

β -Carotene Downregulates the Steady-State and Heregulin- α -Induced COX-2 Pathways in Colon Cancer Cells¹

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ABSTRACT Experimental studies have shown that β -carotene inhibited the growth of colon cancer cells, and human trials have demonstrated that the carotenoid reduces colon cell proliferation of adenomatous polyps; however, molecular mechanisms underlying this chemopreventive activity remain unclear. Because COX-2 has been implicated as a causative factor in colon carcinogenesis, the present study was designed to investigate the relation between the growth-inhibitory effect of the carotenoid and COX-2 expression in colon cancer cells. We evaluated the effects of β -carotene on the growth of human colon adenocarcinoma cells overexpressing (LS-174, HT-29, WiDr) or not expressing (HCT116) COX-2. We also studied COX-2 expression induced by heregulin- α , apoptosis induction, reactive oxygen species (ROS) production, and extracellular signal-regulated kinase 1/2 (ERK1/2) activation. β -Carotene (0.5–2.0 μ mol/L) decreased COX-2 expression ($P < 0.05$) and prostaglandin E₂ (PGE₂) production ($P < 0.05$) in colon cancer cells. This effect was not observed in cells treated with retinoic acid or retinol. The downregulation of COX-2 by the carotenoid occurred in both untreated and heregulin-treated cells. It was accompanied by an increased ability of cells to undergo apoptosis and by a decrease in intracellular ROS production and in the activation of ERK1/2. Moreover, cells not expressing COX-2 were insensitive to the growth-inhibitory and proapoptotic effects of the carotenoid. Here, we report that the suppression of COX-2 by β -carotene may represent a molecular mechanism by which this compound acts as an antitumor agent in colon carcinogenesis. *J. Nutr.* 135: 129–136, 2005.

KEY WORDS: • β -carotene • COX-2 expression • cell growth • apoptosis • colon cancer cells

Accumulating evidence suggests that colorectal tumorigenesis may be regulated by cyclooxygenase (COX)³-2 (1), an inducible enzyme responsible for the conversion of arachidonic acid to prostaglandins. Tsujii and DuBois (2) first reported that cells expressing high levels of COX-2 had increased tumorigenic potential that could be reversed by COX-2 inhibitors. Using an APC knockout mouse model, Oshima et al. (3) demonstrated that COX-2 expression was induced very early in neoplastic progression. Interestingly, in that study, the number and size of intestinal polyps were dramatically reduced by specific COX-2 inhibitors (3). Moreover, recent studies showed increased levels of COX-2 in colorectal carcinomas compared with adjacent normal appearing mucosa (4–6).

COX-2 expression is induced by growth factors such as epidermal growth factor (EGF) or tumor growth factor- α in a

number of cell systems, including rat intestinal epithelial cells (7) and HCA-7 colon cancer cells (8). High levels of this protein have been associated with a decreased ability of cells to undergo apoptosis, suggesting that COX-2 expression may protect cancer cells from apoptosis induced by a variety of stimuli and could enhance cell tumorigenic potential (9). Thus, it has been suggested that COX-2 inhibitors are also able to act as apoptotic inducers (10–12).

Recently, much attention has been devoted to identifying colon cancer chemopreventive agents of dietary origin (13). In particular, evidence from epidemiologic studies showed that a high dietary intake of fruit and vegetables, rich in β -carotene and other carotenoids, is associated with a low risk for colon neoplasia (14–16). Although the ATBC and the CARET trials showed that β -carotene supplements increased the incidence of lung cancer in smokers (17–19), an extensive intervention study in China showed a significant protective effect of β -carotene in combination with vitamin E and selenium on gastrointestinal cancer in a population at high risk (20). Moreover, β -carotene supplementation reduced the rate of colon cell proliferation in patients with adenomatous polyps (21). Interestingly, the carotenoid was reported to accumulate in colonic neoplastic tissues in humans (22). Concomitantly, protection by β -carotene against colon cancer was shown in animal models (23–25) as well as in cultured cells (26–30). In

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³ Abbreviations used: COX, cyclooxygenase; DCF, di(acetoxymethyl ester) analog (C-2938) of 6-carboxy-2[prime],7[prime]-dichlorodihydrofluorescein diacetate; DTT, dithiothreitol; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase 1/2; FCS, fetal calf serum; NDF, neu-differentiation factor; PGE₂, prostaglandin E₂; PI, propidium iodide; ROS, reactive oxygen species; THF, tetrahydrofuran; TUNEL, terminal dUTP nick end labeling.

particular, we observed recently that β -carotene arrested the growth of different human colon adenocarcinoma cells in a manner strictly related to the cell's ability to accumulate the carotenoid and by a mechanism involving both cell cycle arrest and induction of apoptosis (30).

Taken together, these data raise questions about the possibility that the growth-inhibitory and proapoptotic effects of β -carotene observed in experimental and clinical studies may involve a reduction in the expression of COX-2. Therefore, the present study aimed at verifying the effect of β -carotene on the growth of human colon adenocarcinoma cells overexpressing (LS-174, HT-29, WiDr) or not expressing (HCT116) COX-2, COX-2 expression induced by the growth factor heregulin- α , which promotes COX-2 expression through the stimulation of HER2/HER3 receptors, and induction of apoptosis. Moreover, to further elucidate the molecular mechanisms by which β -carotene may regulate COX-2 expression, we also investigated its effect on reactive oxygen species (ROS) production and on the activation of a redox-sensitive upstream signaling enzyme, extracellular signal-regulated kinase 1/2 (ERK1/2).

