

Non-genomic inhibitory signaling of β -carotene in squamous cell carcinoma of the lungs

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Abstract. Studies have suggested that retinoids prevent lung cancer by interacting with nuclear retinoid receptors. However, clinical trials with β -carotene increased lung cancer mortality. We recently showed that β -carotene stimulates the proliferation of small airway-derived adenocarcinoma by increasing cAMP signaling. Here, we have tested the hypothesis that β -carotene may stimulate squamous cell carcinoma cells via similar mechanisms. We determined the effects of β -carotene in cell lines from squamous cell carcinomas and large airway epithelia on proliferation by MTT assays in the presence and absence of inhibitors. Signaling via cAMP/PKA was measured by immunoassays and PKA activation assay. Phosphorylated ERK1/2 was determined by Western blotting. β -carotene significantly inhibited proliferation and phosphorylation of ERK1/2 by $G\alpha_s$ -mediated signaling involving adenylyl cyclase, cAMP, PKA and ERK1/2. These findings introduce a non-genomic inhibitory mechanism of β -carotene and emphasize the need for the development of marker-guided lung cancer prevention.

Introduction

Lung cancer is the most common cause of cancer deaths in men and women worldwide (1,2). Lung cancer is divided into two basic types, non-small cell and small cell. This classification provides a standardized system useful in estimating prognosis and selecting treatment. About 80% of lung cancers are non-small cell. Non-small cell lung cancer (NSCLC) combines three types of lung cancer: squamous cell, adenocarcinoma and large cell carcinoma (3). Adenocarcinoma and small cell carcinoma are among the most common histological types of lung cancer. Adenocarcinoma is

thought to be derived from epithelial cells that line the peripheral small airways, whereas small cell carcinoma and squamous cell carcinoma are thought to be mainly derived from epithelial cells that line centrally located large airways. Small cell carcinoma and squamous cell carcinoma are developing exclusively in smokers while pulmonary adenocarcinoma PAC is additionally found in a significant number of non-smokers. The high mortality rate of lung cancer is due to the frequent lack of responsiveness to existing therapeutic strategies and the absence of effective diagnostic tools for the early detection of premalignant lesions. Effective strategies for the prevention of lung cancer are therefore urgently needed.

Observational epidemiologic studies have consistently shown that individuals eating a diet rich in fruit and vegetables, which are rich in carotenoids, have a lower risk of cancer, particularly lung cancer (4). The natural precursor of vitamin A, β -carotene, has been extensively tested in preclinical systems. Vitamin A deficiency has been shown to cause squamous cell metaplasia in large airways of hamsters, an effect reversed by treatment with retinoids (5). Since these early reports, numerous preclinical studies have shown genomic effects of high concentrations of retinoids via nuclear retinoid receptors that suggested these agents as cancer preventive agents (6). Based on these promising preclinical results, the α -tocopherol, β -carotene supplementation trial (ATBC) and the β -carotene and retinoid efficacy trial (CARET) in smokers and former smokers were conducted. Both trials had to be stopped early because of significant increases in lung cancer and cardiovascular mortality in the β -carotene or retinoid treated groups (7,8).

Studies in our laboratory have shown that the proliferation and migration of human lung cancer cell lines from small airway-derived pulmonary adenocarcinoma (PAC) is stimulated by β -adrenergic receptor signaling and that the tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) acts as an agonist for this receptor family (9,10). Exposure of PAC cells or immortalized human small airway epithelial cells to NNK or a classic β -adrenergic agonist increased intracellular cAMP, resulting in the activation of PKA, CREB and transactivation of the EGFR (11). Studies in a hamster model of NNK-induced small airway-derived PAC have shown that β -adrenergic agonists (12) or the phosphodiesterase inhibitor theophylline (13), that causes intracellular accumulation of cAMP,

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significantly promoted the development of this cancer type. In addition, we have shown that β -carotene or retinol stimulate the growth of immortalized human small airway epithelial cells and small airway-derived PAC cells by the activation of a signaling pathway that included cAMP, PKA, CREB and ERK1/2 (14). Since PAC constituted the largest population of lung cancers in the ATBC and CARET trials, these findings suggest that β -carotene may have promoted the development and progression of PAC by such non-genomic mechanisms in both clinical trials.

