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Direct activation of Constitutive Androstane Receptor by Phthalates

Haishan LI
Chinese Academy of Inspection and Quarantine
Beijing, China
e-mail: lihs@ aqsiqch.ac.cn

Guolin SHEN
Chinese Academy of Inspection and Quarantine
Beijing, China
e-mail:shenguolin801129@163.com

Wenchao AI
Chinese Academy of Inspection and Quarantine
Beijing, China
e-mail :aiwc@aqsiqch.ac.cn

Wenping XIE
Chinese Academy of Inspection and Quarantine
Beijing, China
e-mail:xiewp@aqsiqch.ac.cn

Hui HAN
Chinese Academy of Inspection and Quarantine
Beijing, China
e-mail:hanhui2002@163.com

Huiming CHEN
Chinese Academy of Inspection and Quarantine
Beijing, China
e-mail:chenhm@aqsiqch.ac.cn

***Abstract*—Phthalates are esters of phthalic acid and are mainly used as plasticizers. It was reported that some phthalates activated constitutive androstane receptor (CAR), the major xenobiotic sensor and metabolism regulating nuclear receptor. Here, we compared the effects of CAR activation induced by 15 common used phthalates, and elucidated the species-specific CAR activation by phthalates based on in vitro-in vivo test.**

Keywords-phthalates; constitutive androstane receptor; activation

I. INTRODUCTION

The constitutive androstane receptor (CAR; NR1I3) is a hepatic transcription factor that controls the expression of numerous drug-metabolizing enzymes and transporters in response to xenobiotic exposures [1]. Compared with other nuclear receptors, the hallmark mechanisms of CAR activation lie in two different models: the direct ligand-binding, and the ligand-independent (indirect) pathway [2].

Although human CAR and its rodent orthologs share several common features, dramatic distinction exists amongst these receptors. For instance, TCPOBOP, the most potent mouse CAR ligand identified to date, cannot bind or activate either rat or human CAR [3]. This species-specific ligand profile relies on the sequence divergence in the ligand binding domains of the rodents and human receptors. In addition, significant difference might be observed between *in vitro* screening tests and *in vivo* animal tests, mostly due to complex metabolism in intact body.

Phthalates are esters of phthalic acid and are mainly used as plasticizers. Due to the ubiquity of plastics (and therefore plasticizers) in modern life, the vast majority of people are exposed to some level of phthalates. It was reported that di(2-ethylhexyl) phthalate (DEHP) activated human CAR [4]. However, no systemic *in vitro-in vivo* study was conducted in CAR activation by phthalates.

Here, we compared the effects of CAR activation induced by 15 common used phthalates, i.e. DEHP, butyl benzyl phthalate (BBP), di-n-butyl phthalate (DBP), diisodecyl phthalate (DIDP), diisononyl phthalate (DINP), di(n-octyl) phthalate (DNOP), dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-propyl phthalate (DPP), diisobutyl phthalate (DIBP), di-n-pentyl phthalate (DNPP), di-n-hexyl phthalate (DNHP), dicyclohexyl phthalate (DCP), diisooctyl phthalate (DIOP), and di-n-nonyl phthalate (DNP), and constructed mouse CAR knockout mice and human CAR transgenic mice model, therefore, the systemic *in vitro-in vivo* study elucidated the species-specific CAR activation by phthalates.

II. MATERIALS AND METHODS

A. Chemicals and Biological Reagents

CITCO was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Rifampicin (RIF), TCPOBOP and phthalates were from Sigma (St. Louis, MO). The Dual-Luciferase Reporter Assay System was purchased through Promega (Madison, WI). FuGENE® 6 transfection reagent was obtained from Roche (Basel, Switzerland). Other cell culture reagents were purchased from Invitrogen (Calsbad, CA) or Sigma-Aldrich.

B. Animals and Cell Culture

CAR knockout mice and human CAR transgenic mice were constructed according to reference [5]. About 7-week-old C57BL-6 male mice purchased from Vital River Laboratories (Beijing, China), were housed in controlled conditions (temperature, humidity, 12-hrs light/dark cycles). They were given free access to pellet food and tap water ad libitum, and quarantined for at least 1 week prior to study.

HepG2 cells (human hepatocellular carcinoma cell line) and A431 cells (human epidermoid carcinoma cell line) were

purchased from the American Type Culture Collection and were maintained as an inherent cell line in Eagel's minimal essential medium containing 10% (v/v) fetal bovine serum and penicillin/ streptomycin (100 units/ml and 0.1 mg/ml, respectively) at 37 ℃ in an incubator with 5% CO2 and atmospheric air.

