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To the Graduate Council:

I am submitting herewith a dissertation written by Hussein Abdulhadi Nasser Al-Wadei entitled "Growth Stimulation of Pulmonary Adenocarcinoma and their Cells of Origin by "Chemopreventive" Agents that Increase Intracellular cAMP." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Hildegard M. Schuller, Major Professor

We have read this dissertation and recommend its acceptance:

Potgieter L.N.D, Michael D. Karlstad, Howard K. Plummer

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

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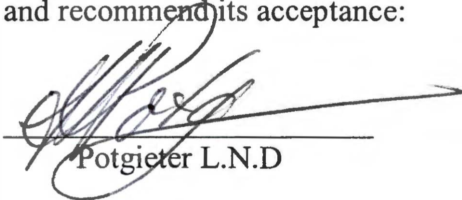

Potgieter L.N.D

Michael D. Karlstad

Howard K. Plummer

Accepted for the Council:

Vice Chanc[illegible]ean of
Graduate Studies

Thesis
2006
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GROWTH STIMULATION OF PULMONARY ADENOCARCINOMA AND THEIR CELLS OF ORIGIN BY "CHEMOPREVENTIVE" AGENTS THAT INCREASE INTRACELLULAR cAMP

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Hussein Abdulhadi Nasser Al-Wadei
December, 2004

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DEDICATION

First of all the author would like to thank almighty Allah (God); the compassionate, the merciful, for all of the blessings that he gives. The author dedicates this dissertation to the soul of his father, ***Abdul-Hadi Naser Al-Wadei***, who devoted his life to his children, who viewed his sons as the best sons in the world, who always scarified and lived away instead of living close to his children, and grand and great grandchildren; however, he always encouraged them to join in the vast world and to live full lives. He asks Allah (God) to protect his father's soul and body from hell fire. Furthermore, the author will keep his father beneficial advice in his mind for ever. He also dedicates this dissertation to his mother ***Fatemah Yahya Al-Wadei***, who at the last moment still ignored to travel to the other world without seeing the picture of her son; who the last thing she did in her very special life was to give her best wishes to her son, daughter-in-law and grandchildren in the United States. This dissertation is also dedicated to his brothers, Hameed, Dr. Khalid, Naser; and his sisters, Horea, and Aisha, for their constant love and assistance, and for teaching him everything is possible. The author presents his heartfelt thanks to all the members of his extended family, such as uncles especially my Uncle Nasser bin Nasser Mofleh, who has encouraged me to finish my higher education and always provide me with valuable life lessons, brothers and sisters-in-law. Loved ones in both families of the author also offered tremendous love and encouragement, which helped him and his family to safely go through many hardships of

the course. Living a fuller life and doing better things is only one way that the author can reward these loved ones.

The author can not wrap up this section without expressing his most sincere dedication to his sweetheart, Aisha Ghilan Abu-Farea, for her omnipresent love and a lifetime of support and encouragement, and his wonderful sons, Mohammed, Yusof, and Hussan; and his daughters, Al-Anood, Abeer, and Rahaf respectively. Their love, their mighty power and their understanding attitude have been the utmost reliable support, providing the best shelter in which the author can live. Their sacrifice to his academic life has been priceless. The author also thanks all of his children for approving continuation of his study leave, and in so doing, suspending their natural right to be cared for by a father.

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If I have omitted someone from this acknowledgment, it is purely unintentional. The number of people I'm thinking about while writing this is very long indeed, and I collectively thank you all even if your names were not mentioned.

ABSTRACT

Lung cancer is the leading cause of cancer mortality for men and women in the United States, with a high mortality rate and a five-year survival rate of less than 15%.Cancer ranks second as a cause of death for Americans after cardiovascular disease. The American Cancer Society (ACS) reported 171,900 new cases of lung cancer for 2003 (ACS, 2003). Peripheral adenocarcinoma (PAC) of the lung has increased dramatically over the last 20 years and is the leading histological type of lung cancer in smokers and nonsmokers in industrialized countries, including the United States. Among the four main histological lung caner types (small-cell carcinoma, squamous-cell carcinoma, adenocarcinoma and large-cell carcinoma), adenocarcinoma that is derived from small-airway epithelia with features of Clara cells accounts for about 35-40% of all lung cancer cases. Unlike other histological lung cancer types, adenocarcinoma also develops in a significant number of non-smokers.

Smoking remains the greatest contributor to the development of lung cancer, with 90% of all lung cancer cases estimated to be smoking related. Cigarette smoke contains about 4,000 toxic chemicals including the highly carcinogenic nitrosamine, 4-(methylnitrosamino)-1- (3-pyridyl)-1-butanone (NNK). NNK is the most potent carcinogen in laboratory animals and has therefore been implicated as a significant cause of tobacco-associated cancers in human.

Dietary and genetically determined factors appear to play an important role in modulating individual susceptibility to smoking-associated cancer and are closely linked to the chemoprevention approach.

Chemoprevention is defined as the use of naturally occurring or synthetic agents to prevent, inhibit or reverse the process of carcinogenesis. This relatively new approach of cancer prevention has precedence in other areas of medicine such as cardiovascular diseases.

Earlier studies have shown the presence of a β-adrenergic/cAMP growth-regulating pathway in PAC. Therefore, β-adrenergic stimulants in various drugs that are used for the treatment of chronic respiratory and cardiovascular diseases have been proposed as potential risk factors for the development of PAC. Furthermore, little is known about the downstream effectors of this pathway and their role in the regulation of proliferation of PAC and their normal cells of origin.

Using assays for the assessment of cAMP production, PKA activity, MAPK activation, CREB activation, and cell proliferation, we have identified a mitogenic pathway, which activates cAMP in cell lines derived from human peripheral adenocarcinomas that express features of Clara cells and their normal cells of origin, small airway epithelial cells (SAEC).

β-carotene and a substance contained in green and black tea, theophylline are widely believed to have cancer preventive effects. However, our current data show that each of these agents increases cAMP/PKA activity, ERK1/2 and CREB resulting in a significant growth stimulation of PAC and SAEC. Accordingly, and pending on the exact level targeted by a given chemopreventive agents, such treatments will likely promote the

development of PAC. While some of these agents may inhibit the metabolic activation of tobacco carcinogens, such as NNK, former smokers who start chemoprevention will not benefit from such effects as they start chemopreventive treatment after discontinuation of exposure to tobacco carcinogens. Currently, there is no agent that has been shown to be effective in preventing lung cancer. Accordingly, the most effective prevention of lung cancer is never to smoke and to avoid exposure to second hand smoke.

Our study findings suggest that the widely advertised cancer preventive agents that currently are still tested by several laboratories as "chemopreventive" agents such as β-carotene and a substance contained in green and black tea, theophylline are unsafe to be used by smokers or by ex-smokers due to their tumor promoting effects via stimulation of cAMP on initiated cells of Clara cell lineage.

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LIST OF ABBREVIATIONS

AA: Arachidonic Acid

AC: Adenylyl Cyclase

AKT (PKB): Protein Kinase B

AMP: Adenosine Monophosphate

ATF-1: Activating-Transcription Fator-1

AP-1: Activator Protein-1

ATBC: Alpha-Tocopherol Beta-Carotene

ATCC: American Type Culture Collection

BCA: Bichinchoninic Acid Assay

BEBM: Bronchial Epithelial cell Basal Medium

BO: Bound Optical density

BPE: Bovine Pituitary Extract, cell culture tested

cAMP: Cyclic Adenosine Monophosphate

c-fos: Nuclear phosphoprotein gene (transcription factor)

c-jun: Nuclear phosphoprotein gene (transcription factor)

c-myc: Nuclear phosphoprotein gene (transcription factor)

Ca^{2+} : Calcium ion

CAMR: Center for Applied Microbiology and Research

CARET: β-Carotene and Retinol Efficacy Trial

COPD: Chronic Obstructive Pulmonary Disease

CP: Chemoprevention

CPA: Chemopreventive Agents

CRE: c-AMP Response Element

CREB: c-AMP Response Element Binding protein

CREM: c-AMP Response Element Modulator

DAG: Diacylglycerol

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DTT: 1, 4-Dithio-DL-threitol Solution

ECACC: European Collection of Cell Cultures

EC: Epicatechin

ECG: Epicatechin gallate

EDTA: EthyleneDiamineTetraacetic Acid

EGC: Epigallocatechin

EGCG: Epigallocatechin-3-gallate

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ERK: Extracellular regulated kinase

EtOH: Ethyl Alcohol

FBS: Fetal Bovine Serum

FCS: Fetal Calf Serum

GA: Gentamycin Sulfate Amphotercin-B

GC: Gallocatechin

GPCRs: G-protein coupled receptors

GSTM1: Glutathione S-transferase M1

GT: Green Tea

GTE: Green Tea Extract

HBSS: HEPES Buffered Saline Solution

HC: Hydrocortisone

HCL: Hydrochloric acid

hEGF: Human Epidermal Growth Factor

HSM: High serum medium

IBMX: IsoButyl-1-MethylXanthine

JAK: Janus Kinase

LSM: Low serum medium

MAPK: Mitogen-Activated Protein Kinase

MEK: MAPK/ERK kinase

MAP/ErK: (Extracellular Signal-Regulated Kinase) Kinase

MTT: 3-(4, 5-dimethyle thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

Na Cl: Sodium Chloride

NaOH: Sodium hydroxide

$Na_3 VO_4$: Sodium Orthovanadate

NCI: National Cancer Institute

NF-AT: Nuclear Factor-AT

NF-B: Nuclear Factor-B

NIH: National Institute of Health

NNK: 4-(methylnitrosamine-)-1-(3-pyridyl)-1-butanone

NSB: Non-specific binding

NSCLC: Non-Small Cell Lung Carcinoma

PAC: Pulmonary Adenocarcinoma

PACC: Pulmonary Adenocarcinoma of Clara cell phenotype

PAC-II: Pulmonary Adenocarcinoma of alveolar type II cell phenotype

PBS: Phosphate Buffered Saline

PDEs: Phosphodiesterase

PDGF: Platelet derived growth factor-A/B

PDK: phosphoinositide-dependent kinase

PI: phosphatidylinositol

PIK-3: phosphatidylinositol 3-kinase

PKA: Protein Kinase-A

PKB (AKT): Protein kinase-B

PKC: Protein kinase-C

PLA: Phospholipase-A

PLC: Phospholipase-C

PLD: Phospholipase-D

PMSF: PhenylMethylSulFonyl Floride

p-Npp: p-nitrophenyl phosphate

Raf-1: Cytoplasmic serine / threonine protein kinase

Rap1: Ras-related G-protein

Ras: Membrane associated GTP protein kinase

rhEGF: Epidermal Growth Factor, Human Recombinant

RH: Relative Humidity

Rpm: Round per minute

RTK: Receptor tyrosine kinase

SAEC: Small Airway Epithelial Cells

SCLC: Small-Cell Lung Carcinoma

SDS-Page: Sodium dodecyl Sulfate-Polyacrylamide gel.

SOS: Son of sevenless (guanaine exchange factor)

SQC: Squamous cell carcinoma

Src: Non-membrane associated tyrosine kinase

STAT: Signal transducers and activators of transcription

TA: Total activity

TGF-α: transforming growth factor-alpha

TGF-β: transforming growth factor-beta

TNS: Trypsin Neutralizing Solution

T3: Triiodothyronine

TBS: Tris-Buffered Saline

TBST: Tris-Buffered Saline Tween-20

TRIZMA: Tris-Hydroxymethyl-aminomethane

WHO: Word Health Organization

PART I:

Introduction

I. Introduction

Peripheral adenocarcinoma (PAC) of the lung has increased dramatically over the last 20 years. PAC is today the leading histological type of lung cancer death in smokers and non-smokers in both men and women of industrialized countries, with a high mortality rate and five-year survival rate of less than 15% after diagnosis (1-4). According to a recent report by the United States Surgeon General, lung cancer in women has increased by 600% since the 1950s and has reached epidemic levels (5, 6). Since the late 1980s, lung cancer in women has surpassed breast cancer as the leading cause of cancer death (7). The American Cancer Society (ACS) reported 171,900 new cases of lung cancer for 2003 and 173,770 new cases and 160,440 deaths are expected in the United States in the year 2004 (8). Lung cancer ranks as a second cause of death after cardiovascular disease (9).

The most important risk factor for lung cancer is cigarette smoking, which accounts for 80-90% of all cases (10, 17). The only effective means of lung cancer prevention is never to smoke or giving up smoking (11). Lung cancer is usually detected at an advanced stage of development when metastasis and drug resistance have already developed (12). The prognosis has not been improved by chemoprevention because of the lack of an effective systemic treatment (13, 14). The incidence of lung cancer has slowly decreased among men in developed countries, whereas it has increased in women (10). However, lung cancer is the main cause of cancer death in both sexes, with an annual mortality rate of 91% (15).

Among the four main histological lung cancer types (small cell carcinoma, squamous cell carcinoma (SQC), adenocarcinoma, and large cell carcinoma), adenocarcinoma that is derived from small airway epithelium with feature of Clara cells accounts for about 35-40% of all lung cancer cases (16). Small cell carcinoma and squamous cell carcinoma are developing exclusively in smokers (3, 4) while PAC is additionally found in a significant numbers of non-smokers (3). Moreover, PAC frequently develops in patients under the age of 50 as opposed to other types of lung cancer that are typically diagnosed in patients of age 60 and older (18).

An alarming increase in teenage smoking will without doubt, raise the lung cancer epidemic even farther in the years to come (19). The development of effective treatments that prevent the progression of precancerous lesions or early stage lung cancers into overt cancers in smokers, who quit, are therefore urgently needed. Mouse models are widely used in preclinical studies to test the efficacy of novel chemopreventive agents. Studies in our laboratory have shown that the growth of human PAC cell lines of Clara cell lineage (PACC) in vitro and of PACC induced in hamsters by the nicotine-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are regulated by beta-adrenergic receptors through the activation of cAMP (20-23). These experiments also demonstrated that the in vitro and in vivo growth of PACC is promoted by the beta-adrenergic agonist epinephrine and by the cyclic nucleotide phosphodiesterase inhibitor theophylline (20-23). These findings suggest an important role of beta-adrenergic receptors and their downstream effector cyclic adenosine monophosphate (cAMP) in the growth regulation of PACC. By contrast, studies of PAC derived from alveolar type II cells in mice and in human squamous cell carcinoma (SQC) have identified signaling via the epidermal

growth factor receptor (EGFr) pathway and a PKC-mediated pathway as an important growth regulating events (24-30).

The major concern with respect to preclinical lung cancer prevention is the fact that, these pathways appear to be antagonistic at the level of cAMP/PKA. While agents that activate or increase intracellular cAMP may inhibit the EGF or PKC pathways via inhibiting Raf-1, these may promote β-adrenergic, cAMP- mediated growth regulating pathways. Accordingly, chemopreventive agents that interact with β-adrenergic/cAMP signaling pathway treatment may prevent the development of alveolar type II cell carcinoma or squamous cell carcinoma while promoting the development of PACC.

In the 1990s, a comprehensive international chemoprevention trial with β-carotene and retinoids was conducted in smokers and ex-smokers (carotene and retinoid efficacy trials, CARET). This trial was based on preclinical studies that had identified significant chemopreventive effects of β-carotene and retinoids on rodent tracheobronchial SQC (31). Five years into the trial, this study had to be discontinued due to a 46% increase in lung cancer mortality and a 26% increase in cardiovascular mortality in groups receiving beta-carotene and retinyl palmitate (14, 32-36).

Another popular "chemopreventive" agent, green tea, has shown mixed results in epidemiological studies with some reports demonstrating a reduction in lung cancer risk (37-42, 44), while others reported no effect or even promoting effects on lung cancer (40, 43, 45-49). Green tea contains significant amounts of theophylline which, due to its inhibiting effects on phosphodiesterase, increases intracellular cAMP (34). Because PACC and cardiovascular function are both under β-adrenergic control and both diseases were presented in the caret trial (20-23), the hypothesis of the current project is that β-

carotene and theophylline (which is contained in tea and asthma medications) promote the growth of PACC via their documented ability to increase intracellular cAMP (33, 34-36).

Using assays for the assessment of cAMP production, PKA activity, MAPK activation, CREB activation and cell proliferation. This project has characterized cAMP, PKA, CREB and ERK1/2 as components of a mitogenic signal transduction pathway in cell lines derived from human PACC and their normal cells of origin, small airway epithelial cells (SAEC). The selective nature of this growth-promoting pathway on PACC and SAECs was confirmed by studies in a cell line derived from human large airway epithelial cells (BEAS-2B). Contrary to PACC and SAECs, these cells responded with a growth inhibition to β-carotene.

Working hypothesis

The central hypothesis of this project is that the pro-vitamin A, β-carotene and a substance contained in green and black tea such as theophylline, promote the growth of the human PACC cell line NCI-H322 and their normal cells of origin, SAEC. Data generated by this project supported the hypothesis and showed that both of these "chemopreventive" agents stimulated cell grown in both cell systems, an effect mediated by activation of cAMP,PKA,CREB and ERK1/2.

Specific aims

1. To test the hypothesis that β-carotene stimulates the growth of pulmonary adenocarcinoma (PAC) cells and their normal cells of origin, small airway epithelial cells (SAEC), and to identify signal transduction components involved in this effect.

2. To test the hypothesis that theophylline stimulates the growth of pulmonary adenocarcinoma (PAC) cells and their normal cells of origin, small airway epithelial cells (SAEC), and to identify signal transduction components involved in this effect.

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PART II:

Literature Review

I. Lung cancer overview

At the beginning of the 20th century, lung cancer was considered a rare disease (1, 2, 27). However, since then the incidence of lung cancer has dramatically increased to become one of the most common malignancies and the leading cause of cancer death with a high mortality rate and five-year survival rates of less than 15% in developed countries (3, 4). New lung cancer cases diagnosed throughout the world in 2002 were estimated to be approximately 1.2 million and more than 90% of these cases are expected to die from this disease (5, 6).

