CURCUMIN AND ITS DERIVATIVES SUPPRESS THE TRANSFORMATION OF AN ARYL HYDROCARBON RECEPTOR

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor and is activated by various environmental contaminants, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyl (PCB), benzo[a]pyrene (BaP), and 3-methylcholanthrene (MC)[1,2]. In addition, certain phytochemicals are known to bind to AhR[3]. These ligands bind to cytosolic AhR, and then the activated AhR translocates into the nucleus, heterodimerizes with the AhR nuclear translocator (Arnt)[4]. This heterodimer binds to specific enhancer sequences adjacent to target promoters termed ‘‘dioxin responsive elements” (DREs), and regulates the expression of target genes including cytochrome P450 (CYP) 1A subfamilies[5].

Studies of the interaction between the AhR and phytochemicals have revealed that certain polyphenols, such as flavonoids and resveratrol, act as agonists or antagonists of the receptor[6,7]. Curcumin, a yellow pigment of *Curcuma longa* (turmeric), is both agonist and antagonist of the AhR. It induced the transformation of the AhR and expression of CYP1A1 in human mammary carcinoma MCF-7 cells, while it suppressed the dimethylbenzanthracene-induced transformation and CYP1A1 expression in the same cells[8]. In addition, curcumin facilitated the nuclear translocation of the AhR as well as the heterodimerization of the AhR with the Arnt in oral squamous cell carcinoma cells[9]. In a cell-free system using guinea pig liver cytosol, curcumin had an antagonistic effect on AhR transformation[10]. In this study, we investigated that the mode of action of curcumin on the transformationand the structure-activity relationship using synthesized curcumin derivatives.

Materials and methods

Materials: Curcumin and its derivatives are kindly provided from Prof. Toshihiko Osawa, Aichi Gakuin University, Japan, and Assoc. Prof. Yoshiyuki Mizushina, Kobe-Gakuin University, Japan. Structures of these compounds are shown in Table 1.

Cell culture and treatment: Mouse hepatoma Hepa-1c1c7 cells were serum-starved using MEM containing 5 mg/mL bovine serum albumin for 14 h and treated with curcumin and its derivatives at the indicated concentrations and/or 0.1 nM TCDD or with 1 µL/mL dimethylsulfoxide (DMSO) alone as a vehicle control[11]. A post-nuclear fraction and nuclear extract were prepared from the cells according to our previous report[12].

Determination of AhR transformation: Rat liver cytosol was used as a source of AhR. AhR transformation was determined by a southwestern enzyme-linked immunosorbent assay (SW-ELISA)[12] and an electrophoretic mobility shift assay (EMSA)[6].

Immunoprecipitation and Western blot: Immunoprecipitation using nuclear extract with anti-phosphothreonine or anti-Arnt antibody and detection of CYP1A1, AhR, Arnt, histone H4, and β-actin by Western blotting were carried out according to a previous report[11].

Measurement of ethoxyresorufin O-deethylase (EROD) activity: EROD activity was measured in Hepa-1c1c7 cells on a 96-well plate after treatment with curcumin and its derivatives and/or TCDD for a 24 h at 37 °C[13].

AhR ligand binding assay: The specific binding of [^{3}H] MC to the AhR was assessed with a ligand binding assay using hydroxyapatite as previously described[13].

Results and discussion

The effects of curcumin on AhR transformation and its downstream event in Hepa-1c1c7 cells

First, we examined that the effects of curcumin on the translocation of the AhR into the nucleus, transformation of the receptor, and expression of CYP1A1 in Hepa-1c1c7 cells (Fig. 1). As expected, TCDD at 0.1 nM promoted the translocation of the AhR into the nucleus as compared with DMSO alone (Fig. 1A). However, co-treatment with 0.1 or 10 µM curcumin plus 0.1 nM TCDD accelerated the translocation. This acceleration was also observed on treatment with 10 µM curcumin alone, indicating that curcumin *per se*