In the current study, we have investigated the effects of β -carotene on cell number, intracellular cAMP, PKA activation and ERK1/2 phosphorylation in immortalized human large airway epithelial cells and in three cell lines derived from human squamous cell carcinomas of the lung. Contrary to the stimulatory effects observed in PAC cells and their cells of origin, β -carotene significantly inhibited the proliferation and phosphorylation of ERK1/2 of squamous cell lung carcinoma and large airway epithelial cells via cAMP-mediated non-genomic signaling.

Materials and methods

Cell lines and tissue culture. The human squamous carcinoma cell lines of the lung (NCI-H226, NCI-H520 and NCI-H2170) and the SV40/adenovirus/12 hybrid virus immortalized human bronchial epithelial cell line BEAS-2B (15) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The three squamous carcinoma cell lines were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) purchased from (ATCC), while the immortalized human bronchial epithelial cell line BEAS-2B were maintained according to the ATCC recommendation in Bronchial Epithelial cell Basal serum-free medium (BEBM) supplemented with 2 ml bovine pituitary extract (BPE), 0.5 ml bovine insulin, 0.5 ml of 10^{-7} M hydrocortisone (HC), 0.5 ml retinoic acid (RA), 0.5 ml transferrin, 0.5 ml triiodothyronine (T3), 0.5 ml epinephrine, 0.5 ml human epidermal growth factor (hEGF) (Cambrex). All cells were grown in an atmosphere of 5% CO₂ and 99% humidified air and maintained in antibiotic-free media. All assays with all cell lines were conducted in basal media without any supplements.

Assessment of cell proliferation by MTT assay. The modulation of cell proliferation by β -carotene (Sigma, St. Louis, MO, USA) in the presence and absence of inhibitors was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma) as previously described according to the manufacturer's instructions (14). The MTT test is based on the NADH-dependent enzymatic reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] in metabolically active cells but not in dead cells, thus measuring the number of viable cells. While both cell proliferation and apoptosis regulate viable cell numbers, the investigated signaling pathways have been clearly linked with DNA synthesis indicative of cell proliferation in previous experiments with different cell lines (23). Cells were seeded into 6-well tissue culture plates (Falcon, Franklin Lakes, NJ, USA) at a density of 50,000 cells per well in their respective

basal media without additives. β -carotene dissolved in DMSO (Fisher Scientific, NJ, USA), was added to the wells to yield the final concentrations specified in the Figure legends and incubated for 72 h. Preliminary tests with DMSO treated versus untreated cells indicated that DMSO had no effect in the MTT assay (data not shown), a finding in accord with the suggestion that DMSO appears to be the most appropriate vehicle for delivering β -carotene to cultured cells (24). Cells treated with DMSO alone served as controls. Pre-incubation with the inhibitor of adenylyl cyclase, SQ22536 (SQ; 50 nM) or cholera toxin (CTX; 100 nM; Calbiochem, Gibbstown, NJ, USA), which downregulates G α_s -coupled receptors, was for 10 min and 4 h, respectively. Three hours before the end of the incubation time, 100 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.5 mg/ml) dissolved in phenol red-free medium was added to allow for the metabolic conversion of the MTT substrate to blue formazan. The media were replaced with isopropanol, and optical density was measured at 570 nm with background subtraction at 630 nm using a uQuant microplate spectrophotometer [MQX200, Bio-Tek Instruments, Inc., Winooski, VT, USA (ELISA reader)]. Data are expressed as mean values and standard errors of four samples per treatment group. Each experiment was repeated twice and yielded similar data. The viability of cells under the exposure conditions used was monitored by trypan blue dye exclusion and was >98%.

cAMP immunoassay. Cell culture, treatment and sample preparation for cAMP immunoassay was conducted in accordance with the manufacturer's instructions as previously described (14). Cells were seeded at 4×10^5 cells per well in their respective complete media in 6-well plates and grown until 65-70% confluence. The completed media was changed to basal media for 24 h. Then cells were washed twice with 1X PBS, pretreated for 30 min with 1 mM IBMX (Sigma). β -carotene (Sigma) dissolved in DMSO was then added to the appropriate basal media to yield a final concentration of 20 nM for the time intervals specified in the Figure legends. After three washes with water, the cells were treated with 0.1 M HCl for 20 min, then lysed by sonication. After centrifugation, the samples were analyzed for cAMP levels using a direct cyclic AMP enzyme immunoassay kit according to the manufacturer's instructions (Assay Designs Inc., Ann Arbor, MI). Briefly, the assay utilizes *p*-nitrophenyl phosphate as a substrate and a polyclonal antibody to cAMP to bind in a competitive way, the cAMP in sample that has cAMP covalently attached to it. Reactions were stopped by the addition of trisodium stop solution and color intensity was measured at 405 nm as a primary reading and 590 nm as a reference reading wavelength with KC junior computer software (Bio-Tek Instruments, Inc.). Data are expressed as mean values and standard errors of triplicate samples per treatment group. The experiments were repeated twice with each repetition yielding similar data.

