an alternative detection method, it is well known that α_{2u}-globulin droplets in the kidneys are negative for PAS reaction, but that they are stained positively by Azan-Mallory staining (U.S. EPA, 1991; Alden *et al.*, 1984). Although these additional stainings can distinguish hyaline droplets resulting from α_{2u}-globulin accumulation from those resulting from other causes, these analyses provide only indirect evidence. Direct evidence of α_{2u}-globulin accumulation in renal hyaline droplets could be required for appropriate risk assess-

Table 2. Grading results of histological/histochemical examination.

Chemical	Staining	Results		
		Control	Low dose	High dose
1,4-Dibromobenzene	HE [1]	–/–/±	++/++/+	++/+++/+++
	Azan-Mallory [2]	–/–/±	++/++/+	++/+++/+++
	Anti-α_{2u}-globulin [2]	–/–/±	++/++/+	++/+++/+++
Dicyclopentadiene	HE	–/–/–	+/++/++	+++/+++/+++
	Azan-Mallory	–/–/–	+/++/++	+++/+++/+++
	Anti-α_{2u}-globulin	–/–/–	+/++/++	+++/+++/+++
3,4-Dimethylaniline	HE	–/–/–	–/–/±	±/±/+
	Azan-Mallory	–/–/–	–/–/±	±/±/+
	Anti-α_{2u}-globulin	–/–/–	–/–/±	±/±/+
1,4-Dicyanobenzene	HE	–/–/–	±/+/+	++/+++/+++
	Azan-Mallory	–/–/–	±/++/+	+++/+++/+++
	Anti-α_{2u}-globulin	–/–/–	±/++/+	+++/+++/+++
Tetrahydrothiophene-1,1-dioxide	HE	+/–/–	+/+/++	++/++/++
	Azan-Mallory	+/–/–	++/+/++	++/++/++
	Anti-α_{2u}-globulin	+/–/–	++/+/++	++/++/++
1,3-Dicyanobenzene	HE	–/–/±	+/±/±	++/++/+++
	Azan-Mallory	–/±/±	+/±/±	++/+++/+++
	Anti-α_{2u}-globulin	–/±/±	+/±/±	++/+++/+++
Acenaphthene	HE	±/–/+	+/–/+	+/+/++
	Azan-Mallory	±/–/+	+/±/+	+/+/++
	Anti-α_{2u}-globulin	±/–/+	+/±/+	+/+/++
3,4-Dichloro-1-butene	HE	–/–/++	+/+/±	++/+/++
	Azan-Mallory	–/–/++	+/+/+	++/+/++
	Anti-α_{2u}-globulin	–/–/++	+/+/+	++/+/++
3a,4,7,7a-Tetrahydro-1*H*-indene	HE	+/+/++	++/++/++	+++/+++/+++
	Azan-Mallory	+/+/++	++/++/++	+++/+++/+++
	Anti-α_{2u}-globulin	+/+/++	++/++/++	+++/+++/+++
3,5,5-Trimethylhexan-1-ol	HE	–/–/±	+/+/++	+++/++/+++
	Azan-Mallory	±/–/±	+/+/++	+++/++/+++
	Anti-α_{2u}-globulin	±/–/±	+/+/++	+++/+++/+++
2,4-Di-tert-butylphenol	HE	–/–/–		–/–/–
	Azan-Mallory	–/–/–		–/–/–
	Anti-α_{2u}-globulin	–/–/–		–/–/–
4-Aminophenol	HE	–/±/–	–/–/–	–/–/–
	Azan-Mallory	–/±/–	–/–/–	–/–/–
	Anti-α_{2u}-globulin	–/±/–	–/–/–	–/–/–

[1] Grading for hyaline droplets.
[2] Grading for positive droplets.
No PAS-positive reaction for the hyaline droplets was observed in any sample.
Low dose for 2,4-di-*tert*-butylphenol was not examined.

