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発行人　須摩春樹
発行所　株式会社　秀潤社
〒101-0054　東京都千代田区神田錦町3-5-1 興和一橋ビル別館
TEL: 03-5281-0551（大代表）　FAX: 03-5281-0550
03-5281-0552（営業部直通）　03-5281-0555（編集部直通）
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Inhibition of estrogen action by 2-phenylchromone as AhR agonist in MCF-7 cells

Joohee Jung [1], Kunie Ishida, Jun-ichi Nishikawa, Tsutomu Nishihara *

Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan

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Abstract

Large amounts of phytoestrogen, a group of estrogen derived from plant sources, are taken from the diet by Asians, but a sign of feminization has not been fully recognized. In this study, we found that some flavonoids inhibited an effect on estrogen action without estrogen receptor (ER) binding. Considering the report that dioxin, an aryl hydrocarbon receptor (AhR) agonist, disrupts the transcriptional activity of ER without binding to the ER, 14 flavonoids were examined for the transcriptional activity of AhR by the yeast reporter assay (AhR). Among them, 2-phenylchromone (flavone, FLA) showed the highest activity. FLA increased the expression of CYP1A1 mRNA, and inhibited the expression of progesterone receptor and pS2 mRNA in MCF-7 cells via non-ER-mediated pathway. Further studies showed that FLA had agonist activity for AhR and enhanced the proteosome-dependent degradation of ERα protein. Thus, FLA inhibited the estrogen action without binding to the ER by acting as a competitive agonist for AhR, which meaning that there can be anti-estrogenic flavonoids such as FLA as well as estrogenic ones such as isoflavones.
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Keywords: 2-phenylchromone; Aryl hydrocarbon receptor; Estrogen receptor; MCF-7 cell

Introduction

Flavonoids are widely present in plants, and possess diverse physiological activities. Numerous reports have implicated flavonoid phytochemicals as possessing hormone-disrupting activity (Diel et al., 2004; Kuo, 2002; Hsieh et al., 1998). Similar to estrogen's role, several of these phytochemicals have been shown to prevent osteoporosis and cardiovascular disease (Lee et al., 2007; Bingham et al., 1998; Humfrey, 1988; Kutzer and Xu, 1997).

Estrogen plays important roles in the function, growth and differentiation of the mammary gland, uterus, and ovary. It also affects other tissues, including the bone, liver, cardiovascular system, and brain. Estrogen acts primarily through the estrogen receptor (ER), which is a member of the nuclear hormone receptor superfamily and a ligand-dependent transcription factor (Evans, 1988; Speroff, 2000). The biological activities of many flavonoids may occur via the ER-mediated pathway. It is well known that soybean and its products contain isoflavones, such as genistein, coumesterol, and diadzein, and Asians including Japanese have taken a large amount of such phytoestrogens from food for several hundreds of years. Nishikawa (2003) estimated that Japanese took phytoestrogen at 15 mg/day, corresponding to about ten times the tolerable daily intake of β-estradiol (E2). Nevertheless, feminine qualities in man did not appear (Iwamoto et al., 2006). It may be due to intake of food containing phytoestrogen along with anti-estrogenic substances. The effect of flavonoids on estrogen action by the ER-mediated pathway has been investigated through the authors (Nishihara et al., 2000), but the elucidation of the action of flavonoids is scant. Some chemicals may bind to other receptors, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Brosens and Parker, 2003; Mikamo et al., 2003). Among them, we had an interest in

* Corresponding author. Present address: School of Pharmacy, Hyogo University of Health Sciences, 3-6, Minatojima-1, Chuo, Kobe, Hyogo 650-8530, Japan. Tel.: +81 78 304 3000; fax: +81 78 304 2700.

E-mail address: nishihara@huhs.ac.jp (T. Nishihara).

[1] Present address: Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan.

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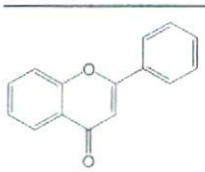
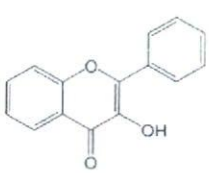
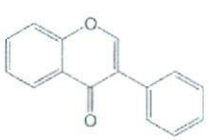
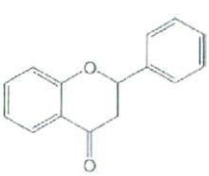
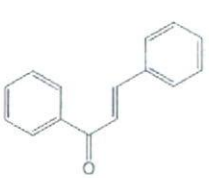
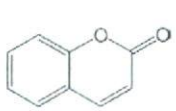
AhR, which is a ligand-activated transcription factor that stimulates gene expression when coupled with AhR nuclear translocator (ARNT) (Carver and Bradfield, 1997; Denison and Whitlock, 1995). TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a ligand of AhR) induces CYP1A1, CYP1A2, and CYP1B1 that hydroxylate E2. Moreover, TCDD and other agonists for AhR disrupt the transcriptional activity of ER by degradation of ER (Buchanan et al., 2000, 2002). Thus, agonists of AhR may mimic the effects of estrogen through the mechanism that is involved in the degradation of ER by a transcriptional active AhR–ARNT complex (Ohtake et al., 2003).

In this study, we investigated the effect of flavonoids on AhR and ER action (Table 1), and found that in MCF-7 cells, 2-phenylchromone (FLA, the structurally most basic compound) inhibited the expression of ER target genes, suggesting antagonist activity of ERα expressed via AhR in an indirect manner.

Table 1
Structures of flavonoids used in this study

Structures	Classifications	Test chemicals
	Flavones	Flavone (FLA)
	Flavonols	Kaempferol Quercetin
	Isoflavones	Biochanin A Daidzein Genistein
	Flavanones	Hesperetin Hesperidin Naringenin
	Chalcones	Phloretin
	Coumarins	Bromofluoro coumarin 2-Bromomethyl-7-methoxycoumarin Coumestrol 4-Methylumbeliferone

Materials and methods

Chemicals

FLA, E2, kaempferol, hesperidin, and α- and β-naphthoflavone (α-NF and β-NF) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), hesperetin and bromofluoro coumarin were from Extrasynthese (Genay Cedex, France), 2-bromomethyl-7-methoxycoumarin was from BD Gentest (CA, USA), 4-methylumbeliferone was from Sigma-Aldrich Co. (MO, USA), and MG-132 was from Calbiochem (Darmstadt, Germany). Biochanin A, coumestrol, daidzein, genistein, naringenin, phloretin, and quercetin were provided by Dr. H. Utsumi (Kyushu University).

Yeast assay for AhR ligand activity

The yeast transformed with the AhR–ARNT complex and xenobiotic-responsive element (XRE) plasmids was used as described by Miller (Miller, 1999). The AhR ligand activity was determined essentially according to the method of Adachi et al. (2001). The yeast strain YCM3 was grown for 5 h at 30 °C in SD medium lacking tryptophan. Test chemicals were added at given concentrations to 5 μl of culture and 200 μl of SD medium containing 2% galactosidase and incubated overnight at 30 °C. After the cell density was determined by reading O.D. at 595 nm, 10 μl of cell suspension was added to 140 μl of *Z*-buffer and β-galactosidase activity was determined by *o*-nitrophenol-β-D-galactopyranoside for 60 min at 37 °C. Absorbance was read at 415 nm.

Cells

MCF-7 cells were grown for routine maintenance in Eagle's minimal essential medium (EMEM) with phenol red (Nissui Pharmaceuticals Co., Tokyo, Japan), supplemented with 10 mM non-essential amino acids (Nacalai Tesque Co., Tokyo, Japan) and 10% dextran-charcoal treated fetal bovine serum (FBS). Cells were maintained in a humidified environment at 37 °C with 5% CO_2 in air.

RNA isolation and RT-PCR

MCF-7 (4×10^5 cells/ml) cells were plated in 35-mm dishes and, after 48 h incubation, treated with chemicals for 24 h. After treatment, the cells were washed twice with PBS and RNA was then isolated using Trizol (Invitrogen, CA). cDNA synthesized from 0.8 μg of total RNA using ReverTra Ace-α™ (TOYOBO, Osaka, Japan) and PCR for progesterone receptor (PR), pS2, CYP1A1, and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) was performed using Ampli Taq (Roche, Basel, Switzerland). The optimum cycle number that fell within the exponential range of response was used for PR (30 cycles), pS2 (21 cycles), CYP1A1 (30 cycles), or G3PDH (17 cycles).

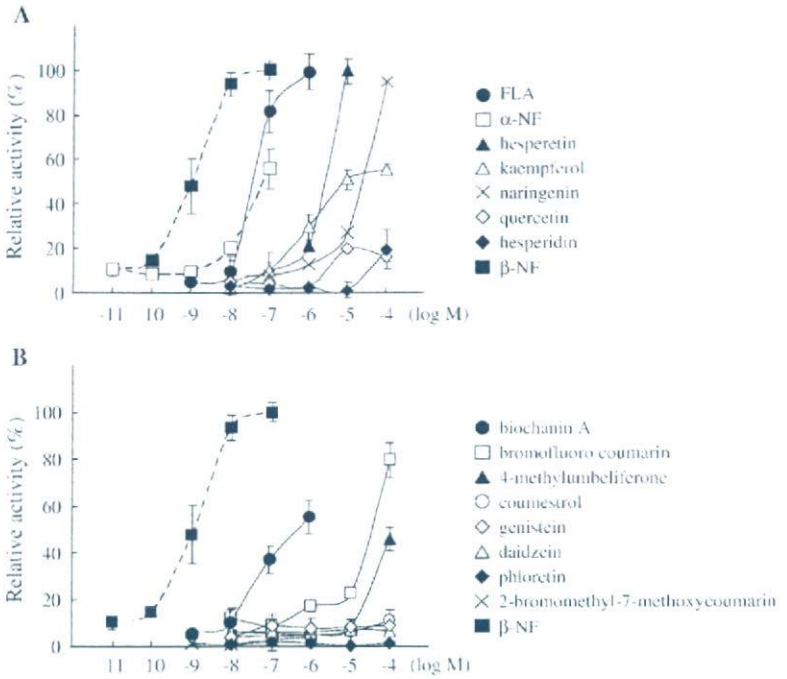


Fig. 1. AhR agonist activity of flavonoids. The test chemicals were applied to the recombinant yeast assay for AhR ligand activity as described in Materials and methods. The test chemicals were divided into two groups; a group included flavones, flavonols, and flavanones (A), and the other group included isoflavones, chalcones, and coumarins (B). Some flavonoids including FLA showed positive activity.

Yeast two-hybrid assay (ER)

We used a yeast two-hybrid assay system with the rat ER (rER) α and the coactivator, TIF2 as described in earlier works (Nishihara et al., 2000; Jung et al., 2004).

