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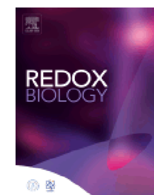

Possible involvement of nuclear factor erythroid 2-related factor 2 in the gene expression of Cyp2b10 and Cyp2a5 ☆

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Cytochrome P450 gene expression is altered by various chemical compounds. In this study, we used nuclear factor erythroid 2-related factor 2 (Nrf2)–deficient (Nrf2$^{-/-}$) mice to investigate the involvement of Nrf2 in Cyp2b10 and Cyp2a5 gene expression. Phorone, an Nrf2 activator, strongly increased Cyp2b10 and Cyp2a5 mRNA as well as Nrf2 target genes, including NAD(P)H-quinone oxidoreductase-1 and heme oxygenase-1, in wild-type mouse livers 8 h after treatment. The phorone-induced mRNA levels in Nrf2$^{-/-}$ mouse livers were lower than that in wild-type mouse livers. Nrf2$^{-/-}$ mice showed attenuated Cyp2b10 and Cyp2a5 induction by phenobarbital, a classical Cyp2b inducer. These findings suggest that the Nrf2 pathway is involved in Cyp2b10 and Cyp2a5 gene expression.

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Introduction

Cytochrome P450s (P450s) are phase I detoxification enzymes that catalyze various reactions such as oxidation, reduction, and dehalogenation to metabolize endogenous and exogenous compounds [1,2]. P450 gene expression can be extensively regulated by a number of factors including xenobiotics (e.g., phenobarbital). Previous studies have investigated the transcriptional activation of P450s and several xenobiotic-activated receptors, such as aryl hydrocarbon receptor, constitutive androstane receptor (CAR), and pregnane X receptor [3], which act as specific transcription factors. Recent investigations have demonstrated that nuclear factor erythroid 2-related factor 2 (Nrf2) functions as a transcription factor for the Cyp2a5 gene [4]; however, its involvement in other P450 genes is unclear.

Nrf2 is transferred to the nucleus when it and its anchoring protein, Kelch-like ECH-associated protein 1, respond to oxidative stressors such as electrophiles. Nuclear Nrf2 heterodimerizes to the small musculoaponeurotic fibrosarcoma oncogene homolog (Maf), a transcriptional activator, and positively regulates gene expression by binding to the antioxidant response element (ARE) located upstream of the target genes [5]. Thus, the Nrf2 system is a gene regulator of biological defense proteins, such as antioxidant proteins, associated proteins of glutathione synthesis, and phase II detoxification enzymes [5], which suggests that this transcription factor has multiple functions. α,β-Unsaturated carbonyl compounds, such as phorone, induce NAD(P)H-quinone oxidoreductase-1 (Nqo1) and heme oxygenase-1 (Hmox1), particularly in the liver [6,7]. These effects are due to Nrf2 activation followed by an increase in ARE binding because Nqo1 [8] and Hmox1 [9] are genes that are known to be regulated by Nrf2. The present study provides evidence that Nrf2 is involved in phorone- and phenobarbital-induced P450 gene expression changes in mouse livers. These results suggest that chemical compounds associated with Nrf2 activation modify Cyp2b10 and Cyp2a5 gene expression.

Materials and methods

Materials

Phorone was from Wako Pure Chemical Industries (Tokyo, Japan). Phenobarbital sodium salt was from Tokyo Chemical Industry (Tokyo,

Abbreviations: ARE, antioxidant response element; CAR, constitutive androstane receptor; P450, cytochrome P450; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Hmox1, heme oxygenase-1; Nqo1, NAD(P)H-quinone oxidoreductase-1; Maf, musculoaponeurotic fibrosarcoma oncogene homolog; Nrf2, nuclear-factor erythroid 2-related factor 2; PBREM, phenobarbital responsive element module; PCR, polymerase chain reaction

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Japan). All other reagents used were of the highest grade commercially available.

