Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 303 (2003) 105–111
BBRC
www.elsevier.com/locate/ybbrc

Gene expression of detoxifying enzymes in AhR and Nrf2 compound null mutant mouse☆

Shuhei Noda,[a,1] Nobuhiko Harada,[a,1] Azumi Hida,[a] Yoshiaki Fujii-Kuriyama,[a] Hozumi Motohashi,[a,*] and Masayuki Yamamoto[a,b]

[a] *Center for Tsukuba Advanced Research Alliance, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan*
[b] *ERATO Environmental Response Project, Japan Science and Technology Corporation, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan*

Received 10 February 2003

Abstract

The arylhydrocarbon receptor (AhR) regulates the expression of cytochrome P450 (CYP)-1 gene family members which catalyze xenobiotic Phase I metabolism, while Nrf2 exerts the concerted regulation of Phase II enzyme genes. We generated AhR and Nrf2 compound null mutant mice to examine the integrated function of AhR- and Nrf2-regulated enzymes in detoxification. Furthermore, we used this mouse model, by administering three different classes of chemical inducers, to examine how xenobiotic metabolism may be influenced in the absence of signals transduced by AhR or Nrf2. The compound mutant mice responded only weakly to AhR ligand or Phase II inducer, while they displayed a clear response to phenobarbital, an inducer of the CYP2B family through another, unrelated transcription factor. Here, we report an initial characterization of the AhR-Nrf2 double mutant mice, which may serve as a simplified bioassay system to evaluate xenobiotic toxicity and metabolic biotransformation of various drugs and environmental chemicals.

Keywords: Xenobiotics; Phase I enzymes; Phase II enzymes; Gene knockout mouse; 3-Methylchoranthrene; Butylated hydroxyanisole; Phenobarbital

The detoxification of foreign substances, although complex, can be considered to comprise two sequential reaction processes; namely, Phase I and Phase II. In Phase I reactions, foreign chemicals are mainly oxidized by cytochrome P450 (CYP) enzymes to become polarized metabolites. Subsequently, Phase II metabolism, catalyzed by enzymes such as glutathione *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase (NQO1), converts the reactive Phase I products to more hydrophilic substances [1].

Four members of the *CYP* gene family, i.e., *CYP1–CYP4*, encode liver-expressed enzymes responsible for metabolizing xenobiotics and endogenous lipophilic substrates. The transcription of many members of the CYP1–CYP4 family can be activated by foreign chemicals through one of the four receptor-dependent mechanisms. The aryl hydrocarbon receptor (AhR) is a basic region-helix-loop-helix (bHLH)-PAS transcription factor that regulates the *CYP1* family genes. When bound by polycyclic aromatic hydrocarbons (PAHs), such as dioxins and 3-methylchoranthrene (3-MC), AhR translocates from the cytoplasm to the nucleus, heterodimerizes with AhR nuclear translocator (ARNT), and activates transcription through the xenobiotic-responsive element (XRE) [2]. The requirement for the AhR in the inducible expression of *CYP1* family genes by PAH was demonstrated in AhR-null mutant mice, which were generated independently by three different laboratories [3–5].

☆ *Abbreviations:* CYP, cytochrome P450; 3-MC, 3-methylchoranthrene; BHA, butylated hydroxyanisole; *t*-BHQ, *tert*-butylhydroquinone; PB, phenobarbital; AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; CAR, constitutive androstane receptor; XRE, xenobiotic-responsive element; ARE, antioxidant-responsive element; GST-P, glutathione *S*-transferase class Pi; NQO1, NAD(P)H:quinone oxidoreductase 1; D-KO, double knockout mouse of AhR and Nrf2; N-KO, Nrf2 knockout mouse; A-KO, AhR knockout mice; WT, wild type.

* Corresponding author. Fax: +81-29-853-7318.
E-mail address: hozumim@tara.tsukuba.ac.jp (H. Motohashi).

[1] These authors equally contributed to this work.

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00306-1

The xenobiotic induction mechanism of CYP2, 3, and 4 involves three distinct orphan nuclear receptors. CYP2B induction by phenobarbital (PB) and other PB-like lipophilic chemicals is mediated by the constitutive androstane receptor (CAR) through its interaction with the PB-responsive enhancer module (PBREM) [6]. Germ line mutation of the murine *CAR* gene revealed that it is essential for the PB-induction of the *CYP2B* family of genes [7]. PXR activates the *CYP3A* family genes in response to diverse chemicals including natural and synthetic steroids [8], whereas PPAR mediates the induction of the *CYP4A* family genes by many acidic chemicals classified as non-genotoxic carcinogens and peroxisome proliferators [9].