Approximately 1,334,100 new cases of cancer were diagnosed and approximately 556,500 died from cancer in the United States in the year of 2003 (7). Lung cancer was the second leading type among new cancer cases (171,900; 13% of the total) and the first in cancer death (157,200; 28% of the total) in the United States (7). For the year 2004, 173,770 new cases of lung cancer and 160,440 deaths are expected in the United States (8, 9). Unfortunately, the National Cancer Institute's stated goal of a 50% reduction in overall cancer mortality by the year 2000 has not been met and the death rates from some of the common cancers continue to rise (10). In addition, the total annual direct and indirect costs of cancer care in the United States have been estimated at more than $96 billion (11). The death rate for lung cancer exceeded the combined total for breast, prostate, and colon cancer in developed countries (7, 12, 13). It is the most frequently diagnosed cancer type and the most common cause of cancer deaths in males and females in the world. This trend is also expected to continue for many years (14). The convergence of lung cancer death rates among men and women born after 1960s support

the idea that males and females may be equally susceptible to developing lung cancer from a given amount of cigarette smoking, years of smoking, earlier age of onset, degree of inhalation, tar and nicotine content, and use of unfiltered cigarettes. Lung cancer in women has increased by 600 % since 1950 and has reached epidemic levels (7, 4, 15), an effect likely associated with the simultaneous observed increase of women who smoke. Lung cancer has surpassed breast cancer as leading cause of cancer death in women since the late 1980s (13). The birth-cohort pattern of lung cancer mortality after 1950 appears to reflect the early impact of teenage cigarette smoking on lung cancer risk on people under the age of 45 years (16). Before that, lung cancer was a rare disease in individuals under 40 years of age. Lung cancer is most frequently diagnosed in patients 50-75 years of age, and the incidence is higher among patients in 65-75 years old than in 55-64 years old group (17). In addition to that, individuals who stop smoking after the age of 50 may retain a substantial risk for lung cancer life-long (18, 19).

Unfortunately, lung cancer is usually diagnosed after the development of metastasis and drug resistance (20). Despite improvement in the diagnosis and treatment, the mortality rate remains higher than 95% within one year of diagnosis (21, 22).

A large number of risk factors have been identified for the development of lung cancer, such as cigarette smoking, airflow obstruction, and exposure to asbestos, radon, arsenic, ionizing radiation, haloethers, polycyclic aromatic hydrocarbons, and nickel (23). Cigarette smoking is the single most extensively documented risk factor for lung cancer, accounting for ~ 90% of cases in men and 70-85% of cases in women (23-28, 20). Genetic risk factors contribute to an individual's susceptibility to lung cancer, which is illustrated by the fact that more than 16% of long-term smokers will develop lung cancer

(29). So far, a recent study reported a relationship between environmental tobacco smoke exposure and increased lung cancer risk among nonsmoking women with a common genetic deficiency in glutathione S-transferase M1 (GSTM1) enzymatic activity because of a genetic polymorphism in the GSTM1 gene (30). Life style factors such as diet are also thought to be important in the modulation of lung cancer risk (29).

The World Health Organization (WHO) distinguishes two major lung cancer families: non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). Among the family of NSCLC, adenocarcinoma (35-40%), squamous cell carcinoma (25-30%) and large cell carcinoma (10-15%) are recognized. Small cell lung cancer often responds to chemotherapy but then relapses while NSCLC is usually non-responsive (31).

Pulmonary adenocarcinoma tends to grow very fast (32). The tumor grows along the alveoli and the tumor cells are bigger than normal cells and tend to cluster together and exhibit epithelial cells (32). It has been suggested that pulmonary adenocarcinoma PAC be reclassified into five cells types: bronchial surface epithelial cell type, with little or no mucus, goblet cell type, Clara cell type, type II alveolar epithelial cell type, and bronchial gland cell type (33). On the other hand, it has been suggested to classify PAC into: hobnail cell type, columnar/cubical cell type, polygonal cell type, and goblet cell type (34). Also in 1999, the WHO classified PAC into five subtypes; acinar, papillary, bronchioalveolar carcinoma, solid adenocarcinoma with mucin, and adenocarcinoma of mixed cell type (35).

PAC has risen dramatically during the last three decades from a very rare type of lung cancer to be the leading type of lung cancer today (2, 28). PAC accounts for about 60% of all lung cancer cases (2, 28, 36, 38) and is particularly prominent in women and

African Americans (37). In the near future, this trend of PAC is expected to cover many geographical regions (1). It has been estimated that the mortality from adenocarcinoma will increase world-wide in contrast to squamous cell carcinoma and small-cell carcinoma for which a decrease is expected.

PAC is the only histological lung cancer type that develops in a significant number of non-smoking individuals implying, that additional factors other than smoking contribute to the continued rise of this cancer type. Studies by Park et al. 1995 (39) and Schuller et al. 1999 (40) showed that the growth of PAC of Clara cell linage in vitro and in an animal model is under beta-adrenergic control and that the tobacco-specific carcinogen NNK [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone] is the most potent stimulator of this proliferative pathway. In addition to that, the chronic treatment of existing respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) by β-adrenergic stimulators might play a significant role in the PAC epidemic. For instance, COPD is one of the most common respiratory conditions of adults in the developed world, which encompasses both chronic bronchitis and emphysema (41). In fact, patients with COPD should be monitored carefully for lung cancer (42).

An alarming increase among teenage smoking will, without doubt, increase the lung epidemic even farther in the years to come (16). The developments of effective treatments that prevent progression of precancerous lesions, or early stage lung cancers, into overt cancer in smokers, who quit, are therefore urgently needed. Mouse models are widely used in preclinical studies to test the efficacy of novel chemopreventive agents. On the other hand, studies of Clara cell-derived PAC induced by NNK in hamsters have shown that these lung tumors express β-1 and β-2 adrenergic receptors and that their

growth is promoted by the β-adrenergic agonist epinephrine and by the cyclic nucleotide phosphodiesterace inhibitor theophylline (43, 44, 45). Those findings suggest an important role of β-adrenergic receptors and their downstream effector cyclic adenosine monophosphate (cAMP) in the growth regulation of lung tumors of Clara cell lineage. In contrast to the hamster, NNK-induced PAC in mice is of alveolar type II cell lineage and their growth is inhibited by agents that stimulate cAMP (46-51, 20). Chemopreventive agents that stimulate β-adrenergic/cAMP signaling may therefore prevent the development of lung cancer in mice while promoting the development of PACC in hamster and man. In support of this hypothesis we have recently shown that green tea which contains the phosphodiesterase inhibitor theophylline as well as injections with theophylline have strong growth promoting effects on experimentally induced PACC in hamsters (45).

II. Chemoprevention (CP)

Currently, in the United States and Canada alone, there are approximately 50 million former smokers and 50 million current smokers (52). As a result of this large population of people at risk, an alternative cancer control method such as chemoprevention needs to be developed to reduce lung cancer mortality, especially for smokers who have followed medical advice to give up smoking. Michael Sporn was the first researcher who described chemoprevention (53) in 1976 and defined it as "the use of

specific natural or synthetic chemical agents to reverse, suppress or prevent carcinogenic progression to invasive cancer." Proof of principle studies by Ki Hong and co-workers (54-56) showed that chemoprevention could prevent cancer in the upper aerodigestive tract. Around 2000 natural and synthetic agents have been shown in experimental systems to have chemopreventive activity. Vitamin A was first noted to be an essential nutrient in 1913, while in 1925 vitamin A deficiency was reported to be associated with changes in epithelial histology. Since that time, vitamin A deficiency has been associated with bronchial metaplasia and an increased incidence of cancer (57-61). The pro-vitamin A, β-carotene, is also one of the most widely studied chemopreventive agents that has been tested in clinical trials (65). Chemoprevention trials for lung cancer have been carried out in phase III clinical trials for over a decade with mostly negative results (62-66). Although, a combination of beta-carotene and vitamin A supplement for an average of four years had no benefit and may have had an adverse effect on the incidence of lung cancer and on the risk of death from lung cancer, cardiovascular disease, and any cause in smokers and workers exposed to asbestos (62).

Chemopreventive agents (CPA) may affect cellular proliferation, differentiation, apoptosis and tumor angiogenesis (67). In primary prevention trials, three large studies demonstrated that neither α-tocopherol nor beta-carotene had preventive effects on lung cancer (68, 62, 63). In the 1990s, a comprehensive international chemoprevention trial was conducted in which smokers and ex-smokers were treated with β-carotene or retinoid. However, the β-Carotene and Retinol Efficacy Trial (CARET) and Alpha-Tocopherol, β-Carotene (ATBC), showed that agents which effectively inhibit lung cancer growth in a preclinical model of hamster tracheo-bronchial organ cultures had the

opposite effect on human lung cancer. Five years into the trials, this study had to be discontinued due to a 46% increase in lung cancer mortality and a 26% increase in cardiovascular mortality in the groups receiving β-carotene and retinyl palmitate (62, 66). As cardiovascular function as well as the growth regulation of PAC are under beta-adrenergic control, we hypothesized that β-carotene caused the dramatic increase of both diseases in this trial because it increases intracellular cAMP by inhibition of the enzyme phosphodiesterase (69). We further hypothesized that beta-carotene would have selective strong promoting effects on the growth of human pulmonary adenocarcinoma cells of Clara cell phenotype (NCI-H322) and their normal cell of origin (SAEC) via its stimulatory effects on cAMP. We have also included theophylline in our experiments because it has the potential to increase intracellular cAMP.

Beta-carotene is a member of the carotenoid family that consists of over 600 compounds found predominantly in fruits and vegetables and is converted by the mammalian organism to vitamin A. This molecule has been reported to have a number of actions, including important antioxidant activity due to its inhibition of radical initiated peroxidation in vitro (70-73). Original epidemiologic data showed a positive association between β-carotene deficiency in the diet and increased risk of lung cancer (58, 59, 60, 72 -75). On the other hand, clinical trials involving β-carotene supplementation revealed unfavorable effects (62-65, 56, 68, 70). Among the possible explanations for this effect inhibition of absorption of other nutrients by large doses of β-carotene and the autocatalytic pro-oxidant activity of β-carotene under high oxygen tension such as that occurring in the lungs of smokers were discussed (74-76). The ability of beta-carotene to increase intracellular accumulation of cAMP by inhibiting phosphodiesterase (69) has not

been considered to date as a cause for the observed lung cancer promoting effects. Also, it has been suggested that β-carotene increases mortality of lung cancer and cardiovascular diseases (73). However, chemoprevention of lung cancer is proving difficult and frustrating (77). In general, lung cancer chemoprevention trials have been disappointing. Several large-scale chemoprevention trials have been performed, including the European Organization for Research and Treatment of Cancer Head and Neck and Lung Cancer Cooperation Groups (EUROSCAN) Trials, the Physician Health Study, the Alpha-Tocopherol and Beta-Carotene trial (ATBC, CARET), which involved thousands of active smokers followed up for over 10 years. Unfortunately, none of those studies have yielded favorable results.

Green Tea (GT) was discovered in the year of 2737 BC by the Chinese emperor Sheng Nung. The traditional use of tea began in China about 4700 years ago and it was often used as medicine. In the 8th century, tea was introduced in Japan and in Europe, and then very rapidly became an important commercial product throughout the world. (78).

Tea is the most ancient and widely consumed beverage in the world next to water, made from leaves of the Camellia sinensis species of the theaceae family (79). Fresh tea leaves are rich in flavanol monomers of the polyphenol family known as catechins. The predominant catechins found in tea are epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). EGCG is the most potent and widely used catechin in tea (80). While some sources additionally contain gallo catechin (GC) all of the catechins are contained in green tea extract GTE that is widely used as a dietary supplement. Green tea constituents have been characterized as antioxidants that scavenge free radicals to protect normal cells. The antioxidant effect is

directly related to the combination of aromatic rings and hydroxyl groups that make up the structure and also a result of binding and neutralizing of free radicals by the hydroxyl groups (81). However, results of epidemiological studies on tea and cancer have been inconsistent; some show consumption of tea is protective, whereas others show it either has no relationship with cancer or even increases the risk of certain cancers (82).

Lung cancer studies have shown an inverse effect with Okinawan tea, yet a tentatively increased risk was shown in another study (83, 84). Although human studies have their limitations, the research has warranted a further look into the effects of green tea and cancer (83). However, early studies focused on the effects of polyphenols and catechins in tea on cancer development, while recent studies have identified anti-mutagenic, anti-proliferative, and anti-neoplastic effects of all tea fractions (85), suggesting that non-catechin components of tea contribute to the cancer modulating effects of tea. Theophylline which is also contained in tea has been given little attention by cancer researchers. This agent is a widely documented inhibitor of phosphodiesterase (85-88, 89) and increases intracellular cAMP via this mechanism. In the current projects experiments on NCI-H322 and SAEC have been performed to test the hypothesis that theophylline which is the main component of green tea, promotes growth-regulating signal transduction.

The principal current use of theophylline began in the early 1970s with reports demonstrating its efficacy as preventive medicine for chronic asthma (90) and by 1980, theopyhlline had become a leading medication for asthma because of its bronchodilating effects (91). Theophylline in fact, has immunomodulatory (92), anti-inflammatory (93,

94, 42), and bronchoprotective effects that potentially contribute to its efficacy as a prophylactic antiasthma drug (95-97).

III. Growth-Regulating Signaling Pathways Expressed in Lung Epithelia and Lung Cancer

The epidermal growth factor receptor (EGFR)

Generally, a signal is transmitted from the cell membrane to the nucleus through interaction of proteins, and conversion of those proteins from inactive to active status. This begins with the interaction of signals with the membrane receptors. This extracellular signal may include growth factors hormones, neurotransmitters, extracellular matrix, and stress signals. This interaction activates the receptor, which then catalyzes a series of reactions beginning with the activation of a secondary protein, often through cofactors such as ATP, GTP, cAMP, or cations. This protein often catalyzes the activation of the third protein, and this pattern continues in a cascade of reaction, usually involving cytoskeleton proteins like beta-catenin and APC, adapter proteins, kinases and phosphatases. The kinases can be either receptor, like the EGF receptor, or non receptor, like Src and Jak. This cascade culminates in the activation or deactivation of transcription factors, like c-myc, or other factors, and translocalization of these factors into the nucleus (89, 101, 103-106).

Cell growth is regulated by several growth factors by activating intracellular signaling pathways after binding to high affinity tyrosine kinase receptors on the cell

surface. Among these, the mitogenic activated protein kinase (MAPK) pathway plays an important role in the transduction of mitogenic signals initiated by growth factors acting on a variety of cell surface receptors. MAP kinases are regulated through many protein phosphorylation cascades (89, 101, 103-106).

Several stimulatory growth factors are found in lung cancers including, epidermal growth factor (EGFR), transforming growth factor alpha (TGFα), insulin growth factor-1/2 (IGF-1/2), platelet derived growth factor-A/B (PDGF-A/B), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor, (HGF), and mammary derived growth factor-1 (MDGF-1) (98, 99). The epidermal growth factor receptor (EGFR) is a 170 KDa transmembrane receptor with tyrosine kinase activity, which has several ligands including EGF, amphiregulin (AR) and TGFα (98). EGF and EGFR are frequently over-expressed in lung cancers (117, 118).

The EGFR signaling pathway demonstrates remarkable flexibility in mediating a host of signaling pathways that regulate various physiological and pathological processes. The EGFR belongs to the receptor tyrosine kinases (RTKs) family. RTKs have an intrinsic enzymatic activity in the cytoplasmic region that is directed against the receptor and downstream signaling molecules (100). The RTKs are involved in a range of cellular physiology including fertilization, proliferation, cell migration and apoptosis. Furthermore, they play an important role in cancer development (101).

The EGFR is expressed in many cell types as well as regulates many cell functions (102, 103), and it has four closely related receptors called the EGFR family (erbB1), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4).

The Ras-mediated mitogen activated protein kinase (MAPK) cascade is one of the best characterized signaling pathways initiated by EGFR activation. The Ras- mediated EGFR-MAPK cascade is initiated when the growth factor Grb- 2 associated SOS (Ras guanine nucleotide exchange factor) is recruited to phosphotyrosine residues of activated EGFR. This association allows the exchange of GDP (inactive form of G-protein) to GTP, which is the active form of G-protein. This is followed by membrane recruitment and activation of the serine/threonine kinase Raf (MAPK kinase kinase), which in turn phosphorylates and activates MEK (MAPK kinase). The dual-specific MEK phosphorylates and activates the serin/ threonine mitogen activated protein kinases (MAPKs) (104). There is increasing evidence that MAP kinase can be activated by a variety of signals, including growth factors (105), phorbol esters (106) and calcium ion (Ca^{2+}) (107).

In summary, the epidermal growth factor (EGF) pathway is found in many cancer cells. In this cascade, EGF binds to its receptor (EGFR) leading to the dimerization of the receptor. This leads to autophosphorylation of the receptor tyrosines, and recruitment of proteins to the receptors, which bind to the receptor via their SH2 domains. One such protein is Grb2-SOS. In turn, this protein activates Ras through conversion of GDP to GTP. Ras then activates Raf. Raf activates MEK which activates MAPK (ERK). MAPK activates factors such as SRF, ATF, c-fos, c-Jun, c-myc leading to gene expression and cell proliferation (104, 108, 109).

Besides the classical Ras-mediated kinase cascade, various signaling molecules could also undergo phosphorylation and activation upon binding to the phosphotyrosine residues on activated EGFR. Activated Phospholipase C (PLC) hydrolyses membrane

bound phosphatidylinositol (PI) yielding diacylglycerol (DAG) and inositol phosphates (110). Inositol phosphate regulates intracellular calcium, which together with DAG, activates protein kinase C (PKC). PKC causes potent activation of ERK through the direct phosphorylation and activation of Raf-1 (111, 112).

Phosphatidylinositol 3-kinase (P13K) binds to phosphorylated tyrosine residues of receptor tyrosine kinases (RTKs) (113). Phosphorylated inositol phospholipids (Phosphatidylinositols) are required for the membrane localization of phosphoinositide-dependent kinase (PDK). PDK phosphorylates and activates protein kinase B (Akt), which is involved in cell proliferation and inhibition of apoptosis (114). Recently Akt/PKB was identified as a promoter of cancer cell invasion through increased motility and metalloproteinase production (114). The requirement of P13K for the induction of DNA synthesis in cells was demonstrated by Roche et al. 1994 (115). Among the proteins that become phosphorylated and activated by EGFR is the Janus kinase 1 (JAK 1) and STAT to the nucleus for gene activation (116, 117).

The epidermal growth factor receptor and lung cancer

An autocrine growth loop is present when both receptor and its ligand are co-expressed in a cell with self-regulated proliferation (118). EGFR has been implicated as a promoter of proliferation and progression of malignant cells via various autocrine growth pathways that have been associated with the pathogenesis of many cancers (119).

Several studies have shown that EGFR plays a major role in the autocrine growth of human non-small cell lung cancer (NSCLC) (120). The family of NSCLC consists of adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and carcinoid (31).

Expression and overexpression of EGFR was found in a greater proportion of squamous and adenocarcinomas than in large cell or small cell carcinoma (120, 121). The co-expression of transforming growth factor-α (TGF-α) and the EGFR exists in many lung cancer cell lines particularly in NSCLC (120, 50). TGF-α is the main and most important autocrine ligand in NSCLC tumor formation. Moreover, activation of point mutations of the Ras gene resulting in constitutively active mitogenic signals were observed in about 30% of NSCLCs (122, 123, 124).

