

A Review of Hazardous Chemical Toxicity Studies Utilizing Genetically-Modified Animals —Their Applications for Risk Assessment—^a

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Abstract: Studies on the mechanisms of chemical toxicity carried out using knockout mice lacking genes of enzymes for drug metabolism or nuclear receptor proteins were reviewed, and the studies were compared with the respective conventional mechanistic studies. While the toxicity of many hazardous chemicals was observed only in wild-type or knockout mice, which clearly showed that their toxicity was involved in the enzyme or receptor, some chemicals exhibited the same degree of toxicity in two genotypes, i.e., in both the wild strain and knockout mice, demonstrating that the enzymes or receptors are not involved in their toxicity. The use of genetically-modified animals presents not only the advantage of simultaneous evaluation of toxicity endpoints and mechanisms, but also suggests significant benefits over conventional methods using several chemicals to elucidate toxicity mechanisms. Elucidation of the mechanism of toxicity will provide useful information for risk assessment, and the use of genetically-modified animals for this purpose will lead to the advancement of this assessment.

Key words: Alcohol hepatitis, Aromatic hydrocarbon receptor, Cytochrome P450, Knockout mice, Mechanism, Peroxisome proliferator-activated receptor, Risk assessment, Species difference

Introduction

Risk assessment of hazardous chemicals requires hazard information on the relevant chemicals, which is mainly obtained by animal experiments, and in some cases, also from epidemiological studies. In the former case, the results of well-conducted animal experiments, where No-Observed-

Adverse-Effect-Level (NOAEL) is often used, need to be extrapolated to humans. For this extrapolation, the elucidation of underlying mechanisms and considerations of uncertainty factors concerning species differences are required because each factor is required for risk assessment calculation such as ADI (acceptable daily intake) or TDI (tolerable daily intake). Traditionally, an uncertainty factor of 100 is used for TDI or ADI calculation to extrapolate from the animal bioassay to human (10-fold factor animal to human and 10-fold factor human-human variability in response)¹.

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Table 1. Xenobiotic-metabolism and nuclear-receptor null mice

Gene	Chemicals	Phenotype	References	Ref.	
Cyp1a2	4-Aminobiphenyl	Carcinogenicity	Kimura <i>et al.</i> (1999)	8	
	Caffeine	Metabolism	Buters <i>et al.</i> (1996)	38	
	Acetaminophen	Hepatotoxicity	Zaher <i>et al.</i> (1998)	39	
	Hexachlorobenzene	Uroporphyrin	Sinclair <i>et al.</i> (2000)	40	
	TCDD	Hepatotoxicity	Smith <i>et al.</i> (2001)	41	
Cyp1b1	7, 12-dimethylbenz[a]anthracene	Lymphoma	Buters <i>et al.</i> (1999)	42	
Cyp2e1	Chloroform	Hepatotoxicity	Constan <i>et al.</i> (1999)	12	
	Carbon tetrachloride	Hepatotoxicity	Wong <i>et al.</i> (1998)	13	
	Acrylamide	Metabolism	Sumner <i>et al.</i> (1999)	15	
	Acrylonitrile	Metabolism	Sumner <i>et al.</i> (1999)	15	
				Chanas <i>et al.</i> (2003)	43
		Methacrylonitrile	Metabolism	Ghanayem <i>et al.</i> (1999)	44
		Benzene	Metabolism, hematotoxicity	Valentine <i>et al.</i> (1996)	14
AhR	Benzo[a]pyrene	Skin or subcutaneous tumor	Shimizu <i>et al.</i> (2000)	45	
	TCDD	Cleft palate, Hydronephrosis	Mimura <i>et al.</i> (1997)	18	
PPAR α	Alcohol	Hepatotoxicity	Nakajima <i>et al.</i> (2004)	29	

