ment, and a reliable detection method for the existence of α_{2u}-globulin is therefore necessary.

Using both immunochemical staining for paraffin-embedded sections and the immuno-electron microscopy technique, we demonstrated that our prepared antibody reacted specifically to α_{2u}-globulin in renal hyaline droplets in the male rats administered d-limonene, a well-known α_{2u}-globulin nephropathy inducer. The dose-dependent positive immuno-reaction of the antibody in both the tissue sections and the homogenates from d-limonene-treated rat kidneys indicated that the antibody could be applicable for semi-quantitative analysis. In addition, computational image analysis revealed that classical visual microscopic grading was also useful for semi-quantitative analysis of α_{2u}-globulin accumulation.

Although immunohistochemical α_{2u}-globulin analysis of the glycolmethacrylate-embedded sections had already been reported by Burnett *et al.* (1989), our method was advantageous from the standpoint of applicability to the paraffin-embedded sections. The paraffin-embedded specimens were usually prepared and stored for the general toxicity studies. In fact, all the sections used in experiment 2 in this study originated from study specimens which were prepared in the Japanese Existing Chemicals Survey Program conducted previously and stored for a long time. It indicated that our method is applicable to specimens derived directly from ordinary toxicology studies retrospectively. Hashimoto and Takaya (1992) previously investigated the application of α_{2u}-globulin immunostaining to paraffin sections by modifying the protocol of Burnett *et al.* (1989). The protocol includes pronase E treatment owing to enhancement of the antigen reactivity and removal of the non-specific reaction. Our method also includes the pronase E treatment, but

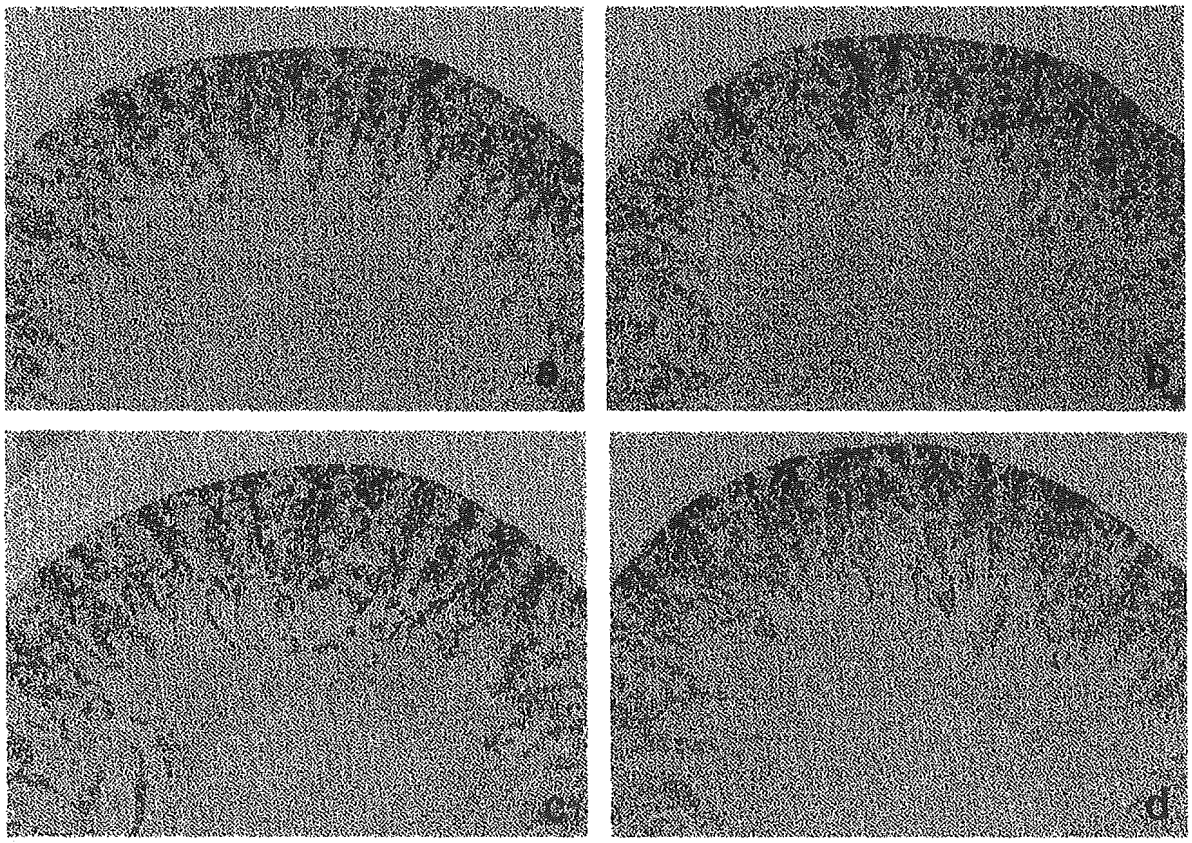

Photo 4. Immunohistochemical features of the anti-α_{2u}-globulin antibody, representing the four grades; minimal (a), slight (b), moderate (c) and severe (d). Original magnification, ×5.

Semi-quantitative immunohistochemical analysis of male rat-specific α_{2u}-globulin accumulation.

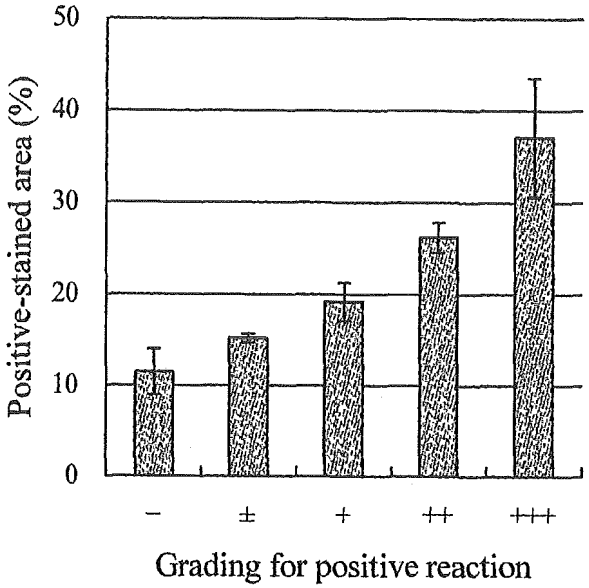

Fig. 2. Correlation between semi-quantitative and quantitative analyses for immuno-stained sections.
Results are expressed as mean ± SD (n=3).

the treatment is performed only in order to enhance the antigen activity and not to remove the non-specific reaction. This may suggest that our prepared antibody has a high specificity for α_{2u}-globulin. Caldwell *et al.* (1999) had conducted a similar quantitative immunohistochemical α_{2u}-globulin analysis, but it seems that the actual analyzed area was limited to narrower fields than in our study.

Urinary immunochemical analysis for detection of α_{2u}-globulin accumulation in male rat kidneys has been developed by Saito *et al.* (1996). Although the convenient urinary analysis is sufficient for detecting CIGA, the detectability is weaker than with kidney soluble protein analysis. The aim of the present analysis is not only to detect CIGA, but also to exclude the α_{2u}-globulin-induced nephrotoxic effects from risk assessment of chemicals. For 10 chemicals suspected of being CIGA, the occurrence of hyaline droplets in the kidneys with treatment was the lowest endpoint. In the process of evaluating chemical toxicity, if the most sensitive nephrotoxicity is concluded to be a neglected effect for human health, the NOAEL could be set based on other kinds of toxicological effects.

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ORIGINAL ARTICLE

Comparative susceptibility of newborn and young rats to six industrial chemicals

Ryuichi Hasegawa, Mutsuko Hirata-Koizumi, Mika Takahashi, Eiichi Kamata, and Makoto Ema
National Institute of Health Sciences, Tokyo, Japan

ABSTRACT **To elucidate the comparative susceptibility of newborn rats to chemicals, newborn and young animals were administered six industrial chemicals by gavage from postnatal days (PND) 4 to 21, and for 28 days starting at 5–6 weeks of age respectively, under the same experimental conditions as far as possible. As two new toxicity endpoints specific to this comparative analysis, presumed no-observed-adverse-effect-levels (pNOAELs) were estimated based on results of both main and dose-finding studies, and presumed unequivocally toxic levels (pUETLs) were also decided. pNOAELs for newborn and young rats were 40 and 200 for 2-chlorophenol, 100 and 100 for 4-chlorophenol, 30 and 100 for p-(α,α-dimethylbenzyl) phenol, 100 and 40 for (hydroxyphenyl)methyl phenol, 60 and 12 for trityl chloride, and 100 and 300 mg/kg/day for 1,3,5-trihydroxybenezene, respectively. To determine pUETLs, dose ranges were adopted in several cases because of the limited results of experimental doses. Values for newborn and young rats were thus estimated as 200–250 and 1000 for 2-chlorophenol, 300 and 500 for 4-chlorophenol, 300 and 700–800 for p-(α,α-dimethylbenzyl) phenol, 140–160 and 1000 for (hydroxyphenyl)methyl phenol, 400–500 and 300 for trityl chloride, and 500 and 1000 mg/kg/day for 1,3,5-trihydroxybenzene, respectively. In most cases, newborn rats were 2–5 times more susceptible than young rats in terms of both the pNOAEL and the pUETL. An exception was that young rats were clearly more susceptible than their newborn counterparts for trityl chloride.**

Key Words: industrial chemicals, newborn rats, susceptibility

INTRODUCTION

In risk assessment of chemicals, the no-observed-adverse-effect-level (NOAEL) determined with repeated dose toxicity studies is generally divided by uncertainty factors (UFs) to obtain the tolerable daily intake (TDI) (Hasegawa *et al.* 2004). UFs include inter- and intraspecies differences, lack of data quality and the nature of observed toxicity. As TDI is an allowable lifetime exposure level for a chemical, at which no appreciable health risk would be expected over a lifetime, the NOAEL must be derived from lifetime exposure studies and appropriate reproductive/developmental studies, or their equivalents. Administration generally starts at the prepubertal stage (4–5 weeks old) or with young adults (10–12 weeks old) in rodent studies. Therefore, the suckling phase is the major remaining period where animals are not directly administered to chemicals. If susceptibility of infant animals to chemicals via direct exposure was evidenced by appropriate comparative studies, the results would preferably be incorporated into the UF as one justification for lack of data quality.

