파라벤류와 트리글로산의 인체 안드로겐 수용체 매개 내분비계 교란작용

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Human Androgen Receptor-Mediated Endocrine Disrupting Potential of Parabens and Triclosan

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ABSTRACT – This study aimed to determine the human androgen receptor (AR)–mediated endocrine disrupting potential of parabens and triclosan in food and household products using a cell-based assay in the OECD TG No.458, the 22Rv1/MMTV_GR-KO transcriptional activation assay. Four parabens (methyl-, ethyl-, propyl-, and butyl-) are determined as AR antagonists in OECD TG No.458. However, their AR antagonistic effects were not exhibited in the presence of the S9 hepatic fraction. Triclosan is also classified as an AR antagonist, and the AR antagonistic effect induced by triclosan significantly decreased in the presence of the phase I + II S9 fraction. Regarding the mechanism of AR antagonism induced by parabens and triclosan, the AR-mediated endocrine disrupting effects were exhibited through suppressing the translocation of ligand-bound AR to the nucleus via blocking of AR dimerization in the cytosol. These results indicate that the four parabens and triclosan have AR-mediated endocrine disrupting potential through an AR antagonistic effect via inhibiting AR dimerization; however, their endocrine disrupting effects deceased in the presence of hepatic metabolic enzymes.

Keywords: Parabens, Triclosan, Androgen receptor, Antagonist, Hepatic metabolites

Parabens and triclosan are chemicals used as microbicides and preservatives in personal care and medical products. Parabens are the alkyl esters of p-hydroxybenzoic acid, and are added to food, pharmaceutical and personal care products as preservatives by their antimicrobial properties[1]. Triclosan is a chemical that has been added to a wide range of personal care and medical products, including toothpaste and soaps, for its anti-microbial properties[2]. In the case of parabens, approximately 8,000 tons are consumed annually, and the domestic SED level is 87.836 μg/kg bw/day[3]. For

triclosan, it has been estimated that globally, 1500 tonnes of triclosan are produced annually, and 132 million liters of triclosan-containing products are used annually in the US alone[4]. Lee's study found that the systemic exposure dose (SED) from the sum of representative cosmetic and oral care products of triclosan in Korea was 0.14337 mg/kg bw/day[5]. The maximum allowable concentration of parabens in cosmetics has been set at 0.4% for individual parabens and 0.8% for mixtures of parabens in Korea and the EU[3]. For triclosan, the maximum allowable concentration in cosmetics in cosmetics and mouthwashes is 0.3% in Korea, the US and the EU[4].

Parabens and triclosan exhibited estrogenic and antiandrogenic properties in *in vitro* model[5]. In animal studies, parabens induced reduction of sperm reserve and quality in the epididymis of male rats, and to be involved in the development of endometriosis and polycystic ovarian syndrome in females[6]. Triclosan has been reported to

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increase uterine weight in female rats and decrease sperm production and male accessory gland weight in male rats[7].

According to Centers for Disease Control and Prevention (CDC), elevated urinary levels of parabens and triclosan were found in samples from all age groups in the United States[8]. They are also included in the UN's list of endocrine disrupting chemicals (EDCs)[9]. EDC is defined as "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action" by the Endocrine Society statement[10]. EDCs are currently recognized to exert their effects by binding to nuclear hormone receptors or to membrane receptors, while additional modes of action have been identified, including oxidative stress, genetic susceptibility, and epigenetic modifications (e.g. DNA methylation)[11,12].

Since the late 1990s, the Organization for Economic Cooperation and Development (OECD) has been developing test guidelines (TG) for the endocrine disrupting potential of various chemicals that can affect humans through the environment and food[13]. The OECD has proposed a conceptual framework for testing and evaluating EDCs that consists of five levels. The second of these five levels have been used to identify the mechanisms or endocrine pathways affected by the collection of *in vitro* test data from chemicals. The androgen receptor transactivation (ARTA) assay using the 22Rv1/MMTV_GR-KO cell line has been approved by the performance-based testing guideline (PBTG) No. 458 for the identification of AR agonists and antagonists by 2020[14].

