DMD Fast Forward. Published on June 5, 2020 as DOI: 10.1124/dmd.120.000010
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Nrf2 Antioxidative System is Involved in Cytochrome P450 Gene Expression and Activity: A Delay in Pentobarbital Metabolism in Nrf2-deficient Mice

Takashi Ashino, Masayuki Yamamoto, Satoshi Numazawa

Division of Toxicology, Department of Pharmacology, Toxicology and Therapeutics, Showa University School of Pharmacy, Tokyo, Japan (T.A., S.N.); Pharmacological Research Center, Showa University, Tokyo, Japan (T.A., S.N.); Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan (M.Y.)

DMD Fast Forward. Published on June 5, 2020 as DOI: 10.1124/dmd.120.000010
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Running title: Nrf2 depletion down-regulates P450 expression and activity

Corresponding author: Takashi Ashino, Ph.D.

Division of Toxicology,

Department of Pharmacology, Toxicology, and Therapeutics,

Showa University School of Pharmacy,

1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

Phone: +81-3-3784-8206

Fax: +81-3-3784-8176

E-mail: ashino@pharm.showa-u.ac.jp

Number of Text pages: 29

Tables: 0

Figures: 8

References: 42

Words in Abstract: 231

Words in Introduction: 671

Words in Discussion: 1460

Abbreviations: ARE/StRE, antioxidant- or stress-response elements; CAR, constitutive androstane receptor; HPLC, high-performance liquid chromatography; Nrf2, NF-E2-related factor 2; P450, Cytochrome P450; PCR, polymerase chain reaction; ROS, reactive oxygen species; UV, ultraviolet; WT, wild-type

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ABSTRACT

NF-E2-related factor 2 (Nrf2) is a transcriptional regulator of biological defense proteins, such as antioxidant proteins and phase II detoxification enzymes. Cytochrome P450 (P450) enzymes have been shown to regulate phase I metabolism of various drugs and are partially regulated by Nrf2; however, the influence of Nrf2 on drug pharmacokinetics is not known. Here, we showed that Nrf2 depletion prolonged the effect of pentobarbital, a sleep-promoting drug. Pretreatment with phenobarbital, a P450 inducer, shortens the sleeping time associated with pentobarbital-induced sedation in wild-type (WT) mice; however, this effect was not observed in $Nrf2^{-/-}$ mice. Further, the blood pentobarbital concentration was higher in $Nrf2^{-/-}$ mice than in WT mice at 30–60 min, and the phenobarbital-induced enhancement of its clearance was attenuated in $Nrf2^{-/-}$ mice compared to WT mice. Total P450 content was decreased in $Nrf2^{-/-}$ mouse livers, and the phenobarbital-induced increase in P450 content was lower in $Nrf2^{-/-}$ mice than WT mice. Cyp1a2, Cyp2a5, Cyp2c29, and Cyp2e1 gene expression levels under physiological conditions and Cyp1a2, Cyp2a5, and Cyp2b10 gene expression levels under phenobarbital-treated conditions were lower in $Nrf2^{-/-}$ mice compared to WT mice. Additionally, pentobarbital metabolism in liver microsomes was attenuated by Nrf2 depletion. Taken together, these findings suggested that Nrf2 influenced pentobarbital pharmacokinetics through the regulation of drug metabolism and P450 gene expression. Thus, Nrf2-mediated regulation of P450 may contribute to the biological defense against increased reactive oxygen species production.

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SIGNIFICANCE STATEMENT

Nrf2 plays a critical role in the cellular defense against oxidative stress. $Nrf2^{-/-}$ mice with reduced ability to eliminate ROS showed a significant delay in emergence from pentobarbital-induced sleep, which was associated with decreased P450 activities and gene expression. Our findings provide that Nrf2 dysfunction or ROS that exceed a threshold level of the eliminating ability of the Nrf2 system may reduce P450 activity.

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Introduction

Cytochrome P450s (P450s) are a large family of heme-containing monooxygenase enzymes that oxidatively metabolize xenobiotics such as drugs and environmental pollutants (Gonzalez, 1988; Isin and Guengerich, 2007). Numerous factors, including xenobiotics (e.g., phenobarbital) and diseases regulate P450 enzymatic activity (Morgan, 1997; Wang and Negishi, 2003; Aitken et al., 2006; Hakkola et al., 2018). Alterations in the gene expression and activity of P450s can affect the pharmacokinetics of administered drugs, leading to changes in the duration and intensity of their intended efficacies. Hypoactivity of P450 enzymes can result in increased blood levels of the therapeutic drugs, followed by adverse events. Therefore, elucidation of the underlying regulatory mechanism of P450s may improve clinical predictions of the pharmacokinetics and adverse effects of these medicines.

Excess reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide, can induce oxidative damage to various cells, followed by the development and progression of multiple organ dysfunction, including atherosclerosis, diabetes mellitus, and cancer (Roberts and Sindhu, 2009). Conversely, ROS have also been recognized as intracellular signaling molecules and are constitutively produced during various biological processes, including energy production, immune response, and wound healing (Griffiths et al., 2017). Additionally, the P450 drug metabolizing system is known to produce ROS both in the presence and absence of substrates (Zangar et al., 2004). Generally, P450-mediated oxidation requires input of two electrons by the flavoprotein NADPH-P450 reductase to activate oxygen and insert an oxygen atom into the substrate molecule (Zangar et al., 2004). ROS accumulation can occur through NADPH consumption by microsomal P450, which is reported to be involved in nonalcoholic fatty liver disease (Aubert et al., 2011), hypertension (Sahan-Firat et al., 2010), and diabetes (Eid et al., 2009). These reports suggested that P450 activity was tightly controlled by regulating its gene expression in ROS-related pathological conditions to protect against organ injury.