Over-expression of EGFR is associated with reduced survival (121, 125, 126), metastasis (127), and resistance to chemotherapy (126-128). In lung cancer, many chemotherapeutic approaches have targeted the EGFR signaling pathway with encouraging preclinical results (131, 132).

Signaling through G-Protein receptors

G protein-coupled receptors (GPCRs) are among the largest family of cell surface receptors. They mediate signaling from a number of stimuli and ligands including peptide and glycoprotein hormones, neurotransmitters, phospholipids and photons. These receptors consist of an extracellular ligand binding domain, a seven transmembrane domain, and heterotrimeric G-proteins (133, 134). The heterotrimeric G-proteins consist of α, β, γ subunits, which dissociate into active G_α-GTP and $G_{\beta\gamma}$-subunits after interaction with ligand-bound receptor. However, there are numerous G_α-proteins, the main classes of which are $G_{\alpha s}$, which activate adenylyl cyclase; the $G_{\alpha i}$, which inhibit adenylyl cyclase; and $G_{\alpha q}$ which activate phospholipase C. Beside the G_α subunits, the $G_{\beta\gamma}$-subunit is also involved in regulating various effectors, including adenylyl cyclase, phospholipase

C and A_2 isoforms, serine threonine kinases, and protein kinase C (134, 135). When an agonist binds to GPCRs, the bound GDP exchanges to GTP on the G-proteins leading to the dissociation of G-protein into active G_{α}-GTP and $G_{\beta\gamma}$ subunits. Once the agonist leaves the receptor, the subunits associate to each other again to form inactive form of G-protein (GDP) bound state (136-138).

From events occurring at the cell membrane to make changes in gene transcription, the cyclic AMP (cAMP)-dependent signaling pathway is a classic signaling pathway that has been well characterized. Once ligand (hormones, neurotransmitters, growth factors) bind to the specific receptors located in the plasma membranes, an allosteric change occurs, allowing it to bind to the G-protein. The G-protein becomes active and GTP binds to G-protein and GDP (inactive form of G-protein) will be released. Now the G-protein can move and transfer a message from the receptor to the catalytic subunit. The G_s protein then stimulates the activation of the enzyme adenylyl cyclase, which converts ATP via hydrolyzation to cAMP. cAMP acts as a second messenger to activate protein kinases which will phosphorylate other proteins such as protein kinase A(PKA). Activation of PKA results in the downstream activation of either CREB or Erk1/2 and finally causes cellular response such as proliferation (139, 89).

***β* -adrenergic signaling and cancer**

Beta-adrenergic receptors are members of the super family of seven-transmembrane G-protein coupled receptors encoded by a gene on chromosome 5 (140). Beta-adrenergic receptors are classified into β_1-, β_2-, and β_3-receptors (140, 141). In the

lungs, β_1- and β_2 receptors are widely distributed in epithelia, smooth muscle cells, and endothelial cells (142, 143). β_3-receptors have not been identified in lung tissues but are abundant in adipose tissues. The functions of β-adrenergic receptors in endothelia, bronchial smooth muscle cells, vascular smooth muscle cells, cardiac muscle, and pulmonary macrophages has been well characterized. It is well established that beta-adrenergic signal transduction pathway plays a key role in cardiovascular disease and asthma (144). It has also been shown that beta-adrenergic receptors regulate secretion of alveolar type II cells, bronchiolar Clara cells and Mucociliary clearance (142, 143).

Elevation of cAMP influences cell physiology, including proliferation, survival, and differentiation, either stimulating or inhibiting the response depending on cell type and context. Conflicting effects reported for cAMP might result from differences in cell types, concentration, and localization of the cAMP dependent protein kinase (145). There are 9 isoforms of adenylyl cyclase enzyme and the tissue specific expression of the specific isoforms determines the relative amount of cAMP in response to stimuli (89). Moreover, the large number of possible combination of different G-protein subunits and effector molecules allows cells to respond in different ways (146). Phosphodiestrase degrades cAMP to 5'-adenosine monophosphate (5'-AMP) terminating the cAMP mediated effect of hormone stimulation.

Cyclic AMP activates protein kinase A (PKA) which phosphorylates numerous proteins, with subsequent modulation of different cellular functions, including gene transcription. Gene transcription is mediated through the PKA-mediated activation of cAMP-responsive transcription factors including cAMP response element binding protein

(CREB), cAMP response element modulator (CREM), and activating transcription factor-1 (ATF-1) (89).

The growth of some types of cancer is stimulated by β-adrenergic signaling. This signaling pathway enhances the growth of human lung adenocarcinoma cell lines (39, 43), pancreas (147) or breast (148). Park et al, 1995 (39) initially demonstrated the mitogenic role of β-adrenergic signal transduction in human PAC of the Clara cell phenotype. This work showed the importance of β-adrenergic stimulators/cAMP in the proliferation of human cancer cell lines derived from peripheral pulmonary adenocarcinoma with features of Clara cells (NCI-H322) and their normal cell of origin immortalized small airway epithelium cells.

Finally, current data imply that PAC of Clara cell phenotype and small airway epithelia cells with characteristics of Clara cells respond to elevated cAMP levels in the same manner. This is likely to have important clinical and epidemiological implications since beta-adrenergic stimulators are active ingredients of drug formulations that are widely used in the management of chronic respiratory diseases and dietary supplements for weight control.

Figure1[1] shows that beta-adrenergic receptors on pulmonary epithelial cells activates GTP-binding proteins (G-proteins) coupled to receptor signaling, resulting in the activation of adenylyl cyclase and cyclic AMP (cAMP), followed by activation of protein kinase A (PKA) depending on the cell type, PKA can activate the transcription factors cAMP response element binding protein (CREB), cAMP response element modulator (CREM), activating transcription factor-1 (ATF-1), activator protein 1 (AP1)

[1] See all figures and figure legends in Appendix.

or nuclear factor κB (NF- κB) which all regulate cell proliferation. Alternatively, PKA can activate the small G-protein RAP-1, leading to the activation of serine/threonine kinase B-RAF, followed by activation of the mitogen-activated protein kinase (MAPK) cascade and transcriptional activation of proliferation. PKA can also activate phospholipase-A 2 (PLA2) to release arachidonic acid (AA) from cell membrane phospholipids. AA itself, as well as its metabolites such as prostaglandins and leukotrienes, can also activate.

In Figure 2 the flow-chart represents a simplified version of beta-adrenergic signaling in NCI-H322 and SAEC cells that has been focus of this project. GTP-binding proteins (G-proteins) coupled to receptor signaling, results in the activation of adenylyl cyclase and cyclic AMP (cAMP), followed by activation of protein kinase A (PKA). In our project, we hypothesized that PKA can activate the transcription factor cAMP response element binding protein (CREB). Alternatively, PKA can activate the small G-protein RAP-1, leading to the activation of serine/threonine kinase B-RAF, followed by activation of the mitogen-activated protein kinase (MAPK) cascade, all of which may lead to the activation of human cancer cell lines derived from peripheral adenocarcinoma with features of Clara cells (NCI-H322) and their normal cells of origin (SAEC) and transcriptional activation of proliferation. Also, depending on cell type, ERK1/2 may activate CREB or CREB may activate ERK1/2.

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APPENDIX

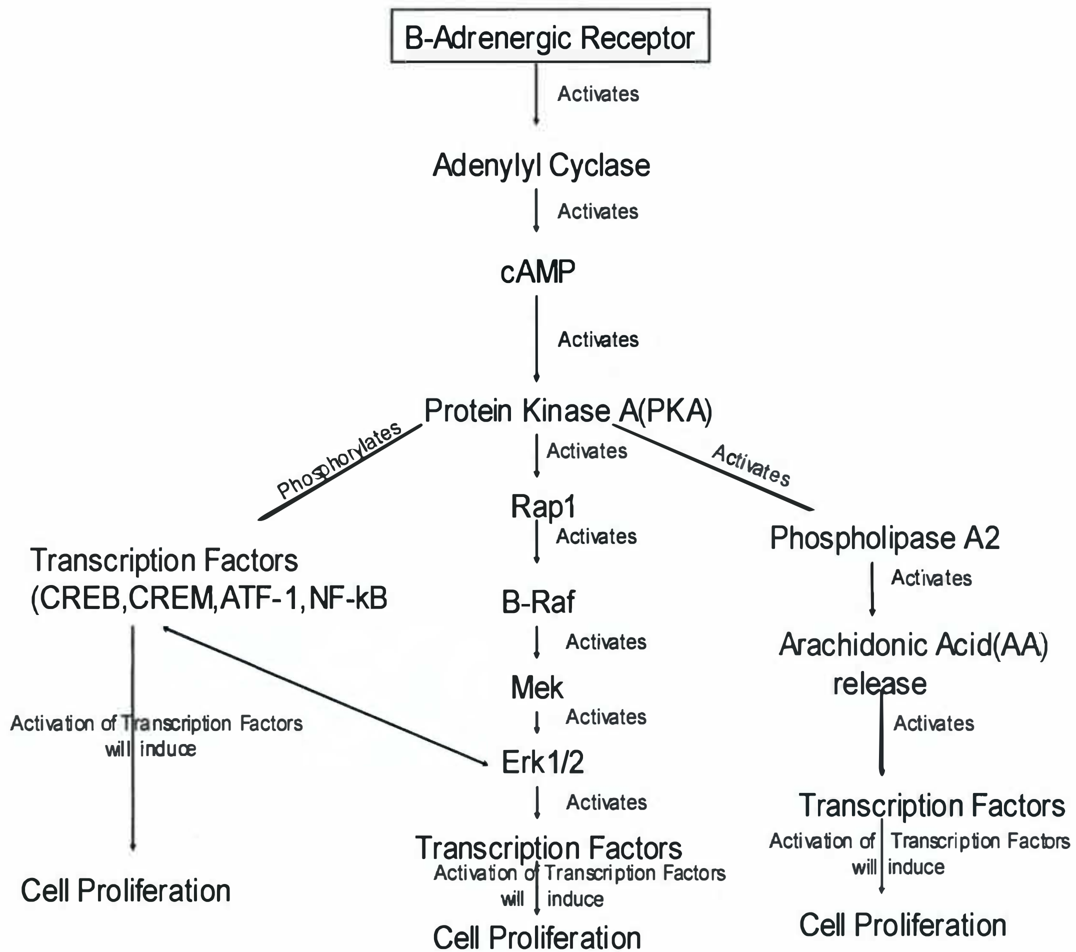

Figure 1. cAMP-dependent signal transduction in response to β-adrenergic receptor stimulation. Binding of agonists to the receptor will activate the enzyme adenylyl cyclase, leading to the activation of the second messenger cAMP which in turn phosphorylates other downstream proteins such as its associated protein kinase A (PKA). In turn, PKA may phosphorylate the transcription factors CREB, CREM, ATF-1, or NF-kB, or activation of PKA may phosphorylate the MAPK cascade through the activation of Rap-1 and b-Raf or it may activate Phospholipase A2 which then leads to release of AA. However, the activation of the transcription factors in all three different ways will cause cellular response such as cell proliferation.

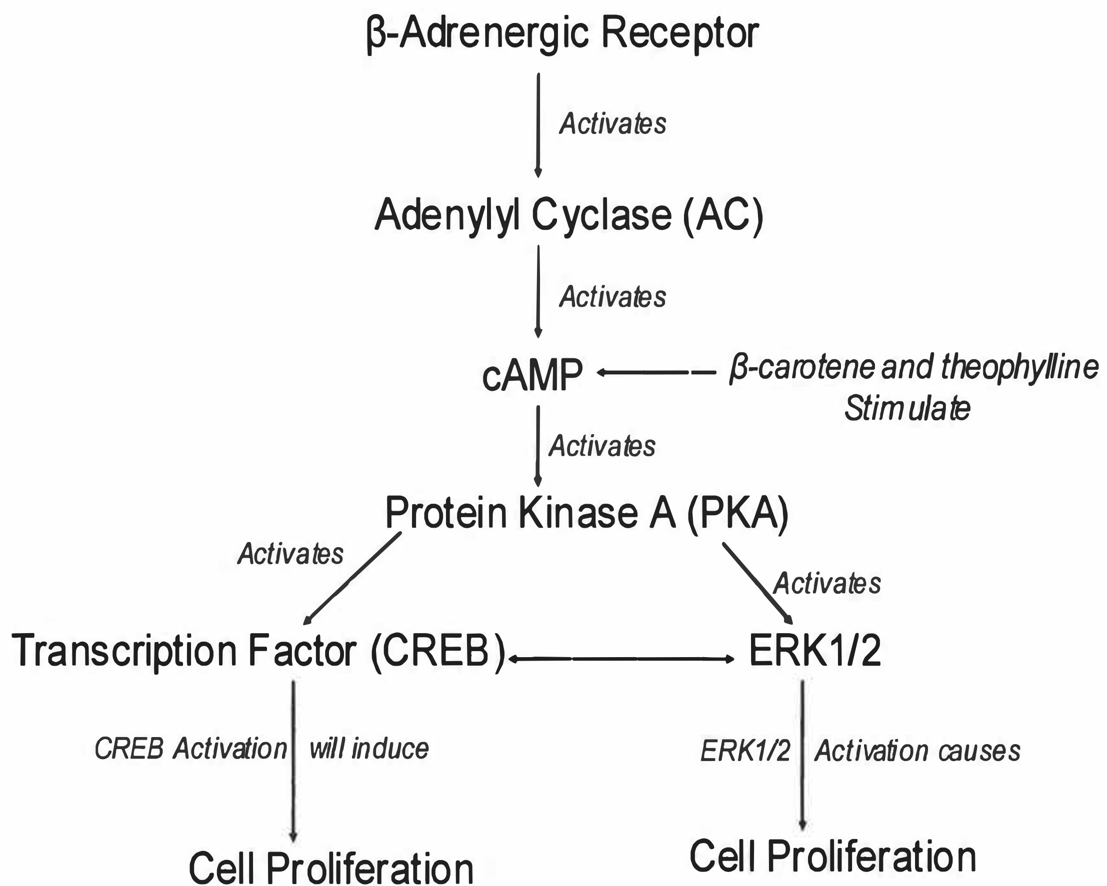

Figure 2. Flow-chart represents a simplified version of β-adrenergic signaling in NCI-H322 and SAEC cells that has been focus of this project. cAMP-mediated signal transduction pathway in response to β-adrenergic receptor stimulation. Once stimuli bind to the receptor in the cell membrane, the enzyme adenyly cyclase will be activated via ATP hydrolyze, which then will activate the second messenger cAMP. cAMP will activate protein kinase A (PKA), which in turn will activate either CREB or Erk1/2 which either one will finally causes cell proliferation.

PART III:

Growth stimulation of human pulmonary adenocarcinoma cells and small airway epithelial cells by β-carotene via activation of cAMP, PKA, CREB and ERK1/2

Part III

Brief explanatory statement

This chapter is a lightly revised version of a manuscript by the same name that has recently been submitted to the journal "Cancer Research". November 4, 2004.

Hussein A. Al-Wadei[1], Takashi Takahashi and Hildegard M. Schuller[1,3]. [1]Experimental Oncology Laboratory, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA and [2]Aichi Cancer Center Research Institute, Nagoya University, Nagoya, Japan.

[3]Author for correspondence and reprint requests:
Dr. H. M. Schuller
Department of Pathobiology
College of Veterinary Medicine
University of Tennessee
2407 River Drive
Knoxville, TN 37996

Phone: 865-974-8217
Fax: 865-974-5616
e-mail: hmsch@utk.edu

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic, (2) development of assay conditions suitable to test the hypothesis that β-carotene stimulates the growth of pulmonary adenocarcinoma cells and their normal cells of origin, (3) conduction of cAMP assays, (4) conduction of PKA activation assays, (5) conduction of Western blots, (6) conduction of MTT assays, (7) analysis and interpretation of all data, (8) statistical analysis of all data, (9) photographic and graphic documentation of results, (10) conduction of comprehensive literature reviews, (11) writing of the manuscript (with some editorial assistance by Dr. Schuller).

Introduction

Lung cancer is the leading cause of cancer deaths in industrialized countries (1-3). Among the four major types of lung cancer recognized by the WHO classification (adenocarcinoma, small cell carcinoma, squamous cell carcinoma, large cell carcinoma) pulmonary adenocarcinoma (PAC) predominates today, accounting for about 60% of all lung cancer cases (2, 4, 5). Smoking is a well documented risk factor for all types of lung cancer (6, 7) while a high fat diet constitutes an additional risk factor for PAC (8-10). Human PAC may be derived from bronchiolar Clara cells or from alveolar type II cells. 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 has been reported to account for about 50% of PAC cases (11). By contrast, electron microscopic investigations have identified 90% of PAC as being derived from Clara cells (12). Recent reports have shown that exposure to cigarette smoke or 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 (13). 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 derive from PAC of Clara cell lineage are regulated by β-adrenergic receptors and that the tobacco-specific carcinogenic nitrosamine 4-(methylnitrosamino)-(3-pyridyl)-1 butanone (NNK) acts as an agonist for this receptor family (14). NNK as well as a stimulator of the classic downstream effector of β-adrenergic receptors, cAMP, significantly stimulated DNA synthesis of these cells in vitro (14, 15). In support of these data, studies in a hamster model of NNK-induced Clara cell-derived PAC have shown that β-adrenergic agonists (16) and the phospodiesterase inhibitor theophylline (17) , which causes intracellular accumulation of cAMP, each significantly promoted the development of this cancer type .

A multinational chemoprevention trial with β-carotene and retinoids (β-carotene and retinoid efficacy "CARET" trial) was conducted in the 1990s in populations at risk for the development of lung cancer because of previous or current exposure to smoking or asbestos (18, 19). This trial was based on preclinical studies that had shown in vivo and in vitro inhibition of chemically induced carcinogenesis in the upper airways of hamsters by the pro-vitamin β-carotene or by synthetic vitamin A analogues of the retinoid family (20-24). In addition, a strong promotion of diethylnitrosamine-induced tracheobronchial carcinoma by vitamin A-deficiency was reported in hamsters (25). The trial had to be discontinued after 5 years due to a 28% increase in lung cancer incidence and mortality (46%) and a 26% increase in cardiovascular mortality (19). Conclusive explanations for this disconcerting result have not been provided to date.

