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Comparative study of the effect of 17 parabens on PXR-, CAR- and PPARα-mediated transcriptional activation

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ABSTRACT

Parabens are widely used as preservatives in personal care products, medicines and foods, resulting in substantial human exposures, even though some harmful effects, such as endocrine-disrupting activity, have been reported. Pregnane X receptor (PXR), constitutive androstane receptor (CAR) and peroxisome proliferator-activated receptor α (PPARα), which are members of the nuclear receptor superfamily, regulate the metabolism of endogenous substrates including hormones. Therefore, we hypothesized that parabens may alter hormone-metabolizing activities by acting on these receptors, and such changes could contribute to the endocrine-disrupting activity. To test this idea, we systematically examined the effects of 17 parabens on these receptors using reporter gene assays. Nine parabens significantly activated human and rat PXR. Parabens with C2–C5 (linear and branched) side chains were most active. Butylparaben and isobutylparaben also significantly activated rat CAR. We found that long-side-chain (C7–C12) parabens showed up to 2-fold activation of PPARα at 10 μM. Furthermore, pentylparaben and hexylparaben showed rat PXR antagonistic activity and rat CAR inverse agonistic activity. The activity of butylparaben towards PXR and CAR was lost after carboxylesterase-mediated metabolism. These findings confirm that parabens influence the activities of PXR, CAR and PPARα, and thus have the potential to contribute to endocrine disruption by altering hormone metabolism.

1. Introduction

Parabens, a homologous series of alcohol esters of 4-hydroxybenzoic acid, have been widely used as preservatives in personal care products, medicines and foods (Soni et al., 2005; Liao and Kannan, 2014). The side chain moiety of parabens influences their properties, and longer-alkyl-chain parabens have more potent antimicrobial activities (Dymicky and Huhtanen, 1979). Humans are routinely exposed to these substances orally or via the skin, but some parabens have been reported to cause adverse effects in mammals. Prenatal and postnatal exposure to butylparaben induced neuro-developmental disorders in the pups of rats (Ali and Elgoly, 2013). Methylparaben promoted UV-induced damage of human skin keratinocytes (Handa et al., 2006). Furthermore, some reports have suggested potential estrogenic activities (Routledge et al., 1998; Okubo et al., 2001; Byford et al., 2002; Lemini et al., 2003; Gomez et al., 2005; Vo et al., 2010; Watanabe et al., 2013) or effects on thyroid hormone levels (Koeppe et al., 2013) in humans. The estrogenic activities of parabens may be associated with the development of human breast cancer (Darbre and Harvey, 2014), and indeed, parabens were detected in human breast tumor tissue (Darbre et al., 2004; Barr et al., 2012).

Hormone receptors such as estrogen receptor (ER) and thyroid hormone receptor are considered to mediate the reported endocrine disrupting effects of parabens, but other mechanisms, such as altered

Abbreviations: BNPP, bis(4-nitrophenyl) phosphate; BZF, bezafibrate; CAR, constitutive androstane receptor; CYP, cytochrome P450; ER, estrogen receptor; 4-HBA, 4-hydroxybenzoic acid; PCN, 5-pregnen-3β-ol-20-one-16α-carbonitrile; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; REC_{20}, 20% relative effective concentration; RIC_{20}, 20% relative inhibitory concentration; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase

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metabolism of hormones, could also play a role. ER and thyroid hormone receptor are members of the nuclear receptor superfamily, which includes other receptors that are involved in hormone metabolism, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR) and peroxisome proliferator-activated receptor (PPAR)α. PXR, CAR and PPARα regulate the expression of drug-metabolizing enzymes such as cytochrome P450s (CYPs), UDP-glucuronosyltransferases (UGTs), glutathione *S*-transferases and sulfotransferases (SULTs) (Kroetz et al., 1998; Kast et al., 2002; Maglich et al., 2002; Omiecinski et al., 2011; Bigo et al., 2013; Kodama and Negishi, 2013). These enzymes play key roles in the metabolism of endogenous substrates, as well as xenobiotics such as drugs and environmental chemicals. For example, CYP3A4, mainly induced by PXR, was reported to mediate 2- and 4-hydroxylation of estradiol (Yu et al., 2005). CAR activation induces UGTs and SULTs, leading to reduced serum thyroid hormone levels, probably due to conjugation of thyroid hormone, in mice (Maglich et al., 2004; Qatanani et al., 2005). PPARα induces gene expression of 17β-hydroxysteroid dehydrogenase type IV in the liver, and thus contributes to the decrease in serum estradiol levels by increasing the conversion of estradiol to estrone in rats and mice (Fan et al., 1998). Therefore, we hypothesized that parabens may alter hormone-metabolizing activities by acting on PXR, CAR and PPARα, and such alterations could contribute to the endocrine disrupting activity. Nevertheless, the effects of parabens on PXR, CAR and PPARα have not yet been systematically examined.

