

Low Concentrations of Beta-carotene Stimulate the Proliferation of Human Pancreatic Duct Epithelial Cells in a PKA-dependent Manner

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Abstract. *Background: Pancreatic ductal adenocarcinoma (PDAC) is among the most common causes of cancer death. Preclinical and clinical studies on the preventive effects of beta-carotene or other retinoids have used dietary supplements that yielded high systemic concentrations (1-50 µM). While some of the preclinical data suggested cancer preventive effects of these agents, they have disappointed in clinical investigations. Materials and Methods: The effects of low concentrations (10 fM-200 nM) of beta-carotene on the proliferation, intracellular cAMP levels, PKA activation status and phosphorylation of EGFR-specific tyrosine kinases and ERK1/2 in immortalized human pancreatic duct epithelial cells was investigated. Results: Our data show significant concentration-dependent and PKA-dependent stimulation of all measured endpoints. Similar responses were achieved with forskolin. Our data indicate that low concentrations of beta-carotene stimulate the proliferation of the putative origin of PDAC, pancreatic duct epithelial cells via cAMP and PKA-dependent transactivation of the EGFR pathway. This could potentially have promoting effects on the development of PDAC.*

Pancreatic cancer is the fifth most common human cause of cancer-related death with a mortality rate near 100% within one year of diagnosis (1). The majority of these malignancies are classified as pancreatic ductal adenocarcinomas (PDACs) due to phenotypic similarities of the cancer cells with pancreatic duct epithelia, their putative origin. The lack of responsiveness of pancreatic cancer to conventional

chemotherapy has prompted efforts for the development of agents that prevent the development of this malignancy in populations at risk. Smoking, diabetes, and pancreatitis are risk factors for pancreatic cancer (2). However, the mechanisms how these risk factors trigger the development of pancreatic cancer are poorly understood.

Vitamin A (retinol) is formed in mammalian cells from beta-carotene, contained in numerous vegetables. The classic concept of vitamin A as an essential protector of epithelial cell integrity (3) has prompted investigations into potential cancer preventive and therapeutic effects of retinoids on pancreatic cancer. The results of these studies, most of which have been conducted with dietary supplements of retinoids that yield high systemic concentrations, remain controversial. It has thus been shown that retinoids reduced the progression of premalignant lesions to overt pancreatic cancer in a rat model of azaserine-induced acinar cell carcinoma (4). By contrast, four different retinoids tested at high or low dose level dietary supplements showed tumor promoting effects on PDAC induced in hamsters by N-nitroso(2-oxopropyl)-amine (5). Similarly, dietary dried cabbage, which yields low systemic concentrations of beta-carotene, significantly promoted the development of PDAC induced in hamsters by this nitrosamine (6). All-*trans* retinoic acid (ATRA, 10 µM), a major metabolite of vitamin A, inhibited the proliferation of the human PDAC cell line, Capan-1, but enhanced cell invasion (7). The same vitamin A metabolite (0.10-10 µM) inhibited proliferation while inducing apoptosis in ten different human PDAC cell lines (8) whereas another laboratory reported cell-cycle arrest in one of these cell lines, panc-1 only when ATRA concentrations of 1-50 µM were used (9). High concentrations (10 µM-10 M) of the retinoids ATRA or 9-*cis*-retinoic acid (9-*cis*-RA) also significantly reduced cell numbers of human pancreatic cancer cell lines (10). However, clinical trials in patients with advanced pancreatic cancer showed no response to 13-*cis*-retinoic acid (13-*cis*-RA) when administered at a dose of 1 mg/kg per day,

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which approximates to a systemic concentration of 46.6 μM in a person of 70 kg bodyweight and a mean total blood volume of 5 liters (11). On the other hand, another trial with a similar regimen reported prolonged stable disease (12), while the administration of 20 mg/day of beta-carotene in the Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) trial in male smokers had no statistically significant effect on the incidence or mortality of pancreatic cancer (13).

