Caffeine stimulates the proliferation of human lung adenocarcinoma cells and small airway epithelial cells via activation of PKA, CREB and ERK1/2

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Abstract. The incidence of pulmonary adenocarcinoma (PAC) has increased dramatically over the last three decades. Recent studies have shown that human PAC cells with phenotypic features of bronchiolar Clara cells and experimentally induced PAC of Clara cell origin are under ß-adrenergic growth control. The phosphodiesterase inhibitor, theophylline, which is contained in tea, asthma/allergy medications and numerous dietary supplements selectively stimulated the growth of this cancer type *in vivo* and *in vitro*. The current study has tested the hypothesis that another environmentally prominent phosphodiesterase inhibitor, caffeine, has similar effects. Using a cell line derived from a human PAC with Clara cell features (PACC) and immortalized human small airway epithelial cells (SAECs), our data show that caffeine activated protein kinase A (PKA), the mitogen-activated kinases ERK1/2, the nuclear transcription factor cyclic AMP response element binding protein (CREB) and stimulated cell proliferation in these cell lines. These findings suggest that exposure to caffeine may contribute to the prevalence of PAC observed today.

Introduction

Lung cancer is the leading cause of cancer death in both, men and women, and cigarette smoking is a dominant risk factor for this disease (1). Pulmonary adenocarcinoma (PAC) accounted for less than 10% of all lung cancer cases 30 years ago but is now the leading type of lung cancer in smokers and non-smokers (2-4). Human PAC may be derived from bronchiolar Clara cells or from alveolar type II cells (5). Immunohistochemistry is primarily used to identify cell lineage of this cancer type by using antibodies to the Clara cell-specific CC10 protein and the alveolar type II cell-specific surfactant. Using this technique, PAC of Clara cell lineage (PACC) has been reported to account for about 50% of PAC cases (6). By contrast, electron microscopic investigations have identified 90% of PAC as being derived from Clara cells (7). Recent reports have shown that exposure to cigarette smoke or to the tobacco-specific carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) down-regulate the expression of the Clara cell-specific CC10 protein in human and animal lungs (8). These findings suggest that identification of PAC cell lineage by immunohistochemistry may result in significant numbers of false negative immunoreactions to the CC10 antibody because of down-regulated CC10 in smokers. Accordingly, a higher proportion of PACs are likely of Clara cell lineage than is generally accepted in the literature.

Studies in our laboratory have shown that the *in vitro* growth of human lung cancer cell lines derived from PACC are regulated by ß-adrenergic receptors and that the tobacco-specific carcinogenic nitrosamine 4-(methylnitrosamino)-(3-pyridyl)-1-butanone (NNK) acts as an agonist for this receptor family (9). NNK as well as a stimulator of the classic downstream effector of ß-adrenergic receptors, cAMP, significantly stimulated DNA synthesis of these cells *in vitro* (9,10). Studies in a hamster model of NNK-induced PACC have shown that ß-adrenergic agonists and the phosphodiesterase inhibitor, theophylline, which causes intracellular accumulation of cAMP, each significantly promoted the development of this cancer type (11,12). Similarly, hamsters given green tea as the single source of drinking fluid in addition to multiple injections with NNK developed significantly more PACC than animals given NNK alone (12). Analysis by HPLC confirmed the presence of significant amounts of theophylline and caffeine in the green tea used in the hamster study. Subsequently, *in vitro* experiments showed that theophylline stimulated the proliferation of human PACC cells and their normal cells of origin, small airway epithelial cells (SAECs) via cAMP-initiated activation of protein kinase A (PKA), mitogen-activated protein kinases ERK1/2 and cAMP response element binding protein (CREB) (13). Both, theophylline and caffeine, are methylxanthines that increase intracellular cAMP levels by inhibiting the enzyme phosphodiesterase (14). The current

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experiments have therefore tested the hypothesis that caffeine has similar stimulating effects on the growth of human PACC cells and SAECs as theophylline.

