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Title: Astaxanthin can alter CYP1A-dependent activities via two different mechanisms:
induction of protein expression and inhibition of NADPH P450 reductase dependent
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Running title: The effects of Ax on xenobiotic metabolisms

Key words: astaxanthin, cytochrome P450 1A, conjugating enzyme, metabolic activation, benzo[a]pyrene, NADPH P450 reductase

Abbreviations;Ax: Astaxanthin

B[a]P: benzo[a]pyrene

CYP: cytochrome P450

DCNB: 1,2-dichloro-4-nitrobenzene

EROD: ethoxyresorufin O-deethylase

GSH: reduced glutathione

GST: glutathione S-transferase

UDPGA: glucuronic acid trisodium salt

UGT: UDP-glucuronosyltransferase

Abstract

Astaxanthin (Ax), a xanthophyll carotenoid, is reported to induce cytochrome P450 (CYP) 1A-dependent activity. CYP1A is one of the most important enzymes participating in phase I metabolism for chemicals, and it can activate various mutagens. To investigate the effect of Ax on the metabolic activation of a typical promutagen, benzo[a]pyrene by CYP1A, we orally administrated Ax (100 mg / kg body weight / day for 3 days) to male Wistar rats. In the treated rat liver, expression of CYP1A1 mRNA, protein, and its activity were significantly increased (5.5-fold, 8.5-fold, and 2.5-fold, respectively). In contrast, the activities of phase II enzymes (glutathione S-transferase and glucuronosyl-transferase) were not modulated by Ax. As a consequence, the mutagenicity of benzo[a]pyrene was more enhanced in Ax-treated rats, compared with controls in the Ames assay. On the other hand, NADPH P450 reductase activity was decreased in liver microsomes from the treated group. This result suggests the possibility that Ax inhibits the electron supply necessary for CYP catalytic activities and decreases CYP1A activity indirectly. In conclusion, Ax intake can alter CYP1A-dependent activities through two different mechanisms: 1) induction of CYP1A1 mRNA, protein expression, and activity; and 2) inhibition of the electron supply for the enzyme.

Introduction

Carotenoids are pigments found widely throughout the world and are consumed by animals including humans as food or supplements every day. Astaxanthin (Ax), one of the xanthophylls, is synthesized by *Haematococcus* spp., and is of great interest for its strong antioxidant effect.

Since the drastic antioxidant effect of β -carotene was published in 1984 [Burton and Ingold, 1984], numerous studies have focused on antioxidant effects exhibited by diverse carotenoids. In particular, previous in vitro studies have demonstrated that Ax can efficiently quench singlet oxygen [Cantrell et al., 2003], trap radicals and prevent peroxidation of the phospholipid membrane [Goto et al., 2001], and protect cells against DNA damage induced by reactive nitrogen species [Santocono et al., 2007]. In addition, Ax has been also reported to suppress increases in body weight and percentage of adipose tissue in mice that resulted from intake of a high-fat diet [Ikeuchi et al., 2007]. Thus, daily supplementation of β -carotene or other carotenoids, especially Ax, is thought to be beneficial for human and animal health as an antioxidant, cancer preventative, and suppressor of obesity.

However, several reports showed unexpected effects of Ax on xenobiotic metabolism. Especially, Ax induced cytochrome P450 (CYP) 1A-dependent activity [Gradelet et al., 1996; Wolz et al., 1999; Jewell and O'Brien, 1999]. The activities of the CYP1A subfamily appear to be one of the most important factors in cancer initiation, since mutagenic and carcinogenic chemicals, e.g., heterocyclic aromatic amines and polycyclic aromatic hydrocarbons, are metabolically activated by the CYP1A subfamily, and become electrophilic intermediates that can damage DNA [Nebert, 1991]. Increased CYP1A1 activity has been shown to be related to a high risk of lung cancer and colorectal cancer [McLemore et al., 1990; Sivaraman et al., 1994]. Although daily intake of Ax is recommended for its strong antioxidant effects, there is very limited information about Ax supplementation effects on CYP1A-related xenobiotic metabolism and metabolic activation of promutagens.