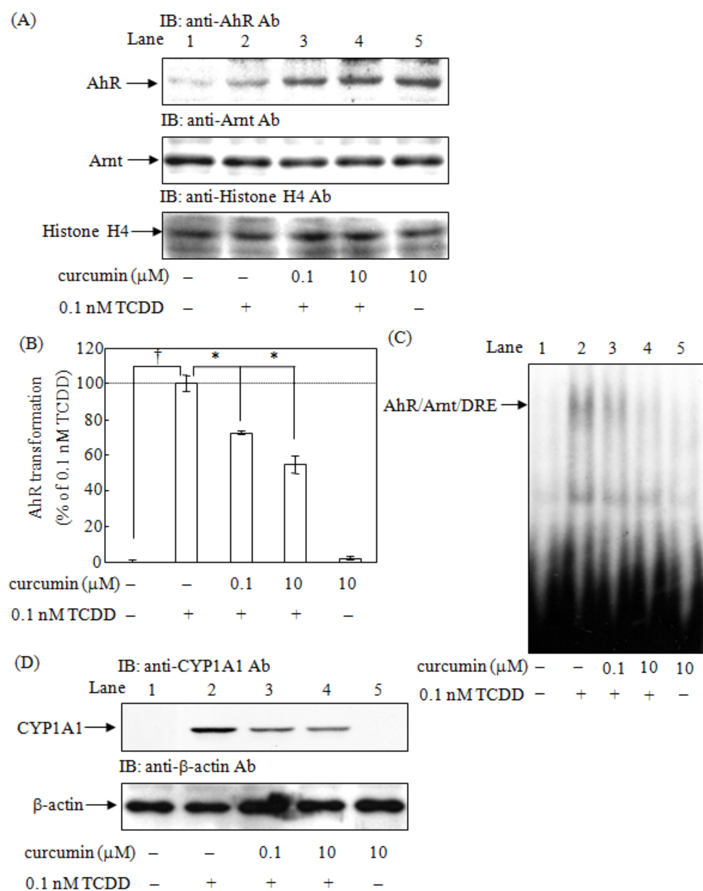

Fig.1. The effects of curcumin on nuclear translocation of the AhR, DRE-binding of the AhR, and CYP1A1 expression in Hepa-1c1c7 cells[11].
After 1 h-treatment with curcumin and/or TCDD to the cells, the AhR, Arnt, and histone H4 in the nucleus were detected by Western blotting (A) and the DRE-binding activity of the AhR was measured by SW-ELISA (B) and EMSA (C). After 24 h, CYP1A1 and β-actin were detected by Western blotting in a post-nuclear fraction (D).

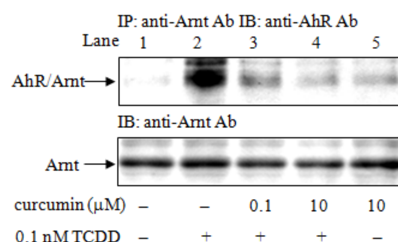

Fig. 2. The effect of curcumin on heterodimerization of the AhR with Arnt in Hepa-1c1c7 cells[11].
After 1 h-treatment with curcumin and/or TCDD to the cells, AhR/Arnt heterodimer was detected in nuclear extract. Nuclear Arnt was also determined by Western blotting.

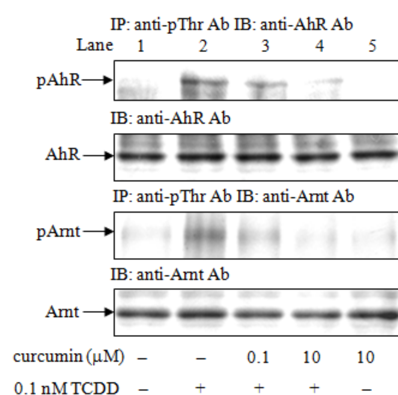

Fig. 3. The effect of curcumin on phosphorylation of the AhR and Arnt in Hepa-1c1c7 cells[11].
After 1 h-treatment with curcumin and/or TCDD to the cell, phosphorylated AhR and Arnt were detected by pThr immunoprecipitation followed by Western blotting with anti-AhR and Arnt antibodies. The constitutive levels of AhR and Arnt were also determined.

promotes the nuclear translocation of the AhR. Under the same conditions, Arnt in the nucleus were not altered. Regarding the binding of the AhR to DRE as an endpoint marker for the transformation, curcumin suppressed the TCDD-induced transformation of the AhR dose-dependently by both SW-ELISA (Fig. 1B) and EMSA (Fig. 1C). Moreover, curcumin dose-dependently suppressed 0.1 nM TCDD-induced CYP1A1 expression (Fig. 1D), and the compound alone did not increase the expression consistent with results of the binding of the AhR to DRE (Fig. 1B and C). The heterodimerization of the AhR with Arnt is essential for the transformation. TCDD at 0.1 nM facilitated the formation of a heterodimer as expected, while co-treatment with curcumin had an inhibitory effect (Fig. 2). Interestingly, 10 μM curcumin alone caused weak heterodimerization.