Materials and methods

Cell lines and tissue culture. The human PAC cell line with characteristics of Clara cells, NCI-H322 (Center for Applied Microbiology and Research; ECACC, Salisbury, Wiltshire, UK) was maintained in RPMI-1640 medium (American Type Culture Collection, Manassas, VA) supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4500 mg/l glucose, and 1500 mg/l sodium bicarbonate. The simian virus 40 (SV40)-immortalized human peripheral airway cell line HPL1D is referred to in this publication as small airway epithelial cells SAEC. These cells were maintained in F-12 Nutrient mixture (HAM; Gibco, Carlsbad, CA) medium supplemented with 1% FBS, 15 mM HEPES pH 7.3, 5 μg/ml insulin, 5 μg/ml transferrin, 10^{-7} M hydrocortisone, $2x10^{-10}$ M triiodothyronine (Cambrex, Walkersville, MD). Both cell lines were maintained in antibiotic-free medium. Assays with NCI-H322 cells were conducted in low-serum (0.1%) RPMI medium while assays with SAECs were conducted in their basal media with 0.05% FBS without other supplements.

PKA activation assay. Following incubation of cells with caffeine (10 pM in PBS at 37˚C) for 5-60 min as specified in the figure legends, PKA activity was assayed in cell lysates using a PepTag assay for non-radioactive detection of activated PKA (Promega Corporation, Madison, WI, USA), following the instruction of the manufacturer. This assay utilizes fluorescent substrate for PKA that changes the peptide's net charge upon phosphorylation of PKA, thus allowing the phosphorylated peptide to migrate to the positive electrode (+), while the non-phosphorylated peptide migrates to the negative electrode (-). Briefly, reactions containing a brightly colored fluorescent peptag A1 peptide (0.4 μg/ml), peptide protection PepTag PKA reaction and PKA activator solutions were incubated in ice for few min before 1-min incubation at 30˚C. After adding samples, cAMP-dependent protein kinase reactions were incubated at room temperature for 30 min, boiled at 95˚C for 10 min, and loaded onto 0.8% agarose gel in 50 mM Tris-HCl (pH 8.0). At this point the qualitative assay is complete, and the protein kinase A activity in samples is determined by examining the gel under UV lights. Densitometric analysis of the bands was conducted after inversion of the images, using NIH Scion software for image quantitation.

Data are expressed as mean values and standard errors of five densitometric readings per band after background subtraction. Each experiment was repeated once with similar results. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparisons test and two-tailed unpaired t-test.

Assessment of total proteins and phosphorylated proteins by Western blotting. To assess the effects of caffeine on the expression and phosphorylation of the mitogen-activated protein kinases ERK1/2 or the cAMP response element binding protein CREB, 500,000 NCI-H322 or SAEC cells were seeded into culture vessels (100 cm^2) containing their respective growth media. When the cells had reached 60-65% confluence, they were rinsed one time with 1X PBS and serum-starved for 24 h. Following removal of the media and replacement with fresh low-serum media, caffeine (dissolved in PBS) was added to the culture vessels (final concentration 10 pM) and cells were incubated from 5 to 60 min as detailed in the figure legends. Cells exposed to the caffeine vehicle (PBS) served as controls. The cultured cells then were washed once with cold PBS, lysed in 20 mM Tris-base, 200 mM NaCl, 1 M sodium fluoride, 0.5 M EDTA, 100 mM Na_3VO_4, 100 mM PMSF, 1 μl pepstatin, 1 μl leupeptin, 1 μl aprotinin, and 0.25% NP-40. Then, protein samples were denatured by boiling at 95˚C for 5 min, separated on 10% SDS-PAGE, and transferred to nitrocellulose. Membranes were blocked with 5% non-fat dry milk, probed with rabbit polyclonal CREB and phospho-CREB antibodies, respectively, and developed by chemiluminescence with ECL reagents membranes were blocked in 10 ml of 5% non-fat dry milk (Kroger) in 1X TBST for 1 h. Membranes were then incubated overnight at 4˚C with primary antibodies at a 1:1000 dilution (rabbit polyclonal for total ERK1/2, rabbit polyclonal for Thr202/Tyr204 phosphorylated Erk1/2; rabbit polyclonal for total CREB, mouse monoclonal for SER33 phosphorylated CREB) (Cell Signaling Technology, Beverly, MA). Densitometric analysis of the bands was conducted, using NIH Scion software for image quantitation.

Data are expressed as mean values and standard errors of five densitometric readings per band. Each experiment was repeated twice and yielded similar data. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparisons test and two-tailed unpaired t-test.