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

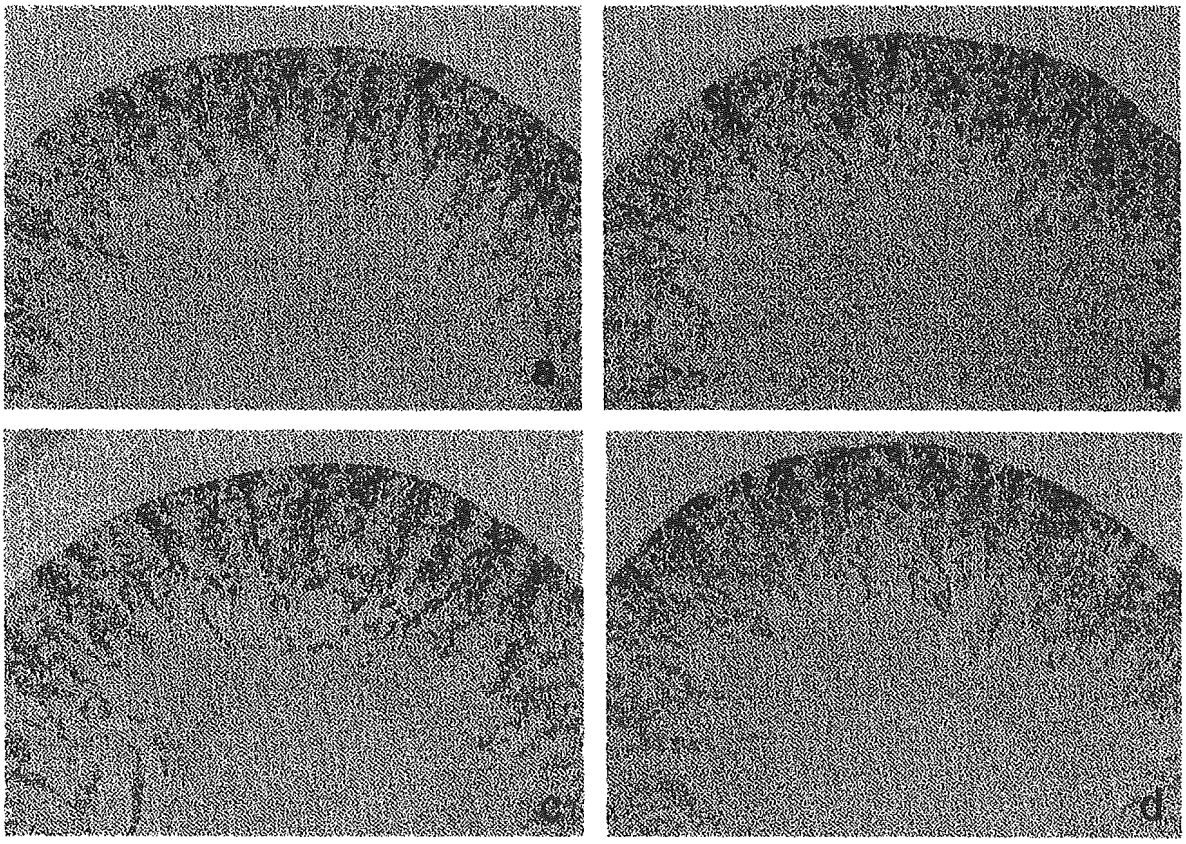

Photo 4. Immunohistochemical features of the anti-α_{2u}-globulin antibody, representing the four grades; minimal (a), slight (b), moderate (c) and severe (d). Original magnification, ×5.

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

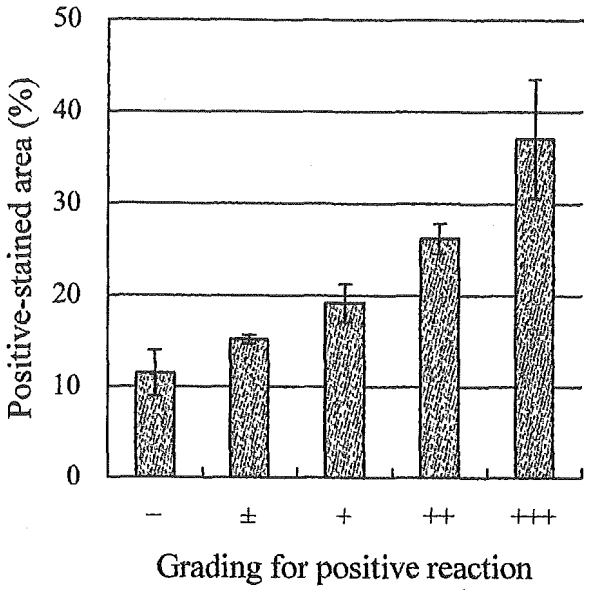

Fig. 2. Correlation between semi-quantitative and quantitative analyses for immuno-stained sections.
Results are expressed as mean ± SD (n=3).

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

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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

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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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Regulation of SR-BI protein levels by phosphorylation of its associated protein, PDZK1

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Scavenger receptor class B type I (SR-BI) is a high-density lipoprotein (HDL) receptor that mediates the selective uptake of HDL cholesterol and cholesterol secretion into bile in the liver. Previously, we identified an SR-BI-associated protein, termed PDZK1, from rat liver membrane extracts. PDZK1 contains four PSD-95/Dlg/ZO-1 (PDZ) domains, the first of which in the N-terminal region is responsible for the association with SR-BI. PDZK1 controls hepatic SR-BI expression in a posttranscriptional fashion both in cell culture and *in vivo*. In this study, we demonstrated that the C-terminal region of PDZK1 is crucial for up-regulating SR-BI protein expression. Metabolic labeling experiments and phosphoamino acid analysis revealed that PDZK1 is phosphorylated at Ser residues within this region. Point-mutation analysis demonstrated that PDZK1 is phosphorylated at Ser-509. Interestingly, a mutant PDZK1, in which Ser-509 was replaced with Ala, lost the ability to up-regulate SR-BI protein. We identified Ser-509 of PDZK1 as the residue that is phosphorylated by the cAMP-dependent PKA *in vitro* as well as in cell culture. Ser-509 of PDZK1 in rat liver was also phosphorylated, as shown by an Ab that specifically detects phosphorylated Ser-509. Administration of glucagon to Wistar rats increased PDZK1 phosphorylation as well as hepatic SR-BI and PDZK1 expression while it decreased plasma HDL levels, indicating that PDZK1 phosphorylation is hormonally regulated. These findings suggest that phosphorylation of PDZK1 has an important role in the regulation of hepatic SR-BI expression and, thus, influences plasma HDL levels.

glucagon | high-density lipoprotein | PKA

Plasma high-density lipoprotein (HDL) has a critical role in cholesterol metabolism, and plasma HDL concentrations are inversely related to the risk of developing cardiovascular disease (1, 2). The protective role of HDL against cardiovascular disease is commonly attributed to its ability to remove excess cholesterol from cells in the arterial wall and transport it to the liver for disposal, which is a process that is called reverse cholesterol transport (3, 4). Scavenger receptor class B type I (SR-BI) is an HDL receptor that is expressed in the liver as well as in steroidogenic tissues at high levels and mediates the selective uptake of HDL cholesterol (5, 6). Overexpression of SR-BI in murine hepatocytes results in the virtual disappearance of plasma HDL and a substantial increase in biliary cholesterol (7–10). Mice with a targeted mutation in the *SR-BI* gene exhibit increased plasma HDL cholesterol levels, increased HDL particle size (11, 12), and impaired biliary cholesterol secretion (13). These studies have established the concept that SR-BI determines the level of plasma HDL by taking up HDL cholesterol into the liver for transport into bile.

