p-Hydroxybenzoate Esters Enhance Mouse Contact Hypersensitivity

Fumitoshi Sakazaki,[a] **Hitoshi Ueno,**[a] **Hisamitsu Nagase,**[b] **and Katsuhiko Nakamuro*,**[a]

[a]*Department of Public Health & Preventive Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, 45–1 Nagaotoge-cho, Hirakata, Osaka 573–0101, Japan and* [b]*Department of Hygienics, Gifu Pharmaceutical University, 5–6–1 Mitahora-higashi, Gifu 502–8585, Japan*

p-Hydroxybenzoate esters (parabens), which are often used in cosmetics as preservatives, have been reported to have estrogenic and anti-androgenic effects and to cause contact dermatitis. In this paper, we evaluate the effects of parabens on contact hypersensitivity, an allergic response to low molecular weight chemicals that causes contact dermatitis. Female BALB/c mice were administered 1200 mg/kg of butylparaben and sensitized by painting 3% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXA) on their backs. Seven days later, the mice were challenged by painting 1% OXA on the ear and the ear thickness was measured. Ear auricles were excised and the RNA expressions of interleukin (IL)-18 and interferon (IFN)-γ were evaluated by reverse transcription-polymerase chain reaction (RT-PCR). Butylparaben enhanced ear swelling at 6 hr after the elicitation of allergy. Butylparaben at a dosage of 600 mg/kg was sufficient to aggravate contact hypersensitivity. Among the six parabens examined, butylparaben exerted the strongest allergy-enhancing activity. Butylparaben also enhanced the RNA expression of IL-18 before the challenge with OXA and the RNA expression of IFN-γ at 6 hr after the challenge. These results suggest that parabens could enhance IL-18 and IFN-γ expression and exacerbate mouse contact hypersensitivity to OXA.

Key words —— *p*-hydroxybenzoate esters, contact allergy, endocrine-disrupting chemicals, interferon-γ, interleukin-18

INTRODUCTION

Contact hypersensitivity is an allergic reaction to low molecular weight chemicals that are applied to the skin, and is a cause of contact dermatitis.[1–3] Contact hypersensitivity can be elicited by nickel in costume jewelry; urushiol, a component of the Japanese lacquer tree *Rhus vernici-flua*; and constituents of cosmetics such as eugenol and geraniol.[4–7] Cosmetics also often contain *p*-hydroxybenzoate esters (parabens) as a preservatives,[8] and these parabens have been reported to cause contact dermatitis.[9–11]

Parabens have been demonstrated to have both estrogenic[12, 13] and anti-androgenic effects,[14] and, among the parabens examined, butylparaben has been found to have the strongest estrogenic activity on rat uterus enlargement.[12] We previously reported that 17β-estradiol augments contact hypersensitivity induced by 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXA) in the mouse auricle, which was used as a model of contact dermatitis.[15–17] In that study, 17β-estradiol administration led to an increase in the expression of interleukin (IL)-18 mRNA on the seventh day after sensitization of mice, in the expression of interferon (IFN)-γ mRNA at 6 hr after allergy elicitation, and, finally, in ear swelling. However, unlike 17β-estradiol, parabens have not been examined for their enhancing effects on contact hypersensitivity.

Accordingly, in the present study, we evaluated the effect of parabens on mouse contact hypersensitivity and the expressions of IL-18 and IFN-γ mRNAs.

*To whom correspondence should be addressed: Department of Public Health & Preventive Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, 45–1 Nagaotoge-cho, Hirakata, Osaka 573–0101, Japan. Tel.: +81-72-866-3122; Fax: +81-72-866-3123; E-mail: nakamuro@pharm.setsunan.ac.jp

MATERIALS AND METHODS

Materials —— Methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, and isobutylparaben were kindly provided by Drs. Kanako Sato and Ken-ichi Oyama of Tokyo Metropolitan Institute of Public Health. The purity of these parabens was assayed and reported by them.[14]

Animal Gonadectomy and Paraben Administration —— The protocol used in the present study was conducted in accordance with the Animal Experiment Guidelines of Setsunan University that were established by revising the guidelines of the Japanese Society for Pharmacology. The study was approved by the Committee for Ethical Use of Experimental Animals at Setsunan University. All efforts were invariably made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. BALB/c mice (Japan SLC Co., Shizuoka, Japan) were treated as previously described.[15–17] Briefly, all treatments and maintenance of animals were conducted in a specific pathogen-free room at $23 \pm 1°C$ and 47–67% humidity, under a 12-hr light-dark cycle (lights on at 7:00 AM). Three-week-old mice were gonadectomized under pentobarbital anesthesia, and parabens dissolved in 100 μl of olive oil was subcutaneously injected. For the experiment with adult mice, mice were ovariectomized at the age 3 weeks, maintained for 4 weeks, and then administered butylparaben twice a week for 4 weeks. At the age 12 weeks, contact hypersensitivity was elicited. As a negative control, the same volume of olive oil alone was administered.