Studies in cell lines from human PDAC and normal duct epithelia have shown that these cells are regulated by beta-adrenoreceptors *via* the release of arachidonic acid and stimulation of cell proliferation (14, 15). The nitrosated carcinogenic nicotine-derivative, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been identified as a high affinity agonist for these receptors (14, 16), suggesting that this potent carcinogen may directly interfere with the beta-adrenoreceptor regulation of PDAC. In support of this hypothesis, treatment of human pancreatic duct epithelial cells with NNK or a classic beta-adrenergic agonist stimulated cell proliferation *via* an increase in intracellular cAMP, resulting in the activation of protein kinase A (PKA) and transactivation of the EGFR and its downstream effectors, ERK1/2 (15, 17). These findings suggest that agents that increase cAMP may stimulate the proliferation of pancreatic duct epithelial cells and the tumors arising from them, thus contributing to the development of pancreatic cancer.

Recent investigations with cell lines derived from human small airway epithelial cells and the lung adenocarcinomas derived from such cells have revealed a novel mechanism of action of low concentrations (1 pM-200 nM) of beta-carotene, retinol, 9-*cis*-RA, and 13-*cis*-RA (18, 19). Each of these agents caused an increase in intracellular cAMP, resulting in the activation of PKA, ERK1/2 and stimulation of cell proliferation (18, 19). In light of the PKA-dependent transactivation of the EGFR pathway discovered in pancreatic duct epithelial cells treated with beta-adrenergic agonists (15, 17), these findings raise concern that low concentrations of beta-carotene may stimulate the growth of these cells by transactivation of the EGFR pathway, thus potentially promoting the development of pancreatic cancer. In the current study, we have therefore investigated the effects of low concentrations of beta-carotene on cell proliferation, intracellular cAMP concentration, activation of PKA, EGFR-tyrosine kinases and ERK1/2 in an immortalized cell line derived from human pancreatic duct epithelial cells.

Materials and Methods

Cell culture. The human pancreatic duct epithelial cells, HPDE6-c7 have been established by transduction of the *HPV16-E6E7* genes into primary cultures of normal pancreatic duct epithelial cells (20). The cells were maintained in keratinocyte serum free medium supplemented with bovine pituitary extract (25 $\mu\text{g}/500\text{ ml}$) and epidermal growth factor (2.5 $\mu\text{g}/500\text{ ml}$) at 37°C with 5% CO_2 .

The cells were washed with phosphate buffered saline (PBS) and maintained in basal medium without additives for 24 h prior to each assay and during the assays.

Assessment of cell proliferation by MTT assay. The effects of beta-carotene (Sigma, St Louis, IL, USA) or the activator of cAMP, forskolin (Sigma) on cell proliferation were assessed using the colorimetric 3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (2) (Sigma). Cells were seeded into 6-well tissue culture plates (Falcon, Franklin Lakes, NJ, USA) at a density of 50,000 cells per well in basal medium without additives. Beta-carotene dissolved in dimethyl sulfoxide (DMSO) or forskolin dissolved in PBS were added to plates immediately to yield the final concentrations specified in Figure 1 and incubated for 72 h at 37°C. Cells treated with DMSO alone served as controls. Pre-incubation of cells with an inhibitor of PKA (H89, 1 μM) was for 10 min. Three hours before the end of the incubation time, 100 μl of 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (0.5 mg/ml) was dissolved in complete medium together with phenol-free medium (Gibco, Carlsbad, CA, USA) and added to the cells for 2-3 h to allow metabolic conversion of the MTT substrate to blue formazan. The media were replaced with isopropanol and optical density at 570 nm was determined using an ELISA reader.

Data are expressed as mean values and standard errors of four samples per treatment group. Each experiment was repeated twice and yielded similar data. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparisons test and two-tailed unpaired *t*-test. The viability of cells under the exposure conditions used was monitored using trypan blue dye exclusion.

CAMP immunoassay. Cells were plated in complete medium at 4×10^5 cells per 6-well plate and grown until 65-70% confluence. The cells were washed with PBS and maintained in basal medium without additives for 24 h. Following two washes with PBS, the cells were then pre-incubated for 30 min with 1mM IBMX (Sigma) and then exposed to beta-carotene (20 nM) in DMSO or forskolin (200 nM) in PBS in fresh basal medium containing 1 mM IBMX for 10 or 30 min. After 3 washes with distilled water, cells were treated with 0.1 M HCl for 20 min then lysed by sonication. After centrifugation, 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, USA). Briefly, the assay utilizes p-nitrophenyl phosphate as a substrate and a polyclonal antibody to cAMP that binds to the cAMP in the sample. Reactions were stopped with trisodium phosphate and color intensity was measured at 405 nm.