PKA activation assay. Following incubation of cells with 20 nM β -carotene for 5 min to 2 h as specified in the Figure legends, PKA activity was assayed in cell lysates using a PepTag assay for non-radioactive detection of activated PKA

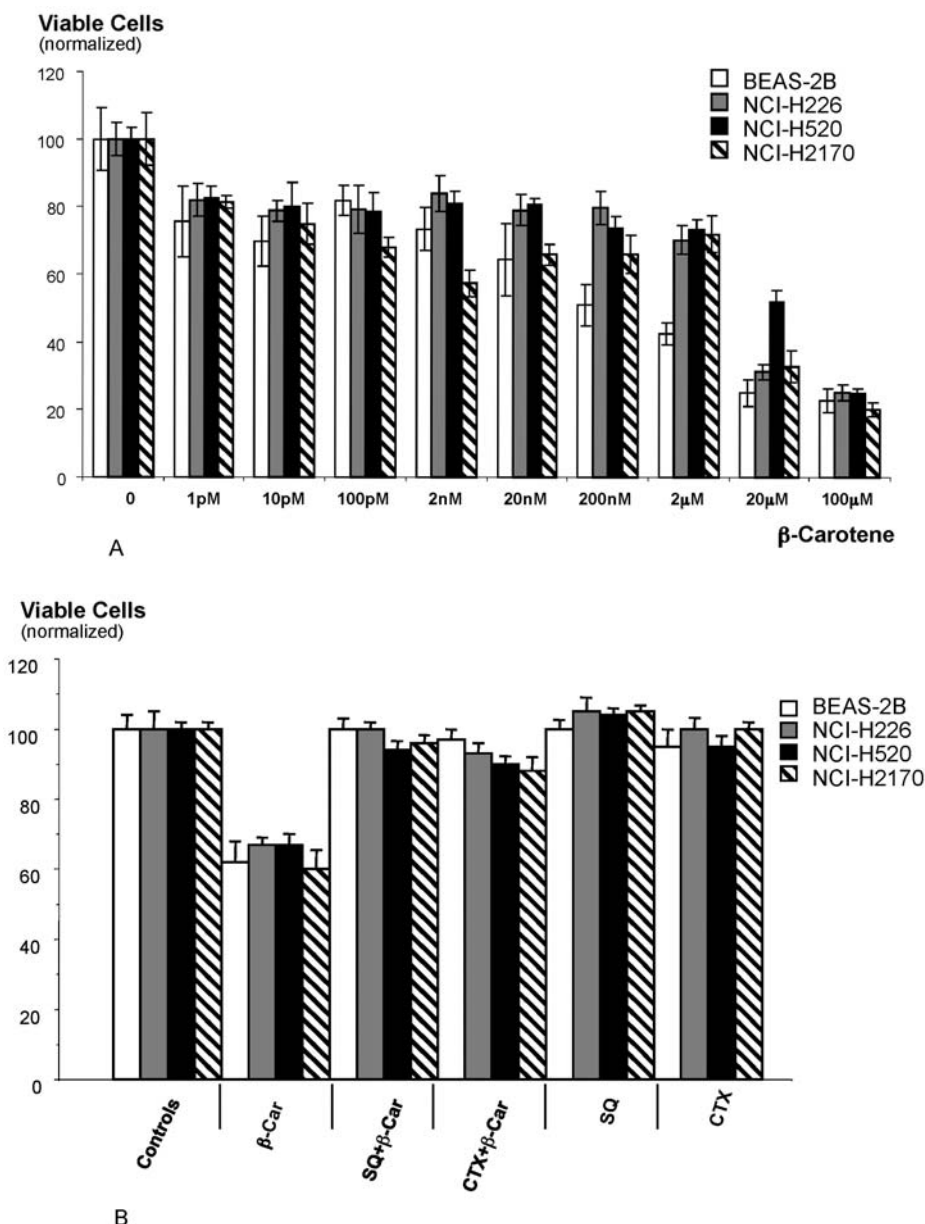


Figure 1. Results of MTT assays for the assessment of cell proliferation in the human large airway epithelial cells, BEAS-2B, and the human squamous cell lung carcinoma cells, NCI-H226, NCI-H520 and NCI-H2170, after exposure for 72 h to β -carotene (A). All cell lines responded with a concentration-dependent reduction in cell numbers. The observed responses were highly significant ($p < 0.001$) at all β -carotene concentrations tested. Preincubation of cells with SQ22536 (SQ, 10 min, 50 nM) or cholera toxin (CTX, 4 h, 100 nM) abrogated ($p < 0.001$) the inhibitory effects of β -carotene (20 nM) in all cell lines (B). Data are mean values and standard errors of four samples per treatment group. Each assay was repeated twice with similar results.

(Promega Corp., Madison, WI, USA), following the instructions 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 (-) (28). Briefly, reactions containing a brightly colored fluorescent PeTag A1 peptide (0.4 μ g/ml), peptide protection, PepTag PKA reaction 5X buffer, and PKA activator 5 x solutions and cAMP-dependent protein kinase, catalytic subunit 2,500 U (control enzyme) were incubated in ice for 5 min followed by 1 min at 30°C. Samples were then added, and the mixtures were incubated at room temperature for 30 min, heated to 95°C for 10 min, and loaded into 0.8%

agarose gel in 50 mM Tris-HCl (pH 8.0). The protein kinase A activity in samples is determined by examining the gel under UV light. The densitometric analysis of each band was quantitatively assessed by image analysis using NIH Scion image analysis software. Five densitometric readings per band were taken and mean values and standard errors were calculated after background subtraction. Each experiment was repeated twice with similar results.

Assessment of total proteins and phosphorylated proteins by Western blotting. To assess the effects of 20 nM β -carotene on the expression and phosphorylation of the mitogen activated protein kinases ERK1/2, 500,000 cells were seeded into culture vessels (100 cm²) containing their respective