It is well established that cardiovascular function is under β-adrenergic control and β-blockers are widely used for the clinical management of hypertension and heart disease (26). In light of our recent in vitro and in vivo findings on the β-adrenergic regulation of PAC, we therefore hypothesized that the chemopreventive agents used in the CARET trial may have promoted the development of PAC via stimulation of a signaling component of its β-adrenergic regulatory pathway. An extensive literature search revealed that β-carotene has been shown to increase intracellular cAMP in neuroblastoma cells (27). Our current experiments have therefore focused on the effects of β-carotene on cAMP and its associated downstream pathways as potential mediators of a stimulating effect on the proliferation of a human Clara cell-derived PAC cell line and its putative cell of origin (immortalized human small airway epithelial cells). Our data provide strong evidence in support of the hypothesis that β-carotene stimulates the growth of these cells via an increase in intracellular cAMP, activation of protein kinase A, activation of the cAMP response element (CREB) and activation of the extracellular signal-regulated kinases (ERK1/2).

Materials and Methods

Cell lines and tissue culture

The human PAC cell line with characteristics of Clara cells, NCI-H322, was purchased from the Center for Applied Microbiology and Research (ECACC, Salisbury, Wiltshire, UK). NCI-H322 cells were maintained in RPMI-1640 medium containing 10mM HEPES; 1mM sodium pyruvate; 2mM L-glutamine; 4500 mg glucose/L; and 1500 mg sodium bicarbonate/L), supplemented with 10% fetal bovine serum (FBS) but without supplement of antibiotics. The Simian virus 40 (SV40)-immortalized human peripheral airway cell line HPL1D (28), referred to in this publication as small airway epithelial cells SAEC, was provided to us by Dr. Takashi Takahashi (Aichi Cancer Center Research Institute, Nagoya University, Chikusa-ku, Nagoya 464, Japan). These cells were maintained in F-12 (HAM) medium buffered with 15mM HEPES (pH 7.3; Gibco Invitrogen-Life Technologies, Carlsbad, CA) and supplemented with 5µg/ml bovine insulin, 5µg/ml human transferrin, 10^{-7} M hydrocortisone, 2 x10^{-10} M triiodo-thyronine (Cambrex Bio Science Walkersville, Inc, Walkersville, MD) and 1% fetal calf serum (FCS; ATCC, Manassas, VA). No antibiotic was added. The SV40/adinovirus/12 hybrid virus immortalized human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained in bronchial epithelial cell basal medium (BEBM) supplemented with 2ml BPE, 0.5ml insulin, bovine (5mg/ml), 0.5ml HC, 0.5 ml retinoic acid, 0.5ml transferrin, 0.5ml T3, 0.5ml epinephrine, 0.5ml HEGF (Cambrex Bioscience Walkersville, MD) and without supplement of antibiotics.

cAMP Immunoassay

The level of cAMP in cell lysates was determined with a direct cyclic AMP Correlate- EIA ™ kit (Assay Design, Ins., Ann Arbor, MI), according to the instructions by the manufacturer. Briefly, in this competitive binding assay the cyclic AMP present in a sample competes with a fixed amount of alkaline phosphatase-labeled cyclic AMP for sites on a rabbit polyclonal antibody to cyclic AMP. During the incubation, the polyclonal antibody is bound to a goat anti-rabbit antibody coated on the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution, p-nitrophenyl phosphate, is added to the wells to determine the bound enzyme activity. The color development is stopped with a solution of trisodium phosphate after an hour of incubation and the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of cyclic AMP in the sample.

NCI H322 cells or SAECs were seeded into 6-well plates (Falcon, Franklin Lakes, NJ, USA) at 4×10^5 cells/ well in their individual media as specified above and maintained in an environment of 37°C, 5% CO_2 until they had reached 65-70 confluence. The media were then removed and replaced by low serum medium (0.1% FEBS) for NCI-H322 cells or basal F-12 medium (0.05% FBS) without additives for SAECs to starve the cells for 24 hours. The cells were then washed twice with 1X PBS and preincubated with fresh RPMI-1640 medium (0.1% FBS) for NCI-H322 cells or F-12 medium (0.05% FBS) for SAECs containing 3-isobutyl-1-methylxanthine (1mM) for 30 minutes. Following removal of this preincubation mix, the cells were incubated for 10 or 30 minutes with β-carotene type 1 (20 nM, Sigma, Louis, MO, USA) in fresh media

containing 1mM IBMX in an atmosphere of 37°C , 5% CO_2. Following 3 washes with sterile distilled water, the cells were then incubated with 600 μl of 0.1M HCL for 20-30 minutes and scraped off the well surfaces with a plastic scraper (Fisher Scientific, GA, USA). The cell lysates were collected into 1.5ml Eppendorf tubes and stored in ice. Following sonication of the cell lysates at 40 rpm for 10 seconds (Fisher Sonic Dismembrator. Model 300), the samples were immediately analyzed using the standard non-acetylated version of the cAMP enzyme immunoassay kit, according to the manufacturer's instructions. Each experiment was repeated three times. Data are expressed as mean values and standard errors of three independent experiments, each conducted with triplicate samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. Following statistical analysis, data were normalized with controls set as 100% for documentation as column graphs.

PKA activation assay

Following incubation of cells with β-carotene (20 nM) for 5 minutes to 2 hours as specified in the figure legends, PKA activity was assayed in cell lysates using a Pep Tag assay for non-radioactive detection of activated PKA (Promega Corperation, Madison, WI, USA), following the instruction of the manufacturer. Tubes containing the catalytic subunit of PKA (provided with the kit) served as positive controls.This assay utilizes fluorescent substrate for PKA that changes the peptide's net charge upon

phosphorylation of PKA, thus allowing the phopsorylated peptide to migrate to the positive electrode (+), while the non-phosphorylated peptide migrates to the negative electrode (-). All samples were kept on ice. At time zero, the samples were removed from the ice and incubated in a water bath at 30°C for 1 minute. A 1-10 µl aliquot from each sample was added to the reaction cocktail (5µl PEP Tag PKA reaction 5X buffer, 5 µl Pep Tag A1 peptide, 5 µl PKA activator 5X solution, 1 µl peptide protection solution) and incubated for 30 minutes at room temperature. The reaction was stopped by placing all samples into a boiling water bath or at 95°C in a heating block for 10 minutes. All samples were stored at –20°C in the dark until loading into 0.8% agarose gels (GIBCOBRL, Grand Island, N.Y, USA; 100 V for 15-18 minutes) in the presence of 50mM Tris-HCL (pH 8) (Fisher Biotech, Fair Lawn, New Jersey), using a Gel XL Plus Electrophoresis System (Labnet International, Inc., Woodbridge, NJ), following the addition of 1 µl of 80% glycerol per sample. An electronic transilluminator camera (Ultra Lum, Inc., Paramaount, CA) was used to view and capture the images for densitometric analysis (Scion software for image quantitation, NIH). Each experiment was conducted independently under identical conditions three times with similar results. Data are expressed as mean values and standard errors of three independent experiments, each conducted with triplicate samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. Following statistical analysis, data were normalized with controls set as 100% for documentation as column graphs.

Western blotting of proteins and phosporylated proteins

To assess the effects of β-carotene 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 hours. Following removal of the media and replacement with fresh low-serum media, β-carotene (20 nM) was added to the culture vessels and cells were incubated from 5 minutes to 2 hours as detailed in the figure legends. Cells exposed to the β-carotene vehicle (dimethylsulfoxide) served as controls. The cultured cells then were washed once with cold PBS, and were mixed with an appropriate volume of cell lysis buffer containing 20mM Tris-base, 200mM NaCL, 1M Sodium Floride, 0.5M EDTA, 100mM Na_3 VO_4, 100mM PMSF, 1μl/ml of pepstatin A, 1μl/ml of leupeptin, 1μl of aprotinin (protease inhibitor, Sigma-Aldrich, St. Louis, MO) and 0.25% NP-40 (Calbiochem, La Jolla, CA). The cells were reincubated with lysis buffer at 4°C for 20-30 minutes in the refrigerator and were then scraped off. The cell lysates were collected into 1.5ml Eppendorf tubes and kept on ice. The lysates were vortexed for 10 minutes and stored in liquid nitrogen for 1 minute then thawed for 2 cycles. The lysates were clarified by centrifugation at 15,000 x g for 30 minutes at 4°C and the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Samples were boiled with 4X SDS sample protein loading buffer containing 200mM Tris-Hcl (pH 6.8), 100mM DTT, 8% SDS, 0.5 ~0.1% bromophenol blue, and 40% glycerol (Sigma-

Aldrich, St. Louis, MO). Prestained protein marker, broad range (New England Biolabs, Inc., Beverly, MA) and 20-30 μg of protein samples were loaded/ lane and running buffer for protein sample separation (Bio-Rad, Alfred Nobel Drive, Hercules, CA). This gel was transferred in methanol (Fisher Scientific), and distilled water at 100V for 1 hour then washed one time with 1X TBST (200mM Tris-HCL and 1.5M NACL per liter) and 0.05% tween-20 (Sigma). Membranes were blocked in 10ml of 5% non-fat dry milk (Kroger) in 1X TBST for 1 hour. Membranes were then incubated over night at 4°C with primary antibodies (rabbit antibody for total ERK1/2, rabbit Thr202/Tyr204 for phospho-Erk1/2, each at a 1: 1000 dilution; Cell Signaling Technology, Beverly, MA). As a control for equal loading of the lanes, a monoclonal anti-actin clone (Ac-74, mouse source, Sigma-Aldrich, St. Louis, MO) was used at a 1:1000 dilution. At the end of the incubation period, five five-minute washes were done with 1X TBST. Membranes were further incubated with the secondary antibodies in 10ml of 5% non-fat dry milk in TBST at room temperature for one hour. The secondary antibodies, Alexflour 680 goat-anti-rabbit IgG (Probes Eugene, Oregon, USA) and IRDye 800 conjugated affinity purified anti-mouse IgG (Rockland Immunochemicals, Inc., Gilbertsville, PA) were used at 1:2500 dilution and 1:5000 dilution respectively. Membranes were then rinsed five times five-minutes each with 1X TBST. An Odyssey scanner and software (Lincoln, Nebraska, USA) were used for membrane scanning and visualization of bands. The same cell lysates and methods were used to assess the effects of β-carotene on CREB and phosphorylated CREB. A rabbit polyclonal CREB antibody (Cell Signaling Technology, Beverly, MA) was used as a primary antibody for total CREB (dilution 1:1000), while a monoclonal mouse antibody that recognizes

SER33 of phosphorylated CREB (Cell Signaling Technology, Beverly, MA) was used to probe for the activated form of CREB (dilution 1:1000). Each Western blot was repeated three times under identical conditions and yielded similar data. Data are expressed as mean values and standard errors of three independent experiments, each conducted with triplicate samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. Following statistical analysis, data were normalized with controls set as 100% for documentation as column graphs.

MTT assay for the assessment of cell numbers

The effects of β-carotene on cell proliferation were assessed by the colorimetric 3-(4, 5-dimethyle thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (2) (Sigma-Aldrich Co., St. Louis, MO, USA. 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-tetrazoliumbromide] 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 for NCI-H322, and 50,000 cells per well for SAEC and BEAS-2B cells. The cells were left to grow in complete media at 37°C with 5% CO_2 for 5 hours to attach. The cells were then switched to fresh low serum media and β-carotene was added at the concentrations specified in the figure legends and incubated for 72 hours. Pre-icubation of cells with inhibitors of adenylyl cyclase (SQ22536, 500

nM) or of ERK1/2 (PD98059, 1 μM) for assays documented in the inset of Figure 5 was for 10 minutes. Fresh low serum medium of each cell line and fresh treatment were added every other day. After treatment for 68 hours 50μl of [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (0.5mg/ml) was dissolved in RPMI-1640 with L-glutamine and phenol-free medium (Gibco Invitrogen Corporation, Grand Island, N.Y, USA)], added to the medium and incubated at 37°C in the incubator for another 2-3, allowing for the metabolic conversion of the MTT substrate to blue formazan. The media were then discarded and 550 μl of Isopropanol (2-propanol UV cutoff 205 nm, Fisher Scientific, GA, USA) was added to the wells. Absorbances at 570 nm and 630 nm in each well were measured on a micro-ELISA reader. Data are expressed as mean values and standard errors of three independent experiments, each conducted with four samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. Following statistical analysis, data were normalized with controls set as 100% for documentation as column graphs. Data in the inset of Figure 5 that illustrates the effects of an adenylate cyclase inhibitor or an inhibitor of MEK on the stimulation of cell proliferation by β-carotene were normalized with β-carotene-stimulated cells set at 100%.

Results

We have previously shown that the proliferation of human PAC cells of Clara cell lineage in vitro and the development of this cancer type in NNK-treated hamsters is under β-adrenergic control (14-17). Moreover, our recent immunohistochemical studies have documented selective over-expression of PKA, phosporylated CREB and phosphorylated ERK1/2 in the NNK-induced PACs (29). The activation of cAMP, PKA and CREB are classic events downstream of β-adrenergic receptor stimulation in many cell types unrelated to cancer (30-32). Similarly, activation of ERK1/2 has been reported as an effector of CREB or PKA in some cells (33-35). Taken together, our published data therefore point to an important role of cAMP, PKA, CREB and ERK1/2 in the growth regulation of PAC of Clara cell linage in humans and in the hamster model. In an effort to understand the reasons for the unfortunate outcome of the CARET trial, we therefore tested the hypothesis that β-carotene may stimulate one or several components of this regulatory pathway.

Exposure of the putative cells of origin of pulmonary adenocaricnoma, SAECs, to β-carotene (20 nM) caused a significant and time-dependent increase in intracellular cAMP as assessed by immunoassay (Figure 1). This response peaked after 10 minutes of exposure with a 1.6-fold increase. The response of the human PAC cell line NCI-H322 to an identical exposure of β-carotene was even more pronounced with a 2.5-fold increase of intracellular cAMP after 10 minutes of exposure (Figure 1). The observed stimulation of cAMP was reproducible as assessed by three independent experiments that yielded

similar results, each with three samples per treatment group. Statistical evaluation of the data by one-way ANOVA, Tukey-Kramer multiple comparison test and paired two-tailed t-test established significant differences between control and treatment groups as specified in the figure legends.

Assessment of PKA activation by a non-radioactive kit that measures the migration of the phosphorylated peptide to the positive electrode(+), while the non-phosphorylated peptide migrates to the negative electrode (-), revealed a time-dependent increase in activated PKA of SAECs exposed to β-carotene (20 nM; Figure 2) with a maximum 2.6-fold increase at the 1 hour time interval (Figure 2). Similarly, the human PAC cell line NCI-H322 responded to 20 nM of β-carotene with a time-dependent increase of PKA activity that peaked at a 1.6-fold increase (Figures 2a, b). In both cell systems, the observed increase in PKA activity was highly significant ($p < 0.001$ by one way ANOVA, Tukey-Kramer multiple comparison test and unpaired, two-tailed t-test from triplicate samples per treatment group of three independent experiments).

The effects of β-carotene (20 nM) on expression levels of the transcription factor CREB and its phosphorylated form were assessed by Western blot analysis (Figure 3). SAECs demonstrated a time-dependent increase in p-CREB protein with the highest level (4.7-fold increase) observed after 120 minutes of exposure (Figure 3). NCI-H322 cells were even more responsive to β-carotene with a 4.8-fold increase in p-CREB observed after 5 minutes of exposure and peak values of 8.7-fold after 1 hour (Figure 3) In neither cell line did exposure to β-carotene increase the levels of unphosphorylated CREB protein (Figure 3). The observed increases in p-CREB expression were highly significant

in both cell systems at all time intervals tested ($p<0.001$ by one way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test from mean values and standard errors of densitometric values from three independent experiments conducted under identical conditions, each with triplicate samples).

The effects of β-carotene (20 nM) on the mitogen-activated kinases ERK1/2 and their phosphorylated forms were also assessed by Western blotting. As exemplified in Figure 4, p-ERK1/2 was significantly increased in SAECs at all time intervals tested with a 2.5-fold increase as early as 5 minutes after exposure to β-carotene and a peak value of 4.6-fold at the 30 minute time interval (Figure 4). The response of the cancer cell line NCI-H322 to β-carotene was even more pronounced with a 4.8-fold increase in p-ERK1/2 expression after 5 minutes of exposure to β-carotene and a peak level of 7.8-fold at the 1 hour time interval (Figure 4). The observed increases in p-ERK1/2 in both cell lines cells were highly significant at all time intervals tested ($p<0.001$) by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test from densitometric values of three independent experiments conducted under identical conditions, each with triplicate samples. Beta-carotene did not increase the expression of unphosphorylated ERK1/2 protein in either cell system at any of the time intervals tested.

The potential role of the observed changes in cAMP, PKA, and phosporylated proteins on cell proliferation was assessed by MTT assays. In our published studies on the growth-stimulating effects of β-adrenergic agonists and cAMP on NCI-H322 cells we used [^{3}H]-thymidine incorporation assays that monitor DNA synthesis (14, 15). Because the MTT assay monitors numbers of viable cells which can be influenced by both, cell

proliferation and apoptosis, the results of the MTT assays were verified by [^{3}H]thimidine incorporation assay with controls versus three concentrations (1 pM, 10 pM, 100 pM) of β-carotene (data not shown). We tested the effects of β-carotene on cell numbers in the MTT assay after 24, 48 or 72 hours of incubation. While the stimulating effects of β-carotene were highly significant at all time intervals tested, the responses after 72 hours of exposure were the most dramatic and are therefore illustrated in Figure 5. Our data show that β-carotene caused a dramatic increase in the numbers of SAECs at all concentrations, an effect that peaked with a 13-fold increase after incubation with a 10 pM concentration. Similarly, the cancer cells NCI-H322 were highly responsive to the growth-stimulating effects of β-carotene, an effect that peaked with a 12.3-fold increase at a 100 pM concentration of β-carotene (Figure 5). The stimulatory effects of β-carotene on cell proliferation in both cell systems were highly significant ($p < 0.001$) by one way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. Pre-incubation of the cells for 10 minutes with the adenylyl cyclase inhibitor SQ2536 (500 nM) reduced the stimulating effects of β-carotene (20 nM) in SAECs and NCI-H322 cells to 27 and 32%, respectively (with fully β-carotene-stimulated cells set at 100%; Figure 5, inset). On the other hand, pre-incubation for 10 minutes with the MEK inhibitor PD98059 (10 μM) completely blocked the response of SAECs to β-carotene while reducing the response of NCI-H322 cells to 28% (Figure 5, inset). These data support our interpretation that the observed stimulating effects of β-carotene on the proliferation of SAECs and NCI-H322 cells was mediated by c-AMP-dependent signaling and activation of ERK1/2.

Because the preclinical studies leading to the CARET trial had emphasized inhibiting effect of β-carotene on the development of chemically induced tumors derived from the upper airways of laboratory animals, we assessed the effects of β-carotene on immortalized human large airway epithelial cells (BEAS-2B) by MTT assay. In support of the original reports in hamster models, our data show a concentration-dependent decrease in cell number when BEAS-2B cells were exposed for 72 hours to β-carotene (Figure 6). The growth-inhibiting effects of β-carotene on these cells was highly significant ($p < 0.001$ by one way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test from 4 samples per treatment group of three independent experiments).