Animals and treatments

All mouse experiments were carried out under the control of the Committee Regulation of Animal Care and Welfare of Showa University. Male C57BL/6 mice (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The Nrf2-deficient ($Nrf2^{-/-}$) mice were established by Itoh et al. [10]. Phorone (2 mmol/kg) was dissolved in corn oil and injected intraperitoneally; a dose of 2 mmol/kg is used for most of these Hmox1 induction studies [6]. Phenobarbital (100 mg/kg) was dissolved in saline and injected intraperitoneally; a dose of 100 mg/kg is used for most of these P450 induction studies [11]. Control mice were injected with respective vehicles in volumes similar to that of the treated mice. All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Showa University (#27003).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from the liver using RNeasy Mini Kit (QIAGEN, Valencia, CA), and first-stranded cDNA was synthesized with a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). The quantitative real-time PCR was performed according to the manufacturer's protocol using StepOne real-time PCR system (Life Technologies, Carlsbad, CA), SYBR Premix Ex Taq (Takara Bio), and TaqMan Fast Universal Master Mix (Life Technologies). The PCR primer and TaqMan MGB Probe were purchased from Takara Bio or Life Technologies. The mRNA levels were measured as the relative ratio to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA.

Statistical analysis

Statistical analysis was performed using the Kruskal–Wallis non-parametric analysis, with the Dunnett test or the Scheffe test for post hoc comparisons. The accepted level of significance was set at $p < 0.05$.

Results

Phorone, an α,β-unsaturated carbonyl compound, induces Nrf2 target genes in mouse livers

Nrf2 transcriptionally regulates Nqo1 and Hmox1 [12,13]. We previously reported that phorone (Fig. 1A) is a potent inducer of Hmox1 in rat livers [6]. However, its effects on other Nrf2 target genes in mouse livers were unclear. Thus, we first performed time-course experiments to confirm phorone inductions of Nqo1 and Hmox1 in wild-type (WT) mouse livers. Phorone (2 mmol/kg) increased Nqo1 mRNA 4 h after treatment and reached 890% of the control level by 8 h (Fig. 1B). Further, phorone increased Hmox1 mRNA 2 h after treatment and reached a peak level of 3800% of the control level by 4 h (Fig. 1C).

Phorone induces Cyp2b10 and Cyp2a5 gene expression in mouse livers

Because phorone induced Nrf2 target genes, we next examined its effect on P450 gene expression. Phorone enhanced the gene expression of various P450 species; in particular Cyp2b10 and Cyp2a5 mRNA were markedly increased 8 h after treatment (1800% and 1100% of the control, respectively) (Fig. 2). Phorone also significantly induced Cyp1a2 and Cyp3a11, although this induction was extremely low as compared with that of Cyp2b10 and Cyp2a5.