One of the limitations of *in vitro* testing is metabolic capacity. The OECD has published a review of metabolizing systems for use with *in vitro* assays to address this[15]. Validation of protocols for ER and AR *in vitro* assays using S9 fractions, the supernatant of liver tissue homogenate containing microsomal and cytosolic components, is under development. A variety of liver fractions can be used to perform metabolic studies. Since the S9 fraction contains both microsomal and cytosolic components, it has the great advantage of mimicking both phase I and phase II metabolism *in vitro*[16].

When xenobiotics such as EDC enter the body, they are eliminated through phase I and phase II metabolism. Through oxidation (via cytochrome P450), reduction and hydrolysis reactions, phase I reactions transform a parent drug into more polar (water-soluble) active metabolites. And phase II reactions use glucuronidation, acetylation, and sulfation to transform a parent drug into more polar (water-soluble) inactive metabolites[17].

Metabolic processes must therefore be considered when studying chemicals. A disadvantage of the S9 fraction compared to microsomal preparations is the internal dilution of the enzyme. However, this can be easily corrected by adjusting the protein level. They require exogenous cofactors such as the β-nicotinamide adenine dinucleotide phosphate regeneration system (NADPH; phase I oxidation), uridine 5'-diphospho-α-D-glucuronic acid (UDPGA; phase II glucuronidation) and glutathione (GSH), and 3'-phosphoadenosine-5'-phosphosulfate (PAPS; phase II sulfation) for activity[18]. In addition, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD) and magnesium chloride (MgCl2) support the NADPH generation system by mediating the reduction of NADP to NADPH[18].

The purpose of this study is to provide evidence for the AR-mediated endocrine disrupting potential of parabens, triclosan and their metabolites according to OECD PBTG No. 458 with the hepatic S9 fraction using the 22Rv1/MMTV_GR-KO cell line.

Materials and methods

Chemicals
Methylparaben, Ethylparaben, Propylparaben, and Butylparaben were purchased from Tokyo Chemical Industry CO., Ltd (Tokyo, Japan). Triclosan was purchased from Sigma-Aldrich (St. Louis, MO, USA). 5α-Dihydrotestosterone (DHT) was purchased from Tokyo Chemical Industry CO., Ltd (Tokyo, Japan) for ARTA agonist assays and AR dimerization assays. Stanozolol (STZ) was purchased from Tokyo Chemical Industry CO., Ltd for ARTA antagonist assays. Bicalutamide (BIC) was purchased from Tokyo Chemical Industry CO., Ltd for AR antagonist control in AR TA antagonist assays. All chemical stock solutions were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

Cell line
The 22Rv1/MMTV_GR-KO cells which were developed in our previous study were cultivated in RPMI 1640 (WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA), 2 mM GlutaMAX[TM] (Thermo Fisher Scientific, Franklin, MA, USA) and 1% Antibiotic-Antibiotic (Thermo Fisher Scientific). The HEK293 cell line stably transfected with AR tagged with Nano-luciferase (NL; Promega, Madison, WI, USA) and HaloTag protein (HL; Promega), as established in a previous study, was used to evaluate the AR homo-dimerization assay[19]. These cells were cultivated in EMEM (WelGENE) supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin (P/S; Thermo Fisher Scientific, MA, USA), 25 μg/mL hygromycin B (InvivoGen, San Diego, CA, USA) and 400 μg/mL G418 (InvivoGen).