growth media. When the adherent cells had reached 60-65% confluence, they were rinsed one time with 1X PBS and switched into basal media. Following removal of the serum-starved media and replacement with fresh basal media, 20 nM β -carotene were added to the culture vessels and cells were incubated from 5 min to 2 h. Cells exposed to the β -carotene vehicle (DMSO) served as controls. The cultured cells then were washed once with cold 1X 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 phenylmethylsulfonyl fluoride, 1 μl pepstatin, 1 μl leupeptin, 1 μl aprotinin and 0.25% NP-40. Protein lysates were centrifuged and the concentration was determined by using the BCA Protein Assay (Pierce). Equal amounts of protein samples and 4 x protein loading buffer were denatured by heating at 95°C for 5 min, separated on 12% sodium dodecyl sulfate-polyacrylamide gel, and transferred to nitrocellulose membrane. After transfer, the membranes were blocked with 5% non-fat dry milk in 1X Tris-buffered saline with Tween-20 (TBST) for 1 h and then incubated over night at 4°C with the primary antibodies. After the incubation with the primary antibodies a 5 washes with TBST for 5 min each has followed. The membranes were then incubated with horseradish peroxidase-labeled secondary antibody (goat anti-mouse or goat anti-rabbit; Cell Signaling) for 1 h at room temperature. Membranes were then washed five times 5-min each with 1X TBST and the Immunoreactive bands were detected by using a chemiluminescent reaction [Enhanced chemiluminescence (ECL); Amersham Biosciences, Piscataway, NJ] through autoradiography on Kodak Bio-Max XAR film. Three separate Western blottings were conducted for each antibody per sample and yielded similar data. Relative densities of the bands were determined by image analysis using National Institutes of Health (NIH) SCION image-analysis software.

Statistical evaluation of data. Statistical analysis of all data was by one-way analysis of variance (ANOVA), Tukey-Kramer multiple comparison test, and two-tailed unpaired t-test.