The genes of Phase II metabolism are also regulated in a concerted manner at the transcriptional level through the antioxidant-responsive element (ARE) or electrophile-responsive element (EpRE) [10,11]. Our previous analysis of nrf2-null mutant mice revealed that Nrf2 is central to ARE-mediated gene expression [12]. Nrf2 belongs to a family of transcription factors containing a basic region-leucine zipper (bZip) motif. The products of Phase I metabolism, as well as Phase II inducers, such as butylated hydroxyanisole (BHA) and Oltipraz, cause Nrf2 to dissociate from a cytoplasmic inhibitor molecule called Keap1, thereby permitting Nrf2 to translocate into the nucleus [13]. Nrf2 activates transcription of Phase II genes through the ARE/EpRE. In the absence of Nrf2, Phase II inducers were ineffective [14], and consequently, the reactive Phase I metabolites are not conjugated for excretion but form electrophiles that may attack intracellular macromolecules including DNA and protein [15,16].

To establish a model mouse system for assessing the integrated function of the AhR battery and Nrf2 battery in detoxification and to understand the contribution of AhR and Nrf2 to xenobiotic metabolism, we generated AhR–Nrf2 compound null mutant mice. Initial characterization of the double mutant mice focused on their responsiveness to three chemicals of different categories, 3-MC as a model AhR ligand, BHA as an activator of Nrf2, and PB as a regulator of the *CYP2B* family genes. Genes for CYP1A1, CYP1A2, NQO1, GST-P, and CYP2B10 were selected for analysis. The AhR–Nrf2 compound null mutant mice respond to neither the AhR ligand nor the Phase II inducer, while they displayed a clear response to PB. Thus, the AhR–Nrf2 double mutant mice serve as a simplified bioassay system for PB and related chemicals under conditions free of AhR and Nrf2 interference.

Materials and methods

Generation of $AhR^{-/-}$::$Nrf2^{-/-}$ mice. AhR- and Nrf2-null mutant mice were previously generated [5,12]. After the crossing of $AhR^{-/-}$ male mouse in 129svJ-C57BL/6 mixed background and a $Nrf2^{-/-}$ female mouse in 129svJ-ICR mixed background, the mice heterozygous for both genes ($AhR^{-/+}$::$Nrf2^{-/+}$) were obtained. One male and two females from the same litter were further intercrossed to produce progeny null for both AhR and Nrf2 genes. Double heterozygous ($AhR^{-/+}$::$Nrf2^{-/+}$) and double homozygous ($AhR^{-/-}$::$Nrf2^{-/-}$) mutant mice obtained from the offspring were bred for 400 days to examine their survival. The double homozygous female animals (D-KO) were exploited for enzyme-induction experiments with chemical inducers at 3–6 months after birth together with the control wild-type (WT), Nrf2 single homozygous (N-KO), and AhR single homozygous (A-KO) females at the same age.

Animals were housed under controlled temperature (23 °C), humidity (40–60%), and lighting (14/10 h light/dark cycle) and provided food (Oriental Yeast, Tokyo) and water ad libitum. AhR genotype was determined by PCR with three primers (5′-CGCGGGCACCATGAGCAG-3′, 5′-TTGAGACTCAGCTCCTGGATGG-3′, 5′-GCGGATTGACCGTAATGGGATAGG-3′) under the condition of 96 °C for 20 s, 62 °C for 30 s, and 72 °C for 45 s. For PCR of Nrf2 genotyping, three primers (5′-TGGACGGGACTATTGAAGGCTG-3′, 5′-GCCGCCTTTTCAGTAGATGGAGG-3′, 5′-GCGGATTGACCGTAATGGGATAGG-3′) were used at 96 °C for 20 s, 59 °C for 30 s, and 72 °C for 45 s.

Survival curve. Cohorts of 32 $AhR^{-/+}$::$Nrf2^{-/+}$ mice and of 65 D-KO mice were followed for 400 days to see their survival. Data were plotted into Kaplan–Meier survival curves.

Treatment with chemical inducers. 3-MC (WAKO Pure Chemical, Tokyo) was administered intraperitoneally at a dose of 80 mg/kg dissolved in corn oil as vehicle. Oral gavage treatment was employed to administer BHA (Sigma, Darmstadt) at a dose of 400 mg/kg dissolved in corn oil as vehicle. PB (Tokyo Kasei Kougyo, Tokyo) was injected intraperitoneally at a dose of 80 mg/kg in saline once a day for three consecutive days. The animals were sacrificed 24 h after the last treatment and the liver was processed for RNA purification.

RNA blot analysis. Total RNA was isolated from the liver with Isogen (Nippon Gene, Toyama) according to the manufacturer's instruction. Fifteen micrograms of purified total RNA was denatured and separated on 1% agarose gel containing formaldehyde, followed by capillary transfer to a nylon membrane (Zeta Probe Blotting Membrane; Bio-Rad Laboratories, CA). cDNA fragments of CYP1A1, CYP1A2, NQO1, GST-P, CYP2B10, and G3PDH were labeled and used as probes. Hybridization and washing were performed under the instruction manual of Zeta Probe Blotting Membrane.