In the context of biological defense, excess ROS can induce oxidative damage; hence, cellular

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redox balance is tightly controlled by various antioxidant systems. NF-E2-related factor 2 (Nrf2), a redox-regulated transcription factor, plays a critical role in the cellular defense system by activating gene expression of biological defense proteins, such as antioxidant proteins, glutathione synthesis-associated proteins, and phase II detoxification enzymes, via its binding to antioxidant- or stress-response elements (ARE/StRE) (Yamamoto et al., 2018). We previously demonstrated that Nrf2 is involved in the expression of Cyp2b10 and Cyp2a5 during the steady state and their induction by phorone, an α,β-unsaturated carbonyl compound, and phenobarbital, a classical P450 inducer (Ashino et al., 2014). Furthermore, oxidative stress-induced transcription of Cyp2a5 is activated through direct binding of Nrf2 to its ARE/StRE in the 5'-flanking region (Abu-Bakar et al., 2007). However, ARE/StRE have not been identified in other P450 genes.

Several studies have reported that inflammatory diseases (e.g., rheumatoid arthritis and chronic heart failure) and infection are risk factors for decreased P450 gene expression and activity (Morgan, 1997; Mayo et al., 2000; Frye et al., 2002; Ashino et al., 2007). In addition, these diseases increase ROS levels, resulting in oxidative stress. We have previously demonstrated that lipopolysaccharide and Bacillus Calmette-Guérin down-regulate the levels of Cyp3a11 and Cyp2c29 in mouse livers (Ashino et al., 2004), as well as induce heme oxygenase-1, a well-known Nrf2 target gene (Oguro et al., 2002)Ashino, unpublished observations). These observations suggest that Nrf2 is involved in the expression of various P450 species. However, the influence of Nrf2 on P450 gene expression, except for Cyp2a5 and Cyp2b10, and drug pharmacokinetics has not been demonstrated.

Pentobarbital, an anesthetic barbiturate, is a widely used pharmacological or toxicological study model. In this study, we demonstrate that Nrf2 depletion prolonged the sleeping time with pentobarbital sedation. Nrf2 depletion maintains high blood levels of pentobarbital, through P450 and ferritin-mediated metabolic suppression, in both physiological and phenobarbital-induced P450 expression conditions. Collectively, Nrf2 depletion-increased ROS was shown to down-regulate P450 gene expression through a

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negative feedback mechanism, thereby decreasing P450 protein levels and their activities and maintaining blood concentrations of the drug. These findings suggest that Nrf2 activation may be used as an indicator of drug metabolizing capacity in oxidative stress-related diseases.

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Materials and Methods

Materials. Pentobarbital and phenobarbital sodium salts were acquired from Tokyo Chemical Industry (Tokyo, Japan). All other reagents used were of the highest grade commercially available.

Animals and treatments. All animal experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committees of Showa University (#29015), in accordance with the Standards Relating to the Care and Management of Experimental Animals in Japan. Male C57BL/6 mice (8-week-old) were purchased from Japan SLC (Shizuoka, Japan). The $Nrf2^{-/-}$ mice were established by Itoh *et al.* (Itoh et al., 1997). Subsequently, C57BL/6 mice were mated with $Nrf2^{-/-}$ mice to generate wild-type (WT) and deficient mice progenies. Pentobarbital (50 mg/kg) and phenobarbital (100 mg/kg; dose used for most P450 induction studies (Ashino et al., 2014)) were dissolved in saline and injected intraperitoneally (i.p.). Control groups were injected with the same volume of vehicle (saline).

Measurement of sleeping time with pentobarbital sedation. All experiments were started between 11:00 am and 2:00 pm. Mice were injected with pentobarbital, and the time to loss of righting reflex was monitored. The awakening time was defined as the time of righting of the animal.

Preparation of liver microsomes and measurement of total P450 content. Microsomal fractions from mouse livers were prepared as described previously (Ashino et al., 2011). Total hepatic P450 content was determined from a carbon monoxide difference spectrum of dithionite-treated microsomes suspended in 0.1 M sodium-potassium phosphate buffer (pH 7.4) and recorded with an MPS-2450 spectrophotometer (Shimadzu, Kyoto, Japan) using a difference extinction coefficient (450–490 nm) of 91 mM^{-1} cm^{-1} as described by Omura and Sato (Omura and Sato, 1964). Microsomal protein concentration was determined

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using the BCA protein assay.

Measurement of blood pentobarbital concentrations. Whole blood samples were collected from different mice under anesthesia by injecting medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg) intraperitoneally because pentobarbital shows no analgesic effect. Plasma samples was prepared from whole blood. Plasma pentobarbital concentrations were determined by using a high-performance liquid chromatography (HPLC)–ultraviolet (UV) assay based on previous reports (Kelner and Bailey, 1983). Briefly, hexobarbital (as an internal standard; 10 µL; 0.1 µg/µL in acetonitrile) and acetonitrile (190 µL) were added to 100 µL of plasma, and the mixture was vortexed for 30 s and centrifuged at 20000 × *g* for 10 min to separate the precipitated proteins. Clear supernatant (50 µL) was injected into the HPLC system. The HPLC system consisted of an LC-10AD liquid chromatograph pump (Shimadzu), an SPD-10A UV-VIS detector (Shimadzu), a SIL-10A auto injector (Shimadzu), a CMB-10A communications bus module (Shimadzu), and a 4.6 × 150 mm Luna 5 µm C18(2) column (Shimadzu GLC, Tokyo, Japan). The mobile phase consisted of methanol, 0.16 M sodium-potassium phosphate buffer (pH 6.6), and tetrahydrofuran (50:43:7), at a flow rate of 1 mL/min. The eluate was monitored at 220 nm. Under the present chromatographic conditions, hexobarbital and pentobarbital were eluted at 5 and 6 min, respectively. Pentobarbital was quantified by comparison with the standard curves (6.25–100 µg/mL) using the area ratio method.