Discussion

The pro-vitamin β-carotene is converted in the mammalian organism to vitamin A. The majority of functions of vitamin A are carried out by its metabolite, retinoic acid (36), a transcriptional activator operating primarily through members of the nuclear receptor family of transcription factors (37). Synthetic retinoids continue to be widely studied as potential preventive and/or therapeutic agents for a variety of cancers (38-42) or disorders of the nervous system such as Parkinson's disease, motoneuron disease and Huntington's disease (37). In addition to the original preclinical studies on the prevention of cancer development from epithelia of the upper airways (20, 43-45), current research primarily concentrates on the use of retinoids for the prevention and/or therapy of head and neck cancer (40) or leukemia (41, 42). It remains undisputed that β-carotene and retinoids do have beneficial effects via inhibition of cell proliferation and/or stimulation of apoptosis in cancers of those cell lineages. In fact our MTT assay with the human large airway epithelial cells BEAS-2B confirm that. However, our data also provide compelling evidence for cell type-specific growth-stimulating effects of β-carotene on human PAC cells and their normal cells of origin (SAECs) via increase in intracellular cAMP, activation of PKA as well as phosphorylation of CREB and ERK1/2. In light of the prevalence of PAC, these novel and hitherto unknown mechanisms of action of β-carotene should be considered as key factors responsible for the unfortunate outcome of the CARET trial. This interpretation gains strong support from the fact that both, lung cancer and cardiovascular deaths were significantly increased in the CARET trial (19).

As we have previously pointed out, PAC and cardiovascular disease not only share the same risk factors (smoking and high-fat diet) but they are also both regulated by β-adrenergic receptor-initiated and cAMP-mediated signal transduction pathways (46).

While it is far from understood which cellular mechanisms caused the increase in intracellular cAMP in response to β-carotene in our experiments, a similar effect of this pro-vitamin has been reported in neuroblastoma cells (27). It has also been shown that exposure of myeloid leukemia cells to a phosphodiesterase inhibitor that increased intracellular cAMP and activated PKA potentiated the cytodifferentiating action of retinoids (41). The dramatic increase in activated PKA, CREB and ERK1/2 observed in SAECs and PAC cells exposed to β-carotene in our experiments is particularly intriguing. While phophorylation of ERK1/2 is a key event downstream of numerous signaling pathways in many types of cancer (47-52), a potential growth-stimulating role of PKA and its downstream transcription factor CREB in cancer cells has been given little attention. To our knowledge, activation of PKA/CREB has only been implicated in the development of endocrine tumors (31). On the other hand, recent immunohistochemical studies conducted by us in NNK-induced PACs in hamsters have shown a strong and selective over-expression of PKA, phosphorylated CREB and phosphorylated ERK1/2 in the tumor tissue (29). Further studies are clearly needed to address the role of the cAMP/PKA/CREB pathway and its potential cross-talk with other signaling pathways in human PAC.

The growth-stimulating effects of β-carotene observed in SAECs and PAC cells are in accord with published data that have documented a stimulation of cell proliferation

of human PAC cells in vitro in response to agents that increase intracellular cAMP (14, 15). A cancer promoting effect of agents that increase intracellular cAMP has also been documented in a hamster model of PAC induced by the tobacco-specific carcinogen NNK (16, 17). By contrast, such agents inhibited the growth of human PAC cells of alveolar type II cell lineage (15) or human small cell lung cancer cells (53) and demonstrated significant cancer preventive effects in a hamster model of NNK-induced neuroendocrine lung tumors (17).

Collectively, our current data and published evidence emphasize the fact that identical pathways can have very different functions in different cell types and in cancers of different cell lineages. Strategies that target regulatory signal transduction pathways for the prevention and therapy of cancer have to carefully consider this. Unless suitable diagnostic tools are developed that identify which signaling pathway is hyperactive in individual patients, clinical trials aimed at modulating signal transduction will continue to yield disappointing results.

Summary

A multinational chemoprevention trial with β-carotene and retinoids (β-carotene and retinoid efficacy "CARET" trial) was conducted in the 1990s in populations at risk for the development of lung cancer because of previous or current exposure to smoking or asbestos. The trial had to be discontinued after 5 years due to a 28% increase in lung cancer incidence and mortality (46%) and a 26% increase in cardiovascular mortality. The current experiments represent a first step towards unraveling the reasons for this disappointing outcome.

Using a cell line derived from a human pulmonary adenocarcinoma of Clara cell lineage and immortalized human small airway epithelial cells; our data show that low concentrations of β-carotene that can be realistically expected in human tissues after oral administration caused a significant increase in intracellular cAMP, activated PKA, as well as in phosporylation of ERK1/2 and CREB. Furthermore, the proliferation of cells was significantly stimulated by identical concentrations of β-carotene as monitored by MTT assays.

These data are in accord with our earlier publications that have identified a beta-adrenergic receptor-initiated growth-regulating pathway with cAMP as a downstream effector in human pulmonary adenocarcinomas of Clara cell lineage in vitro and in a hamster model. In light of the fact that pulmonary adenocarcinoma is the leading type of lung cancer, these findings suggest that the growth promoting effects of β-carotene on this cancer type observed in our experiments was an important factor responsible for the

unfortunate outcome of the CARET trial. This interpretation is supported by the fact that elevated levels of cAMP in the cardiovascular system play a major role in the genesis of cardiovascular disease, which was also greatly promoted in the CARET trial.

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APPENDIX

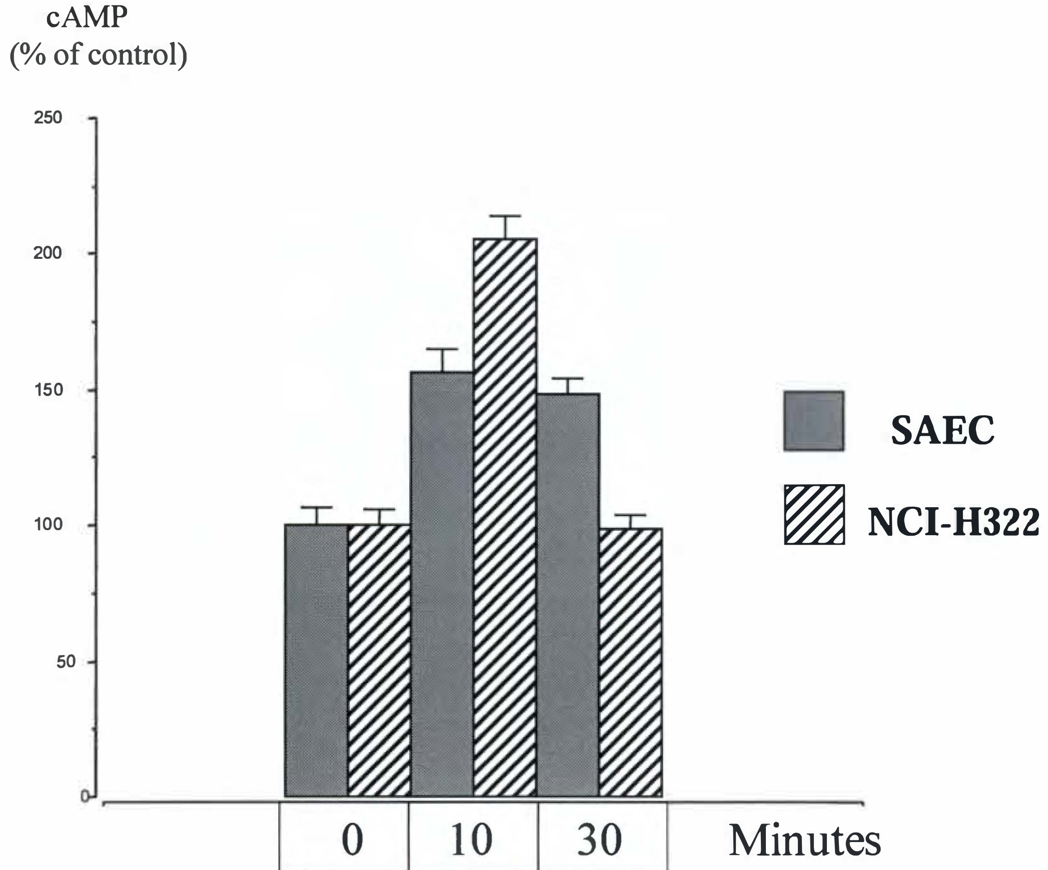

Figure 1. Effects of β-carotene (20 nM) on intracellular cAMP accumulation in SAEC and NCI-H322 cells. Following a 24 hour starvation period, the cells were incubated for 10 or 30 minutes with β-carotene. Cells in the control group were treated with the vehicle of β-carotene (DMSO). Analysis of cAMP was by competitive binding assay as outlined in the Materials and Methods. Bars represent mean values and standard errors of triplicate samples from three independent experiments expressed as normalized data (controls were set as 100-%). Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The increase in intracellular cAMP was significant ($p<0.05$ and 0.01) for SAECs at both time intervals tested and were significant ($p<0.01$) for NCI-H322 cells at the ten minute time interval.

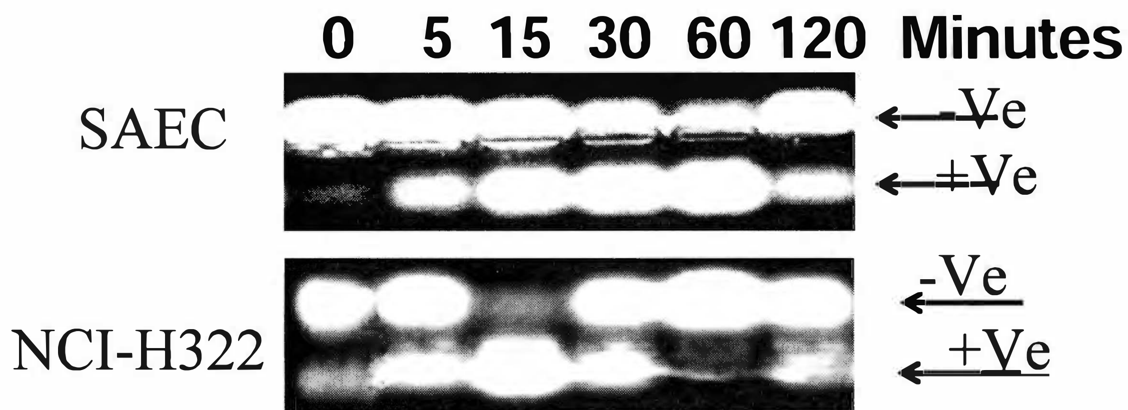

Figure 2A. Agarose gel exemplifying the effects of β-carotene (20 nM) on phosphorylation of PKA in SAECs and NCI-H322 cells. Following a 24 hour starvation period, the cells were exposed to β-carotene for the indicated times. PKA activity was then assayed in cell lysates using a Pep Tag assay for non-radioactive detection of PKA and the samples were separated on an 0.8% agarose gel. Phosphorylated peptide migrated towards the positive electrode (+Ve), while non-phosphorylated peptide migrated towards the negative electrode (-Ve).

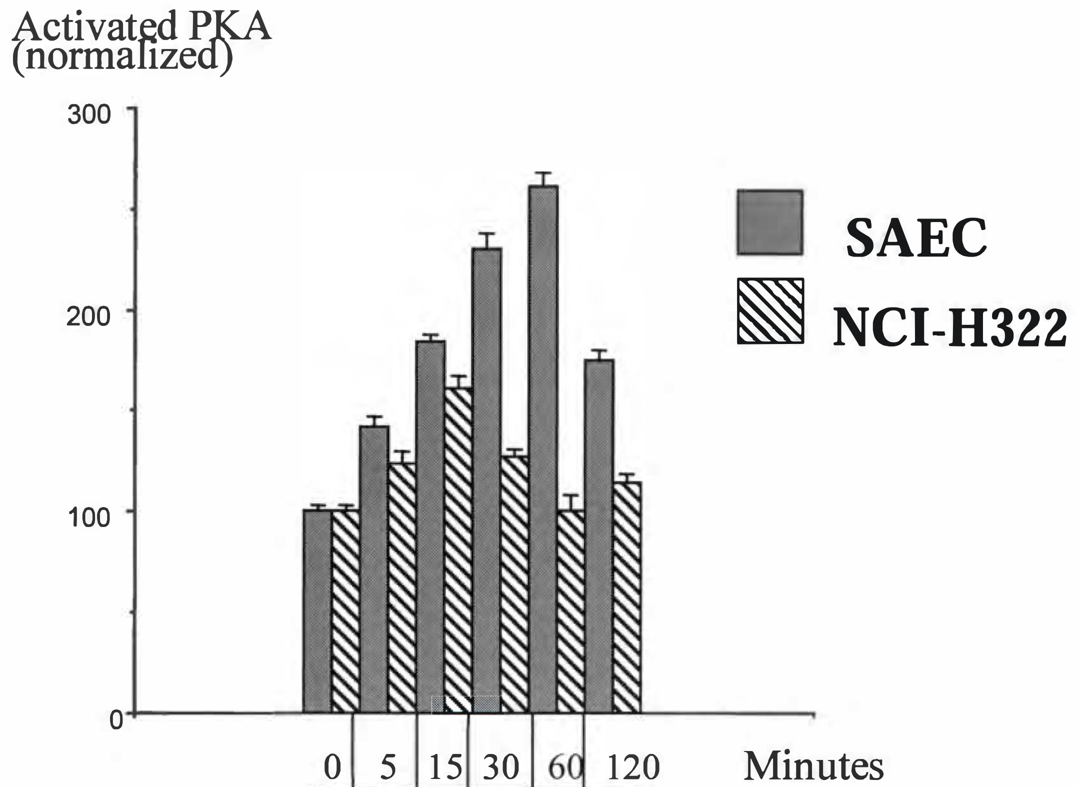

Figure 2B. Bar graph illustrating densitometry values of the bands in Figure 2A. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with triplicate samples. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in phosphorylated PKA were significant ($p<0.001$) at all time intervals tested in the SAECs and after 5, 15, and 30 minutes of exposure in NCI-H322 cells.

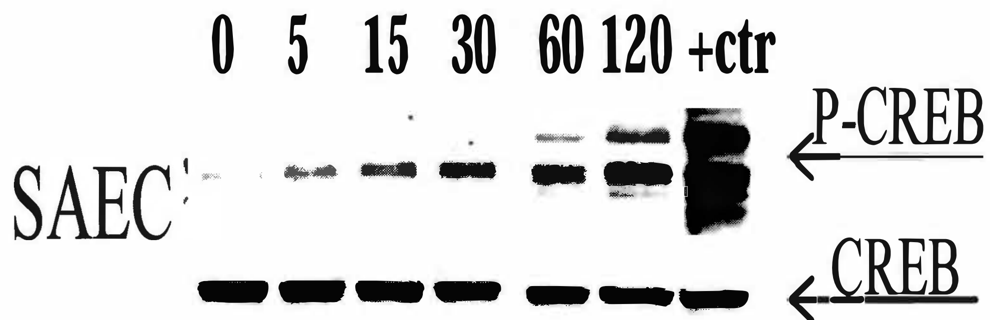

Figure 3A. Western blot exemplifying the effects of β-carotene (20 nM) on the expression of phosphorylated CREB and total CREB protein in SAECs. Following a 24 hour starvation period, cells were exposed to b-carotene for the time intervals indicated. The bands for p-CREB increased in size and density over time whereas no increase was observed in the bands for total CREB protein.

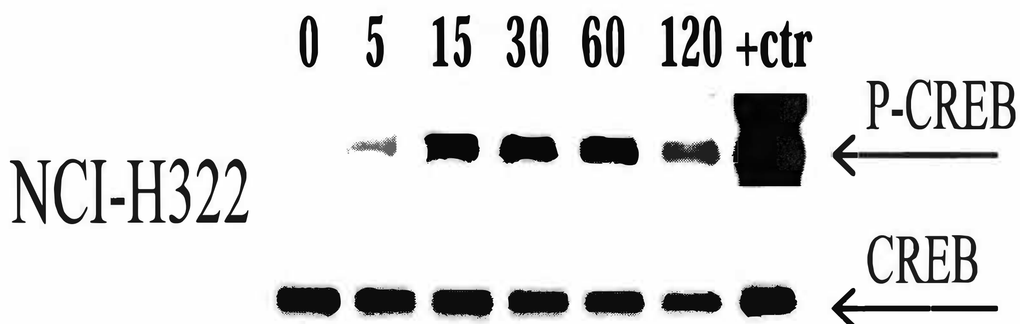

Figure 3B. Western blot exemplifying the effects of β-carotene (20 nM) on the expression of phosphorylated CREB and total CREB protein in NCI-H322 cells. Following a 24 hour starvation period, cells were exposed to β-carotene for the time intervals indicated. The bands for p-CREB increased in size and density over time whereas no increase was observed in the bands for total CREB protein.