Results and discussion

Generation of $AhR^{-/-}$::$Nrf2^{-/-}$ compound mutant mice

$AhR^{-/-}$ male mice and $Nrf2^{-/-}$ female mice were mated to obtain $AhR^{+/-}$::$Nrf2^{+/-}$ double heterozygous mice. $AhR^{-/-}$::$Nrf2^{-/-}$ compound mutant mice were generated from the mating of double heterozygous animals. Approximately half of the double homozygous mutant (D-KO) mice died within one week after birth for unknown reason(s), but the mice that survived this critical period became stable (below). The survival rate was 40% after 400 days (Fig. 1). When pups were examined one week after birth, no significant changes were observed except for the liver, where steatosis developed as previously reported in the analysis of AhR-null mutant mice [4].

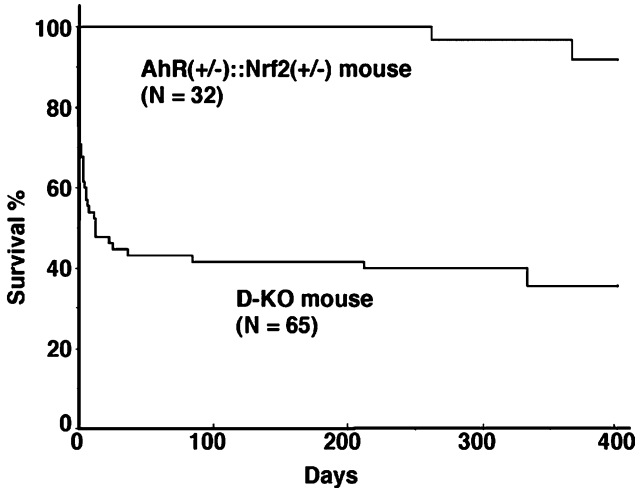

Fig. 1. Long-term survival of the D-KO mice. Sixty-five D-KO (double homozygous) mice and 32 control double heterozygous mice were bred up to 400 days. Survival ratios of each genetic group were plotted into Kaplan–Meier curves.

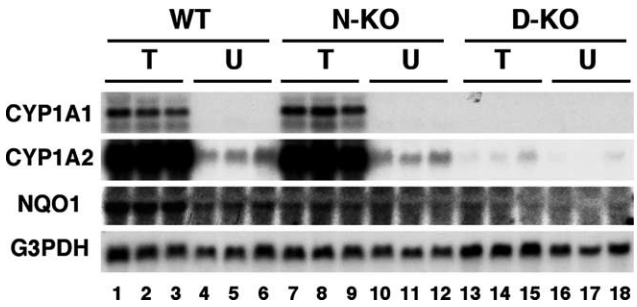

Fig. 2. Expression profiles of detoxifying enzyme genes in WT, N-KO, and D-KO mice induced by 3-MC. WT, N-KO, and D-KO were treated with 3-MC or vehicle as described in Materials and methods. Total RNAs (15 μg) extracted from the liver were subjected to RNA blot analysis. The expression levels of CYP1A1, CYP1A2, and NQO1 mRNAs were examined together with G3PDH mRNA as a loading control. WT mice treated with 3-MC (lanes 1–3) or vehicle (lanes 4–6), N-KO mice treated with 3-MC (lanes 7–9) or vehicle (lanes 10–12), and D-KO mice treated with 3-MC (lanes 13–15) or vehicle (lanes 16–18) were examined. T, treated; U, untreated.

The D-KO survivors, both males and females, were viable and fertile and displayed no apparent phenotypic change. We therefore examined carefully their major organs, including brain, heart, lung, thymus, stomach, intestine, spleen, liver, kidney, bladder, and reproductive organs, both macroscopically and microscopically, but again we could find no apparent abnormalities (data not shown). We envisage that heterogeneity in the genetic background might affect the neonatal viability of each D-KO mouse. However, most importantly for this study, obtaining viable D-KO mice enabled us to analyze the xenobiotic response in a situation where the *AhR* and *Nrf2* genes are simultaneously ablated.

Response to 3-methylchoranthrene

We first treated the D-KO mice with 3-MC, which induces Phase I gene expression through the XRE. Since AhR-null mutant (A-KO) animals have already been reported to be unresponsive to 3-MC treatment [17], we compared the 3-MC response in D-KO animals to those of Nrf2-null mutant (N-KO) and wild type (WT) mice. The expression levels of *CYP1A1*, *CYP1A2*, and *NQO1* genes were examined, which are categorized as the "AhR battery" [18]. The inducible expression of the *CYP1A1* and *CYP1A2* genes was completely abolished in the D-KO mice (Fig. 2, lanes 13–18), while the N-KO mice (Fig. 2, lanes 7–12) displayed a normal response comparable to those of WT mice (Fig. 2, lanes 1–6). This result is consistent with previous reports showing that *CYP1A1* and *CYP1A2* are typical AhR target genes [19,20], and demonstrates that the induction of CYP1A family genes by 3-MC is independent of Nrf2.

