

Fig. 2. Dose-response curves of styrene dimers in a RAR γ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

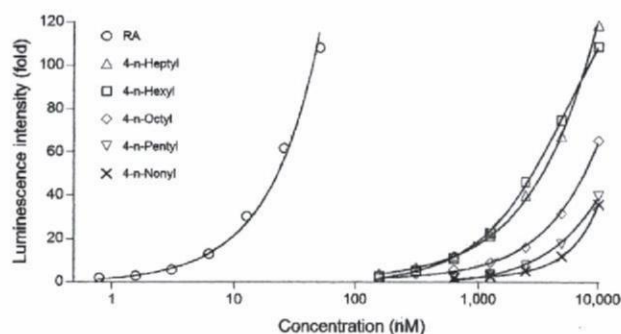


Fig. 3. Dose-response curves of monoalkylphenols having a linear alkyl group in a RAR γ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

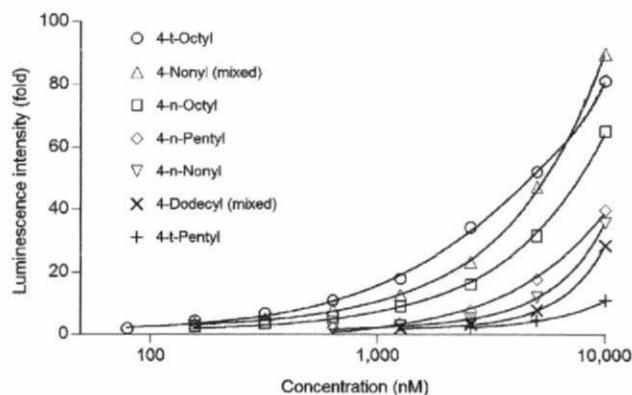


Fig. 4. Activity comparison of monoalkylphenols having a linear alkyl group with a branched group in a RAR γ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

in hazard identification with implications for mechanism of action. As RAs are known to undergo isomerization and oxidation when exposed to light and air (Bempong et al.,

1995), some of the variability in the activity of *all-trans* RA can probably be attributed to the lability of the reagent to, in particular, photodegradation. However, the ready availability of an endogenous ligand for use in the assay more than compensates for this instability.

Many of the compounds active to the RAR γ (organochlorines and styrene dimers were notable exceptions) were *para*-alkyl-substituted phenols. Phenols of this type are manufactured on a very large scale for many industrial purposes and have been reported to possess estrogenic activity, with the degree of activity depending on the length and branching of the alkyl substituent. Of the 4-*n*-alkylphenols, 4-*n*-nonylphenol had the highest binding affinity for the human ER (Tabira et al., 1999). 4-Alkylphenols having an alkyl group composed of 3–12 carbons, including branched groups (both secondary and tertiary), exhibited ER transactivation activity in a recombinant yeast assay (Routledge and Sumpter, 1997). There were some differences in the responsiveness of the present RAR γ assay to alkyl and other phenols from those of the ER. The most potent activator of the RAR γ was 4-*n*-heptylphenol and phenols with four or less carbons in their alkyl groups had no effect regardless of its position or branching. Alkylphenols having a branched alkyl group with many carbons, such as the mixed isomers of nonylphenol and tert-octylphenol, were more potent than analogs with a linear alkyl group, whereas of the pentylphenols with less carbon atoms in their alkyl groups, tert-pentylphenol is weaker than its analog with a linear alkyl group. This suggests that the overall length of a side-chain but not the actual number of carbons it contains may influence the potency of alkylphenols to the RAR γ and that there may be an optimal length.

Ring-chlorinated alkylphenols had somewhat higher activity than their unchlorinated analogs, and bisphenol A, which had no positive effect on the RAR γ , acquired agonistic activity by chlorination. This parallels observations in previous studies of estrogenicity where the estrogenic activities of chlorinated bisphenol A were stronger both *in vitro* and *in vivo* than those of the unchlorinated compound (Takemura et al., 2005; unpublished data, Shiraishi et al., 2000). Halogenation of chemicals might thus activate them or boost their actions on nuclear receptors. This is also indicated by observations in the present study that chlorination and bromination activated 17 β -estradiol (E2) to the RAR γ to a small extent. These findings suggest that not only in industrial manufacturing but in effluent processing, halogenation treatment does not reduce the unexpected activity of chemicals on nuclear receptors, but on the contrary might enhance the harmful effects.

We present here the first evidence that styrene dimers but not trimers have the ability to transactivate the RAR. The dimer 1-phenyltetralin was one of the most active chemicals revealed in this study. Furthermore, it was apparent that the fewer carbons linking the benzene rings of the dimers, the higher the agonistic activity (Figs. 2 and 5). Therefore, limited overall molecular length seems

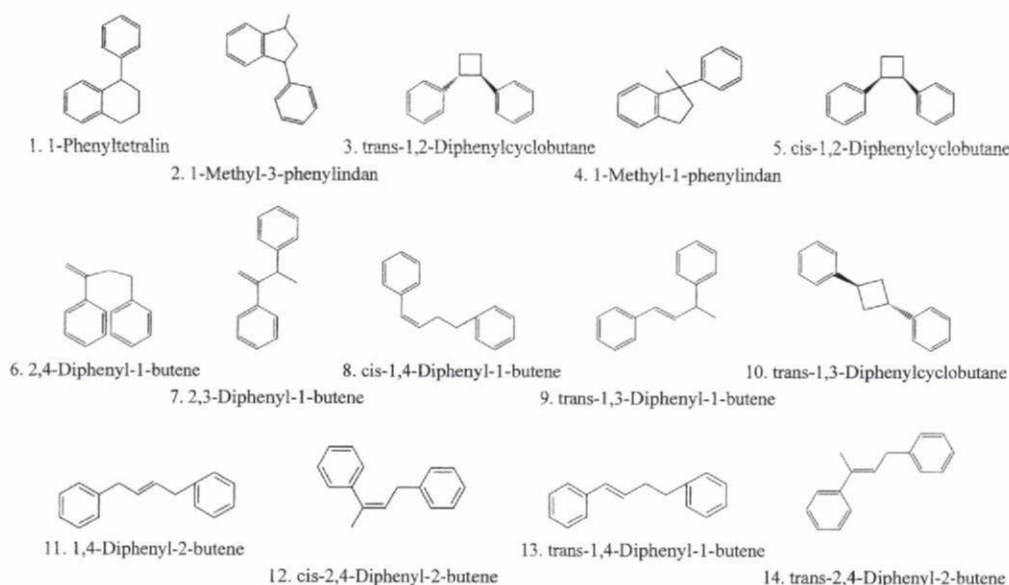


Fig. 5. Structural formulas of styrene dimers.

to correlate with potency in styrene dimers. The lack of activity of styrene trimers may also arise from their length or size as the upper limit for the molecular weights of positive compounds (except halogenated compounds and organophosphates) was 286.5 (*all-trans* retinol), whereas the styrene trimers have molecular weights of 312. It is interesting that 2-terphenyl and 1,3-diphenylpropane have about the same activity as styrene dimers with similar structures (*trans*-1,2-diphenylcyclobutane and *trans*-2,4-diphenyl-2-butene, respectively). The binding affinity of these chemicals for the RAR may depend on molecular length, like alkylphenols as discussed above. Styrene oligomers also have binding affinity for the human ER (Ohyama et al., 2001; Kitamura et al., 2003). Importantly, it has been reported that styrene trimers exhibit *in vivo* disruption of endocrine systems and that embryonic exposure obstructs genital organ development and disrupts the endocrine function of male rat offspring (Ohyama et al., 2007). Polystyrene resins contain substantial amounts of styrene dimers and trimers, and extraction tests with polystyrene food containers have shown that these compounds leak into the food, water and oil with which they are in direct contact (Kawamura et al., 1998a,b,c). Because all styrene dimers tested in this study had a positive effect on the RAR γ and selective ligands for the RAR subtypes are teratogenic in the developing embryo, the developmental toxicity of styrene oligomers via the RAR as well as the ER should be investigated.

Organochlorine pesticides were previously reported to transactivate RAR β and γ , but not RAR α in human RAR reporter cell lines (Lemaire et al., 2005). The tested five organochlorines had dose-dependent effects on the RAR γ and the order of potency was endrin > dieldrin > aldrin > chlordane > endosulfan, although even the activity of endrin was only approximately 1/12000 of that

of the standard RAR agonist, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl-1-propenyl)] benzoic acid. Our results indicated that many organochlorines, including those five compounds examined, also have the potential to activate the RAR γ . The present yeast assay detected the activities of these compounds at lower concentrations than the reported cell line. In addition, our assay results indicated that binding affinity for the RAR partially depends on the number of chlorine atoms in the molecule and that there seems to be an optimal range of their chlorine number. Many studies have shown that a number of pesticides, including organochlorines, possess estrogenic activity (Hodges et al., 2000; Kojima et al., 2004), but their potencies for the ER do not correspond with those for the RAR. For example, the ranking of the activities of organochlorines for the ERs in Chinese hamster ovary cells are *o,p'*-DDT (4.5×10^{-8} M, test compound concentration showing 20% of the agonistic activity of 10^{-10} M 17 β -estradiol) > β -BHC (3.5×10^{-7} M) > methoxychlor (5.6×10^{-7} M) for the ER α , and β -BHC (1.1×10^{-7} M) > *o,p'*-DDT (1.2×10^{-7} M) > δ -BHC (1.1×10^{-6} M) for the ER β (Kojima et al., 2004), whereas none of these compounds showed a significant effect on the RAR γ in our assay system. Because persistent and/or harmful pesticides such as organochlorines are now prohibited in developed countries, but still used in developing regions, detailed investigations of their toxicity via nuclear receptors, as well as a global restriction on their use, are required.