SR-BI contains a large extracellular domain that is anchored in the plasma membrane by transmembrane domains adjacent to short cytoplasmic N- and C-terminal regions (6). We have identified (14) an SR-BI C-terminal binding protein from rat liver membranes and named it CLAMP (C-terminal linking and modulating protein). CLAMP contains four PSD-95/Dlg/ZO-1 (PDZ) domains and associates with the C terminus of SR-BI via its N-terminal first PDZ domain (14). CLAMP is expressed mainly in the liver, kidney, and small intestine, whereas steroidogenic organs that express high levels of SR-BI, such as the adrenal grand, showed no significant CLAMP expression (15, 16). This protein has been shown to interact with a number of membrane-associated transporter proteins from different tissues, such as cMOAT/MRP2 (17), cystic fibrosis transmembrane regulator (CFTR) (16), CIC-3B (18), type IIa Na^+/P_i cotransporter (Na/P_i-IIa) (19), solute carrier SLC17A1 (Na/P_i-I), Na^+/H^+ exchanger (NHE-3), organic cation transporter (OCTN), chloride–formate exchanger (CFEX), and urate–anion exchanger (URAT1) (20). CLAMP is also called diphor-1 (21), PDZK1 (15), CAP70 (16), and NaPi-Cap1 (19), depending on its binding partners. Here, we refer to CLAMP as PDZK1.

In recent studies, Kocher *et al.* showed that PDZK1 knockout mice had significantly increased plasma cholesterol levels (22) as well as dramatically reduced hepatic SR-BI protein levels and abnormally large HDL particles in plasma (23). Similar changes were observed in SR-BI knockout mice (11). These studies established that PDZK1 up-regulates hepatic SR-BI expression at the protein level *in vivo*. Consistent with these observations, we have demonstrated (14) that coexpression of PDZK1 and SR-BI in CHO cells results in increased SR-BI protein levels without affecting the SR-BI mRNA level.

In this study, we searched for other domains, in addition to the N-terminal first PDZ domain in PDZK1, that are required for its SR-BI up-regulating activity. We found evidence that phosphorylation of the PDZK1 C-terminal region is also involved in the up-regulation of SR-BI protein expression.

Materials and Methods

Cells. CHO-K1 cells were grown in Ham's F-12 medium, supplemented with 10% FCS, 50 units/ml penicillin, 50 mg/ml streptomycin, and 2 mM glutamine at 37°C in a humidified 5% CO_2/95% air incubator. Rat hepatoma Fao cells were maintained in Coon's F-12 medium supplemented with 10% FCS, 50 units/ml penicillin, 50 mg/ml streptomycin, and 2 mM glutamine at 37°C in a humidified 10% CO_2/90% air incubator.

Creation of PDZK1 Mutants. PDZK1 mutations were constructed by PCR-mediated mutagenesis. All cDNAs were cloned into pcDNA 3.1/Hygro (Invitrogen), pGEX6P-1 (Amersham Biosciences), or pShuttle-CMV (Q-BIO Gene, Carlsbad, CA) and verified by sequencing. Fusion proteins of GST were expressed in *Escherichia coli* and purified as described (14). The shuttle

Abbreviations: HDL, high-density lipoprotein; PDZ, PSD-95/Dlg/ZO-1; CLAMP, C-terminal linking and modulating protein; pAb, polyclonal Ab; CBB, Coomassie brilliant blue; SR BI, scavenger receptor class B type I.

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vector plasmids were inserted into pAdEasy-1 (Q-BIO Gene). Recombinant adenoviral constructs were transfected into E1-transformed human embryonic kidney (HEK293ΔE1) cells to produce viral particles. The recombinant adenoviruses were purified by cesium chloride ultracentrifugation. As controls, the LacZ virus that carries β-gal cDNA was also constructed and purified as described above.

Transfection of Plasmids and Transduction of Adenoviral Vectors into Cells. CHO-K1 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Constitutively transfected cells were selected with hygromycin. Cultured Fao cells at 70% confluence were infected with the respective adenoviruses at a rate of 5,000 particles per cell in Coon's F-12 medium containing 2% FBS for 2 h. The cultures were supplemented an equal volume of Coon's F-12 medium containing 20% FBS and incubated for an additional 4 days.

Preparation of Rat Liver Membrane Extracts. Livers obtained from male Wistar rats (200–250 g, 8 weeks old) were homogenized with a homogenizing buffer (14), and the homogenate was centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting precipitate was suspended in a homogenizing buffer.

Ab Production. A synthetic phosphopeptide based on residues 503–514 of rat PDZK1 with phospho-Ser-509 was conjugated to keyhole limpet hemocyanin [KLH-CARDRTLS (PO_3H_2) AASHS] and injected subcutaneously into rabbits together with adjuvants. After eight booster injections at weekly intervals, anti-509-P-PDZK1 polyclonal Ab (pAb) was purified from rabbit serum by using a peptide-ligand-affinity column.

Western Blot Analysis. Proteins in the cell lysates or rat liver membrane extracts were separated by SDS/PAGE. The gels were blotted to polyvinylidene difluoride membranes (Millipore). The membranes were treated with anti-SR-BI pAb (NB400-101, Novus Biologicals, Littleton, CO), anti-PDZK1 mAb (14), or anti-509-P-PDZK1. The immunoreactive proteins were visualized by using chemiluminescence and recorded with a digital recorder (LAS-1000, Fuji Film, Tokyo). The relative amounts of proteins were quantified by using IMAGE GAUGE V.3.45 (Fuji Film).