In the latest decade, infant and child health has become a major focus (Landrigan *et al.* 2004), especially since endocrine disrupters became a contentious issue around the world (IPCS 2002). Since there are distinct differences in characteristics from the adult case (Dourson *et al.* 2002), particular attention must be paid to infant and child health. The Japanese government has therefore incorporated the newborn rat study (newborn study) into Existing Chemical Safety Programs as an especial project to comparatively determine susceptibility to 18 industrial chemicals. As the core of this program is to conduct 28-day repeated dose toxicity studies using young rats (young study) with untested chemicals from the existing list, chemicals for newborn studies were selected among the chemicals scheduled for young studies in the same year for the best comparison of data. Furthermore, we have had to newly establish a newborn rat study protocol because of the lack of any standard testing guidelines. Major differences of newborn from young studies are a shorter administration period (18 days only for the suckling phase) and additional examination of early functional, external and sexual development (Koizumi *et al.* 2001). Studies were conducted from 1995 to 1998 and we have already reported the results of comparative analysis for eight chemicals, showing newborn rats to be generally 2–4 fold more susceptible than young rats in most cases on basis of NOAEL and the unequivocally toxic level (UETL), the latter being uniquely defined in this program as doses inducing clear clinical toxic signs, death or critical histopathological damage (Koizumi *et al.* 2001, 2002, 2003; Fukuda *et al.* 2004; Takahashi *et al.* 2004; Hirata-Koizumi *et al.* 2005).

The purpose of this study is to obtain additional information on susceptibility of newborn rats to other chemicals. Here we selected the following six industrial chemicals, mostly phenolic compounds: 2-chlorophenol, 4-chlorophenol, p-(α,α-dimethylbenzyl) phenol (hydroxyphenyl)methyl phenol, trityl chloride and 1,3,5-trihydroxybenzene, because of structural similarity to endocrine-disrupting phenols, bisphenol A (Takahashi & Oishi 2001), and nonylphenol (Lee 1998). These chemicals have been used as an intermediate in dyes and an ingredient in pesticides (2-chlorophenol), an intermediate in dyes, bactericides and an ingredient in cosmetics (4-chlorophenol), an ingredient in surfactants, bactericides, an intermediate in pesticides and plasticizers (p-(α,α-dimethylbenzyl) phenol), an ingredient in resins ((hydroxyphenyl)methyl phenol), an intermediate in medicines (trityl chloride) and an ingredient in medicines, a stabilizer of synthetic rubbers and an adhesive of rubbers (1,3,5-trihydroxybenzene) (Chemical Products' Handbook 2004). Under the same experimental conditions as far as possible, we have examined the repeated dose toxicity of these chemicals in newborn and young rats and compared susceptibility for each. Previously we had applied NOAEL and UETL as estimated doses

Correspondence: Ryuichi Hasegawa, PhD, Division of Medicinal Safety Science, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Email: hasegawa@nihs.go.jp

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or ranges of doses for comparison of chemical susceptibility, but we have decided to employ the new terminology of presumed NOAEL (pNOAEL) and presumed UETL (pUETL) in their place. As a result, in most cases newborn rats were more susceptible to these industrial chemicals than young rats in terms of both pNOAEL and pUETL.

MATERIALS

2-Chlorophenol (CAS no. 95-57-8, Lot no. OJL-15, purity: 99.49%) was obtained from Inui Corporation and prepared in olive oil; 4-chlorophenol (CAS no. 106-48-9, Lot no. PJF-3, purity: 99.29%) from Inui Corporation and in corn oil; p-(α,α-dimethylbenzyl) phenol (CAS no. 599-64-4, Lot no. 101002, purity: 99.88%) from Sun TechnoChemical Inc. in olive oil; (hydroxyphenyl)methyl phenol (CAS no. 1333-16-0, Lot no. S980013, purity: 99.0% [2,2′ isomer 14–18%, 2,4′ isomer 44–48%, 4,4′ isomer 26–32%]) from Mitsui Chemicals, Inc. in 0.5% CMC-Na solution containing 0.1% Tween 80; trityl chloride (CAS no. 76-83-5, Lot no. 1038, purity: 99.5%) from Kurogane Kasei Co. Ltd. in olive oil; and 1,3,5-trihydroxybenzene (CAS no. 108-73-6, Lot no. OS-12074, purity: 99.9%) from Ishihara Sangyou Co., Ltd. in olive oil. Test solutions were prepared at least once a week and were kept cool and in the dark until dosing. The stability was confirmed to be at least seven days under these conditions. All other reagents used in this study were specific purity grade.

METHODS

All animal studies were performed in five testing laboratories contracted to the Japanese Government, after we approved the test protocol.

Animals

Sprague-Dawley SPF rats [Crj:CD(SD)IGS] were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained in an environmentally controlled room at 24 ± 2°C with a relative humidity of 55 ± 15%, a ventilation rate of more than 10 times per hour, and a 12:12 h light/dark cycle. For the studies of newborns, 20 pregnant rats (shipped in at gestation day 14) were allowed to deliver spontaneously. All newborns were separated from dams on postnatal day (PND) 3 and groups of 12 males and 12 females were selected and assigned to each of the four dose groups, including the controls. Twelve foster mothers were selected based on health and nursing conditions, and suckled the four males and four females assigned to each group up to weaning on PND 21 (termination of dosing and autopsy for half of the animals). After weaning, the rest of the animals for the recovery-maintenance group (see Study Design) were individually maintained for nine weeks. In the studies of young, four-week-old male and female rats were obtained and used at ages of 5–6 weeks after acclimation. All animals were allowed free access to a basal diet and water.

Study design (time schedule as described previously [Koizumi *et al.* 2001])

1. 18-day repeated dose study in newborn rats (newborn study)

In a dose-finding study, chemicals were administered by gastric intubation to newborn male and female rats on PNDs 4–21. Animals were examined for general behavior and body weights during the dosing period, and sacrificed at PND 22 for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

In the main study, newborn rats (12/sex/dose) were administered chemicals by gastric intubation on PNDs 4–21, the dosage being set on the basis of results of the dose-finding study. On PND 22, half of the animals were sacrificed and the rest were maintained for nine weeks without chemical treatment, and then sacrificed at 12 weeks of age (the recovery-maintenance group). During the study, general behavior and body weight were examined at least once a day and each week, respectively. In addition, developmental parameters were assessed, such as surface righting and visual placing reflex for reflex ontogeny, fur appearance, incisor eruption and eye opening for external development, and preputial separation, vaginal opening and estrous cycle for sexual development. Urinalysis (color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, volume of the urine and osmotic pressure) was conducted in the late recovery-maintenance period.

At weaning age PND 22 after the last treatment, blood was collected under anesthesia from the abdomen of all animals in the scheduled-sacrifice group. In the recovery-maintenance group, this was conducted at 85 days of age after overnight starvation. Blood was examined for hematological parameters such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count, and for biochemistry (total protein, albumin, albumin/globulin ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase, alanine animotransferase (ALT), alkaline phosphatase, γ-glutamyl transpeptidase (γ-GTP), calcium, inorganic phosphorus, sodium, potassium and chlorine). Prothrombin time and activated thromboplastin time were examined only in the recovery-maintenance group. The brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, ovaries and uterus were weighed, and these, with other macroscopically abnormal organs, were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides). Paraffin sections were routinely prepared and stained with hematoxylin-eosin for microscopic examination. All studies were conducted in compliance with the Good Laboratory Practice Act of the Japanese Government.

2. 28-day repeated dose study in young rats (young study)

In a dose-finding study, chemicals were administered by gastric intubation to five-week-old male and female rats for 14 days. The general behavior, body weight and food consumption were examined, and the animals were sacrificed the day after the last treatment for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

In the main study, 5–6 week old male and female rats were given chemicals by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment (scheduled-sacrifice group). Recovery groups were maintained for two weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. Rats were examined for general behavior, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings in compliance with the Test Guideline in the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

Statistical analysis

Quantitative data were analyzed by Bartlett's test (Bartlett 1937) for homogeneity of distribution. When homogeneity was recog-

nized, Dunnett's test (Dunnett 1964) was conducted for comparison between control and individual treatment groups. If not homogenous, the data were analyzed using Steel's multiple comparison test (Steel 1959) or the mean rank test of the Dunnett type (Hollander & Wolfe 1973). For qualitative data such as histopathological findings, the Mann–Whitney's *U*-test (Mann & Whitney 1947) or the Fisher's exact test (Fisher 1973) were performed.

Adoption of pNOAEL and pUETL

NOAEL is a measure used in toxicity studies for the greatest dose at which no adverse effects are observed. No toxicologically meaningful changes are excluded for any grounds, including increase of relative organ weights without any other related changes. As the present purpose was to elucidate susceptibility of newborn rats to chemicals as compared with young rats as accurately as possible, simple application of NOAELs obtained from newborn and young main studies was considered not to be necessarily appropriate even though the dose setting is pertinent. Therefore, we newly defined a pNOAEL as the most likely estimated no-adverse-effect-dose on the basis of data from both main and dose-finding studies. As urinalysis and histopathological examination were not conducted in both dose-finding studies, and the administration period in young dose-finding study was half of the main study, we carefully weighed how the results from the dose-finding study should be taken into account, especially concerning the type of toxicity. In order to consider equivalently toxic intensity doses for newborn and young rats, we also newly defined a pUETL, although this is not without problems given the limited dose points. Therefore, in the most cases, the appropriate pUETL for either newborn or young rats was chosen first, thereafter the matching pUETL or the range of pUETL was speculated to assess equivalent toxicity, considering the entire body of data.

RESULTS

***2-Chlorophenol* (Table 1)**

The newborn investigation was conducted at doses of 0, 20, 100, and 500 mg/kg for the dose-finding and 0, 8, 50, and 300 mg/kg for the main study. The young investigation was conducted at doses of 0, 100, 200, and 500 mg/kg for the dose-finding and 0, 8, 40, 200, and 1000 mg/kg for the main study.