The present study illustrated that a number of compounds possessed unexpected transcriptional activation effects on the human RAR γ in a yeast two-hybrid system, as we have already shown for the ERs and TRs (Shiraishi et al., 2003; Arulmozhiraja et al., 2005; Morohoshi et al., 2005). It is common for xenobiotics to have low affinity for nuclear receptors relative to the natural ligands, as we

reported here. However, there are a number of examples (principally from the ER, which is better studied than the RAR) where man-made compounds produce adverse effects in living organisms via nuclear receptors. Agonistic ligands for nuclear receptors such as the RARs and ERs have been confirmed the ability to experimentally induce developmental abnormalities (Elmazar et al., 1996, 2001; Kamata et al., 2006), and there are medically or environmentally documented cases of their teratogenicities (Lammer et al., 1985; Fry, 1995; Sumpter, 1998). For example, in previous studies, the REC20 of 2,2',4,4'-tetrachlorobiphenyl-4-ol, one of monohydroxylated polychlorinated biphenyls, was 24 nM in our human ER α yeast assay, and its activity relative to E2 was 1.7% (Arulmozhiraja et al., 2005). *In ovo* exposure to this compound at a dose of 100 ng/g egg or more caused shortening of the oviduct in female Japanese quails and a dose of 500 ng/g or more caused a reduction testis weight in males after sexual maturation (Kamata et al., 2006). This dose range *in ovo* was approximately 100 times higher than a positive control, diethylstilbestrol, producing *in vitro* effect equivalent to E2. Recently, the existence of environmental pollutants having binding affinity for less researched but important nuclear receptors, such as the retinoid X receptors (RXR) and peroxisome proliferator-activated receptors (PPAR), and consequential disorders in organisms has been reported (Nishikawa et al., 2004; Abbott et al., 2007; Takacs and Abbott, 2007). Both tributyltin and triphenyltin used in antifouling paints exhibited agonistic activity in yeast assays transfected with the human RXR (α , β or γ) at a concentration of 10 nM or more, and this concentration was somewhat lower than that of 9-*cis* RA, the natural ligand of RXR (Nishikawa et al., 2004). One injection of 1 μ g triphenyltin/g wet weight induced the differentiation and growth of male genital tracts in female gastropods, *Thais clavigera*, which was also stronger than 9-*cis* RA (Nishikawa et al., 2004). Thus, these reports are typical cases that *in vitro* affinity of chemicals for nuclear receptors is well correlated with their *in vivo* potential, and therefore, measurement of the relative activity of chemicals using *in vitro* assays seems to be valuable to estimate the *in vivo* effects of them.

There is great concern that substances released into the environment and/or used in everyday life may influence human and wildlife health. Detection of chemicals with an affinity for the various receptors is an important step in hazard identification. This data can be used to direct and prioritize additional research and *in vivo* studies on chemicals that bind nuclear receptors for possible receptor-mediated toxicity and endocrine disrupting activity. As described here, yeast assay systems including a RAR assay are useful for high-throughput screening of substances active to nuclear receptors.

5. Conflict of interest

There are no conflicts of interests involved in this study.

Acknowledgement

The authors thank Ms. Miho Yamasaki for skilful technical assistance.

References

- Abbott, B.D., Wolf, C.J., Schmid, J.E., Das, K.P., Zehr, R.D., Helfant, L., Nakayama, S., Lindstrom, A.B., Strynar, M.J., Lau, C.S., 2007. Perfluorooctanoic acid (PFOA)-induced developmental toxicity in the mouse is dependent on expression of peroxisome proliferator activated receptor- α . *Toxicological Sciences* 98, 571–581.
- Allenby, G., Janocha, R., Kazmer, S., Speck, J., Grippo, J.F., Levin, A.A., 1994. Binding of 9-*cis*-retinoic acid and *all-trans*-retinoic acid to retinoic acid receptors α , β , and γ . Retinoic acid receptor gamma binds *all-trans*-retinoic acid preferentially over 9-*cis*-retinoic acid. *Journal of Biological Chemistry* 269, 16689–16695.
- Arulmozhiraja, S., Shiraishi, F., Okumura, T., Iida, M., Takigami, H., Edmonds, J.S., Morita, M., 2005. Structural requirements for the interaction of 91 hydroxylated polychlorinated biphenyls with estrogen and thyroid hormone receptors. *Toxicological Sciences* 84, 49–62.
- Bempong, D.K., Honigberg, I.L., Meltzer, N.M., 1995. Normal phase LC-MS determination of retinoic acid degradation products. *Journal of Pharmaceutical and Biomedical Analysis* 13, 285–291.
- Bernard, B.A., Bernardon, J.M., Delescluse, C., Martin, B., Lenoir, M.C., Maignan, J., Charpentier, B., Pilgrim, W.R., Reichert, U., Shroot, B., 1992. Identification of synthetic retinoids with selectivity for human nuclear retinoic acid receptor gamma. *Biochemistry and Biophysical Research Communications* 186, 977–983.
- Elmazar, M.M., Reichert, U., Shroot, B., Nau, H., 1996. Pattern of retinoid-induced teratogenic effects: possible relationship with relative selectivity for nuclear retinoid receptors RAR alpha, RAR beta, and RAR gamma. *Teratology* 53, 158–167.
- Elmazar, M.M., Ruhl, R., Nau, H., 2001. Synergistic teratogenic effects induced by retinoids in mice by coadministration of a RAR α - or RAR γ -selective agonist with a RXR-selective agonist. *Toxicology and Applied Pharmacology* 170, 2–9.
- Fry, D.M., 1995. Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environmental Health Perspectives* 103 (Suppl. 7), 165–171.
- Hodges, L.C., Bergerson, J.S., Hunter, D.S., Walker, C.L., 2000. Estrogenic effects of organochlorine pesticides on uterine leiomyoma cells *in vitro*. *Toxicological Sciences* 54, 355–364.
- Kamata, R., Takahashi, S., Shimizu, A., Morita, M., Shiraishi, F., 2006. *In ovo* exposure quail assay for risk assessment of endocrine disrupting chemicals. *Archives of Toxicology* 80, 857–867.
- Kawamura, M., Nishi, K., Maehara, T., Yamada, T., 1998a. Migration of styrene dimers and trimers from polystyrene containers into instant foods. *Journal of the Food Hygiene Society of Japan* 39, 390–398.
- Kawamura, M., Sugimoto, N., Takeda, Y., Yamada, T., 1998b. Identification of unknown substances in food contact polystyrene. *Journal of the Food Hygiene Society of Japan* 39, 110–119.
- Kawamura, Y., Kawamura, M., Takeda, Y., Yamada, T., 1998c. Determination of styrene dimers and trimers in food contact polystyrene. *Journal of the Food Hygiene Society of Japan* 39, 199–205.
- Kitamura, S., Ohmegi, M., Sanoh, S., Sugihara, K., Yoshihara, S., Fujimoto, N., Ohta, S., 2003. Estrogenic activity of styrene oligomers after metabolic activation by rat liver microsomes. *Environmental Health Perspectives* 111, 329–334.
- Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., Kobayashi, K., 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by *in vitro* reporter gene assays using Chinese hamster ovary cells. *Environmental Health Perspectives* 112, 524–531.
- Lammer, E.J., Chen, D.T., Hoar, R.M., Agnish, N.D., Benke, P.J., Braun, J.T., Curry, C.J., Fernhoff, P.M., Grix Jr., A.W., Lott, I.T., et al.,

1985. Retinoic acid embryopathy. *New England Journal of Medicine* 313, 837–841.
- Lemaire, G., Balaguer, P., Michel, S., Rahmani, R., 2005. Activation of retinoic acid receptor-dependent transcription by organochlorine pesticides. *Toxicology Applied Pharmacology* 202, 38–49.
- Mispagel, C., Shiraishi, F., Allinson, M., Allinson, G., 2005. Estrogenic activity of treated municipal effluent from seven sewage treatment plants in Victoria, Australia. *Bulletin of Environmental Contamination and Toxicology* 74, 853–856.
- Morohoshi, K., Yamamoto, H., Kamata, R., Shiraishi, F., Koda, T., Morita, M., 2005. Estrogenic activity of 37 components of commercial sunscreen lotions evaluated by *in vitro* assays. *Toxicology in Vitro* 19, 457–469.
- Nishikawa, J., Mamiya, S., Kanayama, T., Nishikawa, T., Shiraishi, F., Horiguchi, T., 2004. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. *Environmental Science and Technology* 38, 6271–6276.
- Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M., Nishihara, T., 1999. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicology and Applied Pharmacology* 154, 76–83.
- Ohyama, K., Satoh, K., Sakamoto, Y., Ogata, A., Nagai, F., 2007. Effects of prenatal exposure to styrene trimers on genital organs and hormones in male rats. *Experimental Biology and Medicine (Maywood)* 232, 301–308.
- Ohyama, K.I., Nagai, F., Tsuchiya, Y., 2001. Certain styrene oligomers have proliferative activity on MCF-7 human breast tumor cells and binding affinity for human estrogen receptor. *Environmental Health Perspectives* 109, 699–703.
- Routledge, E.J., Sumpter, J.P., 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. *Journal of Biological Chemistry* 272, 3280–3288.
- Shiraishi, F., Okumura, T., Nomachi, M., Serizawa, S., Nishikawa, J., Edmonds, J.S., Shiraishi, H., Morita, M., 2003. Estrogenic and thyroid hormone activity of a series of hydroxy-polychlorinated biphenyls. *Chemosphere* 52, 33–42.
- Shiraishi, F., Shiraishi, H., Nishikawa, J., Nishihara, T., Morita, M., 2000. Development of a simple operational estrogenicity assay system using the yeast two-hybrid system. *Journal of Environmental Chemistry* 10, 57–64.
- Sumpter, J.P., 1998. Xenoendocrine disrupters-environmental impacts. *Toxicology Letters* 102–103, 337–342.
- Tabira, Y., Nakai, M., Asai, D., Yakabe, Y., Tahara, Y., Shinmyozu, T., Noguchi, M., Takatsuki, M., Shimohigashi, Y., 1999. Structural requirements of *para*-alkylphenols to bind to estrogen receptor. *European Journal of Biochemistry* 262, 240–245.
- Takacs, M.L., Abbott, B.D., 2007. Activation of mouse and human peroxisome proliferator-activated receptor (α , β/δ , γ) by perfluorooctanoic acid and perfluorooctane sulfonate. *Toxicological Sciences* 95, 108–117.
- Takemura, H., Ma, J., Sayama, K., Terao, Y., Zhu, B.T., Shimoi, K., 2005. *In vitro* and *in vivo* estrogenic activity of chlorinated derivatives of bisphenol A. *Toxicology* 207, 215–221.
- Zile, M.H., 1998. Vitamin A and embryonic development: an overview. *Journal of Nutrition* 128, 455S–458S.
- Zile, M.H., 2001. Function of vitamin A in vertebrate embryonic development. *Journal of Nutrition* 131, 705–708.

