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Ah Receptor-Dependent CYP1A Induction by Two Carotenoids, Canthaxanthin and β-apo-8′-carotenal, With No Affinity for the TCDD Binding Site

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ABSTRACT. The assays of several phase I and phase II xenobiotic-metabolizing enzyme activities, as well as CYP1A immunoblot analysis, were performed in liver microsomes and cytosol of male C57BL/6 mice (Ah receptor-responsive), of male DBA/2 mice (Ah receptor-low responsive) and of female Ah receptor gene knockout mice that were fed diets containing 300 mg/kg of a nonprovitamin A carotenoid, canthaxanthin, or a provitamin A carotenoid, β-apo-8′-carotenal for 14 days, or which were injected i.p. with 3-methylcholanthrene. Previous studies have shown that some carotenoids, such as canthaxanthin and β-apo-8′-carotenal, are strong inducers of liver CYP1A1 and 1A2 when given to rats. In this work, only canthaxanthin induced both CYP1A1 and 1A2 in C57BL/6 mice, whereas β-apo-8′-carotenal induced only CYP1A2 in this strain. Neither of the two carotenoids modified CYP1A1/2 protein contents or enzyme activities in Ah receptor-low responsive DBA/2 or in Ah receptor gene knockout mice. Cytosol prepared from C57BL/6 mice liver tissue was incubated with [^{3}H] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the presence of canthaxanthin or β-apo-8′-carotenal and analyzed by sucrose density gradient sedimentation: neither of the carotenoids, even when present in large excess, competed with TCDD for the TCDD binding site of the cytosolic Ah receptor of C57BL/6 mice. In brief, the carotenoids canthaxanthin or β-apo-8′-carotenal induced *Cyp1a* genes in mice through an Ah receptor-dependent pathway, but did not bind to the Ah receptor. BIOCHEM PHARMACOL **54**;2:307–315, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. carotenoids; 3-methylcholanthrene; CYP1A; induction; Ah receptor; Ah knockout mice

In previous reports [1–3], we have shown that some carotenoids, namely, canthaxanthin, astaxanthin and β-apo-8′-carotenal, proved powerful inducers of liver cytochromes P4501A1 and 1A2 when fed to rats. The induction of Ah receptor-dependent phase II enzyme activities, in particular *p*-nitrophenol uridine diphosphoglucuronosyl transferase (4NP-UGT, EC 2.4.1.17) and quinone reductase (QR, EC 1.6.99.2), was also observed in rats fed with these carotenoids, suggesting a common pattern of induction with some prototypical inducers of CYP1A. The latter, such as 3-methylcholanthrene (3-MC), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or β-naphtoflavone, act as ligands and activators of a cytosolic protein, the Ah receptor (AhR),§ which then activates the transcription of the *CYP1A* gene and other genes [4, 5]. As AhR ligands, the known CYP1A inducers meet relatively strict structural requirements: they are hydrophobic, polycyclic, and planar molecules of a particular size [6]. However, the carotenoids that induce CYP1A are structurally very different. Moreover, other carotenoids such as β-carotene, lycopene, or lutein have no effect [1–3], so that no clear structure–activity relationships can be drawn. The finding that canthaxanthin, astaxanthin and β-apo-8′-carotenal (Fig. 1) induce P4501A1 and 1A2 in rat liver raises the question of whether these compounds are ligands of the Ah receptor, and whether they act through an AhR-dependent pathway.

In this work, we addressed these questions by feeding canthaxanthin or β-apo-8′-carotenal to AhR-responsive C57BL/6 mice, AhR-low responsive DBA/2 mice and AhR gene knockout mice [7]. Liver microsomal cytochrome P450 and the associated reductases were assayed, as well as the activities of several phase I and phase II xenobiotic-metabolizing enzymes. Immunoblots of CYP1A were also performed. In addition, the direct interaction between both carotenoids and the cytosolic AhR of C57BL/6 mice was tested *in vitro*.

We report here that canthaxanthin and, to a lesser degree, β-apo-8′-carotenal, are inducers of liver CYP1A in C57BL/6 mice, but not in AhR-nonresponsive DBA/2 nor