Assessment of cell numbers by MTT assay. The effects of caffeine on cell proliferation were assessed by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma). Briefly, the MTT test is based on the NADH-dependent enzymatic reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide] in metabolically active cells but not in dead cells. Cells were seeded into 6-well tissue culture plates (Falcon, Franklin Lakes, NJ, USA) at a density of 50,000 cells per well. The cells were left to grow in complete media at 37˚C with 5% CO_2 for 5 h to attach. The cells were then switched to fresh low serum (0.1% FBS, NCI-H322) or basal media with 0.05% FBS without other additives (SAECs) and caffeine dissolved in PBS was added to yield the final concentrations specified in the figure legends and incubated for 72 h. Cells treated with PBS alone served as controls. Three hours before the end of the 72-h incubation period, 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (0.5 mg/ml) was dissolved in complete medium together with phenol-free medium (Gibco) and added to the cells to allow metabolic conversion of the MTT substrate to blue formazan. The media was replaced with isopropanol, and optical density at 570 nm was determined using an ELISA reader.

Data are expressed as mean values and standard errors of four samples per treatment group. Each experiment was repeated twice and yielded similar data. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparisons test and two-tailed unpaired t-test.

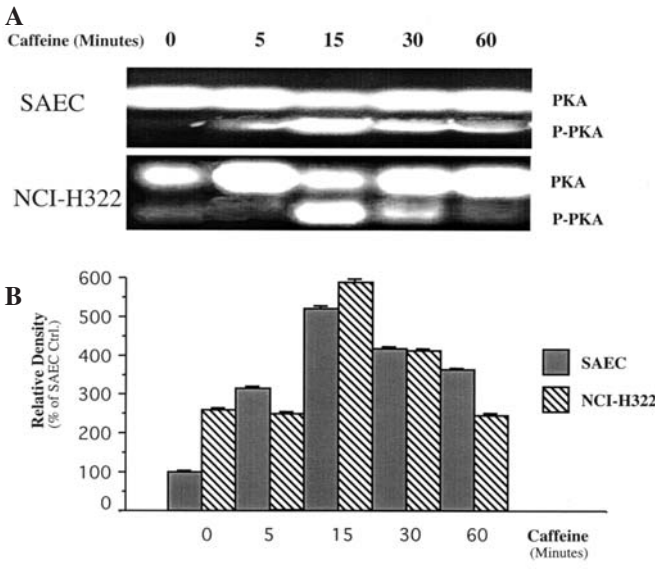

Figure 1. A, Agarose gel showing the effects of caffeine (10 pM) incubated for 5-60 min on phosphorylation of SAEC and NCI-H322. PKA was assayed using a non-radioactive PepTag assay. B, Bar graph demonstrating normalized (controls of SAECs are set as 100%) mean values and standard deviations of five densitometric readings per band. The experiment was repeated twice with similar results. Statistical analysis was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The increases in PKA phosphorylation were significant (P<0.001) at all time intervals tested in SAECs and at the 15- and 30-min time intervals in NCI-H322.

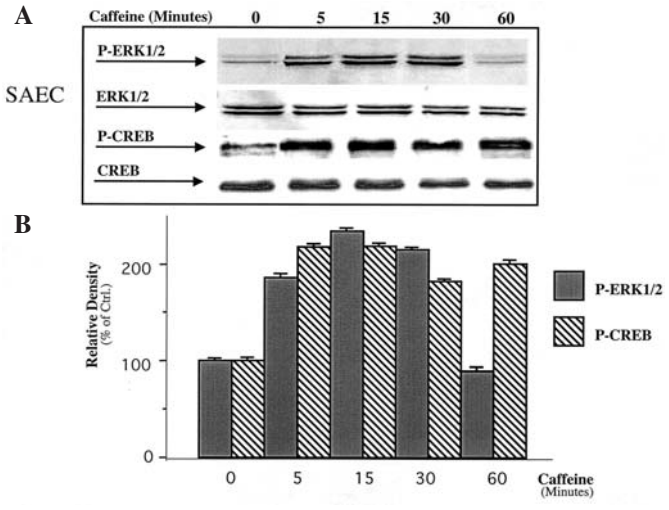

Figure 2. A, Western blot exemplifying the effects of caffeine (10 pM) on the expression of phosphorylated ERK1/2 and phosphorylated CREB in SAECs. The bands for P-ERK1/2 and P-CREB increased in size and density over time while the bands for total proteins (ERK1/2, CREB) remained unchanged. B, Bar graph illustrating normalized (controls are set as 100%) mean values and standard deviations of five densitometric readings per band. The experiment was repeated twice with similar results. Statistical analysis was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The increase in P-ERK1/2 was significant (P<0.001) at the 5-, 15- and 30-min time intervals while the observed increase in P-CREB was significant (P<0.001) at all time intervals tested.