Major toxic effects on the central nervous system (CNS) were found in both sexes of newborn and young rats. In the newborn study, tremors appeared within five minutes and disappeared within four hours in most animals at 300 mg/kg. Hypoactivity and an abnormal gait were also observed in a few cases. The histopathological examination showed slight to moderate basophilic renal tubules in more than half the animals of both sexes, without relative kidney weight changes (increase by 8% for males, 4% for females). In addition to these effects, the body weights of both sexes at this dose were transiently decreased. At 50 mg/kg, only one female showed tremors once from 15 to 30 minutes on day nine after the dosing start. There were no chemical-related changes in developmental parameters. In the young study, most animals of both sexes sporadically showed various effects on the CNS such as tremors, hypoactivity, and an abnormal gait within three hours after dosing at 1000 mg/kg. Most animals also exhibited slight centrilobular hypertrophy of hepatocytes, suggesting a compensatory response to a requirement for hepatic metabolism. In the dose-finding study, no toxic signs were observed, but the information was limited because of the small number of animals, the short administration period, and the lack of histopathological examination. There were no chemical-related abnormalities at 200 mg/kg in the main study.

Although the NOAEL was 8 mg/kg/day for newborn rats based on the main study results, this value was concluded to be too low

Table 1 Toxicity findings for 2-chlorophenol in the newborn and young rat main studies

	Newborn study (mg/kg)					Young study (mg/kg)			
	0	20†	50	100†	300	0	200	500†	1000
Male									
General behavior									
Tremors	0/12	0/4	0/12	0/4	11/12	0/12	0/12	0/3	4/12
Hypoactivity	0/12	0/4	0/12	0/4	2/12	0/12	0/12	0/3	8/12
Abnormal gait	0/12	0/4	0/12	0/4	1/12	0/12	0/12	0/3	4/12
Histopathology									
Renal tubules, basophilic	0/6	no data	0/6	no data	4/6	0/6	0/6	no data	0/6
Centrilobular hypertrophy	0/6	no data	0/6	no data	0/6	0/6	0/6	no data	6/6
Female									
General behavior									
Tremors	0/12	0/4	1/12	0/4	12/12	0/12	0/12	0/3	5/12
Hypoactivity	0/12	0/4	0/12	0/4	3/12	0/12	0/12	0/3	5/12
Abnormal gait	0/12	0/4	0/12	0/4	1/12	0/12	0/12	0/3	7/12
Histopathology									
Renal tubules, basophilic	0/6	no data	0/6	no data	5/6	0/6	0/6	no data	0/6
Centrilobular hypertrophy	0/6	no data	0/6	no data	0/6	0/6	0/6	no data	5/6

Only data for items showing change are included in this table. Data are numbers of animals with the change of the total examined. †indicates dose and data from the dose-finding study. All newborn animals died by the 9th dosing day at 500 mg/kg in the dose-finding study. Body weights of both sexes were only transiently, but not finally reduced, at 300 mg/kg in the newborn main study. Clinical signs in newborn rats were not observed at doses of 20 and 100 mg/kg in the dose-finding study.

because of the absence of clinical signs at 20 and 100 mg/kg in the dose-finding study, and only one female showed tremors once at 50 mg/kg in the main study. The pNOAEL for newborn rats was therefore estimated to be 40 mg/kg/day, a little below the 50 mg/kg. For young rats, the pNOAEL can be considered to be 200 mg/kg/day because of the limited information at 500 mg/kg in the dose-finding study. The toxicity at 300 mg/kg for newborn rats seemed to be slightly higher than that at 1000 mg/kg for young rats, because of the transient depression of body weight found limited to the former cases, although the toxicity profile regarding the CNS was very similar in newborn and young rats. The dose for newborn rats showing the same toxic intensity, as that for young rats at 1000 mg/kg, is considered to be slightly lower than 300 mg/kg, at 200–250 mg/kg/day. Therefore, pUETLs of 200–250 and 1000 mg/kg/day may be considered equivalent doses for newborn and young rats, respectively.

4-Chlorophenol (Table 2)

The newborn investigation was conducted at doses of 0, 20, 100, and 500 mg/kg for the dose-finding and 0, 12, 60, and 300 mg/kg for the main study. With young rats doses of 0, 20, 100, and 500 mg/kg were applied in both dose-finding and main studies.

Toxic effects on the CNS were observed in both sexes of newborn and young rats. Most newborn rats at 500 mg/kg in the dose-finding study showed tremors, hypoactivity, bradypnea and hypothermia, and died. All newborn rats at 300 mg/kg exhibited tremors, mostly within 15 minutes to one hour, but these completely disappeared within four hours after dosing. There were no abnormalities at 100 mg/kg in the dose-finding, and 60 and 12 mg/kg in the main study. No developmental abnormalities were observed at any dose in the newborn dose-finding and main studies. In the young study, tremors, tachypnea and salivation were observed from five to 30 minutes after dosing in most animals in both sexes at 500 mg/kg. There were no other dose-dependent changes at any dose.

The pNOAEL for newborn rats is considered to be 100 mg/kg/day, because CNS toxicity was not observed at 100 mg/kg in the dose-finding study. The pNOAEL for young rats must be set at 100 mg/kg/day, because there were no doses set between 100 and 500 mg/kg. Although the toxicity profile regarding the CNS differed to some extent between newborn rats at 300 mg/kg and young rats at 500 mg/kg with respect to symptom appearance and duration, the same level can be concluded, considering the specific characteristics of the newborn body. Thereby, pUETLs of 300 and 500 mg/kg/day were estimated as appropriate for newborn and young rats, respectively.

p-(α,α-Dimethylbenzyl) phenol (Table 3)

The newborn investigation was conducted at doses of 0, 30, 100, and 300 mg/kg for both dose-finding and main studies. The young investigation was conducted at doses of 0, 250, 500, and 1000 mg/kg for dose-finding and 0, 100, 300, and 1000 mg/kg for the main study.

No newborn animals died although the body weights of both sexes were transiently lowered at 300 mg/kg (8% maximum decrease). General behavior, functional parameters and urinalysis, hematology and biochemistry data were all within normal ranges except for high urinary volume in males and high BUN in females at 300 mg/kg. The relative kidney weights were increased more than double at 300 mg/kg in both sexes, and dilation of tubules and papillary ducts was observed at relatively high grades in kidneys of both sexes, with no complete recoveries even after a nine-week recovery-maintenance period. Such histopathological change in kidneys was also slightly observed at 100 mg/kg in both sexes. In addition, there were effects on the endocrine systems, despite no effects on sexual differentiation. Absolute testicular weights were reduced by 16% at 300 mg/kg and ovary weights by 26% at 100

Table 2 Toxicity findings for 4-chlorophenol in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)		
	0	60	100†	300	0	100	500
Male							
General behavior							
Tremors	0/12	0/12	0/4	12/12	0/12	0/6	12/12
Tachypnea	0/12	0/12	0/4	0/12	0/12	0/6	11/12
Salivation	0/12	0/12	0/4	0/12	0/12	0/6	9/12
Histopathology							
Kidney	0/6	0/6	no data	0/6	0/6	0/6	0/6
Liver	0/6	0/6	no data	0/6	0/6	0/6	0/6
Female							
General behavior							
Tremors	0/12	0/12	0/4	12/12	0/12	0/6	11/12
Tachypnea	0/12	0/12	0/4	0/12	0/12	0/6	9/12
Salivation	0/12	0/12	0/4	0/12	0/12	0/6	8/12
Histopathology							
Kidney	0/6	0/6	no data	0/6	0/6	0/6	0/6
Liver	0/6	0/6	no data	0/6	0/6	0/6	0/6

Data are numbers of animals with the change of the total examined. All newborn males and 3/4 females died at 500 mg/kg in the dose-finding study. †indicates dose and data from the dose-finding study.

Table 3 Major toxicity findings for p-(α,α-dimethylbenzyl) phenol in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)			
	0	30	100	300	0	100	300	1000
Male								
Dead or moribund	0/12	0/12	0/12	0/12	0/14	0/7	0/7	3/14
ALT, γ-GTP	/	–	–	–	/	–	–	↑
BUN, Creatinine	/	–	–	–	/	–	–	↑
Relative liver weight	/	–	–	–	/	–	↑	↑
Relative kidney weight	/	–	–	↑	/	–	–	↑
Stomach, hyperplasia	0/6	0/6	0/6	0/6	0/7	0/7	0/7	1/6
Liver, proliferation bile ducts	0/6	0/6	0/6	0/6	0/7	0/7	0/7	6/6
Kidney, regeneration	0/6	0/6	0/6	0/6	3/7	3/7	5/7	6/6
Kidney, dilatation	0/6	0/6	1/6	6/6	0/7	0/7	0/7	6/6
Female								
Dead or moribund	0/12	0/12	0/12	0/12	0/14	0/7	0/7	1/14
ALT, γ-GTP	/	–	–	–	/	–	–	↑
BUN, Creatinine	/	–	–	↑,–	/	–	–	–
Relative liver weight	/	–	–	–	/	–	–	↑
Relative kidney weight	/	–	–	↑	/	–	–	↑
Stomach, hyperplasia	0/6	0/6	0/6	0/6	0/7	0/7	0/7	3/7
Liver, proliferation bile ducts	0/6	0/6	0/6	0/6	0/7	0/7	0/7	7/7
Kidney, regeneration	0/6	0/6	0/6	0/6	0/7	1/7	0/7	7/7
Kidney, dilatation	0/6	0/6	2/6	6/6	0/7	0/7	0/7	4/7

Only critical data are shown in this table. Data are numbers of animals with the change of the number examined. Slashes and bars mean no statistical significance as compared to controls. ↑ indicates significant increase at $P < 0.05$. Relative kidney weights were increased 2.5- and 2.1-fold for males and females at 300 mg/kg in the newborn study. For the young study, 14 males and 14 females (half for examination of recovery) were assigned to each group but 6 males and 7 females at 1000 mg/kg were re-assigned for 28-day examination because of deaths.

and 300 mg/kg. The absolute ovary weights were still lowered by 32% at 300 mg/kg after the recovery-maintenance period. Increased numbers of atretic follicles were found in ovaries of half of the females at 300 mg/kg at the end of the dosing period, and most females continued to show various changes such as decreased numbers of corpa luteua in the ovaries and hypertrophy of endometrial epithelium in the uteri, after the recovery-maintenance period.