Phosphorylation of the AhR and/or Arnt is also essential for the transformation and certain protein kinases would be involved in their phosphorylation. Therefore, the phosphorylation of the AhR and Arnt was investigated by the pThr immunoprecipitation followed by Western blotting with AhR or Arnt antibody. TCDD stimulated their phosphorylation as expected, while curcumin alone did not (Fig. 3). Co-treatment with curcumin and TCDD inhibited the TCDD-induced phosphorylation of the AhR and Arnt dose-dependently. Under the same conditions, the constitutive levels of the AhR and Arnt were unchanged.

The effects of curcumin on the binding of MC to the AhR and AhR transformation in a cell-free system

Since curcumin itself accelerated the nuclear translocation of the AhR (Fig. 1A), it would bind to the receptor as a ligand. Therefore, it was investigated whether curcumin competitively inhibits the binding of agonist of the AhR in a cell-free system using rat liver cytosol (Fig. 4A). Curcumin at 10 μM inhibited the specific binding of

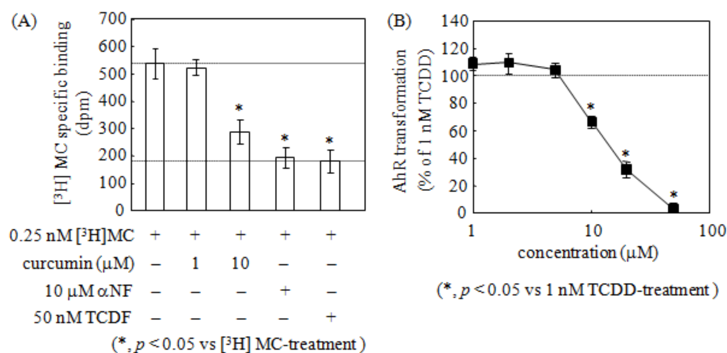

Fig. 4. The effects of curcumin on binding of MC to the AhR and TCDD-induced AhR transformation in a cell-free system[11].
(A) The inhibitory effect of curcumin on the binding of [^{3}H] MC to the AhR was determined by AhR ligand binding. The upper and lower dotted lines represents total and non-specific binding, respectively. (B) After the cytosol was treated with various concentrations of curcumin and 1 nM TCDD, the transformed AhR was determined by SW-ELISA. The dotted line represents 100% of the transformation.

[^{3}H] MC to 30% of that with [^{3}H] MC alone. α-naphthoflavone (αNF), a known antagonist of the AhR, completely inhibited the specific binding at 10 μM to the same level as 50 nM TCDF, which is used for determination of the non-specific binding level. In the cell free system, curcumin suppressed the transformation induced by 1 nM TCDD dose-dependently with the 50% of inhibitory concentration value of 15.4 μM (Fig. 4B). Curcumin alone did not induce the transformation of the AhR from 1 pM to 50 μM (data not shown).

Table 1. Structures of curcumin derivatives and their inhibitory effects on AhR transformation and EROD activity

Curcumin (entry 1)
(1*E*,6*E*)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione