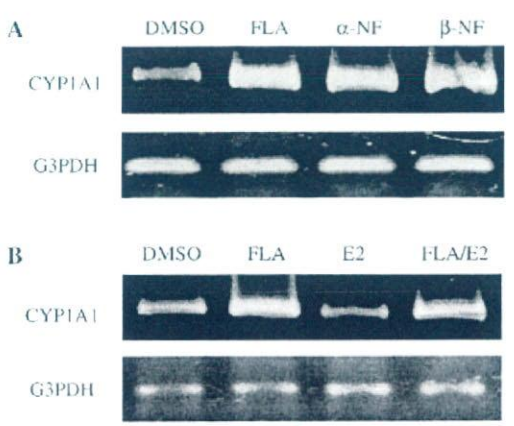


Fig. 2. Effect of FLA on the expression of CYP1A1 mRNA in MCF-7 cells. FLA (10 μM) was incubated with MCF-7 cells for 24 h in the absence (A) and presence (B) of E2 (10 pM). α- and -β NFs (10 μM) were used as positive controls, and DMSO as negative control. The expression of CYP1A1 and G3PDH mRNA (as an internal control) was detected by RT-PCR as described in Materials and Methods. FLA induced the expression as well as α-, β-NFs (A), but it was inhibited by E2 (B).

Estrogen receptor competitive binding assay

The binding activity of chemicals to human ER (hER) α was determined using a fluorescence polarization assay by FP Screen-for-Competitors Kit (ERα, high sensitivity; PanVera, Madison, WI). Briefly, 1 μl of each chemical solution was added to 49 μl of screening buffer in tubes and mixed well by shaking. Then, 50 μl of ERα-fluorescence estrogen (ES1) complex solution was added to the tube, incubated at room temperature for 1 h, and the fluorescence was determined using BEACON2000 (PanVera, Madison, WI). DMSO (0% inhibition) instead of the chemical solution was used as a negative control and 10 μl of ES1 (50 nM) instead of ERα ES1 complex as a

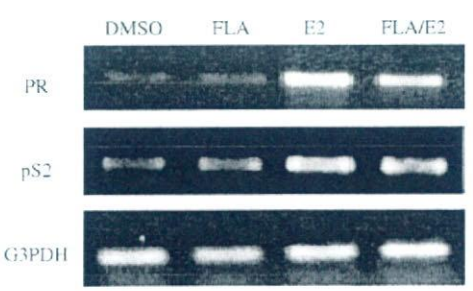


Fig. 3. Effect of FLA on mRNA level of E2-dependent target genes. FLA (10 μM) was incubated with MCF-7 cells for 24 h in the absence and presence of E2 (10 pM). The expression of PR, pS2 and G3PDH (as an internal control) mRNA was detected by RT-PCR. FLA repressed the expression by E2.

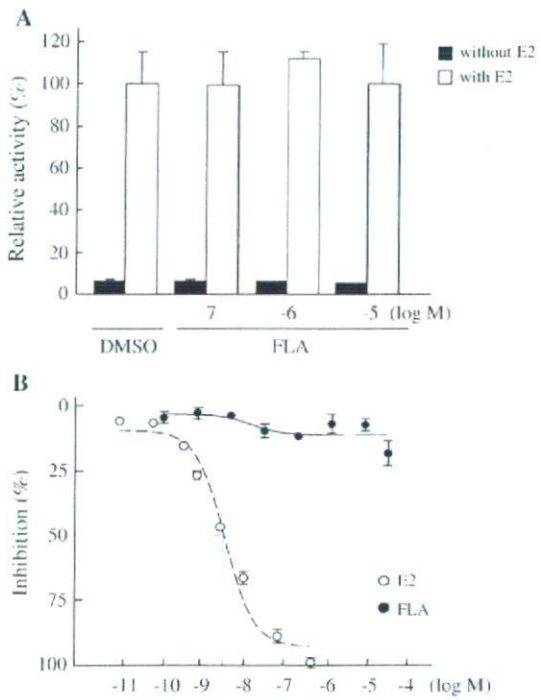


Fig. 4. Estrogen activity of FLA. (A) ERα agonist or antagonist activity of FLA was examined by yeast two-hybrid assay, and the relative activity (%) was calculated as the percentage of E2 (5 nM) activity (100%). (B) Binding affinity of FLA was examined by ERα competitive binding assay as described in Materials and Methods. FLA exhibited no effect on estrogen binding activity to ERα.

positive control (100% inhibition). Curve fitting was performed using GraphPad Prism 2.01 software to obtain IC_{50}.

Protein isolation and Western blots

MCF-7 (4×10^5 cells/ml) cells were plated in 35-mm dishes and, after 48 h, treated with chemicals for the indicated times. After treatment, the cells were washed twice with PBS and then lysed in 70 μl of lysis buffer containing 8 M urea, 1% NP-40, and 2% 2-mercaptoethanol. After removing the cell debris, the supernatants were used for protein concentration assay. The protein was boiled for 2 min, resolved on a 10% SDS-polyacrylamide gel, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore immobilon transfer membrane, MA). After the membrane was blocked using 3% skimmed milk (Yukijirushi, Tokyo, Japan) overnight at 4 °C, it was probed with primary antibodies ERα (1:200 in 1% skimmed milk, Santa Cruz Biotechnology Inc., CA) and G3PDH monoclonal antibodies (1:1000 in 1% skimmed milk, Chemicon International, MA). Following incubation with peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Amersham Pharmacia Biotech, UK).

Results and discussion

In the yeast reporter gene assay, the effect of flavonoids on AhR was evaluated because it had been reported that TCDD and other AhR agonists inhibited the expression of several E2-induced genes without binding to ER (Buchanan et al., 2002). Among 14 tested flavonoids, 7 compounds (FLA, biochanin A, hesperitin, kaempferol, naringenin, bromofluoro coumarin and 4-methylumbeliferone) dose-dependently increased the AhR activity (Fig. 1A and B). On the contrary, Hamada et al. (2006) reported that some of these flavonoids suppressed TCDD-induced CYP1A1 expression in dioxin-responsive HepG2 cells by permeating Caco-2 cell monolayers. The difference may involve the metabolism of flavonoids by drug-metabolizing enzymes in the Caco-2 cells. Furthermore, we investigated FLA, which was particularly responsible for the high activity of AhR. Although FLA was ten times weaker than β-NF (positive control), it was stronger than the other flavonoids and α-NF (a second positive control). Therefore, FLA was chosen for further study. AhR agonists induce the expression of several genes. For example, mRNA levels of CYP1A1 are induced by TCDD and other AhR ligands (Whitlock et al., 1996). RT-PCR assay indicated that in MCF-7 cells, FLA (fold, 2.6±1.0) induced the expression of CYP1A1 mRNA at similar levels to α- and β-NF (folds, 2.2±0.5 and 2.4±0.5) (Fig. 2A). In contrast, E2 inhibits the expression of genes induced by AhR ligands (Stacey et al., 1999). In this study, the induction of CYP1A1 mRNA by FLA (fold, 2.2±0.1) was inhibited in combination with E2 (fold, 1.5±0.4) (Fig. 2B). The expression of G3PDH mRNA was measured as control, and then it was not changed. Thus, the results suggest that FLA acted as an AhR agonist.

When we tested the anti-estrogenic activity of many chemicals including flavonoids by the yeast two-hybrid assay and the competitive ER binding assay, some flavonoids and

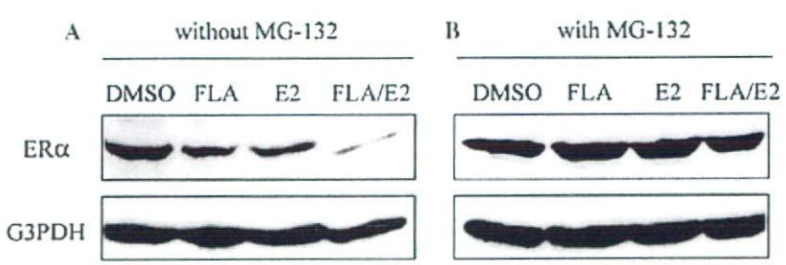


Fig. 5. Effect of FLA on ERα protein level. In the absence (A) or presence (B) of MG-132 (protease inhibitor), FLA (10 μM) was incubated with/without E2 (10 pM) in MCF-7 cells for 24 h. The cell lysates were subjected to SDS-PAGE and Western blot analysis for ERα and G3PDH (as an internal control). FLA induced the degradation of ERα and it was prevented by MG-132.

their metabolites were determined to be ER antagonists as well as agonists (Nishihara et al., 2000; Ahn et al., 2004a,b; Okamoto et al., 2006). As mentioned earlier in this work, however, these assays are insufficient to explain the anti-estrogenic activity of some flavonoids because these assays can measure only when chemicals directly affect the interaction with ERα. When we screened for the active compound by the reporter gene assay using MCF-7 cells in the presence of E2, some chemicals inhibited the transcriptional activity of ER (details not shown). The expressions of PR and pS2 mRNA were induced by E2 depending on the dose, indicating that these expressions demonstrated estrogen activity (Seo et al., 2003; Petz et al., 2002; Kim et al., 2000). As an AhR agonist, FLA was investigated for estrogen action. E2 (1 pM) induces the mRNA expression of PR (fold, 9.6±1.2) and pS2 (fold, 6.3±0.1) in MCF-7 cells. The E2 induced mRNA expressions of PR (fold, 2.1±0.2) and of pS2 (fold, 1.5±0.1) was minimized by treating with FLA, although FLA alone did not affect these expressions (Fig. 3). The expression of G3PDH mRNA was measured as control. To reconfirm the mode of action of FLA on ERα, the binding activity was examined. As shown in Fig. 4A, FLA had neither the agonistic, nor antagonistic activity on ER in a yeast two-hybrid assay using rER. In the competitive binding assay using hER, FLA did not inhibit binding of ER (Fig. 4B). The results suggested that FLA of the basic structure had anti-estrogenic activity without binding to the ER receptor, though several derivatives of the flavone group have estrogenic activity (Innocenti et al., 2007; Hiremath et al., 2000).

AhR agonists induce rapid proteosome-dependent degradation of ER (Wormke et al., 2003). Furthermore, in breast cancer cells, ligand-bound AhR enhances ubiquitinated forms of ERα and proteasome-dependent degradation of ERα to repress the E2-induced transactivation (Wormke et al., 2000). As shown Figs. 1 and 2, FLA was suggested to be a ligand of AhR. Consequently, the protein level of ERα in the presence of FLA was determined in MCF-7 cells. FLA or E2 significantly decreased ERα protein level and FLA together with E2 enhanced this effect. Since TCDD, a ligand of AhR, activates proteasome-dependent degradation of ERα (Ohtake et al., 2003), the effect of protease inhibitor, a MG-132, was determined on ERα protein level. The results show that MG-132 prevented ERα from degradation by FLA (Fig. 5). Moreover, the results indicate that FLA induced the expression of CYP1A1 mRNA to enhance the degradation of ERα protein, and inhibited the expression of PR and pS2 mRNA through the AhR pathway.