Contact Hypersensitivity —— After administration of parabens, mice were sensitized by the topical application of 50 μl of 3% OXA (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) in a 3 : 1 (v/v) mixture of ethanol and acetone. After 7 d, the mice were challenged by applying 7.5 μl of 1% OXA in olive oil to both sides of the right auricle. After taking into consideration the circadian rhythms of mice,[18] the elicitation of allergy was carried out at 8:00 AM. The auricle thickness was measured under ether anesthesia using a digital thickness gauge (Ozaki MFG Co. Ltd., Tokyo, Japan) while taking care to avoid denting the edematous skin. The degree of ear swelling was calculated by subtracting the thickness of the left ear from that of the right ear.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of Cytokine mRNAs —— The right auricles were excised and immersed in buffer RLT of an RNeasy Fibrous Tissue Mini Kit (QIAGEN GmbH, Hilden, Germany). The samples were stored at −80°C until homogenization and RNA extraction following the manufacturer's protocol. An aliquot (1 μg) of each total RNA sample was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, U.S.A.), and 1 μl of the cDNA solution thus obtained was used for PCR using SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan) and a Smart Cycler II System (TaKaRa Bio Inc.). Primer sets for IFN-γ, IL-18, and β-actin (an internal control) were obtained from TaKaRa Bio Inc. The mRNA levels were calculated as ratios relative to the corresponding β-actin mRNA levels.

Statistical Analysis —— The values obtained from 4 or 5 mice were calculated as the means ± standard deviation. The differences among more than 3 groups were examined by one-way analysis of variance (ANOVA) and Scheffe's test. The differences between two group were examined by Fisher's F test and Student's *t*-test: Differences between the standard deviations were evaluated using Fisher's F test, whereas the differences between the means were analyzed by Student's *t*-test. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

In order to investigate the effects of butylparaben on contact hypersensitivity, 1200 mg/kg of butylparaben was administered to mice and contact allergy was elicited by OXA application to their right ears; ear thicknesses were subsequently measured over time (Fig. 1). There was virtually no swelling of ears within the first 3 hr, indicating the absence of immediate-type hypersensitivity. Subsequently, however, the ears started to swell, maintained a maximal thickness from 9 to 24 hr, and thereafter decreased in size. The ears of butylparaben-administered mice exhibited a greater amount of swelling than those of vehicle-administered mice at 6 hr after the elicitation (Fig. 1). In 3-week-old female mice, butylparaben enhanced contact hypersensitivity at an early phase.

We next investigated the effect of butylparaben on contact hypersensitivity in adult mice. Mice were ovariectomized at the age of 3 weeks, main-
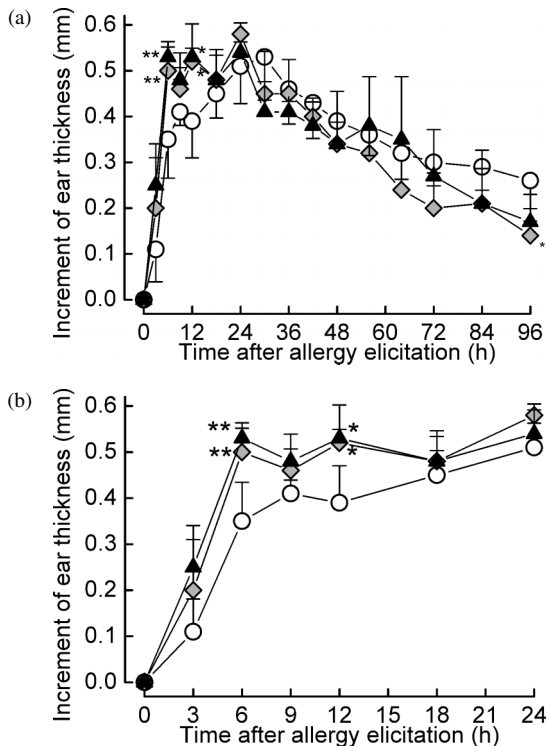
Fig. 1. Degree of Ear Swelling Due to the Contact Hypersensitivity Reaction in 3-week-old Mice

