Carotenoids Up-Regulate Connexin43 Gene Expression Independent of Their Provitamin A or Antioxidant Properties

Li-Xin Zhang, Robert V. Cooney, and John S. Bertram

Molecular Oncology Unit, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, Hawaii 96813

ABSTRACT

Epidemiological evidence and studies in whole animals and cell culture have indicated that carotenoids have cancer chemopreventive action. In mouse C3H10T1/2 cells, this activity is highly correlated with the ability of carotenoids to up-regulate gap junctional intercellular communication. Here, we report that in mouse cells, carotenoids increase the expression of connexin43, a gene that encodes a major gap junction protein. This effect appears unrelated to their provitamin A or antioxidant properties, since carotenoids with and without provitamin A activity increased levels of connexin43 mRNA and protein, whereas the antioxidants methyl-bixin and alpha-tocopherol were inactive. Moreover, the active carotenoid canthaxanthin did not induce the vitamin A-inducible gene retinoic acid receptor-beta. Connexin43 is the first carotenoid-inducible gene described in mammals. By indicating an additional pathway through which carotenoids function, these data provide a mechanistic basis for cancer chemoprevention by carotenoids and may lead to a re-evaluation of carotenoid physiology.

INTRODUCTION

Carotenoids, yellow and red pigments found in vegetables and fruits, are common components of the human diet. Accumulating evidence suggests that carotenoids, particularly beta-carotene, have diverse beneficial roles in animals and humans. Among these are: provitamin A activity (1), cancer chemoprevention (2-4), stimulation of immune responses (5), suppression of atherosclerosis (6), and prevention of cataract formation (7). High dose beta-carotene is clinically approved for use in patients with erythropoietic protoporphyria (8), is currently undergoing clinical trial as a cancer preventive agent (9), and, together with canthaxanthin, is a widely used, United States Department of Agriculture-approved, food coloring agent (10).

Until recently, biological activity of carotenoids in mammals was considered limited to those with provitamin A activity. However, since Peto et al. (11) in 1981 first raised the question, “Can dietary beta-carotene materially reduce human cancer rates?”, the focus of carotenoid research has shifted to include studies of their properties as antioxidants (12, 13). At present, therefore, 2 competing, but not mutually exclusive, hypotheses exist to explain carotenoid action in mammals: (a) conversion to retinoids, vitamin A-like compounds possessing multiple physiological properties; and (b) activity of the intact molecule as a lipid-phase antioxidant. In previous studies using carcinogen-treated C3H10T1/2 cells, we have confirmed that carotenoids can function as cancer preventive agents (14, 15) and have demonstrated a novel action of carotenoids: that of up-regulating gap junctional communication (16). The ability of carotenoids to enhance communication was highly correlated with their ability to inhibit neoplastic transformation, however, their antioxidant capabilities were not. Neither activity was associated with the provitamin status of the various carotenoids tested (16).

Here, we explore the mechanism by which various carotenoids elevate junctional communication. Gap junctions are composed of transmembrane proteins called connexins, which form a family of proteins (17). One member of this family, Cx43, is a major gap junctional protein in various mammalian cells including 10T1/2 cells (18). The data indicate that carotenoids enhance gap junctional communication by increasing the levels of Cx43 mRNA and protein. This effect appears unrelated to their provitamin A activity or antioxidant properties.

MATERIALS AND METHODS

Chemicals. Lycopene and beta-carotene were obtained from Sigma Chemical Co. (St. Louis, MO) and were 90 and 80% pure, respectively, as determined by high-performance liquid chromatography. Canthaxanthin and trans-methyl-bixin were gifts from Hoffmann-LaRoche (Nutley, NJ), and were >99% and 90% pure, respectively. Because of its purity and greater chemical stability, canthaxanthin was used for many of the molecular studies. The synthetic benzoidal derivative of retinoic acid, TTNPB, was a kind gift from Hoffmann-LaRoche. Alpha-tocopherol was purchased from Sigma.