Table 1. Composition of S9 mixture

Reagent	S9 mixture Concentration	Inactive S9 mixture (μL)	Phase I S9 mixture (μL)	Phase I+II S9 mixture (μL)
Rat liver S9	0.1 mg/mL	5	5	5
NADPH	0.002 M	-	20	20
G-6-P	0.03 M	-	20	20
MgCl$_2$	0.05 M	-	20	20
G-6-PD	3 units/mL	-	20	20
GSH	0.02 M	-	-	125
UDPGA	0.005 M	-	-	20
PAPS	2×10^{-5} M	-	-	20
Test medium	-	995	915	750
Total volume		1,000	1,000	1,000

ARTA assay using the 22Rv1/MMTV_GR-KO cell line with rat liver S9 fraction

The protocol for maintaining 22Rv1/MMTV_GR-KO cells was based on OECD PBTG NO. 458. Cells were seeded and pre-incubated in a 5% CO$_2$ at 37°C for 48 h before the chemical exposure. After replacement with media containing chemicals without (agonist assay) or with (antagonist assay) STZ,, the treated plates were incubated in a 5% CO$_2$ at 37°C for 20-24 h.

While the AR agonistic/antagonistic effects of chemicals and their metabolites were investigated using the rat liver S9 fraction (Sigma-Aldrich). The protocol was adapted from previous studies with minor modifications[20]. Briefly, before chemical treatment in conventional AR TA assay, an S9 mixture was pre-incubated with chemicals for 6 h. The composition of S9 mixture is provided in Table 1.

BRET-based human androgen receptor dimerization assay

To confirm AR antagonistic activity by suppressing cytoplasmic homo-dimerization of AR, bioluminescence resonance energy transfer (BRET) assays were performed using the AR-stably transfected HEK293 cell line developed in a previous study[19]. HEK293 cells were treated with chemicals to confirm ligand-mediated AR homo-dimerization through BRET protein-protein interactions. The protocol was adapted from previous studies[19].

Data analysis

The results for each chemical run in each assay were classified as positive/negative according to the OECD TG No. 458 decision criteria for classification. Briefly, for the chemical to be classified as AR agonist, it should elicit a response equal to at least 10% of the maximum response of the reference DHT. For classification as an AR antagonist, the chemical should be able to inhibit the response to STZ by at least 30% (IC_{30}). All data represent the average of three wells in each experiment and are expressed as the mean±standard error of the mean (SEM).

Results and Discussion

To investigate whether parabens and triclosan can potentially disrupt the endocrine system by interfering with the genetic pathways involving the human androgen receptor (AR), we conducted laboratory tests using two methods: the androgen receptor transcriptional activation assay (using the 22Rv1/MMTV_GRKO cell line) and the androgen receptor homo-dimerization assay (using the HEK-293 AR cell line). In addition, we applied the metabolic process in the body using the "rat liver S9 fraction" to assess the potential for endocrine disruption by metabolites.

AR agonistic/antagonistic effect of chemicals

In the agonist assay, all chemicals were not found AR agonistic effectsby OECD TG No.458, 22Rv1/MMTV_GR-KO ARTA assay (data not shown). On the other hand, four parabens were determined as AR antagonist with IC_{50} values of 2.11×10^{-5}, 2.07×10^{-5}, 1.24×10^{-5}, and 4.61×10^{-6}, respectively (Table 2, and Fig. 1). Also, triclosan exhibited AR antagonistic effects with IC_{50} value of 1.31×10^{-6} M (Table 2, and Fig. 1).