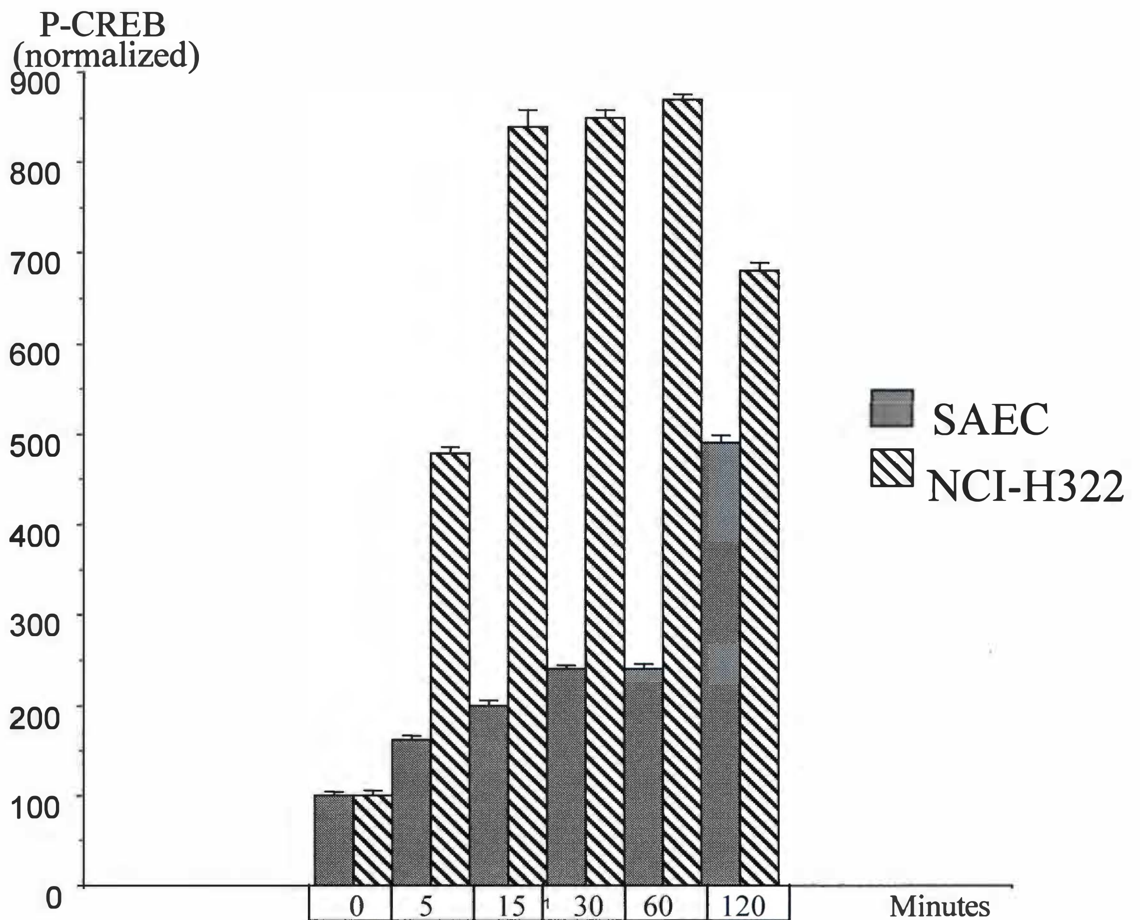

Figure 3C. Bar graph illustrating densitometry values of the bands in Figures 3A and 3B. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with triplicate samples. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in p-CREB were significant ($p<0.001$) in both cell systems at all time intervals tested.

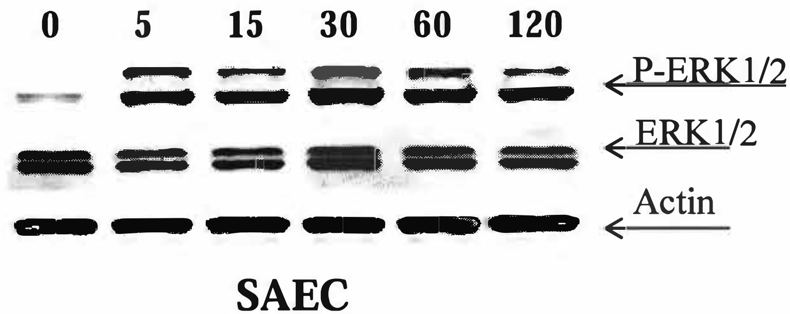

Figure 4A. Western blot illustrating the effects of β-carotene (20 nM) on the expression of ERK1/2 and its phosphorylated form in SAECs. Cells were exposed to β-carotene for the time intervals indicated after a 24 hour starvation period. The bands for p-ERK1/2 increased in size and intensity, an effect that peaked after 30 minutes of incubation whereas the expression of ERK1/2 did not increase.

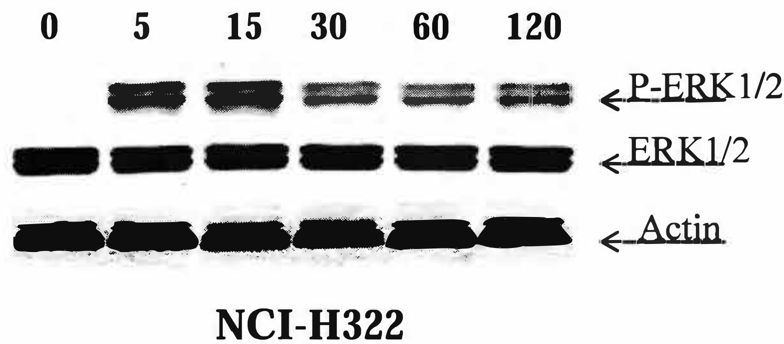

Figure 4B. Western blot illustrating the effects of β-carotene (20 nM) on the expression of ERK1/2 and its phosphorylated form in NCI-H322 cells. Cells were exposed to β-carotene for the time intervals indicated after a 24 hour starvation period. The bands for p-ERK1/2 increased in size and intensity, an effect that peaked after 15 minutes of incubation whereas the expression of ERK1/2 did not increase.

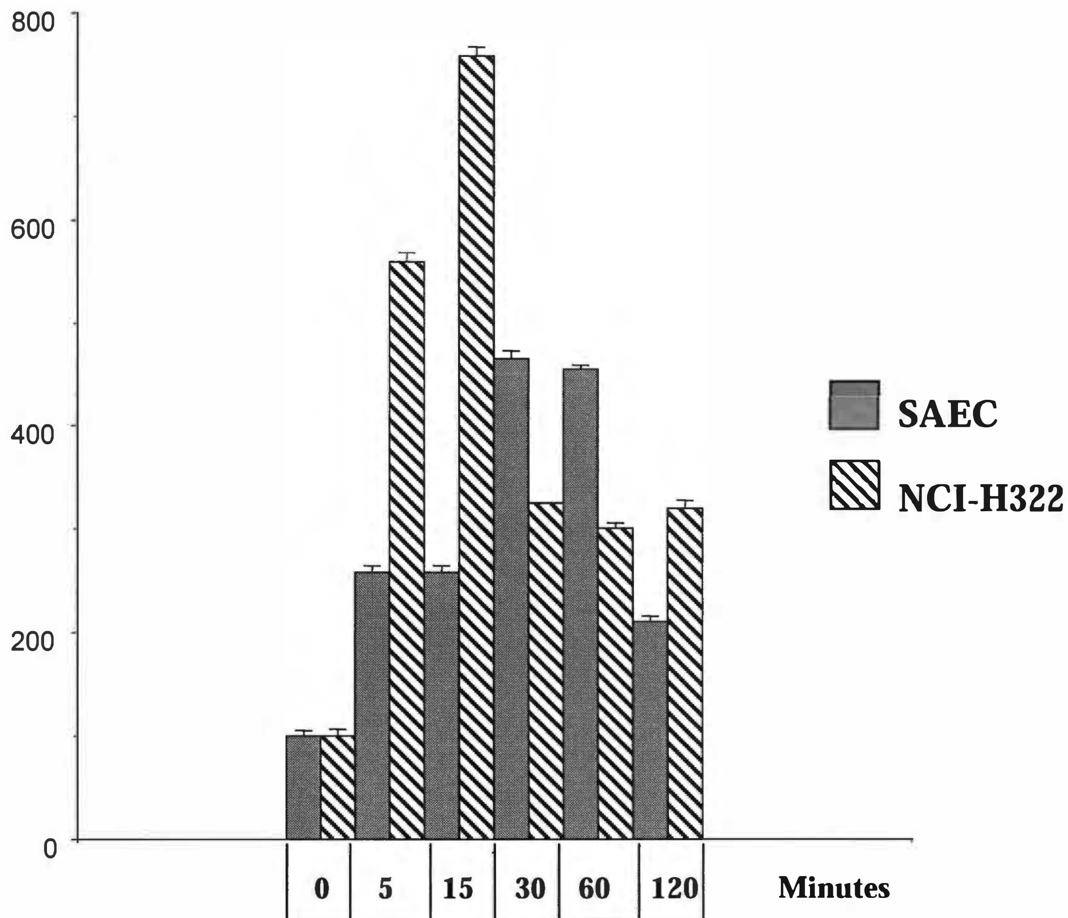

Figure 4C. Bar graph illustrating densitometry values of the bands in Figures 4A and 4B. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with triplicate samples. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in p-ERK1/2 were significant ($p<0.001$) in both cell systems at all time intervals tested.

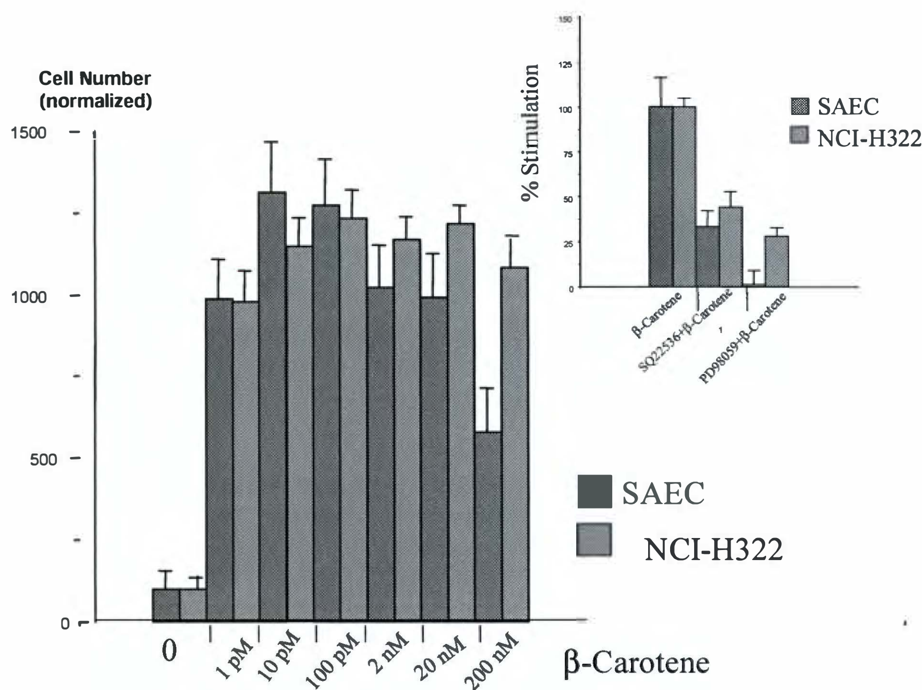

Figure 5. Effects of β-carotene (1pM-200 nM) on cell number in SAECs and NCI-H322 as assessed by MTT assay. The cells were left to grow in complete media for 5 hours to attach. The cells were then switched to fresh low serum media (0.05% FBS) for SAECs and (0.1% FBS) for NCI-H322 cells. Cells were then exposed for 72 hours to β-carotene at the concentrations indicated. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with four samples per group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in cell number were significant ($p<0.001$) at all concentrations tested in both cell systems.

Inset: Inhibitory effects of the adenylase cyclase inhibitor SQ2536 (500 nM) or the MEK inhibitor PD98059 (10 μM) on β-carotene induced cell numbers as assessed by MTT assay. Following a 24 hour starvation period, cells were pre-incubated for ten minutes with the inhibitors and then exposed for 72 hours to β-carotene. The stimulatory response to β-carotene was significantly reduced ($p<0.001$) by both inhibitors in both cell systems. Bars represent normalized (cells treated with β-carotene alone set as 100%) mean values and standard errors of three independent experiments, each with triplicate samples.

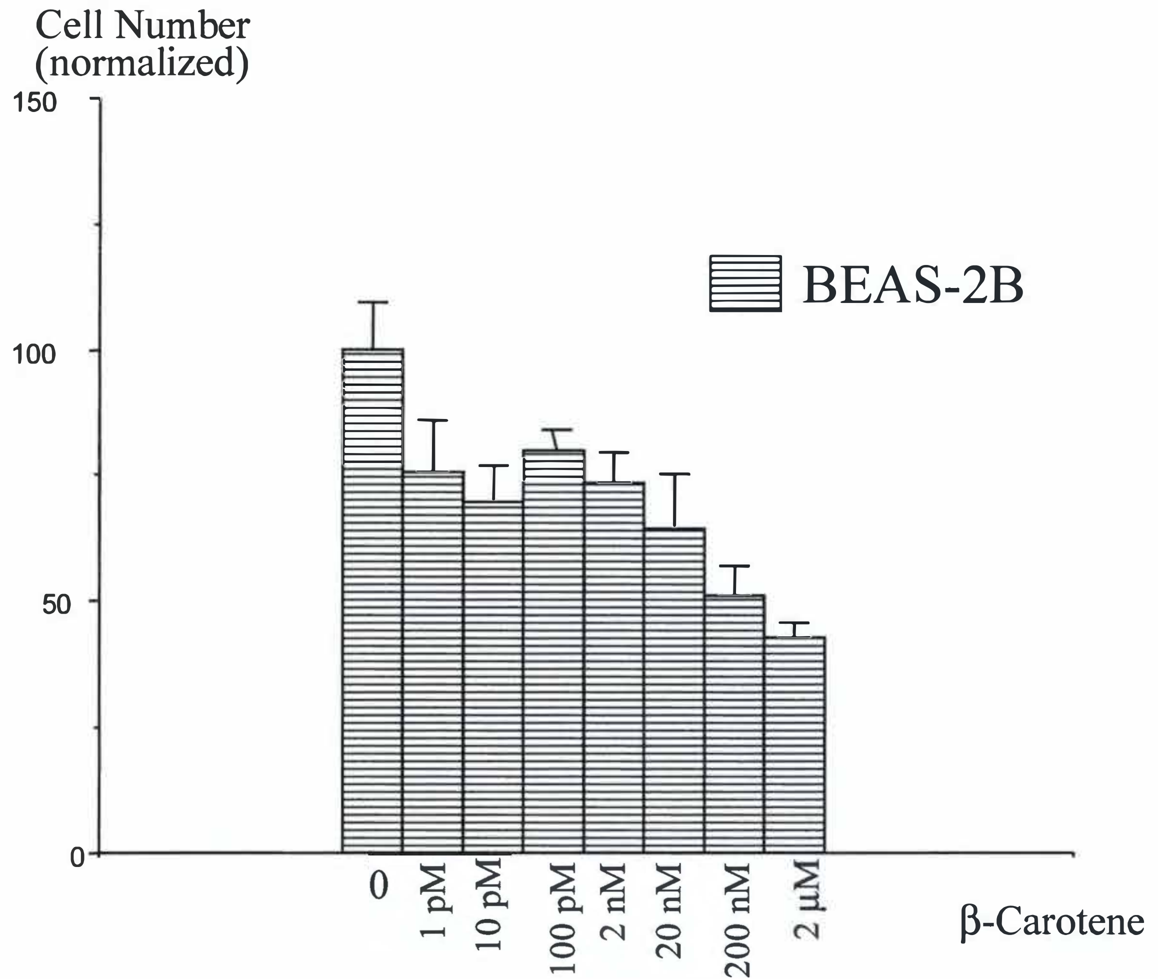

Figure 6. Effects of β-carotene (1pM-2µM) on cell number in BEAS-2B cells as assessed by MTT assay. The cells were left to grow in complete media for 5 hours to attach. The cells were then switched to fresh low serum media (0.05% FBS). Cells were exposed for 72 hours to β-carotene at the concentrations indicated. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with four samples per group. Cell numbers were significantly reduced at all concentrations tested ($p < 0.001$ by one way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test).

PART IV:

Theophylline stimulates cAMP-mediated signaling associated with growth regulation in human cells from pulmonary adenocarcinoma and small airway epithelia

Part IV

Brief explanatory statement

This chapter is a lightly revised version of a manuscript by the same name that has recently been submitted to the journal "International Journal of Oncology".

Hussein, A., N., Al-Wadei[1], Takashi Takahashi and Hildegard M. Schuller[1,3]. [1]Experimental Oncology Laboratory, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA and [2]Aichi Cancer Center Research Institute, Nagoya University, Nagoya, Japan.

[3]Author for correspondence and reprint requests:
Dr. H. M. Schuller
Department of Pathobiology
College of Veterinary Medicine
University of Tennessee
2407 River Drive
Knoxville, TN 37996

Phone: 865-974-8217
Fax: 865-974-5616
e-mail: hmsch@utk.edu

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic, (2) development of assay conditions suitable to test the hypothesis that theophylline stimulates the growth of pulmonary adenocarcinoma cells and their normal cells of origin, (3) conduction of cAMP assays, (4) conduction of PKA activation assays, (5) conduction of Western blots, (6) conduction of MTT assays, (7) analysis and interpretation of all data, (8) statistical analysis of all data, (9) photographic and graphic documentation of results, (10) conduction of comprehensive literature reviews, (11) writing of the manuscript (with some editorial assistance by Dr. Schuller).

Introduction

Preclinical studies in mouse models of lung cancer have demonstrated that green tea as well as black tea inhibit the development of experimentally induced lung adenomas and that this effect is largely caused by polyphenols of the catechin family that have antioxidant, anti-mutagenic, pro-apoptotitc and anti-proliferative effects in this animal model (1-4). However, epidemiological studies on the effects of tea in human populations have been inconclusive with some showing a reduction in lung cancer risk while others demonstrated no effect or even cancer promoting effects (5-7). Unfortunately, none of the epidemiological investigations have specified the effects of tea on histological lung cancer types. Among the four major histological lung cancer types (adenocarinoma, small cell lung carcinoma, squamous cell carcinoma, large cell carcinoma), pulmonary adenocarcinoma (PAC) is the leading type of lung cancer today (8, 9). Even though mouse lung adenoma is a model for human PAC, it is well established that these mouse tumors are derived from alveolar type II cells (10, 11). By contrast, electron microscopic investigations have provided evidence for about 90% of human PACs to be derived from bronchial and bronchiolar Clara cells (PACC) with only about 10% of cases exhibiting characteristics of alveolar type II cells (12). Accordingly, preclinical data generated in the mouse models cannot be extrapolated to about 90% of cases of the leading type of human lung cancer (PACC).

Studies conducted in our laboratory have shown that human cell lines derived from PACC as well as experimentally induced PACC in hamsters are under β-adrenergic growth control (13-16). Pharmacological agonists of β-adrenergic receptors as well as forskolin, which stimulates the downstream effector of this receptor family, cAMP, each demonstrated strong promoting effects on human PACC cell growth in vitro and on the development of PACC in hamsters. We have also shown that a cell line derived from human PAC of alveolar type II cell lineage responded with an inhibition in cell proliferation to forskolin (16). These findings suggest that within the family of human PACs the predominating Clara cell derived tumors respond with increased growth to cAMP whereas the rare PAC of alveolar type II cell lineage is inhibited. Accordingly, any agents that increase intracellular cAMP have the potential to act as tumor promoters for the development of PACC.

Theophylline is a methylxanthine contained in tea products and in numerous medications for the treatment of asthma, cough and the common cold (17). Among the tea products that contain theophylline are grean tea, black tea, mint tea (all in their caffeinated and decaffeinated forms) as well as numerous dietary supplements that are based on green tea extracts and which are widely used as weight control medicines. It is well established that theophylline inhibits the enzyme phopsphodiesterase that mediates the cellular break-down of cAMP (17). Consequently, exposure to theophylline results in intracellular accumulation of cAMP. It is therefore reasonable to assume that signaling pathways downstream of cAMP will be stimulated by theophylline. In light of the tumor promoting effects of cAMP on human and hamster PACC discovered by us, theophylline

may therefore selectively promote the growth and development of this form of lung cancer.