It has been reported that some flavones show estrogenic activity through ER binding so that their intake has a preventive effect against prostate cancer (Raschke et al., 2006) and menopausal syndrome (Miller-Martini et al., 2001), and also has a stimulative effect on endometritis (Cline et al., 2004). Recently the Food Safety Commission of Japan published the upper-limit dose for soy isoflavone supplement to be 30 mg/day. Although Wood et al. (2006) reported that soy isoflavones had anti-estrogenic effects in the postmenopausal breast through ER signaling, we have presented in this work that FLA can be anti-estrogenic via AhR in MCF-7 cells. This means that other AhR agonists in food may potentially affect the action of estrogen.

Acknowledgments

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β-Cryptoxanthin, a novel natural RAR ligand, induces ATP-binding cassette transporters in macrophages

Akira Matsumoto[a,b], *Hajime Mizukami*[b], *Satoshi Mizuno*[c], *Keizo Umegaki*[d], *Jun-ichi Nishikawa*[e], *Koichi Shudo*[f], *Hiroyuki Kagechika*[g], *Makoto Inoue*[a,b,*]

[a] *Laboratory of Medicinal Resources, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan*
[b] *Laboratory of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan*
[c] *Research and Development of Kemin Health Co. Ltd., 600 E. Court Ave., Suite A Des Moines, IA 50309, USA*
[d] *Information Center, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan*
[e] *Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan*
[f] *Research Foundation Itsuu Laboratory, 2-28-10 Tamagawa, Setagaya-ku, Tokyo 158-0094, Japan*
[g] *School of Biomedical Science, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan*

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ABSTRACT

Despite its serious adverse effects, recent accumulating evidence suggests that a physiological retinoic acid receptor (RAR) agonist, all-*trans* retinoic acid (atRA), exhibits preventive effects on atherogenesis. Therefore, the present study was designed to explore novel natural RAR ligands with anti-atherogenic effects in order to identify and develop a drug without severe side effects. Among xanthophylls and carotenoids studied, β-cryptoxanthin and lutein exhibited RAR ligand activity in yeast two-hybrid system that was found to be completely abolished by the RAR pan-antagonist LE540. Furthermore, these molecules can bind the RAR ligand-binding domain in the CoA-BAP system but not RXR ligand-binding domain. These results indicate that both β-cryptoxanthin and lutein serve as ligands for RAR, but not RXR, although their binding affinity was three orders of magnitude lower than that of atRA. Additionally, when applied to macrophages, β-cryptoxanthin indeed was found to induce the ATP-binding cassette transporter A1 (ABCA1) and ABCG1 mRNAs, which exert anti-atherosclerotic effects by preventing cholesteryl ester accumulation in macrophages. The induction of ABCA1 proteins by β-cryptoxanthin as well as atRA was abrogated by LE540. In summary, β-cryptoxanthin appears to be more an efficient provitamin A source than other carotenoids and xanthophylls including β-carotene, since β-cryptoxanthin can act not only as a RAR agonist but also a source of vitamin A. Taking into account that the pharmacodynamics difference between β-cryptoxanthin and atRA, β-cryptoxanthin appears to exert beneficial effects on atherogenesis through RAR activation in the manner different from atRA.

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* *Corresponding author at:* Laboratory of Medicinal Resources, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan. Tel.: +81 52 757 6792; fax: +81 52 757 6793.
E-mail address: minoue@dpc.aichi-gakuin.ac.jp (M. Inoue).
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1. Introduction

Prospective cohort studies show a direct inverse association between a higher intake of fruits and vegetables and the development of cardiovascular diseases, such as coronary heart disease and stroke [1–4]. Among major carotenoids and xanthophylls, such as β-carotene, lycopene, α-carotene, lutein, and β-cryptoxanthin, β-carotene has been studied most extensively, but no benefits have been found for it in randomized trials [5,6]. These studies indicate that the apparent benefits from fruit and vegetable intake are not due to β-carotene itself and may be more properly ascribed to other nutrients or other factors present in these foods.

Atherosclerosis is a complex disease characterized by chronic inflammation [7] and by abnormal and excessive proliferation of vascular smooth muscle cells (VSMCs) [8]. In addition, the presence of activated macrophages and T cells in lesions caused by the disease suggest that cell-mediated immune reactions also occur during the development of atherosclerosis [9]. All-*trans* retinoic acid (atRA), a bioactive retinoic acid produced from vitamin A (retinol), is known to inhibit neointimal formation following balloon withdrawal injury of rat [10–12] or rabbit carotid artery [13]. atRA is also capable of inhibiting VSMC proliferation and promoting VSMC differentiation [14–16]. In regards to macrophages that are intimately involved in atherosclerosis development, atRA has been shown to induce ATP-binding cassette transporter A1 (ABCA1) [17] and a synthetic retinoid (AM80) reduced scavenger receptor expression [18], suggesting that atRA or its derivatives prevent foam cell formation by decreasing intracellular cholesteryl ester accumulation [19]. In addition, it was recently been reported that atRA exerts direct effects on T cells by suppressing T helper (T_h)1 development and enhancing T_h2 development that is essential for antibody responses [20,21]. Since the T_h1-driven immune response has been consistently shown to promote atherosclerosis, immunomodulation through T_h2 should be suitable to help reduce the progression of atherosclerosis. In total, these findings imply that atRA may be capable of attenuating the development of atherosclerosis by suppressing cellular immunity and stimulating humoral immunity.

To date, no clinically proven therapy exists for the successful management of neointimal formation in atherosclerosis or restenosis following angioplasty. However, atRA therapy appears to be a promising attractive approach for the treatment of neointimal formation especially given the clinical applications of atRA have been shown to have some success in the treatment of human diseases such as cancer, psoriasis, and leukemia. However, the therapeutic use of atRA has been excluded from consideration because of serious adverse effects such as skin or liver toxicity, teratogenesis, hypertriglyceridemia and development of resistance to atRA in addition to atRA syndrome characterized by fever, respiratory distress, interstitial pulmonary infiltrates, pleural and pericardial effusion, episodic hypotension, and acute renal failure [22,23].

Retinoid signaling is transduced by two families of nuclear receptors, the retinoic acid receptor (RAR), and the retinoid X receptor (RXR) [24,25]. These receptors belong to the superfamily of nuclear hormone receptors that act as ligand-activated transcription factors. RAR forms a heterodimer with RXR and the ligand–receptor complexes act as inducible transcription regulators of several genes by binding to specific retinoic acid response element. The pleiotropic effects of retinoids are primarily brought about by the existence of two families receptors (RAR and RXR), the RAR isotypes (α, β, and γ), and the RXR isotypes (α, β, and γ) in addition to the cross-talk of these receptors with other signaling pathways and the existence of multiple putative coactivators and corepressors [26]. In addition, since the RAR ligand-binding domain is capable of adopting a conformation exhibiting a large hydrophobic cavity similar to that observed in PPARγ-ligand-binding domain [27], various RAR ligands can induce various conformational changes in RAR, resulting in versatile gene expression and repression via the recruitment of different coactivators or corepressors as PPARγ ligands [28,29]. Furthermore, metabolic enzymes and retinoid binding proteins involved in storage, transport, uptake and sequestration of retinoids play critical roles in determining the availability of bioactive retinoids in cells [30]. This notion may indicate that RAR ligands that are metabolically different from the natural ligands for the RAR, such as atRA, 9-*cis*-RA, and 13-*cis*-RA [31], appear to be useful in treating various diseases.

Consequently, it seems to be very important to explore various RAR ligands that can create beneficial responses in a living body in order to develop therapeutic agents for cancer, atherosclerosis, and rheumatoid arthritis while simultaneously clarifying the signaling pathways associated with RAR. The present study was designed to search for natural RAR ligands among carotenoids and xanthophylls in order to develop potential therapeutic agents for cardiovascular diseases and to clarify the benefits of fruit and vegetable intake to prevent such diseases. That research had culminated in the identification of β-cryptoxanthin as a novel natural RAR agonist and a better provitamin A to activate RAR/RXR heterodimer than β-carotene.

2. Materials and methods

2.1. Chemicals and reagents

β-Cryptoxanthin, zeaxanthin, β-carotene, lycopene, all-*trans* retinoic acid, and 9-*cis* retinoic acid were obtained from Yashima Pure Chemical Co. Ltd. (Tokushima, Japan), Extrasyntheses (Genay, France), Nacalai Tesque Co. (Kyoto, Japan), Wako Pure Chemical Industries (Osaka, Japan), Sigma Chemical Co. (St. Louis, USA), and Sigma Chemical Co. (St. Louis, USA), respectively. Lutein was kindly provided from Koyo Mercantile Co. Ltd. (Tokyo, Japan). RAR antagonist, LE540, was kindly provided from Dr. Kagechika of Tokyo Medical and Dental University. All test chemicals were dissolved in dimethylsulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) and stored at −80 °C until use. Antibody against ABCA1 was kindly provided from Dr. Yokoyama of Medical School of Nagoya City University.

2.2. Animals

Male C57BL/6J mice (6–8 weeks of age) were purchased from Nippon Charles River (Kanagawa, Japan). All animals were

kept in a temperature-controlled room (23 ± 1 °C) with a 12 h light/dark cycle, under specific-pathogen-free conditions and given a sterilized commercial diet (CE-2; Nippon Crea Co., Ltd., Shizuoka, Japan) and water *ad libitum* at the Laboratory Animal Center of Nagoya City University. All animal procedures were approved by the institutional animal care and use committee of Nagoya City University.

2.3. Yeast two-hybrid assay

Yeast expressing GAL4DBD-RARα or γ, GAL4DBD-RXRγ, GAL4AD-TIF2, and *lacZ* reporter plasmids were kindly provided by Dr. Nishikawa of Osaka University [32]. Yeast transformants were grown overnight at 30 °C with vigorous shaking in 5 ml of selective medium without leucine and tryptophan. The yeast was harvested by a centrifugation at 3000 × *g* for 15 min and suspended in fresh medium. The absorbance at 630 nm of the cell suspension was measured with U-2001 spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). All assays were performed by using the yeast suspension with the absorbance at 630 nm of 0.6. Four hundred and fifty microliters aliquots of the yeast suspension were cultured in the presence of 5 μl of test compounds for 4 h at 30 °C with vigorous shaking. The 100 μl of chemically treated yeasts were incubated with 30 μl of Z buffer (0.1 M sodium phosphate (pH 7.9), 10 mM KCl, 1 mM $MgSO_4$) containing 4 mg/ml ZYMOLYASE®-20T (Seikagaku Co., Tokyo, Japan) for 30 min at 30 °C. The enzymatic reaction of β-galactosidase was initiated by the addition of 25 μl of 0.5 mg/ml chlorophenol Red β-D-galactopyranoside dissolved in 0.1 M sodium phosphate buffer (pH 7.9). When the red color developed, 50 μl of 2 M Na_2CO_3 were added to stop the reaction. The absorbances at 540 and 630 nm were measured and the activity was calculated according to the following equation: U = [(absorbance at 540 nm) − (absorbance at 630 nm)]/(absorbance at 630 nm at the start of the assay). β-Galactosidase activity is presented as the means ± S.D. of three determinations.