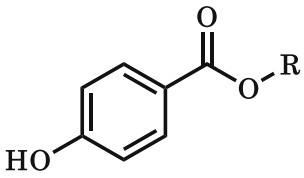
Parabens are mainly metabolized to 4-hydroxybenzoic acid (4-HBA) and alcohols by carboxylesterase in human and rodent (Imai et al., 2006; Jewell et al., 2007a,b; Aubert et al., 2012; Ozaki et al., 2013). The resulting 4-HBA is then conjugated with glucuronic acid, sulfonic acid or glycine (Tsukamoto and Terada, 1960, 1962; 1964; Abbas et al., 2010). It is considered that the toxicity, such as endocrine-disrupting potential, of parabens is decreased by metabolism; for example, Watanabe et al. (2013) reported that the estrogenic activity of butylparaben was decreased after incubation with rat liver microsomes.

In this study, we set out to test our hypothesis that parabens may alter hormone-metabolizing activities via PXR, CAR and PPARα by systematically examining the effects of 17 parabens on human/rat PXR (h/rPXR), rat CAR (rCAR) and rat PPARα (rPPARα). In addition, we investigated the effects of metabolism by rat liver microsomes on the activities of butylparaben towards these receptors. For most of this work, we used rat nuclear receptors, but we also examined human PXR because there are species differences in PXR ligand binding. Our findings confirm that parabens influence the activities of PXR, CAR and PPARα, and thus have the potential to contribute to endocrine disruption by altering hormone metabolism.

2. Materials and methods

2.1. Chemicals

The structure, source and purity of each of the 17 parabens and an authentic sample of their metabolite, 4-HBA, tested in the present study are shown in Fig. 1 and Table 1. Undecylparaben and dodecylparaben were synthesized as reported previously (Fujino et al., 2014). Rifampicin (for biochemistry), bezafibrate (BZF; > 99.3% pure) and Infinity pure dimethyl sulfoxide (DMSO; > 99.5% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5-Pregnen-3β-ol-20-one-16α-carbonitrile (PCN; > 97% pure) and bis(4-nitrophenyl) phosphate (BNPP; > 97% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Artemisinin (> 97% pure) was purchased from Tokyo Chemical Industry Co. Ltd. (TCI; Tokyo, Japan). DMSO was used as a vehicle, and all test compounds used were dissolved in DMSO at a concentration of 30 mM. All compounds were diluted to predetermined concentrations in the appropriate medium immediately before use.



Parabens

Linear alkyl side chain group

R: C_nH_{2n+1} (n=1-12)

methylparaben, ethylparaben, propylparaben, butylparaben, pentylparaben, hexylparaben, heptylparaben, octylparaben, nonylparaben, decylparaben, undecylparaben, dodecylparaben

Branched alkyl side chain group

R:

isopropylparaben isobutylparaben isopentylparaben

Aromatic side chain group

R:

phenylparaben benzylparaben

Fig. 1. Chemical structures of the parabens examined.

Table 1
Source, purity and side chain length of 17 parabens and their metabolite used in this study.

Compound	Source	Purity (%)	Side chain length
methylparaben	Wako	> 99.0	C_1
ethylparaben	Wako	> 99.0	C_2
propylparaben	Wako	> 95.0	C_3
butylparaben	Wako	> 98.0	C_4
pentylparaben	TCI	> 98.0	C_5
hexylparaben	TCI	> 98.0	C_6
heptylparaben	TCI	> 98.0	C_7
octylparaben	FL. Inc.	> 98.0	C_8
nonylparaben	TCI	> 98.0	C_9
decylparaben	This study	> 98.0	C_{10}
undecylparaben	This study	> 98.0	C_{11}
dodecylparaben	TCI	> 98.0	C_{12}
isopropylparaben	Alfa Aesar	> 98.0	C_3 (iso)
isobutylparaben	TCI	> 99.0	C_4 (iso)
isopentylparaben	TCI	> 98.0	C_5 (iso)
phenylparaben	TCI	> 99.0	C_4 (phenyl)
benzylparaben	TCI	> 98.0	C_5 (benzyl)
4-hydroxybenzoic acid	Wako	> 95.0	C_0

Wako; Wako Pure Chemical Industries, Ltd., (Osaka, Japan), TCI; Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan), FL, Inc.; Frinton Laboratories, Inc. (California, USA), Alfa Aesar; Alfa Aesar – A Johnson Matthey Company (Massachusetts, USA).

2.2. Rat PXR, CAR and PPARα reporter gene assays

Rat PXR, CAR and PPARα reporter gene assays were performed as reported previously (Fujino et al., 2016). *Renilla* luciferase activity (thymidine kinase activity) was measured to check the cytotoxicity of the test chemicals in terms of transcriptional activity.

2.3. Human PXR reporter gene assay

Human PXR reporter gene assay was performed as reported, with some modifications (Kojima et al., 2011, 2013, 2016). β-Galactosidase activity was measured to check the cytotoxicity of the test chemicals in

terms of transcriptional activity.

2.4. Animals

Male Sprague Dawley rats (Slc:SD, 210–230 g, Japan SLC, Shizuoka, Japan) were used. The animals were housed at 22 °C and a relative humidity of 55% with a 12-hr light/dark cycle, with free access to tap water and a standard pellet diet MM-3 (Funabashi Farm, Funabashi, Japan). All experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" of Nihon Pharmaceutical University.