Entry #	Position # 3′	4′	3″	4″	3	4	5	1-2 (bond)	6-7 (bond)	LogP	% of inhibition AhR trans-formation	EROD activity
1	OCH_3	OH	OCH_3	OH	O	H	OH	double	double	2.94	40	67
2	OCH_3	$OCOCH_3$	OCH_3	$OCOCH_3$	O	H2	O	double	double	2.06	28	24
3	–	OCH_3	OCH_3	OCH_3	O	$(CH_3)_2$	O	double	double	4.08	78	100
4	OCH_3	OCH_3	OCH_3	OCH_3	O	$(CH_3)_2$	O	double	double	3.82	84	100
5	OCH_3	OH	OCH_3	OH	OH	H_2	OH	double	double	1.63	29	4
6	OCH_3	OH	OCH_3	OH	O	H_2	O	single	single	2.10	48	10
7	OH	OH	OCH_3	OH	O	H_2	OH	single	single	1.51	28	28
8	OCH_3	OH	OCH_3	OH	OH	H_2	OH	single	single	1.83	43	19
9	OCH_3	OH	OCH_3	$OCOCH_3$	O	H_2	O	double	double	2.16	65	68
10	OCH_3	OH	OCH_3	OCH_3	O	H_2	O	double	double	2.73	39	35
11	OCH_3	$OCOCH_3$	OCH_3	$OCOCH_3$	O	H_2	O	single	single	1.91	53	37
12	OCH_3	$OCOCH_3$	OCH_3	$OCOCH_3$	O	H_2	OH	single	single	1.77	49	33
13	OCH_3	$OCOCH_3$	OCH_3	$OCOCH_3$	OH	H_2	OH	single	single	1.65	56	44
14	OCH_3	$OCOCH_3$	OCH_3	$OCOCH_3$	O	$(CH_3)_2$	O	double	double	2.68	41	49
15	OCH_3	OH	OCH_3	OH	O	H	OH	single	single	2.50	42	40

From these results, we confirmed that curcumin had the antagonistic effect both in Hepa-1c1c7 cells and in the cell-free system, although it stimulated the nuclear translocation of the AhR and the heterodimerization of the receptor with Arnt. Moreover, curcumin inhibited the binding of MC to the AhR in the same manner as αNF. Thus, curcumin binds to a ligand-binding domain of the AhR as antagonist, resulting in the induction of nuclear translocation in the same way as TCDD. In the nucleus, curcumin stimulated heterodimerization of the AhR with Arnt as an essential step for the transformation, but did not have the ability to induce the binding of AhR to DRE or CYP1A1 expression. These results suggest that the binding of nontoxic polyphenols to the AhR is different from that of toxic compounds including TCDD in terms of the effect on the AhR-signaling pathway. Therefore, AhR transformation and its downstream events must require not only the nuclear translocation of the receptor but also some essential events in addition to the heterodimerization of the AhR with Arnt.

Phosphorylation of the AhR and/or Arnt is essential for the transformation and its downstream events. In this study, curcumin dose-dependently inhibited the TCDD-induced phosphorylation of the AhR and Arnt, followed by suppression of the heterodimerization of the AhR with Arnt, DRE-binding of the AhR and CYP1A1 expression induced by TCDD. Therefore, the antagonistic effect of curcumin on the transformation is due to inhibition of the phosphorylation of the AhR and/or Arnt. For the phosphorylation of the AhR and Arnt, we obtained evidences that protein kinase C[11] and ERK[14] are involved in this effect (data not shown). Interestingly, curcumin alone caused heterodimerization of the AhR with Arnt, although it did not phosphorylate either protein. Moreover, curcumin did not induce the binding of the AhR to DRE. These results suggest that the translocation of AhR into the nucleus by curcumin has the potentiality to heterodimerize with non-phosphorylated Arnt, and this heterodimer may be structurally unstable or lack DRE-binding activity.

Structure-activity relationships between curcumin derivatives and suppression of AhR transformation
To understand the active moiety of curcumin (entry 1 in Table 1) on the suppression of the AhR transformation, fourteen derivatives (entry 2-15) were introduced. AhR transformation was measured in cell free system, while EROD activity as a downstream event of the transformation was measured in Hepa-1c1c7 cells. Inhibitory effects against the transformation and EROD activity were shown in Table 1 as a % of inhibition. Entry 3 and 4 showed strong inhibitory effects, whereas entry 5 and 7 had weak. Result from quantitative structure-activity relationship analysis showed that hydrophobicity of compound is important. Especially, hydrophobic functional group at C4, C4', and C4" positions strongly affect the inhibitory effects. Further study is needed to clarify whether the effective derivatives possess the inhibitory mechanism in the same manner as curcumin.

Acknowledgements
Part of this study was supported by Creation of Innovation Centers for Advanced Interdisciplinary Research Areas in Special Coordination Funds for Promoting Science and Technology Program by MEXT, Japan. The authors would like to thank Prof. Masaya Kawase, Nagahama Institute of Bio-science and Technology for QSAR analysis. We also appreciate Prof. Toshihiko Osawa, Aichi Gakuin University, Japan, and Assoc. Prof. Yoshiyuki Mizushina, Kobe-Gakuin University, Japan, for providing curcumin derivatives.

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