Pentobarbital metabolic activity in the hepatic microsomes. Pentobarbital metabolic activity assay was performed in a reaction mixture (500 µL) containing hepatic microsomes (500 µg protein), 5 mM $MgCl_2$, pentobarbital (50 µM in Fig. 3 or 6.25–100 µM in Fig. 4), and the NADPH-generating system (5 mM glucose-6-phosphate, 0.6 mM β-nicotinamide adenine dinucleotide phosphate$^+$, and 1 U glucose-6-phosphate dehydrogenase) in 100 mM phosphate buffer (pH 7.4). The reaction mixture was

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incubated at 37°C for 15 min, and the reaction was stopped by adding ice-cold 490 µL acetonitrile. Hexobarbital (10 µL; 1 mM in acetonitrile) was added to the reaction mixture as an internal standard, and the mixture was vortexed for 30 s and centrifuged at 1800 × *g* for 10 min to separate the precipitated proteins. Clear supernatant (50 µL) was injected into the HPLC system. Pentobarbital was quantified as mentioned above by comparing to the standard curves (3.125–100 nmol/reaction mixture). Pentobarbital metabolic activity was calculated from the rate of decrease. Controls without microsomes showed no decrease in pentobarbital.

Quantitative real-time polymerase chain reaction (PCR). Total RNA was isolated from the livers using the PureLink™ RNA Mini Kit (Invitrogen), and first-stranded cDNA was synthesized using the PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). Duplex TaqMan real-time PCR was performed using the TaqMan Fast Advanced Master Mix (Applied Biosystems), a FAM-labeled probe for the target mRNA, and a VIC-labeled probe for the housekeeping gene (β-actin) according to the manufacturer's protocol on a StepOne real-time PCR system (Applied Biosystems). The mRNA levels were measured relative to β-actin mRNA expression in each run. All predesigned PCR primers and TaqMan MGB probes were purchased from Applied Biosystems.

Statistical analysis. Values are expressed as median with interquartile ± 1.5 interquartile range (Fig. 1 and 2A) or mean ± SEM (Fig. 2B, 3, 5, 6, and 7). The values beyond ± 1.5 interquartile range were defined as the outliers. Time-courses were compared among groups by Kruskal–Wallis test and post-hoc Steel's test. Two group means were compared by Mann–Whitney's U test, and three or more group means by Kruskal–Wallis test, followed by post-hoc Scheffe's tests. Statistical significance was accepted at $P < 0.05$.

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Results

Loss of Nrf2 prolongs pentobarbital-induced sleeping time without effects on phenobarbital-induced sleeping time. Alteration of P450 enzymatic activity can affect pharmacokinetics of drugs. We previously demonstrated that Nrf2 is involved in expression of some P450 genes using Nrf2$^{-/-}$ mice (Ashino et al., 2014). Thus, we investigated the changes in drug-metabolizing activity in Nrf2$^{-/-}$ and age-, sex-, and body weight-matched WT mice following pentobarbital (a known substrate for P450s) administration (Fig. 1). The dose of pentobarbital (50 mg/kg) used was that at which the WT mice recovered in approximately 60 min after the loss of righting reflex. Compared to WT mice, Nrf2$^{-/-}$ mice showed a significant delay in emergence from pentobarbital-induced sleep (average: WT, 69 min vs. Nrf2$^{-/-}$, 192 min). We also reported that Nrf2$^{-/-}$ mice showed attenuated Cyp2b10 and Cyp2a5 induction by phenobarbital, a classical P450 inducer (Ashino et al., 2014). In WT mice, pretreatment with phenobarbital for 24 h shortened pentobarbital-induced sleeping time, but this effect was not observed in Nrf2$^{-/-}$ mice.

Clearance of blood pentobarbital is delayed in Nrf2$^{-/-}$ mice both with and without phenobarbital pretreatment. To determine whether Nrf2 deficiency influences pentobarbital pharmacokinetics, we examined time-dependent changes in blood concentrations of pentobarbital in WT and Nrf2$^{-/-}$ mice. As shown in Fig. 2A, 15 min after treatment, there was no significant difference in pentobarbital concentrations between WT and Nrf2$^{-/-}$ mice. At 30 min, pentobarbital blood levels were higher in Nrf2$^{-/-}$ mice than WT mice; the difference was even more pronounced at 60 min, indicating a delay in pentobarbital metabolism in Nrf2$^{-/-}$ mice. Furthermore, we examined the effect of phenobarbital on the pentobarbital metabolic rate in WT and Nrf2$^{-/-}$ mice (Fig. 2B). Phenobarbital pretreatment for 24 h markedly decreased blood pentobarbital levels in WT mice, whereas this effect was attenuated in Nrf2$^{-/-}$ mice.

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Nrf2 depletion decreases hepatic microsomal cytochrome P450 content. Since Nrf2 depletion resulted in sustained high blood levels of pentobarbital, which is metabolized by P450 enzymes, we examined changes in hepatic P450 content. As shown in Fig. 3, physiological and phenobarbital-induced hepatic P450 levels in Nrf2$^{-/-}$ mice were significantly lower than WT mice.

Nrf2 depletion influences P450 expression levels in the liver under physiological and inducing conditions. To determine which P450 enzymes were influenced by Nrf2 depletion, we examined the expression levels of constitutive-type P450 (Fig. 4). Under physiological conditions, Nrf2$^{-/-}$ mice showed low levels of Cyp1a2, Cyp2a5, Cyp2c29, and Cyp2e1 expression compared to WT mice. Under phenobarbital-inducing conditions, although almost all P450 species were increased in both WT and Nrf2$^{-/-}$ mice, Cyp1a2, Cyp2a5, and Cyp2b10 expression levels were lower in Nrf2$^{-/-}$ mice than WT mice. Expression of the Cyp3a11 gene did not show significant change between WT and Nrf2$^{-/-}$ mice before and after phenobarbital treatment.