2.4. Binding assay using CoA-BAP system [33]

Binding assay was performed using Nu ligand kit (Microsystems, Kyoto, Japan) according to the manufacturer's instructions. Briefly, 100 μl of GST-fused nuclear receptor protein dissolved in 0.1 M sodium carbonate buffer (pH 6.8) was added to 96-well plate and incubated overnight at 4 °C. After washing the plate with 120 μl of wash buffer A, alkaline phosphatase-fused TIF2 protein dissolved in wash buffer A and test compounds dissolved in DMSO were added and incubated for 1 h at 4 °C. The plate was then washed with 120 μl of wash buffer B. The enzyme reaction was started at 30 °C by the addition of 100 μl of NPP solution. When the yellow color developed, the reaction was stopped by the addition of 25 μl of 0.5 M NaOH. The absorbance at 405 nm was measured with ARVO™ Wallac 1420 Multilabel Counter (Wallac, Finland).

2.5. Cell culture

Thioglycolate-elicited macrophages were prepared as previously described [34]. Briefly, C57BL/6J mice were intraperitoneally injected with 2 ml of 3% thioglycolate medium (Difco Laboratories, Detroit, MI, USA). Six days later, peritoneal macrophages were harvested from the abdominal cavity, seeded at a concentration of 2×10^6 cells/ml and maintained in RPMI1640 medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin (Invitrogen), and 100 μg/ml of streptomycin (Invitrogen) for 2 h. After non-adherent cells were removed, the resulting macrophages were incubated with various concentrations of the test compounds in RPMI1640 medium supplemented with 0.1% bovine serum albumin for 8 and 20 h in order to prepare mRNA and protein fraction, respectively.

2.6. Western blot analysis

After 20 h incubation, macrophages were harvested and lysed by homogenization in 80 μl of lysis buffer containing 10 mM Tris-base, pH 8.0, 0.1% Triton X-100, 0.15 mM KCl, 5 mM mercaptoethanol, 1.3 mM EDTA, and protease inhibitor cocktail tablets (Roche Diagnostics) on ice. The lysates were palletized by centrifugation (12,000 × *g*, 15 min) at 4 °C, and the supernatant was assayed for protein concentration (Bradford method, Bio-Rad Laboratories). The lysates (protein 30 μg) were suspended in 0.9 M urea, 0.2% (v/v) Triton X-100, and 0.1% (w/v) dithiotheitol and supplied with 10% (w/v) lithium dodecylsulfate and then separated by 8% LDS-PAGE followed by transferring onto a polyvinylidene fluoride membrane (Millipore) in a transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 20% methanol). The membrane was blocked for 3 h in a solution of 5% powdered skim milk in Tris-buffered saline (TBS) and then incubated with anti-ABCA1 antibody diluted 1:1000 in TBS containing 1% of skim milk and 0.05% Tween-20 overnight at 4 °C. The blot was washed in three changes of wash buffer (0.05% Tween-20 in TBS) and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, MA, USA) in 1% powdered skim milk and 0.05% Tween-20 in TBS for 1 h at room temperature. The blot was thoroughly washed in three changes of wash buffer, and ABCA1 was detected using CDP-Star (PE Biosystems, Bedford, MS, USA) as a substrate of alkaline phosphatase. Protein concentration was determined using LAS-3000mini (FUJIFILM, Tokyo, Japan).

2.7. Reverse transcription

After 8 h incubation, macrophages were harvested and total RNA from macrophages was extracted using a RNeasy™ mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instruction. The resulted RNA was treated with DNase I (Invitrogen) to degrade contaminating DNA. The RNA was dissolved in diethyl pyrocarbonate-treated water and quantified by GeneQuant II (GE Healthcare Bio-Science Corp., NJ, USA). To prepare first strand cDNA, 500 ng of total RNA was reverse-transcribed using Revertra Ace (Toyobo Biochemicals, Tokyo, Japan) according to the manufacturer's instructions.

2.8. Quantitative RT-PCR

Reactions were prepared in 96-well optical grade PCR plate in a total of 50 μl containing the following components; 33 μg of cDNA dissolved in 25 μl of water, 25 μl of 2× TaqMan™ Universal Master Mix, 2.5 μl of TaqMan™ gene expression assays containing fluorogenic probes predesigned at Applied

Biosystems. Thermal cycling conditions consisted of an initial step of 2 min at 50 °C and then 10 min at 95 °C, followed by 40 cycles of 15 S at 95 °C and 1 min at 60 °C for ABCA1, ABCG1, and β-actin. Adjustments for baseline and threshold were performed according to the manufacturer's instructions. Levels of ABCA1 and ABCG1 mRNA expression were subsequently normalized relative to β-actin mRNA levels and calculated with delta–delta Ct method.

2.9. *Statistical analysis*

Data was represented as the mean ± S.D. or S.E. as described in the legends. Statistical significance was determined by Dunnett's test using Stat Light software. P-values less than 0.05 were considered significant.

3. Results

3.1. *RAR ligand activity using yeast two-hybrid assay*

The results of the binding activity against RARα and γ for the carotenoids and xanthopylls evaluated are shown in Fig. 1 for the yeast two-hybrid assay described in Section 2. atRA showed dose-dependent increase in β-galactosidase activity at the concentration of 10^{-10} to 10^{-8} M (Fig. 2A). Among carotenoids and xanthophylls tested, β-cryptoxanthin exhibited a dose-dependent increase in β-galactosidase activity in the concentration of 10^{-7} to 10^{-5} M. Lutein also showed increased β-galactosidase activity but at higher concentrations than β-cryptoxanthin. In contrast, β-carotene, zeaxanthin, astaxanthin, and lycopene did not exhibit significant activity. The transcription activation by β-cryptoxanthin and lutein was observed in both RARα and RARγ assays. When the agonist activity against RXRγ, which forms the heterodimer with RAR and transduces the signal, was determined, no activity was detected among any of the carotenoids and xanthophylls tested (Fig. 2B).

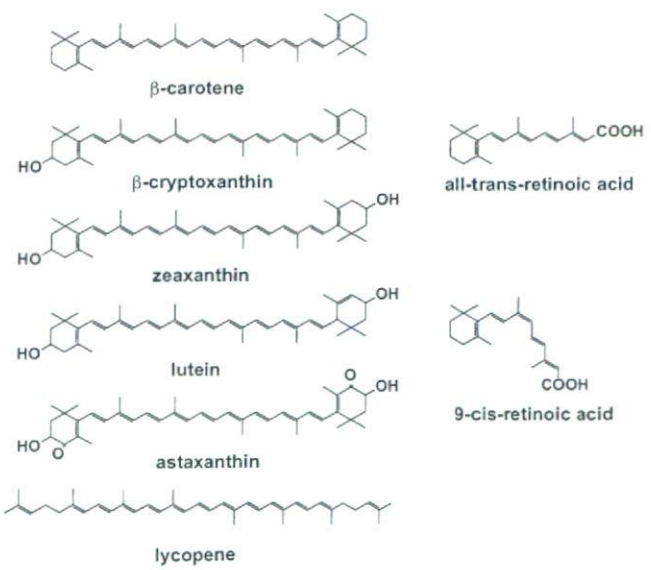

Fig. 1 – Structures of xanthophylls, carotenoids, and retinoic acid used in the present study.

3.2. *Effect of RAR pan-antagonist LE540 on RAR binding activity of β-cryptoxanthin and lutein*

To confirm whether β-galactosidase was induced by β-cryptoxanthin and lutein via RARα or RARγ, the effect of LE540, which is a pan-antagonist for RARs [35], was investigated. As shown in Fig. 3, β-cryptoxanthin induced β-galactosidase activity at the concentrations of 0.1 and 1 μM. This induction was completely inhibited by 10 μM LE540. Lutein induced β-galactosidase activity at 1 μM concentration and 10 μM LE540 abolished that induction. These results indicate that both β-cryptoxanthin and lutein induce β-galactosidase via RARα or γ in this yeast two-hybrid assay.

3.3. *Binding assay for RAR using CoA-BAP system*

To assess the agonist activity of β-cryptoxanthin and lutein and to preclude the possibility that β-cryptoxanthin metabolites or degradation products by yeast bind RARs, the binding activity to RAR was evaluated using the cell-free CoA-BAP system [33]. β-Cryptoxanthin and lutein were found to bind RARα in agreement with the result obtained in yeast two-hybrid assay. Conversely, zeaxanthin did not exhibit comparable binding activity in the CoA-BAP systems (Fig. 4) as was found in yeast two-hybrid assay. The β-cryptoxanthin dose where the binding activity was detected was three orders of magnitude higher than that of atRA. Additionally, when the binding activity to RXR was measured, no activity was found in β-cryptoxanthin and lutein. This corresponds to the results obtained from the yeast two-hybrid assay.

3.4. *Effect of β-cryptoxanthin on ATP-binding cassette transporters in macrophages*

Recently, ATP-binding cassette transporters A1 (ABCA1) and ABCG1 were reportedly induced in macrophages following the activation of RAR/RXR by retinoic acid [17]. To verify that β-cryptoxanthin acts as an RAR agonist in cells, the capacity of β-cryptoxanthin to induce ABCA1 and ABCG1 mRNAs in macrophages was evaluated. As shown in Fig. 5, β-cryptoxanthin increased mRNA levels of ABCA1 and ABCG1 dose-dependently, although the levels were less than those induced by atRA. Furthermore, the concentrations needed to induce both mRNAs were very similar to those found to induce effects in the yeast two-hybrid assay as well as in the CoA-BAP system. When ABCA1 protein levels were assessed by Western blot analysis, β-cryptoxanthin increased protein levels compared with vehicle-treated group, but less than atRA (Fig. 6A). In addition, the induction of ABCA1 protein by β-cryptoxanthin (5 μM) as well as atRA (5 μM) was completely abrogated by LE540 treatment (Fig. 6B). However, β-cryptoxanthin was not capable of increasing ABCG1 protein, although it increased ABCG1 mRNA levels (data not shown).