Metabolic Labeling, Immunoprecipitation, and Phosphoamino Acid Analysis. CHO-K1 cells expressing PDZK1 or mutants of PDZK1 were incubated for 30 min in phosphate-depleted DMEM. Cultures were then labeled in labeling media containing 0.3 mCi (1 Ci = 37 GBq) of [^{32}P]orthophosphate for 4 h. Cells were lysed in immunoprecipitation buffer (0.5% Nonidet P-40/1% Triton X-100/10 mM Tris·HCl, pH 7.4/1 mM EDTA/150 mM NaCl/0.2 mM sodium vanadate/10 mM NaF/1 μg/ml pepstatin/1 μg/ml leupeptin/2 μg/ml aprotinin/1 mM PMSF). Lysates were immunoprecipitated with anti-PDZK1 mAb, as described in ref. 14. The immunoprecipitates were then analyzed by Western blotting and autoradiography. Phosphoamino acid maps of phosphorylated PDZK1 were generated by excising ^{32}P-labeled PDZK1 bands from polyvinylidene difluoride membranes and eluting the proteins with 6 M HCl for 1 h at 100°C. The samples were dried in a SpeedVac concentrator (Savant) and dissolved in first-dimension buffer [2.2% (vol/vol) formic acid/7.8% (vol/vol) acetic acid, pH 1.9], each sample containing three phosphoamino-acid standards (phospho-Ser, phospho-Thr, and phospho-Tyr). Phosphoamino acids were subjected to two-dimensional electrophoresis on TLC cellulose plates (pH 1.9, 1.5 kV for 30 min; pH 3.5, 1.3 kV for 15 min), followed by ninhydrin staining. The TLC plates were scanned with a BAS 2000 imaging system (Fuji Film) to detect labeled phosphoamino acids.

***In Vitro* Phosphorylation of PDZK1 by PKA.** We incubated 1 μg of recombinant GST-fused wild-type PDZK1 protein or GST-fused mutant PDZK1 protein, in which Ser-509 was replaced with Ala (S509A), with 5 units of cAMP-dependent PKA (Sigma) in phosphorylation buffer (50 mM Tris·HCl, pH 7.5/2 mM EDTA/7 mM $MgCl_2$/0.1 mM DTT/1 mM PMSF). We then added [γ-^{32}P]ATP (6 μCi), and the mixture was incubated at 30°C for 30 min. The reaction was stopped by boiling for 5 min in SDS sample buffer. The samples were subjected to SDS/PAGE and then stained with Coomassie brilliant blue (CBB). The gels were scanned with BAS 2000 to detect labeled PDZK1.

Phosphorylation of PDZK1 by PKA in Cell Culture. CHO-K1 cells stably expressing PDZK1 were treated with 10 μM forskolin (a PKA inducer) or 10 μM H-89 (a PKA inhibitor) and then labeled in labeling media containing 0.3 mCi of [^{32}P]orthophosphate for 4 h. Cells were lysed and immunoprecipitated with PDZK1. The immunoprecipitates were separated by SDS/PAGE. The gel was stained with CBB and scanned with BAS 2000 to detect labeled PDZK1.

Glucagon Administration. Male Wistar rats (200–250 g, 8 weeks old) were used. Animals were kept under standardized conditions with free access to water and chow. The light-cycle was from 7 a.m. to 7 p.m. Rats were injected s.c. under light ether anesthesia at 10 a.m. and 4 p.m. with 400 μg (115 nmol) of glucagon or a vehicle on each of 2 consecutive days. The blood was removed by venipuncture of the orbital sinus at 12 a.m., which was 2 h after the last injection. Then, rats were immediately killed, and livers were removed. Plasma was separated by low-speed centrifugation for 10 min at 4°C. Plasma total cholesterol and HDL cholesterol were prepared and assayed by using the cholesterol test kit (Wako Pure Chemical, Osaka).

Quantitative RT-PCR. Total RNA of rat liver was isolated with Isogen reagent (Nippon Gene, Toyama, Japan). Total RNA (1 μg) was reverse-transcribed in the presence of poly(dT) sequences in a total volume of 10 μl. We used 1 μl of this mixture as template in the quantitative RT-PCR. Quantitative RT-PCR reactions were performed by using the Prism 7000 sequence-detection system (Applied Biosystems). The following PCR primers were used: β-actin, 5-CCTTCTACAATGAGCTGCGTGT-3 (forward) and 5-TGGGGTGTTGAAGGTCTCAAAC-3 (reverse); SR-BI, 5-TTCTGGTGCCCATCATTTACC-3 (forward) and 5-AGCCCTTTTTACTACCACTCCAAA-3 (reverse); and CLAMP, 5-TTGAAGTGAATGGAGAAAATGTAG-3 (forward) and 5-TGATACGGCTTCCTGACTTTGTC-3 (reverse). Results were normalized to β-actin data.

Results

The PDZK1 C-Terminal Domain Is Required for Up-Regulation of SR-BI Expression. CHO-K1 cells expressing various deletion mutants of PDZK1 were established (Fig. 1*A*) and transiently transfected with SR-BI. SR-BI protein expression increased 4- to 5-fold in the cells coexpressing SR-BI and full-length PDZK1 (Fig. 1*B*). We also examined the effect of PDZK1 on SR-BI expression using rat hepatoma Fao cells, which intrinsically express SR-BI but not PDZK1. Expression of PDZK1 in Fao cells was achieved by infection with an adenovirus vector. The same degree of up-regulation of SR-BI protein was observed in this hepatoma cell line (Fig. 1*C*). However, none of the PDZK1 deletion mutants lacking the C-terminal region were able to up-regulate the SR-BI protein (Fig. 1*B*). Even the mutant lacking only 66 aa at the C terminus was less effective than the full-length PDZK1. These results suggest that PDZK1 is capable of up-regulating the SR-BI protein and that a region within the C-terminal 66 aa is indispensable for the up-regulation.