Data are expressed as mean values and standard errors of triplicate samples per treatment group. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparisons test and two-tailed unpaired *t*-test. The experiments were repeated twice with each repetition yielding similar data.

PKA activation assay. Following incubation of cells with beta-carotene (20 nM in DMSO) for 5 min to 2 h as specified in Figure 2, PKA activity was assayed in cell lysates using a Pep Tag assay for non-radioactive detection of activated PKA (Promega Corporation, Madison, WI, USA), following the instructions of the manufacturer. Cells treated for 30 min with forskolin (200 nM) served as positive controls. This assay utilizes a fluorescent

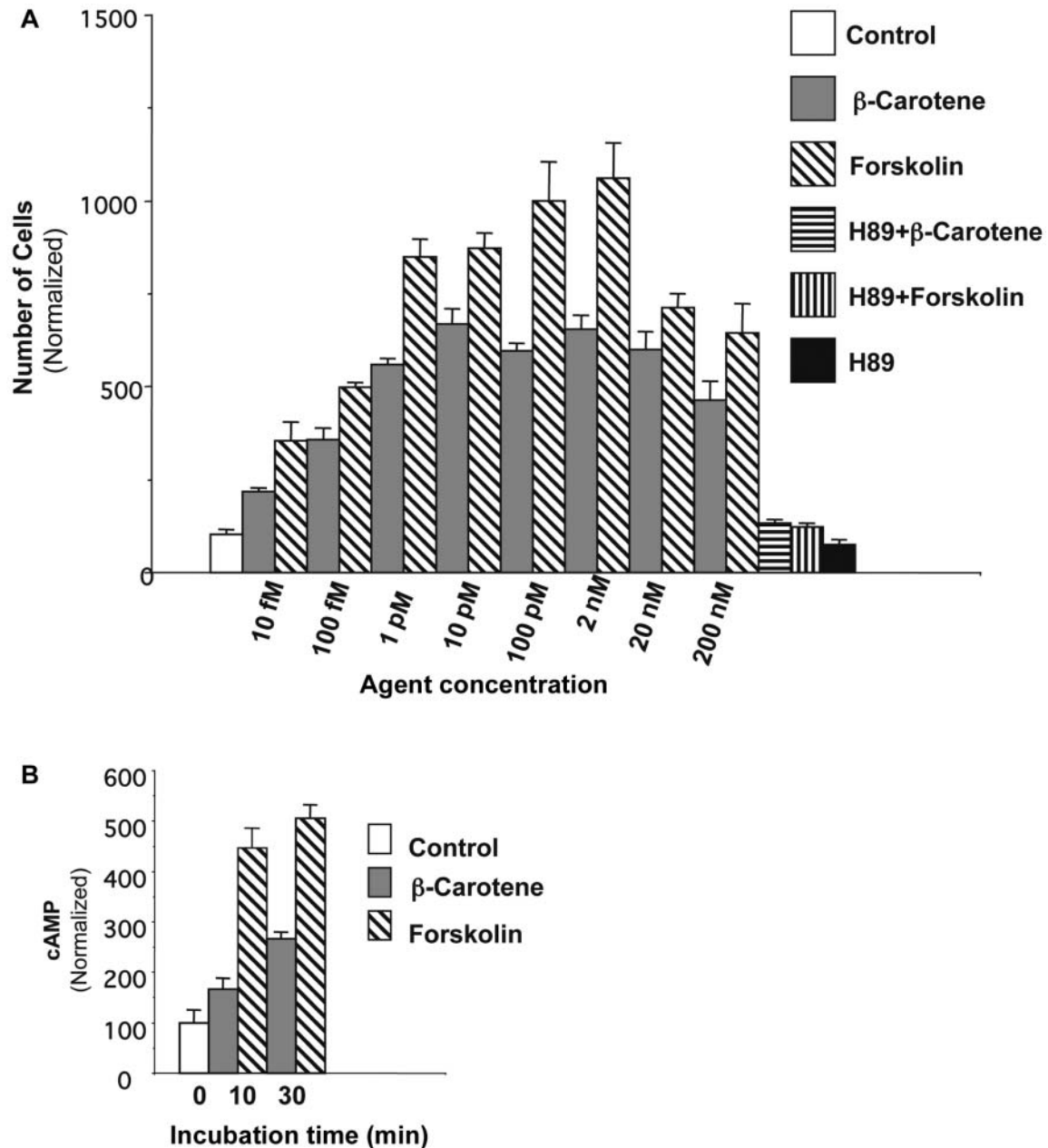


Figure 1. (A) Results of MTT assays, illustrating the effects of beta-carotene and forskolin on the number of HPDE6-c7 cells after incubation for 72 h. Both agents yielded significant ($p < 0.001$) stimulation at all concentrations tested, an effect completely blocked by the PKA inhibitor, H89 (1 μ M). Data are mean values and standard errors of four samples per treatment group. (B) Data of cAMP immunoassays, showing a significant ($p < 0.001$) increase in intracellular cAMP in cells exposed to beta-carotene (20 nM) or forskolin (200 nM) for 10 or 30 min. Data are mean values and standard errors of triplicate samples per treatment group.

substrate for PKA that changes the peptide's net charge upon phosphorylation of PKA, thus allowing the phosphorylated peptide to migrate to the positive electrode, while the non-phosphorylated peptide migrates to the negative electrode. Briefly, reactions containing a brightly colored fluorescent peptag A1 peptide (0.4 μ g/ml), peptide protection, peptag PKA reaction and PKA activator solutions were incubated in ice for a few

minutes before 1 min incubation at 30°C. After adding samples, reactions were incubated at room temperature for 30 min, boiled at 95°C for 10 min then loaded onto 0.8% agarose gel in 50 mM Tris-HCl (pH 8.0). At this point the qualitative assay was complete; the protein kinase A activity in samples was determined by examining the gel under UV light. Each experiment was repeated once with similar results.