Cell Culture. Mouse embryonic fibroblast C3H10T1/2 cells were cultured in Eagle’s basal medium with Earle’s salts (GIBCO), supplemented with 5% fetal bovine serum (Hyclone Laboratories) and 25 micrograms/ml gentamicin sulfate, and were incubated at 37°C in 5% CO2 in air as described previously (15, 19). Unless otherwise mentioned, cells were seeded in plastic culture dishes (Falcon). When confluent, 1 week post-seeding, the medium was changed to 3% fetal bovine serum. Drug treatment started with the first medium change unless otherwise indicated. Cell number was counted by Coulter counter. The murine embryonal teratocarcinoma F9 cell line was kindly supplied by Dr. Loraine J. Gudas (Harvard) and cultured as described (20). Briefly, the cells were cultured in Dulbecco’s modified Eagle’s medium (Flow) containing 10% fetal calf serum (Hyclone Laboratories) and 25 micrograms/ml gentamicin sulfate and were incubated at 37°C in 5% CO2 in air. Cells were seeded in 150-mm plastic culture dishes. Since this transformed cell line rapidly lifts off from the culture dish after attaining confluence, it was treated on the second day after seeding.

Treatment. All carotenoids tested were delivered to cells using tetrahydrofuran as solvent (99.9% with 0.025% butylated hydroxytoluene; purchased from Aldrich Chemical Co.) as described previously (15). TTNPB and alpha-tocopherol were dissolved in acetone unless indicated. The final concentration of acetone or tetrahydrofuran in the culture medium was 0.5%.

Gap Junctional Communication Assay. Cell cultures were grown in 60-mm dishes and treated with drugs after confluence. Gap junctional communication was measured by microinjection of the fluorescent tracer dye Lucifer yellow CH (Sigma) as a 10% solution in 0.33 nM LiCl as described previously (21). Micropipettes were prepared from glass capillary tubes with the aid of a Flaming-Brown micropipette puller (Sutter Instrument Co., Novata, CA), and Lucifer yellow was microinjected into a single cell with an Eppendorf microinjector. The total...
number of fluorescent neighboring cells was counted 10 min after microinjection and served as an index of gap junctional communication. The communicating networks were digitized using a video camera and stored for later analysis.

Isolation of Protein. Cell cultures were grown in 150-mm dishes and were treated after reaching confluence. The cells were washed with ice-cold phosphate-buffered saline containing 1 mM NaF and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 3,000 rpm for 10 min, cell pellets were either frozen at −70°C for future analysis or lysed in 30–50 μl of lysis buffer (1% Nonidet P-40, 0.05 M iodoacetamide, 10 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM leupeptin, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin in borate buffer, pH 8.0) for 1–2 h at 4°C. Lysates were then centrifuged at 10,000 × g for 15 min, and the supernatant was used as the source of Cx43. Protein content was measured with a commercial Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL) according to the manufacture's instructions.

Protein Electrophoresis and Western Blotting. Cell lysates containing an equal amount of protein (usually 35–50 μg/lane) were mixed with 2× SDS sample buffer containing 20% β-mercaptoethanol. After at least 15 min at room temperature, the samples were electrophoresed on a 10% SDS-polyacrylamide mini-gel (Bio-Rad, Richmond, CA) at 200 V. A prestained protein standard was also loaded for indication of both molecular weight and transference efficiency. After electrophoresis, Western blots were performed as previously described using a rabbit polyclonal antibody raised against a 15-mer synthetic polypeptide located in the C-terminal domain of rat heart Cx43 (22).

Immunofluorescent Detection of Gap Junctional Plaques. This analysis was essentially performed as previously described (22) using the same polyclonal anti-Cx43 antibody as described above.