Results

Analysis of cell proliferation by MTT assays revealed significant ($p < 0.001$) and concentration-dependent growth inhibiting effects of β -carotene after 72 h of incubation in the respective basal media at concentrations from 1 pM to 100 μM in the large airway epithelial cells, BEAS-2B, and all three squamous cell lung carcinoma cells (Fig. 1A). Maximum inhibition of proliferation was observed with the highest concentration (100 μM) of β -carotene, with the number of viable BEAS-2B cells reduced to 25% of controls, NCI-H226 cells to 30% while NCI-H520 cells and NCI-H2170 cells were each reduced to 20% of controls (Fig. 1A). Preincubation of the cells for 10 min with the inhibitor of adenylyl cyclase SQ22536 (50 nM) completely abrogated these inhibitory effects of β -carotene (20 nM) in all cell lines (Fig. 1B). Since adenylyl cyclase is the exclusive mediator of intracellular cAMP formation (16), these findings suggest that the observed inhibition of cell proliferation in response to β -carotene was mediated by non-genomic signaling via cAMP.

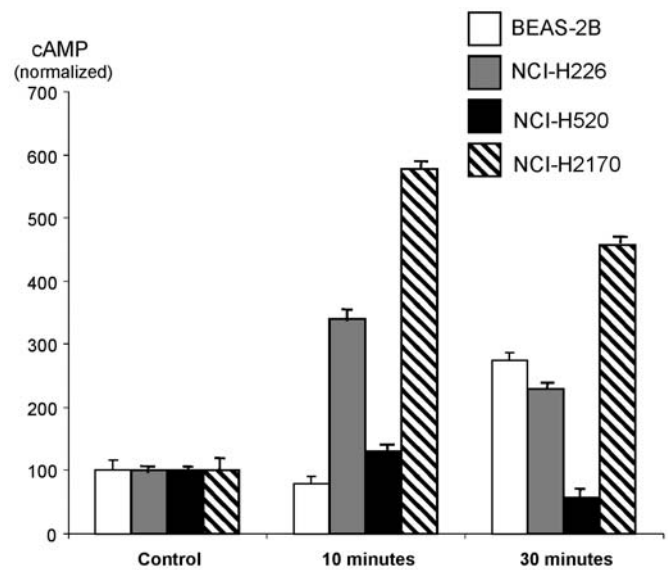


Figure 2. Effects of 20 nM β -carotene on intracellular cAMP as assessed by a cAMP immunoassay in large airway epithelial cells BEAS-2B, squamous cell lung carcinoma cells NCI-H226, NCI-H520 and NCI-H2170 after 10 and 30 min of incubation. β -carotene significantly ($p < 0.001$) increased the concentration of cAMP in all cell lines. Data are mean values and standard errors of triplicate samples per treatment group. Each assay was repeated twice and yielded similar results.

While other mechanisms do exist, the predominating stimulus for the activation of adenylyl cyclase that triggers the formation of cAMP is the binding of an agonist to a $G\alpha_s$ -coupled receptor (16). We therefore preincubated the cells for 4 h with cholera toxin (CTX, 100 nM) to downregulate $G\alpha_s$ in our cells prior to their exposure to β -carotene (20 nM). As Fig. 1B shows, CTX almost completely ($p < 0.001$) blocked the inhibition of cell proliferation by β -carotene. These findings suggest that the observed inhibitory signaling of β -carotene involved binding of this agent to a $G\alpha_s$ -coupled cell membrane receptor.

Our interpretation that inhibition of cell proliferation by β -carotene involved non-genomic signaling via cAMP was additionally supported by the results of the cAMP immunoassays (Fig. 2) and the PKA activation assays (Fig. 3). Each of the four cell lines demonstrated significant ($p < 0.001$) increases in intracellular cAMP after 10 and 30 min of exposure to 20 nM β -carotene, with peak values in all cell lines at either 10 min or at 30 min time interval (Fig. 2). In accord with these findings, the downstream effector of cAMP, PKA, was activated by 20 nM β -carotene in all four cell lines in a time-dependent manner as evidenced by migration of the phosphorylated peptide to the positive electrode (Fig. 3A). Quantitative analysis of PKA activity by densitometry of the bands (Fig. 3B) revealed that the increase in activated PKA levels was highly significant ($p < 0.001$) in all cell lines after 15, 30 and 60 min of exposure to β -carotene (20 nM), with the highest levels (2.8-fold increase over controls) observed at 15 min in NCI-H226 cells.