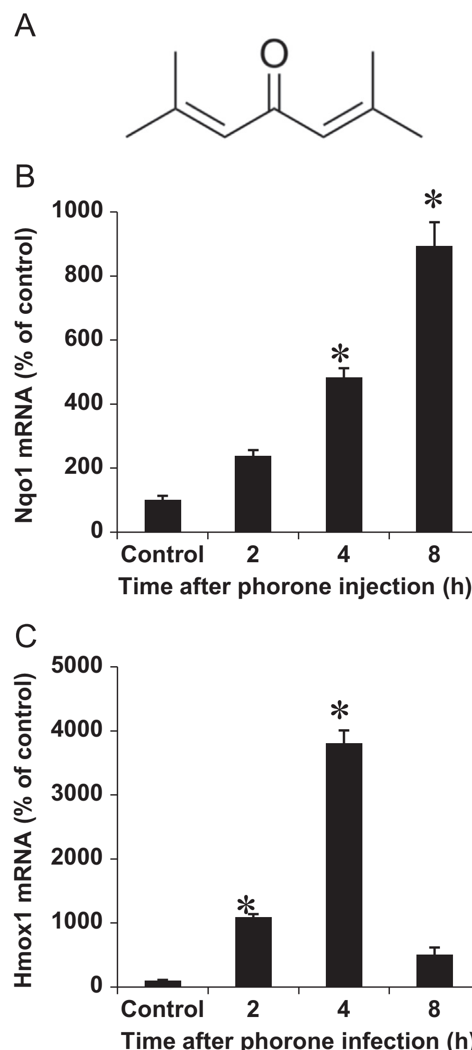

Fig. 1. The effects of phorone on Nqo1 and Hmox1 gene expression in mouse livers. (A) Structure of phorone. (B and C) Mice were injected intraperitoneally with phorone or corn oil, and their livers were excised at the times indicated. The mRNA levels were determined by real-time PCR and semiquantified by normalizing to Gapdh mRNA. Values represent the mean ± S.E.M. ($n = 3$). The significant difference was assessed by the Kruskal–Wallis non-parametric analysis followed by the Dunnett test. *$P < 0.05$ vs. control mice.

Attenuation of Cyp2b10 and Cyp2a5 induction by phorone in $Nrf2^{-/-}$ mouse livers

Phorone robustly induced Cyp2b10 and Cyp2a5 in WT mouse livers (Fig. 2). Therefore, to elucidate the Nrf2 involvement in Cyp2b10 and Cyp2a5 induction, we investigated the amount of Cyp2b10 and Cyp2a5 mRNA increased by phorone using $Nrf2^{-/-}$ mice. As shown in Fig. 3, Cyp2b10, Cyp2a5, and Nqo1 mRNA levels of the controls were lower in $Nrf2^{-/-}$ mice livers compared with those in WT mice livers. Phorone significantly increased Cyp2b10 (1200% of the control), Cyp2a5 (300% of the control), and Nqo1 mRNA (720% of the control) in WT mouse livers at 8 h after treatment (in a way similar to what is shown in Figs. 1B and 2). Cyp2b10 mRNA levels in phorone-treated $Nrf2^{-/-}$ mouse livers were significantly lower than that in the corresponding WT mouse livers (23% of the phorone-treated WT mouse livers) (Fig. 3A),

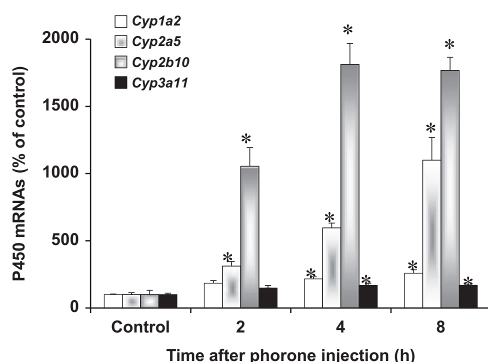

Fig. 2. The effects of phorone on P450 gene expression in mouse livers. Mice were injected intraperitoneally with phorone or corn oil, and their livers were excised at the times indicated. The mRNA levels were determined by real-time PCR and semiquantified by normalizing to Gapdh mRNA. Values represent the mean ± S.E. M. (n=3). The significant difference was assessed by the Kruskal–Wallis non-parametric analysis followed by the Dunnett test. *P < 0.05 vs. control mice.

and phorone failed to induce Cyp2a5 and Nqo1 in Nrf2$^{-/-}$ mouse livers (Fig. 3B and C).

Nrf2 is involved in phenobarbital-induced Cyp2b10 and Cyp2a5 gene expression in mouse livers

Phenobarbital (Fig. 4A) is a classical Cyp2b10 inducer. To further assess the involvement of Nrf2 in Cyp2b10 and Cyp2a5 induction, we treated WT and Nrf2$^{-/-}$ mice with phenobarbital and measured Cyp2b10 and Cyp2a5 mRNA levels in the livers. Phenobarbital markedly increased Cyp2b10 (8540% of the controls), Cyp2a5 (420% of the controls), and Nqo1 mRNAs (160% of the controls) 12 h after treatment in WT mice (Fig. 4B–D). Nrf2$^{-/-}$ mice showed significantly suppressed phenobarbital-induced Cyp2b10 gene expression (43% of the phenobarbital-treated WT mice). Similar to the phorone treatment, phenobarbital failed to induce Cyp2a5 and Nqo1 in Nrf2$^{-/-}$ mouse livers.