Table 2. AR antagonistic effect of chemicals

Chemicals	IC_{30} value (M)	IC_{50} value (M)
Methylparaben	7.43×10^{-6}	2.11×10^{-5}
Ethylparaben	6.87×10^{-6}	2.07×10^{-5}
Propylparaben	3.85×10^{-6}	1.24×10^{-5}
Butylparaben	2.54×10^{-6}	4.61×10^{-6}
Triclosan	4.66×10^{-7}	1.31×10^{-6}
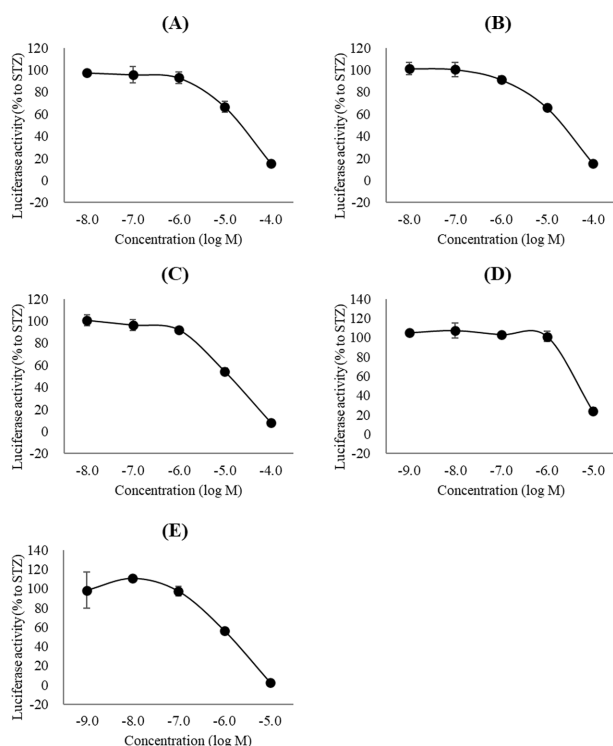
Fig. 1. AR antagonistic effects of parabens and triclosan in AR TA assay using 22Rv1/MMTV_GR-KO cell line. (**A**) Methylparaben, (**B**) Ethylparaben, (**C**) Propylparaben, (**D**) Butylparaben, (**E**) Triclosan. Luciferase activities are expressed at the % of the activity for 1 nM STZ. Shown are mean values±SD.

Phase I and II hepatic metabolism of chemicals on ARTA

None of the 4 parabens showed an antagonistic effect in the presence of the S9 fraction, regardless of the presence of any cofactors. The decreased efficacy of parabens is not due to their undergoing hepatic metabolism during phase I or II. It seems that parabens are metabolized by esterase and aromatase, which is present in the S9 fraction and does not require cofactors[20-22]. It is therefore reasonable to assume that inhibition of esterase and/or aromatase eliminates the anti-androgenic effect.

In case of triclosan, it had reduced inhibitory activity in phase I+II metabolites induced by the S9 fraction. The results of the experiments performed without the S9 fraction were found to be identical to the control experiments performed with the S9 fraction but without any cofactors. This suggests that the observed changes in potency are due to the metabolic process rather than the binding of the test compounds to the S9 proteins. This decrease in anti-androgenic activity is probably due to the fact that most *in vitro* compounds are excreted as hydrophilic metabolites that can be easily eliminated by phase I and phase II metabolism[23]. It is important to note that structurally related

Table 3. Variations in AR antagonistic effects by Phase I and II metabolism in ARTA assay

Chemicals	IC$_{30}$[a] value (M)		
	S9 Inactive	S9 Phase I	S9 Phase I+II
Methylparaben	-	-	-
Ethylparaben	-	-	-
Propylparaben	-	-	-
Butylparaben	-	-	-
Triclosan	3.30×10^{-7}	3.21×10^{-7}	-
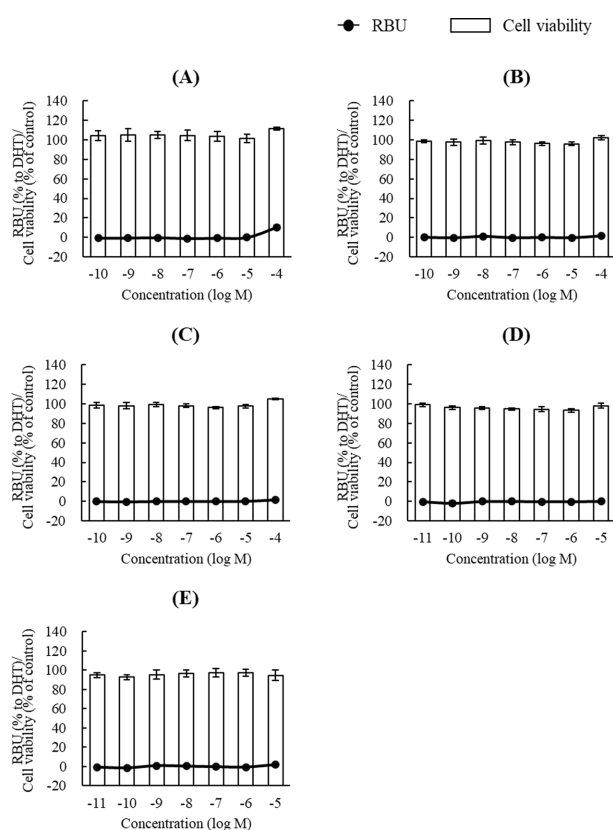
Fig. 2. AR dimerization affinities of antagonists in BRET-based AR dimerization assay using HEK293-AR cell line. (**A**) Methylparaben, (**B**) Ethylparaben, (**C**) Propylparaben, (**D**) Butylparaben, (**E**) Triclosan. RBU is expressed at the % of the activity for 10 nM DHT. Shown are mean values ±SD. RBU: relative BRET units.