In contrast, the gene induction of *NQO1* was abolished both in the N-KO mice and D-KO mice (Fig. 2). This results shows that Nrf2 is indispensable for the induction of *NQO1* gene by 3-MC. Although the *NQO1* gene has been included in the "AhR battery," some controversy remains regarding its regulatory mechanism [21]. It has been shown that *NQO1* gene expression is strongly induced in C57BL/6 mice possessing high affinity AhR, but not in DBA/2 mice possessing low affinity AhR [22], suggesting that the induction of NQO1 by 3-MC correlates with AhR activity. The existence of an XRE motif in the upstream regulatory region of the *NQO1* gene supports the conclusion of this observation [21]. However, the true significance of the XRE motif for the gene expression has not yet been established. Importantly, the regulatory region of the *NQO1* gene also contains an ARE, and one report showed that the ARE, not the XRE, is critical for NQO1 induction by TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), a model AhR ligand [23].

A plausible mechanism whereby AhR ligands induce expression of the *NQO1* gene may involve an increase in oxidative stress capable of promoting Nrf2 activation, which in turn could increase the expression of the *NQO1* gene. Consistent with this view, the *GST-P* gene, a recognized ARE-dependent gene, shows a similar pattern of gene induction by 3-MC (data not shown). An alternative possibility is that the AhR activates *NQO1* gene transcription synergistically with Nrf2, although no report has shown that Nrf2 activity depends on the availability of the AhR in the neighboring XRE.

Response to BHA

BHA is a mono-functional inducer of detoxifying enzymes, capable of inducing Phase II enzymes but not Phase I enzymes. This ability of BHA to induce Phase II gene expression may result from its oxidative

metabolism to several active polar species including *t*-BHQ (*tert*-butylhydroquinone) as it has been reported to occur in the presence of microsomes [24]. To confirm the Nrf2-dependency of BHA action, we examined the response of AhR–Nrf2 double mutant mice to BHA treatment in comparison to those of WT, N-KO, and A-KO animals. WT and N-KO mice were obtained as littermates of D-KO mice from the double heterozygous mating, whereas A-KO mice were prepared independently from different parents due to breeding problems in the original mating.

NQO1 and *GST-P* were chosen as typical genes activated by BHA administration. In accordance with our expectation, the expression level of *NQO1* mRNA was clearly increased in the livers of WT and A-KO mice by 5.5- and 3.0-fold, respectively (Fig. 3, lanes 19–30), but induction was abolished in the N-KO and D-KO mouse livers (Fig. 3, lanes 1–18). *GST-P* mRNA was strongly induced 14- and 4.6-fold in WT and A-KO mice (Fig. 3, lanes 19–30), but weakly induced 1.6- and 2.0-fold in N-KO and D-KO mice, respectively (Fig. 3, lanes 1–18). These results confirm the fact that Nrf2 makes a major contribution to the inducible expression of *NQO1* and *GST-P* genes. It is interesting that in the A-KO mice

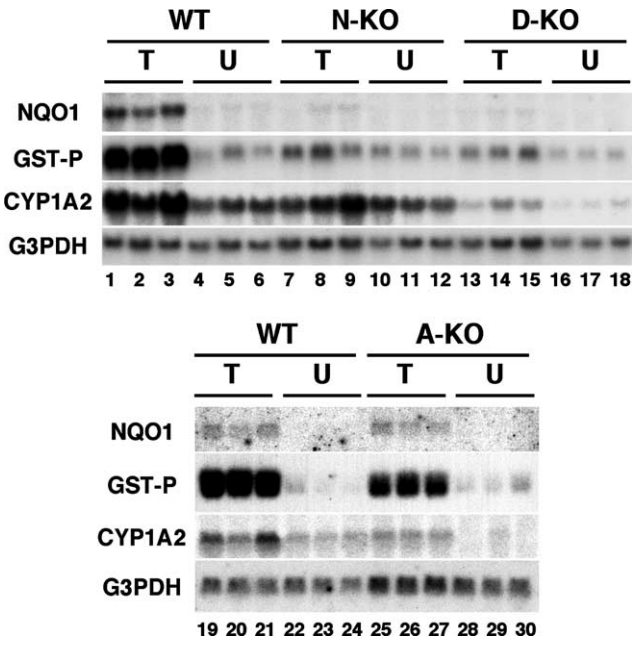

Fig. 3. Expression profiles of detoxifying enzyme genes induced in WT, N-KO, D-KO, and A-KO mice by BHA. WT, N-KO, D-KO, and A-KO mice were treated with BHA or vehicle. Total RNAs (15 μg) extracted from the liver were subjected to RNA blot analysis. The expression levels of NQO1, GST-P, and CYP1A2 mRNAs were examined together with G3PDH mRNA as a loading control. WT mice treated with BHA (lanes 1–3, 19–21) or vehicle (lanes 4–6, 22–24), N-KO mice treated with BHA (lanes 7–9) or vehicle (lanes 10–12), D-KO mice treated with BHA (lanes 13–15) or vehicle (lanes 16–18), and A-KO mice treated with BHA (lanes 25–27) or vehicle (lanes 28–30) were examined. T, treated; U, untreated.