Nrf2 depletion down-regulates ferritin gene expression. P450 enzymes are capable of performing catalysis through reduction or oxidation of their heme iron. Thus, the supply of heme to the apoprotein of P450 enzymes is important for their catalytic activity. Ferritin, an intracellular iron storage protein composed of ferritin heavy (H) and light (L) chains, has been postulated to act as an intermediate for heme synthesis (Richardson and Ponka, 1997). As shown in Fig. 5, under physiological conditions, gene expression levels of both ferritin heavy (H) and light (L) chains were lower in Nrf2$^{-/-}$ mouse livers than WT. Further, phenobarbital induced expression of both ferritin heavy (H) and light (L) chains. However, expression levels of ferritin heavy (H) chain, and not ferritin light (L) chain, was lower in Nrf2$^{-/-}$ mice than WT mice, even in phenobarbital induced conditions.

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Pentobarbital metabolic activity is decreased in Nrf2$^{-/-}$ mouse livers. Nrf2 depletion decreased the levels of several P450 molecular species and ferritin gene expression. We therefore examined pentobarbital metabolic activity in liver microsomes of phenobarbital-treated and untreated WT and Nrf2$^{-/-}$ mice (Fig. 6). Consistent with sleeping time and blood pentobarbital levels, pentobarbital metabolic activity in uninduced liver microsomes was significantly lower in Nrf2$^{-/-}$ mice compared to WT mice. Further, phenobarbital enhanced pentobarbital metabolic activity in both WT and Nrf2$^{-/-}$ mouse liver microsomes at 24 h after treatment. However, pentobarbital metabolic activity in phenobarbital-treated Nrf2$^{-/-}$ mouse liver microsomes was significantly lower than WT microsomes. To characterize the mode of inhibition of pentobarbital metabolic activity in Nrf2$^{-/-}$ mice, we performed kinetic analysis (Fig. 7). Michaelis–Menten kinetics (Fig. 7A) and Lineweaver–Burk plots (Fig. 7B) showed that pentobarbital metabolic activity was noncompetitively suppressed in Nrf2$^{-/-}$ mouse livers, with Km values of approximately 25 μM (Fig. 7C), suggesting that Nrf2 depletion did not affect the affinity of P450 enzymes to pentobarbital.

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Discussion

In clinical reports, patients under oxidative stress conditions have been shown to suppress P450 metabolizing activities, including mephenytoin (a substrate for CYP2C19) and caffeine (a substrate for CYP1A2) in patients with congestive heart failure (Frye et al., 2002). Further, patients after surgical operations have been shown to suppress erythromycin metabolic capacity (a substrate for CYP3A4) (Haas et al., 2003). Other reports have implicated ROS in the transcriptional regulation of P450s (Barouki and Morel, 2001), suggesting that ROS-induced oxidative stress may have a role in suppressing drug metabolizing activity. At present, the role of oxidative stress defensive transcription factor Nrf2 on drug pharmacokinetics is unclear. This study revealed a novel role for Nrf2 in which it modulates drug pharmacokinetics by regulating P450 expression. In WT mice, Nrf2-activated antioxidant proteins eliminated ROS, thereby maintaining P450 expression and activity (Fig. 8, upper). In contrast, $Nrf2^{-/-}$ mice had a reduced ability to eliminate ROS, resulting in elevated ROS levels that suppressed P450 expression and activity through a negative feedback mechanism (Fig. 8, lower). Thus, our results suggest that Nrf2 modulates the duration and intensity of drug effects in order to maintain redox balance.

The length of pentobarbital-induced sleeping time is inversely related to the rate of drug metabolism, and its duration of action can be altered by induction or inhibition of P450s (Sirisangtragul and Sripanidkulchai, 2013; Kovacs et al., 2018). In this study, Nrf2 depletion prolonged sleeping time by sustaining high blood levels of pentobarbital under physiological conditions. These results indicate that pentobarbital metabolism is delayed by Nrf2 depletion, as evidenced by suppressed pentobarbital metabolism in the liver microsomes of $Nrf2^{-/-}$ mice. Barbiturates are metabolized by several P450 species, including CYP2B6 and CYP3A4 in humans (Tsuji et al., 1996; Sirisangtragul and Sripanidkulchai, 2013). Since P450 enzymes show broad substrate specificities, other P450 species may contribute to pentobarbital metabolism. In this study, Nrf2 depletion decreased total P450 content and down-regulated gene expression of several P450 species, such as Cyp1a2, Cyp2a5, Cyp2c29, and Cyp2e1, under

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physiological conditions. Consistent with our observations, portal vein ligation of the liver transiently elevated pentobarbital-induced sleeping time, which was associated with decreased P450 activities and gene expression of CYP1A, CYP2B, and CYP2C in rats (Kovacs et al., 2018). Collectively, the sustained efficacy of pentobarbital by Nrf2 depletion is attributed to P450 down-regulation.

This study also evaluated changes in pentobarbital pharmacokinetics using the P450 inducer phenobarbital in Nrf2$^{-/-}$ mice. Phenobarbital shortened pentobarbital-induced sleeping time. This was abolished by Nrf2 depletion. Consistent with sleeping time, phenobarbital-enhanced pentobarbital metabolic activity was suppressed by Nrf2 depletion both *in vivo* and *in vitro*. Phenobarbital is a classical inducer of the drug metabolizing P450 genes, especially the CYP2B subfamily. In this study, phenobarbital induced expression of all P450 genes, except for Cyp2e1 in WT mice. Cyp1a2, Cyp2a5, and Cyp2b10 gene expression levels in Nrf2$^{-/-}$ mice were significantly lower than WT mice even under the phenobarbital-induced conditions, which differed from physiological conditions. These results suggest that although P450 activities were lower in Nrf2$^{-/-}$ mice under both physiological and induced conditions, the contributing P450 species were different. Phenobarbital substantially induced Cyp2b10 compared to other P450 genes, and Cyp2b10 levels were decreased in Nrf2$^{-/-}$ mice compared to WT mice. Phenobarbital-induced sleeping time was previously reported to be associated with CYP2B activity (Sirisangtragul and Sripanidkulchai, 2013). Taken together, the phenobarbital-shortened pentobarbital efficiency and its inhibition in Nrf2$^{-/-}$ mice may depend on CYP2B activity.