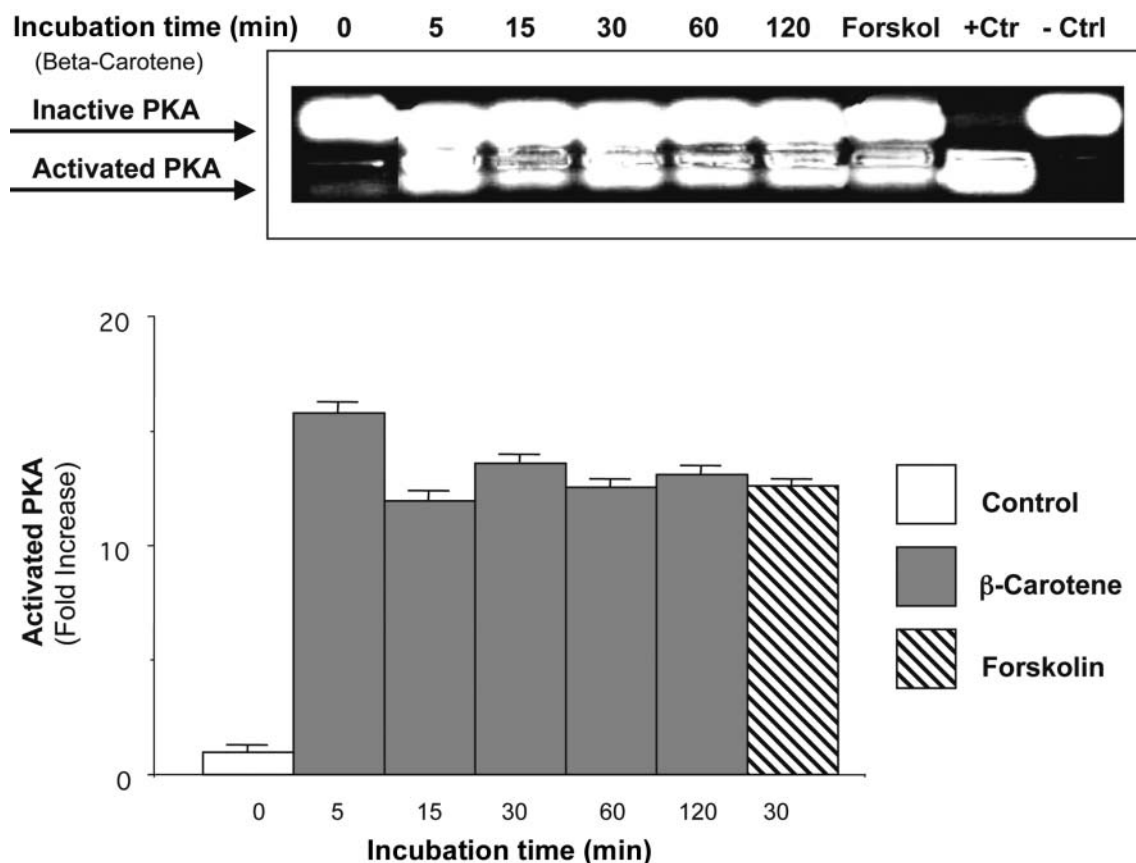


Figure 2. The levels of activated PKA in HPDE6-c7 cells in response to beta-carotene (20 nM) or forskolin (200 nM) were assessed using a nonradioactive Pep Tag assay kit. Beta-carotene significantly ($p < 0.001$) induced PKA activation at all time intervals tested. The classic inducer of cAMP, forskolin, served as positive control. Additional positive and negative controls (+Ctrl, -Ctrl) were provided by the kit. The data in the graph are mean values and standard errors of five densitometric readings per band.

Assessment of total proteins and phosphorylated proteins by Western blotting. To assess the effects of beta-carotene (20 nM) or forskolin (200 nM) on the expression and phosphorylation of the mitogen activated protein kinases ERK1/2 and of the EGFR-specific tyrosine kinases 992 or 1068, 500,000 cells were seeded into culture vessels (100 cm²) containing complete medium. When the cells had reached 60-65% confluence, they were rinsed once with PBS and placed into basal medium without additives for 24 h. Following removal of the media and replacement with fresh basal medium, beta-carotene in DMSO or forskolin in PBS were added to the culture vessels to yield final concentrations of 20 nM or 200 nM, respectively. Cells were then incubated from 5 min to 2 h at 37°C as detailed in Figure 3. Cells exposed to the retinoid vehicle (DMSO) served as controls. The cultured cells then were washed once with cold PBS, lysed in 20 mM