there was a 45% (from 5.5- to 3.0-fold) decrease in *NQO1* induction and 67% (from 14- to 4.6-fold) decrease in *GST-P* induction. This may reflect an inability of the A-KO mice to metabolize BHA to active metabolites, such as *t*-BHQ, and suggests that, in vivo, AhR-dependent *CYP* genes make an active contribution to the metabolism of BHA. It further suggests that *GST-P* induction is partially mediated by mechanisms independent of both AhR and Nrf2.

CYP1A2 is a known Phase I enzyme but was induced by BHA treatment in all the mice examined (Fig. 3). The relative induced expression levels of *CYP1A2* gene are 1.0, 0.73, 0.17, and 0.29 in WT, N-KO, D-KO, and A-KO mice, respectively (Fig. 3, lanes 1–3, 7–9, 13–15, 19–21, and 25–27). They are the highest in WT, intermediate in N-KO and in A-KO, and the lowest in D-KO. This result suggests that both Nrf2 and AhR are necessary for the higher levels of the induced expression of the *CYP1A2* gene. The relative basal expression levels are 1.0, 1.0, 0.1, and 0.3 in WT, N-KO, D-KO, and A-KO mice, respectively (Fig. 3, lanes 4–6, 10–12, 16–18, 22–24, and 28–30). They are high in WT and N-KO, and low in D-KO and A-KO. This result indicates that the AhR is critical for maintaining the basal expression levels of the *CYP1A2* gene.

When the *CYP1A2* locus in the mouse genomic database was examined, several AREs and XREs were found in the sequence surrounding the *CYP1A2* gene (data not shown). Although none of the AREs have been experimentally tested, we surmise that some of the AREs in this locus might be responsible for the gene activation by BHA. On the contrary, XREs within this locus were well examined and characterized. Since several XREs are present in the mouse *CYP1A2* locus and the corresponding human locus [25], the AhR may, through the XREs, direct basal levels of expression of the *CYP1A2* gene in addition to its induction. However, from an analysis of the human *CYP1A2* gene in a transient overexpression assay, no apparent XRE was found within the regions important for basal constitutive transcription [26], whereas functional XREs were identified in the upstream regions required for the response to 3-MC [20]. Assuming that the regulatory mechanisms are conserved between human and mouse, the contribution made by the AhR to the constitutive expression of the*CYP1A2* gene might be indirect.

Response to PB

PB activates transcription of the *CYP2B* family of genes. This induction has been shown to be under the regulation of CAR transcription factor and PBREM sequence [6]. In this study, we examined how the lack of both AhR and Nrf2 affects the PB-mediated induction of CYP2B10. We found that *CYP2B10* gene expression was induced in the liver of mice of all genotypes tested

(Fig. 4). However, the relative induced expression levels are 1.0, 0.6, 4.1, and 2.9 in WT, N-KO, D-KO, and A-KO, respectively (Fig. 4, lanes 1–3, 7–9, 13–15, 19–21, and 25–27). The magnitude of induction is far greater in A-KO and D-KO than in WT and N-KO mice, indicating that the *AhR* gene disruption leads to the super-induction of *CYP2B10* gene expression. This result indicates that AhR directly or indirectly represses the PB-mediated induction of *CYP2B10* gene.

One possible explanation is an indirect effect of AhR disruption. PB metabolism may be retarded in the absence of AhR, resulting in the higher effective PB concentration in A-KO and D-KO livers. Supporting this contention, an elongated sedative effect of PB was observed in D-KO and A-KO animals (data not shown).

Alternative explanation would be a rather direct repressive effect of AhR on *CYP2B10* gene transcription. It is intriguing to note two recent reports related to the regulation of *CYP2B* family genes, in which transcriptional coactivator SRC-1 was reported to enhance the AhR transcriptional activity [27] and also to potentiate the CAR activity for the transcription of the rat *CYP2B1* gene [28]. The AhR potentiation appears to be the consequence of direct interaction of SRC-1 and AhR [27] or SRC-1 and ARNT [29], while the CAR potentiation requires flanking regions of PBREM core sequence as well as the core sequence itself, suggesting the requirement of additional factor(s) for the enhancement of CAR activity by SRC-1 coactivator [28]. These studies thus imply that AhR and CAR share or compete for the common coactivator SRC-1, which may explain the super-induction of the *CYP2B10* gene expression by PB in the absence of AhR.