MATERIALS AND METHODS

Cell culture. The LS-174 human colon adenocarcinoma cancer cell line (American Type Culture Collection) and the WiDr human colon adenocarcinoma cancer cell line (American Type Culture Collection) were cultured in RPMI 1640 medium (Gibco Biocult). HT-29 human colon adenocarcinoma cells (American Type Culture Collection) were grown in MEM medium. HCT116 colon carcinoma cells were cultured in McCoy's 5a. Cells were maintained in log phase by seeding twice a week at a density of 3×10^5 cells/L at 37°C under 5% CO₂/air atmosphere. In all of the experiments, the medium was supplemented with 1% (v/v) fetal calf serum (FCS; Flow) and 2 mmol/L glutamine. FCS was added at a concentration of 1% (v/v) to minimize the effects of other growth-promoting substances normally contained in serum. β -Carotene (Fluka Chemika-bioChemika), all-trans-retinoic acid (Sigma-Aldrich), and all-trans-retinol (Sigma-Aldrich) were delivered to the cells (10^9 cells/L) using tetrahydrofuran (THF) as a solvent, containing 0.025 g/L BHT to avoid the formation of peroxides. The purity of β -carotene was verified to be 97% (27). The stock solutions of the compounds were prepared immediately before each experiment. From the stock solutions, aliquots of β -carotene, retinol, or retinoic acid were rapidly added to the culture medium to give the final concentrations indicated. The amount of THF added to the cells was not >0.1% (v/v). Control cultures received an amount of solvent (THF) equal to that present in carotenoid-treated cultures. Cells treated with THF and untreated cells did not differ in cell number or viability. β -Carotene, as well as retinol and retinoic acid, was added to the cells for 24 h, and the medium was not further replaced throughout the experiments. Experiments were routinely carried out in triplicate. After the incubation, cells were harvested and quadruplicate hemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

Cell cycle analysis. Cell cycle distribution was analyzed by flow cytometry, as previously described (31). Aliquots of 10^6 cells were harvested by centrifugation ($600 \times g$ for 5 min), washed in PBS, and fixed with ice-cold 70% ethanol. The cells were incubated at 4°C for 30 min and then were centrifuged at $2500 \times g$ for 10 min. The pellet was resuspended in 0.5 mL PBS and 0.5 mL DNA-Prep stain (Coulter Reagents), containing 1 g/L RNase and 50 g/L propidium iodide (PI). All of the tubes were incubated for 30 min in the dark at 4°C. The DNA content of cells stained with PI was measured with a FACScan instrument using Multicycle AV software.

Apoptosis detection. The percentage of apoptotic cells was determined by in situ terminal dUTP nick end labeling (TUNEL) (29). The percentage of TUNEL-positive apoptotic cells (labeling index) was counted at 400X magnification. In the absence of terminal deoxynucleotidyl transferase, no unspecific staining was observed. For

each slide, 3 randomly selected microscopic fields were observed and at least 100 cells/field were evaluated.

Caspase-3 activity assay. The activity of caspase-3 was determined as indicated by Palozza et al. (32). Briefly, after a 24-h treatment, cells (2×10^6) were lysed in 50 mmol/L Tris-HCl buffer, pH 7.5, containing 0.5 mmol/L EDTA, 0.5% IGEPAL, and 150 mmol/L NaCl; cell lysate was incubated with 50 μ mol/L fluorogenic substrate, Ac-DEVD-AMC (Alexis Biochemicals), in a reaction buffer [10 mmol/L HEPES, pH 7.5, containing 50 mmol/L NaCl and 2.5 mmol/L dithiothreitol (DDT) for 120 min at 37°C. The release of AMC was measured with excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer.

Western blot analysis of COX-1, COX-2, p-ERK and total ERK expression. Cells (10×10^6) were lysed in ice-cold buffer (1 mmol/L MgCl₂, 350 mmol/L NaCl, 20 mmol/L HEPES, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L Na₄P₂O₇, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L aprotinin, 1.5 mmol/L leupeptin, 1 mmol/L Na₃VO₄, 20% glycerol, 1% NP-40) and cell lysate was centrifuged for 10 min at 4°C ($10,000 \times g$) to obtain the supernatants, which were used for Western blot analysis with anti-COX-2 (clone C-20, catalog #1745, Santa Cruz Biotechnology), anti-COX-1 (clone C-20, catalog #1752, Santa Cruz Biotechnology), anti-total ERK (clone K-23, catalog #94, Santa Cruz Biotechnology), and anti p-ERK (clone E-4, catalog #7383, Santa Cruz Biotechnology) monoclonal antibodies. The blots were treated as previously described (32). The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

Measurement of ROS. Cells treated with varying concentrations of the carotenoid were harvested to evaluate ROS production using DCF (Molecular Probes) as previously described (27). Before the addition of the fluorescent probes, 2×10^6 cells were washed to eliminate the amount of the carotenoid that was not cell associated. Fluorescent units were measured in each well after 30 min of incubation with DCF (10 μ mol/L) using a Cytofluor 2300/2350 Fluorescence Measurement System (Millipore). β -Carotene at a concentration of 1–2 μ mol/L did not alter the basal fluorescence of DCF.