2.5. Metabolism of butylparaben by rat liver microsomes

Rat liver microsomes were prepared as described elsewhere (Kitamura et al., 2003). Butylparaben (1 μmol) was incubated with rat liver microsomes (5 mg protein) and an NADPH-generating system (1 μmol of NADPH, 5 μmol of D-glucose-6-phosphate disodium salt and 1 unit of glucose-6-phosphate dehydrogenase) in the presence or absence of BNPP (1 μmol). The final volume of the incubation mixture was 10 ml in 0.1 M K,Na-phosphate buffer (pH 7.4). Rat microsomes boiled for 10 min at 90 °C were used as the control. Incubation was continued for 20 min at 37 °C. After incubation, the mixture was extracted with 25 ml of ethyl acetate. The extract was evaporated to dryness and the residue was dissolved in DMSO at a concentration of 30 mM, determined as initial concentration of unchanged form (the residue contains remaining unchanged form and formed metabolites). The extract samples were used to examine the effects of metabolism on the activities towards PXR, CAR and PPARα. The assay method was the same as described above.

2.6. Statistical analysis

An analysis of variance (ANOVA) followed by Bonferroni correction was used to evaluate the differences in transcriptional levels between experimental and control (0.1% DMSO alone) groups or among experimental groups.

3. Results

3.1. Effects of 17 parabens on hPXR-, rPXR-, rCAR- and rPPARα-mediated transcription

The effects of 17 parabens (12 linear alkyl, 3 branched alkyl and 2 aromatic parabens) on the transcriptional activities of hPXR (Fig. 2A), rPXR (Fig. 2B), rCAR (Fig. 2C) and rPPARα (Fig. 2D) were investigated using gene reporter assays. Rifampicin, a positive control for hPXR, enhanced luciferase activity 4.5-fold compared to DMSO-treated cells (control) at the concentration of 10 μM (Fig. 2A). Nine parabens significantly increased the activation of hPXR, and butylparaben and isopentylparaben showed more than 2-fold increases compared with the control at the concentration of 30 μM (Fig. 2A). 4-HBA which is the common metabolite of the parabens, showed only weak hPXR activation (not significant).

PCN, a positive control for rPXR, enhanced luciferase activity 2.7-fold compared to the control at the concentration of 1 μM (Fig. 2B). Nine of the 17 parabens also showed significant rPXR activation (Fig. 2B), but they were not all the same as in the case of hPXR – there was a species difference between human and rat. Ethyl-, butyl-, decyl- and isobutylparaben showed 2-fold or greater enhancement at the highest concentration (10 or 30 μM). Interestingly, pentylparaben and hexylparaben enhanced the activity at lower concentrations, but significantly decreased it at higher concentration (30 μM). 4-HBA showed no effect on rPXR in this assay (Fig. 2B).

Artemisinin, a positive control for rCAR, enhanced luciferase activity 1.9-fold compared to control at the concentration of 30 μM (Fig. 2C). Butyl-, isobutyl-, isopentyl- and phenylparaben showed significant activation, and butyl-, isobutyl- and phenylparaben were as potent as the positive control (artemisinin). Hexylparaben significantly decreased the activity at the concentration of 30 μM. 4-HBA had no effect on rCAR-mediated transcription (Fig. 2C).

BZF, a positive control for rPPARα, enhanced luciferase activity 2.9-fold compared to the control at the concentration of 30 μM (Fig. 2D). Fourteen of the 17 parabens increased rPPARα-mediated gene transcription. In particular, long-side-chain (C7–C12 linear alkyl chain) parabens showed up to 2-fold activation at 10 μM, but could not be tested at higher concentration, as they were cytotoxic at 30 μM (data not shown). Furthermore, the activities of pentyl-, heptyl- and isopentylparaben enhanced the activity in a dose-dependent manner (Fig. 2D). 4-HBA had no effect on rPPARα (Fig. 2D).

In this study, even though PXR, CAR and PPARα activation by hexyl-, heptyl-, octyl-, nonyl-, decyl-, undecyl- and dodecylparaben was observed, these parabens proved to be cytotoxic at the concentration of 30 μM, based on measurements of β-galactosidase or thymidine kinase activity (data not shown). Thus, we could not determine the PXR, CAR and PPARα activities of these parabens at 30 μM.

3.2. The effects of pentylparaben, hexylparaben and heptylparaben on rPXR and rCAR-mediated transcription

As some parabens appeared to show antagonistic and inverse agonistic activities towards rPXR and rCAR, respectively, at high concentration, we examined their effects in more detail. PXR antagonistic effects were examined in the presence of a positive control for rPXR, PCN (0.3 μM), and CAR inverse agonistic activity was examined in the presence of a positive control for rCAR, artemisinin (10 μM). Pentylparaben tended to suppress the rPXR activity of 0.3 μM PCN at 30 μM. Hexylparaben significantly suppressed the activity of PCN at 10–30 μM (Fig. 3A). Hexylparaben also increased rCAR activation additively with artemisinin at the concentration of 1 μM, but significantly suppressed the activity of artemisinin at 30 μM (Fig. 3B). Pentylparaben additively enhanced rCAR activation by 10 μM artemisinin in the concentration range of 3–10 μM, but tended to suppress the activity at 30 μM. Heptylparaben had no significant effect on rPXR, and its effect on rCAR was not examined due to cytotoxicity at high concentration (data not shown).