Isolation of Total RNA and Poly(A)^+ RNA. After treatment, cells were harvested with cold phosphate-buffered saline containing 10 mM EDTA and pelleted by centrifugation at 3000 rpm for 10 min. The total RNA was isolated by using RNA isolation solvent RNAzol B kit (TM Cinna Scientific, Inc., Friendswood, TX). The poly(A)^+ RNA was isolated from above total RNA by using PolyATract mRNA isolation system III kit (Promega, Madison, WI).

Agarose Gel Electrophoresis and Northern Blotting. Total RNA (10 μg) or poly(A)^+ RNA (4.5 μg) was separated by 1% agarose/2.2 M formaldehyde gel electrophoresis and blotted overnight onto a Hybond-N+ nylon membrane (Amersham, Oakville, Ontario, Canada). The cDNA probes were radiolabeled with [α-32P]dCTP (300 Ci/mmol) using the Oligolabeling kit (Pharmacia, Piscataway, NJ). The G2A cDNA clone of rat heart Cx43 was a kind gift from Dr. E. Beyer, Harvard University (18). The cDNA clone of human RAR-β was a gift from Dr. Chambón (23). The cDNA clone for glyceraldehyde-3-phosphate dehydrogenase was from American Type Culture Collection. The labeled cDNA was purified from unincorporated radioactivity by passing it through a commercially available column (Schleicher and Schuell). The membranes were hybridized with labeled probes for 24 h at 42°C followed by 2 stringent washes with 75 mM NaCl, 15 mM Tris, and 1 mM EDTA, pH 7.5/0.1% SDS/0.1% Na-FPI at 65°C for 20 min. The final post-hybridization wash was with 600 mM NaCl, 120 mM Tris, and 8 mM EDTA, pH 7.5, at room temperature for 20 min followed by autoradiography with Kodak X-Omat AR film at −70°C using double intensifying screens. In some experiments, membranes were reprobed after first stripping off the radioactivity by boiling in 0.5% SDS for 1 h. The adequacy of stripping was confirmed autoradiographically. Membranes were reprobed as described above.

RESULTS

We have previously found that carotenoids enhance junctional communication in 10T1/2 cells and that this activity was highly correlated with their ability to inhibit carcinogen-induced neoplastic transformation in these cells (16). Here, we further explore the mechanism by which selected carotenoids (structures shown in Fig. 1) up-regulate junctional communication. As shown in Fig. 2A, a 7-day treatment of 10T1/2 cells with β-carotene, canthaxanthin, or lycopene at concentrations of 10−5 M resulted in large increases in Cx43 levels in comparison with control solvent-treated cultures. This combination of concentration and time had previously been shown to cause a dramatic increase in functional communication as measured by dye micro-injection (Fig. 3) (16). In contrast, treatment with methyl-bixin [a carotenoid also active in protecting from membrane oxidation (16)], or with α-tocopherol [a highly potent lipid-phase antioxidant (16)], did not alter Cx43 levels. In carotenoid- and in retinoid-treated cultures (included as positive controls), the immunoreactive bands were observed as doublets. It appears likely that in accordance with previous observations in retinoid-treated 10T1/2 cells (22), the upper band represents...
UP-REGULATION OF CONNEXIN43 GENE EXPRESSION BY CAROTENOIDs

Fig. 3. Increased gap junctional communication is accompanied by an increased number and size of immunofluorescent plaques in regions of cell-cell contact. Confluent cultures were treated with 10^{-5} M canthaxanthin for 7 days. A and B, photomicrographs of dye transfer. A, solvent control; B, canthaxanthin (stars indicate the injected cell). Junctional permeability was assayed by microinjection of the fluorescent dye Lucifer yellow CH (10% in 0.33 M LiCl) into a single cell as described (21). C-F, immunofluorescent detection of junctional plaques induced by canthaxanthin. C and E, control, fluorescent and respective phase image; D and F, canthaxanthan treated, fluorescent and respective phase image (arrows indicate junctional plaques). 10T1/2 cells were seeded on Permanox plastic slides and treated as in A and B. Cultures were fixed and labeled with the same Cx43 antibody as used in Western blotting, and then with FITC labeled goat anti-rabbit IgG F(ab')2 fragment as described (22).

a phosphorylated form of Cx43. In other cells, Cx43 phosphorylation on serine residues is associated with gap junctional competence (24). The inability of α-tocopherol or methyl-bixin to increase Cx43 levels indicates that the increases observed in β-carotene-, canthaxanthin-, and lycopene-treated cultures are not related to their antioxidant properties, nor to their provitamin-A activities, since, of the 3 active compounds, only β-carotene is known to be converted to retinoids in mammals (1).