4. Discussion

In the present study, it has clearly demonstrated that β-cryptoxanthin and lutein, which are major nutrients in fruits and vegetables, are novel natural ligands for RARs and that β-

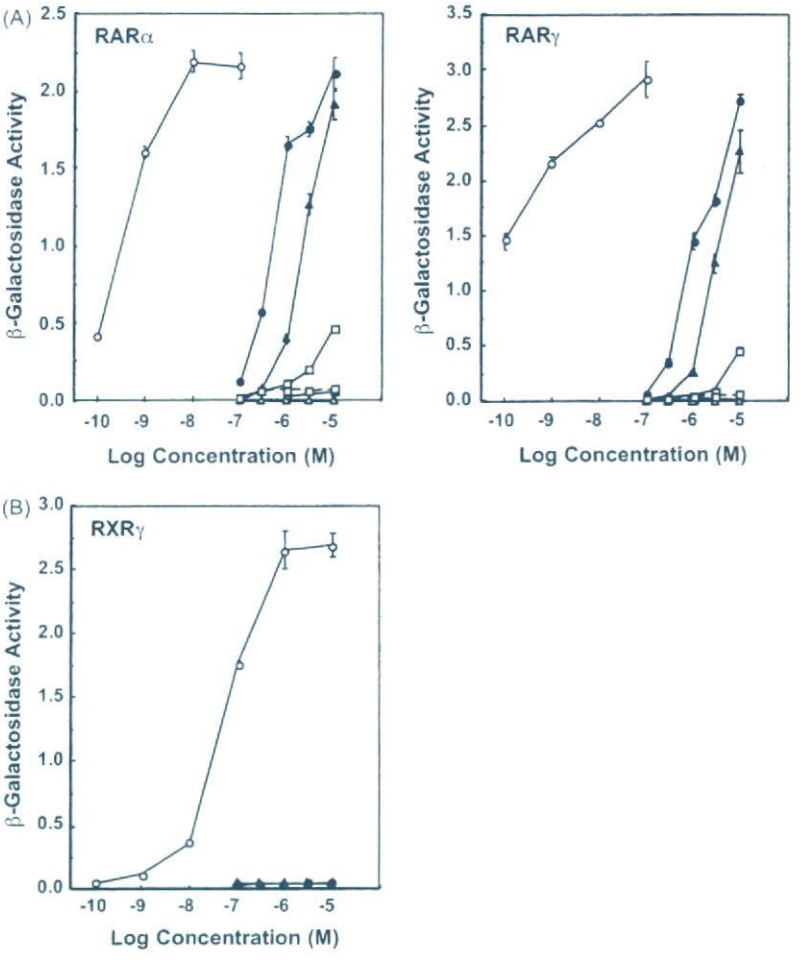

Fig. 2 – Yeast two-hybrid assay for RARα or RARγ activity. Yeast strain Y190 was transformed with GAL4 activation domain fused to TIF2 and GAL4 DNA binding domain fused to agonist-binding domain of RARα or RARγ. Chemicals were added to yeast cultures in doses ranging from 10^{-10} to 10^{-5} M. Following 4 h incubation the cultures were then assayed for β-galactosidase activity. Open circle in (A) and (B): atRA and 9-*cis* RA, respectively, closed circle: β-cryptoxanthin, open triangle: astaxanthin, closed triangle: lultein, open square: β-carotene, closed square: zeaxanthin, open square with broken line: lycopene. The values are represented as means ± S.E. of three determinants from a representative of three independent experiments, which showed similar results.

cryptoxanthin is a more potent ligand than lutein. Simultaneously, it has been shown that β-carotene, zeaxanthin, astaxanthin, and lycopene failed to show similar activity. An RAR pan-antagonist, LE540, completely abolished the β-crypotoxanthin- or lutein-dependent interaction between GAL4-RAR LBD and GAL4-TIF2 in the yeast two-hybrid assay, indicating that β-cryptoxanthin and lutein serve as an RAR agonist. The agonist activity was also supported by the finding that β-cryptoxanthin induces ABCA1 and ABCG1 mRNAs and ABCA1 protein in macrophages. To eliminate the possibility that metabolites or degradation products of β-cryptoxanthin act as RAR agonist, the binding assay was performed in cell-free CoA-BAP system. β-Cryptoxanthin exhibited binding activity against RAR LBD in a dose-dependent manner similar with the finding in the yeast two-hybrid assay. These data led to the conclusion that β-cryptoxanthin and lutein act as natural RAR agonists and may have preventive effects on atherosclerosis and restenosis.

β-Carotene is known to be cleaved in the intestinal mucosa at higher efficiency than any other organs. A responsible enzyme for cleaving β-carotene is β-carotene 15,15′-dioxygenase [36], which has a low level of activity toward provitamin A sources aside from β-carotene. The resulting all-*trans*-retinol (vitamin A) is first esterified to give an all-*trans*-retinyl ester, which is stored in the liver. In plasma, the vitamin A concentration is usually maintained at about 1 μM and the biologically active retinoid, atRA, is produced as the need arises in cells where the metabolic enzymes, aldehyde dehydrogenase and retinal dehydrogenase exist. The plasma concentration of atRA is therefore maintained at around a 12 nM level [37] and about 15% of this form is converted to 9-*cis* retinoic acid [38]. When considering the individual carotinoid and xanthopyll concentrations in plasma, β-cryptoxanthin has been shown to reach a concentration than other major carotenoids and xanthophylls, including β-carotene, α-carotene, lycopene, lutein, and zeaxanthin, assuming that the

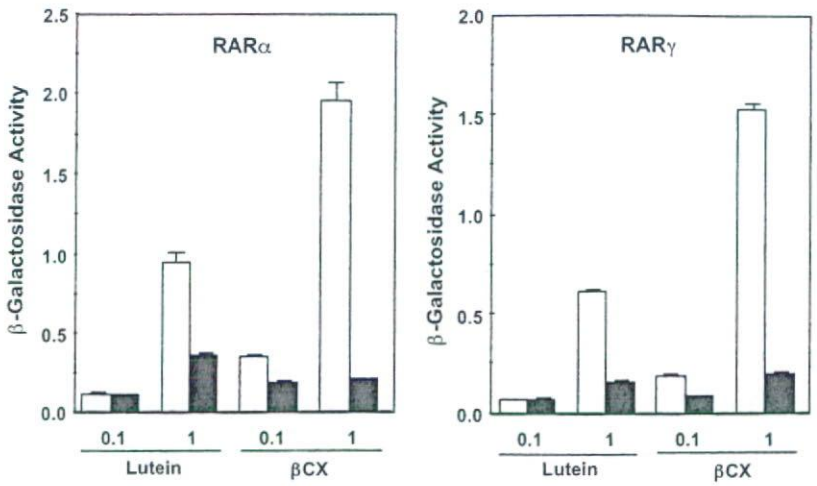

Fig. 3 – Effect of RAR pan-antagonist, LE540, on the transcriptional activity of β-cryptoxanthin or lutein against RARα or RARγ. Following 30 min incubation of 10 μM LE540 with yeast cultures, β-cryptoxanthin or lutein was added to yeast cultures at the indicated concentrations (μM) and incubated for 4 h. The cultures were then assayed for β-galactosidase activity. Open column: without LE540, closed column: with LE540. The values are represented as means ± S.E. of three determinants from a representative of three independent experiments, which showed similar results.

equal amounts of each of these carotenoid is present in the diet [39]. In actuality, β-cryptoxanthin reaches to 0.4–1.1 μM concentrations in the plasma after the intake of orange juice rich in cryptoxanthin [40,41]. In addition, the β-cryptoxanthin concentration in adipose tissue is also higher than that of other carotenoids and that the tissue distribution of β-cryptoxanthin is definitely different from that of atRA. The results of this study have shown that a 5×10^{-7} M of β-cryptoxanthin is sufficient to transactivate RAR and act as an RAR agonist, although the RAR-binding affinity of β-cryptoxanthin is three orders of magnitude lower than that of atRA. These results led to the hypothesis that the difference in the pharmacokinetics between β-cryptoxanthin and atRA makes β-cryptoxanthin an RAR agonist with physiological effects distinct from atRA. Although the preventive effects of vegetable and fruit intake against cardiovascular diseases have been considered based the known anti-oxidative effects of carotenoids found in vegetables and fruits, the result presented here strongly suggest that a β-cryptoxanthin signaling mechanism via RAR/RXR heterodimer contributes to the preventive effects by enhancing the expression of anti-atherogenic molecules such as ABCA1 and ABCG1.

This study did not reveal a structure–activity relationship that can explain why β-cryptoxanthin exhibited better binding activity to RAR in comparison to the other carotenoids and xanthopylls tested in this study. However, this difference may potentially be explained based upon the polarity and hydrophobicity properties of these compounds. It is reported that

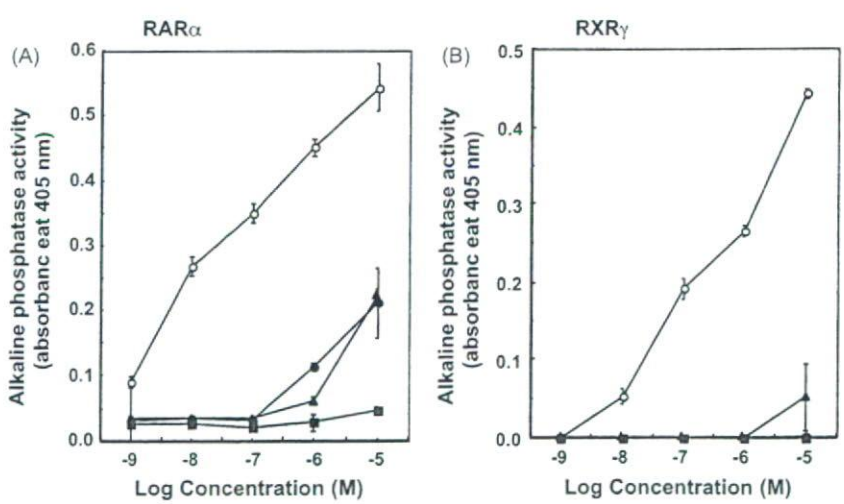

Fig. 4 – Binding activity of β-cryptoxanthin and lutein against RARγ in *in vitro* CoA-BAP system. Agonist-dependent interactions between GST-RARγ and TIF2-BAP were determined as alkaline phosphatase activity according to the method described in Section 2. Open circle in (A) and (B): atRA and 9-*cis* RA, respectively, closed circle: β-cryptoxanthin, closed triangle; lutein, closed square: zeaxanthin. The values are represented as means ± S.E. of three determinants.