By the 1980's, studies on the mechanisms of toxicity had become extremely complicated. To elucidate the involvement of metabolic enzymes in the development of toxicity of chemicals, many kinds of measures had been introduced as follows: partial hepatectomy²⁾ or pre-administration of enzyme inducers such as phenobarbital (which induces CYP2B1), 3-methylcholanthrene (which induces CYP1A1)^{2,3)} or alcohol (which induces CYP2E1)³⁾ to decrease or increase expression of the target enzyme; and co- or pre-administration of drug metabolizing enzyme inhibitors to change its activity. Administration of a specified chemical after using such inhibitors or inducers may lead to erroneous determination of the effects since the influences of the remaining inhibitors or inducers are not completely excluded. For example, the inhibition action of alcohol remaining in the body may overlook the effect of alcohol-induced enzymatic activation⁴⁾.

In the late 1990s, knockout animals lacking the gene of drug metabolizing enzymes were developed, and have been applied to elucidate mechanisms of chemical toxicity^{5,6)}. The advantage of using these genetically-modified animals is that functional analysis of the target gene, analysis of the toxic mechanism and toxic assessment, can all be performed simultaneously. Recently, epidemiological studies dealing with genetic polymorphisms have been carried out. These genetically-modified animals would present further advantages demonstrating scientific proof of these molecular epidemiological studies. However, one drawback is the very high cost of breeding and maintaining the lines.

Species-specific differences in kinetics of inhaled

chemicals can be estimated to some extent through analyzing the blood or urine levels of chemicals in humans and animals. However, it is extremely difficult to thoroughly elucidate species-specific differences in dynamics because studies using human tissues pose ethical issues. Recent development of knockin animals carrying human genes has brought new hope to this issue: besides knocking out or transferring target genes, small animals carrying a human target gene (knockin animals) have been developed, allowing probes for deeper scientific information about the function of human genes. For example, Gonzalez' group at the US NIH have developed a transgenic mouse carrying human PPAR α (humanized PPAR α mouse), and reported the absence of a species-specific difference in PPAR α function regarding the induction of β -oxidation enzyme⁷⁾. Thus, the combination of several genetically-modified animals may promote studies on species-specific differences.

Studies on mechanisms using cytochrome P450 gene knockout mice

Table 1 shows the results of studies using gene knockout animals reported in PubMed. As indicated in the references, the earliest such study was reported in 1996. CYP1A2 and CYP2E1 have been studied most frequently. Arylamine is one chemical for which carcinogenicity has been demonstrated in humans. Cigarette smoke contains 4-ABP (4-aminobiphenyl), a chemical belonging to arylamine, and CYP1A2 reportedly catalyzes the metabolism of the chemical

(N-hydroxylation). Nevertheless, no differences in the frequency of carcinogenesis due to 4-ABP were observed between the wild strain and *cyp1a2*-null mice, suggesting the possibility that a pathway other than CYP1A2 is responsible for the development of cancer⁸).

In the early 1990s, we reported that CYP2E1 is involved in liver toxicity caused by chloroform⁹⁻¹¹). Since gene-modified animals were not available at that time, the experiment was conducted by the pre-administration of inducers to induce target enzymes. Pretreatment of alcohol to rats to induce CYP2E1, followed by exposure to chloroform, resulted in the development of liver damage, while control rats without pretreatment did not develop the damage at the exposure to chloroform. This suggested the involvement of CYP2E1 in the liver toxicity caused by chloroform. In this case, however, concern remained with regard to the potential effects of the presence of residual alcohol in the body. It was recently reported that the absence of liver toxicity by chloroform was observed in CYP2E1 knockout mice, whereas the toxicity was found in the wild-type mice, which leads to the conclusion that active metabolites were generated in the process of chloroform metabolism, and that CYP2E1 was involved in that metabolism¹²). No liver damage was observed due to no active metabolite production in the knockout mice, since the mice lacked CYP2E1.