Western blottings which monitored the expression of phosphorylated and unphosphorylated ERK1/2 proteins over time in response to 20 nM β -carotene revealed a pronounced reduction in the activation of P-ERK1/2 bands in BEAS-2B

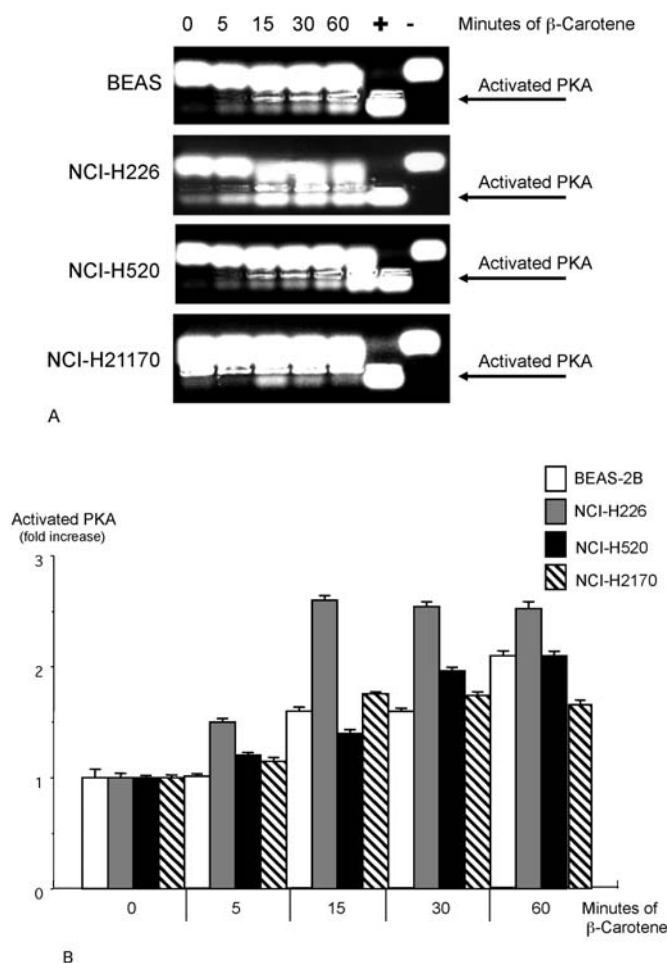


Figure 3. PKA activation over time in response to 20 nM β -carotene in large airway epithelial cells BEAS-2B, squamous cell lung carcinoma cells NCI-H226, NCI-H520 and NCI-H2170 as assessed by a non-radioactive PKA activation assay (A). Quantitative assessment of PKA activity by densitometry of the bands is illustrated in (B). Columns in the graph are mean values and standard errors of five densitometric readings per band. The increases in PKA activity were highly significant ($p < 0.001$) in all cell lines.

and all squamous lung carcinoma cells NCI-H226, NCI-H520 and NCI-H2170 cells at all time intervals (5-60 min; Fig. 4A). Quantitative assessment of p-ERK1/2 levels by densitometry of the bands revealed that the observed reduction in p-ERK1/2 protein was highly significant ($p < 0.001$) at all time intervals tested in all four cell lines (Fig. 4B). These findings are in accord with the inhibition of cell proliferation observed in the MTT assays in response to β -carotene (Fig. 1A).

Discussion

Our data provide evidence, for the first time, that β -carotene induces an inhibitory intracellular non-genomic signaling cascade that involves the stimulation of a G_{α_s} -coupled cell membrane receptor, resulting in the activation of adenylyl cyclase, formation of cAMP and activation of PKA and inhibition of ERK1/2 phosphorylation and cell proliferation. Unlike the classic transcriptional effects of retinoids via nuclear retinoid receptors (17,18), the observed signaling