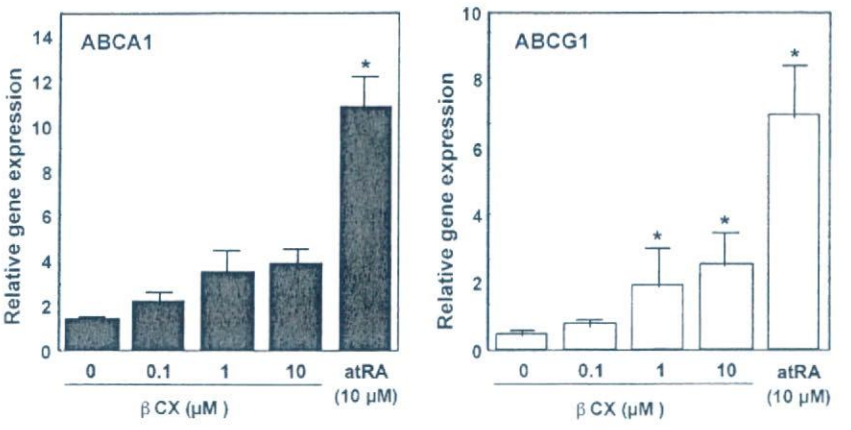

Fig. 5 – Effect of β-cryotoxanthin on induction of ABCA1 or ABCG1 mRNA in mouse peritoneal macrophages. Thioglycolate-elicited peritoneal macrophages were seeded at a concentration of 2.0×10^6 cells/ml and treated with β-cryptoxanthin or atRA at the indicated concentrations for 8 h. mRNA levels of ABCA1 and ABCG1 were determined by quantitative RT-PCR as described in Section 2. Data were normalized using the β-actin levels and are expressed relative to the cells treated with vehicle (DMSO). The values are represented as means ± S.D. of 3–4 culture dishes. Statistically significant differences from the cells treated with vehicle are indicated followed by Dunnett's test (*$p < 0.05$).

lutein and zeaxanthin, which are dipolar xanthophylls, appear to adopt an orientation mainly perpendicular to membrane surfaces because the hydrophilic groups at the opposite ends of the molecule are attracted to the polar zones of the membrane at the membrane surfaces [42]. For carotenes lacking such polar groups, such as β-carotene and lycopene, orientation in the lipid membrane environment seems to be exclusively governed by van der Waals interactions with the hydrocarbon acyl chains of lipid molecules thereby resulting in random orientations of these carotenoid in membranes. In the case of β-cryptoxanthin, a monopolar xanthophyll, the hydrophilic portion seems to be attracted to the membrane surface with hydrophobic end floating in the core of the membrane [43]. In fact, although RAR does not exist in membrane, such difference in molecular polarity may influence the interaction between ligands and RAR in RAR ligand-binding pocket. Recently, a zeaxanthin-binding protein was isolated and identified as a Pi isoform of glutathione S-transferase [44]. Thus, there is a possibility that a β-cryptoxanthin-specific protein exists and transactivates RAR in a ligand-dependent manner. Of particular interest from the present work is that β-carotene failed to show any ligand activity at physiological or pharmacological concentrations. In addition, it has been reported that the carboxyl group of retinoid cannot be replaced by typical bioisosteric functional groups, such as sulfonamide and tetrazole [45]. This suggests that β-cryptoxanthin may possess a structural uniqueness as a RAR ligand.

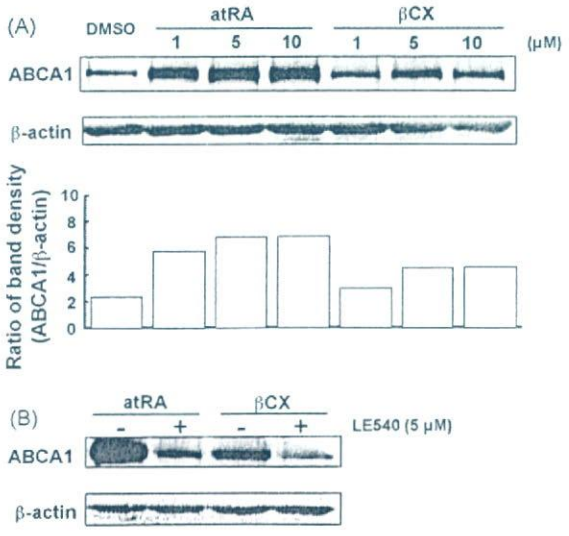

Fig. 6 – Induction of ABCA1 protein by β-cryptoxanthin. Thioglycolate-elicited peritoneal macrophages were seeded at a concentration of 2.0×10^6 cells/ml and treated with increasing concentrations of β-cryptoxanthin or atRA for 20 h in the absence (A) or presence (B) of an RAR pan-antagonist LE540 (5 μM). ABCA1 protein was determined by Western blot analysis as described in Section 2. The figure is a representative of three independent experiments, which show similar results.

To date, there are only a limited numbers of reports concerning the biological activities of β-cryptoxanthin. Among them, β-cryptoxanthin has been found to possess a unique anabolic effect on bone calcification [46], stimulation of bone formation, and inhibition of bone resorption [47]. The authors of those resports denied the possibility that β-cryptoxanthin shows such effects via RAR activation, although the evidence is not completely clear. However, Lian et al. reported that β-cryptoxanthin suppresses the growth of human bronchial cells by upregulating RARβ expression [48]. They also indicated that β-cryptoxanthin can transactivate RAR-mediated transcription activity of the retinoic acid response element. However, they concluded that the metabolites of β-cryptoxanthin might induce RARβ expression based upon the need for a higher effective dose of β-cryptoxanthin than atRA for the induction of RARβ expression or transactivation of RARs. In contrast, the use of the CoA-BAP system employed in the present study to assay the ligand

activity against RARα reduces the potential that metabolites or degradation products of β-cryptoxanthin are responsible for such activity. Furthermore, in yeast two-hybrid assay, β-cryptoxanthin induced β-galactosidase via RARs at the concentration of 5×10^{-7} M. Furthermore, the RAR pan-antagonist, LE540, effectively inhibited β-galactosidase induction by β-cryptoxanthin. Taken together, these results indicate that β-cryptoxanthin itself, not its metabolites, acts as an RAR ligand and stimulates RAR-mediated transcription activity at physiological concentrations.

Considering that the prevalence of acute and chronic side effects has limited wide applicability of vitamin A and its retinoic acid derivatives as therapeutic drugs of choice, novel natural ligands against RAR should pave the way for not only understanding the mechanism underlying the pleiotropic effects of RA, but also developing the therapeutic agents with fewer side effects for atherosclerosis, cancer and other related conditions. In this respect, β-cryptoxanthin appears to be a molecule that warrants further investigation.

Recent epidemiological studies reveal that a moderate increase in β-cryptoxanthin intake is associated with a reduced risk of developing rheumatoid arthritis and lung cancer [49,50]. The results reported here led to the hypothesis that β-cryptoxanthin is capable of stimulating differentiation of lung cancer cells and modulating immune response through Th2 cells via RAR. Epidemiologic studies indicate that appropriate vegetable and fruit intake may be helpful in preventing cancer and cardiovascular diseases. Given the fact that vegetables and fruits contain β-cryptoxanthin, this molecule may indeed be the source of that anti-atherogenic and anti-tumor activity by acting through an RAR signaling mechanism.

In conclusion, the present study has shown that β-cryptoxanthin, a xanthophyll, exhibits agonist activity against RAR as well as exhibiting anti-atherogenic effect on macrophages by inducing ABCA1 and G1 expressions. Since natural RAR ligands other than retinol metabolites have not previously been found, β-cryptoxanthin, a provitamin A carotenoid that exhibits RAR agonist activity and acts as an RAR agonist which has hydrophobicity and exhibits a metabolic fate different from atRA, should be potential candidate for preventive or therapeutic agents against cardiovascular diseases.

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Histone acetyltransferase MOZ acts as a co-activator of Nrf2–MafK and induces tumour marker gene expression during hepatocarcinogenesis

Kumiko OHTA*, Megumi OHIGASHI*, Ayako NAGANAWA*, Hiromi IKEDA†, Masaharu SAKAI†, Jun-ichi NISHIKAWA*, Masayoshi IMAGAWA‡, Shigehiro OSADA*‡[1] and Tsutomu NISHIHARA*

*Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-Oka, Suita, Osaka 565-0871, Japan, †Department of Biochemistry, Graduate School of Medicine, Hokkaido University, N15, W7, Kita-ku, Sapporo 060-8638, Japan, and ‡Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan

HATs (histone acetyltransferases) contribute to the regulation of gene expression, and loss or dysregulation of these activities may link to tumorigenesis. Here, we demonstrate that expression levels of HATs, p300 and CBP [CREB (cAMP-response-element-binding protein)-binding protein] were decreased during chemical hepatocarcinogenesis, whereas expression of MOZ (monocytic leukaemia zinc-finger protein; MYST3) – a member of the MYST [MOZ, Ybf2/Sas3, Sas2 and TIP60 (Tat-interacting protein, 60 kDa)] acetyltransferase family – was induced. Although the MOZ gene frequently is rearranged in leukaemia, we were unable to detect MOZ rearrangement in livers with hyperplastic nodules. We examined the effect of MOZ on hepatocarcinogenic-specific gene expression. GSTP (glutathione S-transferase placental form) is a Phase II detoxification enzyme and a well-known tumour marker that is specifically elevated during hepatocarcinogenesis. GSTP gene activation is regulated mainly by the GPE1 (GSTP enhancer 1) enhancer element, which is recognized by the Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2)–MafK heterodimer. We found that MOZ enhances GSTP promoter activity through GPE1 and acts as a co-activator of the Nrf2–MafK heterodimer. Further, exogenous MOZ induced GSTP expression in rat hepatoma H4IIE cells. These results suggest that during early hepatocarcinogenesis, aberrantly expressed MOZ may induce GSTP expression through the Nrf2-mediated pathway.

Key words: glutathione S-transferase placental form (GSTP), hepatocarcinogenesis, histone acetyltransferase (HAT), MafK, monocytic leukaemia zinc-finger protein (MOZ), nuclear factor-erythroid 2 p45 subunit-related factor 2 (Nrf2).

INTRODUCTION

Acetylation is an important post-translational modification known to occur in histones, transcription factors and other proteins [1]. Histone acetylation is catalysed by HATs (histone acetyltransferases), which transfer an acetyl group from acetyl-CoA to lysine residues in histones. Lysine acetylation destabilizes the nucleosome structure and promotes the accessibility of transcription factors to a genetic locus. In agreement with these phenomena, acetylated chromatin has been associated with a transcriptionally activated state [2].

The well-characterized transcriptional co-activators CBP [CREB (cAMP-response-element-binding protein)-binding protein], p300, GCN5 (positive general control of transcription-5), and P/CAF (p300/CBP-associated factor) have intrinsic HAT activity [3–6]. HATs are divided into several groups on the basis of their similarity in homologous regions including acetyl-CoA-binding motifs [1,7]. For example, p300 and CBP, and P/CAF and GCN5 share a remarkable degree of similarity throughout their sequences respectively, and they play distinct but functionally overlapping roles [1,8,9]. Another group of evolutionarily related HATs is the MYST [MOZ (monocytic leukaemia zinc-finger protein), Ybf2/Sas3, Sas2 and TIP60 (Tat-interacting protein, 60 kDa)] family. MYST proteins not only contribute to transcriptional activation, but they also have diverse roles in various nuclear processes, including cell-cycle progression, DNA repair, DNA replication and gene silencing [10–16].

Recent studies indicate that some HATs play roles in tumour suppression and that loss or misregulation of these activities may lead to cancer [17]. Development of liver cancer is controlled by several transcriptional factors, such as c-Jun, Foxm1b (forkhead box m1b) and p53 [18,19]. These factors are acetylated by p300 and CBP, which thereby modulate the transcriptional activity of these factors [1,19]. Hyperacetylation of histones in the promoter region of c-Jun is detected [20]. In addition, p53 recruits p300 to nucleosomal histones within the p21 promoter *in vitro* [21]. These results suggest that HATs may be involved in hepatocarcinogenesis, but the underlying mechanism has not been addressed. Here, we show that MOZ (also known as MYST3), a member of the MYST family of HATs, is induced during hepatocarcinogenesis. MOZ frequently is rearranged in leukaemia [10,22–27], and it regulates transcription mediated by the haemopoietic transcriptional factor AML1 (acute myeloid leukaemia 1) and the MOZ fusion protein, which is generated by translocation, down-regulates in haemopoiesis and leads to leukaemogenesis [28].