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§*Abbreviations:* AhR, Ah receptor; ALDH1, cytosolic aldehyde dehydrogenase class 1; ALDH3, cytosolic aldehyde dehydrogenase class 3; BROD, benzoxyresorufin O-dearylase; EROD, ethoxyresorufin O-deethylase; GST, glutathione S-transferase; 4HBP-UGT, 4-hydroxybiphenyl uridine diphosphoglucuronosyl transferase; 3-MC, 3-methylcholanthrene; MROD, methoxyresorufin O-demethylase; 4NP-UGT, *p*-nitrophenol uridine diphosphoglucuronosyl transferase; PROD, pentoxyresorufin O-dealkylase; QR, NADPH quinone reductase; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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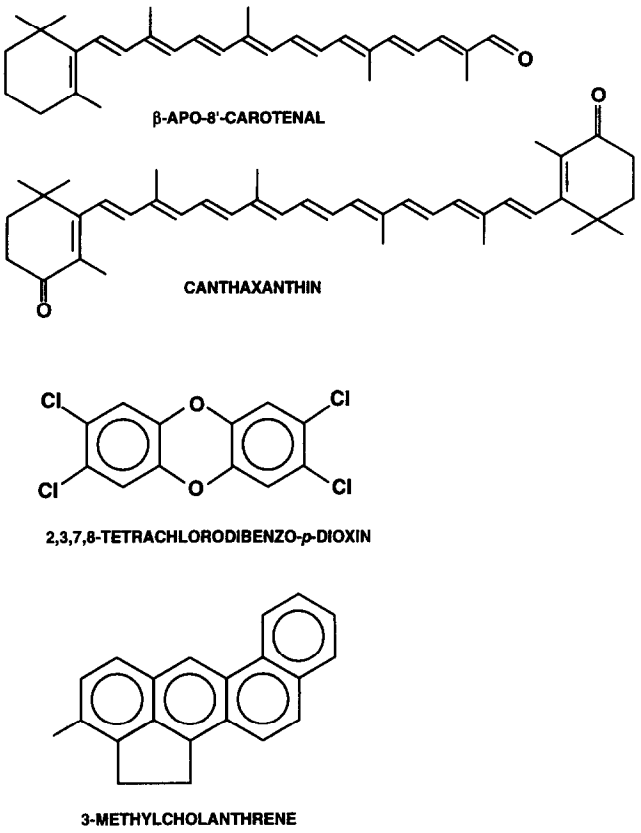

FIG. 1. Chemical structures of carotenoids, of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and of 3-methylcholanthrene (3-MC).

in AhR gene knockout mice, strongly suggesting the implication of the Ah receptor. However, these carotenoids did not compete with TCDD when tested *in vitro* with purified Ah receptor from C57BL/6 mice. These results show that the carotenoids studied are likely to activate the Ah receptor, despite their lack of binding to the TCDD site of action.

MATERIALS AND METHODS

Chemicals

Ten percent canthaxanthin cold water-dispersible powder, placebo powder (containing the same ingredients as the 10% canthaxanthin powder, i.e. sucrose, corn starch, gelatin, ascorbyl palmitate, vegetable oil, and DL-α-tocopherol) and 20% β-apo-8′-carotenal oil suspension were provided by Hoffmann–LaRoche (Basel, Switzerland). Pure carotenoids used as HPLC standards were also provided by Hoffmann–LaRoche. Unlabeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin (34.7 Ci/mmol) were purchased from Chemsyn Science Laboratories (Lenexa, KS). 3-Methylcholanthrene, Hepes, dextran, and activated charcoal were obtained from Sigma (St. Louis, MO). Peroxidase-conjugated rabbit anti-mouse immunoglobulins and swine antirabbit immunoglobulins were from DAKO (Copenhagen, Denmark). The polyclonal anti-rat P4501A1/2 and P4502B1/2 antibodies were a generous gift from Dr J. Magdalou (INSERM, URA 597, Centre du Médicament, Nancy, France).

Animals and Diets

In a first experiment, 12 male Ah receptor-responsive C57BL/6 mice and 12 male Ah receptor-low responsive DBA/2 mice, 24–27 days old (IFFA CREDO, Lyon, France) were fed semiliquid purified diets *ad lib* for 7 days, as described previously [1]. Twelve mice of each strain were then alloted to four groups of three, designated as C (control), CX (canthaxanthin), AC (β-apo-8′-carotenal) and MC (3-methylcholanthrene). The CX and AC groups were given diets containing 300 mg/kg of canthaxanthin or β-apo-8′-carotenal, respectively, for 14 days. Canthaxanthin was added to the diet as 10% powder. The oil suspension of β-apo-8′-carotenal was mixed with the corn oil included in the diet. Placebo powder was added (3 g/kg) to the diets of groups C, MC, and AC in order to equalize the diet compositions. The carotenoid or placebo powders were first dispersed in water, then the dispersion was thoroughly mixed with corn oil. The resulting emulsionlike mixtures were stored frozen and added to the diets, which were prepared daily. The mice in the MC groups were injected i.p. with 3-MC (50 mg/kg bodyweight, 3 consecutive days, the last injection 24 hr before sacrifice).

In a second experiment, three female AhR-responsive C57BL/6 mice (Charles Rivers, Cléon, France) and three female AhR gene knockout (Ah−/−) mice (from our laboratory, Toulouse), 24–27 days old, were fed the semiliquid purified diet supplemented with 300 mg canthaxanthin/kg *ad lib* for 14 days. The homozygous Ah−/− mice used in this study were predominantly of C57BL/6 genetic background; they were selected following two rounds of crossbreeding with C57BL/6 females.

In both experiments, drinking water was supplied *ad lib*. Food intake was recorded daily; the mice were weighed twice a week.