Measurement of prostaglandin E₂ (PGE₂) production. Cells were plated at a density of 1.7×10^5 cells/cm² in a medium containing 1% (v/v) FCS. After 24 h, cells were treated with varying β -carotene concentrations (0.5–2.0 μ mol/L) in the absence or presence of heregulin- α (0.5 μ g/L). The medium was harvested 24 h later and PGE₂ levels were measured by RIA, as previously described (33). Neither β -carotene nor heregulin- α were cross-reactive with the PGE₂ antibody (data not shown). Briefly, 25 μ L of culture medium were diluted to 250 μ L with 0.025 mol/L phosphate-buffer (pH 7.5) and mixed with 63 kBq of [³H]PGE₂ and appropriately diluted (1:120,000) antiserum to give a final volume of 1.5 mL. A duplicate standard curve (ranging from 2 to 400 pg/tube, with an effective concentration of 28 pg/tube) was run with each assay. Separation of antibody-bound PGE₂ was obtained with activated charcoal (Sigma), which absorbs 95–98% of free PGE₂. After centrifugation at $600 \times g$ for 10 min at 4°C, supernatant solutions were decanted directly into 10 mL of liquid scintillation fluid. Radioactivity was measured by liquid scintillation counting. The results are expressed as pg PGE₂/10⁵ cells.

Extraction and analysis of β -carotene. β -Carotene was extracted with 1 volume methanol and 3 volumes hexane:diethyl ether (1:1) from 10×10^6 cells after 24 h of treatment with β -carotene and analyzed by HPLC, as described earlier (27).

Statistical analysis. Three separate cultures per treatment were utilized for analysis in each experiment. Values are presented as means $\pm$ SEM. Multifactorial 2-way ANOVA was used to assess differences among the treatments (absence or presence of heregulin- α) and the carotenoid concentrations (Fig. 2B, 3C, 4B, Tables 1 and 2). When the F-test was significant ($P < 0.05$), post-hoc comparisons were made using Tukey's Honestly Significant Differences test. One-way ANOVA was used to assess differences among the treatments or the concentrations (Figs. 1, 2A, 3A, 3B, 4A, 6, 7). When differences were significant ($P < 0.05$), post-hoc comparisons of means were made using Fisher's test. Differences were analyzed using Minitab Software.

supplementation with 0.5 and 2 μmol/L β-carotene, respectively. Increasing the concentration of β-carotene significantly decreased COX-2 levels in both untreated (Fig. 1B) and heregulin-α-treated (Fig. 1C) LS-174 cells. The growth factor was used at a concentration of 0.5 μg/L (Fig. 1C), which was the most effective in inducing COX-2 expression (Fig. 1A). On the other hand, COX-2 expression did not differ between untreated and vehicle control cells (data not shown), suggesting that THF at concentrations up to 0.5% (v:v) did not modify the levels of this protein.

We also measured the levels of COX-1 in LS-174 cells after treatment with varying β-carotene concentrations for 24 h. In β-carotene-treated cells, COX-1 expression was 98.0 ± 0.5 , 99.2 ± 0.5 , and $101.0 \pm 0.6\%$ with respect to control cells, at β-carotene concentrations of 0.5, 1 and 2 μmol/L, respectively, indicating that there was no effect of the carotenoid treatment and suggesting that the carotenoid selectively interfered with COX-2 expression. Such results were not modified by treating LS-174 cells with β-carotene in the presence of heregulin-α (0.5 μg/L) (data not shown).

Cell growth. Heregulin-α treatment led to a dose-dependent increase in the number of LS-174 cells, at least up to the concentration of 0.5 μg/L (Fig. 2A). At higher concentrations of the growth factor (1 μg/L), a decrease in cell number was observed, which paralleled the reduction of COX-2 expression. Increasing the concentration of β-carotene significantly