In this study, Nrf2$^{-/-}$ mice reliably showed prolonged pentobarbital-induced sleeping time; however, the mechanism underlying the wide range of individual differences in the sleeping time in Nrf2$^{-/-}$ mice under physiological conditions remains unclear. It has been reported that a congenital intrahepatic shunt is present at a constant rate in Nrf2-disrupted C57BL/6J mice (Skoko et al., 2014). This intrahepatic shunt would be expected to change blood flow, which may result in the individual differences in sleeping time of Nrf2$^{-/-}$ mice. However, individual differences in blood concentration of pentobarbital were not

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observed in Nrf2$^{-/-}$ mice. Furthermore, individual differences in sleeping time were also not observed in the phenobarbital-administered Nrf2$^{-/-}$ mice, which may contribute in part to the wide range of individual differences in the sleeping time in Nrf2$^{-/-}$ mice under physiological conditions.

Expression of P450 genes is influenced by various physiological changes and exogenous factors. Several pathophysiological conditions, such as infection and inflammation, have been shown to suppress expression of several P450 isoforms (Morgan, 1997). While this is well-studied in terms of regulation of inflammatory cytokines such as IL-1β, IL-6, and TNFα (Ashino et al., 2004), little is known about the underlying mechanism of P450 down-regulation, which could involve ROS generation by inflammatory cytokine signaling. Indeed, CYP1A1 and CYP2E1 gene expression levels have been shown to be reduced by ROS production in response to H_2O_2 treatment (Morel and Barouki, 1998; Morel et al., 2000), suggesting that other P450 species are also repressed by ROS. Further, this study showed that the affinity of metabolic enzymes to pentobarbital was not altered by Nrf2 depletion. Taken together, an increase in ROS by Nrf2 depletion may negatively regulate P450 gene expression, thereby suppressing their activities without affecting the P450 structural conformation.

Transcriptional activation of P450s has been mainly investigated in the context of several xenobiotic-activated receptors, such as aryl hydrocarbon receptor, constitutive androstane receptor (CAR), and pregnane X receptor (Rushmore and Kong, 2002), which act as specific transcription factors. Previous investigations have demonstrated that Nrf2 functions as a transcription factor for the Cyp2a5 gene by directly binding to the promoter region (Abu-Bakar et al., 2007; Wu et al., 2012; Ashino et al., 2014). This study also showed that Cyp2a5 gene expression was regulated by Nrf2, indicating Cyp2a5 as the first phase I xenobiotic-metabolizing gene identified under the regulation of the Nrf2 pathway with a potential role in the adaptive response to cellular stress. Although the Nrf2-binding site ARE/StRE has not been identified on Cyp1a2, Cyp2c29, and Cyp2e1 genes, their expression levels were down-regulated in Nrf2$^{-/-}$ mice. Thus, Nrf2 depletion-suppressed ROS elimination is likely involved in this regulation.

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Phenobarbital is the prototype of a large group of structurally diverse xenobiotic chemicals that induce the subset of the P450 genes such as CYP2B, CYP2C, and CYP3A subfamilies, with CYP2B genes being the most effectively induced (Honkakoski et al., 1998). While constitutive expression of the CYP2B subfamily is very low, their expression levels are dramatically increased by specific inducers. Phenobarbital has been demonstrated to transactivate the CYP2B gene through nuclear translocation of CAR, in which it forms a heterodimer with the retinoid X receptor and activates the phenobarbital responsive element module (Honkakoski et al., 1998; Kawamoto et al., 1999; Moore et al., 2000). It has been reported that Nrf2 activators, such as trans-stilbeneoxide and oltipraz, also induce Cyp2b10 gene expression and activate the CYP2B6 promoter (Slitt et al., 2006; Merrell et al., 2008). However, ARE/StRE have not been detected in its promoter regions, indicating no direct transcriptional target of Nrf2. Induction of Cyp2b10 by Nrf2 activators is inhibited in $CAR^{-/-}$ mice, but not $Nrf2^{-/-}$ mice (Merrell et al., 2008). While it remains unclear how Nrf2 activators activates CAR, these reports suggest that several Nrf2 activators can activate CAR, followed by Cyp2b10 induction. Thus, suppressed induction of Cyp2b10 in $Nrf2^{-/-}$ mice may be involved in the increase in ROS levels due to Nrf2 depletion.

This study also showed that Nrf2 depletion down-regulated ferritin gene expression, a Nrf2 target gene. The iron storage protein, ferritin, has been postulated to act as an intermediate for heme synthesis (Richardson and Ponka, 1997). The liver is one of the body compartments with the highest rate of heme synthesis. More than 50% of heme synthesized in the liver is used to synthesize P450s (Vinchi et al., 2014). Therefore, it is speculated that ferritin expression in livers may influence P450 maturation and activation through heme incorporation. It has been reported that the CYP2A6 activity is related to serum ferritin levels, which are correlated to intracellular ferritin levels (Satarug et al., 2004). These results suggest that Nrf2 plays an important role in the variance of pharmacokinetics of administered drugs, at least, in part, by regulating ferritin expression that may contribute to a delay in pentobarbital metabolism in $Nrf2^{-/-}$ mice.

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In conclusion, this study revealed a role of Nrf2 as a maintenance factor of drug metabolizing enzyme P450s through transcriptional modulation of gene expression. Although, further studies are required to elucidate the underlying mechanisms of P450 down-regulation in biological defense, we suggest that the Nrf2 system inhibits an increase in ROS by regulating the metabolic cycle of P450s, thereby maintaining homeostasis against oxidative damage. Therefore, Nrf2 dysfunction or ROS that exceed a threshold level of the eliminating ability of the Nrf2 system may reduce P450 activity. Our findings also highlight the Nrf2 system as a predictive factor for the duration and intensity of intended drug efficacies.

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Acknowledgments

The authors thank K. Higeno for his technical assistance. The authors would like to thank Editage (www.editage.com) for the English language review.

Declaration of competing interest

There is no conflict of interest.

Authorship Contributions

Participated in research design: Ashino.