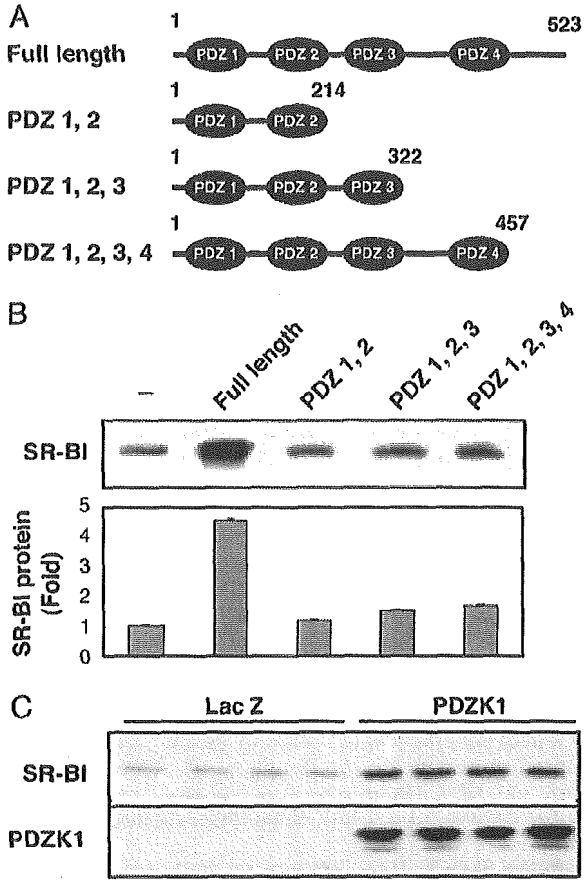

Fig. 1. The C-terminal region of PDZK1 is required for SR-BI up-regulation. (*A*) Schematic representation of various deletion mutants of PDZK1. (*B*) Western blot analysis of SR-BI from CHO-K1 cells constitutively expressing various deletion mutants of PDZK1 and transiently transfected with SR-BI. The graph represents relative quantities of SR-BI proteins in these Western blot analyses. (*C*) Western blot analysis of SR-BI and PDZK1 from cell lysates of Fao cells transduced with the recombinant adenovirus construct of PDZK1. LacZ is a control. The data represent at least three independent experiments.

PDZK1 Is Phosphorylated at the Ser Residue in the C-Terminal Region. PDZK1 possesses multiple potential phosphorylation sites within the C-terminal 66 aa, as indicated by the NetPhos database (available at www.cbs.dtu.dk/services/NetPhos). To examine whether the C-terminal region of PDZK1 is phosphorylated in cells, we constructed three C-terminal deletion mutants (namely, Δ457–523, Δ478–523, and Δ500–523), corresponding to deletions of the indicated amino acids. The C-terminal deletion mutants and full-length PDZK1 were transfected into CHO-KI cells and metabolically labeled with [^{32}P]orthophosphate. The expressed proteins were immunoprecipitated with anti-PDZK1 mAb. Western blotting with the anti-PDZK1 mAb revealed that similar levels of wild-type and mutant PDZK1 proteins were expressed (Fig. 2*A*). A ^{32}P-labeled protein band comigrating with PDZK1 was detected in the CHO cell extract, indicating that PDZK1 is a phosphorylated protein. However, all of the tested C-terminal deletion mutants resulted in significant reduction of ^{32}P-labeling. These data demonstrate that the phosphorylation of PDZK1 occurs in a region within the C-terminal 66 aa.

We then analyzed phosphoamino acids of PDZK1 labeled with [^{32}P]orthophosphate. As shown in Fig. 2*B*, PDZK1 was phosphorylated on Ser residues but not on Thr or Tyr residues.

Ser-509 of PDZK1 Is Phosphorylated. Based on the database search for potential phosphorylation sites within the C-terminal 66 aa, we

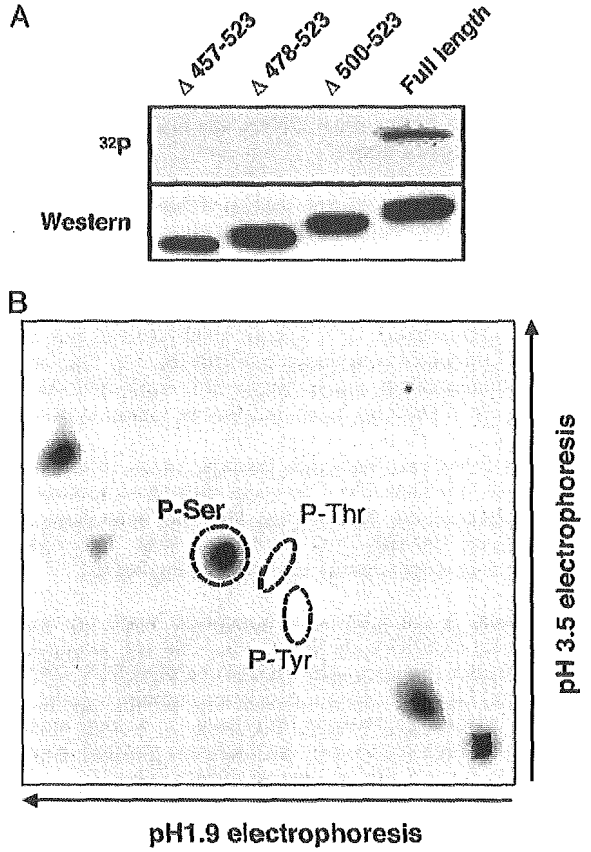

Fig. 2. PDZK1 is a phosphorylated protein. (*A*) Autoradiography (*Upper*) and the Western blot analysis of PDZK1 (*Lower*). CHO-KI cells were transiently transfected with an expression plasmid of C-terminal deletion mutants of PDZK1 (Δ457–523, Δ478–523, and Δ500–523) and incubated with [^{32}P]orthophosphate. (*B*) Phosphoamino acid analysis of ^{32}P-labeled PDZK1. Circles indicate where the phosphoamino acid standards migrated. P-Ser, phospho-Ser; P-Thr, phospho-Thr; P-Tyr, phospho-Tyr.

chose three potential sites of Ser phosphorylation (namely, Ser-509, Ser-516, and Ser-518). To determine whether these Ser residues were phosphorylated, we constructed respective Ala substitution mutants as shown in Fig. 3*A*. Transfection with these mutants into CHO-KI cells and metabolic labeling with ^{32}P revealed that S509A was no longer phosphorylated in transfected cells, whereas S516A