Benzo[a]pyrene (B[a]P), a typical environmental promutagen, is found in grilled food, water and tobacco smoke. B[a]P is metabolized by CYP1A and its metabolites exert strong mutagenicity. The toxic intermediates of B[a]P activated by CYP1A are subsequently conjugated by phase II enzymes, including glutathione S-transferase (GST) or UDP-glucuronosyl-transferase (UGT) isoforms, and become detoxified. In this study, we initially performed enzymatic analysis of CYP1A and phase II enzymes, and then

determined that the effects of Ax on the mutagenicity caused by B[a]P occurred as a result of metabolic activation by CYP1A and detoxification by phase II enzymes.

The aim of this study is to investigate potential adverse effects of Ax through the alteration of xenobiotic metabolism. In this study, we finally found bilateral characteristics of Ax in the modulation of the mechanism of CYP-dependent activity.

Materials and Methods

Chemicals

S9 cofactor NADPH, glucose 6-phosphate (G6P), and glucose 6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan), bovine serum albumin, cytochrome c, and dimethyl sulfoxide (DMSO) from Sigma Chemical Co. (St. Louis, MO, United States), and resorufin, ethoxyresorufin, reduced glutathione (GSH), glucuronic acid trisodium salt (UDPGA), 1-chloro-2,4-dinitrobenzene (CDNB) from Merck Ltd. (Tokyo, Japan), and 1,2-dichloro-4-nitrobenzene (DCNB) and *p*-nitrophenol (PN) from Kanto Chemical Co., Inc. (Tokyo, Japan). Anti-rat CYP1A1 and NADPH-P450 reductase antibodies from goat were obtained from Daiichi Pure Chemicals (Tokyo, Japan) and anti-goat IgG and anti-rabbit IgG conjugated with horseradish peroxidase were acquired from Santa Cruz Biotechnology (Santa Cruz, MO, United States).

Animals and treatment

Eight-week-old male Wistar rats with a body weight of 201.1 ± 8.7 g (mean $\pm$ SD) (SLC, Hamamatsu, Japan) were housed at $24 \pm 1^\circ\text{C}$ with a 12-h dark cycle, and given laboratory feed (MR stock, Nosan Co., Yokohama, Japan) and water ad lib for 1 week. For animal treatment, we used coconut oil (Coconad MT, Kao Co. Ltd., Tokyo, Japan) containing 5%

Ax which is extracted from biomass production of *H. pluvialis*. Rats were orally administered 5% Ax oil or only coconut oil (negative control) at 2 ml/kg body weight for 3 days. The daily Ax supplementation was 100 mg/kg body weight. Each group contained 4 rats, respectively. Twenty-four hours after the last administration, rats were asphyxiated with carbon dioxide, and their livers were removed, then immediately frozen in liquid nitrogen and stored at -80°C before use. There were no significant differences in body weight and weights of various organs between control and Ax-treated groups. All experiments using animals were performed under the supervision and approval of the Institutional Animal Care and Use Committee of Hokkaido University (No. 8095).

RNA extraction and cDNA synthesis

Total RNA was isolated from rat liver using TRI Reagent (guanidium/phenol, Sigma Bioscience). Briefly, tissue samples were homogenized in 1 ml of TRI Reagent, and 0.2 ml of chloroform was added to each sample. The mixtures were shaken for 15 sec, followed by centrifugation at 4°C and 15,000 g for 20 min. The organic layer was transferred to a new set of tubes, and 1 ml of isopropanol was added to each sample. After 5–10 min incubation at room temperature, the samples were centrifuged at 4°C and 15,000 g for 10 min. The RNA pellets were washed with 70% ethanol. RNA was dissolved in deionized distilled water (DDW). The prepared RNA was checked by electrophoresis to show that the RNA

integrity was uncompromised, and then further checked by measuring the optical density. The optical density of all RNA samples was 1.8-2.0 based on the 260/280 ratio. RNA was stored at -80°C.