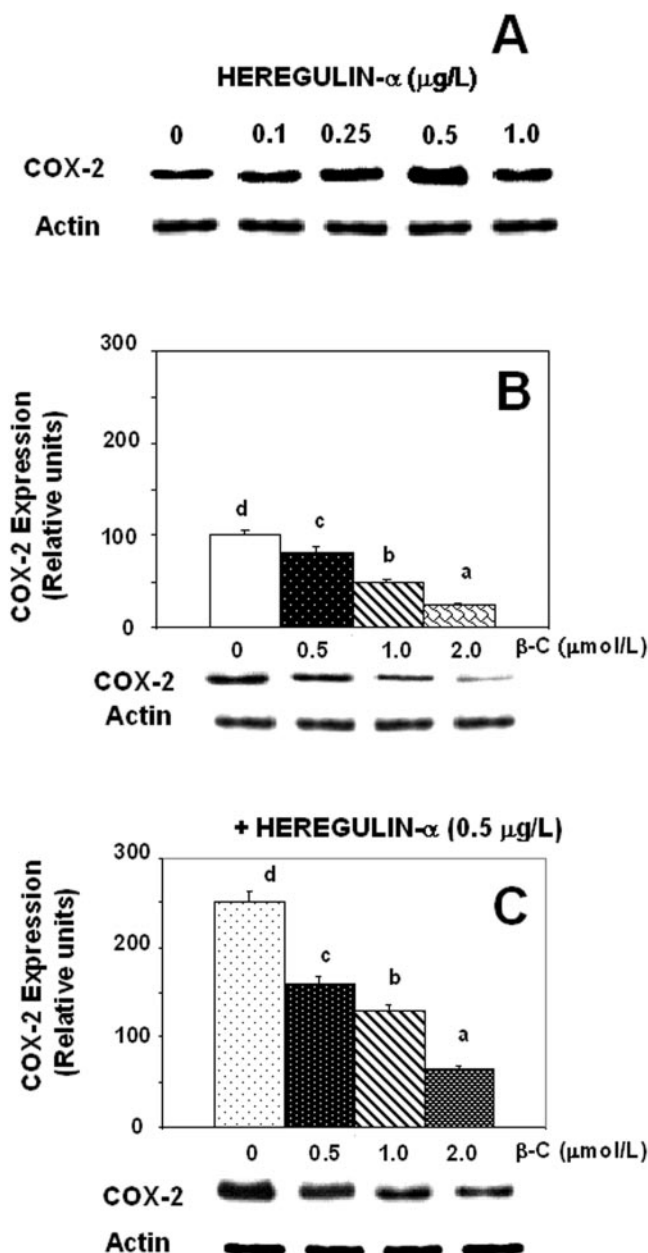


FIGURE 1 COX-2 expression in LS-174 cells treated for 24 h with heregulin-α (panel A), β-carotene (β-C) (panel B), and a combination of heregulin-α and β-carotene (panel C). Panel A shows a representative Western blot analysis of 3 different experiments. Panels B and C show representative Western blot analyses and histograms of densitometric analyses of the autoradiographs. Values are means $\pm$ SEM, $n = 3$. Means without a common letter differ, $P < 0.05$ (Fischer's test).

RESULTS

COX-2 expression in LS-174 cells. Exposure of LS-174 cells to heregulin-α, the human homologue of the rat neu differentiation factor (NDF), progressively increased COX-2 expression in a dose-dependent manner (Fig. 1A). Such an effect was observed up to 0.5 μg/L heregulin. At this concentration, heregulin-α increased COX-2 levels ~250% relative to baseline levels. No further increase, and even a decrease occurred at higher concentrations of the growth factor (1.0 μg/L). Cells acquired β-carotene in a nearly linear manner from 0.02 to 0.08 nmol β-carotene/10⁶ cells after 24 h of

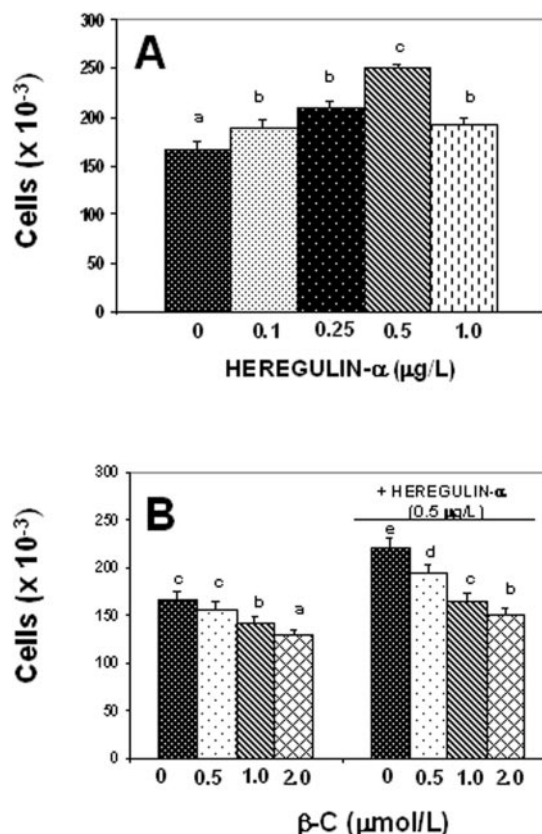


FIGURE 2 Cell growth of LS-174 cells treated for 24 h with heregulin-α (panel A), and β-carotene (β-C), alone and in combination with heregulin-α (panel B). Values are means $\pm$ SEM, $n = 3$ (panel A) and 6 (panel B). Panel A: means without a common letter differ, $P < 0.05$ (Fischer's test). Panel B: the treatment $\times$ concentration interaction was significant, $P < 0.05$. Means without a common letter differ, $P < 0.05$ (Fischer's test).

TABLE 1

Effect of a 24-h β -carotene treatment on cell cycle distribution of LS-174 cells in the absence or presence of heregulin- α^1

β -Carotene $\mu\text{mol/L}$	- Heregulin- α			+ Heregulin- α^2		
	G0/G1	S	G2/M	G0/G1	S	G2/M
	%					
0	70.0 $\pm$ 5.0	23.0 $\pm$ 1.9 ^A	7.0 $\pm$ 0.7 ^a	63.0 $\pm$ 6.0	30.0 $\pm$ 3.0 ^B	7.0 $\pm$ 0.6 ^a
0.5	69.0 $\pm$ 6.0	23.0 $\pm$ 2.0 ^A	8.0 $\pm$ 0.8 ^a	62.8 $\pm$ 6.0	29.0 $\pm$ 2.5 ^B	8.2 $\pm$ 0.9 ^a
1.0	68.0 $\pm$ 5.0	22.0 $\pm$ 2.0 ^A	10.0 $\pm$ 1.0 ^b	61.9 $\pm$ 6.0	27.0 $\pm$ 3.0 ^B	11.1 $\pm$ 1.2 ^b
2.0	66.0 $\pm$ 5.0	22.0 $\pm$ 2.0 ^A	12.0 $\pm$ 2.0 ^b	61.7 $\pm$ 6.0	26.0 $\pm$ 3.0 ^B	12.3 $\pm$ 1.4 ^b

¹ Values are means $\pm$ SEM, $n = 3$. Means for a cell cycle phase with superscripts without a common letter differ: uppercase, $P < 0.01$; lowercase $P < 0.05$ (Tukey's test).