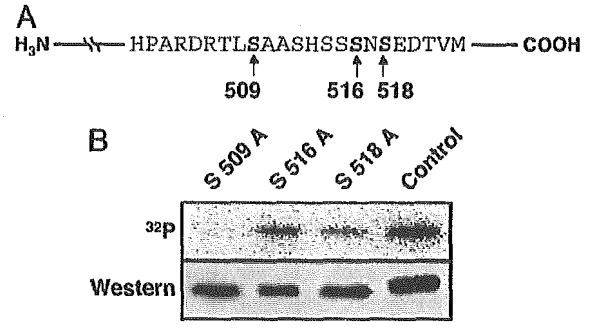

Fig. 3. Ser-509 of PDZK1 is phosphorylated. (*A*) Schematic representation of potential sites of Ser phosphorylation in the PDZK1 C-terminal region. (*B*) Autoradiography (*Upper*) and the Western blot analysis of PDZK1 (*Lower*). CHO-KI cells were transiently transfected with various single-point mutants of PDZK1 (S509A, S516A, and S518A) and labeled with [^{32}P]orthophosphate.

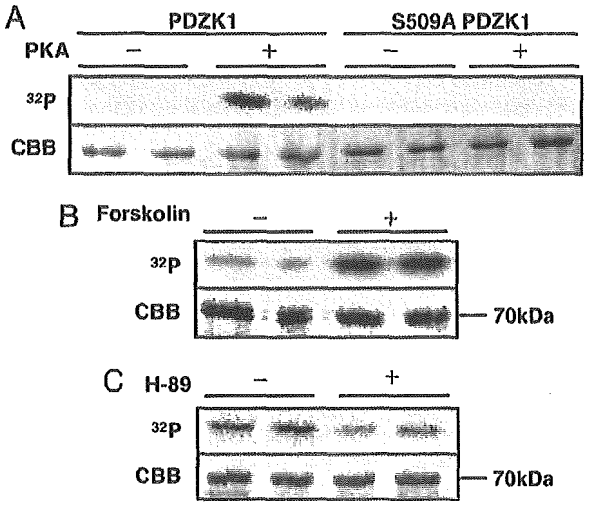

Fig. 4. PKA phosphorylates PDZK1. (*A*) Recombinant GST-PDZK1 or GST-S509A mutant fused protein expressed by *E. coli* was incubated with or without PKA in the presence of [γ-^{32}P]ATP. (*B* and *C*) Metabolic labeling with [^{32}P]orthophosphate of CHO-K1 cells constitutively expressing PDZK1 were performed in the absence or presence of 10 μM forskolin (*B*) or 10 μM H-89 (*C*). Autoradiography (*Upper*) and CBB staining (*Lower*) are shown. The data represent at least three independent experiments.

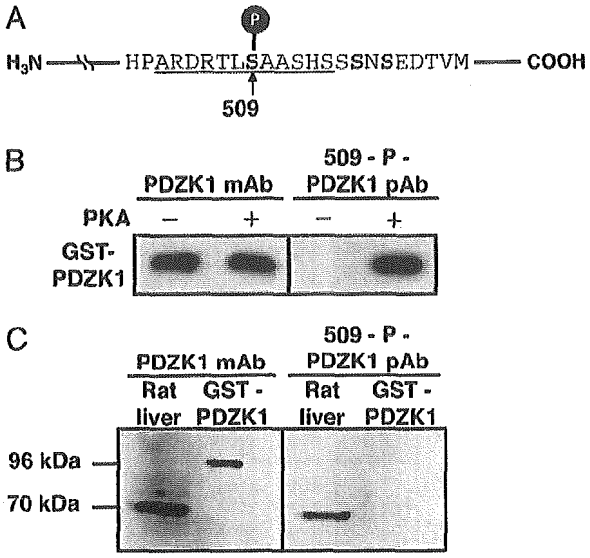

Fig. 5. PDZK1 expressed in the liver is phosphorylated *per se*. (*A*) Preparation of Abs against phospho-Ser-509. The underlined phosphorylated peptide was synthesized for immunization. (*B*) The recombinant PDZK1 expressed by *E. coli* was incubated with or without PKA and then subjected to Western blotting using anti-PDZK1 mAb (*Left*), anti-509-P-PDZK1 pAb (*Right*). (*C*) Western blot analysis of PDZK1 from rat liver membrane extract (70 kDa) and the recombinant GST-PDZK1 fused protein (96 kDa) using anti-PDZK1 mAb (*Left*) and anti-509-P-PDZK1 pAb (*Right*).

and S518A were phosphorylated (Fig. 3*B*), thus implicating Ser-509 as a critical site of PDZK1 phosphorylation.

PDZK1 Ser-509 Is Phosphorylated by PKA *in Vitro* and in Cell Culture. According to ref. 24, Ser-509 was found to be a potential PKA target Ser. To examine whether Ser-509 can be directly phosphorylated by PKA, we incubated the recombinant PDZK1 with the purified catalytic subunit of PKA in the presence of [γ-^{32}P]ATP. We found that PKA phosphorylated PDZK1 *in vitro*, whereas PKA did not phosphorylate the S509A mutant to any extent (Fig. 4*A*). Next, we treated CHO-K1 cells that constitutively express PDZK1 with the PKA inducer forskolin or the PKA inhibitor H-89 in the presence of [^{32}P]orthophosphate. Endogenous PDZK1 was then immunoprecipitated and subjected to CBB staining and autoradiography. The CBB-stained $\approx$70-kDa band was found to be PDZK1, as judged from the fact that this $\approx$70-kDa band in SDS/PAGE (Fig. 4 *B* and *C Lower*) reacted with anti-PDZK1 Ab by Western blot analysis and could not be detected by performing the same procedures with parental CHO-K1 cells (data not shown). Phosphorylation levels of PDZK1 were significantly increased by forskolin (Fig. 4*B*) and reduced by H-89 (Fig. 4*C*). These *in vitro* and cell-culture experiments demonstrated that PDZK1 Ser-509 is phosphorylated by PKA.