Enzyme Assays

At the end of the experiments, the mice were sacrificed without having previously been starved, and their livers rapidly removed and weighed. Liver microsomal and cytosolic fractions were prepared as described by Siess *et al.* [8], and aliquots were stored at −80°C. The protein contents of microsomes and cytosols, the microsomal total cytochrome P450, and the associated NADH and NADPH cytochrome *c* reductase activities, as well as some phase I and phase II enzyme activities, were determined as described previously [2]. The phase I activities assayed were markers of cytochrome P450 isoenzymes: ethoxyresorufin O-deethylase (EROD), marker of P4501A1; methoxyresorufin O-demethylase (MROD), marker of P4501A2; pentoxyresorufin O-depentylase (PROD), marker of P4502B1,2; and benzoxyresorufin O-dearylase (BROD), unspecific marker of

TABLE 1. Effects of dietary carotenoids and of i.p. injection of 3-methylcholanthrene on microsomal cytochrome P450 content, on the wavelength of the CO-P450 peak, and on the activities of NADPH and NADH cytochrome c reductases (means of three mice ± SEM)

Treatment	Strain	P450 (nmol/mg protein)	λ(CO-P450) (nm)	NADPH cyt c reductase (nmol/min/mg protein)	NADH cyt c reductase (nmol/min/mg protein)
C	C57BL/6	0.720 ± 0.025	449.8 ± 0.05	136 ± 19	573 ± 32
	DBA/2	0.614 ± 0.030	450.1 ± 0.09	164 ± 18	559 ± 12
CX	C57BL/6	1.254 ± 0.068*	448.7 ± 0.07*	142 ± 17	714 ± 31
	DBA/2	0.756 ± 0.031†	449.9 ± 0.02†	154 ± 18	557 ± 11†
AC	C57BL/6	0.883 ± 0.047*	448.6 ± 0.11*	133 ± 15	622 ± 60
	DBA/2	0.700 ± 0.051†	450.0 ± 0.10†	145 ± 11	565 ± 28
MC	C57BL/6	1.278 ± 0.071*	448.4 ± 0.06*	166 ± 18	636 ± 62
	DBA/2	0.836 ± 0.064†	449.8 ± 0.12†	154 ± 15	503 ± 97

C: control; AC; β-apo-8′-carotenal; CX, canthaxanthin; MC, 3-methylcholanthrene.
* Within each strain, significantly different from the control group C (one-sided Dunnett's test, $P \leq 0.05$).
† Within each treatment, significantly different from the C57BL/6 strain (t-test, $P \leq 0.05$).

P4501A, 2B, and 3A. The phase II activities assayed were 4NP-UGT, 4-hydroxybiphenyl uridine diphosphoglucuronosyl transferase (4HBP-UGT), cytosolic glutathione S-transferase (GST, EC 2.5.1.18), NAD(P)H quinone reductase (QR), and aldehyde dehydrogenase class 1 and class 3 (ALDH1 and ALDH3, EC 1.2.1.3).

The assays of NADH and NADPH cytochrome c reductases, of phenoxazone O-dealkylases (EROD, PROD, BROD, and MROD) and of phase II enzymes (4NP-UGT, 4HBP-UGT, GST, QR, ALDH1, and ALDH3) were adapted for automatic measurement using a Cobas Fara II Centrifugal Analyser (Roche Instruments, Basel, Switzerland).

Immunochemical Detection of P450 Proteins

Immunoblot analyses of CYP1A1/2 and CYP2B1/2 were performed according to Beaune *et al.* [9]. Microsomal proteins were resolved by electrophoresis on sodium dodecyl sulphate polyacrylamide gel as described by Laemmli [10] and then electrotransferred to nitrocellulose sheets by the general procedure of Towbin *et al.* [11]. CYP1A1 and CYP1A2 were identified by probing the nitrocellulose sheets with a polyclonal antibody crossing with both 1A isoforms, using an immunoperoxidase staining technique according to Guengerich *et al.* [12]. The same technique was used for CYP2B1 and CYP2B2, with a polyclonal anti-CYP2B antibody.

Binding Assays

The binding assays of carotenoids to the TCDD binding site of murine Ah receptor were performed according to Lesca *et al.* [13]. Cytosol was prepared from C57BL/6 mouse liver tissue by centrifugation of the homogenate at 9000 × *g* for 20 min, and the resulting supernatant for 1 hr at 100,000 × *g* in HEDG buffer (25 mM HEPES, pH 7.4, 1.5 mM EDTA, 1 mM dithiothreitol and 10% v/v glycerol). Competition experiments were carried out by incubating 400 μL cytosol with 50 nmol [^{3}H]TCDD for 1 hr at 4°C, under subdued light in presence of 100 μM canthaxanthin, 100 μM β-apo-8′-carotenal or 50 μM nonradiolabeled TCDD. Labeled and unlabeled TCDD were dissolved in dimethyl sulfoxide, and carotenoids in tetrahydrofuran, before being added to samples. After incubation, unbound and loosely bound radioligands were removed by adding the samples to a dextran-charcoal pellet (10 mg charcoal/mg dextran, pelleted from HEDG buffer). Dextran-charcoal was resuspended in the samples on a vortex mixer, incubated for 15 min into ice, and then removed by centrifugation at 4000 × *g* for 15 min. Aliquots (300 μL) of cytosol were layered onto linear sucrose density gradients (10–30%) prepared in HEDG buffer. Gradients (4.8 mL) were centrifuged in a near-vertical tube rotor at 372,000 × *g* for 2 hr (Beckman NVT90, Palo Alto, CA). After centrifugation, 22 fractions (8 drops per fraction) were collected from each gradient, and radioactivity in each fraction was determined by liquid scintillation counting (Ready protein, Beckman).