² Heregulin- α concentration was 0.5 $\mu\text{g/L}$.

reduced cell number in both untreated and heregulin- α -treated LS-174 cells (Fig. 2B).

Cell cycle progression. In the absence of β -carotene, most of the LS-174 cells (70%) were in G0/G1 phase (Table 1). This effect was probably due to the low concentration of FCS in the medium (1%, v:v). The addition of heregulin- α at 0.5 $\mu\text{g/L}$ for 24 h increased the proportion in S phase ($P < 0.01$). Carotenoid addition progressively increased the percentage of cells in G2/M phase (Table 1); this effect was significant when the concentration was $\geq 1 \mu\text{mol/L}$ ($P < 0.05$) and occurred in the absence as well as in the presence of heregulin- α .

Induction of apoptosis. Heregulin- α treatment dose dependently decreased caspase-3 activity, at least up to the concentration of 0.5 $\mu\text{g/L}$ (Fig. 3A). Similar results were obtained when determining the percentage of apoptotic cells using the TUNEL method (data not shown). Treatment of LS-174 colon cancer cells with NS-398, which selectively inhibits COX-2 activity, induced a dose-dependent increase in apoptosis (Fig. 3B). When β -carotene was added to the cells in the absence or presence of heregulin- α (Fig. 3C), a dose-dependent increase ($P < 0.001$) in caspase-3 activity was observed. Similar results were obtained using the TUNEL method. The percentage of apoptosis in LS-174 cells treated with the carotenoid in the absence of heregulin- α was 1.4 ± 0.1 , 3.2 ± 0.3 , and 5.8 ± 0.5 at β -carotene concentrations of 0, 1, and 2 $\mu\text{mol/L}$, respectively; in the presence of heregulin- α , it was 1.0 ± 0.1 , 2.3 ± 0.2 , and 3.4 ± 0.3 at β -carotene concentrations of 0, 1, and 2 $\mu\text{mol/L}$, respectively. It is interesting to note that LS-174 cells stimulated by heregulin- α were less sensitive to apoptosis induction by the carotenoid than unstimulated LS-174 cells, supporting the hypothesis that the levels of COX-2 may be extremely important in determining cell susceptibility to apoptosis.

ROS production. We first measured the levels of ROS in cells stimulated by heregulin- α alone (Fig. 4A); then we measured the levels of ROS in cells treated with the carotenoid in the absence or presence of heregulin- α (Fig. 4B). A progressive increase in ROS production was observed in LS-174 cells after heregulin- α treatment, at least up to the concentration of 0.5 $\mu\text{g/L}$. On the other hand, a clear decrease in intracellular ROS production was observed in β -carotene-enriched LS-174 cells in the absence and presence of the growth factor (0.5 $\mu\text{g/L}$).

ERK1/2 activation. Phosphorylation levels of mitogen-activated protein kinase in untreated (Fig. 5A) and heregulin- α -treated (Fig. 5B) LS-174 cells were measured after the addition of varying β -carotene concentrations for 24 h. Control cells showed a low phosphorylation of ERK1/2; however,

this was deeply increased by heregulin- α addition (0.5 $\mu\text{g/L}$). The presence of the carotenoid in the culture medium significantly decreased ERK phosphorylation in a dose-dependent manner in both untreated and heregulin- α -treated cells, suggesting that the downregulation of COX-2 expression by

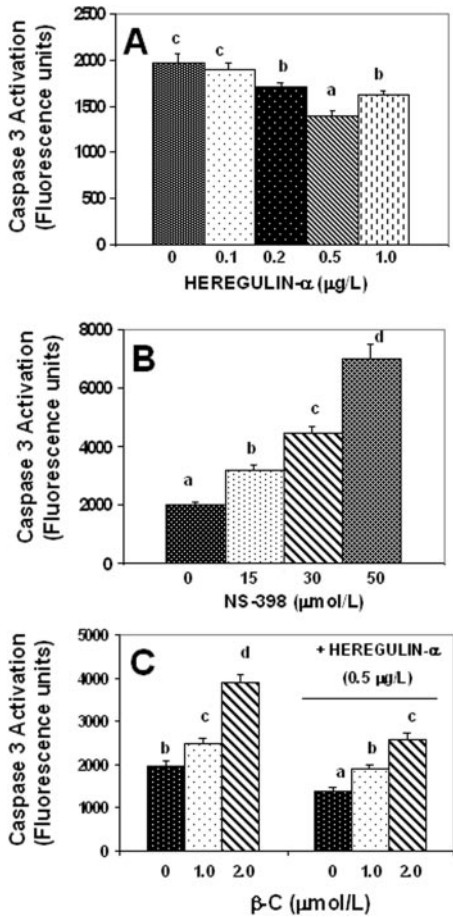


FIGURE 3 Apoptosis induction, measured as caspase-3 activation in LS-174 cells treated for 24 h with heregulin- α (panel A), NS-398 (panel B) and β -carotene (β -C), alone and in combination with heregulin- α (panel C). Values are means $\pm$ SEM, $n = 3$ (panel A) and 6 (panel B). Panels A and B: Means without a common letter differ, $P < 0.05$ (Fischer's test). Panel C: the treatment $\times$ concentration interaction was significant, $P < 0.05$. Means without a common letter differ, $P < 0.05$ (Fischer's test).