For cDNA synthesis, 1 µg of total RNA was used with 0.5 µl of 10 pmol oligo dT. The mixture was heated at 70°C for 10 min and quickly chilled on ice for 1 min. The cDNA was synthesized using Rever Tra Ace kit (Toyobo, Osaka, Japan) in a volume of 20 µL including 4 µL of 5 × RT buffer, 8 µL of dNTP, and 1 µL of Rever Tra Ace. The mixture was incubated at 42°C for 50 min and then heated at 99°C for 5 min. The concentration of synthesized cDNA was measured using a NanoDrop 1000 (Nano-Drop Technologies, Wilmington, DE, USA) and diluted with DDW. The cDNA was stored at -20°C.

Real-time PCR

The mRNA expression levels were quantified by a real-time reverse transcriptase-PCR (RT-PCR) assay, based on the 5' nuclease activity of the Taq polymerase with an Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). In this study, we applied the comparative CT quantification ($\Delta\Delta C_t$ method) of qrtPCR for comparing changes in gene expression of CYP1A1, GSTA1, and UGT1A6. Relative quantification was performed using glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) as the endogenous control gene. The probes and primers used were pre-designed transcripts (so-called inventoried assays) validated by Applied Biosystems bioinformatics design pipelines. The gene bank accession numbers, and Applied Biosystems assay IDs, respectively, were NM_012540.2 and Rn00487218_m1 (Cyp1a1), NM_031509.2 and Rn00755117_A1 (Gsta1), NM_057105 and Rn00756113_AH (Ugt1a6), and NM_017008.3 and Rn99999916_s1 (GAPDH). Each sample was analyzed in duplicate. An identical set of PCR cycle parameters that was validated by Applied Biosystems was used for all genes: 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

Preparation of liver S9 fraction and microsomes

Rat liver was perfused with 3 volumes of 100 mM potassium phosphate buffer at pH 7.4 and homogenized using a Teflon-glass homogenizer in ice. The homogenates were centrifuged at 9,000g for 20 min. The post-mitochondrial supernatants (S9 fraction) obtained were further centrifuged at 105,000g for 60 min. The microsomal pellet resulting from the second centrifugation was resuspended in 1.15% (w/v) KCl and centrifuged again for 60 min at 105,000g for washing. The washed microsomal fractions were then suspended in 100 mM potassium phosphate buffer (pH 7.4). All fractions were stored at -80°C. The protein concentration was determined by the method of Lowry [Lowry et al., 1951], using bovine serum albumin as the standard.

Western blotting

Aliquots of 27 µg of microsomal protein from treated and control rats were applied to 10% sodium dodecyl-sulfate polyacrylamide gels and separated by electrophoresis using a Protean 2 mini 1-D cell (BioRad, Hercules, CA, USA). Western blot analysis was performed according to previously reported method [Laemmli, 1970]. Proteins were transferred electrophoretically to nitrocellulose membranes. After blocking of membranes with 5% skimmed milk, the CYP1A1/2 and NADPH-P450 reductase were identified with specific antibodies and immunostained with diaminobenzidine as the substrate. After scanning of the membrane using a computer, the digital images were analyzed by the image analysis program, NIH image [Lennard, 1990]. This experiment was repeated twice.

CYP1A-dependent activity

Ethoxyresorufin O-deethylase (EROD) activity was determined by a fluorescence intensity assay using the general principles described by Crespi et al. [Crespi et al., 1997]. Reaction mixtures were 1 mL solution/tube containing 10 mM G6P, 10 mM MgCl₂, 500 µg microsomal protein and 10 µM ethoxyresorufin in 100 mM potassium phosphate buffer at pH 7.4. The tubes were preincubated in a dark room for 5 min at 37°C, and the reaction was started by adding 20 µL of a mixture of 50 mM NADPH and 200 U G6PDH and

stopped with 4 mL of ice cold methanol. The fluorescence of resorufin was detected with an excitation wavelength of 530 nm and emission wavelength of 590 nm. This experiment was repeated twice.