Conducted experiments: Ashino.

Contributed new reagents or analytic tools: Yamamoto.

Performed data analysis: Ashino and Numazawa.

Wrote or contributed to the writing of the manuscript: Ashino.

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References

Abu-Bakar A, Lamsa V, Arpiainen S, Moore MR, Lang MA, and Hakkola J (2007) Regulation of CYP2A5 gene by the transcription factor nuclear factor (erythroid-derived 2)-like 2. *Drug Metab Dispos* **35:**787-794.

Aitken AE, Richardson TA, and Morgan ET (2006) Regulation of drug-metabolizing enzymes and transporters in inflammation. *Annu Rev Pharmacol Toxicol* **46:**123-149.

Ashino T, Arima Y, Shioda S, Iwakura Y, Numazawa S, and Yoshida T (2007) Effect of interleukin-6 neutralization on CYP3A11 and metallothionein-1/2 expressions in arthritic mouse liver. *Eur J Pharmacol* **558:**199-207.

Ashino T, Oguro T, Shioda S, Horai R, Asano M, Sekikawa K, Iwakura Y, Numazawa S, and Yoshida T (2004) Involvement of interleukin-6 and tumor necrosis factor alpha in CYP3A11 and 2C29 down-regulation by Bacillus Calmette-Guerin and lipopolysaccharide in mouse liver. *Drug Metab Dispos* **32:**707-714.

Ashino T, Ohkubo-Morita H, Yamamoto M, Yoshida T, and Numazawa S (2014) Possible involvement of nuclear factor erythroid 2-related factor 2 in the gene expression of Cyp2b10 and Cyp2a5. *Redox Biol* **2:**284-288.

Ashino T, Sugiuchi J, Uehara J, Naito-Yamamoto Y, Kenmotsu S, Iwakura Y, Shioda S, Numazawa S, and Yoshida T (2011) Auranofin protects against cocaine-induced hepatic injury through induction of heme oxygenase-1. *J Toxicol Sci* **36:**635-643.

Aubert J, Begriche K, Knockaert L, Robin MA, and Fromenty B (2011) Increased expression of cytochrome P450 2E1 in nonalcoholic fatty liver disease: mechanisms and pathophysiological role. *Clin Res Hepatol Gastroenterol* **35:**630-637.

Barouki R and Morel Y (2001) Repression of cytochrome P450 1A1 gene expression by oxidative stress: mechanisms and biological implications. *Biochem Pharmacol* **61:**511-516.

Eid AA, Gorin Y, Fagg BM, Maalouf R, Barnes JL, Block K, and Abboud HE (2009) Mechanisms of podocyte injury in diabetes: role of cytochrome P450 and NADPH oxidases. *Diabetes* **58:**1201-1211.

Frye RF, Schneider VM, Frye CS, and Feldman AM (2002) Plasma levels of TNF-alpha and IL-6 are inversely related to cytochrome P450-dependent drug metabolism in patients with congestive heart failure. *J Card Fail* **8:**315-319.

Gonzalez FJ (1988) The molecular biology of cytochrome P450s. *Pharmacol Rev* **40:**243-288.

Griffiths HR, Gao D, and Pararasa C (2017) Redox regulation in metabolic programming and inflammation. *Redox Biol* **12:**50-57.

Haas CE, Kaufman DC, Jones CE, Burstein AH, and Reiss W (2003) Cytochrome P450 3A4 activity after surgical stress. *Crit Care Med* **31:**1338-1346.

Hakkola J, Bernasconi C, Coecke S, Richert L, Andersson TB, and Pelkonen O (2018) Cytochrome P450 Induction and Xeno-Sensing Receptors Pregnane X Receptor, Constitutive Androstane Receptor, Aryl Hydrocarbon Receptor and Peroxisome Proliferator-Activated Receptor alpha at the Crossroads of Toxicokinetics and Toxicodynamics. *Basic Clin Pharmacol Toxicol* **123 Suppl 5:**42-50.

Honkakoski P, Zelko I, Sueyoshi T, and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* **18:**5652-5658.

Isin EM and Guengerich FP (2007) Complex reactions catalyzed by cytochrome P450 enzymes. *Biochim Biophys Acta* **1770:**314-329.

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, and Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* **236:**313-322.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* **19:**6318-6322.

Kelner M and Bailey DN (1983) Reversed-phase liquid-chromatographic simultaneous analysis for thiopental and pentobarbital in serum. *Clin Chem* **29:**1097-1100.

Kovacs T, Deri M, Fulop A, Palhazy T, Hafra E, Sirok D, Kiss AF, Lotz G, Szijarto A, and Monostory K

(2018) Isoform-Dependent Changes in Cytochrome P450-Mediated Drug Metabolism after Portal Vein Ligation in the Rat. *Eur Surg Res* **59:**301-319.

Mayo PR, Skeith K, Russell AS, and Jamali F (2000) Decreased dromotropic response to verapamil despite pronounced increased drug concentration in rheumatoid arthritis. *Br J Clin Pharmacol* **50:**605-613.

Merrell MD, Jackson JP, Augustine LM, Fisher CD, Slitt AL, Maher JM, Huang W, Moore DD, Zhang Y, Klaassen CD, and Cherrington NJ (2008) The Nrf2 activator oltipraz also activates the constitutive androstane receptor. *Drug Metab Dispos* **36:**1716-1721.

Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL, and Kliewer SA (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275:**15122-15127.

Morel Y and Barouki R (1998) Down-regulation of cytochrome P450 1A1 gene promoter by oxidative stress. Critical contribution of nuclear factor 1. *J Biol Chem* **273:**26969-26976.

Morel Y, de Waziers I, and Barouki R (2000) A repressive cross-regulation between catalytic and promoter activities of the CYP1A1 and CYP2E1 genes: role of H(2)O(2). *Mol Pharmacol* **57:**1158-1164.

Morgan ET (1997) Regulation of cytochromes P450 during inflammation and infection. *Drug Metab Rev* **29:**1129-1188.