Mice were ovariectomized and then administered a single dose of vehicle, 0.32-mg/kg 17β-estradiol, or 1200-mg/kg butylparaben. Contact hypersensitivity in the ear was elicited by OXA and the thickness of the ears was measured. Circles (○), diamonds (◆), and triangles (▲) represent vehicle-, 17β-estradiol-, and butylparaben-administered mice, respectively. Results are presented as the mean ± S.D. (n = 4–5/group). (a) Entire the result. (b) Results in the earlier phase *i.e.* 0–24 hr after the elicitation of allergy. Differences between the control and 17β-estradiol groups and between the control and butylparaben groups were examined for significance. $^*p < 0.05$, $^{**}p < 0.01$.

tained for 4 weeks, and then administered butylparaben twice a week for 4 weeks. At the age 12 weeks, contact hypersensitivity was elicited in the mice (Fig. 2). The uteri of the butylparaben-administered mice were significantly larger than that of the control mice. The results of contact hypersensitivity showed that butylparaben enhanced the earlier phase of contact hypersensitivity in adult mice at the age of 12 weeks, which is similar to the results obtained with a single application in 3-week-old mice.

In order to evaluate the dose-dependent relationship between butylparaben and contact hypersensitivity, butylparaben at doses ranging from 0 to 1200 mg/kg was administered to 3-week-old mice (Fig. 3). The results showed that the enhancement of contact allergy by butylparaben was greatest at a dose of 600 mg/kg.

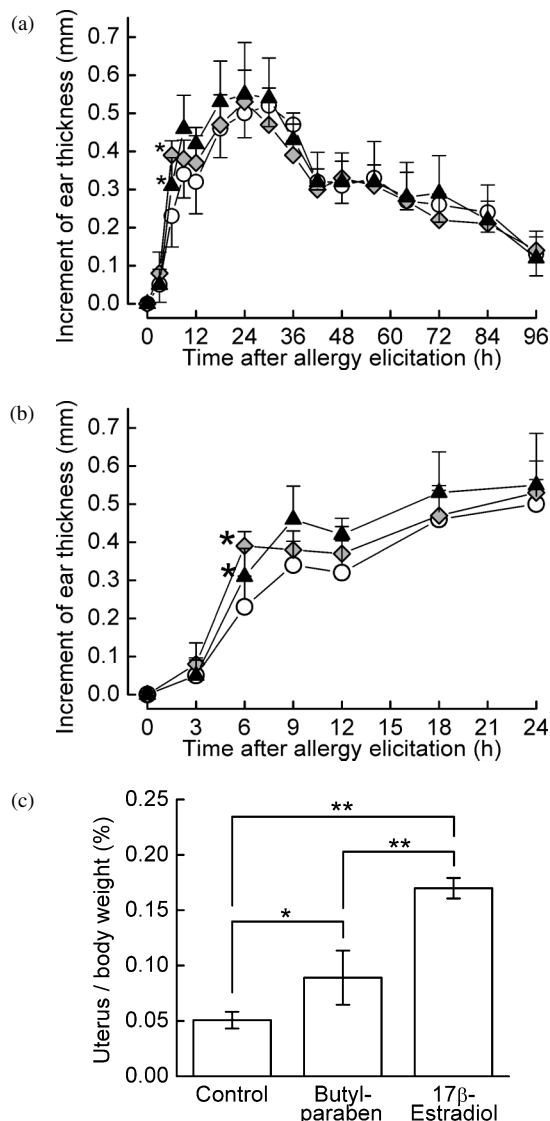The structure-activity relationship between parabens and contact hypersensitivity-induce edema

Fig. 2. Degree of Ear Swelling Due to the Contact Hypersensitivity Reaction and Uterus Weight of 12-week-old Mice

Mice were ovariectomized and administered vehicle, 0.32-mg/kg 17β-estradiol, or 1200-mg/kg butylparaben for 4 weeks. Contact hypersensitivity in the ear was elicited by OXA and the thickness of the ears was measured. Results are presented as the mean ± S.D. (n = 4–5/group). (a) Entire the result of ear swelling. (b) Results in the earlier phase *i.e.* 0–24 hr after the elicitation of allergy. Circles (○), diamonds (◆), and triangles (▲) represent vehicle-, 17β-estradiol-, and butylparaben-administered mice, respectively. Differences between the control and 17β-estradiol groups and between the control and butylparaben groups were examined for significance. $^*p < 0.05$. (c) Uterus weight. Differences between each group were examined for significance. $^*p < 0.05$, $^{**}p < 0.01$.