In the young study, two males and one female died, and one male was killed in a moribund condition at 1000 mg/kg. The final body weights were reduced by 18%, limited to males. On urinalysis, both sexes showed irregularly sized particles of a black substance, accompanied by 2–4 fold elevation of urine volume. Clear changes of several biochemical parameters such as ALT, γ-GTP, BUN, and creatinine, increases of relative liver and kidney weights, and histopathological changes in the forestomach (squamous hyperplasia), liver (bile duct proliferation), and kidney (regeneration of tubular epithelium and dilatation of tubules) were also observed at 1000 mg/kg. A dose of 300 mg/kg was considered to cause slight toxicity, because the abnormal urinary contents described above were found in half of both sexes and a slightly elevated incidence of mild regeneration of the tubular epithelium was noted in male kidneys. After the two-week recovery period, the pathological changes in male kidneys at 1000 mg/kg continued to be evident. There were no signs of toxicity at 250 and 500 mg/kg in the dose-finding study although the administration period was only half and urinalysis and histopathological examinations were not performed.

The pNOAEL of 30 mg/kg/day for newborn rats is clear and one of 100 mg/kg/day for young rats is reasonable because of slight toxicity at 300 mg/kg in the main study and limited information at 250 mg/kg in the dose-finding study. Toxicity for newborn rats was evident at 300 mg/kg as all animals of both sexes showed histopathological changes in kidneys, with increased relative weights. However, the degree of toxicity for young rats at 1000 mg/kg was obviously much stronger than that of newborn rats at 300 mg/kg, which appeared to be equivalent to doses of 700–800 mg/kg in young rats. Therefore, pUFTLs of 300 and 700–800 mg/kg/day may be appropriate for newborn and young rats, respectively. It should be specially noted that this chemical may have endocrine disrupting properties, especially against females, when given only during the suckling phase.

(Hydroxyphenyl)methyl phenol (Table 4)

The newborn investigation was conducted at doses of 0, 20, 60, and 200 mg/kg for dose-finding and 0, 16, 40, and 100 mg/kg for the main study. The young study was conducted at doses of 0, 100, 500, and 1000 mg/kg for dose-finding and 0, 8, 40, 200, and 1000 mg/kg for the main study.

Common changes were limited to depression of body weight and death at high doses in newborn and young rats. The highest dose of 100 mg/kg in the newborn main study did not cause any changes, but half the animals at 200 mg/kg in the newborn dose-finding study died, without accompanying liver weight changes in surviving

Table 4 Major toxicity findings for (hydroxyphenyl)methyl phenol in the newborn and young rat main studies

	Newborn study (mg/kg)			Young study (mg/kg)			
	0	100	200†	0	40	200	1000
Male							
Dead or moribund	0/12	0/12	3/6	0/12	0/12	0/12	0/12
Final body weight	/	–	↓	/	–	–	↓
Total cholesterol	/	–	↑	/	–	–	↓
Relative liver weight	/	–	–	/	–	–	↑
Stomach, hyperplasia	0/6	0/6	no data	0/6	0/6	0/6	6/6
Liver, centrilobular hypertrophy	0/6	0/6	no data	0/6	0/6	2/6	4/6
Female							
Dead or moribund	0/12	0/12	3/6	0/12	0/12	0/12	1/12
Final body weight	/	–	(↓)	/	–	–	(↓)
Total cholesterol	/	–	–	/	↓	↓	↓
Relative liver weight	/	–	–	/	–	↑	↑
Stomach, hyperplasia	0/6	0/6	no data	0/6	0/6	0/6	6/6
Liver, centrilobular hypertrophy	0/6	0/6	no data	0/6	0/6	0/6	4/6

Only critical data are shown in this table. † indicates a dose from the dose-finding study. Numbers are for animals with the feature in the total examined. Slashes and bars mean no statistical significance as compared with controls. ↑ indicates significant increase $P < 0.05$. ↓indicates significant decrease at $P < 0.05$. () indicates that statistical significance was not obtained. Final body weights of surviving newborn males at 200 mg/kg in the dose-finding study were reduced by 30% (14% for females, not significant), respectively. Final body weights of young male rats at 1000 mg/kg in the main study were decreased by 11.8% (5.7% for females, not significant). Increase of relative liver weights was 13% in females at 200 mg/kg, and 16 and 27% in males and females at 1000 mg/kg in the young main study.

animals. There were no chemical-related changes with other examinations, including developmental parameters. In the young study, one female became moribund and the final body weights of males were decreased at 1000 mg/kg. All animals of both sexes at this dose showed squamous hyperplasia of the forestomach or limiting ridge with ulceration, and two-thirds of the animals featured centrilobular hypertrophy of hepatocytes with decrease of total cholesterol (29–51% drop) and increase of relative liver weight. At 200 mg/kg, low incidences of centrilobular hypertrophy in the livers of males and slight increase of liver weights in females with low total cholesterol (45% drop) were found. No toxicity was apparent at 40 mg/kg in the main study. No toxicity was also found at 100 mg/kg in the dose-finding study, but a histopathological examination was not conducted. There were no abnormalities on hematological examination and urinalysis at any dose.

The pNOAEL is considered to be 100 mg/kg/day for newborn rats and 40 mg/kg/day may be appropriate for young rats because of the limited information at 100 mg/kg in the dose-finding study. Although toxicity at 1000 mg/kg for young rats was evident, the dose inducing the same effects in newborn rats was clearly less than 200 mg/kg, because half of the animals died at this dose. We speculate that the dose range for one death in 12 newborn rats would be within 140–160 mg/kg. It is clear that the dose–response curve is much steeper for newborn than young rats. Based on our consideration, pUETLs of 140–160 and 1000 mg/kg/day may be equivalent for newborn and young rats, respectively.

Trityl chloride (Table 5)

The newborn investigation was conducted at doses of 0, 20, 60, 200, and 600 mg/kg for dose-finding and 0, 12, 60, and 300 mg/kg for the main study. The young investigation was conducted at doses of 0, 30, 100, 300, and 1000 mg/kg for dose-finding and 0, 12, 60, and 300 mg/kg for the main study.

Common effects were observed in livers of newborn and young rats. In the newborn study, increase of relative liver weights were shown at 60 mg/kg and more in both sexes and centrilobular hypertrophy of hepatocytes was noted in 300 mg/kg females. In the dose-finding newborn study, one female died and increase of relative liver weights of both sexes at 600 mg/kg was more evident with low body weights (11.3% drop for males, 13.8% for females). There were no chemical-related changes with other examinations, including developmental parameters. In the young study, both sexes at 60 mg/kg showed a high incidence of centrilobular hypertrophy of hypetocytes with limited increases of relative liver weights (10–14%). At 300 mg/kg, soft feces and mucosal thickening of cecum in most animals were observed in addition to more extensive hepatic changes. Although relative kidney weights were increased at 300 mg/kg in males and 60 and 300 mg/kg in females, there were no renal histopathological findings. Hematological and blood chemical examinations revealed several slight to moderate changes (56% as the maximum) in fibrinogen, ALT, total cholesterol and glucose, as well as prolongation of prothrombin and activated thromboplastin times, at 300 mg/kg.

pNOAELs of 60 and 12 mg/kg/day for newborn and young rats appear appropriate because of the lack of information at higher doses in the dose-finding study, which showed no toxicity but without histopathological examination. The dose of 300 mg/kg in the young main study was a clear toxic level, but intensity was much stronger than that at 300 mg/kg in the newborn main study, while less that at 600 mg/kg in the dose-finding study. Based on these data, the toxicity with 300 mg/kg for young rats is considered to be within the range with 400–500 mg/kg for newborn rats.

Table 5 Major toxicity findings for trityl chloride in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)			
	0	60	300	600†	0	12	60	300
Male								
Death	0/12	0/12	0/12	0/6	0/12	0/6	0/12	0/12
Final body weight	/	–	–	↓	/	–	–	↓
ALT, Total cholesterol	/	–	–	–	/	–	–	↑
Relative liver weight	/	↑	↑	↑	–	–	↑	↑
Relative kidney weight	/	–	–	–	–	–	–	↑
Cecum, thickening	0/6	0/6	0/6	no data	0/6	0/6	0/6	5/6
Liver, centrilobular hypertrophy	0/6	0/6	0/6	no data	0/6	0/6	3/6	6/6
Female								
Death	0/12	0/12	0/12	1/6	0/12	0/6	0/12	0/12
Final body weight	/	–	–	↓	/	–	–	–
ALT, Total cholesterol	/	–	–	–	/	–	–	-,↑
Relative liver weight	/	↑	↑	↑	–	–	↑	↑
Relative kidney weight	/	–	–	–	–	–	↑	↑
Cecum, thickening	0/6	0/6	0/6	no data	0/6	0/6	2/6	5/6
Liver, centrilobular hypertrophy	0/6	0/6	4/6	no data	0/6	0/6	5/6	6/6

Only critical data are shown in this table. †indicates a dose from the dose-finding study. Numbers are for animals with the feature in the total examined. Slashes and bars mean no statistical significance as compared to controls. ↑ indicates significant increase $P < 0.05$. ↓indicates significant decrease at $P < 0.05$. Relative liver weights were increased by 11% for males and 8% for females at 60 mg/kg, and 29% for both sexes at 300 mg/kg in the newborn main study and by 44% for males and 46% for females at 600 mg/kg in the newborn dose-finding study. Body weight depression in males (13%) and an increase of relative liver weights (32% for males, 40% for females) were observed at 300 mg/kg in the young main study.

Therefore, pUETLs of 400–500 and 300 mg/kg/day are proposed as appropriate for newborn and young rats, respectively.

1,3,5-Trihydroxybenzene (Table 6)

The newborn investigation was conducted at doses of 0, 100, 500, and 1000 mg/kg for dose-finding and at 0, 20, 100, and 500 mg/kg for the main study. The young investigation was conducted at doses of 0, 100, 250, 500, and 1000 mg/kg for dose-finding and at 0, 30, 100, 300, and 1000 mg/kg for the main study.