Oguro T, Takahashi Y, Ashino T, Takaki A, Shioda S, Horai R, Asano M, Sekikawa K, Iwakura Y, and Yoshida T (2002) Involvement of tumor necrosis factor alpha, rather than interleukin-1alpha/beta or nitric oxides in the heme oxygenase-1 gene expression by lipopolysaccharide in the mouse liver. *FEBS Lett* **516:**63-66.

Omura T and Sato R (1964) The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J Biol Chem* **239:**2370-2378.

Richardson DR and Ponka P (1997) The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. *Biochim Biophys Acta* **1331:**1-40.

Roberts CK and Sindhu KK (2009) Oxidative stress and metabolic syndrome. *Life Sci* **84:**705-712.

Rushmore TH and Kong AN (2002) Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. *Curr Drug Metab* **3:**481-490.

Sahan-Firat S, Jennings BL, Yaghini FA, Song CY, Estes AM, Fang XR, Farjana N, Khan AI, and Malik KU (2010) 2,3',4,5'-Tetramethoxystilbene prevents deoxycorticosterone-salt-induced hypertension: contribution of cytochrome P-450 1B1. *Am J Physiol Heart Circ Physiol* **299:**H1891-1901.

Satarug S, Ujjin P, Vanavanitkun Y, Nishijo M, Baker JR, and Moore MR (2004) Effects of cigarette smoking and exposure to cadmium and lead on phenotypic variability of hepatic CYP2A6 and renal function biomarkers in men. *Toxicology* **204:**161-173.

Sirisangtragul W and Sripanidkulchai B (2013) Moduratory effect of Thai traditional medicine (Yahom Tultavai) on hepatic cytochrome P450 enzymes and pentobarbital-induced sleeping in mice. *Afr J Tradit Complement Altern Med* **10:**128-136.

Skoko JJ, Wakabayashi N, Noda K, Kimura S, Tobita K, Shigemura N, Tsujita T, Yamamoto M, and Kensler TW (2014) Loss of Nrf2 in mice evokes a congenital intrahepatic shunt that alters hepatic oxygen and protein expression gradients and toxicity. *Toxicol Sci* **141:**112-119.

Slitt AL, Cherrington NJ, Dieter MZ, Aleksunes LM, Scheffer GL, Huang W, Moore DD, and Klaassen CD (2006) trans-Stilbene oxide induces expression of genes involved in metabolism and transport in mouse liver via CAR and Nrf2 transcription factors. *Mol Pharmacol* **69:**1554-1563.

Tsuji R, Isobe N, Kurita Y, Hanai K, Yabusaki Y, and Kawasaki H (1996) Species difference in the inhibition of pentobarbital metabolism by empenthrin. *Environ Toxicol Pharmacol* **2:**331-337.

Vinchi F, Ingoglia G, Chiabrando D, Mercurio S, Turco E, Silengo L, Altruda F, and Tolosano E (2014) Heme exporter FLVCR1a regulates heme synthesis and degradation and controls activity of cytochromes P450. *Gastroenterology* **146:**1325-1338.

Wang H and Negishi M (2003) Transcriptional regulation of cytochrome p450 2B genes by nuclear receptors. *Curr Drug Metab* **4:**515-525.

Wu KC, Cui JY, and Klaassen CD (2012) Effect of graded Nrf2 activation on phase-I and -II drug metabolizing enzymes and transporters in mouse liver. *PLoS One* **7:**e39006.

Yamamoto M, Kensler TW, and Motohashi H (2018) The KEAP1-NRF2 System: a Thiol-Based

Sensor-Effector Apparatus for Maintaining Redox Homeostasis. *Physiol Rev* **98:**1169-1203.

Zangar RC, Davydov DR, and Verma S (2004) Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicol Appl Pharmacol* **199:**316-331.

DMD Fast Forward. Published on June 5, 2020 as DOI: 10.1124/dmd.120.000010
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Figure Legends

Fig. 1. Pentobarbital-induced sleeping time in WT and Nrf2$^{-/-}$ mice treated with or without phenobarbital. Mice were pretreated with phenobarbital (100 mg/kg, i.p.) or saline. After 24 h, the mice were injected with pentobarbital (50 mg/kg, i.p.), and sleeping time was monitored (n = 15, *$P < 0.05$). Horizontal lines and boxes indicate median with interquartile range. Whiskers indicate ± 1.5 interquartile range, and points beyond whiskers indicate outliers.

Fig. 2. Rate of decline in blood pentobarbital concentrations in WT and Nrf2$^{-/-}$ mice treated with or without phenobarbital. A, Mice were injected with pentobarbital (50 mg/kg, i.p.), and their plasma samples were prepared from whole blood at the indicated time points. Line graphs represent averaged data expressed as plasma pentobarbital concentrations. Horizontal lines and boxes indicate median with interquartile range. Whiskers indicate ± 1.5 interquartile range, and points beyond whiskers indicate outliers (n = 10, *P < 0.05). **B**, Mice were pretreated with phenobarbital (100 mg/kg, i.p.) or saline. After 24 h, the mice were injected with pentobarbital (50 mg/kg, i.p.), and their plasma samples were prepared at 60 min post-pentobarbital. Bar graphs represent averaged pentobarbital concentrations expressed as the percentage of the respective controls. Numerals indicate plasma pentobarbital concentrations (μg/mL). Values represent the mean ± SEM (n = 11, *$P < 0.05$).

Fig. 3. Microsomal P450 content in liver microsomes of WT and Nrf2$^{-/-}$ mice treated with or without phenobarbital. Mice were injected with phenobarbital (100 mg/kg, i.p.) or saline, and the livers were excised 24 h after treatment. Total P450 content in the liver microsomal proteins was assayed from the carbon monoxide difference spectrum of the reduced protein. Values represent mean ± SEM (n = 4–6, *$P < 0.05$).