Tris-base, 200 mM NaCl, 1 M sodium fluoride, 0.5 M EDTA, 100 mM Na₃VO₄, 100 mM PMSF, 1 µl pepstatin, 1 µl leupeptin, 1 µl aprotinin, and 0.25% NP-40. Protein samples were denatured by boiling at 95°C for 5 min, separated on 12% SDS-PAGE and then transferred to nitrocellulose. Membranes were blocked with 5% non-fat dry milk and then incubated over night at 4°C with the following primary antibodies at a 1:1000 dilution: rabbit polyclonal for total ERK1/2,

rabbit polyclonal for Thr202/Tyr204 phosphorylated Erk1/2 (Cell Signaling Technologies, Beverly, MA, USA), rabbit polyclonal EGFR and rabbit polyclonal anti-phosphorylated EGFR specific to tyrosines 992 and 1068 (Cell Signaling Technology), or mouse polyclonal anti-phosphorylated tyrosine 1173 (Sigma). Three densitometric readings per band (NIH Scion image analysis software) were taken and mean values and standard errors were calculated. Each experiment was repeated twice and yielded similar data. Statistical analysis of data was by one-way ANOVA, Tukey-Kramer multiple comparisons test and two-tailed unpaired *t*-test.

Results

Analysis of cell proliferation using MTT assays revealed a concentration-dependent increase in the number of viable cells upon exposure for 72 h to beta-carotene at concentrations from 10 fM to 200 nM (Figure 1A). This response peaked at the 10 pM concentration with a 6.6-fold increase. The classic activator of cAMP, forskolin, also yielded concentration-dependent increases in cell numbers,