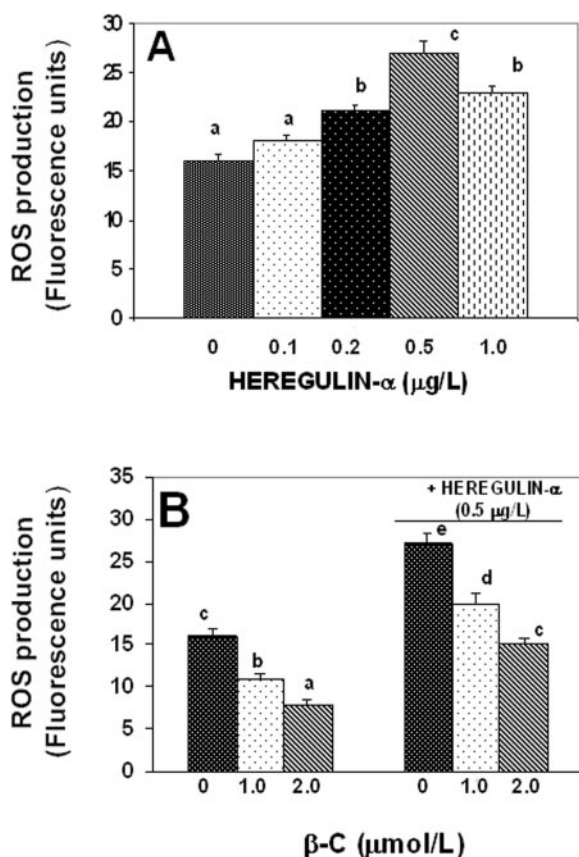


FIGURE 4 ROS production in LS-174 cells treated for 24 h with heregulin-α (panel A), and β-carotene (β-C), alone and in combination with heregulin-α (panel B). Values are means ± SEM, *n* = 3 (panel A) and 6 (panel B). Panel A: Means without a common letter differ, *P* < 0.05 (Fischer's test). Panel B: the treatment × concentration interaction was significant, *P* < 0.05. Means without a common letter differ, *P* < 0.05 (Fischer's test).

β-carotene was mediated, at least in part, through the ERK signaling pathway.

PGE₂ production. Heregulin-α (0.5 μg/L) stimulated production of PGE₂ in LS-174 cells, as reflected by the appearance of PGE₂ in the cell medium after 24 h (Table 2). COX-2 expression indicated that the carotenoid was able to reduce PGE₂ production in a dose-dependent manner in untreated LS-174 cells or those treated with the growth factor (0.5 μg/L) for 24 h.

Effect of retinol and retinoic acid on COX-2 expression. We also compared the potency of β-carotene, retinol, and retinoic acid in lowering COX-2 expression in LS-174 cells. The compounds were all added to the cells at the same concentration of 1 μmol/L for 24 h and their effects on COX-2 expression were measured (Fig. 6). Western blot analyses of COX-2 revealed that COX-2 expression was decreased by treatment with β-carotene, whereas retinol or retinoic acid treatment had no effect.

Effect of β-carotene on COX-2 expression and cell growth in other colon cancer cells. The relation between the inhibition of COX-2 expression and cell growth by β-carotene was also studied in other human colon cancer cells, such as HT-29 and WiDr cells, which overexpress this protein, and HCT-116 cells, which do not express it. Different concentrations of β-carotene, ranging from 1 to 10 μmol/L, were added to the cells. LS-174, HT-29, and WiDr cells were inhibited by the

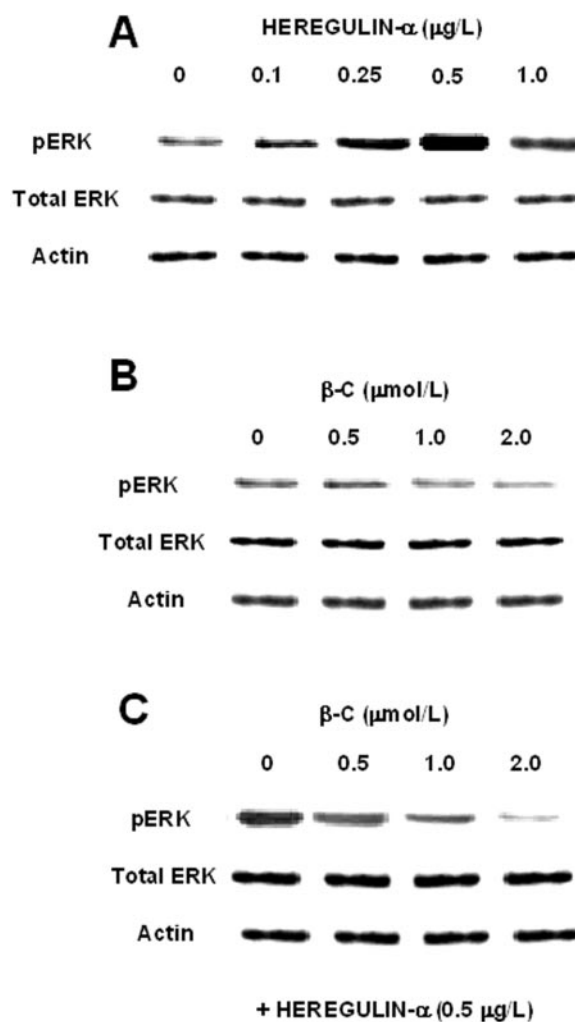


FIGURE 5 Representative Western blot analyses of pERK1/2 and total ERK1/2 expression in LS-174 cells treated for 24 h with heregulin-α (panel A), β-carotene (β-C) (panel B), and a combination of heregulin-α and β-carotene (panel C).