We found that the *CYP1A2* gene was also induced by PB in all the mice examined 1.8-, 2.6-, 5.5-, and 4.4-fold in WT, N-KO, D-KO, and A-KO, respectively (Fig. 4). This result indicates that both AhR and Nrf2 are dispensable for the inducible expression of the *CYP1A2* gene by PB. The higher fold induction in the absence of AhR (D-KO and A-KO) is attributed to the lower basal expression levels (discussed above). Recent report showed that the *CYP1A2* induction by PB is abolished in the CAR-null mutant mice [30]. Since the mechanism of PB-mediated induction of *CYP1A2* gene has not been elucidated yet, we examined the mouse *CYP1A2* gene locus in the database for PBREM. We found one PBREM consensus sequence at 2.6-kbp upstream of the transcription initiation site and are planning to test the significance of this element for the PB-mediated induction of the *CYP1A2* gene. The *CYP1A2* induction is suggested to be one cause for the increased acetaminophen toxicity by pretreatment with PB [30].

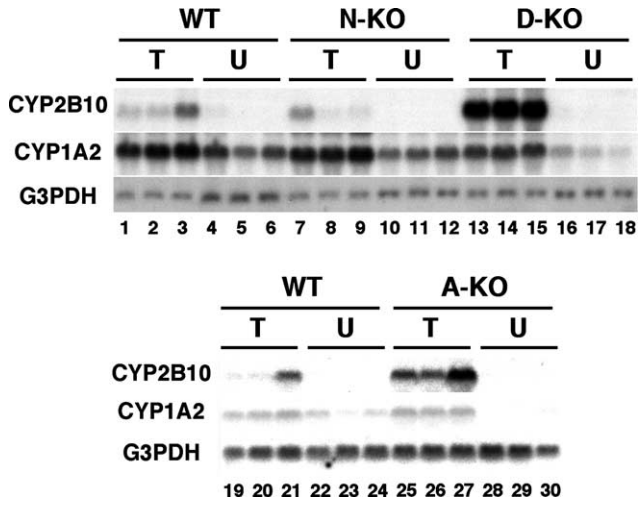

Fig. 4. Expression profiles of detoxifying enzyme genes induced in WT, N-KO, D-KO, and A-KO mice by PB. WT, N-KO, D-KO, and A-KO mice were treated with PB or vehicle. Total RNAs (15 μg) extracted from the liver were subjected to RNA blot analysis. The expression levels of CYP2B10 and CYP1A2 mRNAs were examined together with G3PDH mRNA. WT mice treated with PB (lanes 1–3, 19–21) or vehicle (lanes 4–6, 22–24), N-KO mice treated with PB (lanes 7–9) or vehicle (lanes 10–12), D-KO mice treated with PB (lanes 13–15) or vehicle (lanes 16–18), and A-KO mice treated with PB (lanes 25–27) or vehicle (lanes 28–30) were examined. T, treated; U, untreated.

Prospective uses of $AhR^{-/-}$::$Nrf2^{-/-}$ compound mutant mice

As a mouse model, the AhR–Nrf2 D-KO mouse line could find applications in numerous fields including pharmacology and environmental and agricultural toxicology. In this study, we established an AhR–Nrf2 D-KO mouse line and confirmed that these mice lack the ability to respond to both 3-MC and BHA, a typical AhR ligand and a Phase II inducer, respectively (Fig. 5). It is conceivable that the D-KO mice are virtually devoid of the major PAH-metabolizing pathway. As our aim

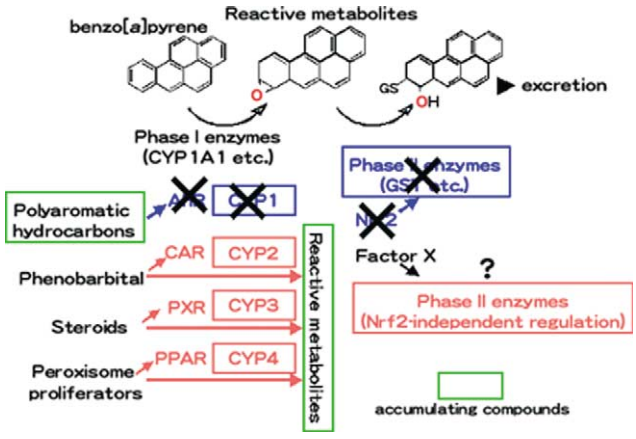

Fig. 5. Schematic illustration of the metabolic and detoxifying pathways remaining in D-KO mice. AhR- or Nrf2-dependent response is abolished and the disrupted pathways are colored in blue. The remaining pathways independent of AhR and Nrf2 function are colored in red. Compounds accumulating in D-KO mice are surrounded by green squares.

was to exploit the D-KO mice in toxicological and pharmacological studies, we wish to propose here some possible applications for the AhR–Nrf2 D-KO mutant animals (Fig. 5). Using this mouse model it would be possible to clarify the contribution that the AhR battery and Nrf2 battery genes make to the detoxification pathway. Further, an analysis of xenobiotic metabolism could be made in the absence of the gene inductions, normally directed by AhR and Nrf2.