PDZK1 Expressed in Liver Is Phosphorylated. Although mutagenesis studies using CHO-K1 cells showed the importance of the Ser site for PDZK1 phosphorylation, it was not clear whether Ser-509 was phosphorylated *in vivo*. To answer this question, we established specific Abs against synthetic peptides corresponding to amino acids 503–514 of rat PDZK1 in which Ser-509 was phosphorylated (Fig. 5*A*). By using a phospho-specific Ab (anti-509-P-PDZK1) that was developed to detect Ser-509 phosphorylation, a prominent immunoreactive band was detected in the recombinant PDZK1 when the recombinant PDZK1 was incubated with PKA, whereas no anti-509-P-PDZK1 immunoreactivity was detected when the recombinant PDZK1 was incubated without PKA (Fig. 5*B*). In a Western blot analysis (Fig. 5*C*), anti-509-P-PDZK1 detected PDZK1 in rat liver extract but not the recombinant PDZK1 produced by *E. coli*. These results showed that Ser-509 of PDZK1 expressed in rat liver was phosphorylated.

SR-BI Protein Level Is Affected by the PDZK1 Phosphorylation State. Because we found that PDZK1 is phosphorylated at position Ser-509, we examined next whether PDZK1 phosphorylation could regulate SR-BI protein expression. Wild-type PDZK1 and the PDZK1 S509A mutant were overexpressed in rat hepatoma Fao cells by using recombinant adenovirus vectors (Fig. 6). The amount of SR-BI protein increased $\approx$4-fold in Fao cells transduced with an adenovirus vector harboring PDZK1 (Ad-PDZK1), as compared with Ad-LacZ. However, transduction of Fao cells with Ad-PDZK1 S509A mutant did not affect the amount of SR-BI protein. These results suggested that PDZK1 phosphorylation of Ser-509 is one of the key factors regulating SR-BI protein levels.

Glucagon Increases PDZK1 Phosphorylation as Well as Hepatic SR-BI Expression. Next, we investigated physiological conditions regulating PDZK1 phosphorylation in rat liver. Reports (25–28) have indicated that the administration of glucagon to laboratory

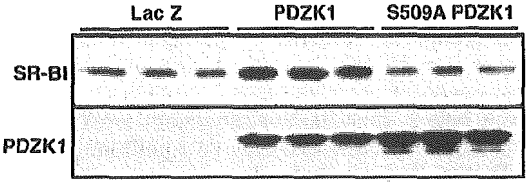

Fig. 6. SR-BI protein level is regulated by PDZK1 through phosphorylation. Western blot analysis of SR-BI (*Upper*) and PDZK1 (*Lower*) from cell lysates of Fao cells transduced with the recombinant adenovirus of PDZK1 or S509A PDZK1. LacZ is a control. The results of this figure are representative of at least three independent experiments.

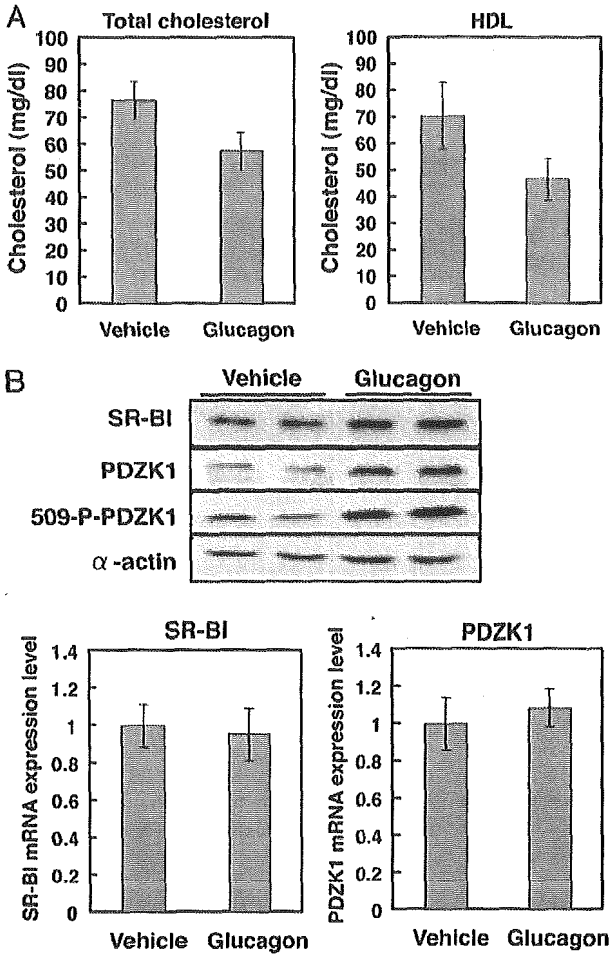

Fig. 7. Glucagon increases PDZK1 phosphorylation and hepatic SR-BI expression. Five rats were injected twice daily with 400 μg of glucagon or a vehicle for 2 days. Rats were killed 2 h after the last injection. (*A*) Total and HDL cholesterol levels. (*B*) Western blot analysis of SR-BI, PDZK1, phosphorylated PDZK1, and α-actin from rat liver homogenates of two representative animals. (*C*) Quantitative analysis of SR-BI and PDZK1 transcripts. Total RNA purified from rat liver was subjected to real time RT-PCR for measuring SR-BI and PDZK1 transcripts, as described in *Materials and Methods*.

animals as well as humans decreases plasma HDL cholesterol levels. To our knowledge, no information is available regarding the effects of glucagon on hepatic SR-BI *in vivo*. The actions of glucagon are mediated by the glucagon receptor linked to a heterotrimeric G-protein complex, leading to increased cellular levels of cAMP and activation of PKA. We hypothesized that the reduction of plasma levels of HDL by glucagon is due to the up-regulation of SR-BI protein expression through PDZK1 phosphorylation by PKA.