The induction of Cx43 by canthaxanthan was found to be time- and dose-dependent (Fig. 2, B and C). When confluent 10T1/2 cells were treated with 10^{-5} M drug, an increase in Cx43 level was detected within 2 days of treatment and progressively increased thereafter. Induction of Cx43 preceded the previously reported time-course for induction of communication (16). Dose-response studies with canthaxanthin demonstrated that a concentration of 3 x 10^{-7} M produced a minimally detectable increase in Cx43 and that higher concentrations resulted in progressively increased levels (Fig. 2C). This concentration range spans the minimum to maximum biological response range for induction of gap junctional communication and inhibition of neoplastic transformation (15, 16). The increase in the Cx43 level after canthaxanthin treatment was accompanied by an increase in functional communication as detected by dye-transfer measurements (Fig. 3A and B), and by a dramatic increase in immunoreactive plaques in regions of cell-cell contact as detected by immunofluorescent microscopy (Fig. 3, C and D). Thus, carotenoid-induced Cx43 becomes localized where junctional plaques are expected to form.

Total RNA from control and carotenoid-stimulated 10T1/2 cells was analyzed by Northern blotting to determine whether the increased protein levels of Cx43 were the result of increased levels of Cx43 mRNA. Blots were probed with a full length Cx43 cDNA cloned from rat heart (18). Under high stringency conditions, a 3.1-kilobase band was detected (Fig. 4A), which is the reported transcript size of Cx43 (18). The steady-state level of Cx43 mRNA was markedly elevated by β-carotene, canthaxanthan, and to a lesser extent by lycopene. Induction of Cx43 mRNA by canthaxanthan was detected within 1 day of treatment and increased progressively over the 7-day observation period (Fig. 4B). This increase preceded the induction of Cx43.
canthaxanthin elevated Cx43 mRNA levels, but only TTNPB also limit their clinical usefulness. Several problems have hindered the further understanding of carotenoid physiology and pharmacology. Principal among these are: (a) lack of animal models that mimic humans; (b) difficulties in delivering these highly lipophilic compounds to cultured cells; and (c) uncertainties regarding the choice of biological endpoint to be studied. An additional problem is that β-carotene, the most widely studied carotenoid, is the major metabolic precursor of retinoids, compounds with multiple physiological functions, including cancer chemoprevention (1–16).

The present studies, by demonstrating overlapping biological activities for some (cancer chemoprevention, Cx43 expression, and junctional communication) but not all (RAR-β induction) of the actions of carotenoids and retinoids may have relevance to the different clinical properties of these compounds. If, as seems likely, clinical toxicity of retinoids is related to activation of retinoic acid responsive elements in diverse retinoid-responsive genes, then administration of carotenoids (which do not activate RAR-β; Fig. 5) in sufficiently high concentrations would circumvent toxicity yet result in effective cancer chemoprevention. This analysis assumes that the enhanced junctional communication induced by retinoids and carotenoids is causal to cancer chemoprevention: as discussed below, there is evidence obtained from studies in cultured cells to support this statement. Furthermore, in a recent clinical trial, all-trans-retinoic acid, when applied topically, was shown to up-regulate expression of Cx43 in human epidermis (32), demonstrating that this action of retinoids is not limited to cells in culture.