In support of this hypothesis, our current data in cell lines derived from human PACC and its cell of origin (SAEC) show that theophylline increased intracellular cAMP resulting in the activation of its downstream kinase, protein kinase A (PKA) and its associated transcription factor cAMP response element binding protein (CREB) as well as phosphorylation of the mitogen-activated protein kinases ERK1/2.

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 (Gibco Invitrogen-Life Technologies, Carlsbad, CA) supplemented with 10% FBS, 10 mM Hepes, 1mM sodium pyruvate, 2mM L-glutamine, 4500 mg/l glucose, and 1500mg/l sodium bicarbonate. The Simian virus 40 (SV40)-immortalized human peripheral airway cell line HPL1D (18), referred to in this publication as small airway epithelial cells SAEC, was provided to us by Dr. Takashi Takahashi (Aichi Cancer Center Research Institute, Nagoya University, Chikusa-ku, Nagoya, Japan). These cells were maintained in F-12 (HAM) medium buffered with 15mM HEPES (pH 7.3; Gibco Invitrogen-Life Technologies, Carlsbad, CA) and supplemented with 5μg/ml bovine insulin, 5μg/ml human transferrin, 10^{-7} M hydrocortisone, 2 $\times 10^{-10}$ M triiodothyronine (Cambrex Bio Science Walkersville, Inc, Walkersville, MD) and 1% fetal calf serum (FCS; ATCC, Manassas, VA). No antibiotic was added.

cAMP Immunoassay

Cells were plated at 4×10^5 cells per well in 6-well plates and grown in F-12 media with supplements and 1% FBS for SAECs or in RPMI-1640 containing 10% FBS until 65-70 % confluence. Then cells were then washed twice with 1X PBS and then fed

with low serum (0.05% FBS) for SAECs and (0.1% FBS) for NCI-H322 cells or no-serum medium for 24 hours starvation. Fresh low serum media of F12 (0.05% FBS) or RPMI media (0.1% FBS) containing 1mM IBMX, a phosphodiesterase inhibitor was added to all treatment and control group and pre-incubated for 30 minutes. After removal of this pre-incubation mix, the cells were incubated and treated with the concentrations of theophylline indicated in the figure legend in the appropriate medium containing 1mM IBMX for 10 minutes. After three washes with water, cells were incubated with 0.1 M HCL lysis buffer for 20-30 minutes, and then the cells were scraped by plastic scraper and collected into 1.5ml eppendorf tubes, then lysed by sonication. Samples were then vortexed immediately before being analyzed for cAMP levels using a direct cyclic AMP enzyme immunoassay kit according to the manufacturer instructions (Assay Designs Inc). Briefly, the assay utilizes p-nitrophenyl phosphate as a substrate and a polyclonal antibody to cAMP to bind, in a competitive manner, the cAMP in sample that has cAMP covalently attached to it. Reactions were stopped with trisodium phosphate and color intensity was measured at 405 nm.

Data are expressed as mean values and standard errors of three independent experiments, each conducted with triplicate samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test.

PKA activation assay

Following incubation of cells with theophylline (10 pM) for 5 minutes to 1 hour as specified in the figure legends, PKA activity was assayed in cell lysates using a Pep Tag assay for non-radioactive detection of activated PKA (Promega Corperation, 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 (0.4μg/μl), peptide protection and PKA activator solutions were incubated in ice for few min before 1min incubation at 30°C. After adding samples, 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 was determined by examining the gel under UV lights. Densitometric analysis of the bands was conducted using NIH Scion software for image quantitation.

Data are expressed as mean values and standard errors of three independent experiments, each with triplicate samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test.

Assessment of total proteins and phosporylated proteins by Western blotting

To assess the effects of theophylline (10 pM) 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 hours. Following removal of the media and replacement with fresh low-serum media, theophylline (10 pM) was added to the culture vessels and cells were incubated from 5 minutes to 1 hour as detailed in the figure legends. 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, 1μl 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 phosphorylated CREB antibodies, respectively, and developed by chemiluminescence with ECL reagentsMembranes were blocked in 10ml of 5% non-fat dry milk (Kroger) in 1X TBST for 1 hour. Membranes were then incubated over night 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). Equal loading of lanes was confirmed by blotting for actin using mouse actin monoclonal antibody (Sigma-Aldrich Co., St. Louis, MO, USA).

Data are expressed as mean values and standard errors of three independent experiments, each conducted with triplicate samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. Following statistical analysis, data were normalized with controls set as 100% for documentation as column graphs.

Assessment of cell numbers by MTT assay

The effects of theophylline on cell proliferation were assessed by the colorimetric 3-(4, 5-dimethyle thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (2) (Sigma-Aldrich Co., St. Louis, MO, USA). 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-tetrazoliumbromide] 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 for NCI-H322, and 50,000 cells per well for SAEC. The cells were left to grow in complete media at 37°C with 5% CO_2 for 5 hours to attach. The cells were then switched to fresh low serum media and theophylline was added at the concentrations specified in the figure legend and incubated for 72 hours. Fresh low serum medium of each cell line and fresh treatment were added every other day. After treatment for 68 hours 50μl of [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (0.5mg/ml) was dissolved in RPMI-1640 with L-glutamine and phenol-free medium (Gibco Invitrogen Corporation,

Grand Island, N.Y, USA)], added to the medium and incubated at 37°C in the incubator for another 2-3, allowing for the metabolic conversion of the MTT substrate to blue formazan. The media were then discarded and 550 μl of Isopropanol (2-propanol UV cutoff 205 nm, Fisher Scientific, GA, USA) was added to the wells. Absorbances at 570 nm and 630 nm in each well were measured on a micro-ELISA. Data are expressed as mean values and standard errors of three independent experiments, each conducted with four samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test.

Results

Exposure of the putative cells of origin of pulmonary adenocaricnoma, SAECs, for ten minutes to theophylline caused a significant and concentration-dependent increase in intracellular cAMP as assessed by immunoassay (Figure 1). This response peaked at the 10, pM concentration a 2.2-fold increase. Similarly, the human PAC cell line NCI-H322 responded with a 2.1-fold increase in intracellular cAMP when exposed to 10 pM theophylline (Figure 1). The observed stimulation of cAMP was reproducible as assessed by three independent experiments that yielded similar results, each with three samples per treatment group. Because cAMP levels peaked in both cell systems after expoure to 10 pM theophylline, this concentration was used in assays for the assessment of PKA activation and phosphorylation of CREB and ERK1/2. Statistical evaluation of the data by one-way ANOVA, Tukey-Kramer multiple comparison test and paired two-tailed t-test established significant differences between control and treatment groups at all theophyline concentrations tested in both cell systems.

Assessment of PKA activation by a non-radioactive kit that measures the migration of the phosphorylated peptide to the positive electrode(+), while the non-phosphorylated peptide migrates to the negative electrode (-), revealed a time-dependent increase in activated PKA of SAECs exposed to theophylline (10 pM; Figure 2) with a maximum 5-fold increase at the 5 minute time interval (Figure 2). Similarly, the human PAC cell line NCI-H322 responded to 10 pM of theophylline with a time-dependent increase of PKA activity that peaked with a 4.97--fold increase at the 15 minute time interval (Figure 2). In SAECs, the observed increase in PKA activity was highly

significant at all time interval tested ($p < 0.001$ by one way ANOVA, Tukey-Kramer multiple comparison test and unpaired, two-tailed t-test from triplicate samples per treatment group of three independent experiments). In NCI-H322 cells, no increase in PKA activity was observed after 5 minutes of exposure to theophylline whereas the increase in PKA acitity was highly significant ($p<0.001$) after expoure for 15, 30, or 60 minutes.

The effects of theophylline (10 pM) on expression levels of the transcription factor CREB and its phosphorylated form were assessed by Western blot analysis (Figure 3). SAECs demonstrated a time-dependent increase in p-CREB protein with the highest level (5.35-fold-fold increase) observed after 15 minutes of exposure (Figure 3). NCI-H322 cells were similarly responsive to theophylline with a 5.45-fold increase in p-CREB observed after 15 minutes of exposure (Figure 3). In neither cell line did exposure to theophylline increase the levels of unphosphorylated CREB protein (Figure 3). The observed increases in p-CREB expression were highly significant in both cell systems at all time intervals tested ($p<0.001$ by one way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test from mean values and standard errors of densitometric values from three independent experiments conducted under identical conditions, each with triplicate samples).

The effects of theophylline (10 pM) on the mitogen-activated kinases ERK1/2 and their phosphorylated forms were also assessed by Western blotting. As exemplified in Figure 4, p-ERK1/2 was significantly increased in SAECs after 5, 15 or 30 minutes of exposure to theophylline with peak values (3.5-fold increase) at the 5 minute time interval (Figure 4). The response of the cancer cell line NCI-H322 to theophylline was

similar, with a 3.3-fold increase in p-ERK1/2 expression after 5 minutes of exposure (Figure 4). The observed increases in p-ERK1/2 in both cell lines cells were highly significant ($p<0.001$) for SAECs at them 5, 15 and 30 minute time intervals and for NCI-H322 at all time points tested (one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test from densitometric values of three independent experiments conducted under identical conditions, each with triplicate samples). Theophylline did not increase the expression of unphosphorylated ERK1/2 protein in either cell system at any of the time intervals tested.

The potential role of the observed changes in cAMP, PKA, and phosporylated proteins on cell proliferation was assessed by MTT assays. In our published studies on the growth-stimulating effects of β-adrenergic agonists and cAMP on NCI-H322 cells we used [^{3}H]-thymidine incorporation assays that monitor DNA synthesis (13, 16). Because the MTT assay monitors numbers of viable cells which can be influenced by both, cell proliferation and apoptosis, the results of the MTT assays were verified by [^{3}H]thimidine incorporation assay with controls versus three concentrations (1 pM, 10 pM, 100 pM) of theophylline (data not shown). We tested the effects of theophylline on cell numbers in the MTT assay after 24, 48 or 72 hours of incubation. While the stimulating effects of theophylline were highly significant at all time intervals tested in SAECs, the responses after 72 hours of exposure were the most dramatic and are therefore illustrated in Figure 5. Our data show that theophylline caused a dramatic increase in the numbers of SAECs particularly at the low concentrations, an effect that peaked with a 2.9-fold increase after incubation with a 1 pM concentration (Figure 5). Under the condition of our experiments, the cancer cell line NCI-H322 were less responsive, with a small but significant increase

in cell numbers at 10 and 100 nM concentrations of theophylline. The stimulatory effects of theophylline on cell proliferation in SAECs were highly significant ($p < 0.001$) while very significant ($p < 0.01$) in NCI-H322 cells by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test.

Discussion

Theophylline is contained in green tea, black tea and mint tea as well as in a host of asthma therapeutics, over the counter cold medicines (17) and dietary supplements that target weight loss via green tea extracts. Even though the conflicting data on the tumor modulating effects of tea (5, 6) implicate that ingredients in this family of beverages may have beneficial effects on some cancers while promoting others, the potential tumor modulating effects of theophylline have not been given attention in past research. The discovery that the leading type of lung cancer, PACC, is regulated by a pathway initiated by β-adrenergic receptors and involving the stimulation of cAMP (13, 14, 16, 19) strongly suggests that phosphodiesterase inhibitors such as theophylline , which increase intracellular levels of cAMP, may act as selective promoters of this cancer type. In support of this hypothesis, our current data show that theophylline significantly increased intracellular cAMP, activated PKA, phosphorylated CREB and phosphorylated ERK1/2 in SAECs and NCI-H322 cells. SAECs additionally responded with a highly significant increase in cell number suggestive of increased cell proliferation. The lack of proliferative response of NCI-H322 cells to theophylline is a reflection of a very strong autocrine growth stimulation via production of EGF that has developed in this cell line under prolonged in vitro passages. Unfortunately, early passage material of this cell line is no longer available.

The growth-stimulating effects of theophylline observed in SAECs and PAC cells are in accord with published data that have documented a stimulation of cell proliferation of human PAC cells in vitro in response to agents that increase intracellular cAMP (13,

16). A cancer promoting effect of agents that increase intracellular cAMP has also been documented in a hamster model of PAC induced by the tobacco-specific carcinogen NNK (14, 19). By contrast, such agents inhibited the growth of human PAC cells of alveolar type II cell lineage (16) or human small cell lung cancer cells (20) and demonstrated significant cancer preventive effects in a hamster model of NNK-induced neuroendocrine lung tumors (19).

Collectively, our current data and published evidence emphasize the fact that identical pathways can have very different functions in different cell types and in cancers of different cell lineages. Strategies that target regulatory signal transduction pathways for the prevention and therapy of cancer have to carefully consider this. Unless suitable diagnostic tools are developed that identify which signaling pathway is hyperactive in individual patients, clinical trials aimed at modulating signal transduction will continue to yield disappointing results.

Summary

The methylxanthine theophylline is contained in tea and in numerous asthma and cold medications. Theophylline inhibits the enzyme phosphodiesterase, thereby preventing the intracellular break-down of cAMP. The resulting increase in intracellular cAMP reduces smooth muscle tone, thus dilating the airways. Epidemiologic studies on preventive effects of tea on the development of lung cancer have yielded mixed results, with some studies demonstrating a reduction in lung cancer risk whereas others showed evidence for cancer promotion. On the other hand, preclinical studies in mouse models of lung cancer or in vitro systems have consistently demonstrated strong cancer preventive effects of tea and of polyphenols contained in tea.

Investigations conducted in our laboratory have recently shown that cell lines derived from human pulmonary adenocarcinomas of Clara cell lineage (PACC) and experimentally induced PACCs in a hamster model are under β-adrenergic growth control. Beta-adrenergic agonists as well as forskolin, which activates cAMP, had strong growth-promoting effects on human PACC cells and on the hamster PACCs. The current project therefore tests the hypothesis that theophylline activates growth-stimulating signaling in human PACC cells and their normal cells of origin, small airway epithelial cells (SAEC).

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APPENDIX

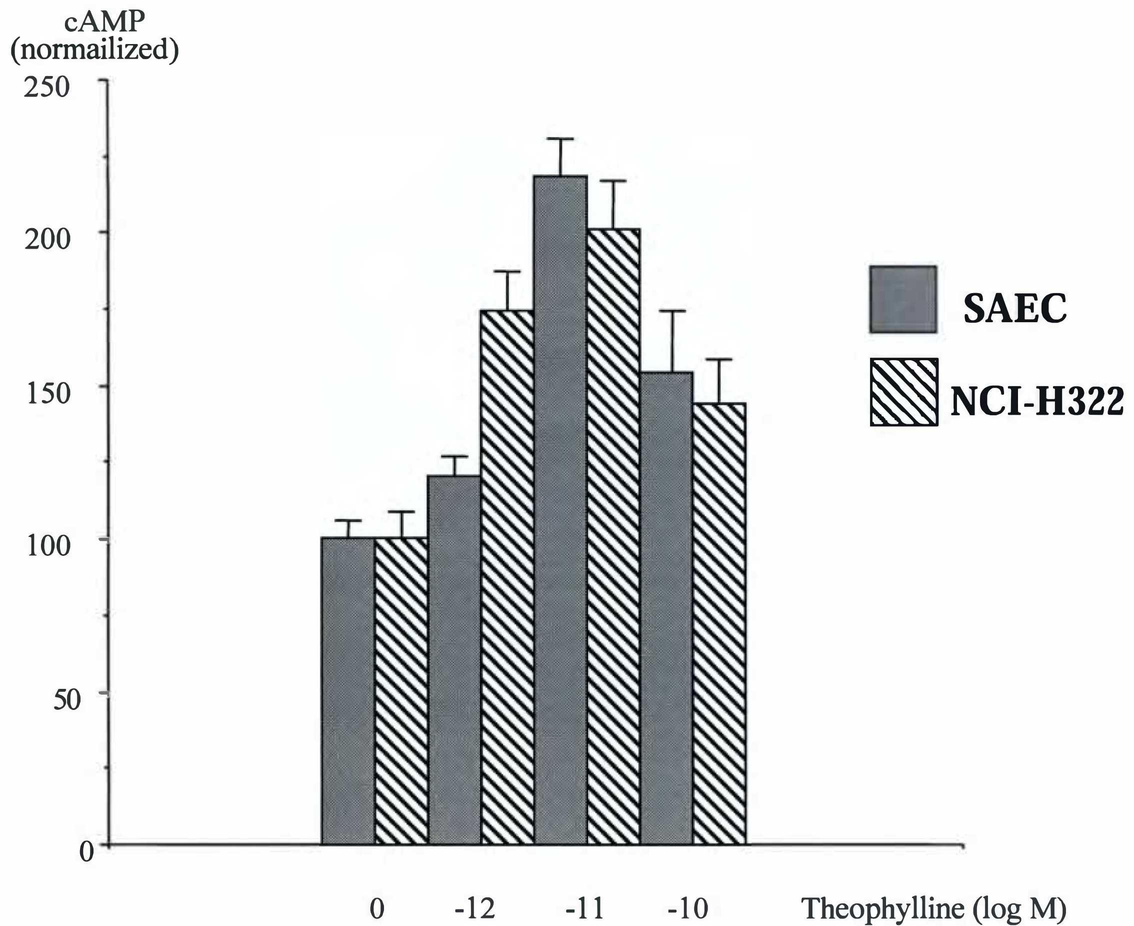

Figure 1. Effects of theophylline (10 minutes) on intracellular cAMP accumulation in SAEC and NCI-H322 cells. Following a 24 hour starvation period, the cells were incubated for 10 minutes with theophylline at the indicated concentrations. Analysis of cAMP was by competitive binding assay as outlined in the Materials and Methods. Bars represent mean values and standard errors of triplicate samples from three independent experiments expressed as normalized data (controls were set as 100- %). Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The increase in intracellular cAMP was significant ($p<0.01$) at all theophylline concentrations tested in both cell systems.