特集 環境化学物質の作用メカニズムを解き明かす

重金属汚染による生物攪乱作用の分子基盤

Molecular Targets of Organotin Compounds in Endocrine Disruption

中西 剛 西川淳一

Tsuyoshi Nakanishi, Jun-ichi Nishikawa

重金属は生物に対し強い毒性を示すものが多いが、一般的にこのような低分子化合物の毒性は作用点が多岐に渡っており、分子レベルでの毒性発現機構の解明は困難であることが多い。一方で、近年の内分泌攪乱物質問題で話題となった有機スズ化合物は、貝類などの特定の生物種に特徴的な生殖毒性を誘引するが、最近、有機スズ化合物が核内受容体であるRXRやPPAR γ の強力なアゴニストとして作用することで、その毒性を発揮することが明らかとなってきた。本稿では、有機スズ化合物の核内受容体を介した生物攪乱作用について概説する。

key words

有機スズ, アロマトーゼ, インボセックス, RXR, PPAR γ , 内分泌攪乱

中西 剛 大阪大学大学院薬学研究所 毒理学分野 E-mail: nakanishi@phs.osaka-u.ac.jp
1998年3月大阪大学大学院薬学研究所修了(薬学博士)後、同年4月より助手として現研究室に兼任。2007年4月より助教。

西川淳一 武庫川女子大学薬学部 衛生化学研究室 E-mail: nisikawa@mukogawa-u.ac.jp
1987年3月大阪大学大学院薬学研究所修了(薬学博士)。2007年4月より教授として現研究室に兼任。

はじめに

重金属は生物に対し強い毒性を示すものが多く、鉱山や工場、産業廃棄物などから排出される重金属が、しばしば水源や土壌などの環境中に放出されてヒトの健康に影響を与えるなど、公害の原因となったりする。我々日本人にとって重金属毒性と言えば、おそらく多くの人がイタイタイ病や水俣病といった公害を思い浮べるだろう。現在では、これら公害は過去に起こった歴史上の出来事であり、その作用機構の解明を含め、すでに解決した問題であるかのように認識されているかもしれない。しかしながら、低分子化合物の毒性発現には多くの分子や作用が関わっていると考えられ、その分子レベルでの作用機構解明は一般的に非常に困難である。イタイタイ病や水俣病においても、これらの原因物質がカドミウムやメチル水銀であることは明らかとなっているが、その分子レベルでの毒性発現機構について解明されたとは言いがたいのが現状である。

一方で、近年話題となった内分泌攪乱物質(endocrine disrupting chemical; EDC)問題においても、船底塗料や漁網防汚剤などに使用されてきたトリブチルスズ(tributyltin; TBT)やトリフェニルスズ(triphenyltin; TPT)に代表される有機スズ化合物(図1)が、貝類に対してではあるものの、極低濃度で雌を雄性化し、繁殖不能にする状態(インボセックス)を誘導する^{1)~3)}ことから、ヒトを含めた生物へのEDC作用が懸念されてきた。このような有機スズ化合物の貝類への影響は、EDC問題が提唱される以前から問題視されていたが、EDC問題が社会問題化してか

らは、有機スズ化合物の性ステロイドホルモンの受容体や合成経路に対する影響を中心に研究が行われてきた。これまでも有機スズ化合物の毒性発現機構については様々な仮説が提唱されてきたが、有機スズ化合物においても他の重金属化合物と同様に、インボセックスの分子メカニズムやそれ以外の生物への毒性発現作用については不明な点が多く残されていた。しかしながら最近、これらの有機スズ化合物については、その毒性発現に関わる分子メカニズムが明確化しつつある。本稿では、有機スズ化合物の生物攪乱作用とその分子メカニズムについて、筆者らが最近得た知見を含めて紹介したい。

I. 有機スズ化合物のアロマトーゼ阻害説

有機スズ化合物は、スズ原子にアルキル基やフェニル基が共有結合する構造を有し、官能基が1個結合するモノ体から4個結合するテトラ体まで多くの化合物から構成される人工化合物群である(図1)。これらの有機スズ化合物は、プラスチックの可塑剤や化学反応の触媒として各種化学工業で使用されるとともに、その殺生物能を利用して農業や木材防腐剤、船底塗料、漁網防汚剤として使用されてきた。しかしながら、世界各地でこれらの化合物の水域汚染(主に海洋汚染)が顕在化し、また有機スズ化合物が貝類の雌に対してペニス様の突起を発生させるインボセックスを誘導することが明らかとなってからは、有機スズ化合物の水棲生態系へのみならず、汚染した海産物を食したヒトへの影響も問題視されるようになってきた。さらに有機スズ化合物は、元来毒性が強いことから、比較的古くから哺乳動物に対し

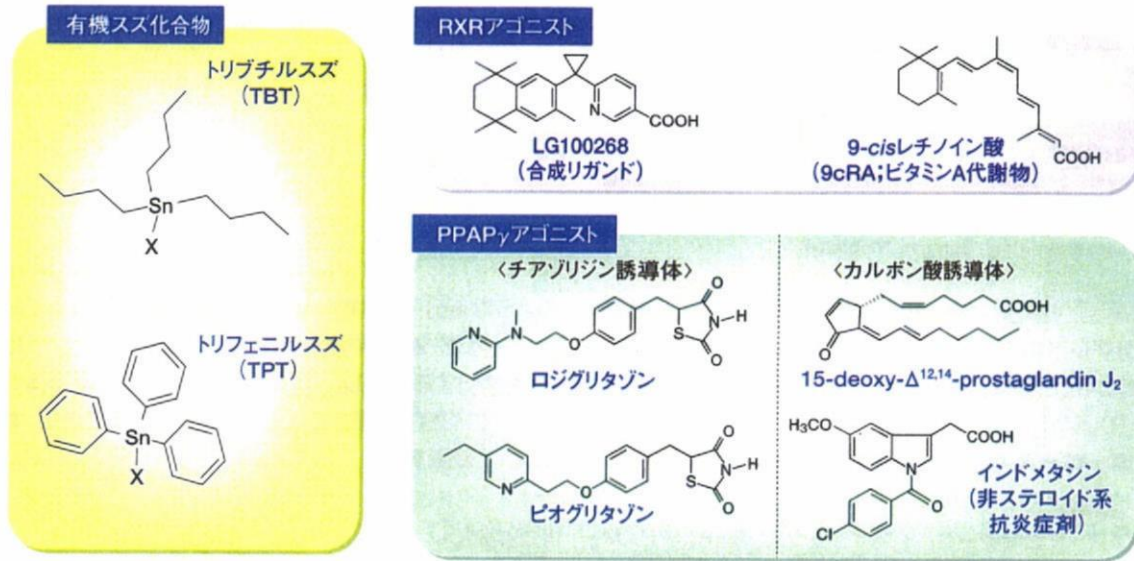


図1. RXR, PPAR γ アゴニストと有機スズ化合物の化学構造

TBTやTPTなどの有機スズ化合物は、スズ原子(Sn)にアルキル基やフェニル基が共有結合する構造を有している。官能基が1個結合するモノ体から4個結合するテトラ体まで存在している。RXRアゴニストは、一般的に極性官能基と疎水性炭化水素基をリンカーで連結したような構造を有しているが、有機スズ化合物はそのような構造を有していない。またPPAR γ アゴニストであるインスリン抵抗性糖尿病治療薬のようなチアゾリジン構造も有していない。

て神経毒性や免疫毒性などを示すことが知られていた⁴⁾が、その毒性発現機構についてはこれまでに統一した見解は得られていない。

一方で、有機スズ化合物の毒性発現には、いくつかのユニークな点が認められる。それは、①特定の生物種(貝類)に対して明確な生殖異常(インボセックス)を誘導する、②EDC問題においては、大部分の化合物が雌性化またはエストロゲン様作用が疑われているのに対し、雄性化作用が疑われている、③雄性化作用が疑われているが、エストロゲンレセプター(estrogen receptor:ER)やアンドロゲンレセプター(androgen receptor:AR)にはまったく親和性を示さない、④非特異的な細胞毒性を示す濃度よりも、かなり低濃度でインボセックスを誘導する、という点である。有機スズ化合物のインボセックスにおけるメカニズムについては様々な仮説が提唱されているが、②や③の観点から、有機スズ化合物はホルモンレセプターに直接作用するのではなく、ステロイドホルモン代謝に影響を与えることで誘導されるという説が有力視されていた^{1), 2)}。

エストロゲンおよびアンドロゲンは、コレステロールを出発物質とし、モノオキシゲナーゼであるシトクロムP450と水酸基またはケト基の酸化または還元を触媒する脱水素酵素により生成されるが、有機スズ化合物はアンドロゲンからエストロゲンへの変換酵素であるアロマトラーゼの活性を阻害するのではないかと考えられた(図2)。すなわち、アロマトラーゼの酵素活性を阻害することで、体内のエストロ

ゲン濃度の上昇を抑制し、アンドロゲンの濃度を上昇させる結果、雄性化を引き起こすのではないかとという“アロマトラーゼ阻害説”である²⁾。では、有機スズ化合物は本当にアロマトラーゼの活性を阻害するのであろうか?