3.3. Effect of rat liver microsomal metabolism of butylparaben on its activities towards PXR, CAR and PPARα

Butylparaben showed PXR, CAR and PPARα transcriptional activation (Fig. 2). Since butylparaben is readily hydrolyzed upon incubation with rat liver microsomes (Ozaki et al., 2013), we examined the effects of metabolism on its activities towards PXR, CAR and PPARα. The rPXR and rCAR activities of butylparaben were lost upon incubation with rat liver microsomes. The loss of activity was blocked by BNPP, which is a carboxylesterase inhibitor. Boiled microsomes had no effect on the activities. On the other hand, PPARα activation by butylparaben was not abrogated by incubation with microsomes or BNPP (Fig. 4).

3.4. Relative activities of parabens towards PXR, CAR and PPARα

To assess the relative activities of parabens towards PXR, CAR and PPARα, we calculated the 20% relative effective concentration (REC_{20}) and 20% relative inhibitory concentration (RIC_{20}). The values of REC_{20} and RIC_{20} for PXR, CAR and PPARα activities of parabens and their metabolite are summarized in Table 2. Eight parabens showed more than 20% of the activity of 10 μM rifampicin towards hPXR. The REC_{20} values for hPXR were in the range of 6.7–28 μM. The order of hPXR activation in terms of REC_{20} was isopentyl- ~ pentyl- > isobutyl- > butyl- > nonyl- > ethyl- ~ isopropyl- ~ propylparaben. Eleven parabens showed more than 20% of the rPXR activity of 1 μM PCN. The