was then evaluated using the following 6 parabens: methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, and isobutylparaben (Fig. 4). The results revealed that methylparaben, isopropylparaben, and isobutylparaben also had notable affects on ear swelling, and butylparaben enhanced ear edema to the greatest extent.
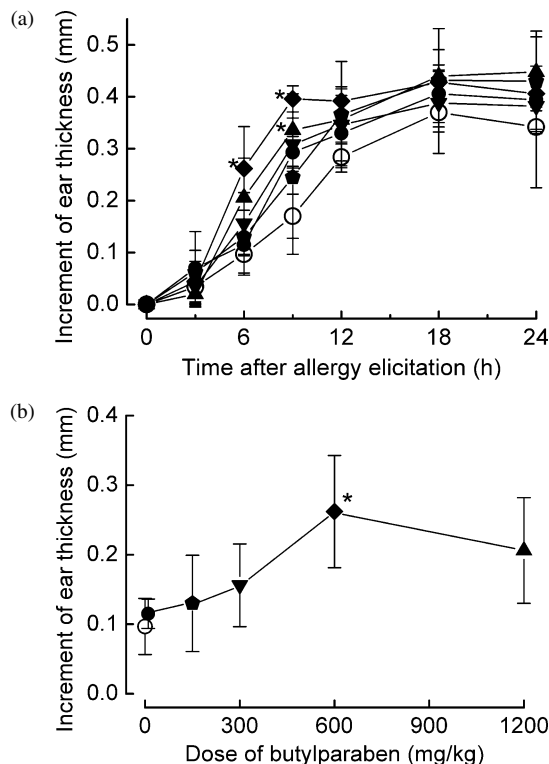
(a)

(b)

Fig. 3. Dose Dependency of Degree of Ear Swelling Due to the Contact Hypersensitivity Reaction

Mice were ovariectomized and received a single administration of butylparaben (0 to 1200 mg/kg). Contact hypersensitivity in the ear was elicited by OXA and the thickness of the ears was measured. Results are presented as the mean ± S.D. ($n = 4$–5/group). Open circles (○), 0 mg/kg; closed circles (●), 10 mg/kg; pentagons (⬠), 150 mg/kg; inverted triangles (▼), 300 mg/kg; diamonds (◆), 600 mg/kg; triangles (▲), 1200 mg/kg. (a) Time-course study of ear swelling. (b) Dose dependency of the ear swelling at 6 hr after the elicitation of allergy. Differences between the control and butylparaben groups were examined for significance. $^{*}p < 0.05$.

(a)

(b)

Fig. 4. Structure-activity Relationship Among Six Parabens and the Degree of Ear Swelling Due to the Contact Hypersensitivity Reaction

Mice were ovariectomized and then received a single administration of 600-mg/kg paraben. Contact hypersensitivity in the ear was elicited by OXA and the thickness of the ears was measured. Results are presented as the mean ± S.D. ($n = 4$–5/group). Circles (○), vehicle; triangles (▲), methylparaben; inverted-triangles (▼), ethylparaben; left-pointing triangles (◀), propylparaben; right-pointing triangles (▶), isopropylparaben; diamonds (◆), butylparaben; pentagons (⬠), isobutylparaben. (a) Time-course study of ear swelling. (b) Degree of the ear swelling at 6 hr after the elicitation of allergy. Differences between the vehicle and paraben groups were examined for significance. $^{*}p < 0.05$.

Finally, we investigated the effects of butylparaben on the mRNA expressions of IL-18 and IFN-γ. Butylparaben enhanced IL-18 mRNA expression before the elicitation of an allergic response, and enhanced IFN-γ mRNA expression at 6 hr after elicitation of hypersensitivity (Fig. 5).