Carbon tetrachloride is a liver-damaging substance as is chloroform, and CYP2E1 plays an important role. During the 1980s, we confirmed the relationship between carbon tetrachloride and CYP2E1 by pre-administering alcohol, an inducer of this enzyme. While a single exposure of 0 to 100 ppm carbon tetrachloride to control rats did not cause hepatopathy, 10 ppm carbon tetrachloride to rats that had been administered alcohol to induce CYP2E1 caused hepatopathy¹¹). In this example, the effects of residual ethanol could not be excluded. Wong *et al.* conducted an experiment about liver damage caused by carbon tetrachloride using *cyp2e1*-null mice¹³). They observed liver damage in wild strain but not in knockout mice, demonstrating the role of CYP2E1 in the hepatopathy caused by carbon tetrachloride, and the results supported our findings.

Studies on the role of CYP2E1 in myelotoxicity of benzene have been conducted by repeating alcohol administration and exposure to benzene⁴). The alcohol-treated group showed a more significant reduction in white blood cell counts caused by benzene exposure than the untreated group, indicating an important role of CYP2E1 induction by alcohol treatment in the myelotoxicity caused by benzene. As with the studies mentioned above, the effects of residual alcohol on the bone

marrow could not be completely excluded. Velenteine *et al.* studied the role of CYP2E1 in benzene-inducing bone marrow damage using *cyp2e1*-null mice¹⁴). They exposed wild strain and *cyp2e1*-null mice to benzene at ≤ 200 ppm, 6 h daily for 5 d. Cytotoxicity (cellularities) and genotoxicity (micronuclei frequencies) due to benzene exposure were more pronounced in the wild strain than in the knockout mice. Benzene metabolism was significantly lower in the knockout mice than in the wild strain mice, revealing that CYP2E1 primarily controlled the metabolism involved in genotoxicity and carcinogenicity of benzene.

The carcinogenic mechanisms of 7,12-dimethylbenz[a]anthracene (DMBA) were investigated using *cyp1b1*-null mice. *Cyp1b1*-null mice showed a lower occurrence of lymphoma or dermatopathy as compared with the wild strain mice, demonstrating the important role of CYP1B1 in metabolic activation of DMBA.

The relationship between the acrylonitrile metabolism and CYP2E1 was also studied. Sumner *et al.* measured the CN ion level of the metabolites excreted into the urine using wild strain and *cyp2e1*-null mice administered acrylonitrile¹⁵). The wild strain mice showed an increase in the amount of excreted CN ions with the dose increasing, while the knockout mice showed little excretion. CYP2E1 was demonstrated to be the primary metabolic enzyme for acrylonitrile.

Studies on Mechanisms Using Nuclear Receptor Gene knockout Mice

Both AhR (aromatic hydrocarbon receptor) and PPAR α (Peroxisome proliferator-activated receptor) are ligand-dependent nuclear receptors. Genetically-modified animals with respect to these nuclear receptor genes have been developed, and a considerable number of investigations have been conducted^{16, 17}).

Mimura *et al.*¹⁸) administered 40 $\mu\text{g}/\text{kg}$ of TCDD (by gavage) to GD (gestational day) 12.5 pregnant wild strain mice and AhR knockout mice to investigate the role of AhR in dioxin-induced deformation, namely effects on hydronephrosis and cleft palate. All mice were dissected at GD 18.5, and the deformation of fetuses was investigated. Cleft palate was observed in 100% of the fetuses of the wild strain mice administered dioxin, but not in those of knockout mice administered dioxin in the same manner. Hydronephrosis developed in a fraction of the unadministered wild strain mice, and the incidence increased with dioxin administration, but not in the knockout mice. Although the involvement of AhR was indicated, the associated target genes have not yet been discovered.