C. Transactivation Assays in Cell Lines

HepG2 cells in 24-well plates were transfected with human CAR expression vector, and CYP2B6-2.2kb reporter construct using Fugene 6 Transfection Kit following the manufacturer's instruction. Twenty four hours after transfection, cells were treated with solvent (0.1% DMSO) or test compounds at indicated concentrations [6]. Twenty-four hours later, cell lysates were assayed for firefly activities normalized against the activities of cotransfected Renilla luciferase using Dual-Luciferase Kit. Data were represented as mean ±S.D. of three individual transfections.

D. Egfr Assays

A431 cells were seeded (2 ×105 cells per well) in 96-well plates and stabilized for 24 h. After this time, cells were pretreated for 1 h with serum-free Opti MEM medium, after which cells were treated with the tested compounds (at concentrations of 10 μM) and reference compounds CITCO (1 mM), erlotinib (10 mM, an EGFR inhibitor), as well as phenobarbital (an indirect CAR activator, 1 mM) for 24 h.

E. Animal Treatment

Mice were dosed daily by gavage with corn oil (vehicle), CITCO (20 mg/kg/d), or TCPOBOP (3 mg/kg/d) for 3 days, and livers were removed 24 h after the last dose. The ratios of liver weight and body weight were calculated.

III. RESULTS AND DISCUSSION

Results were summarized in table 1. In EGFR assays, which is one known indirect way of CAR activation, all tested phthalates showed no effect. This evidence supported direct activation of CAR for phthalates.

Compared with report from Zhang et al. [7], which used in vitro binding assay, our results based on in vitro reporter assays showed significant difference.

It was first report that some phthalates activated mouse CAR or human CAR in vivo test based on knockout and transgenic mouse model.

As expected, nine of 15 tested phthalates induced hepatic hyperplasia in wild type mouse. In contrast, only two phthalates, BBP and DEP, didn't induce hepatic hyperplasia in human transgenic CAR mouse. It is needed to measure the expression levels of the typical target genes to confirm the activation of CAR in mouse model.

TABLE I. SUMMARY OF IN VITRO-IN VIVO TEST FOR CAR ACTIVATION BY PHTHALATES

Test substance	In vitro test			In vivo test (Ratios of liver/body weight)		
	Reporter assay[a]	EGFR Assay[b]	Binding Assay[c]	Wild type	knockout	Human CAR transgenic
DEHP	2.55±0.55	NA	29.51	5.52±0.23**	4.63±0.15	5.33±0.18**
BBP	1.23±0.35	NA	0.28	5.34±0.34*	4.56±0.12	4.56±0.21
DBP	1.00±0.10	NA	0.35	5.09±0.27*	4.85±0.14*	5.45±0.15**
DIDP	1.04±0.40	NA	NA	4.66±0.13	4.24±0.09	5.19±0.22**
DINP	1.26±0.29	NA	NA	4.84±0.19	4.45±0.11	5.50±0.27**
DNOP	2.85±0.74	NA	NA	5.50±0.24**	4.21±0.10	5.01±0.20*
DMP	1.33±0.35	NA	11.75	5.07±0.21*	4.26±0.13	5.00±0.15*
DEP	0.97±0.08	NA	1.45	5.13±0.16*	4.40±0.16	4.44±0.14
DPP	1.21±0.05	NA	0.72	4.91±0.15	4.31±0.15	5.19±0.15**
DIBP	1.31±0.33	NA	0.83	4.98±0.17*	4.45±0.07	5.11±0.16**
DNPP	2.04±0.41	NA	1.41	5.44±0.23**	4.31±0.12	5.25±0.18**
DNHP	4.02±0.94	NA	NA	4.93±0.18	4.02±0.10	5.43±0.24**
DCP	3.26±0.47	NA	10.23	4.66±0.16	4.06±0.11	4.93±0.15**
DIOP	3.89±0.61	NA	NA	4.94±0.15*	4.34±0.09	5.77±0.30**
DNP	1.47±0.13	NA	NA	4.59±0.20	4.08±0.20	4.91±0.13*
Negative contro	1.00±0.02 DMSO	NA DMSO	NA	4.55±0.18 Corn oil	4.40±0.10 Corn oil	4.61±0.12 Corn oil
Positive control	23.20±5.08 CITCO	9.56μM Iressa	0.49 CITCO	5.51±0.26** TCPOBOP	4.37±0.12 TCPOBOP	5.69±0.21** CITCO

[a]Expressed as relative transactivation levels.
[b]Expressed as IC50 values for A431 cells growth.
[c]Expressed as REC10 values for binding, and data from reference [7].
*p<0.05
**p<0.01

IV. CONCLUSIONS

Some phthalates activated mouse CAR or human CAR, and showed direct activation way. In addition, significant species differences were observed for certain phthalates, such as BBP.

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