Common changes were observed in the thyroids and liver. The only toxic change in newborn main study was hypertrophy of thyroid follicular cells with increase in relative thyroid weights in both sexes at 500 mg/kg. Increased relative liver weights in females were not accompanied by any histopathological changes. Although decrease of adrenal weight and histopathological alterations such as vacuolization and pigmentation were noted at the end of the dosing and recovery-maintenance periods, these were always slight and not dose-dependent. There were no chemical-related changes with other examinations, including developmental parameters, in newborn rats. In the young study, similar effects on the thyroids and liver were found at 1000 mg/kg, but the incidence of thyroid histopathological changes was slightly less than in newborn animals at 500 mg/kg.

pNOAELs of 100 and 300 mg/kg/day for newborn and young rats can be considered appropriate because of the lack of data with dose settings between 100 to 500 mg/kg in the newborn, and no histopathological examination at 500 mg/kg in the young dose-finding study. The degree of toxicity at 1000 mg/kg for young rats was almost equal to that at 500 mg/kg for newborn rats. Therefore, pUETLs of 500 and 1000 mg/kg/day are proposed as equivalents for newborn and young rats, respectively.

DISCUSSION

More than 100 000 industrial chemicals are now in use around the world and sufficient toxicity information is available for only a small proportion. The Japanese government started the Existing Chemical Safety Program to obtain minimal toxicity data sets from 28-day toxicity studies using young rats for high production volume chemicals lacking toxicity information. For the present six targeted chemicals, we found toxicity information for only two chemicals by literature search. Daniel *et al.* (1993) reported no toxic effects of 2-chlorophenol on oral administration to male and female Sprague Dawley rats at up to 257 mg/kg for 10 days or 150 mg/kg for 90 days. Our results were consistent with their data, as we found no toxicity at 500 mg/kg in young dose-finding study (14 days administration) and at 200 mg/kg in the young study (28 days), while further providing information on CNS effects at higher doses. As for (hydroxyphenyl)methyl phenol, consisting of bisphenol D, E, and F isomers, bisphenol F has been reported to have estrogenic potential evidenced by several *in vitro* and *in vivo* experiments (Hashimoto *et al.* 2001; Yamasaki *et al.* 2002; Stroheker *et al.* 2003). However, we could not establish any such activity in this study. Our results are reasonable because oral administration of bisphenol F increased relative uterus weights only at more than 100 mg/kg, but not 50 mg/kg given during PNDs 22–25 (Stroheker *et al.* 2003), while our highest dose of (hydroxyphenyl)methyl phenol was equivalent to 30 mg/kg of bisphenol F.

Table 6 Major toxicity findings for 1,3,5-trihydroxybenzene in the newborn and young rat main studies

	Newborn study (mg/kg)			Young study (mg/kg)		
	0	100	500	0	300	1000
Male						
Relative organ weight						
Liver	/	–	–	/	–	↑
Thyroids	/	–	↑	/	–	(↑)
Histopathology						
Liver	0/6	0/6	0/6	0/6	0/6	0/6
Thyroids, hypertrophy	0/6	0/6	4/6	0/6	0/6	2/6
Female						
Relative organ weight						
Liver	/	–	↑	/	–	↑
Thyroids	/	–	(↑)	/	–	(↑)
Histopathology						
Liver	0/6	0/6	0/6	0/6	0/6	0/6
Thyroids, hypertrophy	0/6	0/6	5/6	0/6	0/6	4/6

Only critical data are shown in this table. Slashes and bars mean no statistical significance as compared with controls. ↑ indicates significant increase $P < 0.05$ (except in parentheses where statistical significance was not attained). Numbers are for animals with the feature in the total examined. Increase of relative organ weights at 500 mg/kg in the newborn main study was observed for thyroids (39% for males, 24% for females) and liver (9% for females). Increase of relative organ weights at 1000 mg/kg in the young main study was observed for thyroids (14% for males, 19% for females) and liver (23% for males and 9% for females).

Table 7 Comparative susceptibility of newborn and young rats to the six chemicals

	Newborn study		Young study		pNOAEL	pUETL
	pNOAEL	pUETL	pNOAEL	pUETL	Young/Newborn	Young/Newborn
	mg/kg/day		mg/kg/day			
2-Chlorophenol	40	200–250	200	1000	5.0	4.0–5.0
4-Chlorophenol	100	300	100	500	1.0	1.7
p-(α,α-Dimethylbenzyl) phenol	30	300	100	700–800	3.3	2.3–2.7
(Hydroxyphenyl) methyl phenol	100	140–160	40	1000	0.4	6.3–7.1
Trityl chloride	60	400–500	12	300	0.2	0.6–0.8
1,3,5-Trihydroxybenzene	100	500	300	1000	3.0	2.0

Although there has been no reports for p-(α,α-dimethylbenzyl) phenol, it causes endocrine disruption and possible antiestrogenic activity, when administered to newborn female rats in this study. Therefore, further studies on this chemical should be conducted to elucidate the mechanisms, because the present investigation did not indicate any effects on sexual differentiation such as preputial separation, vaginal opening and the estrous cycle.

For our focus on the comparative sensitivity of newborn and young rats to chemicals, two toxicity endpoints, pNOAEL and pUETL, were newly defined as appropriate, considering the entire data sets from both main and dose-finding studies. We believe that this alternative assessment approach allowed us to make more realistic comparisons between newborn and young rats under the same experimental conditions as far as possible.

The ratios of pNOAELs for chemicals between newborn and young rats may provide an additional UF value in risk assessment according to susceptibility of newborn rats, because regulatory limit values for chemicals to protect public health of humans, including infants, are derived from the division of NOAEL by UFs. The data in Table 7 indicate newborn rats to be 1–5 times more susceptible to four of the tested chemicals, 2- and 4-chlorophenols, p-(α,α-dimethylbenzyl) phenol and 1,3,5-trihydroxybenzene, than young rats in terms of the pNOAELs, similar to the results of previous analyzes of five phenolic chemicals, 4-nitro-, 2,4-dinitro-, 2,4,6-trinitro-, 3-methyl- and 3-amino-phenols (Koizumi *et al.* 2001, 2002, 2003; Takahashi *et al.* 2004). Immaturity in the detoxification potential of phase 1 and phase 2 enzymes in newborn animals may be the major cause of higher toxicity in newborn rats (Rich & Boobis 1997; Gow *et al.* 2001), because these chemical classes are probably direct toxicants. In the case of (hydroxyphenyl)methyl phenol, the pNOAEL (100 mg/kg/day) for newborn rats was 2.5 times higher than that (40 mg/kg/day) for young rats, but it can be speculated that values are in practice rather similar because the toxicity for young rats at the high dose, 200 mg/kg, was only slight (Table 4). As for trityl chloride, newborn rats were obviously less susceptible (0.2 for the pNOAEL ratio). Similar results were

also reported from our previous analysis for bromoalkanes (Hirata-Koizumi *et al.* 2005) and may be explained by mechanisms of action and metabolic characteristics of newborn rats. As this class of chemicals possibly requires metabolism to act as toxicants, the relatively mature metabolic enzyme status of young rats would be expected to provide toxic intermediates by metabolic activation to a greater extent than in newborn rats, as evidenced by data for previously reported chemicals (Onkenhout *et al.* 1986; Kennedy *et al.* 1993). Other compounds such as acetaminophen, bromobenzene, and carbon tetrachloride have also been shown to not produce liver injury in neonatal animals at doses that are hepatotoxic to adults (Gregus & Klaassen 1998).

The ratios of pUETLs, doses inducing the same degree of toxicity in newborn and young rats, were almost the same as for pNOAELs with the direct toxicants, as shown in Table 7. However, newborn rats were considerably more susceptible to (hydroxyphenyl)methyl phenol when considering the pUETL, due to the much steeper dose–response curve in newborn rats, with a 100 mg/kg/day pNOAEL and half the animals dying at 200 mg/kg, compared with a 40 mg/kg/day pNOAEL and only one death in 12 animals at 1000 mg/kg for young rats. Although young rats showed stomach hyperplasia in addition to hepatotoxicity at 1000 mg/kg, the cause of newborn deaths at 200 mg/kg was unclear. With regard to trityl chloride, the pUETL for young rats was almost the same as for newborn although the latter were less susceptible. Such an anomaly has also been found for bromoalkanes previously analyzed. Another example of a chemical for which susceptibility differs at low and high doses is chlorpyrifos, the maximum tolerated dose in 17-day-old rats being reported to be five times less than that in adults following oral exposure (Moser & Padilla 1998), but the differential sensitivity not appearing in low-dose exposure (Pope & Liu 1997). Thus as there are several chemicals of which dose–response curve in newborn rats was obviously steeper than that in young rats, pUETL ratios should be also taken into account for the susceptibility of newborn rats as the second endpoint marker.

In conclusion, newborn rats were 2–5 times more susceptible than young rats in terms of both the pNOAEL and the pUETL in most cases. One exception was that young rats were clearly more susceptible than their newborn counterparts for trityl chloride.

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Peroxisome proliferator-activated receptor α plays a vital role in inducing a detoxification system against plant compounds with crosstalk with other xenobiotic nuclear receptors

Kiyoto Motojima and Toshitake Hirai

Department of Biochemistry, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan

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Detoxification; drug–drug interaction; PPAR; P450; xenobiotic nuclear receptor

Correspondence
K. Motojima, Department of Biochemistry, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan
Tel./Fax: +81 424 95 8474
E-mail: motojima@my-pharm.ac.jp

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Peroxisome proliferator-activated receptor α (PPARα) is thought to play an important role in lipid metabolism in the liver. To clarify the extrahepatic and/or unknown function of PPARα, we previously performed a proteome analysis of the intestinal proteins and identified 17β-hydroxysteroid dehydrogenase type 11 as a mostly induced protein by a PPARα ligand [Motojima, K. (2004) *Eur. J. Biochem.* 271, 4141–4146]. Because of its supposed wide substrate specificity, we examined the possibility that PPARα plays an important role in inducing detoxification systems for some natural foods by feeding mice with various plant seeds and grains. Feeding with sesame but not others often killed PPARα knockout mice but not wild-type mice. A microarray analysis of the sesame-induced mRNAs in the intestine revealed that PPARα plays a vital role in inducing various xenobiotic metabolizing enzymes in the mouse intestine and liver. A PPARα ligand alone could not induce most of these enzymes, suggesting that there is an essential crosstalk among PPARα and other xenobiotic nuclear receptors to induce a detoxification system for plant compounds.