CookeやHeidrichらのグループは、ヒトアロマトラーゼタンパク質を用いて、TBTがアロマトラーゼ活性を基質競合的に阻害することを報告している^{5), 6)}。筆者らもヒト絨毛細胞株のミクロソーム分画を用いて、同様の検討を行ったが、確かにTBTおよびTPTともにアロマトラーゼの活性を阻害する⁷⁾。しかし、いずれの実験においても、その作用濃度は数 μ M~数十 μ Mとかなり高濃度であり、通常の動物細胞は完全に死滅する濃度である⁷⁾。またこの他にも、高濃度のTBTおよびTPTが、アロマトラーゼ以外のステロイドホルモン合成関連酵素の活性を阻害するという報告が多数存在する^{8), 9)}ことを考慮すると、有機スズ化合物のアロマトラーゼに対する酵素特異性についてはかなり疑問が残るところである。

インボセックスのアロマトラーゼ阻害説は、同じ水棲動物である魚類が、性ステロイドホルモンにより雌雄の表現型が決定されることに加え、貝類にTBTを投与すると体内のテストステロン濃度が上昇したり、またテストステロンやアロマトラーゼ阻害剤などで処理をするとインボセックスが誘導されるという結果に基づいている^{1), 2)}。しかしながら、同じ水棲動物でもステロイドホルモンの生理的意義は、脊椎動物と無脊椎動物では大きく異なっていると考えられており、少なくとも無脊椎動物においては、古典的ステロイド

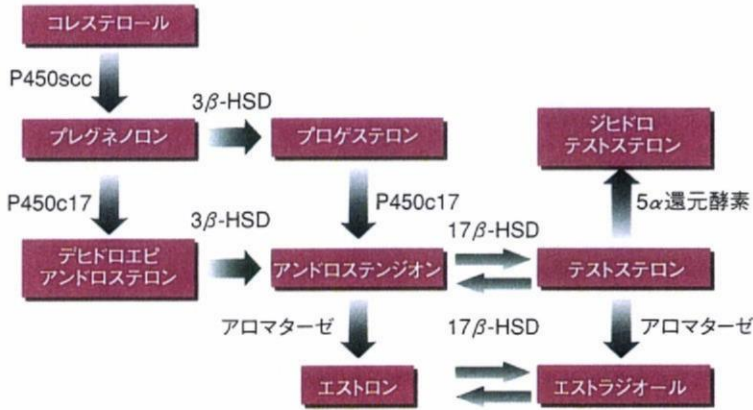


図2. コレステロールから性ステロイドホルモンへの合成経路

性ステロイドホルモンは、コレステロールを出発点としてモノオキシゲナーゼであるシトクロムP450と、水酸基またはケト基の酸化または還元を触媒する脱水素酵素により生成される。アロマターゼはアンドロゲンをエストロゲンに変換する律速酵素であることから、性ステロイドホルモンのバランスは主にアロマターゼにより保たれていると考えられる。HSD; hydroxysteroid dehydrogenase.

ホルモン（エストロゲン，アンドロゲン，プロゲステロン，糖質コルチコイド，鉱質コルチコイド）の受容体は存在しないと報告されている¹⁰⁾。また貝類において，アロマターゼは同定されておらず，ERやARの存在が不確かな貝類に，果たして脊椎動物と同様の古典的ステロイドホルモンの合成および代謝経路が本当に存在するのか？といった疑問もある。貝類のインボセックスにおいては，これまでに提唱された説の中で，“アロマターゼ阻害説”が圧倒的な支持を得てきた感がある。しかしながら，本説はこのような不確かな根拠の上に存在していることから，その信憑性についてはもう少し慎重に判断されるべきではないだろうか？

II. ヒト胎盤のアロマターゼ発現に対する有機スズ化合物の影響

アロマターゼ活性が阻害されることで，体内の性ステロイドホルモンのバランスが崩れ，雌が雄性化するというインボセックスにおける“アロマターゼ阻害説”で想定されたような現象は，じつはヒトの発生段階においても認められる。ヒトの場合，妊娠期のエストロゲン産生（アンドロゲン代謝）の場合は，卵巣から胎盤へと移行し，胎児-胎盤ユニットを形成することで，胎児および母体中の性ステロイドホルモンのバランスを維持している。そのホルモンバランスの維持において，特に重要な分子が胎盤のアロマターゼである。現に，胎盤のアロマターゼが欠損した女兒においては，体内のアンドロゲン濃度が上昇し，ペニス様の突起が発生する仮性半陰陽（外性器は男性型であるが，内生殖器は女性型）となる¹¹⁾。このことは，性ステロイドホルモンのバランスや胎盤のアロマターゼが，ヒト胎児の生殖器官形成に非常に重要であること

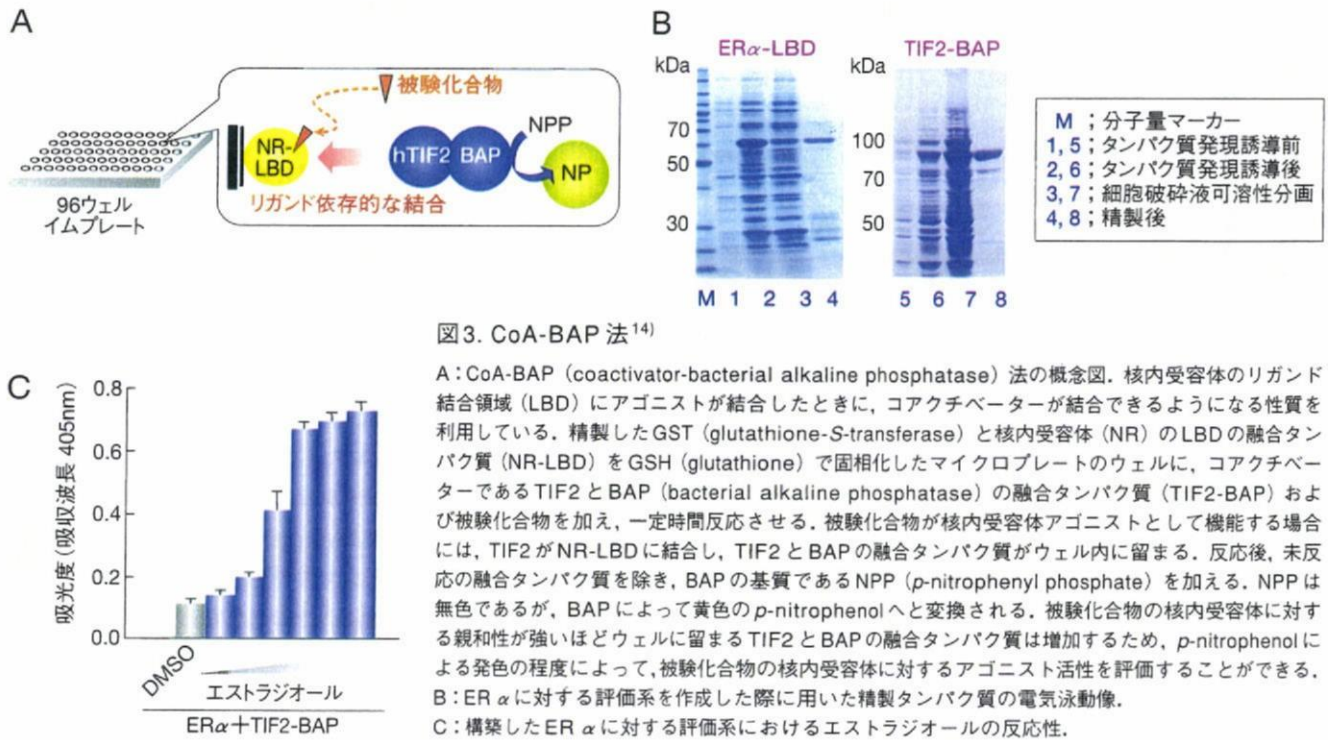
を示しており，化学物質が胎児に直接的な影響を与えなくとも，胎盤の内分泌機能を修飾することで胎児に少なからず影響を与える可能性を示唆している。

そこで筆者らは，有機スズ化合物をはじめとする様々な化学物質のヒト胎盤内分泌機能への影響を，非特異的な細胞毒性を示さない濃度域で検討を行った。その結果，TBTとTPTなどの有機スズ化合物が，濃度依存的にヒト絨毛細胞株のアロマターゼ活性とそのmRNAの発現を上昇させることが確認された^{7), 12)}。さらにこれらの有機スズ化合物は，ヒト胎盤においてエストロンを活性型エストロゲン（エストラジオール）に変換する酵素（17β-hydroxysteroid dehydrogenase type I; 17β-HSD I）をも活性化し，エストラジオール産生を促進することも確認された^{7), 13)}。これらの結果は，これまでに貝類で予測されていたものとはまったく正反対

であり，第I章で述べた“アロマターゼ阻害説”に対する疑問を支持する結果であると言える。さらにEDC問題において有機スズ化合物は，雄性化を引き起こす典型的な化学物質であると認識されていたが，少なくともヒト胎盤に対してはエストロゲン産生を亢進することから，すべての生物種に対し雄性化作用を示すわけではないことが明らかとなった。このことは，有機スズ化合物がアロマターゼ以外の分子を標的として毒性を誘発する可能性を示唆している。