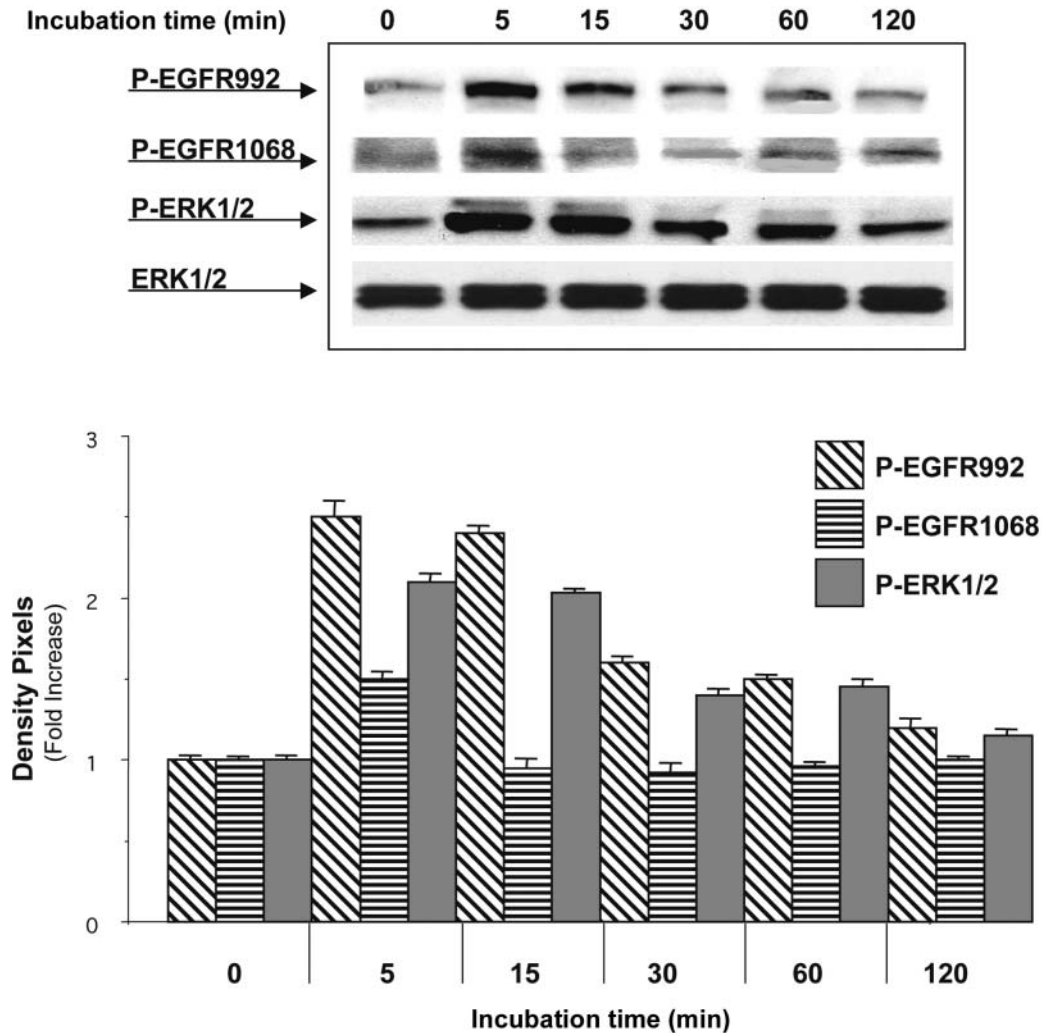


Figure 3. Western blot showing the effects of beta-carotene (20 nM) on the levels of phosphorylated EGFR992, EGFR1068 and ERK1/2. A significant ($p < 0.001$) induction of all three phosphorylated proteins was observed after 5 min of incubation. Data in the graph are mean values and standard errors of three densitometric readings per band.

suggesting that the response to beta-carotene may be mediated by stimulation of cAMP. In support of this interpretation, beta-carotene and forskolin each increased the levels of intracellular cAMP as monitored by immunoassays in cells exposed for 10 or 30 minutes (Figure 1B). The observed increases in cell numbers and cAMP were significant ($p < 0.001$) for both agents at all concentrations tested. Preincubation of the cells for ten minutes with the PKA inhibitor, H89 (1 μ M), prior to the addition of beta-carotene (20 nM) or forskolin (20 nM) completely blocked the stimulation of cell proliferation (Figure 1A). These findings indicate that the observed increases in cell numbers depended on the activation of PKA.

To verify this interpretation, we conducted PKA activation assays. As Figure 2 shows, exposure of the cells to beta-

carotene (20 nM) from 5 min to 2 h significantly ($p < 0.001$) increased the levels of activated PKA at all time intervals tested, with peak levels (12-fold) observed at 5 min. Cells treated with forskolin (200 nM) for 30 min showed a similar level of PKA activity (Figure 2). We previously showed that the induction of intracellular cAMP in response to betaadrenoreceptor stimulation phosphorylated the EGFR at tyrosines 992, 1068, and 1173 and their downstream effectors, ERK1/2 in the human pancreatic duct epithelial cells HPDE6-c7 (15).