Statistical Analysis

Enzyme activity data were submitted to a two-way analysis of variance. Log transforms were used for EROD, MROD, BROD, and PROD in order to homogenize the group variances. The Dunnett's test and the *t*-test were used to compare the treatment means within each strain, or the strain means within each treatment, respectively. Calculations were performed with the SAS system (Cary, NC).

RESULTS

The effects of carotenoid treatments on liver XME activities in C57BL/6, DBA/2, and AhR gene knockout mice are presented in Tables 1–4. In the first experiment, canthaxanthin (CX group) induced a significant increase in the liver cytochrome P450 content (×1.7) and a shift in the P450 absorption peak wavelength to 448.7 nm, but did not

TABLE 2. Effects of dietary carotenoids and of i.p. injection of 3-methylcholanthrene on the activities of the phase I enzymes EROD, MROD, PROD, and BROD (means of three mice ± SEM)

Treatment	Strain	EROD	MROD	PROD	BROD
		(pmol/min/mg protein)			
C	C57BL/6	40 ± 11	134 ± 25	3.7 ± 1.2	12.0 ± 1.7
	DBA/2	24 ± 5	93 ± 17	2.0 ± 0.6	16.3 ± 4.7
CX	C57BL/6	760 ± 110*	597 ± 64*	29.7 ± 3.5*	64.0 ± 11*
	DBA/2	42 ± 9†	144 ± 27†	1.3 ± 0.3†	12.0 ± 1.1†
AC	C57BL/6	103 ± 10*	305 ± 34	12.3 ± 1.7*	28.3 ± 4.1
	DBA/2	35 ± 6†	116 ± 7†	7.7 ± 3.5	12.7 ± 0.3†
MC	C57BL/6	2180 ± 852*	420 ± 201*	17.7 ± 8.2*	56.0 ± 34*
	DBA/2	84 ± 55†	56 ± 14†	2.0 ± 1.0†	8.3 ± 2.4†

C: control; AC: β-apo-8′-carotenal; CX, canthaxanthin; MC, 3-methylcholanthrene.
* Within each strain, significantly different from the control group C (one-sided Dunnett's test, $P \leq 0.05$).
† Within each treatment, significantly different from the C57BL/6 strain (t-test, $P \leq 0.05$).

modify the activities of NADH and NADPH cytochrome *c* reductases (Table 1). Canthaxanthin also induced the phenoxazone dealkylase activities EROD, MROD, PROD, and BROD (×19, ×4.4, ×5.3, and ×8, respectively, vs. control group C) (Table 2). These activities were also increased, but less markedly, in β-apo-8′-carotenal-fed C57BL/6 mice (AC group). EROD and PROD were significantly augmented by a factor of 2.6 and 3.3, respectively, while the increase in MROD and BROD was not significant. 3-MC–injected C57BL/6 mice (MC group) showed the same induction profile as those fed canthaxanthin (Tables 1–2): EROD, MROD, PROD, and BROD were multiplied by a factor of 54.5, 3, 4.7, and 4.8, respectively. Neither the carotenoids nor 3-MC modified the measured phase II enzyme activities (4NP- and 4HBP-UGT, GST, QR, ALDH1, and 3) in C57BL/6 mice (Table 3). No effect on phase I or phase II enzyme activities was observed in DBA/2 mice fed canthaxanthin or β-apo-8′-carotenal, or injected with 3-MC.

The second experiment clearly showed that the increase in phase I activities produced by feeding C57BL/6 mice with canthaxanthin was completely absent in the Ah receptor-deficient strain of mice (Table 4).