carotenoid in a dose-dependent manner. LS-174 cells were the most responsive to the carotenoid, whereas WiDr cells were the least responsive. After 24 h of incubation at a β-carotene concentration of 2.5 μmol/L, which represents the minimum

TABLE 2

Effect of a 24-h β-carotene treatment on PGE₂ production of LS-174 cells in the absence or presence of heregulin-α¹

β-Carotene μmol/L	PGE ₂	
	– Heregulin-α	+ Heregulin-α ²
	pg/10 ⁵ cells	
0	14.0 ± 1.4 ^d	35.0 ± 3.49
0.5	11.2 ± 1.0 ^c	24.2 ± 2.5 ^f
1.0	7.3 ± 0.7 ^b	17.0 ± 1.5 ^e
2.0	4.2 ± 0.4 ^a	10.0 ± 1.2 ^c

¹ Values are means ± SEM, *n* = 3. The treatment × concentration interaction was significant, *P* < 0.05. Means without a common letter differ: *P* < 0.001 (Tukey's test).

² Heregulin-α concentration was 0.5 μg/L.

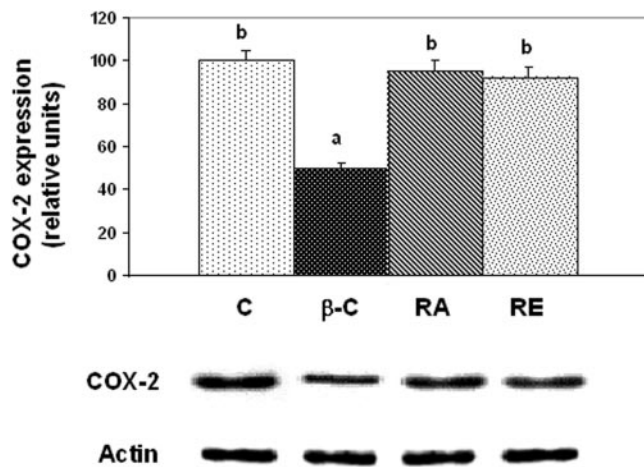


FIGURE 6 Representative Western blot analysis of COX-2 expression and a histogram of the densitometric analyses of the autoradiographs in LS-174 cells treated for 24 h with β -carotene (β -C), retinoic acid (RA), and retinol (RE) at the same concentration of 1 μ mol/L. Values are means $\pm$ SEM, $n = 3$. Means without a common letter differ, $P < 0.05$ (Fischer's test).

level responsible for growth-inhibitory effects in WiDr cells, the growth-inhibition by the carotenoid, as a percentage of the respective control, was: 34.0 ± 0.9 , 25.0 ± 1.7 , and 4.0 ± 0.4 in LS-174, HT-29, and WiDr cells, respectively. Concomitantly, COX-2 inhibition by the carotenoid, measured as a percentage of the respective control, under the same experimental conditions indicated above, was 70.0 ± 7.0 , 51.0 ± 6.0 , and 8.7 ± 0.8 in LS-174, HT-29, and WiDr cells, respectively. These data suggest a direct relation between the growth-inhibitory effects of the carotenoid and its ability to decrease the levels of COX-2. On the other hand, HCT-116 cells, which do not express COX-2, were resistant to the growth-inhibitory effects of the carotenoid, at least in the range of carotenoid concentrations (1–10 μ mol/L) used in this study.

DISCUSSION

In this study, COX was measured under steady-state conditions as well as after stimulation by heregulin- α , a growth factor of the EGF family known to increase selectively COX-2 expression in colon cancer cells (34). For this purpose, we used LS-174 colon cancer cells, which express HER2 and HER3 receptors (34). It was reported recently that the binding of NDF- β 1/hergulin to HER2/HER3 receptors induced the activation of the COX-2 promoter, the expression of COX-2, and the accumulation of PGE₂, a primary product of the COX enzymes, in colorectal cancer cells (34). In agreement with these findings, in our cell model, heregulin- α activated LS-174 colon cancer cells to express COX-2 and to produce PGE₂. Because no significant changes in COX-1 expression were observed in LS-174 cells after heregulin treatment, it seems likely that increased PGE₂ content reflected changes in COX-2 activity. Compared with unstimulated cells, LS-174 cells treated with heregulin- α exhibited an increase in S-phase together with a decrease in the percentages of apoptotic cells and an induction of COX-2 expression. The mitogenic action of heregulin was also reported in other colorectal cancer cells (34,35) as well as in other cell models, including melanoma (36), vestibular sensory epithelia (37), and breast cancer cells (38). In addition, the growth stimulatory and invasive effects