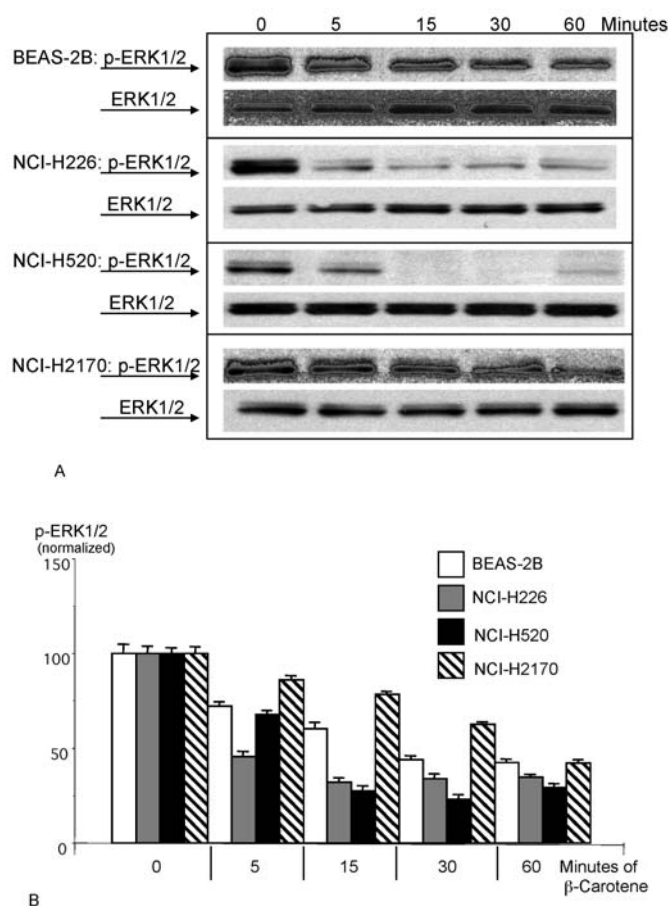


Figure 4. Western blotting (A), indicating the effects of 20 nM β -carotene on the expression of phosphorylated and unphosphorylated ERK1/2 proteins. β -carotene decreased the expression of P-ERK1/2 in large airway epithelial cells BEAS-2B and squamous cell lung carcinoma cells NCI-H226, NCI-H520 and NCI-H2170. Quantitative assessment of p-ERK1/2 by densitometry of the bands is illustrated in (B). Columns in the graph are mean values and standard errors of five densitometric readings per band. The decreases p-ERK1/2 protein were highly significant ($p < 0.001$) in all cell lines and correlate with the inhibition of cell proliferation by β -carotene illustrated in Fig. 1.

responses were rapid, resulting in significant increases of intracellular cAMP and activated PKA within 10-15 min. Moreover, the abrogation of the resulting stimulated cell proliferation via pharmacological downregulation of G_{α_s} by CTX in conjunction with a similar effect of the adenylyl cyclase inhibitor SQ clearly point to a direct interaction of retinoid with a G_{α_s} -coupled receptor. Our data, were generated in three cell lines derived from human squamous cell carcinomas of the lung and in an immortalized cell line from human large airway epithelial cells, considered the origin of this type of lung cancer (19) and are in accord with a publication that reported the stimulation of cellular differentiation in human tracheo-bronchial epithelial cells via cAMP signaling independent of nuclear retinoid receptors (20). Non-genomic cAMP signaling induced by β -carotene, retinol, 9-cis-retinoic acid, 13-cis-retinoic acid or all-trans-retinoic acid has also been reported by us in human small airway epithelial cells (synonym: bronchiolar epithelial cells) and in small airway-derived pulmonary adenocarcinomas (14,21). However, contrary to our current observations in

large airway epithelial and squamous cell carcinoma cells, the retinoid-induced cAMP signaling significantly stimulated ERK1/2 phosphorylation and cell proliferation. These seeming discrepancies are due to inherent cell type-specific differences in the physiological growth regulation of large airway epithelial cells versus small airway epithelial cells that are apparently maintained by the tumor cells derived from these different epithelia. Large airway epithelial cells and squamous cell carcinoma cells are regulated by nicotinic acetylcholine receptors (22,23) that activate a signaling cascade via the EGF receptor (EGFR)→tyrosine kinase→ras→raf→ERK1/2, a pathway frequently inhibited by PKA via blockage of raf (24). The β -carotene-induced inhibition of p-ERK1/2 and cell proliferation observed by us in our current study may thus have involved inhibition of raf by PKA. By contrast, small airway epithelial cells and the adenocarcinomas derived from them are under positive growth control by β -adrenergic receptors that activate G_{α_s} -dependent signaling via adenylyl cyclase/cAMP/PKA (11). In conjunction with our current findings, these data emphasize the need for the development of marker-guided cancer prevention. The arbitrary long-term treatment with any agent that modulates signal transduction of individuals at risk for lung cancer should be avoided as it can lead to the selective promotion of certain types of cancer. Instead, tests need to be developed that allow the identification of hyperactive and hypoactive signaling pathways prior to and during cancer preventive treatments.

Acknowledgements

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