Phase II activities

GST activities were assayed using CDNB and DCNB as substrates. The incubation mixtures consisted of 100 mM potassium phosphate buffer (pH 7.4), 0.5 mM GSH, and 0.75 mM substrate (CDNB or DCNB) in a final volume of 2 mL. The enzymatic reactions were initiated by adding 10 μ L (for CDNB) or 100 μ L (for DCNB) of the S9 fraction containing 40–70 mg protein/mL. The absorbance at 340 nm (for CDNB) and at 345 nm (for DCNB) was recorded continuously for 1 min at 37°C using a Hitachi U-3300 double-beam spectrophotometer (Hitachi High-Tech Fielding Corporation, Tokyo, Japan). CDNB and DCNB conjugation activities were calculated by subtracting data obtained from a non-enzymatic reaction, using an extinction coefficient of 9.6 or 8.5 / mM / cm, respectively. UGT activity toward PN was assayed spectrophotometrically. The incubation medium contained about 0.5 mg microsomal protein, 5 mM MgCl₂, 3 mM UDPGA, 0.25 mM PN, and 0.02% Triton-X in 0.1 M Tris-HCl buffer (pH 7.4). To detect the glucuronidation of PN, the absorbance at 400 nm was recorded continuously for 1 min at 37°C using a Hitachi U-3300 double-beam spectrophotometer. PN conjugation activities

were calculated by subtracting data obtained from a non-enzymatic reaction, using an extinction coefficient of 18.2 / mM / cm. All assays were performed under conditions of linear reactions with respect to time and protein concentrations. These experiments were repeated twice.

Mutagenic activation

The Ames test was performed using liver S9 fractions (20 mg protein/mL) from control and Ax-treated rats according to the preincubation method of Ames [Ames et al., 1975] using the *Salmonella* typhimurium strain TA98 with minor modifications as follows. Five hundred microliters of the liver S9 fraction containing the complete NADPH generating system and NADH, 100 µL of an overnight culture of TA98 and coenzymes (30 mM GSH, 15 mM UDPGA, both of them, or 100 mM potassium phosphate buffer as a control) were added to the test tubes. Then, 50 µL of B[a]P (2.5 µg) as the mutagen or DMSO as a negative control was added, and the tubes were incubated for 25 min at 37°C in a rotary water bath. The reaction was stopped by addition of 2 mL of top agar, and the contents were poured onto a minimum glucose medium plate. The plates were incubated for 48 h at 37°C, and the number of revertant colonies was counted manually. We repeated each experiment at least twice.

Incorporation of Ax to liver microsomes

Forty microliters of Ax diluted in Coconad MT (0.02 - 20 mM) was added to 360 μ L of microsomal suspension (10 mg protein/mL) and was gently mixed for 5 min to incorporate the carotenoid into the microsomal membrane. After centrifuging at 22,500g at 0°C for 15 min, the free Ax and supernatant were removed and Ax-colored microsomes were resuspended in 360 μ L of 0.1 M potassium phosphate buffer (pH 7.4). According to previous report, approximately 10 % of Ax is incorporated into microsomal membrane [Socaciu et al., 2000]. Therefore, Ax incorporated samples were considered to contain Ax from 0.0002 – 0.2 mM. The samples were flash frozen by liquid nitrogen and stored at -80°C.

NADPH-P450 reductase activity

NADPH-P450 reductase activity was measured according to the method of Omura and Takesue [Omura and Takesue, 1970] with some modifications. Cytochrome c reduction by NADPH-P450 reductase was monitored at 550 nm and 37°C. The incubation mixture contained 100 mM potassium phosphate buffer (pH 7.4), 20 μ M cytochrome c, 50 μ M NADPH, 1 mM KCN, and 25 μ g microsomal protein. The amount of reduced cytochrome c was calculated by subtracting data obtained from a non-enzymatic reaction, using an extinction coefficient of 21 / mM / cm. We also checked that Ax had no effects on

absorbance at 550 nm. This experiment was repeated twice.