Although pentachlorophenol (PCP) was registered as a pesticide or herbicide, the registration was invalidated in 1990. PCP is still assumed to be a priority substance as an environmental pollutant in risk assessment. Experimental and epidemiological reports have suggested that PCP reduces thyroid hormone (T4)^{19,20}. We hypothesized that PCP binds to AhR-inducing glucuronic acid conjugate enzyme which is thought to be one of the target gene products, increasing the activation of T4-glucuronidation, and resulting in enhanced excretion of T4 and reducing the levels in serum. In our experiment, four oral doses of PCP were administered to wild strain and AhR knockout mice daily for 28 d. This experiment demonstrated that the level of T4 was reduced in mice with both genotypes (unpublished observation). PCP was found to induce UDP-glucuronic acid conjugate enzyme activity in wild strain mice, but not in knockout mice, suggesting no involvement of AhR in the T4 level reduction. It is known that T4 is reduced by dioxin only in wild-type mice, not in AhR-knockout mice. Our results showed that the T4 level reduction caused by PCP was due to mechanisms other than those involving dioxin. Mechanisms underlying the effect of PCP on thyroid hormone will be investigated in future studies.

PPAR α binds to ligands such as fatty acid forming a heterodimer with RXR, and to nuclear peroxisome response elements, resulting in the induction of target gene expression¹⁷. The representative target gene is the β -oxidative enzyme gene, while many other target genes are known as well.

Many environmental chemicals can bind to PPAR α . Trichloroacetic acid, one of the metabolites from trichloroethylene, is a ligand of PPAR α . We administered trichloroethylene to male and female wild strain and PPAR α knockout mice (0.75 g/kg for 2 wk, by gavage) to investigate the induction of peroxisome and mitochondrial-oxidative enzyme, which are target gene products of PPAR α ²³. The wild strain mice showed induction of β -oxidation system enzymes, but not the knockout mice. In addition, the induction of the enzymes was stronger in male than in female mice, indicating sex differences in the transcriptional activation of PPAR α .

2,4-dichlorophenoxyacetic acid, monoethylhexylphthalate (MEHP), a metabolite of the representative plasticizer, 2-ethylhexylphthalate (DEHP), are ligands of PPAR α ²¹. Once DEHP is taken into the body, an ester bond is cleaved by lipase yielding 2-ethyl-1-hexanol and MEHP. This monocarboxylic acid formed binds to PPAR α , or is further metabolized to MEHP glucuronide that is excreted from the body. In addition, MEHP is ω -oxidized to yield dicarboxylic

acid, and undergoes further metabolism, entering a complex metabolic pathway²².

Male and female 129/sv wild-type and PPAR α -knockout mice were exposed to DEHP to observe the influence on reproduction. The wild strain mice showed decreased survival rates in F1 and F2 generations due to DEHP exposure. Subsequent studies revealed that the reduced survival rate following DEHP exposure resulted from embryo resorption and the death of newborn mice; in particular, an increased death rate by the 2nd postnatal day. In contrast, these phenomena were not observed in the PPAR α knockout mice (unpublished observation). The results suggest that PPAR α is involved in the increase of embryo resorption and mortality rate of newborn pups. The target genes involved in these phenomena are currently under investigation.

These studies suggest the involvement of PPAR α in the reduced fertility of mice caused by DEHP. In this regard, the question arises as to whether human PPAR α functions in an analogous way. Reportedly, human PPAR α differs from mouse PPAR α in both function and expression level^{24,25}. In this case, it appears extremely difficult to determine the uncertainty factor in the risk assessment calculations. As described above, however, transgenic mice carrying human PPAR α have recently been developed, which are expected to be useful for determination of at least some human PPAR α functions.

If positive and negative results are obtained in mice carrying mouse PPAR α and human PPAR α , respectively, upon exposure to a certain chemical, toxicity may develop in mice, but would be unlikely in humans, i.e., the risk in humans may be significantly lower. If toxicity is observed in both genotype mice, similar toxic expression might be observed in both mice and humans. Here, risk assessment using the uncertainty factor described above may be available, although the reasonableness of the current uncertainty factor of ten may need to be reexamined. Transgenic mice carrying human genes are most useful in the case when epidemiological phenomena cannot be reproduced in common animal experiments; for example, positive findings in human epidemiological investigations, but negative in animal experiments. Transgenic mice carrying human genes can be a powerful tool for the solution. Therefore, risk assessment of chemicals should be advanced through extensive use of these animals.