III. 核内受容体アゴニストとしての有機スズ化合物とアロマターゼ発現

ステロイドホルモン受容体をはじめとする核内受容体は，リガンド誘導性転写調節因子であり，ステロイドホルモンなどの脂溶性リガンドの情報を受け，特定の標的遺伝子群の発現を転写レベルで制御する。またこれらの内因性脂溶性リガンドは，それぞれの産生および代謝を互いに制御し合うことによって，生殖系や内分泌系などのホメオスタシスを維持していると考えられる。したがって，化学物質がERやAR以外の核内受容体に作用した場合においても，そのホメオスタシスが破綻する可能性は十分に考えられる。このような観点から，筆者らは，転写共役活性化因子と大腸菌アルカリホスファターゼとの融合タンパク質，および各種核内受容体のリガンド結合領域（ligand binding domain; LBD）を含むタンパク質とを利用した無細胞系リガンドスクリーニング法（CoA-BAP法；図3）¹⁴⁾を用いて，EDC作用が疑われている40種類の化学物質についてリガンドスクリーニングを行った¹⁵⁾。その結果，TBTおよびTPTが，ビタミンAの代謝物（9-*cis* レチノイン酸；9cRA）をアゴニス

図3. CoA-BAP法¹⁴⁾

A: CoA-BAP (coactivator-bacterial alkaline phosphatase) 法の概念図。核内受容体のリガンド結合領域 (LBD) にアゴニストが結合したときに、コアクチベーターが結合できるようになる性質を利用している。精製したGST (glutathione-S-transferase) と核内受容体 (NR) のLBDの融合タンパク質 (NR-LBD) をGSH (glutathione) で固相化したマイクロプレートのウェルに、コアクチベーターであるTIF2とBAP (bacterial alkaline phosphatase) の融合タンパク質 (TIF2-BAP) および被験化合物を加え、一定時間反応させる。被験化合物が核内受容体アゴニストとして機能する場合には、TIF2がNR-LBDに結合し、TIF2とBAPの融合タンパク質がウェル内に留まる。反応後、未反応の融合タンパク質を除き、BAPの基質であるNPP (*p*-nitrophenyl phosphate) を加える。NPPは無色であるが、BAPによって黄色の*p*-nitrophenolへと変換される。被験化合物の核内受容体に対する親和性が強いほどウェルに留まるTIF2とBAPの融合タンパク質は増加するため、*p*-nitrophenolによる発色の程度によって、被験化合物の核内受容体に対するアゴニスト活性を評価することができる。

B: ER α に対する評価系を作成した際に用いた精製タンパク質の電気泳動像。

C: 構築したER α に対する評価系におけるエストラジオールの反応性。

トとするRXR (retinoid X receptor) と、インスリン抵抗性糖尿病治療薬であるチアゾリジン誘導体をアゴニストとするPPAR γ (peroxisome proliferator-activated receptor γ) のアゴニストとしての作用を有する可能性を見いだした¹⁵⁾。これらの知見をもとに、ヒトやマウスの細胞を用いた検討を行ったところ、TBTとTPTは各受容体を介する転写を活性化し、そのEC₅₀は10~20nMであった¹²⁾、¹⁵⁾。また、精製タンパク質を用いた各受容体のLBDに対する解離定数の検討においては、RXR α , β , γ に対しては既知のアゴニストである9cRAの各々約5, 15, 11倍¹²⁾、PPAR γ に対してはチアゾリジン誘導体であるロジグリタゾンとはほぼ同等であった (中西ら; 未発表データ)。既知のPPAR γ やRXRアゴニストとは構造がまったく異なるTBTやTPTが、これほどまでに強力なアゴニスト活性を示すのは驚きである (図1)。さらにTBTやTPT以外の有機スズ化合物についても検討を行ったところ、スズ原子に結合している官能基の構造や官能基の数によって、各受容体に対する転写活性化能や親和性が変動し、そのアゴニスト活性には明確な構造相関が認められた¹²⁾。

有機スズ化合物のこのようなアゴニスト活性が与える細胞機能への影響についても検討を行った。PPAR γ とRXRは互いにヘテロ二量体を形成し、各々のアゴニスト依存的に転写を活性化することで、支配遺伝子の発現や脂肪細胞分化を誘導するが、TBTとTPTは前述の結果を反映して、マウスの脂肪細胞分化をPPAR γ /RXRの支配遺伝子の発現

上昇を伴って誘導することも確認された¹⁵⁾。では、ヒト胎盤に対する影響はどうであろうか? ヒト絨毛細胞株に、PPAR γ アゴニストを添加しても、アロマターゼのmRNA発現や胎盤型のアロマターゼプロモーターの転写活性には影響は認められない¹²⁾。このことは、ヒト胎盤のアロマターゼの発現にはPPAR γ /RXRは関わっていないことを示唆している。しかしながら、RXR特異的アゴニストを添加した場合には、ヒト絨毛細胞株のアロマターゼ発現が上昇するうえ、胎盤型のアロマターゼプロモーターの活性も上昇する¹²⁾。RXRは、PPAR γ 以外にもPPAR α , PPAR β , FXR (farnesoid X receptor), LXR (liver X receptor) ともヘテロ二量体を形成したり、またRXR自身でホモ二量体も形成する。これらの二量体も、PPAR γ /RXRと同様にRXRアゴニストで転写が活性化されるが、胎盤のアロマターゼ発現には前述のヘテロ二量体は関与していない¹²⁾。したがって、RXRアゴニストによるヒト胎盤でのアロマターゼ発現誘導は、RXRホモ二量体を介した作用であると考えられる。このことは、有機スズ化合物によるアロマターゼの発現誘導においても同様であり、有機スズ化合物はRXRホモ二量体を介して、ヒト胎盤のアロマターゼ発現を誘導するものと考えられる (図4)¹²⁾。

IV. 核内受容体を介した有機スズ化合物の毒性

冒頭にも述べたとおり、有機スズ化合物は前述のEDC作用以外にも様々な毒性を誘導することが報告されているが、

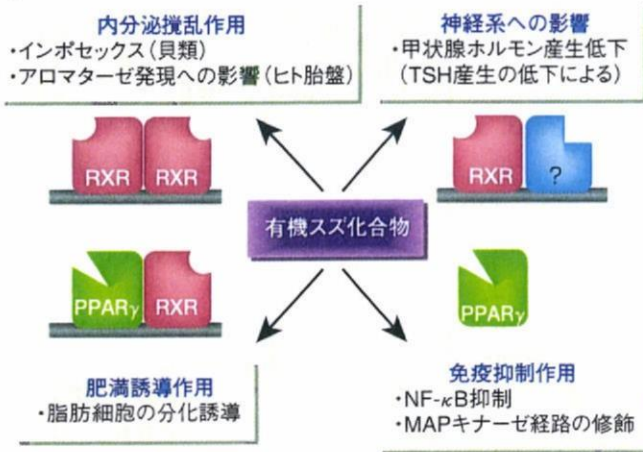


図4. 核内受容体を介した有機スズ化合物の様々な生物攪乱作用
 有機スズ化合物はPPAR γ やRXRを介することで、これらの受容体を有する動物に様々な影響を与える可能性がある。

これらの毒性発現においてもPPAR γ やRXRが関わっている可能性がある(図4)。TBTは、ラットにおいて甲状腺刺激ホルモン(thyroid stimulating hormone; TSH)の産生低下を伴う甲状腺機能低下症を引き起こすことが報告されている⁴⁾が、その一方で、RXR特異的アゴニストがマウスやラットにおいて同様の作用を引き起こす¹⁶⁾ことから、TBTによるTSHや甲状腺ホルモンの産生低下はRXRを介して誘導されている可能性が考えられる。また有機スズ化合物は、免疫抑制作用やアレルギー反応の誘導などの免疫毒性も有する⁴⁾が、PPAR γ が転写制御因子であるNF- κ B(nuclear factor- κ B)の転写活性を抑制したり、MAPキナーゼ(mitogen-activated protein kinase)経路によるシグナル伝達を修飾したりすることで、免疫担当細胞のサイトカイン産生などに影響を与えることが報告されている¹⁷⁾ことから、有機スズ化合物による免疫毒性においてはPPAR γ が関わっている可能性が考えられる。さらに有機スズ化合物は、前述のとおり、PPAR γ /RXRを介してマウスの脂肪細胞分化を誘導する¹⁵⁾が、妊娠マウスにTBTを投与すると胎仔の肝臓などで脂肪組織の過形成が認められることも報告されていることから、最近では肥満誘導因子として作用する可能性も指摘されている¹⁸⁾。これらの知見は、有機スズ化合物がPPAR γ やRXRを介することで、これらの受容体を有する動物に様々な影響を与える可能

性があることを示唆している。

V. インボセックスとRXR

RXRは、核内受容体ファミリーの中でも例外的に種を超えて保存されている受容体であり、昆虫類などの下等無脊椎動物においてもUSP(ultraspiracle)と呼ばれるオルソログが存在する。しかしながら、これら受容体のLBDは動物種によって大きく異なり、哺乳動物の内因性アゴニストとされている9cRAなどに対してUSPは応答しない。その一方で、最近イボニシなどの貝類においては、脊椎動物のRXR-LBDと相溶性の高いRXRが存在することが報告された(図5)^{19), 20)}。これらの貝類のRXRは、ヒトのRXRと同じく9cRAや有機スズ化合物と高い結合能を有する。また興味深いことに、これらの貝類に9cRAを投与すると、有機スズ化合物と同様にインボセックスが誘導されることから(図5)、貝類のインボセックスはRXRを介して誘導されている可能性が示唆された(図4)^{19), 20)}。有機スズ化合物を妊娠マウスなどに投与しても、インボセックスのような表現型は認め