Western blot analysis of microsomal proteins (Fig. 2) showed that the observed increases in phenoxazone dealkylase activities were accompanied by a strong rise in immunodetected P4501A1 and 1A2 isoform levels in C57BL/6 mice fed with canthaxanthin (CX group) or injected with 3-MC (MC group). The two isoforms appeared as two well-separated bands. The two bands were of approximately the same intensity in 3-MC-injected mice, whereas in canthaxanthin-fed mice, the lower band, corresponding to CYP1A2, was more intense than the upper band, corresponding to CYP1A1. β-Apo-8′-carotenal (AC group) increased the expression of CYP1A2 protein in C57BL/6 mice but did not induce CYP1A1. In AhR gene knockout mice fed with canthaxanthin, no CYP1A1 band appeared. Moreover, no CYP1A2 band was detected in these mice under assay conditions, as opposed to either control or treated C57BL/6 and DBA/2 mice. 3-MC, but not the carotenoids, slightly increased the P4501A2 isoform band in the DBA/2 strain. Immunoblot analysis did not reveal any induction of CYP2B1 or CYP2B2 isoforms in C57BL/6 or DBA/2 mice fed with canthaxanthin or β-apo-8′-carotenal, or injected with 3-MC (data not shown). The observed increases in PROD and BROD by carotenoid and

TABLE 3. Effects of dietary carotenoids and of i.p. injection of 3-methylcholanthrene on the activities of some phase II enzymes: Microsomal 4NP-UGT and 4HBP-UGT and cytosolic GST, QR, and ALDH3 (means of three mice ± SEM)

Treatment	Strain	4NP-UGT	4HBP-UGT	GST	QR	ALDH3
		(nmol/min/mg protein)			(nmol/min/mg protein)	
C	C57BL/6	34.8 ± 6.1	41.4 ± 5.0	3440 ± 199	112 ± 14	2.2 ± 0.7
	DBA/2	23.6 ± 3.6	40.9 ± 4.1	2084 ± 268†	77 ± 10	1.4 ± 0.4
CX	C57BL/6	30.3 ± 3.5	37.7 ± 2.6	3886 ± 253	110 ± 11	3.0 ± 0.6
	DBA/2	22.8 ± 0.9	41.8 ± 2.0	2560 ± 159†	94 ± 7	3.2 ± 0.1*
AC	C57BL/6	29.9 ± 2.3	37.2 ± 2.0	2938 ± 131	100 ± 8	2.6 ± 0.2
	DBA/2	24.3 ± 2.6	44.4 ± 1.6†	2006 ± 62†	133 ± 15*	2.5 ± 0.7
MC	C57BL/6	34.4 ± 3.2	34.6 ± 2.0	3501 ± 86	143 ± 12	1.9 ± 0.3
	DBA/2	18.7 ± 2.4†	36.8 ± 2.0	2525 ± 156†	117 ± 12*	1.8 ± 0.2

C: control; AC; β-apo-8′-carotenal; CX, canthaxanthin; MC, 3-methylcholanthrene.
* Within each strain, significantly different from the control group C (one-sided Dunnett's test, $P \leq 0.05$).
† Within each treatment, significantly different from the C57BL/6 strain (t-test, $P \leq 0.05$).

TABLE 4. Activities of phase I (EROD, MROD, PROD, and BROD) and phase II (microsomal 4NP-UGT and 4HBP-UGT and cytosolic GST, QR, and ALDH3) liver drug-metabolizing enzymes in Ah-responsive (C57BL/6) or Ah receptor gene knockout mice (AHR −/−) fed canthaxanthin (means of three mice ± SEM)

	AHR −/−	C57BL/6
EROD (pmol/min/mg protein)	14 ± 6	1537 ± 292*
MROD (pmol/min/mg protein)	58 ± 16	1759 ± 300*
PROD (pmol/min/mg protein)	13 ± 3	50 ± 10*
BROD (pmol/min/mg protein)	20 ± 5	83 ± 15*
4NP-UGT (nmol/min/mg protein)	27 ± 2	30 ± 4
4HBP-UGT (nmol/min/mg protein)	39 ± 5	52 ± 5
GST (nmol/min/mg protein)	1408 ± 324	2083 ± 103
QR (nmol/min/mg protein)	98 ± 33	141 ± 8
ALDH3 (nmol/min/mg protein)	4.5 ± 2.3	7.7 ± 0.5

* Significantly different from the Ah −/− strain (t-test, $P \leq 0.05$).

3-MC treatments were most likely the result of the induction of CYP1A isoforms.

When C57BL/6 liver cytosol was incubated with [^{3}H]TCDD and analyzed on a sucrose density gradient, a peak of radioactivity reflecting the TCDD-AhR complex was obtained in the 9S region (maximum at fraction 11 in our assay, Fig. 3). As expected, the peak almost totally