The effects of alcohol consumption on health and alcohol-related problems are critical health care issues. Obstacles have existed over the study of alcohol where alcoholic liver injury is not clearly induced in small animals. Lieber *et al.* successfully developed alcoholic liver injury in baboons²⁶,

which are not commonly used in most institutions. In addition, it was reported that hepatic damage in a small animal was first observed by adding alcohol in conjunction with a high-fat diet, that is, via a dramatic change in nutritional status²⁷).

Foreseeing the importance of PPAR α receptors in fat metabolism²⁸), we assumed that alcoholic liver injury can easily be developed in PPAR α knockout mice, in which the fat metabolism is largely retarded. Alcohol was administered to both wild strain and PPAR α knockout mice (4% Lieber diet). After a month-acclimated breeding, a 4% alcohol diet was provided for six months. Although the usual Lieber diet contains 5% alcohol, this amount was reduced to 4% in both genotype mice, since some knockout mice were observed to die with a 5% diet²⁹).

Alcoholic administration was found to induce mild hepatomegaly and elevated ALT and AST in wild strain mouse liver, but these changes were more marked in the PPAR α knockout mice. In contrast, no differences were observed between the two genotypes regarding increase in serum and hepatic triglyceride levels. The pathological findings of fat droplets were substantially similar to the hepatic triglyceride levels: alcohol administration resulted in no appreciable change in the fat droplet levels of the two mice genotypes after 6 months. The most significant difference between the livers of the two mice genotypes was the emergence of inflammatory cells, fibrosis and apoptosis positive cells. Inflammatory cells could not be observed in the wild strain mice, but an aggregated necrotic layer was observed around the hepatic central vein of the PPAR α knockout mice. Fibrosis around the inflammatory cells in the PPAR α knockout mice was also observed. Apoptosis-positive cells were found scattered in the livers of the PPAR α knockout mice, but scarcely in the wild strain mice. Electron microscopy revealed obviously-enlarged mitochondria in the livers of the knockout mice due to alcohol administration, but not in the wild strain mice. Administered alcohol slightly increased alcohol metabolic enzyme (ADH, CYP2E1) levels in mice of both genotypes. In contrast, acetaldehyde metabolic enzyme (ALDH) displayed a significantly greater decrease in the knockout mice, suggesting that acetaldehyde may easily accumulate in these mice. The enzymatic activity or expression level of each of the oxidative stress-eliminating enzymes (GPx, SOD, GST, and catalase), with the exception of GST, was found to be lower in the livers of the PPAR α knockout mice. In the livers of the wild strain mice, the enzymatic activity or expression level of each of these oxidative stress eliminating enzymes either remained unchanged or was slightly reduced. Thus, PPAR α knockout

mice became extremely sensitive to oxidative stress due to long-term alcohol administration.

The level of p65, a subunit of NF- κ B, was increased by ethanol administration in the livers of mice of both genotypes. This increase was greater in PPAR α knockout mice, resulting in significantly more severe inflammation, and was consistent with observed increased ALT value and pathological findings.

HGF α and TGF β are involved in liver enlargement and fibrosis, respectively^{30,31}), and their expression is considered to be a marker of the degree of hepatic fibrosis because marked increase in HGF α inhibits the effect of TGF β 1 and could suppress a more severe course of fibrosis³²). No significant change in either expression was observed in the wild strain mice following alcohol administration, but the expressions were obviously raised in the PPAR α knockout mice, indicating high expressions of factors involved in increased fibrosis in the mice. These observations are consistent with the pathological changes in the liver.

Bax, Bit, and tBit, descriptors for mitochondrial permeability transition^{33,34}), are factors in the stimulation of apoptosis and mitochondrial enlargement. These stimulatory factors were increased only in ethanol-administered knockout mice, while Bcl-2 and Bcl-xL displaying a tendency to inhibit apoptosis were decreased, indicating involvement of these functions in the stimulation of apoptosis and mitochondrial enlargement.