Discussion

The Nrf2 pathway mediates the antioxidant response, which is an important cellular defense mechanism that leads to the induction of several chemoprotective and antioxidative genes, including Nqo1, Hmox1, glutathione *S*-transferases, and multidrug resistance-associated proteins [14,15]. In the present study, we demonstrated that in mouse livers, the redox-sensitive transcription factor Nrf2 is involved in the gene expression of phase I drug-metabolizing enzymes Cyp2b10 and Cyp2a5. These results suggest that Nrf2 functions as a key regulator for the cellular detoxification of xenotoxins and in the cellular redox homeostasis.

Because α,β-unsaturated carbonyl compounds, including phorone, have an electrophilic carbon, these compounds deplete a major intracellular antioxidant glutathione and induce oxidative stress, thereby activating Nrf2 [7,16]. Endogenous α,β-unsaturated carbonyl compounds, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, a prostaglandin D_2 dehydration product, and 4-hydroxy-2-nonenal, a lipid peroxidation product, also activate Nrf2 and potently induce antioxidant proteins [7,13]. In this study, we demonstrated that phorone induces Nqo1 and Hmox1 but failed to induce Nqo1 mRNA in Nrf2$^{-/-}$ mouse livers, suggesting that phorone activates the Nrf2 pathway. Furthermore, we found that phenobarbital significantly induces Nqo1 gene expression. However, its induction was not observed in Nrf2$^{-/-}$ mouse livers. Thus, these results suggest that phenobarbital also activates Nrf2 in mouse livers.