DISCUSSION

Routledge *et al.* evaluated the estrogenic activity of parabens with regard to enlargement of the rat uterus and reported that butylparaben has the strongest activity.[12] In that examination, the uterus weight of the rats that were administered the highest dose, 1200 mg/kg body weight, was approximately half that of the mice administered 0.4 mg/kg 17β-estradiol. Similarly, in the present study, the uterus weight of the mice that were administered 1200 mg/kg butylparaben was approximately half
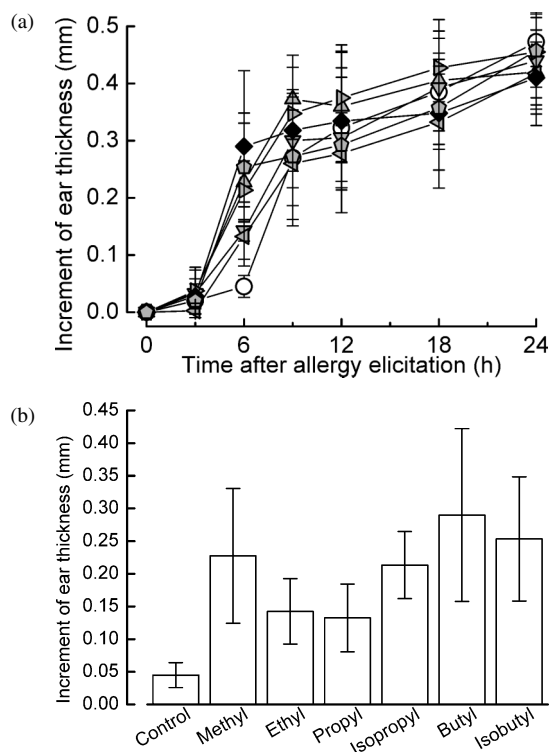
that of the mice administered 0.32 mg/kg 17β-estradiol (Fig. 2a). The reason why parabens exert a weaker uterotropic activity might due to their agonistic activity against estrogen receptors or to the pharmacokinetic properties of parabens—parabens will be hydrolyzed immediately when they are administered transdermally.[11] The fate of parabens administered subcutaneously, however, remains unknown. Nevertheless, in our experiments, butylparaben enlarged the uterus of mice to a certain extent. This result indicates that not all of the administered butylparaben was metabolized and that some remained to exert estrogenic activity.

Parabens have allergenicity and the ability to cause contact hypersensitivity.[9–11] In addition to these direct effects, we evaluated the effects of parabens on the allergic response to OXA. Parabens enhanced the ear swelling at 6 hr after the elicita-
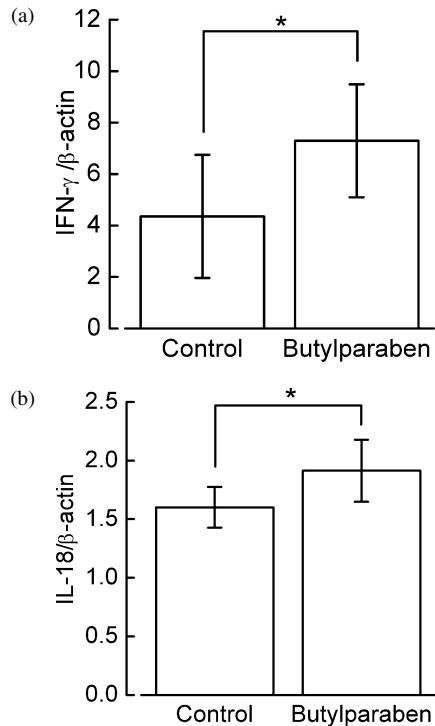
(a)

(b)

Fig. 5. The Effects of Butylparaben on mRNA Expression Levels in Ears

Mice were ovariectomized and then received a single administration of vehicle or 600-mg/kg butylparaben. The mice were sensitized with OXA and the ear auricles were excised. Quantification of mRNA levels was performed by real-time RT-PCR. The mRNA levels are expressed as ratios relative to the corresponding β-actin mRNA levels. Results are presented as the mean ± S.D. ($n = 5$–7/group). Differences between the control and butylparaben groups were examined for significance. $^{*}p < 0.05$. (a) IFN-γ. Ear auricles were excised at 6 hr after the elicitation of the allergy. (b) IL-18. Ear auricles were excised on the seventh day after the sensitization of mice.

tion of allergy to the same extent as 17β-estradiol (Fig. 1). It is observed that the degree of contact hypersensitivity induced by OXA sometimes varied (Figs. 1 and 2 *vs.* Figs. 3 and 4). Butylparaben, however, reproducibly induced contact hypersensitivity at 6 hr after the elicitation of allergy. Butylparaben tended to enhance the edema at 24 hr after the challenge in Fig. 3a while such tendency is not observed in Figs. 1, 2a, and 4a. The reproducibility of enhancement of edema by butylparaben is confident at 6 hr and doubtful at 24 hr after the elicitation of allergy. This difference of reproducibility was also observed when 17β-estradiol enhances the contact hypersensitivity. In our experiments in which mice were administered 17β-estradiol, edema was dominant to cell infiltration at 6 hr after the elicitation of allergy.[15] This suggests that parabens also enhance edema in the early phase of contact hypersensitivity.