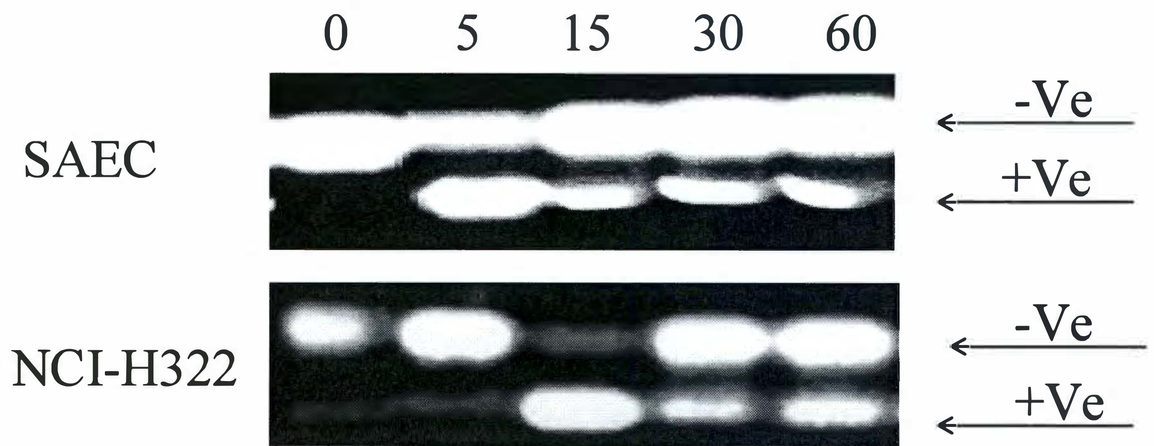

Figure 2A. Agarose gel exemplifying the effects of theophylline (10 pM incubated for 5 to 60 minutes) on phosphorylation of PKA in SAECs and NCI-H322 cells. Following a 24 hour starvation period, the cells were exposed to theophylline for the indicated times. PKA activity was then assayed in cell lysates using a Pep Tag assay for non- radioactive detection of PKA and the samples were separated on an 0.8% agarose gel. Phosphorylated peptide migrated towards the positive electrode (+Ve), while non-phosphorylated peptide migrated towards the negative electrode (-Ve).

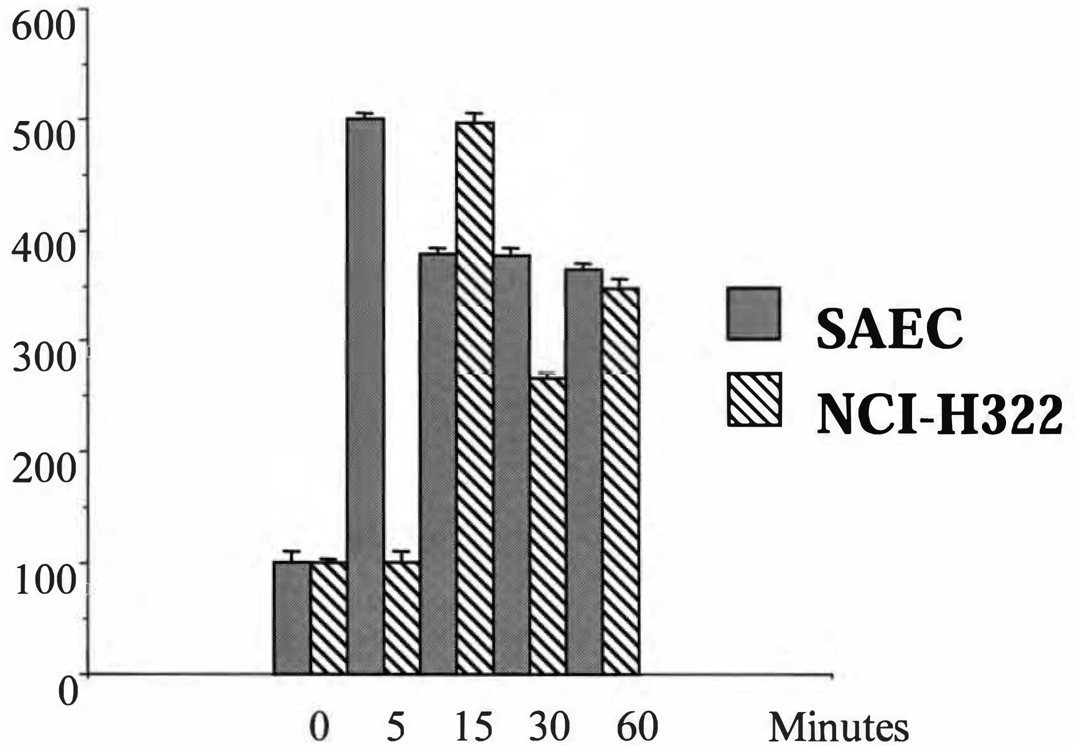

Figure 2B. Bar graph illustrating densitometry values of the bands in Figure 2A. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with triplicate samples. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in phosphorylated PKA were significant ($p<0.001$) at all time intervals tested in the SAECs and after 15, 30 and 60 minutes of exposure in NCI-H322 cells.

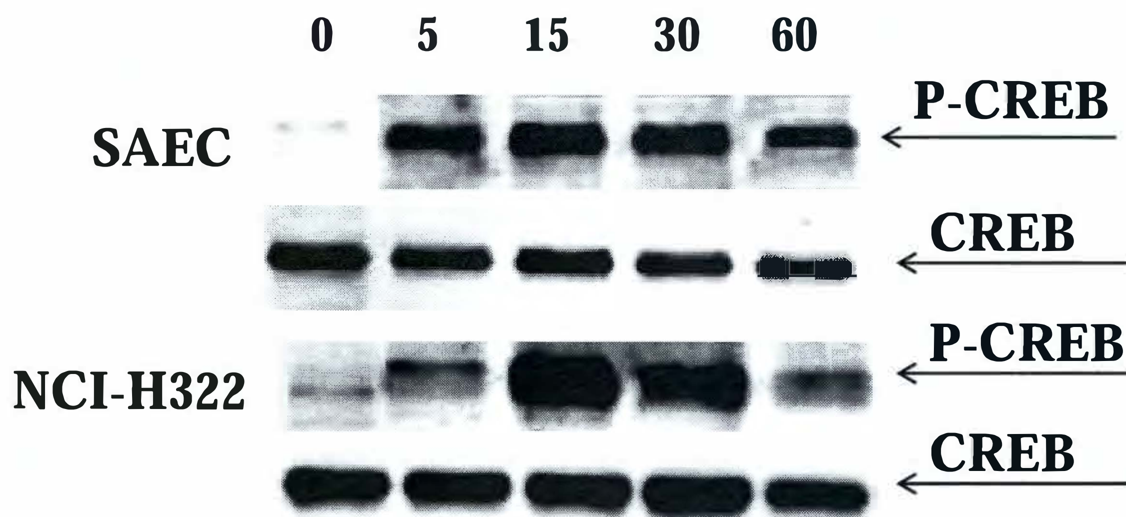

Figure 3A. Western blots exemplifying the effects of theophylline (10 pM) on the expression of phosphorylated CREB and total CREB protein in SAECs or NCI-H322 cells. Following a 24 hour starvation period, cells were exposed to theophylline for the time intervals indicated. The bands for p-CREB increased in size and density over time whereas no increase was observed in the bands for total CREB protein.

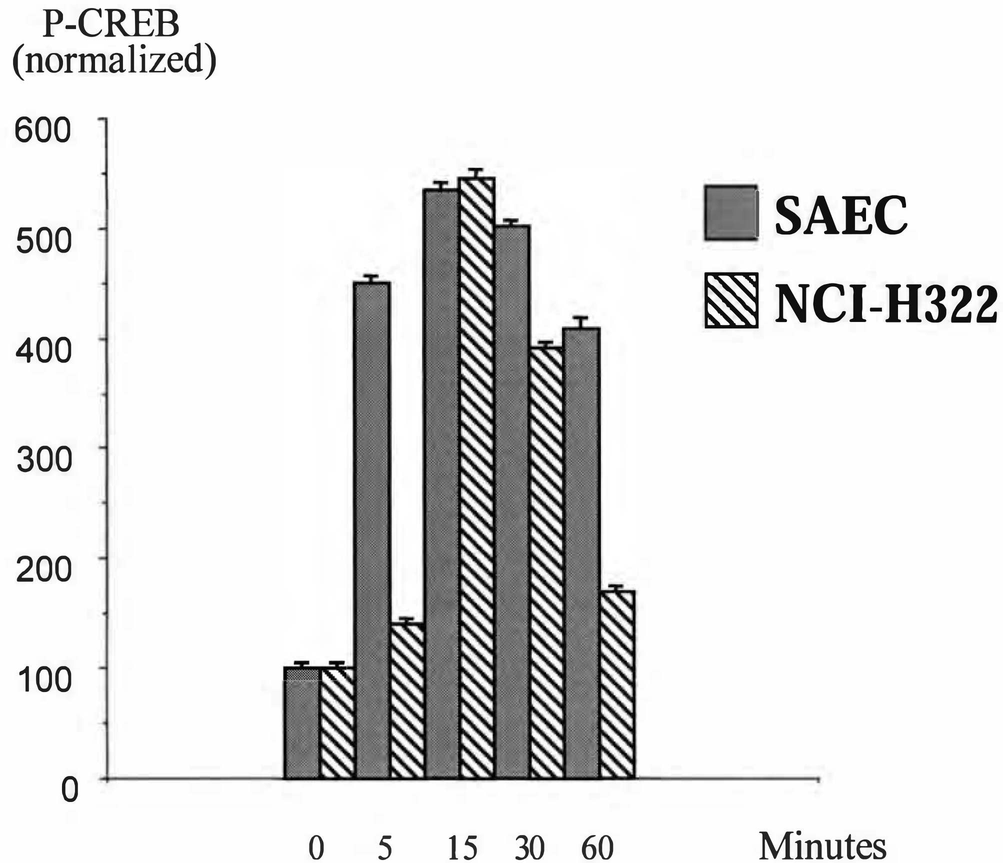

Figure 3B. Bar graph illustrating densitometry values of the bands in Figures 3A. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with triplicate samples. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in p-CREB were significant ($p<0.001$) in both cell systems at all time intervals tested.

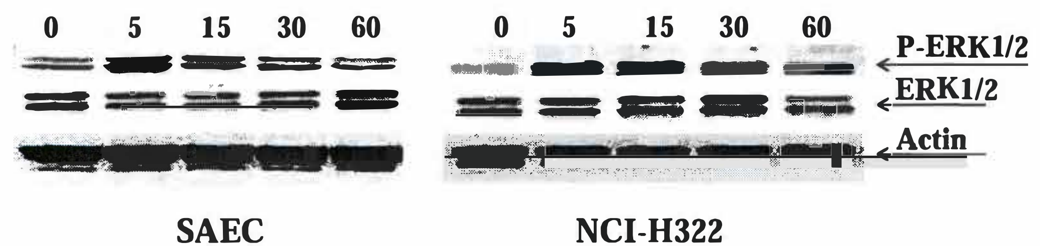

Figure 4A. Western blots illustrating the effects of theophylline (exposures from 5 minutes to 60 minutes, 10 pM) on the expression of ERK1/2 and its phosphorylated form in SAECs and NCI-H322 cells. Cells were exposed to theophylline for the time intervals indicated after a 24 hour starvation period. The bands for p-ERK1/2 increased in size and intensity, an effect that peaked after 5 minutes in both cell systems whereas the expression of ERK1/2 did not increase.

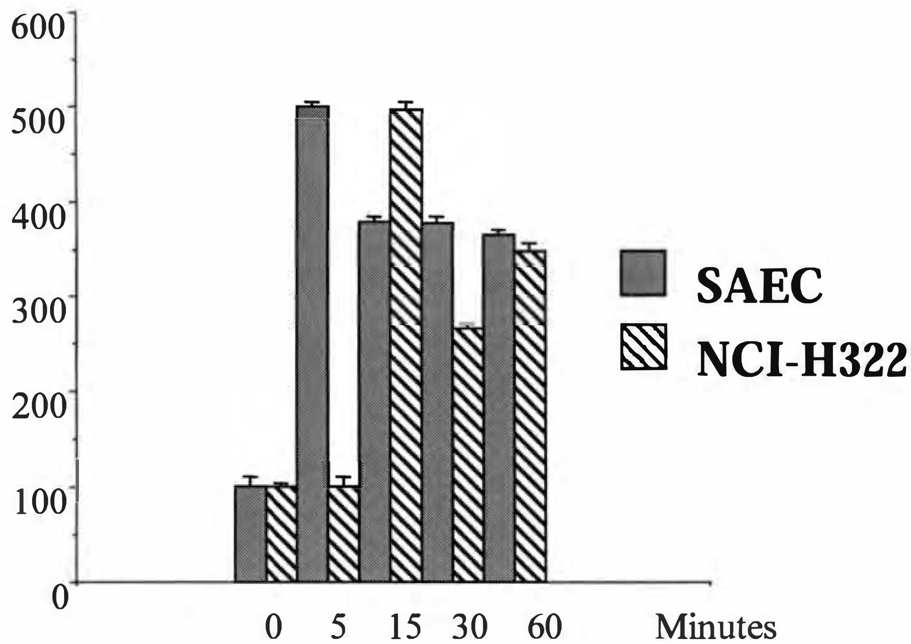

Figure 4B. Bar graph illustrating densitometry values of the bands in Figure 4A. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with triplicate samples. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in p-ERK1/2 were significant ($p<0.001$) in NCI-H322 cells at all time intervals tested and in SAECs after 5, 15, and 30 minutes.

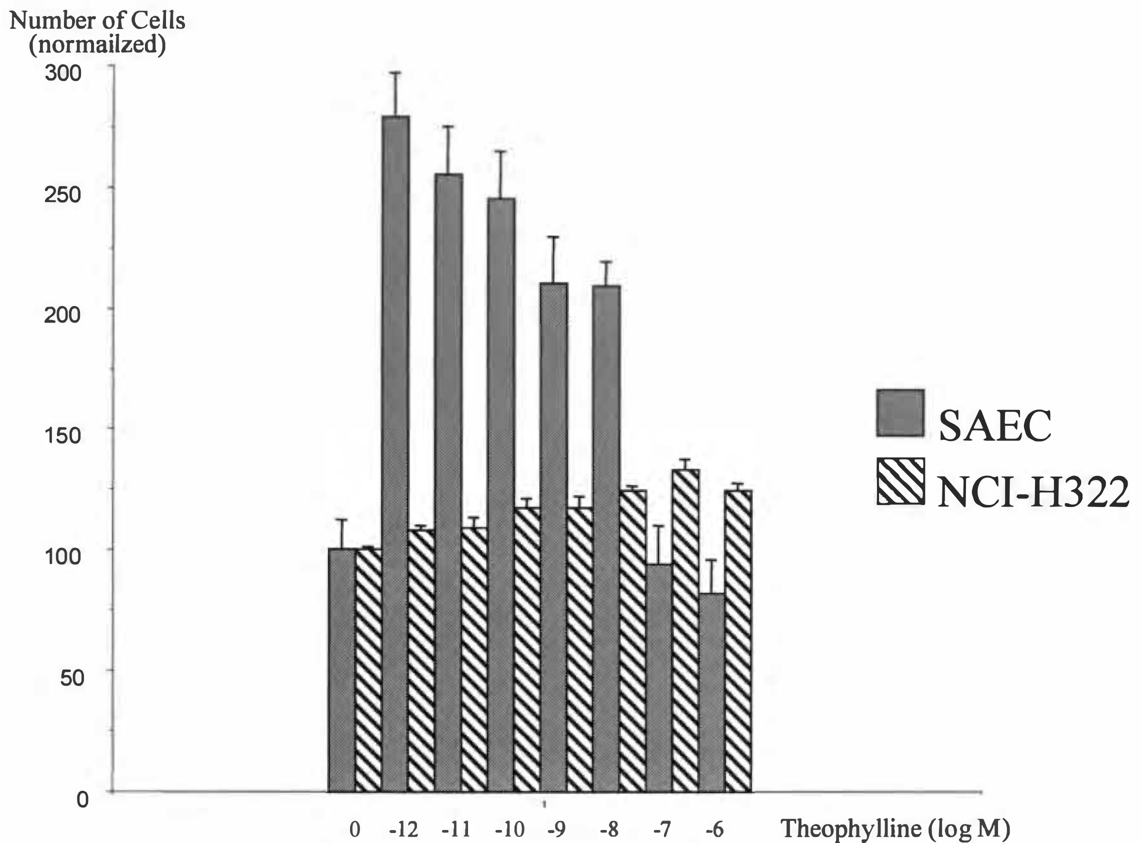

Figure 5. Effects of theophylline (1pM-1 μM) on cell number in SAEC and NCI-H322 cells as assessed by MTT assay. The cells were left in complete media for 5 hours to attach. The cells were then switched to fresh low serum media (0.05% FBS) for SAECs and (0.1% FBS) for NCI-H322. Cells were then exposed for 72 hours to theophylline at the concentrations indicated. Bars represent normalized (control values set at 100%) mean values and standard errors of three independent experiments, each with four samples per group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparison test and two-tailed unpaired t-test. The observed increases in SAECs were significant ($p<0.001$) at concentrations from 1 pM to 10 nM. The PACs were less responsive, with a small but significant ($p<0.01$) increase at the 10 and 100 nM concentrations.

CURRICULUM VITAE

Hussein Abdul-Hadi Nasser Al-Wadei was born on August 15th, 1967 in Yemen in a village called Wadea, Hashad. He received his elementary, secondary (middle) and part of his high school in Taif, Saudi Arabia, and then he went back to his native country to finish the last year of high school. Following high school graduation, he was awarded a scholarship from the Saudi government to study Veterinary Medicine at King Faisal University, Saudi Arabia from 1988 to 1992, where he obtained a Bachelor of Veterinary Medicine degree (DVM). He graduated with the highest academic record (cumulative grade point average) of the year in the College, for which he was rewarded the second Dean's honor, because of his performance and an excellent grades. The author finished his DVM degree within five years, which is the minimum time allowed to complete the degree. The language of his study was in English.

From May 1992 until July 1994 he began working at Al Omeri and Barakah Poultry Farms Company in his native country, Yemen, where he served as a monitor of broiler houses with a capacity of nearly 300,000 chickens; technical advisor; and finally, head of the veterinary department of the company.

From August 1994 to May 2000, he was employed by the Department of Animal Science, College of Agriculture, Sana'a University, Yemen and worked as a teaching assistant. He has taught various animal sciences and related subjects of veterinary medicine to under graduate students of Agriculture. He has advised students in carrying out research projects for the partial fulfillment of their graduation. He has also worked as

technical supervisor of the college farm, which contained cows, goats, sheep, poultry, and rabbits.

From July 1994 to May 2000 he worked with Nakhlan Poultry Company, Sana'a as head of the veterinary department for management and technical aspects in the company, also he has responsible for customers' farms.

In January 2002, he was admitted as a Ph.D. student in Pathology Department, Comparative and Experimental Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, USA. Under the supervision of Dr. Hildegard M. Schuller, the author completed his Ph.D. degree in fall of 2004, with a thesis entitled ***"Growth Stimulation of Pulmonary Adenocarcinoma and their Cells of Origin by Agents that Increase Intracellular cAMP"***. At present, he is the father of three sons and three daughters. He can be reached by email at:

husseinawadei@yahoo.com or Hussein_a_w32@hotmail.com

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