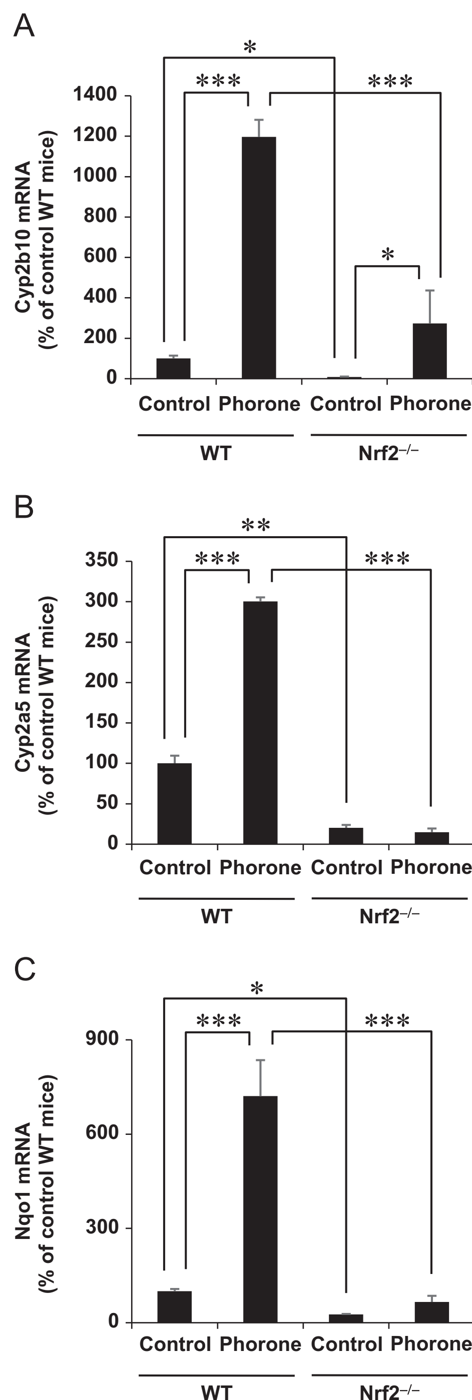

Fig. 3. Phorone suppressed the induction of Cyp2b10, Cyp2a5, and Nqo1 mRNA in Nrf2$^{-/-}$ mouse livers. (A–C) Mice were injected intraperitoneally with phorone or corn oil, and their livers were excised 8 h after treatment. The mRNA levels were determined by real-time PCR and semiquantified by normalizing to Gapdh mRNA. Values represent the mean ± S.E.M. (n=4–5). The significant difference was assessed by the Kruskal–Wallis non-parametric analysis followed by the Scheffé test (*P < 0.05, **P < 0.01 and ***P < 0.001).

The mechanism by which xenobiotics induce Cyp2b gene expression has been well described. On being exposed to an inducer, CAR translocates into the nucleus forming a heterodimer with the retinoid X receptor and activates the phenobarbital responsive element module (PBREM), which is located upstream of the Cyp2b gene family [17]. CAR is either directly controlled by an agonist ligand, such as 1,4-bis [2-(3,5-dichloropyridyloxy)]benzene [18], or indirectly controlled by inducers, such as phenobarbital [17,19,20]. The overall mechanism that