Statistical analysis

The results of enzymatic activities, mRNA expression, protein expression, and Ames assays are expressed as mean $\pm$ SE or SD. The data from the group treated with Ax were compared with data from the control group. Statistical significance was assessed using Student's t-test or Dunnett's test. Differences of $p < 0.05$ were considered to be statistically significant.

Results

CYP1A1 mRNA, protein expression, and its dependent activity were induced by Ax treatment

In Figure 1, Ax did not change mRNA expression of GSTa1 or UGT1a6 in rat liver. However, Ax significantly induced CYP1A1 mRNA (5.5-fold, $p<0.05$), and a great increase in CYP1A protein expression in rat liver (8.5-fold, $p<0.01$) was observed as shown in Figure 2.

In the case of ethoxyresorufin O-deethylation, this activity is dependent on CYP1A, especially CYP1A1. As shown in Figure 3-A, the CYP1A-dependent EROD activity was 102.1 ± 9.6 pmol/min/mg protein in Ax-treated rats and 42.0 ± 3.0 pmol/min/mg protein in controls. Compared to the control group, Ax-treated rat liver showed significantly higher CYP1A-dependent activity by 2.5-fold ($p<0.01$), but it was lower than that expected from the changes in the protein expression level (8.5-fold). While CYP1A-dependent activity was induced by 2.5-fold in Ax-treated rat liver, the activity of GST toward CDNB or DCNB was not modulated by treatment with Ax (Figure 3-B). In addition, activities of UGT toward PN did not differ between control and Ax-treated groups as shown in Figure 3-B.

Ax altered metabolic activation and detoxification of B[a]P

The mutagenicity caused by B[a]P is determined by a balance of CYP1A-dependent activation and GST- and UGT-dependent conjugation. In Figure 4, in both control and Ax-treated groups, addition of coenzymes contributed to a reduction of the mutagenicity caused by B[a]P. GSH (30 mM) reduced the number of revertant colonies/plate from 109 to 35 (31.5%) and from 170 to 62 (36.5%) in control and Ax-treated groups, respectively. UDPGA (15 mM) reduced the number to 88 (80.2%) in control and to 131 (77.1%) in Ax-treated groups. The combination of 30 mM GSH and 15 mM UDPGA reduced the number of revertant colonies to 20 (18.0%) and 56 (33.1%) in control and Ax-treated groups, respectively. However, clear increases in the number of revertant colonies induced by B[a]P were observed in Ax-treated rat liver S9, even in the presence of GSH or UDPGA.

NADPH P450 reductase activity was reduced in Ax-treated rat liver microsome

NADPH P450 reductase transfers electrons from NADPH to CYPs, cytochrome c, or cytochrome b5. Since the electron supply by this enzyme is the key factor of rate limitation for catalytic activities of CYPs (see Scheme 1), we measured the NADPH P450 reductase dependent activities. The protein expression of the enzyme was significantly increased by

Ax treatment by 1.7-fold as shown in Figure 5-A. However, as shown in Figure 5-B, the activity of NADPH P450 reductase normalized with the protein expression level was 23.0 ± 1.2 nmol/min/mg protein in the control group, and 11.0 ± 0.4 nmol/min/mg protein in the Ax-treated group. The activity of NADPH P450 reductase in Ax-treated rat liver was significant lower than the control group (48%, $p < 0.001$).

In Figure 6, we demonstrated the activity of NADPH P450 reductase using rat liver microsomes incorporated Ax in vitro. Ax inhibited the cytochrome c reduction dependent the enzyme in a dose dependent manner. One micromolar of Ax in the reaction significantly reduced the activity to 56% ($p < 0.05$) when the value of only vehicle containing liver microsomes was set to 100%.

Discussion

Diet and dietary factors are believed to be responsible for a major number of cancers worldwide. B[a]P, a strong mutagen that is found in coal tar, tobacco smoke, and also in grilled food, is metabolized by members of the CYP superfamily, notably CYP1A, and transformed to mutagens (B[a]P-7-8-diol-9-10-epoxide and B[a]P-radical cation) that can damage DNA. These genotoxic intermediates are conjugated by GST and UGT, resulting in reduced mutagenicities. Thus, the activities of CYP1A and phase II enzymes are critical determinants for the mutagenicity caused by B[a]P.