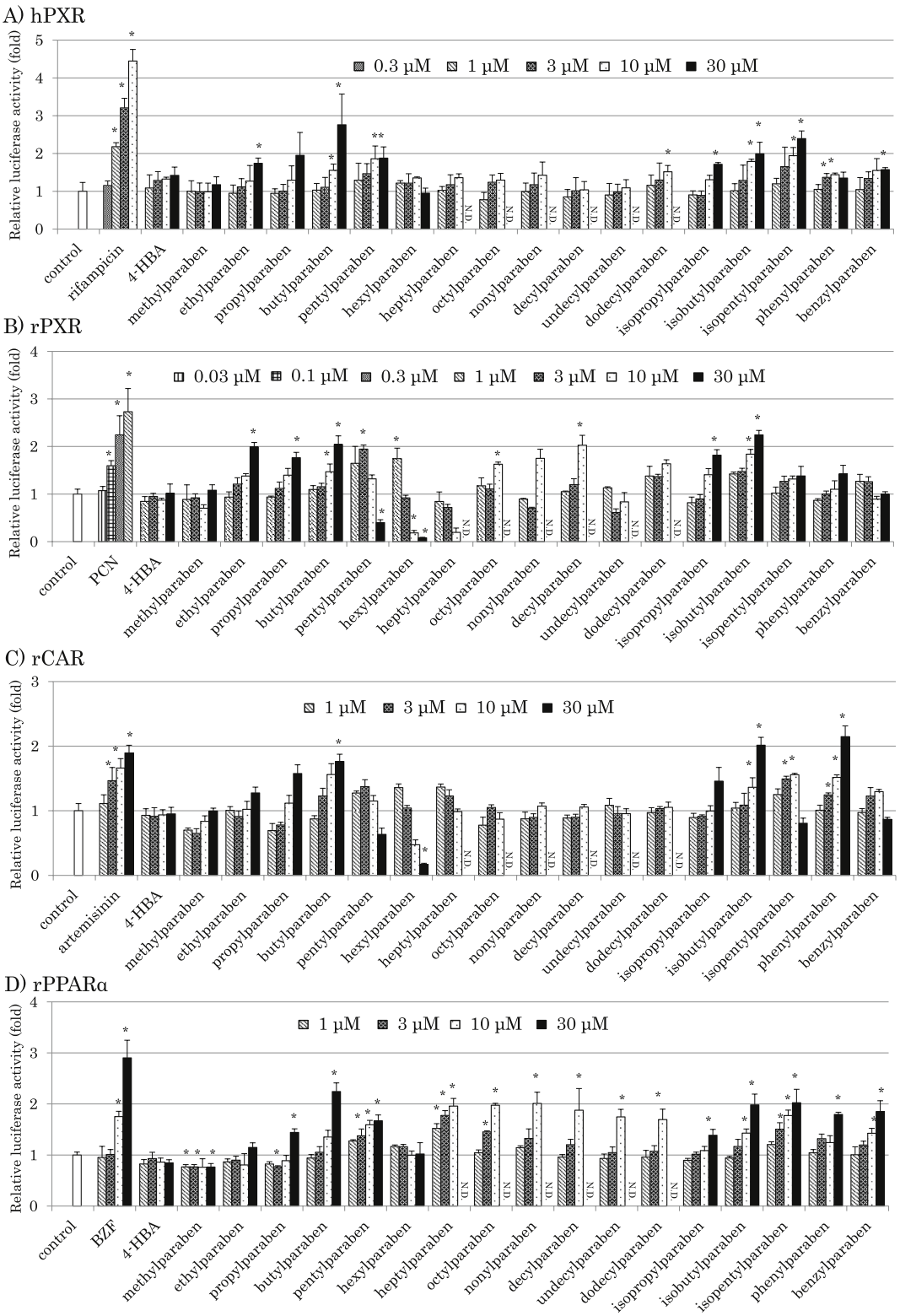


Fig. 2. Activities of parabens towards hPXR (A), rPXR (B), rCAR (C) and rPPARα (D). The PXR, CAR and PPARα activities of parabens are shown as relative activity (fold) with respect to the vehicle control. Each value represents the mean ± SD of 3 experiments. *$p < 0.05$ indicates a significant difference from the appropriate vehicle control. N.D.: not determined, PCN: 5-pregnen-3β-ol-20-one-16α-carbonitrile, BZF: bezafibrate, 4-HBA: 4-hydroxybenzoic acid.

REC_{20} values of rPXR were in the range of 0.90–20 μM, and isobutylparaben showed the lowest value (0.90 μM). Parabens with a C2–C4 alkyl side chain (linear or branched) and phenylparaben showed more than 20% of the rCAR activity of 30 μM artemisinin. The REC_{20} values of butylparaben and phenylparaben were similar to that of artemisinin. Heptylparaben showed the lowest REC_{20} value for rPPARα agonistic activity (0.52 μM), followed by isopentyl-, octyl-, pentyl-, nonyl-, decyl- and undecylparaben (2.2 μM, 2.5 μM, 2.9 μM, 3.2 μM, 4.4 μM and 5.4 μM, respectively). These seven parabens showed lower values of REC_{20} than the positive control (BZF). Three parabens showed