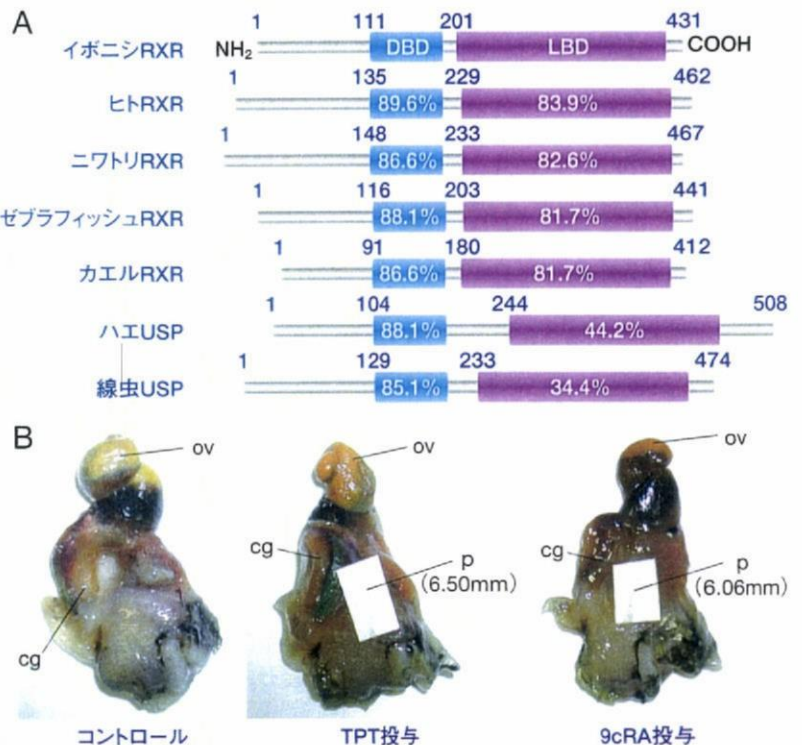


図5. 貝類のRXRと9cRAによるインボセックスの誘導

A: 様々な生物種におけるRXR(USP)の構造の比較。すべての生物種においてDNA結合領域(DBD)の相溶性は高いが、LBDの相溶性はRXRとUSPでは大きく異なる。

B: 9cRAとTPTによるイボニシのインボセックスの写真。各被験物質(1 μ g/g wet wt.)をそれぞれ足部に注射して、人工海水中で1カ月間飼育後、取り上げて解剖した。ペニス部分には、ペニスを際立たせるために白い紙をのせている。cg; capsule gland(卵嚢腺), ov; ovary(卵巣), p; penis(ペニス: 数値はペニス長)。Nishikawa J, et al: Environ Sci Technol (2004) 38: 6271-6276 を一部改変。

写真提供: 国立環境研究所 堀口敏宏先生

られないが、おそらくそれは、生殖器官形成などにおけるRXRの生理的意義が、生物種によって大きく異なるからであると考えられる。

おわりに

本稿では、有機スズ化合物の貝類とヒトアロマトーゼへの影響を中心に、その分子メカニズムについて概説した。有機スズ化合物は、EDC作用が疑われている化学物質の中でも、きわめて低濃度で明確なEDC作用を誘導するが、それは核内受容体アゴニストとして作用することに起因することが明らかとなった。本来アゴニストとなることを意図されていない合成化学物質が、このように生理的アゴニストや合成アゴニストに匹敵するような影響を示す例はきわめ

てまれである。有機スズ化合物は、生物種によって誘発する表現型は異なるものの、様々な生物種に対してRXRまたはPPAR γ を介した毒性を引き起こす可能性が考えられる。今後は、PPAR γ やRXRなどの核内受容体に対する化学物質の作用や、様々な生物種におけるこれらの核内受容体の生理的意義を解明することによって、有機スズ化合物の生物攪乱作用がより明確になることを期待したい。

謝辞 本稿で紹介した成果は、大阪大学大学院薬学研究科 田中慶一先生（現・大阪大谷大学薬学部）のご指導とご助言により成し遂げられた成果であり、ここに深謝致します。また、研究を遂行してくれた廣森洋平君をはじめとする多くの学生に深謝致します。本稿を執筆するにあたり、イボニシの写真のご提供を賜りました国立環境研究所 堀口敏宏先生に深謝致します。

文献

- 1) Matthiessen P, et al: Environ Toxicol Chem (1998) 17: 37-43
- 2) Bettin C, et al: Helgol Meeresunters (1996) 50: 299-317
- 3) Horiguchi T, et al: Environ Pollut (1997) 95: 85-91
- 4) Benya TJ: Drug Metab Rev (1997) 29: 1189-1284
- 5) Cooke GM: Toxicol Lett (2002) 126: 121-130
- 6) Heidrich DD, et al: Steroids (2001) 66: 763-769
- 7) Nakanishi T, et al: J Clin Endocrinol Metab (2002) 87: 2830-2837
- 8) Doering DD, et al: Steroids (2002) 67: 859-867
- 9) Lo S, et al: J Steroid Biochem Mol Biol (2003) 84: 569-576
- 10) Escriva H, et al: Proc Natl Acad Sci USA (1997) 94: 6803-6808
- 11) Shozu M, et al: J Clin Endocrinol Metab (1991) 72: 560-566
- 12) Nakanishi T, et al: Mol Endocrinol (2005) 19: 2502-2516
- 13) Nakanishi T, et al: Biochem Pharmacol (2006) 71: 1349-1357
- 14) Kanayama T, et al: J Biochem (Tokyo) (2003) 133: 791-797
- 15) Kanayama T, et al: Mol Pharmacol (2005) 67: 766-774
- 16) Macchia PE, et al: Am J Physiol Endocrinol Metab (2002) 283: E326-331
- 17) Zhang X, et al: Int Immunopharmacol (2002) 2: 1029-1044
- 18) Grun F, et al: Mol Endocrinol (2006) 20: 2141-2155
- 19) Nishikawa J, et al: Environ Sci Technol (2004) 38: 6271-6276
- 20) Castro LF, et al: Aquat Toxicol (2007) 85: 57-66

for beginners

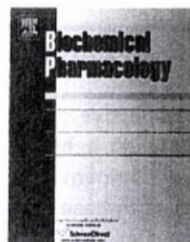
本稿では誌面の都合上、核内受容体の転写調節機能に関する基本的な事項の説明については割愛したが、以下の総説を参考にされたい。
・「核内受容体と創薬」山岡一良ら: 実験医学 (増刊) 24: 152-160 (2006)



ELSEVIER

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

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,*}

^aLaboratory of Medicinal Resources, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan

^bLaboratory of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

^cResearch and Development of Kemin Health Co. Ltd., 600 E. Court Ave., Suite A Des Moines, IA 50309, USA

^dInformation Center, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan

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

^fResearch Foundation Itsuu Laboratory, 2-28-10 Tamagawa, Setagaya-ku, Tokyo 158-0094, Japan

^gSchool of Biomedical Science, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan

ARTICLE INFO

Article history:

Received 18 January 2007

Accepted 16 April 2007

Keywords:

β -Cryptoxanthin

Lutein

Xanthophyll

Retinoic acid receptor

Retinoid X receptor

ATP-binding cassette transporter

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.

© 2007 Elsevier Inc. All rights reserved.

* 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).

0006-2952/\$ – see front matter © 2007 Elsevier Inc. All rights reserved.

doi:10.1016/j.bcp.2007.04.014

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 reported that atRA exerts direct effects on T cells by suppressing T helper (T_H)1 development and enhancing T_H 2 development that is essential for antibody responses [20,21]. Since the T_H 1-driven immune response has been consistently shown to promote atherosclerosis, immunomodulation through T_H 2 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 PPAR γ -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 PPAR γ 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 ($23 \pm 1^\circ\text{C}$) 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-RAR α or γ , GAL4DBD-RXR γ , 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 $3000 \times g$ 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 $_2$ CO $_3$ 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: $U = \{(\text{absorbance at } 540 \text{ nm}) - (\text{absorbance at } 630 \text{ nm})\} / (\text{absorbance at } 630 \text{ nm at the start of the assay})$. β -Galactosidase activity is presented as the means \pm 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 $\mu\text{g}/\text{ml}$ 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 ($12,000 \times g$, 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) dithiothreitol 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 $2 \times$ 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 \pm 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 RAR α and γ for the carotenoids and xanthophylls 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 RAR α and RAR γ assays. When the agonist activity against RXR γ , 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).

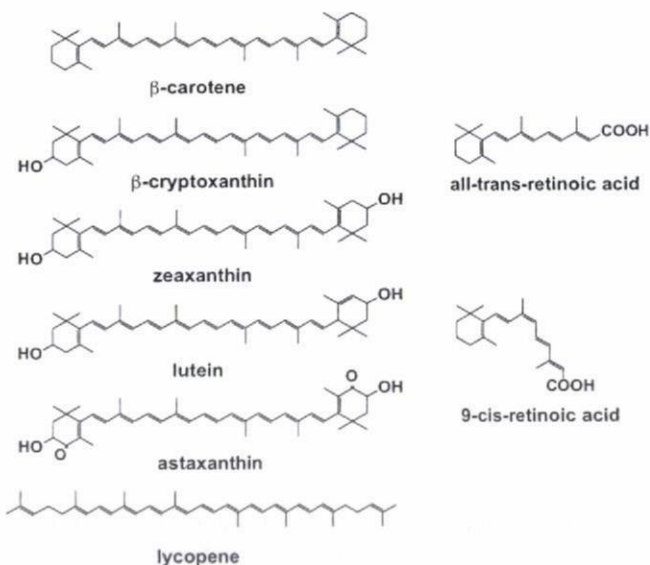


Fig. 1 – Structures of xanthophylls, carotenoids, and retinoic acid used in the present study.