In this study, liver microsomes from Ax-treated rats, 100 mg / kg body weight / day for 3 days, showed significantly higher CYP1A-dependent EROD activity than that from control rats (Figure 3-A). Thus, we postulate that B[a]P was more strongly activated in treated rat liver. On the other hand, both GST- and UGT-conjugating activities did not differ between the Ax-treated and control groups (Figure 3-B). Therefore, an obvious increase of the number of revertant colonies induced by B[a]P was observed in Ax-treated rats, compared with the control in the Ames assay (Figure 4).

However, the induction of EROD activity by Ax observed in our study was much weaker

than those in other studies. Previous studies reported that feeding 300 ppm of Ax in their diet induced EROD (CYP1A1) and MROD (CYP1A2) by 26.8- and 10.8-fold, respectively [Gradelet et al., 1996]. Also, Ax at about 45 mg/kg body weight for 16 days induced EROD activity by 56.1-fold [Jewell and O'Brien, 1999], and a dose of 30 mg/kg body weight Ax for 4-5 days induced it by 17.1-fold [Wolz et al., 1999]. In all of these three reports, authors orally administrated Ax to rats and investigated EROD activity using liver microsomes as same as our study. However, it should be noted that there was a clear difference between our mode of administration and other reports. While we administered Ax to rats in the form of a highly fluid oil once a day, the previous three studies provided the carotenoid powder-containing diet as pellets, and their rats ate it throughout the day. Therefore, it is expected that the concentrations of Ax in our study were much lower and the rats' exposure to this compound was much shorter than in the previous studies [Gradelet et al., 1996; Wolz et al., 1999; Jewell and O'Brien, 1999]. In fact, we observed feces colored by Ax from the treated rats, so that the amount of Ax absorbed was thought to be much lower than expected from the administrated concentration.

Petri and Lundebye (2007) reported the amount of Ax in rat tissues after feeding them an Ax containing diet [Petri and Lundebye, 2007]. Approximately 105 ng/g wet weight Ax was detected in rat liver after feeding about 30 mg /kg body weight/day of an Ax

powder-containing diet for 7 or 14 days in their study. Moreover, they suggested that Ax is rapidly eliminated and is difficult to accumulate for long periods of time, at least in the case of rats. In addition, we believe that the administration method determines the concentration of Ax in tissues, leading to differences in the inducible effect of CYP1A-dependent activity.

On the other hand, we revealed that Ax can increase the transcription of CYP1A1 mRNA and protein expression, leading to the induction of CYP1A dependent EROD activity. However, although the amount of CYP1A protein was increased by 8.5-fold in Ax-treated rat liver, CYP1A-dependent EROD activity was induced by only 2.5-fold, compared to the control group. There seems to be a big gap between protein expression level and its dependent activity in the Ax-treated group.

In this regard, we propose that Ax inhibited CYP dependent activity by reducing the electron supply necessary for CYP. The monooxidation of a substrate by CYP occurs in tandem with the reduction of CYP by NADPH P450 reductase as shown in Scheme 1. Thus, the activity of CYP is greatly affected by the electron supply offered by NADPH P450 reductase. Epigallocatechin gallate and tannic acid, which are ingredients contained in plants, e.g., tea, cocoa, bean, grape, and so on, are reported to be strong non-selective inhibitors of CYP activities in rat and human liver microsomes [Muto et al., 2001; Yao et al., 2008]. In particular, CYP1A is non-competitively inhibited by tannic acid without

modulating the structure of the enzymes [Yao et al., 2008]. Considering that 20 μ M of tannic acid inhibits NADPH P450 reductase activity by ~55% and CYP1A2 dependent enzymatic activity by 90% [Yao et al., 2008], the inhibitory effect of tannic acid on CYP1A activities might be partially attributed to the inhibition of electron transfer from NADPH to CYP by NADPH P450 reductase.