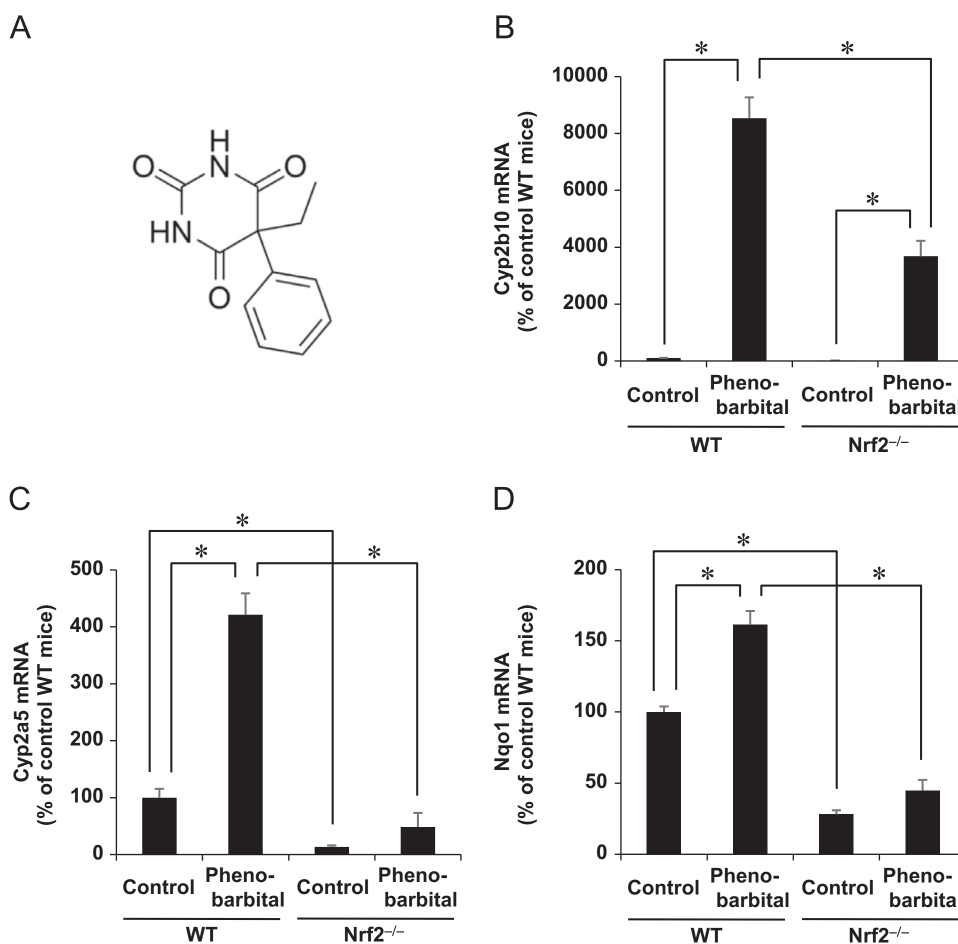

Fig. 4. Nrf2 is involved in phenobarbital-induced Cyp2b10, Cyp2a5, and Nqo1 gene expression in mouse livers. (A) Structure of phenobarbital. (B–D) Mice were injected intraperitoneally with phenobarbital or saline, and their livers were excised 12 h after treatment. The mRNA levels were determined by real-time PCR and semiquantified by normalizing to Gapdh mRNA. Values represent the mean ± S.E.M. (n=3–6). The significant difference was assessed by the Kruskal–Wallis non-parametric analysis followed by the Scheffé test (*$P<0.05$).

causes phenobarbital-mediated nuclear translocation of CAR, a key step in the transactivation of target genes, is unclear. In contrast, Nrf2 activators, such as *trans*-Stilbene oxide and oltipraz, activate the CYP2B6 promoter- (including PBREM) luciferase reporter construct [21,22]. In this study, phorone significantly induced Cyp2b10 gene expression in mouse livers, and this suggests that phorone activates CAR, resulting in the activation of PBREM. However, the mechanism for CAR activation by Nrf2 activators and phenobarbital is still unclear. Furthermore, we showed that Cyp2b10 induction by phorone and phenobarbital is attenuated in $Nrf2^{-/-}$ mouse livers, suggesting that the Nrf2 pathway may also be involved in phorone-induced Cyp2b10 gene expression.

The present study demonstrated that phorone robustly increases Cyp2a5 mRNA in mouse livers. Abu-Balar et al. reported that Cyp2a5 is induced by cadmium chloride, and its induction is directly controlled by Nrf2 [4]. This study is the first example of a P450 gene that is regulated by Nrf2. We also report here on the complete failure of phorone to induce Cyp2a5 in $Nrf2^{-/-}$ mouse livers. In line with our data, it has been previously shown that non-alcoholic fatty liver disease mediates oxidative stress followed by Cyp2a5 induction via Nrf2 activation [23]. These results suggest that phorone-induced oxidative stress enhances Cyp2a5 gene expression, which is regulated by the Nrf2 system. Furthermore, we found that phenobarbital significantly induces Cyp2A5 in mouse livers. Consistent with our observations, Salonpaa et al. reported that phenobarbital induced Cyp2a5 in mouse primary hepatocytes. However, little is known about the involvement of CAR in phenobarbital-induced Cyp2a5 gene expression. Thus, using gene-deficient mice, we demonstrated that Nrf2 depletion inhibits phenobarbital-induced Cyp2a5 gene expression. These results suggest that phenobarbital activates Nrf2, thereby inducing Cyp2a5.

In conclusion, the present study demonstrated that phorone, an Nrf2 activator, and phenobarbital, a CAR activator, induced both Cyp2b10 and Cyp2a5, and these inductions are involved in the Nrf2 pathway. It is expected that the Nrf2 pathway regulates the expression of drug metabolizing enzymes and antioxidant proteins along with the maintenance of cellular homeostasis. Nrf2 and CAR primarily play a role in induction of similar phase I and phase II drug metabolizing enzymes [24]. However, the presence of Nrf2 binding domains (corresponding to ARE in the Cyp2b10 promoter region) and CAR binding domains (corresponding to PBREM in the Cyp2a5 promoter region) has not been explained. These reports suggest that there is an interaction between Nrf2 and CAR, which is involved in the inducible regulation of drug metabolizing enzymes. Thus, further studies are required to elucidate the interaction between Nrf2 and CAR in the regulation of P450 gene expression.

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