In the current study, we therefore tested the hypothesis that beta carotene transactivates the EGFR/ERK1/2 pathway in a PKA-dependent manner in these cells. Cells were exposed from 5 min to 2 h to beta-carotene (20 nM) and cell lysates were processed for the detection of P-

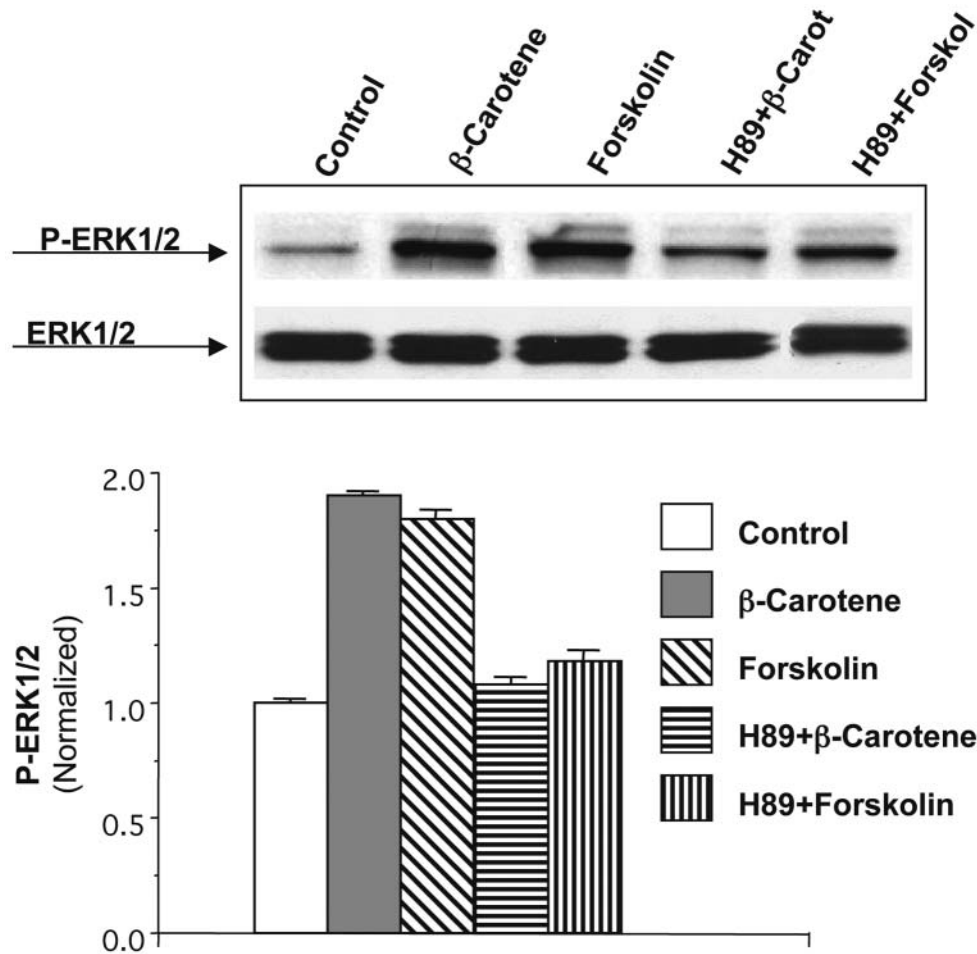


Figure 4. Western blot illustrating similar levels of P-Erk1/2 induction in cells exposed for 5 min to beta-carotene (20 nM) or forskolin (200 nM). These responses were significantly ($p < 0.001$) inhibited by pre-incubation of the cells for 10 min with the PKA inhibitor H89 (1 μ M). Data in the graph are mean values and standard errors of triplicate densitometric readings per band.