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 RAR α or RAR γ , 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 μ M. This induction was completely inhibited by 10 μ M LE540. Lutein induced β -galactosidase activity at 1 μ M concentration and 10 μ M LE540 abolished that induction. These results indicate that both β -cryptoxanthin and lutein induce β -galactosidase via RAR α 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 RAR α 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 μ M) as well as atRA (5 μ M) 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 -

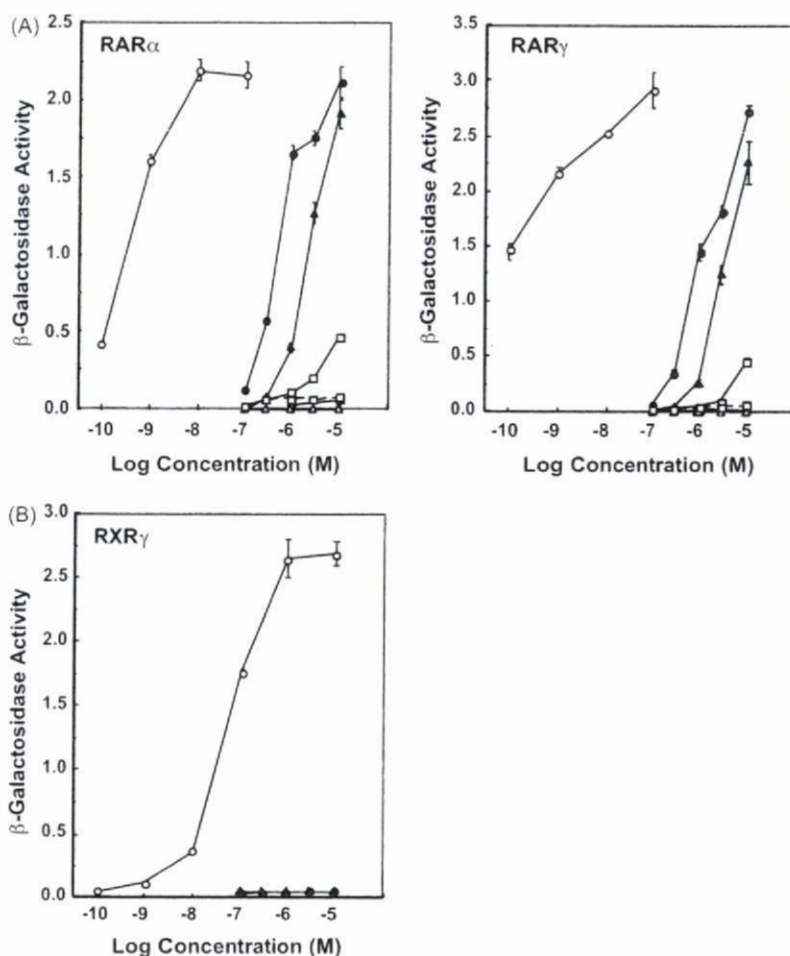


Fig. 2 – Yeast two-hybrid assay for RAR α or RAR γ activity. Yeast strain Y190 was transformed with GAL4 activation domain fused to TIF2 and GAL4 DNA binding domain fused to agonist-binding domain of RAR α or RAR γ . Chemicals were added to yeast cultures in doses ranging from 10^{-10} to 10^{-5} M. 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: lutein, open square: β -carotene, closed square: zeaxanthin, open square with broken line: lycopene. The values are represented as means \pm 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 -cryptoxanthin- 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 $1 \mu\text{M}$ 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 carotenoid and xanthophyll 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

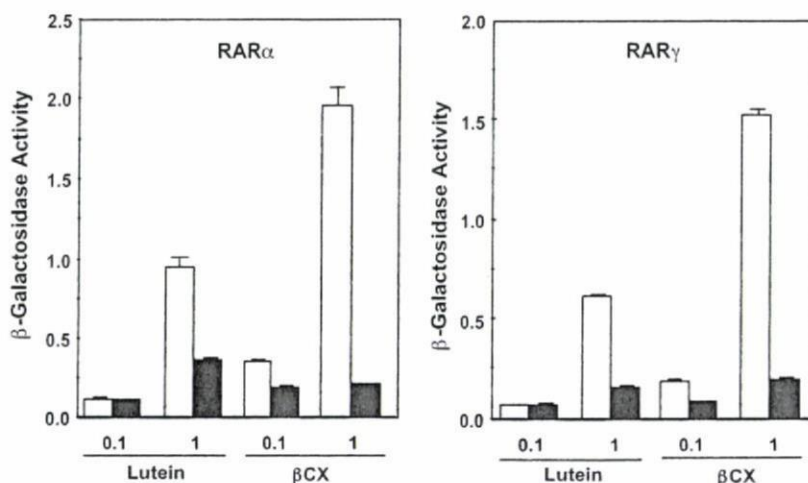


Fig. 3 – Effect of RAR pan-antagonist, LE540, on the transcriptional activity of β -cryptoxanthin or lutein against RAR α or RAR γ . Following 30 min incubation of 10 μ M LE540 with yeast cultures, β -cryptoxanthin or lutein was added to yeast cultures at the indicated concentrations (μ M) and incubated 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 \pm 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 μ M 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 on 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

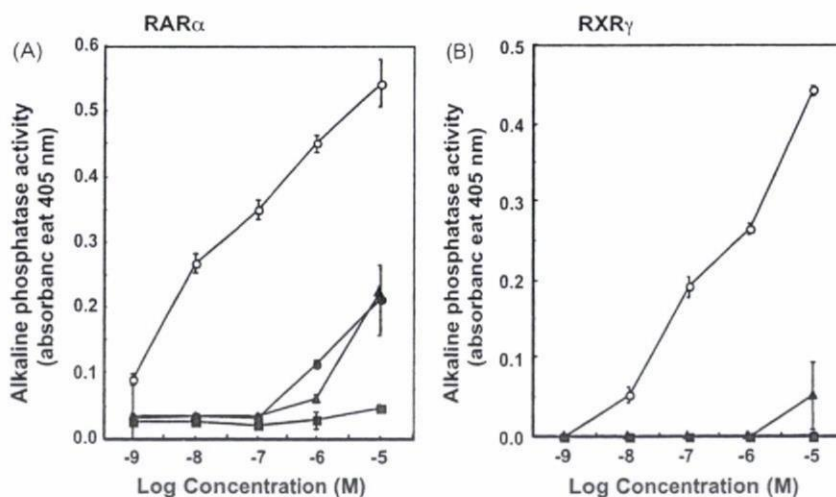


Fig. 4 – Binding activity of β -cryptoxanthin and lutein against RAR γ in *in vitro* CoA-BAP system. Agonist-dependent interactions between GST-RAR γ and TIF2-BAP were determined as alkaline phosphatase activity according to the method described in Section 2. Open circle in (A) and (B): atRA and 9-cis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

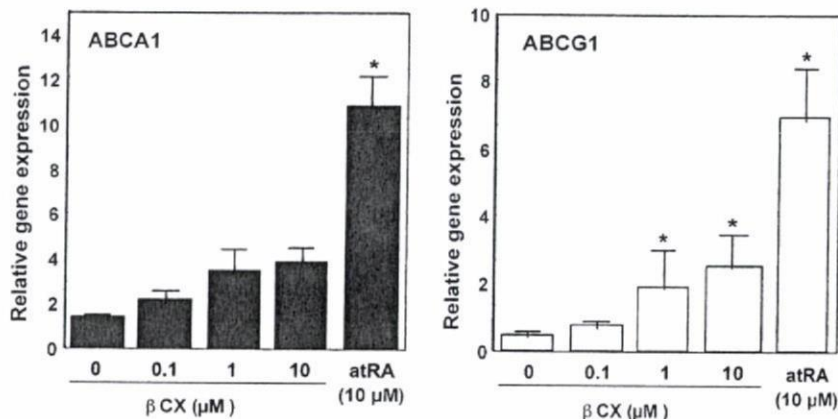


Fig. 5 – Effect of β -cryptoxanthin on induction of ABCA1 or ABCG1 mRNA in mouse peritoneal macrophages. Thioglycolate-elicited peritoneal macrophages were seeded at a concentration of 2.0×10^6 cells/ml and treated with β -cryptoxanthin or atRA at the indicated concentrations for 8 h. mRNA levels of 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 \pm 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.

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 reports 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 RAR β 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 RAR β expression based upon the need for a higher effective dose of β -cryptoxanthin than atRA for the induction of RAR β expression or transactivation of RARs. In contrast, the use of the CoA-BAP system employed in the present study to assay the ligand

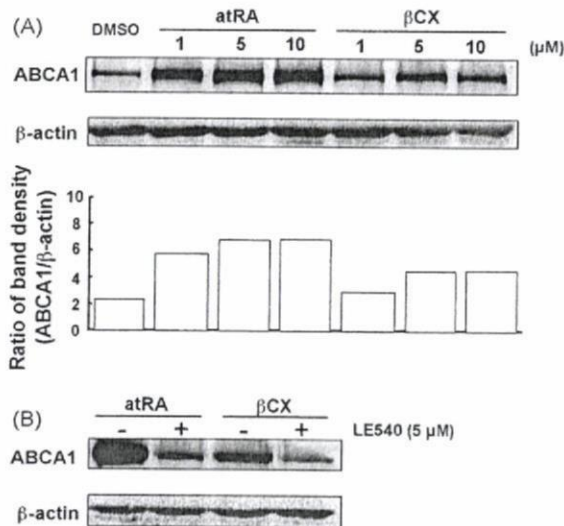


Fig. 6 – Induction of ABCA1 protein by β -cryptoxanthin. Thioglycolate-elicited peritoneal macrophages were seeded at a concentration of 2.0×10^6 cells/ml and treated with increasing concentrations of β -cryptoxanthin or atRA for 20 h in the absence (A) or presence (B) of an RAR pan-antagonist LE540 (5 μ M). 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.

activity against RAR α 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.

Acknowledgments

We are grateful to Dr. Zoraida DeFreitas for her helpful discussion of results. We are also grateful to Dr. Shinji Yokoyama for providing us with anti-ABCA1 antibody. This study was supported by a grant from The Uehara Memorial Foundation.