Multiple lines of evidence support the concept that gap junctions serve as conduits for the passage of growth regulatory molecules between communicating cells. This topic has been studied carotenoid, is the major metabolic precursor of retinoids, and junctional communication induced by retinoids and carotenoids is causal to cancer chemoprevention: as discussed below, there is evidence obtained from studies in cultured cells to support this statement. Furthermore, in a recent clinical trial, all-trans-retinoic acid, when applied topically, was shown to up-regulate expression of Cx43 in human epidermis (32), demonstrating that this action of retinoids is not limited to cells in culture.

Multiple lines of evidence support the concept that gap junctions serve as conduits for the passage of growth regulatory molecules between communicating cells. This topic has been studied carotenoid, is the major metabolic precursor of retinoids, and junctional communication induced by retinoids and carotenoids is causal to cancer chemoprevention: as discussed below, there is evidence obtained from studies in cultured cells to support this statement. Furthermore, in a recent clinical trial, all-trans-retinoic acid, when applied topically, was shown to up-regulate expression of Cx43 in human epidermis (32), demonstrating that this action of retinoids is not limited to cells in culture.

The ability of carotenoids to up-regulate expression of a gene, Cx43, also up-regulated by retinoids suggests that both act through a common mechanism. This concept is reinforced by the structural similarities between carotenoids and retinoids [lycopene is the immediate precursor to β-carotene in plants, whereas β-carotene is a precursor to retinal and retinoic acid in mammals (1)]. To test this concept, we investigated the ability of canthaxanthin to up-regulate expression of RAR-β, a gene known to contain a retinoic acid responsive element in its promoter region (25) and whose expression can be up-regulated by retinoids in diverse cell types (26–29). We chose canthaxanthin for these studies because of its chemical purity and its greater chemical stability than β-carotene and lycopene (15), and because it has been widely used as a non-provitamin A carotenoid. As shown in Fig. 5, a 3-day treatment of 10T1/2 cells with the synthetic retinoid TTNPB at 10^{-8} M strongly increased the level of RAR-β mRNA, whereas treatment with canthaxanthin at 10^{-5} M for 7 days had no effect. This was also the case in murine embryonic teratocarcinoma F9 cells, in which both TTNPB and canthaxanthin elevated Cx43 mRNA levels, but only TTNPB induced RAR-β mRNA (Fig. 5).

**DISCUSSION**

The demands to reduce human cancer morbidity and mortality place a heavy burden on prevention. Of over 1000 compounds tested, the retinoids and carotenoids have obtained most attention and possess great promise for cancer chemoprevention (30). However, the high toxicity of retinoids greatly limits their use (30). Although carotenoids have no known toxicity (31), their lower potency as chemopreventive agents may also limit their clinical usefulness. Several problems have been...
the subject of several reviews (33–36). In 10T1/2 cells, up-regulation of communication between homologous cells (i.e., normal:normal or transformed:transformed) by retinoids correlates with enhanced growth control (21, 37, 38), conversely, tumor promoters both in vitro and in vivo cause rapid uncoupling of communicating cells (34–36). Tumor cells are in general deficient in junctional communication (34), however communication with normal 10T1/2 cells can be restored by elevation of intracellular cAMP: in this situation, tumor cells become growth arrested in proportion to the increase in heterologous communication (21). Furthermore, over-expression of Cx43 in transformed cells by gene transfection causes a partial reversion of the transformed phenotype (39). In contrast, induction of transformation by v-src is temporally associated with the phosphorylation of tyrosine residues on Cx43 and inhibition of communication (40). Finally, in the 10T1/2 model system we have demonstrated a highly significant statistical correlation between the ability of diverse retinoids and carotenoids to inhibit chemically induced neoplastic transformation and their ability to stimulate junctional communication (16, 21). These observations have led to the proposal that retinoids and carotenoids inhibit transformation in the 10T1/2 cell system by placing carcinogen-initiated cells in extensive communication with surrounding noninitiated cells, thereby preventing their transformation (16, 21). Whether the antiproliferative action of this enhanced communication is adequate to explain this inhibition is as yet unknown. The present studies provide a mechanistic basis for enhanced communication induced by carotenoids.