In the present study, we found a significant decrease of NADPH P450 reductase dependent cytochrome c reduction in Ax-treated rat liver microsomes (Figure 5-B). In addition, we also confirmed that the inhibition effect was not due to the reduction of protein expression of NADPH P450 reductase, as judged from Western blotting analysis (Figure 5-A). When adding Ax to rat liver microsomes in vitro, Ax reduced cytochrome c reduction in a dose dependent manner (Figure 6), clearly indicating that Ax has an effect on electron transfer on the microsomal membrane. In a previous study, NADPH P450 reductase activity or its protein expression were not modulated by Ax treatment [Gradelet et al., 1996]. On the other hand, β -ionone increased protein expression of NADPH P450 reductase, leading to induction of the enzymatic activity [Jeong et al., 1998]. Since metabolites of Ax in rat are reported to possess β -ionone architecture, it might be expected that Ax and its metabolites show opposite effects on the expression or activity of NADPH P450 reductase.

Ax has a high affinity for phospholipids and exists mainly within the membranes of microsomes in hepatocytes. Over 50% of Ax deposited in liver tissues was detected in the microsomal fraction and 15% was in the mitochondrial fraction in chicken after a single feeding of Ax (45 mg / kg) [Takahashi et al., 2004]. Thus, it is expected that also in our study Ax was contained in the microsomal membrane and reduced the electron supply for CYP, at least partially contributing to the decrease of CYP enzymatic activities.

There is much debate on the mechanism of carotenoids in the prevention of cellular phospholipid membrane damage. Antioxidants such as carotenoids have been reported to reduce and remove free radicals and lead to the prevention of lipid peroxidation. The peroxidation of the phospholipid membrane, especially microsomes, is deeply involved in the electron transfer that occurs during the reduction and oxidation of CYPs. Nakagawa et al. reported that β -carotene and Ax provide antioxidant protection against NADPH-dependent microsomal lipid peroxidation at a moderately low concentration (0.4 μ M), and they hypothesized that the antioxidative function of carotenoids is essentially involved in the very initial stage of microsomal membrane phospholipid peroxidation [Nakarawa et al., 1997]. Our findings provide additional support to reveal the exact mechanism of the antioxidant effect exerted by carotenoids.

The importance of carotenoids as bioactive molecules remains unknown to a large degree. As reported by numerous researches, carotenoids notably β -carotene contribute to the protection against oxidative stress because of its strong antioxidant effect. Carotenoid consumption is of great interest in disease prevention studies and is even recommended for human health. However, in contrast, recent studies reveal undesirable effects caused by carotenoids [Albanes et al., 1996; Omenn et al., 1996; von Helden et al., 2009]. Two large-scale intervention trials, the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study (ATBC study) and the Carotene and Retinol Efficacy Trial (CARET study), investigated the effect of β -carotene supplementation alone (ATBC) or in combination with vitamin A (CARET) in subjects at risk for lung cancer owing to smoking or asbestos exposure. The effect of α -tocopherol, the predominant form of vitamin E, was also explored in the ATBC study. Unexpectedly, β -carotene supplementation increased lung cancer incidence in both the ATBC and the CARET study, whereas vitamin E supplementation did not show any effect [Albanes et al., 1996; Omenn et al., 1996]. In 2009, it was reported that β -carotene metabolites enhanced inflammation-induced oxidative DNA damage in lung epithelial cells [von Helden et al., 2009]. Thus, these reports clearly show the possibilities that carotenoids may exhibit not only antioxidant effects but also unexpected adverse effects. Actually, the information about their bioactivities rather than their antioxidant effects is very limited. Therefore, we should focus not only on beneficial activities, but also on other effects

especially their impact on xenobiotic metabolism. Actually, we clearly showed that Ax treatment enhanced the mutagenicity caused by B[a]P as an example of an undesirable effect at least under experimental conditions. The present study demonstrated the two-sided effects of Ax on xenobiotic metabolism: 1) induction of CYP1A1 mRNA, protein expression; and 2) reduction of electron supply, which is necessary for CYP enzymatic activity. However, most biological functions of carotenoids are still shrouded in darkness. Further study will shed light on the additional role of carotenoids, for example the transactivation mechanism of CYP1A1 mRNA by Ax.