According to the generally accepted view, peroxisome proliferator-activated receptor α (PPARα) plays an important role in lipid catabolism in the liver [1]. However, this view has been established mainly by the studies carried out using rodent models where PPARα is overexpressed in the liver [2], and there is a possibility that our knowledge on the physiological role of PPARα is biased against its extra-hepatic functions. In humans, it is known that PPARα is highly expressed in the bladder, colon, heart and muscle, with the levels being higher or comparable with that in the liver (http://www.ncbi.nlm.nih.gov/niGene/ESTProfileViewer.cgi?uglist=Hs.275711). To clarify the extrahepatic function of PPARα, we performed a differential proteome analysis of the proteins induced in the mouse intestine by a PPARα ligand in a receptor dependent manner, and we found that 17β-hydroxysteroid dehydrogenase type 11 (17β-HSD11) was much more efficiently induced in the intestine than in the liver by a PPARα ligand, Wy-14 643 [3]. Because of the wide substrate specificity of 17β-HSDs [4,5], we have been interested in the possibility that 17β-HSD11 in the epithelium of the intestine metabolizes potentially toxic compounds included in the natural diet [6], and that PPARα plays an essential role in the induction.

In the present study, we screened plant grains and seeds to identify a possible source of toxic compounds to induce 17β-HSD11 in the intestine by feeding PPARα wild-type and knockout mice as the natural

Abbreviations
Ah, aromatic hydrocarbon; AKR, aldo-keto reductase; CAR, constitutive androstane receptor; CTE-1, cytosolic thioesterase I; Cyp, cytochrome P450; DR, direct repeat; GST, glutathione S-transferase; HSD, hydroxysteroid dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; PPAR, perisome proliferator-activated receptor; PXR, pregnane X receptor; UGT, UDP-glucuronosyltransferase.

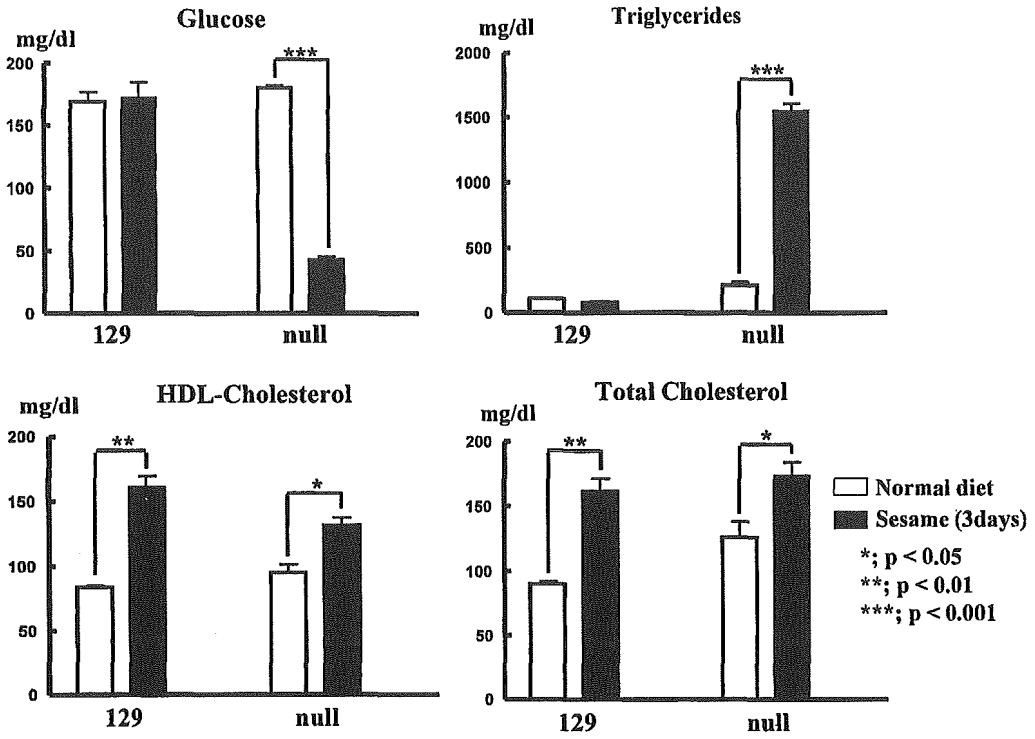

Fig. 1. Metabolic responses to the sesame diet are altered in PPARα null mice. The serum levels of glucose, total cholesterol, HDL-cholesterol and triglycerides were determined in PPARα null (Null) and age-matched (5 weeks) 129/J (129, wild type) male mice fed with a normal laboratory diet (open bar) or sesame seeds (closed bar) for three days. Results are mean ± S.E. of four animals in each group. Statistical evaluation was performed with analysis of two-way ANOVA.

diet. Unexpected observation in the present study is that sesame caused severe faulty lipid metabolism often leading the knockout mice to death. Proteome and transcriptome analyses showed that sesame induced several detoxification enzymes including 17β-HSD11 in the intestine and liver in either a PPARα-dependent or -independent manner. Our new approach revealed a new and essential physiological role of PPARα beyond its important role in energy metabolism.

Results and Discussion

Knocking out of PPARα has not been reported to be lethal to mice under various experimental conditions [7,8]. Because these experiments were carried out using laboratory diets, we considered the possibility that some natural foods might contain compounds that can be detoxified by the induced 17β-HSD11. To test this idea, pairs of wild-type and PPAR-null mice [7] were separately fed with several kinds of natural grains or seeds for one week. Some plant foods differentially affected a little and others largely on the serum parameters, such as glucose, triglycerides, and cholesterol levels, between wild-type and PPARα-null mice but all survived after one week4 treatment except the PPARα-null mice fed with sesame. Feeding with sesame often killed PPARα null mice in four to five days. At day 3 after starting the sesame diet, metabolic responses in PPARα null mice were remarkably different from those in wild-type mice as shown in Fig. 1. In addition to a large increase in the levels of triglycerides, a significant decrease in the glucose levels were observed. The glycogen in the liver of the null mice was also decreased to less than 1 $ng \cdot mg^{-1}$ tissue (in contrast to 10–15 $ng \cdot mg^{-1}$ with wild-type mice fed with sesame) although their liver was extremely fatty. Essentially the same results were obtained with various brands of raw sesame on the market. Utilization of fatty acids as an energy source and gluconeogenesis in the liver of the knockout mice seemed to be blocked by an unknown mechanism and we conceive that the cause of death would be hepatotoxicity and/or hypoglycemia. Actually feeding the mice with sesame caused hepatotoxicity as indicated by measuring the plasma alanine trasaminase (ALT) activities. At day 3 after starting the diet, ALT activities went up from 12.5 ± 5.0

(IU/L, ± S. D) to 26.3 ± 2.5 in wild-type mice and from 13.8 ± 4.8 to 253.3 ± 169.3 in PPARα null mice. However, it is not known at present whether it was a direct cause of death or not. In any case, it had not been observed that PPARα plays a vital role at the whole body level of mice under certain natural conditions until we fed the knockout mice with plant grains and seeds instead of laboratory test diets.

To examine whether feeding with sesame induced 17β-HSD11 and others not, the intestinal and liver proteins of mice fed with various plant seeds were examined by western blotting. As shown in Fig. 2, a low level expression of 17β-HSD11 was detected only in the intestinal protein sample from the wild-type mice fed with sesame, but all the plant seeds induced various levels of 17β-HSD11 in the liver during this period. A low level expression of the enzyme in the intestine was also observed in the intestine of the knockout mice (Fig. 2C), indicating that expression of 17β-HSD11 is regulated not only by PPARα but also by other unknown factors. These data suggested that the lethal effect of sesame on PPARα knockout mice cannot be simply explained by the lack of PPARα-dependent induction of 17β-HSD11.

In addition to 17β-HSD11, SDS/PAGE analysis of the proteins from the intestine of the mice fed with sesame showed a strongly induced protein band having a molecular weight of 24 kDa both in wild and PPARα-null mice (Fig. 3A). Peptide mass fingerprinting analysis of the digested 24 kDa protein-derived peptides showed that the masses of 7 among 20 peptides were consistent with those calculated from the peptide sequences from glutathione S-transferase μ1 (GST'1) (Accession NP_034488.1), and the masses of 5 peptides matched those from GSTμ3 (Accession NP_034489.1) (Fig. 3B). The induction of 17β-HSD11 and GSTμ1, μ3 proteins in the intestine of both wild-type and PPARα null mice was confirmed by Northern blot analysis (not shown). Thus the induction of 17β-HSD11 and GSTs by sesame was also observed in PPARα null mice and this conclusion did not directly match our first speculation that PPARα-inducible 17β-HSD11 in the intestine played a critical role in detoxification of toxic compounds in foods.

The above results indicated that other PPARα-dependent pathways are vital for detoxification and led us to perform transcriptional profiling studies with RNA isolated from the intestines of wild-type male mice fed with sesame for one week in comparison with control RNA from mice fed a normal laboratory diet. The relevant mRNAs that were detected as having been induced by feeding with sesame in the intestine using Agilent's Whole Mouse Genome Oligo micro-

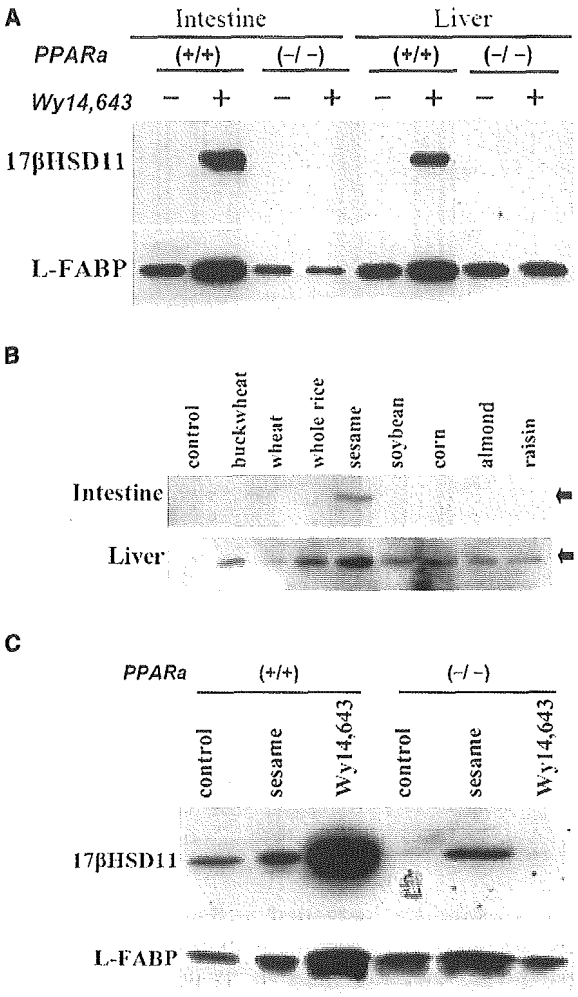

Fig. 2. 17β-HSD11 is induced in mouse liver and intestine by a PPARα agonist Wy-14 643 and by sesame seeds. A,B: Immunoblot analysis of 17β-HSD11 induction in the mouse liver and intestine by Wy-14 643 or by various plant seeds and grains. Normal mice were fed with a control diet, a diet containing 0.05% Wy-14,643, or untreated various plant seeds and grains for 7 days. The postnuclear fractions of the tissues were separated by SDS/PAGE and probed with anti17β-HSD11 antibody or control anti-(L-FABP) antibody. C: Induction of 17β-HSD11 in the intestine of PPARα knockout mouse by sesame. The levels of induction of 17β-HSD11 in the intestine were compared between the mice fed with a diet containing Wy-14 643 and those fed with sesame.