Results

PKA is the classic downstream effector activated by cAMP in mammalian cells (15). In light of the documented ability of caffeine to inhibit phosphodiesterases that mediate the cellular breakdown of cAMP (14), we first tested the hypothesis that caffeine activates PKA in PACC and SAECs. As shown in Fig. 1, SAECs exposed to the solvent of caffeine, PBS, demonstrated no detectable PKA activity whereas PBS-exposed NCI-H322 cells had significant levels of basal PKA activity. Both cell lines responded to caffeine (10 pM) with significant (P<0.01) increases in PKA activity, with peak levels (5.2-fold increase for SAECs, 2.3-fold increase for NCI-H322 cells) observed after 15 min of exposure (Fig. 1).

Activation of PKA generally leads to phosphorylation of the transcription factor CREB in mammalian cells (15). On the other hand, cross-talk between the cAMP/PKA/CREB pathway and the mitogen-activated kinase (MAPK) pathway, including the extracellular signal regulated kinases (ERK1/2) has been reported in several cell types (16,17). We therefore tested the hypothesis that caffeine activates CREB and ERK1/2 in PACC and SAECs. Western blot analysis showed that caffeine (10 pM) significantly (P<0.01) increased the levels of phosphorylated ERK1/2 of SAECs in a time-dependent manner (Fig. 2), with peak levels (2.4-fold) observed after 15 min of exposure. Similarly, caffeine significantly (P<0.01) induced the expression levels of phosphorylated CREB over time in these cells, with the highest levels (2.3-fold) observed after 5 and 15 min of exposure (Fig. 2). The expression levels of unphosphorylated ERK1/2 or unphosphorylated CREB remained unchanged, confirming equal loading of protein. The PACC cell line NCI-H322 demonstrated a similar response

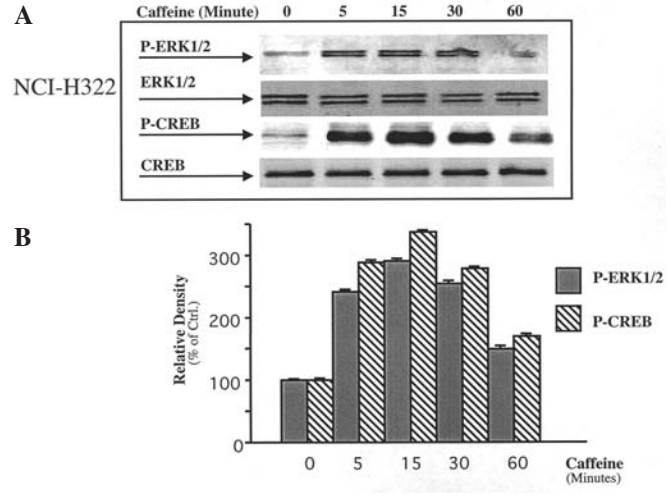

Figure 3. A, Western blot exemplifying the effects of caffeine (10 pM) on the expression of phosphorylated ERK1/2 and phosphorylated CREB in NCI-H322. The bands for P-ERK1/2 and P-CREB increased in size and density over time while the bands for total proteins (ERK1/2, CREB) remained unchanged. B, Bar graph illustrating normalized (controls are set as 100%) mean values and standard deviations of five densitometric readings per band. The experiment was repeated twice with similar results. Statistical analysis was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The increase in P-ERK1/2 and P-CREB was significant (P<0.001) at all time intervals tested.

to caffeine (10 pM). Induction of P-ERK1/2 was highly significant (P<0.001) after 5, 15 and 30 min of exposure to caffeine, with peak levels (2.8-fold increase) at the 15-min time interval (Fig. 3) in these cells. Induction of phosphorylated CREB by caffeine in these PACC cells was even more