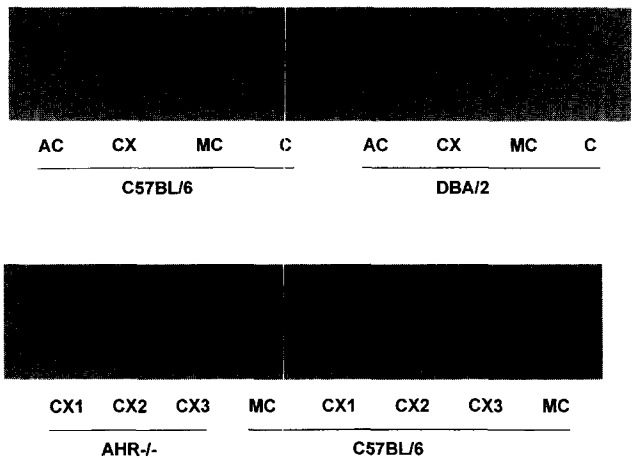

FIG. 2. Upper half: Western blot analysis of hepatic microsomes from C57BL/6 and DBA/2 mice fed canthaxanthin, β-apo-8′-carotenal or control diet, or from C57BL/6 and DBA/2 mice i.p. injected with 3-methylcholanthrene. Lower half: Western blot analysis of hepatic microsomes from C57BL/6 and Ah receptor gene knockout mice fed canthaxanthin (CX) or injected with 3-methylcholanthrene (MC); CX1, CX2, CX3 are three different mice. For each sample, 50 μg of microsomal protein were resolved by electrophoresis in an SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose. Immunoblots were performed with anti-CYP1A1/2 antibody (diluted 1/700).

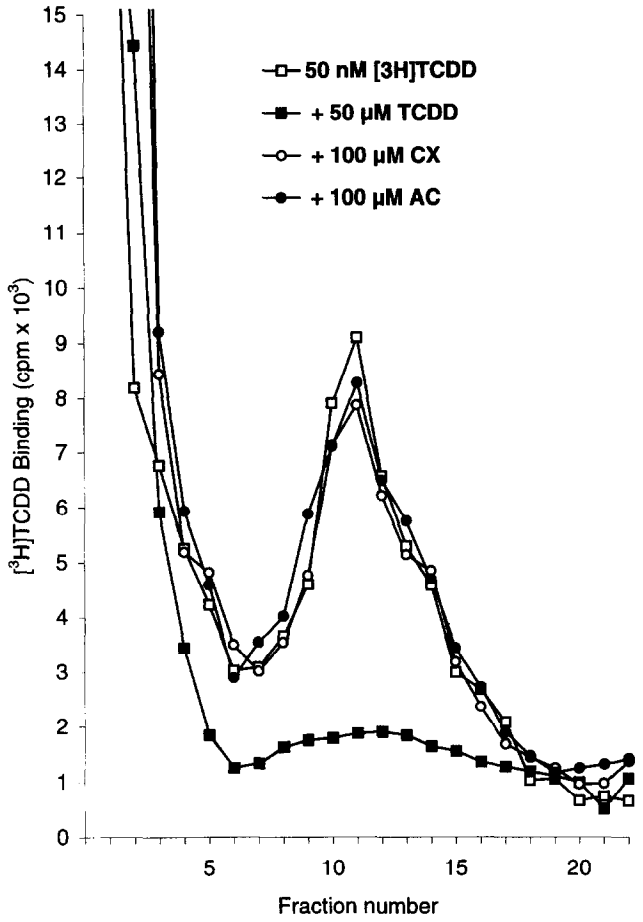

FIG. 3. Specific binding of [^{3}H]TCDD to the Ah receptor of C57BL/6 mice and lack of competitive effect of canthaxanthin and β-apo-8′-carotenal. 0.3 mL cytosol aliquots from C57BL/6 mice liver were incubated for 2 hr at 4°C with 50 nM [^{3}H]TCDD alone (□) or in the presence of either 50 μM TCDD (■) or 100 μM canthaxanthin CX (○) or β-apo-8′-carotenal AC (●). The specific binding of [^{3}H]TCDD was determinated by velocity sedimentation on 10–30% sucrose gradient.

disappeared when the incubation was carried out in the presence of a 1000-fold excess of unlabeled TCDD. In contrast, when canthaxanthin or β-apo-8′-carotenal was used as competitor, even in 2000-fold excess with respect to the radiolabeled ligand, the peak was not affected, showing that the carotenoids did not directly compete with [^{3}H]TCDD for the specific binding site of the C57BL/6 mouse Ah receptor.

DISCUSSION

This study clearly demonstrates that two carotenoids, canthaxanthin and β-apo-8′-carotenal, induce EROD, MROD, and other phase I enzymes in C57BL/6 mice, but do not modify phase II xenobiotic-metabolizing enzyme activities. No activities were modified in AhR-low responsive DBA/2 mice or in AhR gene knockout mice fed with these carotenoids. Immunoblot analysis clearly showed the strong induction of the CYP1A1 and CYP1A2 isoforms in

C57BL/6 mice fed with canthaxanthin and the induction of the CYP1A2 isoform in C57BL/6 mice fed with β-apo-8′-carotenal. Enzyme assays and Western blot analysis correlate nicely and suggest that the effect of canthaxanthin on liver XME in C57BL/6 mice closely resembles that of 3-MC. However, neither of these two carotenoids succeeded in competing with TCDD for the binding to the Ah receptor of C57BL/6 mice. The use of a genetically engineered mouse model deficient in Ah receptor allowed us to demonstrate that these carotenoids act through an Ah receptor-dependent signal transduction pathway.