The mechanism of alcohol-induced liver damage observed in the wild-type and PPAR α -knockout mice is summarized in Fig. 1. Alcoholic liver injury was arrested at fatty liver in the wild strain mice, and did not subsequently progress to hepatopathy, while in knockout mice lacking PPAR α receptors, liver injury did not halt at fatty liver, instead progressing at least to fibrosis, demonstrating a significant involvement of PPAR α receptor, particularly with regard to alcohol-induced steatohepatitis or fibrosis.

The mechanism of preventive action of PPAR α against the progression of alcoholic liver injury is intriguing. Ultimately, I κ B α is induced by ligand binding of PPAR α , after which the induced I κ B α binds to NF- κ B, and the complex cannot enter the nucleus. As a result, the expression of p65 can not be induced³⁵). Thus, wild strain mice carrying PPAR α genes prevent development of inflammation. However, in knockout mice lacking PPAR α gene, stresses such as alcohol can induce the inflammatory factor p65 and stimulate inflammation. These results demonstrate the importance of PPAR α functions in the progression of alcoholic liver injury.

In demonstrating the importance of PPAR α functions in the development of alcoholic liver injury in mice, it would

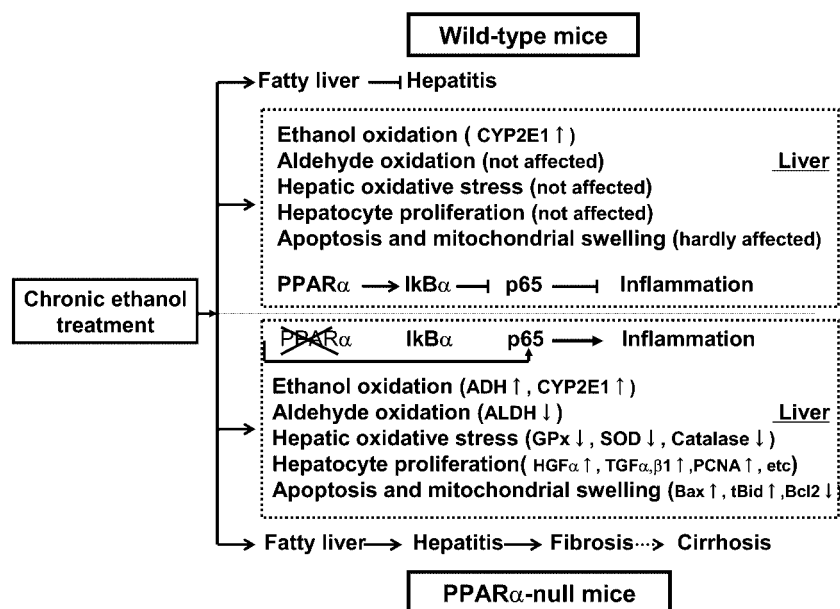


Fig. 1. Proposal mechanisms underlying the alcohol-induced steatohepatitis and fibrosis in the liver of wild-type and PPAR α -knockout mice.

\uparrow , increase in the expression; \downarrow , decrease in the expression.

be of interest to determine whether the same phenomena can also be observed in humans. In particular, several polymorphisms of PPAR α genes have been reported, and some were related with remarkable change in the binding affinity for ligands^{36,37}. Determining the relationship between these polymorphisms and alcoholic fatty liver, and probably the accumulation of dietary fat, is very useful for elucidating the functions of these genes and in health management. New health directives might be necessary for people carrying PPAR α polymorphisms with reduced function in order to balance levels of alcohol consumption and nutrition.

We conclude that the elucidation of the mechanisms of toxicity development has been considerably advanced by the use of knockout mice, or knockin mice carrying human genes, which provide useful information for risk assessment calculation such as ADI or TDI.

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