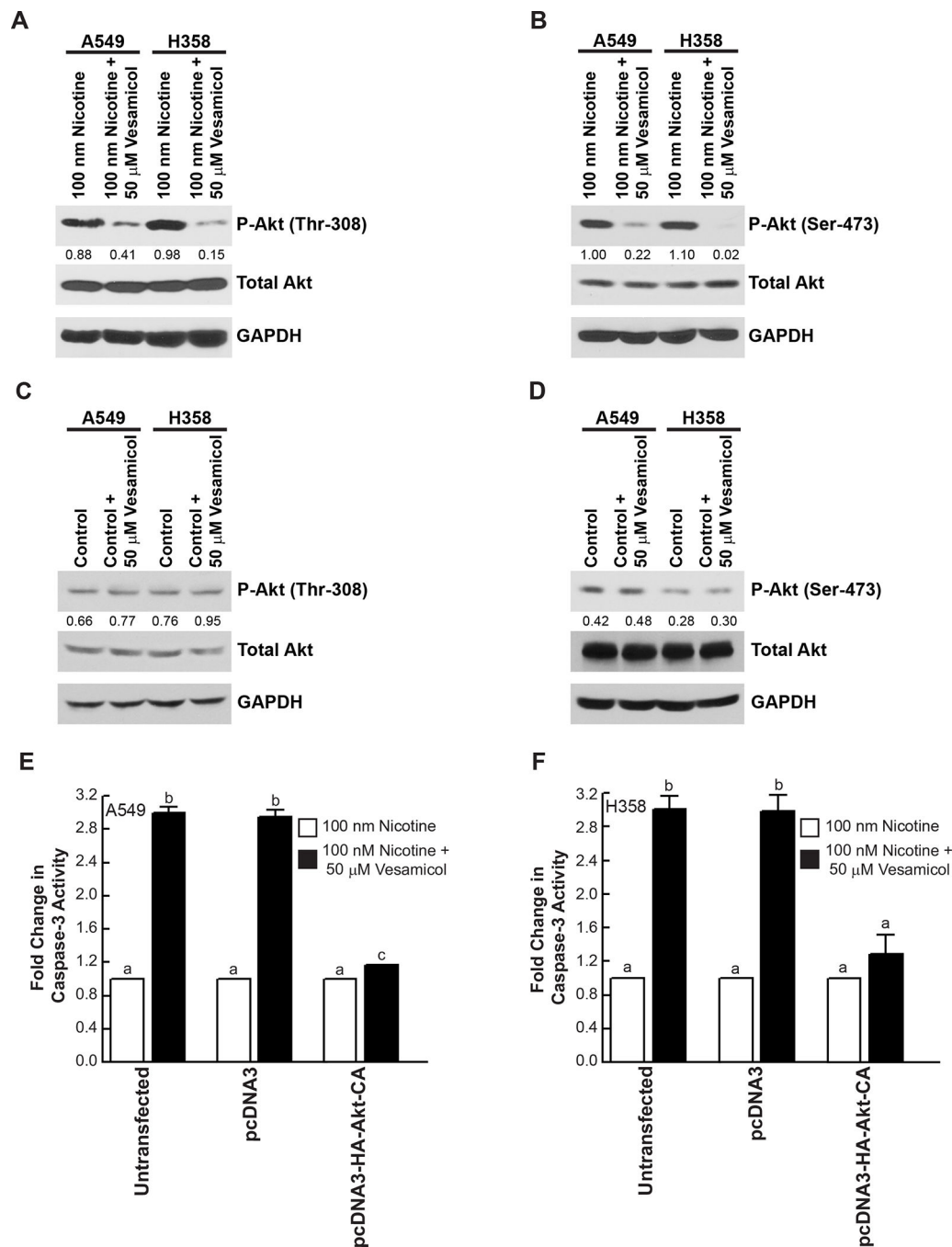


Figure 6. The apoptotic activity of vesamicol is mediated by the Akt pathway. A, the treatment of A549 and H358 human BAC cells with nicotine and vesamicol inhibited Akt phosphorylation at Thr-308. B, vesamicol decreased the levels of phospho-Akt (Ser-473) in nicotine-treated A549 and H358 cells. C, vesamicol had no effect on the levels of phospho-Akt (Thr-308) in untreated A549 and H358 cells. D, the treatment of quiescent A549 and H358 cells 50 μmol/L vesamicol does not affect phospho-Akt (Ser-473) levels. E, the transfection of pcDNA3-HA-Akt-CA (T308D, S473D) reverses the apoptotic activity

of vesamicol, whereas transfection of the empty vector had no effect on vesamicol-induced apoptosis in A549 human BAC cells. F, the transfection experiment was repeated in H358 cells and similar results were obtained. Data represent mean \pm SEM from 2 independent experiments. Each sample was analyzed in duplicate. Results indicated by a different letter are significantly different ($P < 0.05$).