of NDF/hergulin were reported to be completely blocked by a specific COX-2 inhibitor (34). On the other hand, few have reported that heregulin blocks apoptosis in colon cancer cells (39). Interestingly, both of these effects seem to be strongly dependent on the concentration of heregulin used. Although the mechanism is unclear, high levels of the growth factor can be found in the growth-arrested state (39). In our study also, when the concentration of heregulin was increased to 1 μ g/L, a decrease in cell growth and an increase in apoptosis was found in LS-174 cells. The finding that high levels of COX-2 were accompanied by a reduced ability of LS-174 cells in undergoing apoptosis seems to support the hypothesis that this protein plays a key role as an intracellular antiapoptotic agent. In agreement with these findings, in our study, the selective COX-2 inhibitor NS-398 stimulated apoptosis in LS-174 cells. Such an effect was clearly dose dependent. Although it was suggested that COX-2 selective inhibitors may decrease proliferation and induce apoptosis by COX-2 independent mechanism(s) (40,41), several observations demonstrated that decreases in COX-2 protein expression can significantly increase the levels of apoptosis induced by COX-2 inhibitors (10–12).

Our findings in several human colon cancer cell lines, differentially expressing COX-2, provide valuable information for a key role of β -carotene in the COX-2 pathway and suggest a possible mechanism for the chemopreventive and antiproliferative effects of this molecule in cancer cells. The carotenoid decreased the expression of COX-2 but not that of COX-1 in LS-174 cells, indicating that this is a specific effect and not merely a consequence of generalized changes in either transcriptional activity or in RNA stability. Interestingly, COX-2 inhibition occurred at very low concentrations of the carotenoid, which may be achievable in vivo. Without dietary supplementation, serum β -carotene concentrations of humans vary markedly but are typically between 0.25 and 1.0 μ mol/L. Moreover, after consumption of carotenoid supplements or carotenoid-rich fruits and vegetables, serum β -carotene concentrations between 2 and 13 μ mol/L can be achieved (42,43).

In addition, the carotenoid was able to downregulate baseline and heregulin- α -induced expression of COX-2 and PGE₂ content in colon cancer cells. Recently, it was reported that COX-2 expression can be regulated through different mitogen-activated protein kinase signaling pathways and that the particular signaling pathway involved is dependent on the type of stimuli (44,45). Our data clearly show that the induction of COX-2 by heregulin was accompanied by the activation of the ERK1/2 signaling pathway and that such an activation was strongly inhibited by β -carotene in LS-174 cells. It was reported recently that ERK can phosphorylate and activate transcriptional factors, such as CRE/ATF, E-box, nuclear factor-interleukin 6, and nuclear factor- κ B, which can be responsible for COX-2 induction (46–48).

In our cell model, it was shown clearly that the carotenoid selectively inhibited intracellular COX-2 expression and, concomitantly, altered the kinetics of the growth of cultured colon cancer cells. It is interesting to note that the carotenoid inhibited cell growth by both inhibiting cell cycle progression and inducing apoptosis. These data agree with other observations showing that the carotenoid was able to act as a potent growth-inhibitory agent in colon cancer cells by inhibiting cell cycle progression and/or by inducing apoptosis (30). It is interesting to note, however, that, in this study, the carotenoid exhibited proapoptotic effects at very low β -carotene concentrations with respect to those used in other experimental studies. β -Carotene-treated WiDr colon adenocarcinoma cells showed apoptosis induction at a carotenoid concentration of

~36 μmol/L (27), and β-carotene treated HL-60 cells had the same response at ~10 μmol/L (28). In a previous study, we reported that apoptosis induction in LS-174 cells can be reached at β-carotene concentration of ~5 μmol/L (30). It should be noted, however, that in that study, the cells grew in deprivation of serum; therefore, the concentration of the carotenoid required to induce apoptosis was much lower than that necessary in medium at 10%, (v:v) FCS. Although no evidence for a role of COX-2 in the growth-inhibitory effects of the carotenoid was reported previously, we demonstrated recently that COX-2 expression may be modulated by the carotenoid in immortalized and transformed cells exposed to cigarette smoke condensate (49).

Because COX is the rate-limiting enzyme in PG production from arachidonic acid and ROS are generated as a side product of this reaction (50), we also measured ROS levels in cells treated with the carotenoid. According to the dose-dependent decrease in the expression of COX-2, a dose-dependent decrease in ROS production was observed in cells treated with varying β-carotene concentrations. Because the production of ROS by the peroxidase function of COX may be necessary for cell proliferation, its inhibition by β-carotene, directly or through COX inhibition, may be a potential mechanism in bringing about its growth-inhibitory effects in our system. Therefore, this study suggests that 2 distinct mechanisms may be potentially implicated in the proapoptotic effects of the carotenoid in colon cancer cells with the first one involving an increase in ROS production and occurring at high β-carotene concentrations (27) and the second involving the modulation of COX-2 expression and occurring also at low carotenoid concentrations.

Our results also suggest that β-carotene may exert its effects on COX-2 without being converted to retinoids because no changes in the levels of COX-2 were observed after treatment with retinol and retinoic acid.

In conclusion, this study demonstrated that β-carotene can modulate the COX-2 pathway. In particular, we provided evidence showing that the downregulation of COX-2 by the carotenoid occurred under steady-state conditions as well as after growth factor stimulation; it was accompanied by growth-inhibitory effects and by a decreased production of PGE₂. Finally, it occurred through an inhibition of the ERK signaling pathway. Although further work with in vitro and in vivo models is required to verify the relevance of these findings, the present study is the first report showing β-carotene as a natural COX-2 inhibitor, able to decrease concomitantly both COX-2 activity and expression.

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