array are listed in Table 1. As predicted, many mRNAs involved in the lipid/xenobiotic metabolism and stress/inflammation [9–11] showed increased levels; several subfamily members of Cyp2c and other types of Cyps, oxidative enzymes, phase II detoxification enzymes such as UGTs, AKRs, GSTs, transporters, heat shock proteins and resistin. The first identified UGT1A9 as a PPARα and PPARα target

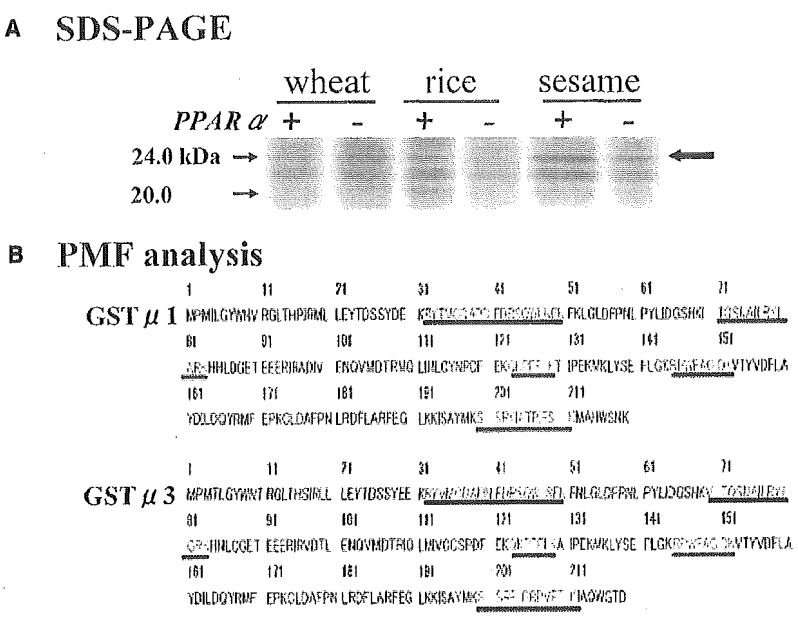

Fig. 3. Sesame-induced 24 kDa proteins are glutathione-S-transferases. The intestinal proteins were separated by SDS/PAGE and the sesame-induced 24 kDa protein band (A) was analyzed by peptide mass fingerprinting after digestion by lysyl-endopeptidase. Masses of the peptides underlined were matched with those obtained by MALDI-TOF mass spectrometry (B).

gene [12], however, was not induced by sesame. Fatty aldehyde dehydrogenase (Aldh3a2), that has been proposed as a key component of the detoxification pathway of aldehydes arising from lipid peroxidation events [13], was not induced in the intestine either. The induced mRNA profile was completely different from those recently reported as induced in rat liver by sesamine, a functional lignan in sesame. Kiso *et al.* described the increase in the levels of a set of lipid- and alcohol-metabolizing enzyme mRNAs including Cyp4A1, Cyp2B1,2 and aldehyde dehydrogenase 1A1, 7 subfamily members [14,15]. These differences suggest that the changes detected in this study had been induced not by sesamine but by other unidentified molecules in sesame.

To confirm the sesame-induced changes in the levels of several mRNAs detected by a microarray analysis and to examine their dependency on PPARα, total RNA from the intestines and livers of wild-type and PPARα null mice fed with control diet or sesame was analyzed by Northern blotting using each specific cDNA mostly corresponding to the 3′-noncoding region of respective mRNA. As shown in Fig. 4, robust induction of several Cyp2c and Cyp2b members by sesame both in the intestine and liver was confirmed, and it completely disappeared in PPARα null mice, indicating that PPARα played an essential role in induction of these Cyps by sesame.

However, the increases in the levels of other detoxifying enzyme mRNAs such as Cyp4a10, Cyp3a44, UGT2b5 and 2b37, and AKR1b8 and 1b7 were not so significant as observed in the array analysis. Induction of Cyp4a10, PDK4, and CTE-1 mRNA [16,17] was far less than that by a PPARα agonist Wy-14 643. 17β-HSD11 was an extreme example, because its increase at the protein level was detected by western blotting (Fig. 2b) but its mRNA was not revealed by the array analysis (Table 1) and the increase in mRNA level was not evidently confirmed by Northern blotting (Fig. 4). Thus the comprehensive analysis employed in this study alone may not collect all the molecular changes induced by feeding sesame and the critical PPARα-dependent transcriptional event leading to the sesame-induced death remains unclear. It is of interest that Shankar *et al.* reported a possible role of PPARα activation in hepatoprotective response against hepatotoxicants under the diabetic condition [18]. If so, PPARα may be involved not only in the induction of detoxification system but also in further adaptive steps.

Sesame seeds, like other botanicals [19–21], should contain a large number of compounds that affect cell function via gene transcription or metabolic inhibition. Further detailed transcriptional profiling coupled with differential metabolome analysis of the whole metabolites between wild-type and PPARα null mice are in progress in our laboratory and collaborating laboratories.

Interestingly, sesame strongly induced Cyp2c29, 2c38, and 2b9 in the intestine and liver in a PPARα-dependent manner, but a PPARα ligand Wy-14 643 had no effect at all although the known PPARα target

Table 1. Genes induced in wild-type mouse intestine by sesame.

Accession no.	Protein name (symbol)	Fold change
Lipid/Xenobiotic metabolism and transport		
gb|D17674	Cyp2c29	8.5
gb|AF047725	Cyp2c38	7.6
gb|BC057912	Cyp2c37	5.6
gb|BC057911	Cyp2c39	5.6
gb|U04204	Aldo-ketoreductase family 1, member B8 (Akr1b8)	5.2
gb|BC028261	Cytosolic acyl-CoA thioesterase (Cte1)	5.2
gb|BC022752	Solute carrier family 37, member 2	4.5
ref|NM_053215	UDP-glucuronosyltransferase family 2, memberB37(Ugt2b37)	4.5
gb|AK008688	Cyp2c18	3.7
gb|X06358	UDP-glucuronosyltransferase family 2, member 5 (Ugt2b5)	3.7
gb|BC010824	Cyp2c55	3.5
gb|NM_010000	Cyp2b9	3.2
gb|AK002528	Cyp4a10	2.7
gb|AB039380	Cyp3a44	2.7
ref|NM_008181	Glutathione S-transferase, alpha 1 (Gsta1)	2.6
gb|AF231120	Solute carrier family 40, member 1 (Slc40a1)	2.5
gb|M21856	Cyp2b10	2.5
gb|M21855	Cyp2b13	2.5
gb|J05663	Aldo-ketoreductase family 1, member B7 (Akr1b7)	2.4
gb|BC054119	Solute carrier family 16, member 9 (Slc16a9)	2.3
gb|X99715	Cyp2b20	2.2
gb|D42048	Squalene epoxidase (Sqle)	2.1
gb|BC028535	Glutathione S-transferase 2 (Gst2)	2.1
gb|BC009805	Glutathione S-transferase, alpha 3 (Gsta3)	2.0
gb|AK003312	Retinol binding protein 2, cellular (Rbp2)	2.0
Proteases		
gb|BC056210	Elastase 3B (Ela3b)	6.5
ref|NM_011645	Trypsin 3 (Try3)	5.4
gb|XM_133021	Carboxypeptidase A2	4.1
gb|X04574	Serine protease 2 (Prss2)	3.9
gb|AK038356	Serine protease 7 (Prss7)	3.0
gb|AB016228	Chymotrypsin-like (Ctrl)	2.6
Stress/Inflammation		
gb|M12571	Heat shock protein (hsp68)	6.3
gb|BC054782	Heat shock protein 1 A	5.4
gb|AJ536019	Resistin-like gamma (Retnlg)	3.2
gb|AK005475	DnaJ (Hsp40) homologue, subfamily B, member 9	2.6
Miscellaneous		
gb|AF028071	Calbindin 3 (Calb3)	9.5
gb|BC012221	Major urinary protein 1 (Mup1)	4.7
gb|BC026134	Pyruvate dehydrogenase kinase 4 (Pdk4)	3.8
gb|M29546	Malic enzyme, supernatant (Mod1)	3.2
gb|AF000581	Nuclear receptor coactivator 3 (Ncoa3)	2.7

genes such as Cyp4a10 and CTE-1 were activated in wild-type mice as expected. These data clearly show that the Cyp genes are not directly regulated by PPARα. Expressions of the corresponding human CYP2C9, 2B6 and 3A4 to these mouse Cyps were reported be regulated by the constitutive androstane receptor (CAR) [22,23]. Jackson *et al.* [24]. proposed an imperfect DR4 element as an essential element for CAR-dependent transcriptional activation of Cyp2c29 and 2b10 genes, although no detailed mechanism has yet been elucidated. Thus the indirect but essential involvement of PPARα in the induction of these Cyps can be at the activation step of CAR. The mouse CAR is localized in the cytosol, at least in the case of primary hepatocytes, and then activated after unknown complex processes. Further analysis is clearly