An interesting aspect of the analysis of the D-KO mouse is the change in the metabolic pathway of various xenobiotic or pharmaceutical compounds. We are curious about the qualitative and quantitative alteration of metabolites in each organ. Observed changes in the metabolism of xenobiotics in the D-KO mice could reveal the integrated and coordinated functions of the AhR-regulated genes and the Nrf2-regulated genes to the detoxification processes.

This mouse model could also simplify investigations into the gene-induction response of regulators other than AhR–Nrf2, unveiling novel process that may otherwise be buried under major pathways of metabolism. Another tempting experiment is to use the D-KO mice to test the effect of chronic toxicity by PAHs. The PAHs should accumulate within the body of D-KO mice over long periods of time, since in theory PAHs should only be slowly metabolized in the absence of AhR and Nrf2.

Concluding remarks

Ingestion of numerous, sometimes harmful, xenobiotics is inevitable. Even food naturally contains many compounds requiring detoxification, in addition to artificial chemicals used in the growing and processing of foodstuffs, such as remnants of agricultural pesticides. Inhalation of air pollutants from combusted fuel and the emissions from factories account for further exposure to toxic chemicals. Therefore, a growing need exits to develop a human-oriented bioassay system capable of evaluating the toxicity of xenobiotics with reasonable sensitivity and specificity. Our $AhR^{-/-}$::$Nrf2^{-/-}$ compound mutant mouse line should provide such a unique and effective assay system in toxicological evaluations.

Acknowledgments

We are grateful to Drs. Vincent Kelley, Tomonori Hosoya, Jun-sei Mimura, and Satoru Takahashi for discussion, and Masahiko Negishi for CYP2B10 cDNA. We thank Ms. Naomi Kaneko for histological analysis and Reiko Kawai for mouse breeding. This work was supported by grants from ERATO (M.Y.), the Ministry of Education, Science, Sports and Culture (H.M. and M.Y.), JSPS-RFTF (M.Y.), CREST (M.Y. and Y.F.-K.), PROBRAIN (H.M.), and Special Coordination Fund for Promoting Science and Technology (H.M.).

References

[1] P. Talalay, Mechanisms of induction of enzymes that protect against chemical carcinogenesis, Adv. Enzyme Regul. 28 (1989) 237–250.
[2] K. Sogawa, Y. Fujii-Kuriyama, Ah receptor, a novel ligand-activated transcription factor, J. Biochem. (Tokyo) 122 (1997) 1075–1079.
[3] P.M. Fernandez-Salguero, T. Pineau, D.M. Hilbert, T. McPhail, S.S. Lee, S. Kimura, D.W. Nebert, S. Rudikoff, J.M. Ward, F.J. Gonzalez, Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor, Science 268 (1995) 722–726.
[4] J.V. Schmidt, G.H. Su, J.K. Reddy, M.C. Simon, C.A. Bradfield, Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development, Proc. Natl. Acad. Sci. USA 93 (1996) 6731–6736.
[5] J. Mimura, K. Yamashita, K. Nakamura, M. Morita, T.N. Takagi, K. Nakao, M. Ema, K. Sogawa, M. Yasuda, M. Katsuki, Y. Fujii-Kuriyama, Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor genes, Genes Cells 2 (1997) 645–654.
[6] P. Honkakoski, I. Zelko, T. Sueyoshi, M. Negishi, The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene, Mol. Cell. Biol. 18 (1998) 5652–5658.
[7] P. Wei, J. Zhang, M. Egan-Hafley, S. Liang, D.D. Moore, The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism, Nature 407 (2000) 920–923.
[8] S.A. Kliewer, J.T. Moore, L. Wade, J.L. Staudinger, M.A. Watson, S.A. Jones, D.D. McKee, B.B. Oliver, T.M. Willson, R.H. Zetterstrom, T. Perlmann, J.M. Lehmann, An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway, Cell 92 (1998) 73–82.
[9] A.C. Bayly, N.J. French, C. Dive, R.A. Roberts, Non-genotoxic hepatocarcinogenesis in vitro: the FaO hepatoma line responds to peroxisome proliferators and retains the ability to undergo apoptosis, J. Cell Sci. 104 (1993) 307–315.
[10] R.S. Friling, A. Bensimon, T. Tichauer, V. Danial, Xenobiotic-inducible expression of murine gluthatione *S*-transferase Ya subunit gene is controlled by as electrophile-responsive element, Proc. Natl. Acad. Sci. USA 87 (1990) 6258–6262.
[11] T.H. Rushmore, M.R. Morton, C.B. Pickett, The antioxidant responseive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity, J. Biol. Chem. 266 (1991) 11632–11639.
[12] K. Itoh, T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements, Biochem. Biophys. Res. Commun. 236 (1997) 313–322.
[13] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J.D. Engel, M. Yamamoto, Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, Genes Dev. 13 (1999) 76–86.
[14] M. McMahon, K. Itoh, M. Yamamoto, S.A. Chanas, C.J. Henderson, L.I. McLellan, C.R. Wolf, C. Cavin, J.D. Hayes, The cap'n'collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes, Cancer Res. 61 (2001) 3299–3307.
[15] A. Enomoto, K. Itoh, E. Nagayoshi, J. Haruta, T. O'Connor, T. Harada, M. Yamamoto, High sensitivity of Nrf2 knockout mice to acetoaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes, Toxicol. Sci. 59 (2001) 169–177.