Phenoxazone dealkylation activities have been shown to be relatively specific markers of some P450 isozymes, although specificity varies somewhat from one species to another [14]. In particular, both CYP1A1 and CYP1A2 contribute to the induction of EROD, MROD, and BROD activities in liver microsomes from rats or mice treated with CYP1A inducers. However, rat CYP1A1 preferentially catalyses EROD, while CYP1A2 preferentially catalyses MROD [15]. In mice, contradicting results have been published concerning the catalytic specificities of CYP1A isozymes. Tsyrlov and Duzchak [16] found that EROD activity was mainly catalyzed by CYP1A2 in liver microsomes of benzo(a)pyrene-induced C57BL/6 mice; in contrast, Nerurkar *et al.* [17] concluded that MROD was a specific substrate for CYP1A2 activity in TCDD-induced NIH/Swiss mice, as in rats. Our results in C57BL/6 mice fed with β-apo-8′-carotenal, showing a two- to threefold increase in EROD, MROD, PROD, and BROD activities when CYP1A2 protein is induced, but without CYP1A1 induction, indicates that CYP1A2 catalyses these four activities at approximately the same rate in this strain of mice, whereas the strong increase in EROD (×55) in 3-MC–injected mice suggests that CYP1A1 preferentially catalyses EROD activity.

The induction of EROD and MROD activities by 3-MC in C57BL/6 mice, but not in DBA/2 mice, is in agreement with previous reports [14, 18, 19]. Similarly, our results regarding the noninduction of phase II enzymes by 3-MC are consistent with those of Aström *et al.* [20], who found that the treatment i.p. of male C57BL/6 and DBA/2 mice with 3-MC (20 mg/kg, once daily for 5 days) did not significantly modify the activity of GST in either strain, and only slightly induced (in C57BL/6 mice) or slightly decreased (in DBA/2 mice) 4NP-UGT activity. In both mouse strains, canthaxanthin had similar effects to those of 3-MC: it induced liver CYP1A1 and CYP1A2 in C57BL/6 mice, but not in DBA/2 mice, and did not modify the activities of the measured phase II xenobiotic-metabolizing enzymes.

In our previous reports [1–3], we have shown that canthaxanthin, astaxanthin, and β-apo-8′-carotenal are strong inducers of rat liver CYP1A1 and CYP1A2, of the associated reductases and of phase II enzyme activities, especially 4NP-UGT, 4HBP-UGT, and NAD(P)H quinone reductase, as is 3-MC. In responsive C57BL/6 mice, dietary canthaxanthin and β-apo-8′-carotenal are much less powerful inducers than 3-MC. Moreover, whereas β-apo-8′-carotenal induced both CYP1A1 and CYP1A2 forms in rat liver [3], it only induced the CYP1A2 isoform in C57BL/6 mice.

DBA/2 mice have an altered Ah receptor with a low binding affinity for polycyclic aromatic hydrocarbons [21]. They respond, however, to TCDD, the most powerful inducer. The absence of effect of dietary canthaxanthin or β-apo-8′-carotenal in the AhR-low responsive DBA/2 mice strongly suggests that CYP1A induction by carotenoids is mediated by the Ah receptor. The complete absence of inductive effect of canthaxanthin in Ah receptor gene knockout mice, in contrast to C57BL/6 mice and to the rat, clearly confirms the key role of the Ah receptor in CYP1A induction by carotenoids.

The classical CYP1A inducers, ligands of the Ah receptor, induce both CYP1A1 and CYP1A2 in rats and responsive mice. The 5′-flanking region of the *CYP1A1* gene contains several replicates of a xenobiotic-responsive element (XRE), to which a protein heterodimer, including the activated Ah receptor, binds to activate the transcription of the gene [5]. CYP1A2 induction is also transcriptional, but the mechanism is less known and appears more complex. Induction of human *CYP1A2* by 3-MC involves both AhR-dependent and AhR-independent responsive elements in the 5′-flanking region of the gene [22]. Moreover, known specific CYP1A2 inducers, namely, piperonyl butoxide and acenaphtylene, have recently been shown to induce CYP1A2 and CYP1B1 mRNAs, *but not* CYP1A1 mRNA, in the liver of AhR knockout mice [23], demonstrating that CYP1A2 (and CYP1B1) can be induced through an AhR-independent pathway. Our results show that induction of CYP1A2 by canthaxanthin and β-apo-8′-carotenal is mediated, in contrast, by an AhR-dependent pathway, as is the case for 3-MC. In addition, the fact that canthaxanthin and 3-MC induce both CYP1A1 and CYP1A2 in C57BL/6 mice, whereas β-apo-8′-carotenal only induces CYP1A2, suggests that the mechanisms of induction of the two isozymes by carotenoids are not identical, though both AhR-dependent.