GSTP [GST (glutathione S-transferase) placental form] is a Phase II detoxification enzyme and a well-known tumour marker that is specifically elevated during chemical hepatocarcinogenesis in the rat [29]. GSTP gene expression is regulated mainly through

Abbreviations used: AAF, 2-acetylaminofluorene; AML1, acute myeloid leukaemia 1; bZIP, basic region leucine zipper; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; DEN, diethylnitrosoamine; DTT, dithiothreitol; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCN5, positive general control of transcription-5; GST, glutathione S-transferase; GSTP, GST placental form; GPE, GSTP enhancer; HA, haemagglutinin; HAT, histone acetyltransferase; HBO1, HAT binding to ORC1 (origin recognition complex subunit 1); ING, inhibitor of growth; MOZ, monocytic leukaemia zinc-finger protein; MORF, MOZ related factor; MYST, MOZ, Ybf2/Sas3, Sas2 and TIP60; Nrf2, nuclear factor-erythroid 2 p45 subunit-related factor 2; ORF, open reading frame; P/CAF, p300/CBP-associated factor; PH, partial hepatectomy; PHD, plant homeodomain; RT, reverse transcriptase; TIF2, transcriptional intermediary factor 2, TIP60, Tat-interacting protein, 60 kDa; TRE, PMA ('TPA')-responsive element.

[1] To whom correspondence should be addressed (email osada@phar.nagoya-cu.ac.jp).

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GPE (GSTP enhancer), located approx. 2.5 kb upstream from the cap site, and the silencer [30,31]. GPE1, a strong enhancer element in GPE, is responsible for GSTP gene expression during hepatocarcinogenesis *in vivo* [32,33]. Recently, we showed that a heterodimer comprising Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) and MafK binds to GPE1 and enhances GSTP promoter activity [34]. Nrf2, a member of bZIP (basic region leucine zipper) family of transcription factors, induces Phase II detoxifying and antioxidative genes [35]. Nrf2 plays a crucial role in early defence against chemical stress and carcinogenesis [36].

To characterize the roles of HATs during hepatocarcinogenesis, we examined their expression profiles and showed that expression of MOZ was induced under these conditions. Further, we found that MOZ acted as a co-activator of the Nrf2–MafK heterodimer and induced expression of GSTP. These results suggest that MOZ induces GSTP expression through the Nrf2-mediated pathway during early hepatocarcinogenesis.

EXPERIMENTAL

Chemical hepatocarcinogenesis of rats

Carcinogenic experiments were performed according to the Solt–Farber protocol [37]. Experiments were initiated by intraperitoneal injection of DEN (diethylnitrosoamine; 200 mg/kg) into 5-week-old Wister rats. After the animals had been fed basal diets for 2 weeks, diets were changed to basal diets containing 0.02 % AAF (2-acetylaminofluorene). Three weeks after the DEN injection, a PH (partial hepatectomy) was performed; livers were extirpated 7 weeks after the DEN injection. Control rats were injected with saline and fed basal diets. All animal care and handling procedures were approved by the Animal Care and Use Committee of Osaka University.

Preparation of nuclear extracts, cytosol fractions and RNA from rat liver

Procedures for preparation of nuclear extracts and cytosol fractions from rat liver were described previously [38]. Livers were homogenized in a sucrose-containing buffer, and nuclei were purified by centrifugation. Nuclear proteins were extracted with 0.55 M KCl and centrifuged at 40000 ***g*** for 60 min at 4 °C. The supernatants were used for the HAT assay and Western blot analysis. Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.A.) in accordance with the manufacturer's recommendations.

Western blotting and antibodies

Proteins were resolved using SDS/PAGE, transferred to nitrocellulose or PVDF membrane and detected using the ECL® (enhanced chemiluminescence) Western blotting analysis detection system (Amersham Biosciences, Piscataway, NJ, U.S.A.). For the generation of antibodies against the N- and C-terminal regions of MOZ, nucleotides corresponding to amino acid residues 1–331 and 1717–1998 respectively were cloned into pET-28a (Novagen, Darmstadt, Germany). The resulting His_6-tagged fusion polypeptides were expressed in bacteria and purified over nickel-nitrilotriacetic acid–agarose (Qiagen, Hilden, Germany). These proteins were injected into rabbits, and antibodies were affinity-purified using Protein A–Sepharose (Amersham Biosciences). The anti-P/CAF antibody was a gift from Dr Y. Nakatani (Harvard Medical School, Boston, MA, U.S.A.). The following antibodies were commercially available: anti-p300 (N-15, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-CBP (A-22, Santa Cruz Biotechnology), anti-GCN5 (N-18, Santa Cruz Biotechnology), anti-TIP60 (Upstate Biotechnology, Lake Placid, NY, U.S.A.), anti-MORF (MOZ-related factor; C-15, Santa Cruz Biotechnology), anti-MYST (Upstate Biotechnology), anti-GSTP (Biotrin, Dublin, Ireland), anti-HA (haemagglutinin) (6B12, Babco, Berkeley, CA, U.S.A.), and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (MAB374, Chemicon, Temecula, CA, U.S.A.).

Plasmid construction

The rat MOZ expression plasmid pCI-MOZ has been described previously [39]. Mutants within the PHD (plant homeodomain) finger and the MYST regions of the *Moz* gene (pCI-MOZ-PHDmut and pCI-MOZ-MYSTmut) were generated using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) following the manufacturer's recommended protocols. All mutations were verified by sequencing over the region of change. For construction of Myc-tagged MOZ-expressing plasmids, the MOZ ORF (open reading frame) was subcloned into the EcoRI–NotI site of pCMV-Myc (Clontech, Franklin Lakes, NJ, U.S.A.). For construction of −2.5GST-luciferase, the fragment from −2.5 kb to −91 kb of the GSTP gene [30] was inserted into the SacI site of −91GST-luciferase [38]. To generate −2.15GST-luciferase (the GPE deletion reporter plasmid), −2.5GST-luciferase was digested with SmaI and AccI, blunted with Klenow fragment (Toyobo, Osaka, Japan), and then self-ligated. The Nrf2 expression plasmid (pAβ2-Nrf2), including the human β-actin promoter and enhancer, and GPE1 reporter plasmid (GPE1-luciferase) were described previously in [40]. The HA-tagged rat MafK expression plasmid (pCMV-HA-MafK) was generated by PCR amplification of the MafK ORF [40] using primers that incorporate SalI and NotI at the 5′ and 3′ ends respectively. The PCR product was cloned into the SalI–NotI site of pCMV-HA (Clontech). The non-tagged MafK expression plasmid pRSV-MafK contained MafK cDNA controlled by the *Rous sarcoma* virus long terminal repeat. For construction of MafK/GEX-KG, MafK cDNA was cloned into pGEX-2T (Amersham Biosciences).

Cell culture

Rat hepatoma H4IIE cells and mouse embryonic carcinoma F9 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FBS (fetal bovine serum). HeLa cells were cultured in minimal essential medium supplemented with 10 % FBS.

Transfection and reporter gene assays

Transfection of H4IIE and F9 cells was performed using FuGENE™ 6 (Roche, Indianapolis, IN, U.S.A.) in accordance with the manufacturer's instructions. For H4IIE cells, all transfections included 100 ng of the reporter plasmid, with or without 1 μg of the MOZ expression plasmid (pCI-MOZ). The amount of plasmid in the transfection was kept constant by using empty pCI vector. Transfectants were harvested 48 h after transfection. The luciferase assay was performed as described previously [38] and protein concentrations were determined by the method of Bradford. Luciferase activities were normalized to the protein amount. In some experiments, the transfection efficiency was checked by co-transfection with pRSV-GAL, a eukaryotic expression plasmid that contained the *Escherichia coli* β-galactosidase structural gene controlled by the *Rous sarcoma* virus long terminal repeat. β-Galactosidase activity was assayed as described in [38]. We confirmed that the variation of transfection efficiency was <15 %. Relative luciferase activity was estimated by the luciferase activity from −2.5GST-luciferase in the absence of MOZ.

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For F9 cells, all transfections contained 100 ng of reporter plasmid (GPE1-luciferase) and 5 ng of *Renilla* luciferase plasmid phRL-tk (Promega, Madison, WI, U.S.A.) as an internal control to normalize for transfection efficiency, with or without 1 μg of the MOZ expression plasmid (pCI-MOZ) in the presence or absence of 5 ng of the Nrf2 expression plasmid (pAβ2-Nrf2). The amount of plasmid in the transfection was kept constant by using empty pCI vector. At 48 h after transfection, cells were harvested and assayed for luciferase activity using the Dual-luciferase Reporter Assay System (Promega) in accordance with the manufacturer's recommendations. Reported values are relative to the activity of GPE1-luciferase without transfection of Nrf2 and MOZ. All experiments were repeated at least three times with two or three different preparations of DNA.

GST pull-down assay

The recombinant plasmid was transformed into BL21(DE3)pLysS cells. Transformants were grown overnight at 30 °C in Luria–Bertani medium containing 100 μg/ml ampicillin. The culture then was diluted 25-fold and grown to an attenuance (D_{600}) of 0.4; at that time, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. The cells were allowed to grow for an additional 4.5 h and then were harvested by centrifugation; resuspended in a buffer containing 0.15 M KCl, 50 mM Tris (pH 8.0), 10 % (v/v) glycerol, 0.1 % Tween 20, 1 mM DTT (dithiothreitol) and 1 mM PMSF, and disrupted by sonication. After centrifugation at 7000 ***g*** for 10 min, the supernatant was cross-linked to glutathione–Sepharose 4B with dimethylpimelimidate. ^{35}S-labelled MOZ proteins were produced using pCI-MOZ, pCI-MOZ-PHDmut and pCI-MOZ-MYSTmut as templates by *in vitro* transcription–translation with the TNT T7-coupled reticulocyte lysate system (Promega). A 5 μl aliquot of the reticulocyte lysate reaction containing ^{35}S-labelled MOZ proteins was incubated for 3 h at 4 °C in a buffer containing 0.15 M KCl, 50 mM Tris (pH 7.6), 10 % (v/v) glycerol, 1 mM DTT and 1 mM PMSF with GST fusion proteins. After extensive washes, bound proteins were separated by SDS/PAGE and detected by autoradiography.

Immunoprecipitation assay

Myc-tagged MOZ expression plasmid (pCMV-Myc-MOZ) was co-transfected into HeLa cells with HA-tagged MafK (pCMV-HA-MafK) or non-tagged MafK (pRSV-MafK) by the calcium phosphate co-precipitation method [41]. The cells were harvested 48 h after transfection, and nuclear extracts from the transfected HeLa cells were prepared as described in [38]. Nuclear extracts were diluted by adding nuclear lysis buffer containing 20 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM spermidine, 1 mM DTT, 10 % glycerol, 1 mM PMSF, 1 μg/ml pepstatin A and 1 μg/ml leupeptin (final KCl concentration, 0.15 M). To immunoprecipitate HA-tagged protein, we incubated extracts with anti-HA antibody immobilized on Sepharose beads overnight at 4 °C. For control experiments, control mouse IgG coupled with Sepharose was used. After extensive washes, bound proteins were separated by SDS/PAGE and detected by Western blotting.