EGFR992, P-EGFR1068, PEGFR1173, P-ERK1/2 and ERK1/2 by Western blotting. As Figure 3 shows, the cells responded with an increase in the expression levels of P-EGFR992, PEGFR1068 and P-ERK1/2. Peak levels of all three phosphorylated proteins were observed after 5 min of exposure (P-EGFR992: 2.5-fold; P-EGFR1068: 1.5-fold; PERK1/ 2: 2.1-fold). By contrast, no increase in the phosphorylation status of EGFR1173 was detected at the time intervals tested (data not shown). We then treated the cells with beta-carotene (20 nM) or forskolin (200 nM) for 5 min with or without a 10 min pre-incubation with H89 (1 μ M) and monitored the expression levels of PERK1/ 2 by Western blotting. As shown in Figure 4, forskolin evoked a similar level of P-ERK1/2 induction as beta-carotene, indicating that this response was cAMP-mediated. In addition, H89 completely blocked P-ERK1/2 induction in response to betacarotene or forskolin (Figure 4). These data support the

hypothesis that the observed activation of the EGFR/ERK1/2 pathway and resulting cell proliferation in response to beta-carotene was cAMP- and PKA-dependent.

Discussion

Our data show, for the first time, that low concentrations of beta-carotene similar to systemic levels after the ingestion of vegetables rich in beta-carotene stimulate the proliferation of human pancreatic duct epithelial cells *via* cAMP/PKA mediated cellular signaling mechanisms that involve the transactivation of the EGFR/ERK1/2 pathway. Identical growth-stimulating signaling induced by beta-carotene, 9-cis-RA, or 13-cis-RA has recently been reported in human small airway epithelial cells and lung adenocarcinomas with phenotypic features of these cells (18, 19). On the other hand, it has been shown that cAMP/PKA signaling in response to 9-

cis-RA or 13-cis-RA inhibited the phosphorylation of ERK1/2 and cell proliferation in human large airway epithelial cells and small cell lung cancer cells (19). Similar to the current study, the experiments in lung cells were conducted with low concentrations (1 pM-200 nM) of retinoids. It hence appears that in addition to the cancer preventive effects of high retinoid concentrations *via* interactions with nuclear retinoid receptors, low concentrations of these agents stimulate cAMP/PKA at the cell membrane level. In turn, the intracellular events downstream of cAMP/PKA appear to be cell type-specific, with some cells responding with a transactivation of the EGFR pathway and increased cell proliferation while others demonstrate inhibition of the EGFR pathway and cell proliferation.

The EGFR and its associated Ras→Raf→MEK→ERK1/2 signaling pathway is frequently overexpressed in human PDAC and is therefore thought to play an important role in the development of this malignancy (21). The recent observation that beta-adrenergic signaling transactivates this pathway in human pancreatic duct epithelial cells (15) in conjunction with the identification of the tobacco-specific carcinogen NNK as an agonist for beta-adrenoreceptors (14, 16) has provided an important mechanistic explanation for the well documented association between smoking and pancreatic cancer. In addition, nicotine stimulates the release of the physiological agonists for beta-adrenoreceptors, epinephrine and norepinephrine (22, 23), thus further contributing to the hyperstimulation of beta-adrenoreceptors in smokers. In turn, the chronically hyperstimulated adrenoreceptors continuously transactivate the EGFR/ERK pathway, thus providing PDAC cells with a growth advantage.

It is generally accepted that smoking-associated beta-adrenoreceptor hyperstimulation is causally linked with the development of cardiovascular disease (24). In fact, betablockers and aspirin are the leading drugs for the prevention and therapy of this disease complex (25, 26). Recently emerging evidence suggests that beta-adrenoreceptor hyperstimulation also plays a crucial role in the development of the leading solid human cancers, adenocarcinoma of the lung (16, 27), pancreas (14, 15), colon (28), prostate (29), mammary gland (30, 31) and stomach. The beta-carotene-induced cAMP/PKA-mediated transactivation of the EGFR pathway in human pancreatic duct epithelial cells observed in the current study represents an additional stimulation of downstream effectors of beta-adrenoreceptors. While this may result in additive growth stimulation, the increase in intracellular cAMP may additionally sensitize beta-adrenoreceptors to agonists, thus rendering the receptors responsive to below threshold concentrations of nicotine-evoked catecholamines or NNK. The wide-spread chronic use of non prescription dietary supplements containing beta-carotene, retinol, or other retinoids as well as long-term diet plans with selective high retinoid-containing components

should be discouraged. Instead of the intended cancer preventive effects, such measures may in fact promote the development of adenocarcinomas that are under beta-adrenergic growth control. On the other hand, retinoids may well serve as valuable adjuvants for the treatment of cancers or precancerous lesions regulated by signaling pathways that are inhibited by cAMP/PKA.

Acknowledgements

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