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Figure legends

Figure 1. Expression level of CYP1A1, GSTa1, and UGT1a6 in rat liver treated with vehicle or Ax.

Rats were orally administered Ax (100 mg/kg body weight) or coconut oil (vehicle) for 3 days and then their livers were collected. Real-time PCR analysis was performed as described in the Materials and Methods section. mRNA expression levels were normalized by GAPDH mRNA, and the value of control was set to 1.0 (opened column; control, filled column; Ax-treated group). Each value represents the mean $\pm$ SEM of 4 rats. *Significant differences (Student's t-test, $p < 0.05$) between control and Ax-treated rat groups.

Figure 2. Results of Western blotting of CYP1A1/2.

Western blotting analysis was performed with liver microsomes from control (opened column) and Ax-treated rats (filled column) as described in the Materials and Methods section. Protein expression of CYP1A1/2 was normalized by control data. Each value represents the mean $\pm$ SEM of 4 rats. ****Significant differences (Student's t-test, $p < 0.0001$) between control and Ax-treated rat groups.

Figure 3. CYP1A activity and conjugating activity of GST and UGT.

(A) Ethoxyresorufin O-deethylase activity was measured using liver microsomes from controls (opened column) and Ax-treated rats (filled column) at 10 μ M ethoxyresorufin as described in the Materials and Methods section. Each value represents the mean $\pm$ SEM of 4 rats. **Significant differences (Student's t-test, $p < 0.01$) between control and Ax-treated rat groups. (B) The conjugating activities of GST toward CDNB or DCNB and UGT toward PN were measured using the S9 fraction (GST) and microsomes (UGT) from control (opened column) and Ax-treated rats (filled column) as described in the Materials and Methods section. Each value represents the mean $\pm$ SEM of 4 rats.

Figure 4. Mutagenic activation of B[a]P with and without coenzymes for GST and UGT.

Mutagenicity was assayed using *S. typhimurium* TA98 with the liver S9 fraction from control (opened column) or Ax-treated rats (filled column). Details are described in Materials and Methods. Each value represents the mean $\pm$ SEM of 4 rats. *** ****Significant differences (Student's t-test, $p < 0.01$, $p < 0.001$, respectively) between control and Ax-treated rat groups.

Figure 5. The effect of Ax supplementation on NADPH P450 reductase.

(A) Western blotting analysis was performed with liver microsomes from control (opened

column) and Ax-treated rats (filled column) as described in the Materials and Methods section. Protein expression of NADPH P450 reductase was normalized by control data. Each value represents the mean $\pm$ SEM of 4 rats. **Significant differences (Student's t-test, $p < 0.001$) between control and Ax-treated rat groups. (B) NADPH P450 reductase dependent cytochrome c reduction was measured with liver microsomes from control (opened column) and Ax-treated rats (filled column) as described in the Materials and Methods section and was normalized to the protein expression level of NADPH P450 reductase. Each value represents the mean $\pm$ SEM of 4 rats. ***Significant differences (Student's t-test, $p < 0.001$) between control and Ax-treated rat groups.

Figure 6. NADPH P450 reductase-dependent cytochrome c reduction.

NADPH P450 reductase dependent cytochrome c reduction was measured with rat liver microsomes containing Ax (1-1000 nM in reaction) as described in the Materials and Methods section. Each value represents the mean $\pm$ SD of 3 tubes. *Significant differences (Dunnett's test, $p < 0.05$) among vehicle only and Ax containing microsomes.

Scheme 1. Metabolism of a substrate by CYP.

Monooxidization of a substrate (R) by CYP is occurs in tandem with the reduction of CYP by NADPH P450 reductase.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Scheme 1