[16] Y. Aoki, H. Sato, N. Nishimura, S. Takahashi, K. Itoh, M. Yamamoto, Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust, Toxicol. Appl. Pharmacol. 173 (2001) 154–160.
[17] T. Shimada, K. Inoue, Y. Suzuki, T. Kawai, E. Azuma, T. Nakajima, M. Shindo, K. Kurose, A. Sugie, Y. Yamagishi, Y. Fujii-Kuriyama, M. Hashimoto, Arylhydrocarbon receptor-dependent induction of liver and lung cytochromes P450 1A1, 1A2, and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in genetically engineered C57BL/6J mice, Carcinogenesis 23 (2002) 1199–1207.
[18] D.W. Nebert, A.L. Roe, M.Z. Dieter, W.A. Solis, Y. Yang, T.P. Dalton, Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis, Biochem. Pharmacol. 59 (2000) 65–85.
[19] A. Fujisawa-Sehara, M. Yamane, Y. Fujii-Kuriyama, A DNA-binding factor specific for xenobiotic responsive elements of P-450c gene exists as a cryptic form in cytoplasm: its possible translocation to nucleus, Proc. Natl. Acad. Sci. USA 85 (1988) 5859–5863.
[20] L.C. Quattrochi, T. Vu, R.H. Tukey, The human CYP1A2 gene and induction by 3-methylchoranthrene. A region of DNA that supports AH-receptor binding and promoter-specific induction, J. Biol. Chem. 269 (1994) 6949–6954.
[21] L.V. Favreau, C.B. Pickett, Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants, J. Biol. Chem. 266 (1991) 4556–4561.
[22] J.A. Robertson, H.C. Chen, D.W. Nebert, NAD(P)H:menadione oxidoreductase. Novel purification of enzyme cDNA and complete amino acid sequence, and gene regulation, J. Biol. Chem. 261 (1986) 15797–15799.
[23] V. Radjendirane, A.K. Jaiswal, Antioxidant response element-mediated 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induction of human NAD(P)H:quinone oxidoreductase 1 gene expression, Biochem. Pharmacol. 58 (1999) 1649–1655.
[24] S.W. Cummings, G.A. Ansari, F.P. Guengerich, L.S. Crouch, R.A. Prough, Metabolism of 3-*tert*-butyl-4-hydroxyanisole by microsomal fractions and isolated rat hepatocytes, Cancer Res. 45 (1985) 5617–5624.
[25] F.J. Gonzalez, S. Kimura, D.W. Nebert, Comparison of the flanking regions and introns of the mouse 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible cytochrome P1-450 and P3-450 genes, J. Biol. Chem. 260 (1985) 5040–5049.
[26] I. Chung, E. Bresnick, Regulation of the constitutive expression of the human CYP1A2 gene: cis elements and their interactions with proteins, Mol. Pharmacol. 47 (1995) 677–685.
[27] M.B. Kumar, G.H. Perdew, Nuclear receptor coactivator SRC-1 interacts with the Q-rich subdomain of the AhR and modulates its transactivation potential, Gene Expr. 8 (1999) 273–286.
[28] R. Muangmoonchai, D. Smirlis, S.C. Wong, M. Edwards, I.R. Phillips, E.A. Shephard, Xenobiotic induction of cytochrome P450 2B1 (CYP2B1) is mediated by the orphan nuclear receptor constitutive androstane receptor (CAR) and requires steroid co-activator 1 (SRC-1) and the transcription factor Sp1, Biochem. J. 355 (2001) 71–78.
[29] T.V. Beischlag, S. Wang, D.W. Rose, J. Torchia, S. Reisz-Porszasz, K. Muhammad, W.E. Nelson, M.R. Probst, M.G. Rosenfeld, O. Hankinson, Recruitment of the NCoA/SRC-1/p160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex, Mol. Cell. Biol. 22 (2002) 4319–4333.
[30] J. Zhang, W. Huang, S.S. Chua, P. Wei, D.D. Moore, Modulation of acetaminophen-induced hepatotoxicity by the xenobiotic receptor CAR, Science 298 (2002) 422–424.