To determine the influence of glucagon on hepatic SR-BI expression and PDZK1 phosphorylation, rats were injected twice daily with 400 μg of glucagon or a vehicle for 2 days before they were killed. Total plasma cholesterol levels and plasma HDL cholesterol levels were decreased significantly, as reported in ref. 28 (Fig. 7*A*). Upon glucagon treatment, the phosphorylated form of PDZK1 was increased by 3.6 ± 0.2, whereas SR-BI and PDZK1 protein levels were increased by 1.8 ± 0.3 and 1.8 ± 0.4, respectively. Glucagon treatment did not affect SR-BI and PDZK1 mRNA levels (Fig. 7*C*), indicating the posttranscriptional regulation of hepatic SR-BI and PDZK1 expression by this hormone. These results suggested that PDZK1 phosphorylation and protein expression were hormonally regulated, and that up-regulation of hepatic SR-BI expression by glucagon is at least partly due to elevation of PDZK1 phosphorylation and/or its protein expression.

Discussion

We previously showed that coexpression of PDZK1 and SR-BI in CHO cells resulted in up-regulation of SR-BI protein expression without affecting the SR-BI mRNA level (14). Moreover, Kocher *et al.* (23) demonstrated that there is a 95% reduction in SR-BI protein expression in the livers of PDZK1-deficient mice compared with wild-type controls. These *in vitro* and *in vivo* data indicate that PDZK1 controls hepatic expression of SR-BI in a posttranscriptional fashion. In this study, we demonstrated that the phosphorylation of Ser-509 in the C-terminal region of PDZK1 is required for SR-BI up-regulation in cultured hepatoma cells. By using a phospho-specific Ab developed to detect Ser-509 phosphorylation, we showed that Ser-509 of hepatic PDZK1 is phosphorylated.

Three lines of evidence suggest that Ser-509 of PDZK1 is phosphorylated by PKA. First, the sequence around Ser-509 matches the potential phosphorylation site of PKA. Second, the recombinant PDZK1 is phosphorylated by PKA *in vitro*. Third, the phosphorylation level of PDZK1 in cultured cells is increased by a PKA activator and reduced by a PKA inhibitor. It has been demonstrated that the dual-specific A kinase-anchoring protein 2 (D-AKAP2), a member of the PKA-anchoring protein family (AKAP) binds to the fourth PDZ domain of PDZK1 in the kidney (20, 29), which is close to the Ser-509 position. D-AKAP2 is also expressed in the liver (30). It remains to be determined whether it interacts with PDZK1 in the liver.

The sequence around Ser-509 of PDZK1 also matches the consensus phosphorylation motif for p70 S6 kinase (p70S6K) (24). Interestingly, the C-terminal region of p70S6K contains a sequence capable of binding to a PDZ domain. Indeed, p70S6K has been reported (31) to interact with a PDZ domain-containing protein, called Neurabin, that exists in neuronal cells. In preliminary experiments, we found that p70S6K can phosphorylate Ser-509 in PDZK1 *in vitro*. Stimulation of hepatoma cells with insulin is known to activate p70S6K (32). Therefore, it is plausible that diverse extracellular events can lead to PDZK1 phosphorylation, thus controlling SR-BI expression levels.

The phosphorylation level of PDZK1 was reduced by approximately one half when Ala was substituted for Ser-518 in ^{32}P-incorporation experiments (Fig. 3*B*). Therefore, it is possible that PDZK1 is also phosphorylated at position Ser-518. However, the Ser-509-to-Ala substitution leads to complete suppression of phosphorylation, which indicates that Ser-518 is phosphorylated after Ser-509 is phosphorylated. Whether the PDZK1 C-terminal region has other phosphorylation sites besides the Ser-509 location remains to be determined.

Glucagon, being a major regulator of plasma glucose concentration (33), has an important role in carbohydrate metabolism. The effects of glucagon are mediated by the cAMP second-messenger system, including PKA. Glucagon also influences cholesterol metabolism. Conditions with increased glucagon levels are associated with reduced plasma cholesterol levels (34, 35), and the administration of glucagon to laboratory animals as well as humans decreases plasma cholesterol levels (25–28). However, the physiological role of this phenomenon remains unclear. Glucagon has been shown to increase the number of hepatic low-density lipoprotein (LDL) receptors and concomitantly decrease plasma cholesterol and apoprotein B levels (28). According to ref. 28, the plasma HDL cholesterol level was decreased also by glucagons treatment, as we observed in this study (Fig. 7*A*). Our results show that glucagon increases expression of the HDL receptor SR-BI in

the liver. Overexpression of hepatic SR-BI leads to reduced plasma HDL levels (7, 8), which supports the hypothesis that increased SR-BI expression is responsible for reduced plasma HDL levels. Furthermore, glucagon was shown to enhance cholesterol uptake into bile in rats (36). HDL cholesterol is known to be a major substrate source for bile acid production in both rats and humans (37). Overexpression of SR-BI markedly promotes the hepatic uptake of HDL cholesterol, thus facilitating the secretion of cholesterol into bile (7, 10). One explanation for our results is that glucagon administration to rats leads to enhanced PDZK1 phosphorylation via PKA, which subsequently leads to enhanced SR-BI protein expression, regulated at a posttranscriptional level. However, it cannot yet be proven that the phosphorylation of PDZK1 leads to the described SR-BI elevation because glucagon treatment leads not only to an elevation of SR-BI but simultaneously to a 2-fold elevation of PDZK1, respectively. Use of mouse models such as PDZK1 knockout mice and SR-BI knockout mice, as well as transgenic animals expressing only the PDZK1 mutant where Ser-509 is substituted by Ala, would bring us further toward solving this problem.

In conclusion, we demonstrated in this study that phosphorylation of the PDZK1 C-terminal region is crucial for up-regulation of SR-BI protein expression and that PDZK1 phosphorylation is hormonally regulated. A major question that remains to be answered is how the phosphorylation of the PDZK1 C-terminal region is involved in the up-regulation of SR-BI levels.

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