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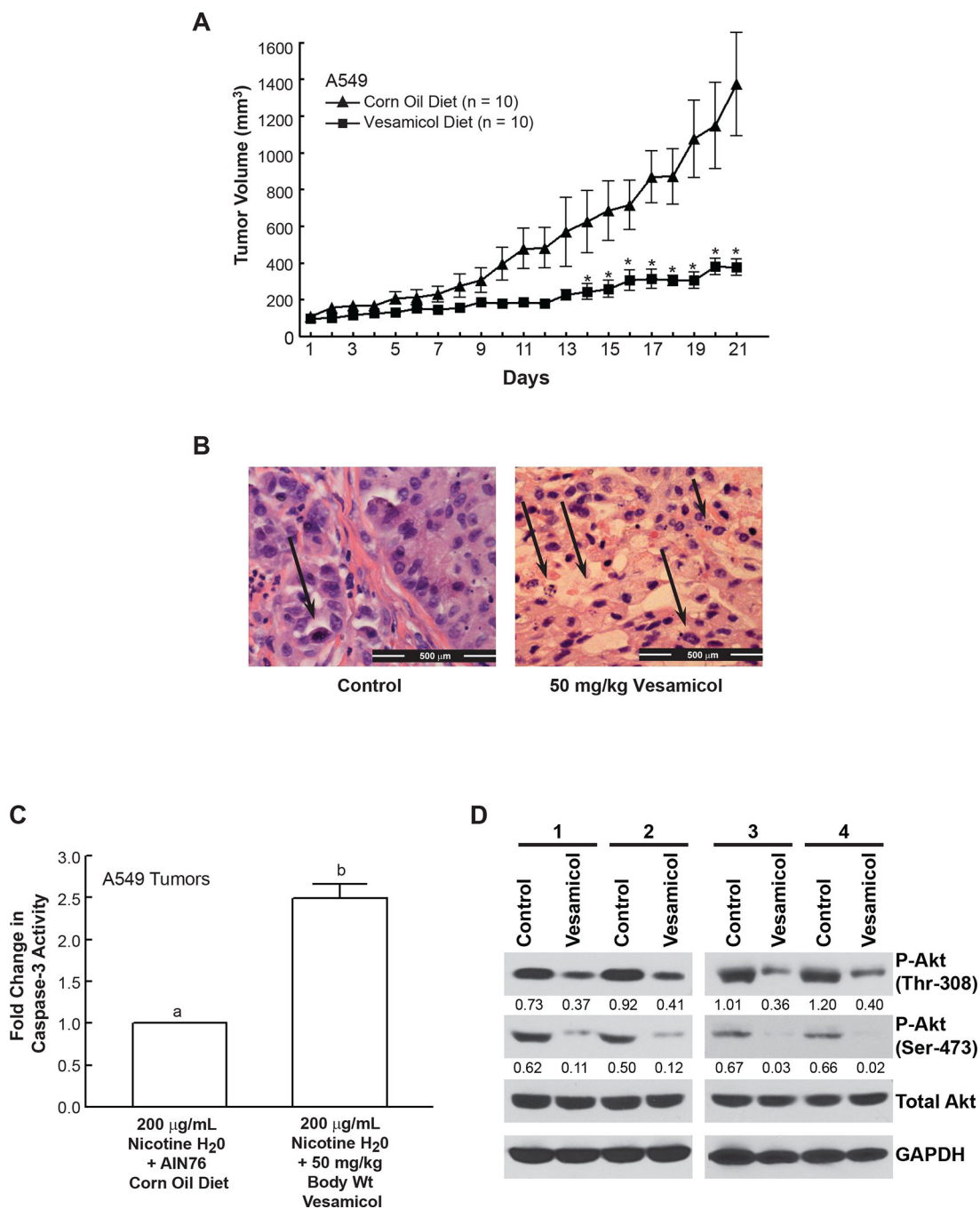


Figure 7.

Vesamicol induces apoptosis of human BAC tumors *in vivo*. A, the administration of vesamicol (50 mg vesamicol/kg food) decreased the growth rate of A549 human BAC tumors xenografted in nude mice. The nude mice experiments comprised 8 mice per group. B, the tumors from the mice were excised and stained with H&E. The black arrow shows the presence of apoptotic bodies in the tissue sections. The vesamicol-treated tumors (right) showed a greater number of apoptotic bodies relative to the mice administered vehicle (left). Scale bar, 500 μ m. C, an aliquot of the tumors from nude mice were frozen and lysates

were made. Caspase-3 activity of these lysates was measured. The vesamicol-treated tumors had higher caspase-3 activity (indicating higher apoptosis) than the tumors in the control group. D, Western blotting showed that vesamicol-treated tumors showed lower levels of phospho-Akt (Thr308) and phospho-Akt (Ser473). The Western blotting was quantitated using NIH ImageJ 1.46p. Data represent mean \pm SEM. Results indicated by a different letter or an asterisk are significantly different ($P < 0.05$).