substances, including endogenous steroids and EDCs, undergo extensive metabolism by phase I and II enzymes in the liver. These metabolic processes may result in activation, inactivation or altered activity of the compounds[15].

The absence of metabolism in *in vitro* endocrine activity assays may lead to false positive or false negative results. It is therefore essential to incorporate metabolism into *in vitro* endocrine activity assays, for example by using subcellular enzyme fractions. Evaluating the metabolic activity of EDCs

using the liver S9 fraction system would be a valuable approach.

BRET-based AR dimerization assay of the chemicals

The chemical-AR homodimerization assay was performed to determine which of the genetic pathways mediated by the androgen receptor (AR) showed impaired androgen activity. The assay was performed for parent chemicals only, not for metabolites. The 10 nM DHT was used as a positive control. All parabens and triclosan showed a negative response. This indicates that these AR antagonists had an antagonistic effect. AR didn't dimerize with these chemicals, so they prevent translocation to the nucleus and inhibit transcriptional activity. These pathways are important mechanisms of endocrine disruption[24].

In this study, we tried to assess the AR-mediated endocrine disrupting potentials of parabens and triclosan, and their hepatic metabolites in *in vitro* system. However, th there are no *in vivo* or *in vitro* international standard test guideline methods, to identify the potential metabolic disrupting effects of chemicals. Therefore, the potential metabolic disrupting effects of parabens and triclosan should be clarify by validated *in vitro* system or animal study.

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국문요약

본 연구는 OECD TG No. 458, 22Rv1/MMTV_GR-KO 전사 활성화 분석법을 포함한 세포 기반 분석법을 사용하여 식품 및 생활용품에 포함된 파라벤과 트리클로산의 인간 안드로겐 수용체를 매개하는 내분비계 교란 가능성을 확인하는 것을 목표로 한다. 4가지 파라벤(메틸-, 에틸-, 프로필-, 부틸-)은 OECD TG No.458에서 AR 길항제로 확인된 반면, 파라벤의 AR 길항 효과는 S9 간 분획물이 있는 경우 나타나지 않았다. 트리클로산 역시 AR 길항제로 분류되었으며, 트리클로산에 의해 유도된 AR 길항 효과는 S9 간 분획물이 존재할 때 제 1상+2상 대사에서 유의하게 감소되었다. 파라벤과 트리클로산에 의해 유도되는 AR 길항 기전은 세포질 내 AR 이량화를 차단하여, 리간드 결합 AR이 핵으로의 전위를 억제함으로써 AR 매개 내분비 교란 효과를 나타냈다. 이러한 결과는 4가지 파라벤과 트리클로산이 AR 이량화 저해를 통한 AR 길항 효과를 나타내는 AR 매개 내분비 교란 가능성을 가지고 있으나, 간 대사 효소가 존재할 경우 내분비 교란 효과는 감소됨을 시사한다.

Conflict of interests

The authors declare no potential conflict of interest.

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