Induction of endogenous GSTP expression by MOZ in rat hepatoma H4IIE cells

Rat hepatoma H4IIE cells were transfected with various amounts of the MOZ expression plasmid pCI-MOZ by using the FuGENE™ 6 reagent in 35 mm plates. The total amount of plasmid DNA was adjusted by supplementing with empty pCI vector to 1 μg. After 36 h, cell lysates were prepared with a buffer comprising 25 mM Tris phosphate (pH 7.8), 2 mM cyclohexane-1,2-diaminetetra-acetic acid, 10 % glycerol, 2 mM DTT and 1 % Triton X-100. Cell lysates were separated by SDS/PAGE (15 % gel), and expression of endogenous GSTP and GAPDH was detected by Western blotting.

RESULTS

Activity and expression profiles of HATs during hepatocarcinogenesis

To evaluate the activity and expression profiles of HATs during hepatocarcinogenesis, we performed chemical carcinogenesis in the rat liver in accordance with the Solt–Farber protocol [37]. This model of liver chemical carcinogenesis is a widely used system for the study of molecular and cellular processes leading to cancer. In this protocol, rats were fed a combination of DEN and AAF and then underwent PH. At the end of 7 weeks, the livers had large numerous hyperplastic nodules, and the rats were killed (Figure 1A). We prepared four types of control experiments: rats underwent saline injection; were injected with DEN; underwent AAF feeding; underwent PH but were not treated with DEN and AAF. We checked the reproducibility of the carcinogenic experiments. Western blotting analysis of the cytosol fractions with an anti-GSTP antibody revealed that GSTP was induced at 7 weeks after combined treatment with DEN, AAF and PH, but no GSTP was detected in any of the control rats (Figure 1B).

We first investigated the HAT activity of nuclear extracts during hepatocarcinogenesis. The assay using core histones or nucleosome histones as substrates revealed that HAT activity in livers with hyperplastic nodules was indistinguishable from that in control rat livers (results not shown). For determination of the expression profiles of HATs during hepatocarcinogenesis, we performed Western blot analysis using nuclear extracts and specific antibodies to each of the HATs (Figure 1B). The HATs best characterized as transcriptional co-activators are p300, CBP, P/CAF and GCN5. The expression levels of P/CAF and GCN5 showed no change during hepatocarcinogenesis, but expression of both p300 and CBP decreased. Next, we observed the expression levels of the MYST-type acetyltransferases, which are involved in a wide range of regulatory functions [1]. Expression of TIP60 was unchanged during hepatocarcinogenesis, whereas MOZ expression increased. MORF was not detected (results not shown). Among those we assayed, MOZ was the sole HAT whose expression was positively correlated with GSTP expression during hepatocarcinogenesis.

Induction of the intact form of MOZ during hepatocarcinogenesis

MOZ belongs to the MYST family of HATs and frequently is rearranged in leukaemia [10]. MOZ fusion partners include CBP, p300 and TIF2 (transcriptional intermediary factor 2); all of these proteins are also known to be transcriptional co-activators [10,24–26]. MOZ is a transcriptional regulator in haemopoiesis, and MOZ fusion proteins antagonize MOZ function and lead to leukaemogenesis [26,28]. Using Western blotting and RT (reverse transcriptase)–PCR analyses, we assessed whether MOZ was translocated and thus fused with these transcriptional co-activators during hepatocarcinogenesis (Figure 2 and results not shown). We generated specific antibodies against the N- and C-terminal regions of rat MOZ, and we also used the anti-MYST antibody, which recognizes a motif (amino acids 671–685) in the MYST region of MOZ. These three antibodies recognize different parts of MOZ. Western blot analysis revealed that MOZ induced in livers with hyperplastic nodules and recognized by the three different antibodies were all the same size (Figure 2A), as

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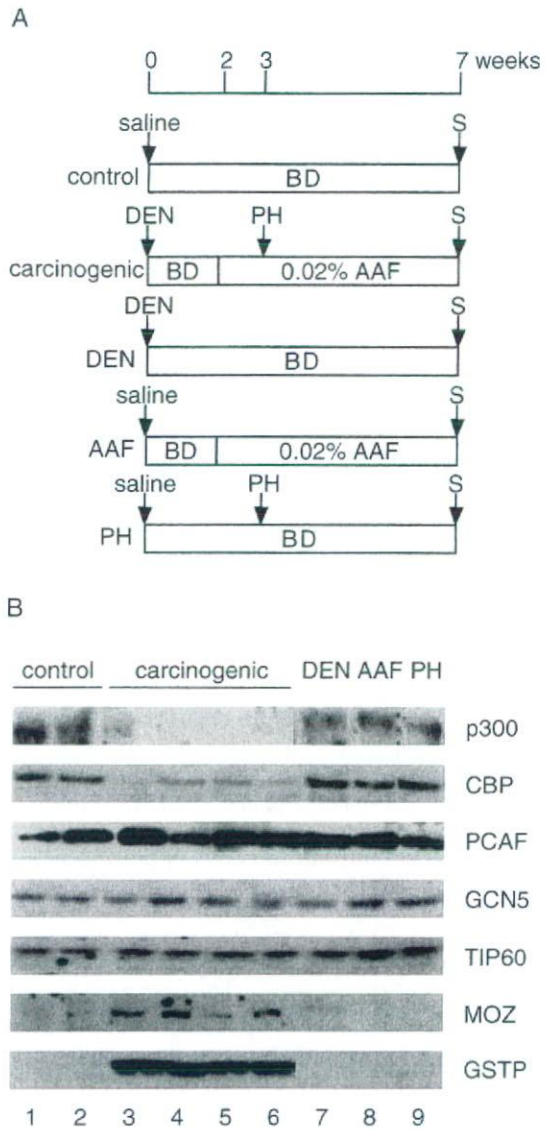

Figure 1 Expression profiles of HAT during hepatocarcinogenesis

(**A**) The Solt–Farber protocol for chemically induced hepatocarcinogenesis in rats [37]. BD, basal diet; S, times at which rats were killed. (**B**) Expression profiles of HATs were investigated in control livers and those with hyperplastic nodules. Nuclear extracts were prepared from livers of various rats, and immunoblot analysis was performed with specific antibodies, as described in the Experimental section. GSTP in the cytosol fraction was also detected (bottom). The fractions shown in lanes 1 and 2 were from control rats; lanes 3–6, rats having livers with hyperplastic nodules; lane 7, rat treated with DEN only; lane 8, rat treated with AAF only; lane 9, rat underwent PH only.

was the less-abundant MOZ in the control rat liver. We characterized additional fusion partners, including p300, CBP and TIF2. The sizes of these proteins in livers with hyperplastic nodules were the same as those in control livers (Figures 1B and 2B). These results suggested that the intact form of MOZ was induced and that translocation of MOZ did not occur during chemical hepatocarcinogenesis in rats. To confirm these results, we performed RT–PCR with three sets of primers spanning the MOZ regions in which rearrangement occurred frequently [10,24–26]. Sequencing of PCR products revealed that MOZ rearrangement did not occur during hepatocarcinogenesis (results not shown). We further examined the MOZ–CBP chimaeric transcript by hemi-nested PCR, but the amplification product was not detected

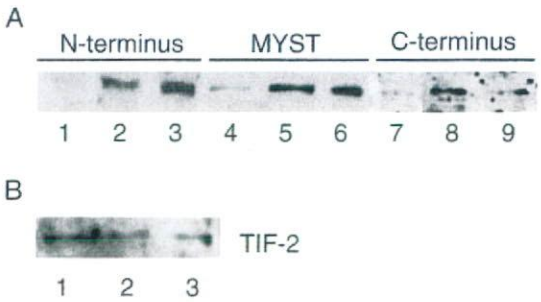

Figure 2 Induction of the intact form of MOZ during hepatocarcinogenesis

(**A**) Nuclear extracts were prepared from control (lanes 1, 4 and 7) and livers with hyperplastic nodules (lanes 2, 3, 5, 6, 8 and 9), separated by SDS/PAGE (7.5% gel), and immunoblotted using polyclonal antibodies against the N- (lanes 1–3) or C- (lanes 7–9) terminal region of MOZ or the anti-MYST antibody (lanes 4–6). (**B**) The putative MOZ fusion partner, TIF-2, was detected with the anti-TIF-2 antibody. The fraction shown in lane 1 is from control; those in lanes 2 and 3 were from livers with hyperplastic.

(results not shown). These results indicate that the intact form of MOZ was induced and that MOZ translocation did not occur during the early stages of hepatocarcinogenesis.

Activation of GSTP promoter activity by MOZ through the GPE

MOZ functions as a transcriptional co-activator and participates as a mediator in haemopoiesis [28,42]. To characterize the effect of MOZ on hepatocarcinogenesis-specific gene expression, we asked whether exogenous MOZ would enhance GSTP promoter activity. GSTP is strongly and specifically expressed during chemical hepatocarcinogenesis and is considered to be an excellent tumour marker [29]. The transcriptional regulatory region of the rat GSTP gene includes enhancer and silencer elements [30,31]. To examine the effect of MOZ on GSTP promoter activity, −2.5GST-luciferase (which has the entire GSTP regulatory region and promoter) was co-transfected with MOZ expression plasmid or control empty vector into rat hepatoma H4IIE cells (Figure 3A). MOZ enhanced GSTP promoter activity (Figure 3B). Luciferase activity in the presence of various concentrations of MOZ was assayed, and MOZ demonstrated dose-dependent enhancement of GSTP promoter activity (Figure 3C). To more closely define the MOZ response element, we used two reporter plasmids: −2.15GST-luciferase, which lacked the GPE, and −91GST-luciferase, which lacked both the GPE and silencer regions (Figures 3A and 3B). These reporter plasmids were not transactivated, thereby suggesting that MOZ activates GSTP promoter activity through the GPE.

MOZ interacts with MafK both *in vitro* and *in vivo*

The GPE1 element in GPE is a key control element responsible for GSTP expression in preneoplastic tissue. GPE1 is similar in sequence to ARE (antioxidant-response-like element), MARE (Maf recognition element) and TRE [PMA ('TPA')-responsive element] [30,33,34]. A recent study showed that the Nrf2–MafK heterodimer binds to GPE1 and regulates GSTP promoter activity [34]. To determine the mechanism of the MOZ-associated enhancement of GSTP promoter activity, we tested whether MOZ could bind Nrf2 and MafK. We previously showed that MOZ interacted with c-Jun through the bZIP domain *in vitro* [39]; Nrf2 and MafK also have bZIP domains. To determine MOZ binding partners, we performed an *in vitro* pull-down assay using ^{35}S-labelled full-length MOZ. We fused the Nrf2 DNA-binding domain to maltose-binding protein, incubated it with ^{35}S-labelled MOZ, and precipitated it with amylose resin, but interaction

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