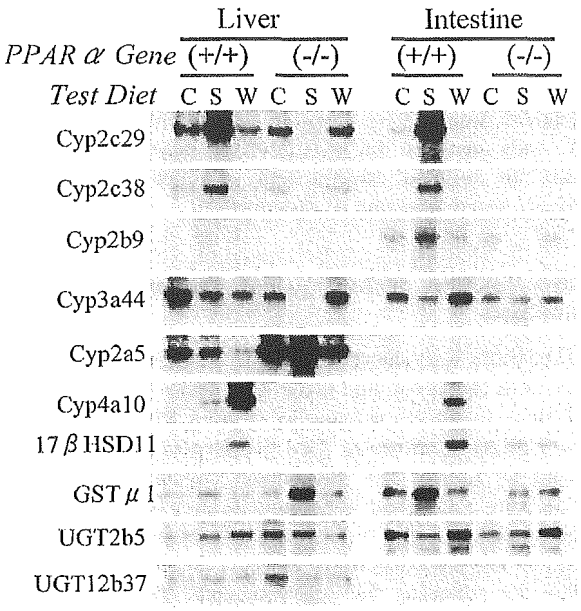

Fig. 4. Direct and indirect involvement of PPARα in induction of detoxifying enzyme mRNAs. Northern blot analysis of total RNA from the livers and intestine of wild-type or PPARα null mice fed either a control diet (C), sesame (S), or diet containing 0.05% Wy-14 643 (W) for three days. Representative data from several independent experiments are shown.

necessary to obtain direct evidence of the involvement of PPARα in the activation step of CAR. Another possibility for the indirect but essential involvement of PPARα in the induction is that some of them are regulated by overlapping transcriptional programs mediated by an axis of PPARα-RXR-LXR as suggested by Anderson *et al.* [25]. Our observations of indirect but essential involvement of PPARα in the transcriptional activation of several Cyp genes should provide an important clue to elucidate the activation processes and the complex network among the xenobiotic nuclear receptors [26–30].

At least some of the detoxifying enzymes in the intestine and liver must be induced by complex functional interactions among xenobiotic receptors. One receptor may be involved in producing the metabolites/ligands for the next receptor that will be involved in inducing the enzymes for further metabolism. Disturbance of this network by genetic mutation, transcriptional repression or metabolic inhibition should severely affect metabolism of xenobiotics and 'parabiotics' if it goes beyond compensating capacity coming from overlapping functions of metabolizing enzymes (Fig. 5). In addition to these phase I and phase II enzymes, phase III transporters play an important role in efflux mechanisms and their expression should be regulated similarly by the network of various nuclear receptors, although significant induction of phase III transporters by sesame was not observed by the microarray analysis in this study. Our present finding with sesame and PPARα knockout mice will be the first example of severe disturbance of the network leading to death by incomplete detoxification of natural compounds. The present data suggest an indirect interaction between PPARα and CAR, and further analysis of CAR-independent changes may reveal interactions between PPARα and other xenobiotic nuclear receptors.

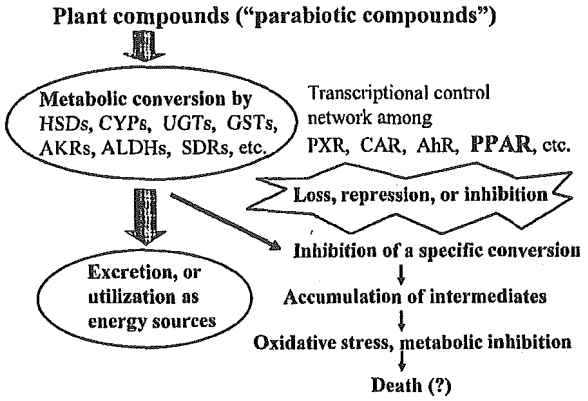

Fig. 5. A proposed model depicting the metabolic conversion of plant compounds in animals and the mechanism by which toxic molecules are produced.

In this study, we showed that PPARα is a xenobiotic receptor, in addition to PXR, CAR and Ah, playing an essential, direct and indirect role in inducing various xenobiotic metabolizing enzymes. Involvement of PPARα in the metabolism of 'parabiotic' substrates from plants as well as endobiotic substrates suggests its wider and more extensive role in energy metabolism from food intake to fat storage than that recently proposed [30]. Our approach to study the physiological role of so-called xenobiotic metabolizing enzymes by using natural foods can be applicable to those studies on other enzymes because most of these enzymes in animals should have evolved through the food chain, including various plants. In this connection, the species differences in the detoxifying systems especially between human and rodents may be explained by food differences between rodents' totally wild life and our agrarian civilization. Eating sesame, however, is common among rodents and humans, and a similar detoxifying system to that discovered in mice must be present in humans. We finally emphasize our finding that the intestine is an important organ for the 'parabiotic' metabolism, and the possibility that significant induction of several metabolizing enzymes by plant

foods in the intestine can occur also in humans. The corresponding human CYPs are well known as the most clinically important members to metabolize many prescribed drugs [31,32] and the possibility that expression of these CYPs not only in the liver but also in the intestine is vigorously regulated by plant foods should be carefully examined to understand food-drug and drug–drug interactions.

Experimental procedures

Animal studies and tissue homogenization

All animal procedures were approved by the Meiji pharmaceutical University Committee for Ethics of Experimentation and Animal Care. Normal male 129/J and C57BL and PPARα-null mice [7] were kept under a 12 h light-dark cycle and provided with food and water *ad libitum*. Rodent Laboratory Diet EQ 5L37 (PMI Nutrition International, SLC, Shizuoka, Japan) was used as a normal diet (control). Natural untreated plant seeds and grains were purchased at a local food store. The mice were killed by cervical dislocation, and portions of the intestine and liver were removed and rapidly homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan).

Serum parameters

Whole blood of mice was collected in 1.5-mL tubes. After clotting at room temperature for 15 min, the samples were centrifuged at 1000 *g* for 5 min. The supernatant was collected and frozen in liquid nitrogen. Serum triglyceride, total cholesterol, alanine transaminase (ALT), glucose and HDL-cholesterol levels were measured with kits (R-Liquid S-TG, R-Liquid T-Cho, R-Liquid S-ALT, R-Liquid S-Glu-HK (Kyokuto Seiyaku, Tokyo, Japan) and Determiner L HDL-C (Kyowa Medics, Tokyo, Japan), respectively), using an autoanalyzer (Kyokuto Seiyaku). Statistical evaluation was performed with analysis of two-way ANOVA.

Western blot and peptide mass fingerprinting analysis

The post nuclear fractions were prepared as described [3] and probed with an antibody raised in rabbits against synthetic peptide corresponding to amino acids 95–109 of 17β-HSD11 (gi|16716597| ref|NP_444492.1|). Liver-type fatty acid binding protein (L-FABP), which is expressed both in the liver and intestine and induced by a PPARα ligand [33], was also detected as a control using an aitibody against L-FABP. Peroxidase-conjugated goat anti-(rabbit IgG) Ig (ICN Pharmaceuticals, Aurora, Ohio) was used for the secondary antibody, and the immunocomplex was detected by an enhanced chemiluminescent kit (Super Signal West Pico, Pierce, Richmond, IL, USA). To identify the 24 kDa protein induced by sesame, the peptides produced by digestion with endoproteinase Lys-C were analyzed by MALDI-TOF mass spectrometry, and the resultant spectra were analyzed by using the MS-FIT search program as described [3].

RNA isolation, microarray and northern blot analyses

Total RNA was isolated as described [34] from the tissues of 2–3 mice per group and mixed for further use. For microanalysis, total RNA was purified by using RNase-free DNase Set (Qiagen, Chatsworth, CA). Its integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA amplification and labeling was performed according to the manufactures' protocol. Hybridization was performed using Agilent's In Situ Hybridization Plus kit following the user's manual. The arrays were scanned by the Agilent dual-laser DNA microarray scanner and analyzed by Agilent FEATURE EXTRACTION software (G2567AA). Statistical evaluation was performed by the algorithm developed by Agilent for the array analysis, and the genes upregulated by feeding sesame more than twofold with *P*-values less than 0.05 were considered.

For Northern blot analysis, RNA was not treated with DNase and analysis was carried out essentially as described previously using Express Hyb hybridization solution (Clontech, Palo Alto, CA, USA) [34]. The cDNAs used for probes were described previously [34] or obtained by PCR of cDNA synthesized from poly(A) RNA isolated from the liver of Wy14,543-fed mice using primer pairs designed mostly in the 3′-noncoding regions of the mRNAs. The PCR primers were as follows: 5′-CCCCTTACAGCTCTG CTTCATT-3′ and 5′-TCAAGAATGGATACACATAAA CACAAGGA-3′ for Cyp2c29; 5′-CCAGCTCTGCTTCAT TCCTCTCT-3′ and 5′-CGCAGGAATGGATAAACATA AGCA-3′ for Cyp2c38; 5′-ACTTCTCTGTGGCAAGCCC TGTTG-3′ and 5′-TCCACTAGCACAGATCACAGATC ATGG-3′ for Cyp2b9; 5′-TGCAGAACTTCCACTTCAA ATCCA-3′ and 5′-AATTTCCCCCTTCTCTGGCTACC-3′ for Cyp2a5; 5′-TTGTTCTAAAAGTTGTGCCACGG GATG-3′ and 5′-AGAGATGATCCCATGAGAAACGG TGAA-3′ for Cyp3a44; 5′-AGATCATCATTCCTTGGCA CTGG-3′ and 5′- ATTGCAGAAAGGAGGGAAGATGG -3′ for Cyp4a10; 5′-CCAGTTGAGTGACGAGGAG ATGG-3′ and 5′-TCTGCATGCCCTCAAATGTTACC-3′ for Akr1b8; 5′-ACCACTCTCTGGATGTGATTGGA-3′ and 5′- TCAAGAACATTTTATTTCCCACATTTT-3′ for Ugt2b5; 5′-ATTGCCCATATGGTGGCCAAAGGAG-3′ and 5′- GGCTGCCACACAAGCGAGTAGGAAT-3′ for Ugt2b37; 5′-GGGAAGGACATGAAGGAGAGAGC-3′ and 5′-GCTGCCAGGCTGTAGGAACTTCT-3′ for Gstal.