The evidence that both provitamin A and non-provitamin A carotenoids increased levels of Cx43 mRNA and protein, whereas other antioxidants, such as α-tocopherol and the carotenoid methyl-bixin, were ineffective, implies that induction of Cx43 gene expression by carotenoids is independent of their provitamin A or antioxidant properties. However, both retinoids and carotenoids reversibly inhibit the postinitiation phase of carcinogenesis (14, 41), enhance junctional communication (Fig. 3) (16, 21), and increase Cx43 mRNA and protein levels (Fig. 2 and 4) (22). These remarkable similarities between carotenoids and retinoids suggest a common mechanism, perhaps involving a low rate of conversion of carotenoids into active retinoids in cell culture by chemical oxidation (1) or other processes. This rate (should it occur in vivo) would be insufficient to support growth (the classical assay for vitamin A activity) since there is no evidence that canthaxanthin or lycopene can be converted to retinoids in mammals (1). Furthermore, we had previously been unable to detect the conversion of 1,4C-labeled β-carotene to retinoids in 10T1/2 cells (42). The recent discovery of high affinity (10−9 to 10−8 M) nuclear RARs (26, 43, 44) raises the possibility that a very low level of conversion of carotenoids to retinoids (not detectable because of the low specific activity of available [14C]-β-carotene) could activate RARs. However, the demonstration of a dissociation between the action of canthaxanthin and TTNPB on RAR-β expression in both 10T1/2 and F9 cells (Fig. 5) strongly indicates that the induction of Cx43 gene expression by canthaxanthin is independent of conversion to retinoids capable of activating RARs and inducing expression of RAR-β. We cannot rule out the possibility that carotenoids or carotenoid metabolites are activating a novel receptor, perhaps one of the RXR family of receptors (45–47), with ligand binding affinity for retinoids and carotenoids, capable of inducing Cx43 but not RAR-β gene expression. The knowledge that these receptors activate distinct classes of responsive elements and thus differentially activate distinct genes could explain our results. It is of interest that 9-cis-retinoic acid has recently been reported to be a physiological ligand for RXR II (47); 9-cis-β-carotene is found in human serum (48) and in preliminary studies was found to be more potent than all-trans-β-carotene in inhibiting transformation and stimulating communication in 10T1/2 cells.4 It is not known whether 9-cis-β-carotene can be converted to the corresponding retinoid or whether RXR responsive elements exist in the promoter region of the Cx43 gene.

The present data demonstrate that certain carotenoids have a hitherto undetected ability to regulate gene expression unrelated to their activity as provitamin A carotenoids or antioxidants. Since the biological activity of carotenoids in mammals was considered limited to these activities, these data indicate that carotenoid physiology in mammals or the classification of provitamin A carotenoids needs to be re-evaluated.

We have recently extended these studies to human dermal fibroblasts in culture and find that, as in 10T1/2 cells, certain carotenoids induce junctional communication and Cx43 gene expression,5 demonstrating that this novel action of carotenoids is not limited to the 10T1/2 cell model system. It remains to be determined whether carotenoids can up-regulate Cx43 expression in a relevant whole animal model.

The independence of carotenoid action on Cx43 expression from their provitamin A or antioxidant activities may provide important insights into carotenoid physiology, and in view of the increasing clinical use of these compounds, their pharmacology. In this context, it should be noted that serum levels of β-carotene that have been reported after high-dose administration to humans are comparable to the concentrations used in our cell culture studies (49).

REFERENCES


4 J. S. Bertram and Doering, unpublished observations.
5 L.-X. Zhang and J. S. Bertram, unpublished observations.


Carotenoids Up-Regulate Connexin43 Gene Expression Independent of Their Provitamin A or Antioxidant Properties

Li-Xin Zhang, Robert V. Cooney and John S. Bertram


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/52/20/5707

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.