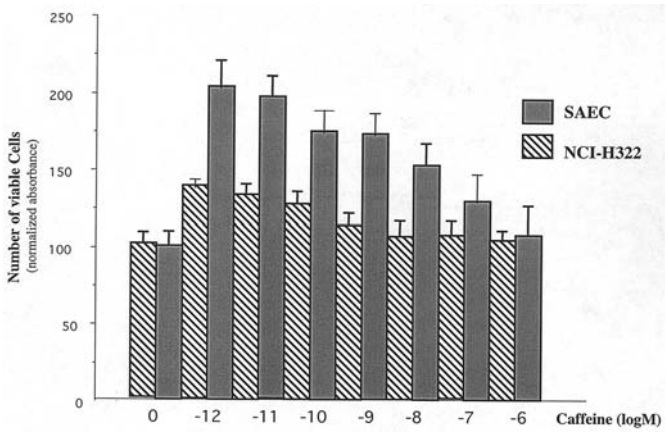

Figure 4. Effects of caffeine (1 pM-1 μM) on cell number in SAECs as assessed by MTT assay. The cells were allowed to attach to the culture vessel surfaces in complete media at 37˚C in 5% CO_2 for 5 h. The cells were then switched to low serum medium and exposed for 72 h to caffeine at the concentrations indicated. Bars represent normalized (control values set at 100%) mean values and standard errors of four samples per treatment group. The experiments were repeated twice with similar data. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in SAEC cell number were significant (P<0.001) at concentrations from 1 pM to 10 nM. NCI-H322 cells were less responsive and yielded a small but significant (P<0.01) increase at the 1, 10 and 100 pM concentrations of caffeine.

pronounced (P<0.001 after 5, 15 and 30 min), with a 3.3-fold increase after 15 min of exposure to caffeine.

Assessment of cell proliferation by MTT assays showed that caffeine increased the numbers of viable cells in both cell lines in a concentration-dependent manner after 72 h of exposure, with the lowest concentrations (1 pM, 10 pM) yielding the most pronounced responses (Fig. 4). NCI-H322 cells which demonstrated high base level proliferation in serum-free medium were less responsive than the immortalized SAECs. In both cell lines, the proliferative response to caffeine was highly significant (P<0.001) at the 4 lowest concentrations of caffeine (1 pM, 10 pM, 100 pM, 1 nM).

Discussion

The phosphodiesterase inhibitor caffeine is contained in coffee, tea, caffeinated beverages and in numerous dietary supplements that promise weight loss while boosting energy. Both, tea and caffeine have been shown to prevent the development of lung cancer in mice and rats (18,19). However, the use of these agents for cancer prevention has recently been challenged by reports that showed a significant and selective promotion of NNK-induced PACC in hamsters by green tea or the phosphodiesterase inhibitor theophylline contained in tea while the development of neuroendocrine carcinomas was inhibited (12). Moreover, a significantly elevated lung cancer risk has recently been reported in individuals who consumed 2-3 cups of regular coffee daily (20). Experiments with the PACC cell line, NCI-H322 and their putative normal cells of origin, SAECs, showed that theophylline stimulated the proliferation of both cell lines via cAMP-initiated activation of protein kinase A (PKA), mitogen-activated protein kinases ERK1/2 and cAMP response element binding protein (CREB) (13). Our current studies with caffeine in these two cell lines yielded similar responses. Low concentrations of caffeine that can be realistically expected in individuals who consume moderate amounts of coffee or tea significantly stimulated the proliferation of PACC and SAECs in MTT assays. Moreover, caffeine caused a significant and time-dependent increase in PKA activity, P-ERK1/2 and P-CREB. Collectively, these data suggest that caffeine may promote the development of PACC in humans.

These findings are in accord with our recently published observations that theophylline (13), ß-carotene (21) and dexamethasone (22) all stimulated the proliferation of PACC and SAECs *in vitro* via activation of PKA, CREB and ERK1/2. Consequently, all of these agents have to be considered as potential tumor promoters for the development of PACC. This interpretation contrasts sharply with the widely held belief that tea (18,19), caffeine (19), glucocorticoids (23) and ß-carotene (24,25) may be useful as cancer preventive agents. We have recently shown that cAMP/PKA selectively stimulate the growth of SAECs and PACC while inhibiting the growth of large airway epithelial cells (9,10,21). On the other hand, cAMP/PKA has been shown to inhibit the proliferation of small cell lung cancer cells (26) and PAC of alveolar type II cell phenotype (10). Collectively, these observations clearly illustrate that the cAMP/PKA pathway stimulates proliferation in some cells and the cancers derived from them while inhibiting others. Attempts to arrive at successful cancer preventive strategies as well as efforts to treat cancer with agents that inhibit components of signal transduction pathways have to take the highly cell type-specific function of signal transduction pathways into account. Unlike diagnostic tools are developed that allow for the identification of hyperactive pathways in each individual patient prior to the assignment to such therapies such efforts will continue to fail and may instead even promote the development of certain cancers.

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