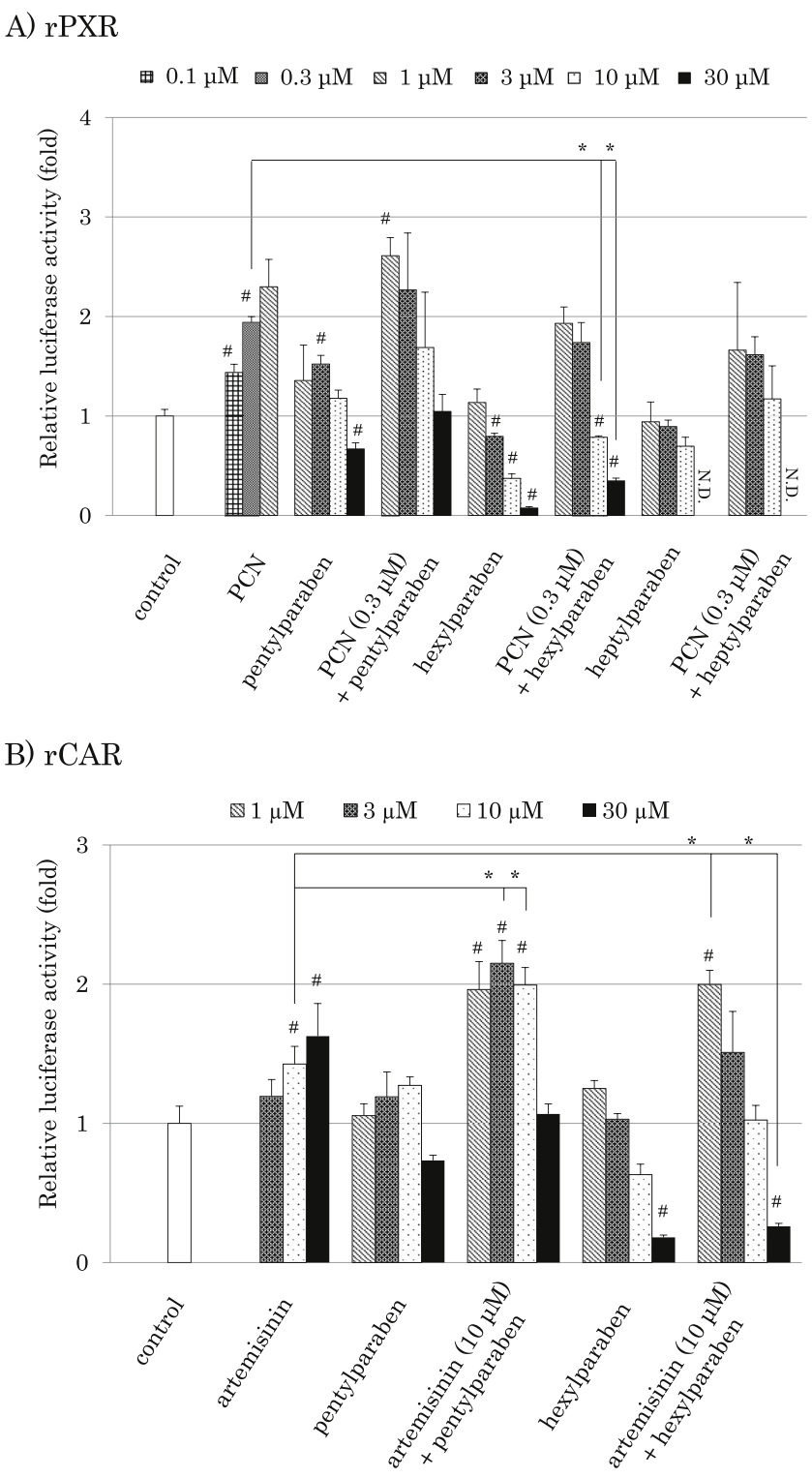


Fig. 3. Effects of pentylparaben, hexylparaben and heptylparaben on rPXR (A) and rCAR (B) activity in the absence or presence of the positive control. The rPXR activities of pentylparaben, hexylparaben and heptylparaben in the absence or presence of 0.3 μM PCN are shown as relative activity (fold) with respect to the vehicle control. The rCAR activities of pentylparaben and hexylparaben in the absence or presence of 10 μM artemisinin are shown as relative activity (fold) with respect to the vehicle control. Each value represents the mean ± SD of 3 experiments. $^{\#}p < 0.05$ indicates a significant difference from the appropriate vehicle control. $^{*}p < 0.05$ indicates a significant difference from the positive control (0.3 μM PCN or 10 μM artemisinin). N.D.: not determined, PCN: 5-pregnen-3β-ol-20-one-16α-carbonitrile.

rPXR antagonistic activity, with less than 80% of the activity of the control (0.1% DMSO). Heptylparaben showed the lowest RIC_{20} value for rPXR antagonistic activity (3.4 μM), followed by hexyl- and pentylparaben (4.6 μM and 22 μM, respectively). Pentyl- and hexylparaben showed rCAR inverse agonistic activity, although heptylparabenwas was cytotoxic in the relevant concentration range. Hexylparaben showed a lower RIC_{20} value for rCAR inverse agonistic activity than pentylparaben (4.8 μM and 21 μM, respectively).

4. Discussion

We investigated the effects of 17 parabens and a metabolite on h/rPXR, rCAR and rPPARα in detail, based on our hypothesis that parabens may alter hormone-metabolizing activities by acting on these receptors, and such changes could contribute to the endocrine disrupting activity, in addition to the direct effects on hormone receptors. We found that the PXR, CAR and PPARα activities of 17 parabens and 4-HBA showed dependence on the side-chain structure (Fig. 2). The relationship between the structure and estrogenic activity of 17 parabens

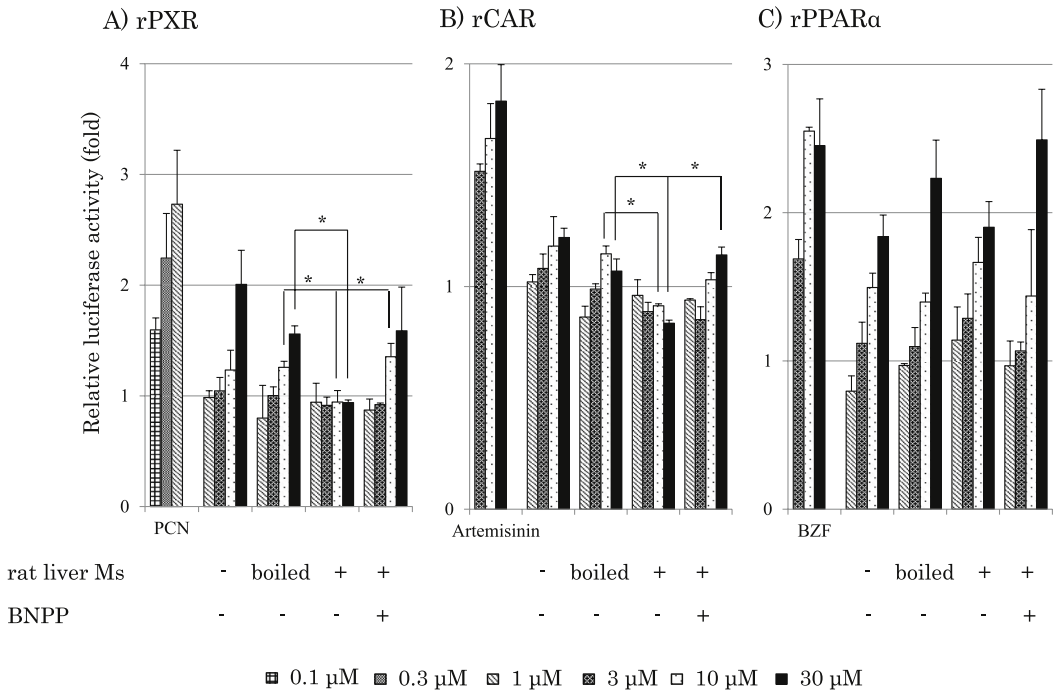


Fig. 4. Rat PXR (A), CAR (B) and PPARα (C) activation by butylparaben after incubation with or without native rat liver microsomes or boiled rat liver microsomes, and in the absence or presence of the inhibitor BNPP. Butylparaben was incubated with or without native rat liver microsomes or boiled rat liver microsomes, and in the absence or presence of BNPP in the presence of NADPH, and the extract of the incubation mixtures was assayed. Activities were expressed as relative activity (fold) with respect to the vehicle control. Each value represents the mean ± SD of 3 experiments. *$p < 0.05$ indicates a significant difference from experiments with boiled microsomes at the same concentration. PCN: 5-pregnen-3β-ol-20-one-16α-carbonitrile BZF: bezafibrate BNPP: bis-4-nitrophenyl phosphate.