Butylparaben enhanced contact hypersensitivity in 3-week-old young mice as well as in 12-week-old adult mice (Figs. 1, 2b). This result suggests

that parabens may have the ability to enhance contact dermatitis both in children having immature immune and endocrine systems and in adults having mature immune and endocrine systems. Parabens added to cosmetics might also exacerbate contact dermatitis in adult women.

Enhancement of contact hypersensitivity by butylparaben was strongest at a dose of 600 mg/kg, and the effect become weaker at a higher dose (1200 mg/kg) (Fig. 3). This suggests that at higher doses the toxicity of parabens might suppress their allergy-enhancing effects. The body weights of 1200 mg/kg butylparaben-administered mice were similar to that of the control mice though skin lesions were observed around the part where 1200 mg/kg of butylparaben was administered for four weeks. On the basis of the growth depression in male rats that were administered parabens in food, the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) suggests that the maximum acceptable daily intake (ADI) of ethylparaben and methylparaben should be 10 mg/kg·d.[19,20] And, indeed, in the present study, we were unable to demonstrate an enhancement of contact sensitivity using a 10 mg/kg dose of butylparaben. The JECFA does not, however, set an ADI for propylparaben because propylparaben has been reported affecting on tissues of reproductive organs in male rats at dietary doses of 10 mg/kg·d and no-observable-effect-level (NOEL) of propylparaben has not been identified.[20] Furthermore, the JECFA does not set an ADI for butylparaben since there are no safety concerns at the current levels of intake when it is used as a flavoring agent.[21] Our result suggests that butylparaben at doses of less than 10 mg/kg might not enhance contact dermatitis. However, at a dose of 600 mg/kg, butylparaben does enhance contact hypersensitivity. This result might suggest that the ADI for butylparaben should be lower than 6 mg/kg·d. In this regard, further studies should be undertaken in order to determine the effects of transdermal administration on sensitized skin and of long-term administration.

Among the parabens examined, butylparaben has strongest uterotropic activity in rats.[12] In our observations on hypersensitivity, although isobutylparaben, isopropylparaben, and methylparaben enhanced hypersensitivity to a relatively high extent, butylparaben enhanced hypersensitivity to the greatest extent (Fig. 4). These results indicate that parabens probably enhance hypersensitiv-

ity, at least partly, through their estrogenic activity. As mentioned above, transdermally administered parabens are hydrolyzed immediately.[11] The structure-activity relationship should be further investigated considering hydrolysis and pharmacokinetic characters in addition to estrogenic activity.

In our previous report,[22] 17β-estradiol enhances the expression of IL-18 mRNA on the fourth and the seventh day after the sensitization of mice, in other words, before the elicitation of allergy. Besides, 17β-estradiol does not enhance IL-18 mRNA expression after the challenge because the IL-18 mRNA expression of control mice is also induced by the elicitation of allergy. Further, we have previously reported that the expression of IFN-γ mRNA of 17β-estradiol-administered mice is enhanced at 6 hr and decreased to the same level with the control mice at 9 hr after the challenge.[15] Similar to our previous observations on 17β-estradiol,[15, 22] in mice that were administered butylparaben, the expression of IFN-γ mRNA was increased 6 hr after challenge (Fig. 5b) and the expression of IL-18 mRNA was increased on the seventh day after the sensitization of mice (Fig. 5a). These results suggest that butylparaben might enhance the expression of IL-18 after the sensitization reaction and also enhance the expression of IFN-γ mRNA to promote the dropsical swelling of auricles. We suspect that the time-course of the expression of IL-18 and IFN-γ mRNA expressions of butylparaben-administered mice are similar to that of 17β-estradiol-administered mice. That should be further examined.

17β-Estradiol depresses contact hypersensitivity in MRL mice, whereas it enhances contact hypersensitivity in BALB/c mice.[23] Butylparaben might also depress contact hypersensitivity under certain conditions. A chemical substance that causes contact dermatitis can induce a Th2 response,[24] and this may provide an explanation for the two apparently conflicting effects of 17β-estradiol on contact hypersensitivity. Further investigations regarding the detailed mechanism of how butylparaben affects contact allergy are clearly needed.

In summary, we found that parabens, including butylparaben, enhanced the early phase of contact hypersensitivity in mice and that butylparaben enhanced the expression of IL-18 mRNA after the sensitization of mice and enhanced the expression of IFN-γ mRNA after the elicitation of allergy.

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