REFERENCES

- [1] Voutilainen S, Nurmi T, Mursu J, Rissanen TH. Carotenoids and cardiovascular health. *Am J Clin Nutr* 2006;83:1265-71.
- [2] Joshipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, et al. Fruit and vegetable intake in relation to risk of ischemic stroke. *JAMA* 1999;282:1233-9.
- [3] Joshipura KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, et al. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann Intern Med* 2001;134:1106-14.
- [4] Bazzano LA, He J, Ogden LG, Loria CM, Vupputuri S, Myers L, et al. Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first national health and nutrition examination survey epidemiologic follow-up study. *Am J Clin Nutr* 2002;76:93-9.
- [5] Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, et al. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N Engl J Med* 1999;334:1145-9.
- [6] Greenberg ER, Baron JA, Karagas MR, Stukel TA, Nierenberg DW, Stevens MM, et al. Mortality associated with low plasma concentration of beta carotene and the effect of oral supplementation. *JAMA* 1996;275:699-703.
- [7] Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352:1685-95.
- [8] Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999;340:115-26.
- [9] Hansson GK, Libby P, Schönbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* 2002;91:281-91.
- [10] DeRose JJJr, Madigan J, Umame JP, Prystowsky JH, Nowygrod R, Oz MC, et al. Retinoic acid suppresses intimal hyperplasia and prevents vessel remodeling following arterial injury. *Cardiovasc Surg* 1999;7:633-9.
- [11] Miano JM, Kelly LA, Artacho CA, Nuckolls TA, Piantadosi R, Blaner WS. All-trans-retinoic acid reduces neointimal formation and promotes favorable geometric remodeling of the rat carotid artery balloon withdrawal injury. *Circulation* 1999;98:1219-27.
- [12] Lee CW, Park SJ, Park SW, Kim JJ, Hong MK, Song JK. All-trans-retinoic acid attenuates neointima formation with acceleration of reendothelialization in balloon-injured rat aorta. *J Korean Med Sci* 2000;15:31-6.
- [13] Herdeg C, Oberhoff M, Baumbach A, Schroeder S, Leitritz M, Blattner A, et al. Effects of local all-trans-retinoic acid delivery on experimental atherosclerosis in the rabbit carotid artery. *Cardiovasc Res* 2003;57:544-53.
- [14] Neuville P, Yan Zq, Gidlöf A, Pepper MS, Hansson GK, Gabbiani ZG, et al. Retinoic acid regulates arterial smooth muscle cell proliferation and phenotypic features in vivo and in vitro through an RAR-a-dependent signaling pathway. *Arterioscler Thromb Vasc Biol* 1999;19:1430-6.
- [15] Miano JM, Topouzis S, Majesky MJ, Olson EN. Retinoid receptor expression and all-trans retinoic acid-mediated growth inhibition in vascular smooth muscle cells. *Circulation* 1996;93:1886-95.
- [16] Kosaka C, Sasaguri T, Komiyama Y, Takahashi H. All-trans retinoic acid inhibits vascular smooth muscle cell proliferation targeting multiple genes for cyclins and cyclin-dependent kinases. *Hypertens Res* 2001;24:579-88.
- [17] Costet P, Lallanne F, Gerbod-Giannone MC, Molina JR, Fu X, Lund EG, et al. Retinoic acid receptor-mediated induction of ABCA1 in macrophages. *Mol Cell Biol* 2003;23:7756-66.
- [18] Takeda N, Manabe I, Shindo T, Iwata H, Iimuro S, Kagechika H, et al. Synthetic retinoid Am80 reduces scavenger receptor expression and atherosclerosis in mice by inhibiting IL-6. *Arterioscler Thromb Vasc Biol* 2006;26:1177-83.
- [19] Langmann T, Liebisch G, Moehle C, Schifferer R, Dayoub R, Heiduczek S, et al. Gene expression profiling identifies retinoids as potent inducers of macrophage lipid efflux. *Biochim Biophys Acta* 2005;1740:155-61.

- [20] Iwata M, Eshima Y, Kagechika H. Retinoic acids exert direct effects on T cells to suppress T_H1 development and enhance T_H2 development via retinoic acid receptors. *Int Immunol* 2003;15:1017-25.
- [21] Hoag KA, Nashold FE, Gorman J, Hayes C. Retinoic acid enhances the T helper 2 cell development that is essential for robust antibody responses through its action on antigen-presenting cells. *J Nutr* 2002;132:3736-9.
- [22] David M, Hodak E, Lowe NJ. Adverse effects of retinoids. *Med Toxicol Adverse Drug Exp* 1988;3:273-88.
- [23] Frankel SR, Eardley A, Lauwers G, Weiss M, Warrell Jr RP. The "retinoic acid syndrome" in acute promyelocytic leukemia. *Ann Intern Med* 1992;117:292-6.
- [24] Petkovich M, Brand NJ, Krust A, Chambon P. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 1987;330:444-50.
- [25] Giguere V, Ong ES, Segui P, Evans RM. Identification of a receptor for the morphogen retinoic acid. *Nature* 1987;330:624-9.
- [26] Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB J* 1996;10:940-54.
- [27] Uppenberg J, Svensson C, Jaki M, Bertilsson G, Jendeborg L, Berkenstam A. Crystal structure of the ligand binding domain of the human nuclear receptor PPAR γ . *J Biol Chem* 1998;273:31108-12.
- [28] Kim KR, Lee JH, Kim SJ, Rhee SD, Jung WH, Yang SD, et al. KR-62980: a novel peroxisome proliferator-activated receptor γ agonist with weak adipogenic effects. *Biochem Pharmacol* 2006;72:446-54.
- [29] Allen T, Zhang F, Moodie SA, Clemens LE, Smith A, Gregoire F, et al. Halofenate is a selective peroxisome proliferator-activated receptor γ modulator with antidiabetic activity. *Diabetes* 2006;55:2523-33.
- [30] Napoli JL. Biosynthesis and metabolism of retinoid acid: roles of CRBP and CRABP in retinoic acid homeostasis. *J Nutr* 1993;123:362-6.
- [31] Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, et al. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 1992;68:397-406.
- [32] Nishikawa J, Saito K, Goto J, Daikayama F, Matsuo M, Nishihara T. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol Appl Pharmacol* 1999;154:76-83.
- [33] Kanayama T, Mamiya S, Nishihara T, Nishikawa J. Basis of a high-throughput method for nuclear receptor agonists. *J Biochem* 2003;133:791-7.
- [34] Ono Y, Inoue M, Mizukami H, Ogihara Y. Suppressive effect of Kanzo-bushi-to, a Kampo medicine, on collagen-induced arthritis. *Biol Pharm Bull* 2004;27:1406-13.
- [35] Umamiya H, Fukasawa H, Ebisawa M, Eyrolles L, Kawachi E, Eisenmann G, et al. Regulation of retinoid actions by doazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers. *J Med Chem* 1997;40:4222-34.
- [36] Redmond TM, Gentleman S, Duncan T, Yu S, Wiggert B, Gantt E, et al. Identification, expression, and substrate specificity of a mammalian β -carotene 15, 15'-dioxygenase. *J Biol Chem* 2001;276:6560-5.
- [37] De Leenheer AP, Lambert WE, Claeys I. All-trans-retinoic acid: measurement of reference values in human serum by high performance liquid chromatography. *J Lipid Res* 1982;23:1362-7.
- [38] Urbach J, Rando RR. Isomerization of all-trans retinoic acid to 9-cis-retinoic acid. *Biochem J* 1994;299:459-65.
- [39] El-Sohemy A, Baylin A, Kabagambe E, Ascherio A, Spiegelman D, Campos H. Individual carotenoid concentrations in adipose tissue and plasma as biomarkers of dietary intake. *Am J Clin Nutr* 2002;76:172-9.
- [40] Wingerath T, Stahl W, Sies H. β -Cryptoxanthin selectively increases in human chylomicrons upon ingestion of tangerine concentrate rich in β -cryptoxanthin esters. *Arch Biochem Biophys* 1995;324:385-90.
- [41] Riso P, Visioli F, Gardana C, Grande S, Brusamolino A, Galvano F, et al. Effects of blood orange juice intake on antioxidant bioavailability and on different markers related to oxidative stress. *J Agric Food Chem* 2005;53:941-7.
- [42] Gruszecki WI, Strzalka K. Carotenoids as modulators of lipid membrane physical properties. *Biochim Biophys Acta* 2005;1740:108-15.
- [43] Wisniewska A, Widomska J, Subczynski WK. Carotenoid-membrane interactions in liposomes: effect of dipolar, monopolar, and nonpolar carotenoids. *Acta Biochim Pol* 2006;53:475-84.
- [44] Bhosale P, Larson AJ, Frederick JM, Southwick K, Thulin CD, Bernstein PS. Identification and characterization of a Pi isoform of glutathione S-transferase (GSTP1) as a zeaxanthin-binding protein in the macula of the human eye. *J Biol Chem* 2004;279:49447-54.
- [45] Kagechika H, Shudo K. Synthetic retinoids: recent developments concerning structure and clinical utility. *J Med Chem* 2005;48:5875-83.
- [46] Yamaguchi M, Uchiyama S. Effect of carotenoid on calcium content and alkaline phosphatase activity in rat femoral tissues in vitro: the unique anabolic effect of β -cryptoxanthin. *Biol Pharm Bull* 2003;26:1188-91.
- [47] Uchiyama S, Yamaguchi M. Inhibitory effect of β -cryptoxanthin on osteoclast-like cell formation in mouse marrow cultures. *Biochem Pharmacol* 2004;67:1297-305.
- [48] Lian F, Hu KQ, Russell RM, Wang XD. β -Cryptoxanthin suppresses the growth of immortalized human bronchial epithelial cells and non-small-cell lung cancer cells and up-regulates retinoic acid receptor β expression. *Int J Cancer* 2006;119:2084-9.
- [49] Yuan JM, Stram DO, Arakawa K, Lee HP, Yu MC. Dietary cryptoxanthin and reduced risk of lung cancer: the Singapore Chinese health study. *Cancer Epidemiol Biomark Prev* 2003;12:890-8.
- [50] Pattison DJ, Symmons DPM, Welch MLA, Bingham SA, Day NE, Silman AJ, et al. β -cryptoxanthin and inflammatory polyarthritis: results from a population-based prospective study. *Am J Clin Nutr* 2005;82:451-5.

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

Kumiko OHTA*, Megumi OHGASHI*, 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, diethylnitrosamine; 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).