As Ah receptor ligands, the classical CYP1A1/2 inducers, such as 3-MC, TCDD, β-naphtoflavone or indolo-3,2(b)-carbazole, share several structural features: they are hydrophobic, planar, or coplanar molecules, of polycyclic structure, whose size allows them to fit into a 6.8 × 13.7 Å rectangle [6]. From a structural point of view, the inability of canthaxanthin and β-apo-8′-carotenal to compete with TCDD for the specific binding site of the C57BL/6 mice AhR could be expected, because the carotenoids are far from meeting the above requirements: they are not polycyclic compounds, are not planar, because the cyclohexene rings form an angle with the plane of the conjugated polyene chain, and are much larger in size (approximately 30 Å for canthaxanthin) [24]. Recently, the transcriptional induction of CYP1A1 and CYP1A2 by a thiazolium compound, YH439, has been demonstrated in rats and in cultured hepatoma cells, but not in Ah knockout mice or in

murine hepatoma cells without a functional AhR, which proves that the induction was mediated by an AhR-dependent pathway [25]. YH439 is neither a polycyclic nor a planar molecule, but has a planar region fitting the 6.8 × 13.7 Å rectangle. However, its ability to bind the Ah receptor has yet to be demonstrated. Indeed, benzimidazole compounds such as omeprazole, thiabendazole, lansoprazole, oxfendazole, cambendazole, and benzimidazole carbamate (but not benzimidazole) have been shown to be CYP1A1 inducers in human or rabbit hepatocytes [26–30] and in human Caco-2 cells [31] through an Ah receptor-mediated mechanism [31], but they have no affinity for the TCDD binding site of the Ah receptor [26, 27, 29, 31], which suggests that they activate the AhR without binding to it. Moreover, evidence for the ligand-independent activation of the Ah receptor has recently been uncovered [32, 33].

In terms of structure–activity relationships, the question arises as to why some carotenoids (canthaxanthin, astaxanthin and β-apo-8′-carotenal) act as CYP1A inducers while others (β-carotene, lutein and lycopene) do not, because no evident structural feature separates the former from the latter. One hypothesis holds that a metabolite common to the three active molecules might be the vector of the observed induction. Little is known, however, concerning the metabolism of carotenoids in mammals. Provitamin A carotenoids such as β-carotene or β-apo-8′-carotenal are metabolized in liver or intestine through central or excentric cleavage to yield retinal and retinoic acid [34]. Such cleavage reactions have not been shown, however, for canthaxanthin or astaxanthin in mammals, although some authors have speculated on the formation of 4-oxo-retinoic acid as an active metabolite from canthaxanthin [35]. 4-Oxo-retinoic acid is a physiological metabolite of retinoic acid and can thus also be formed from retinol, β-carotene or β-apo-8′-carotenal. In summary, known or putative metabolites that can be formed from the inducer carotenoids canthaxanthin, astaxanthin, or β-apo-8′-carotenal can also be derived from β-carotene or vitamin A, which have no inducing effect. Thus, no simple hypothesis concerning the metabolism of carotenoids to retinoids can explain why some carotenoids induce CYP1A while others do not. Retinoic acid and other retinoids regulate cell growth and division through their binding to specific receptors, RAR and RXR, which modulate gene expression [36]. One of these receptors, RXR, can form heterodimers with other receptors, such as thyroid hormone or vitamin D receptors, allowing regulations of hormone or vitamin action by retinoids [36]. Interestingly, recent studies have shown that retinoic acid can antagonize the effects of TCDD: retinoic acid is a potent antagonist of TCDD-induced human keratinocyte differentiation [37], suppresses the differentiation-induced increase of AhR mRNA in keratinocytes [38], and antagonizes both basal and induced CYP1A1 in human keratinocytes [39]. These findings support an antagonistic effect of retinoic acid on CYP1A1 induction, although the recently reported presence of a retinoid-responsive element in the promoter region of the human *CYP1A1* gene [40] would suggest the possibility of upregulation of *CYP1A1* by retinoic acid. However, other retinoids might have different effects on CYP1A. Thus, 13-*cis* retinoic acid, a retinoid used in dermatology, has been shown to increase some CYP-dependent enzyme activities in rat liver, including EROD [41]. Whatever the mechanism involved, the possibility that carotenoids activate the Ah receptor through retinoid metabolite(s) cannot be excluded and merits further investigation. In particular, the effects of retinoids on CYP1A expression, as well as their affinity for the binding site of the AhR, need further research.

Apart from the question of their metabolism, the finding that some carotenoids are potent inducers of liver CYP1A1 and CYP1A2, but have no affinity for the TCDD binding site of the AhR, suggests alternative mechanisms possibly accounting for AhR activation: (1) binding to a second binding site, distinct from the TCDD binding site; and (2) weakening of the interaction forces, which, in the absence of ligand, maintain the complex of AhR ligand-binding subunit with HSP90 in a quiescent state. Our results call for further experiments: (1) to determine whether carotenoids (or retinoid metabolites) can bind the Ah receptor ligand-binding subunit, using labeled carotenoids; (2) to search for other effects of carotenoids (or retinoids) on the native receptor to understand how they can activate AhR without binding to it; and (3) to find evidence for the binding of a carotenoid (or retinoid)-activated AhR complex to DNA using the gel retardation assay.

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