was previously examined by means of reporter gene assay for ERα and ERβ (Watanabe et al., 2013). Those activities showed a bell-shaped curve with respect to the linear alkyl chain length, i.e., medium-size side-chain parabens (C5–C7) showed higher ERα and ERβ activities. In our study, parabens with a short side-chain (C2–C5, linear or branched) tended to show high h/rPXR and rCAR activities. But, in contrast, rPPARα activity was markedly high among long-side-chain parabens (C7–C12) (Fig. 2). These differences presumably reflect the different structures of the receptor binding pockets. The ligand-binding pocket of hPXR is 1200 $Å^3$ in size (Watkins et al., 2001), whereas the binding pocket of CAR is about half as large. In addition, the ligand-binding pocket of PXR is more flexible than that of CAR, and consequently PXR

Table 2
REC_{20} and RIC_{20} values of parabens for PXR, CAR and PPARα activation.

Compound	REC_{20} (± SD, μM)				RIC_{20} (± SD, μM)	
	hPXR	rPXR	rCAR	rPPARα	rPXR	rCAR
positive control	0.56 ± 0.047	0.059 ± 0.027	1.6 ± 0.96	5.5 ± 0.52	=	=
4-HBA	–	–	–	–	=	=
methylparaben	–	–	–	–	=	=
ethylparaben	27 ± 4.9	9.3 ± 9.7	19 ± 9.1	–	=	=
propylparaben	28 ± 17	4.9 ± 1.8	11 ± 5.3	27 ± 2.9	=	=
butylparaben	8.2 ± 4.1	5.9 ± 1.7	2.1 ± 0.12	10 ± 1.5	=	=
pentylparaben	6.9 ± 2.6	=	=	2.9 ± 1.5	22 ± 2.2	21 ± 2.6
hexylparaben	–	=	=	–	4.6 ± 0.51	4.8 ± 0.46
heptylparaben	–	=	=	0.52 ± 0.32	3.4 ± 1.5	=
octylparaben	–	5.1 ± 1.0	–	2.5 ± 0.08	=	=
nonylparaben	17 ± 4.2	7.0 ± 1.7	–	3.2 ± 1.1	=	=
decylparaben	–	3.7 ± 1.2	–	4.4 ± 0.98	=	=
undecylparaben	–	–	–	5.4 ± 0.53	=	=
dodecylparaben	–	2.5 ± 1.6	–	5.6 ± 0.73	=	=
isopropylparaben	28 ± 2.7	7.1 ± 1.3	17 ± 4.8	33 ± 12	=	=
isobutylparaben	7.2 ± 1.7	0.90 ± 1.0	5.0 ± 0.90	8.5 ± 2.6	=	=
isopentylparaben	6.7 ± 9.3	8.1 ± 5.8	–	2.2 ± 0.69	=	=
phenylparaben	–	20 ± 4.1	2.1 ± 0.59	13 ± 2.6	=	=
benzylparaben	–	–	–	11 ± 6.4	=	=

Positive control: refampicin (hPXR), PCN (5-pregnen-3β-ol-20-one-16α-carbonitrile) (rPXR), artemisinin (rCAR), BZF (bezafibrate) (rPPARα).
REC_{20}: 20% relative effective concentration; concentration of test compound showing 20% of the agonistic activity of refampicin (10 μM), PCN (1 μM), artemisinin (30 μM) and BZF (30 μM).
RIC_{20}: 20% relative inhibitory concentration; concentration of test compound showing 20% of the antagonistic or inverse agonistic activity of control (0.1% DMSO).
–: No effect ($REC_{20} > 30$ μM).
= : Not calculated 4-HBA: 4-hydroxybenzoic acid.

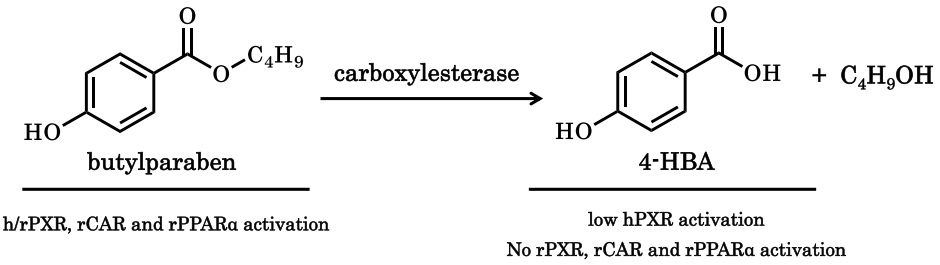


Fig. 5. Metabolic pathway of butylparaben, and the effects of butylparaben and 4-HBA on PXR, CAR and PPARα activities. The metabolic pathway is taken from Imai et al. (2006) and Ozaki et al. (2013).
4-HBA: 4-hydroxybenzoic acid.

is more promiscuous than CAR (Suino et al., 2004). This is consistent with our findings that more of the parabens examined in this study showed activating activity towards PXR than towards CAR. On the other hand, PPARα is activated by fatty acids, so the higher activities of long side-chain parabens (C7–12) may be due to the similarity of these side chains to fatty acids. In addition, methyl-, ethyl-, propyl- and butylparaben were previously reported to show PPARγ activation, and were suggested to have adipogenic potential (Pereira-Fernandes et al., 2013).

PPARα is known to modulate lipid homeostasis, including fatty acid β-oxidation, triglyceride storage, lipid transport, acyl-CoA formation, and cholesterol metabolism. PPARα agonists, such as fatty acids and fibrates, decrease plasma triglyceride levels and increase plasma high-density lipoprotein-cholesterol levels in humans (Rakhshandehroo et al., 2010; Yu et al., 2015). Our results support the idea that daily exposure to long-side-chain parabens might alter lipid homeostasis through PPARα.

There are species differences in the ligand binding of nuclear receptors. For example, rifampicin activates hPXR but not rodent PXR, whereas PCN activates rodent PXR but not hPXR (Kojima et al., 2011). Here, we used both hPXR and rPXR, and confirmed the existence of a marked species difference (Fig. 2A and B). Interestingly, 4-HBA showed hPXR activity, but not rPXR activity. 4-HBA is commonly considered to be safe, so this result is could be important for the safety assessment of parabens. In addition, hexylparaben and heptylparaben had little effect on hPXR activation, whereas they decreased rPXR activity. Octylparaben and decylparaben showed activity towards rPXR, but not hPXR. It seems that rPXR may be structurally more flexible than hPXR.

We found that pentylparaben and hexylparaben exhibit rPXR activation at low concentration, and rPXR antagonistic activity at high concentration (Fig. 3A). Only a few PXR antagonists have been reported so far (Mani et al., 2013; Skledar et al., 2016). One of the new brominated flame retardants, bis(2-ethylhexyl) 2,3,4,5-tetrabromophthalate showed both agonistic and antagonistic activity towards hPXR in a dose-dependent manner in luciferase reporter assay (Skledar et al., 2016). Further, pentylparaben and hexylparaben also showed CAR inverse agonistic activity (Fig. 3B). CAR characteristically has a high constitutive activity, which is repressed by inverse agonists such as androgens (Baldwin and Roling, 2009). Further studies are needed to examine whether or not these activities are physiologically significant.

We also examined the effects of the extracted metabolite of butylparaben on the nuclear receptors, because the estrogenic activity of parabens was reported to decrease after incubation with rat liver microsomes (Watanabe et al., 2013) (Fig. 4). A metabolic study with rat liver microsomes indicated that butylparaben is rapidly metabolized to 4-HBA by carboxylesterase, with loss of the activities against rPXR and rCAR. In contrast, the rPPARα activity was retained. However, the extract of rat liver microsomal incubation mixture showed activity towards rPPARα even in the absence of butylparaben (data not shown). Therefore, the effect of metabolism on the rPPARα activity may be masked. Overall, we found that the PXR, CAR and PPARα activities of parabens were almost always higher than that of their common metabolite, 4-HBA, suggesting that parabens are detoxified by metabolism. The metabolic pathway of butylparaben, and the effects of butylparaben and 4-HBA on PXR, CAR and PPARα activities are summarized in Fig. 5. But, it should be noted that parabens have been detected in human urine, blood, breast and milk in unchanged form regardless of age or sex (Darbre and Harvey, 2014). This may be explained in part by transesterification of parabens by carboxylesterase in the presence of alcohols (Fujino et al., 2014). Thus, parabens may not be efficiently detoxified in the body.

Thus, our present findings confirm that parabens influence the activities of PXR, CAR and PPARα, and so have the potential to contribute to endocrine disruption by altering hormone metabolism. However, further experimental studies in vivo are needed to examine the PXR-, CAR- and PPARα-mediated effects of parabens, in order to predict whether these effects pose possible risks to human health.

5. Conclusion

The effects of 17 parabens on h/rPXR, rCAR and rPPARα were examined in this study. Parabens showed various side-chain-structure-dependent effects on these receptors, including agonistic activities (h/rPXR, rCAR: C2–C5 side-chain, rPPARα: C7–C12 side-chain), and rPXR antagonistic activity (pentylparaben and hexylparaben), and rCAR inverse agonistic activity (pentylparaben and hexylparaben). The activity of butylparaben towards PXR and CAR was lost after incubation with rat liver microsomes. Our findings demonstrate that parabens can alter the activities of PXR, CAR and PPARα, and thus have the potential to contribute to endocrine disruption by altering hormone metabolism.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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