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Fig. 4. Gene expression of various P450 molecular species in livers of WT and Nrf2$^{-/-}$ mice treated with or without phenobarbital. Mice were injected with phenobarbital (100 mg/kg, i.p.) or saline, and the livers were excised at the indicated times. Various P450 mRNA levels were determined by real-time PCR and semi-quantified by normalizing to β-actin mRNA expression. Values represent mean ± SEM (n = 5). $^{\#}P < 0.05$ vs. respective controls (0 h). $^{*}P < 0.05$ vs. WT.

Fig. 5. Ferritin gene expression in livers of WT and Nrf2$^{-/-}$ mice treated with or without phenobarbital. Mice were injected with phenobarbital (100 mg/kg, i.p.) or saline, and the livers were excised at the indicated times. Ferritin heavy (H) and light (L) chain mRNA levels were determined by real-time PCR and semi-quantified by normalizing to β-actin mRNA expression. Values represent mean ± SEM (n = 5). $^{\#}P < 0.05$ vs. respective controls (0 h). $^{*}P < 0.05$ vs. WT.

Fig. 6. Pentobarbital metabolic activity in liver microsomes from WT and Nrf2$^{-/-}$ mice treated with or without phenobarbital. Mice were injected with phenobarbital (100 mg/kg, i.p.) or saline, and the livers were excised 24 h after treatment. Pentobarbital metabolic activity was determined from the rate of decrease in pentobarbital by incubation with the liver microsomes. Values represent mean ± SEM (n = 4–6, $^{*}P < 0.05$).

Fig. 7. Kinetic analysis of pentobarbital metabolic activity of liver microsomes from WT and Nrf2$^{-/-}$ mice treated with or without phenobarbital. A–B, Michaelis-Menten kinetics (**A**) and Lineweaver–Burk plots (**B**) of pentobarbital metabolic activity in the liver microsomes of WT and Nrf2$^{-/-}$ mice at 24 h after phenobarbital treatment (100 mg/kg, i.p.). Microsomal protein was isolated from three different mouse livers and combined. [S] and v indicate pentobarbital concentration (μM) and pentobarbital metabolic activity (nmol/min/mg protein), respectively. C, The Vmax and Km values were

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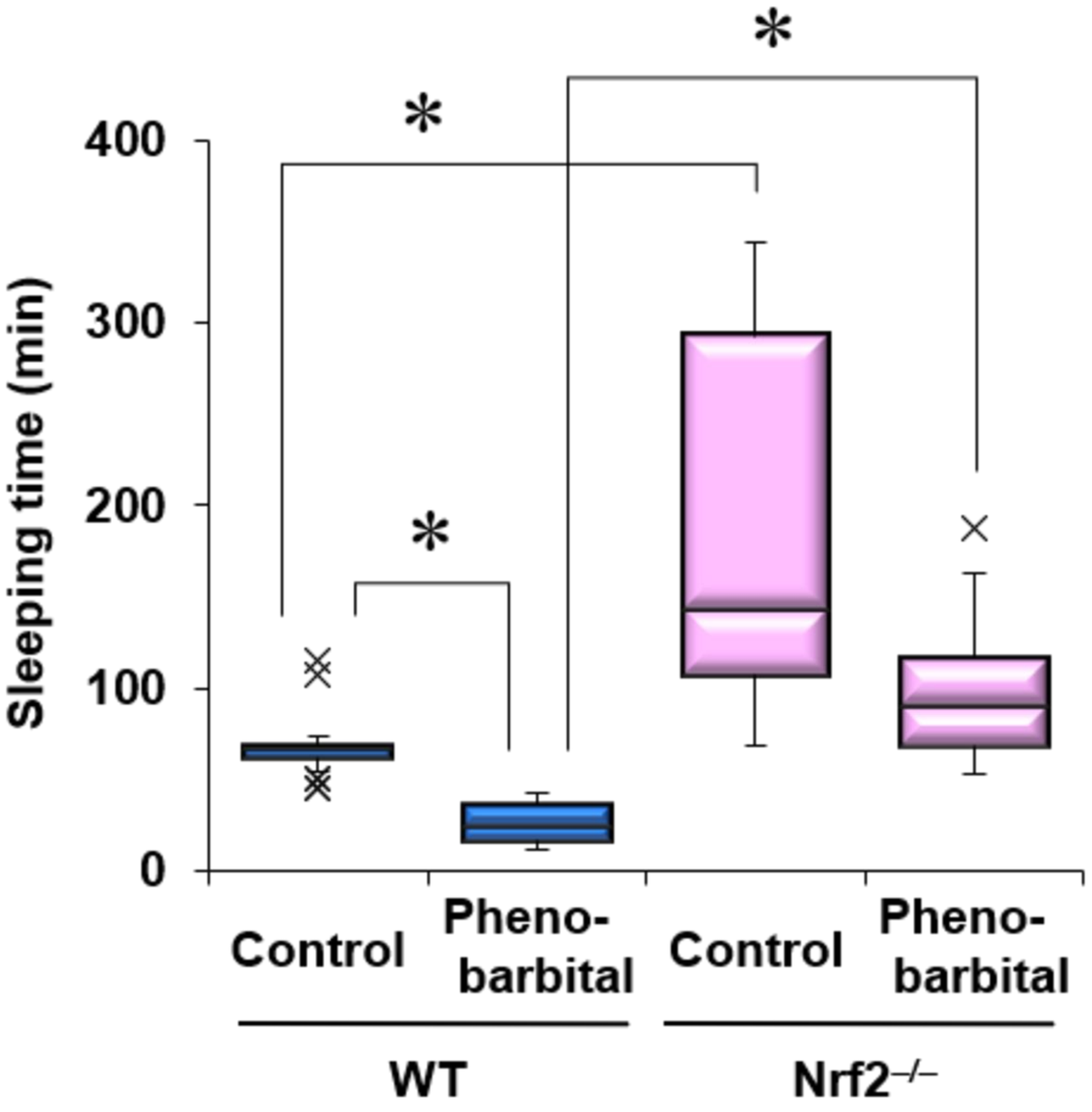
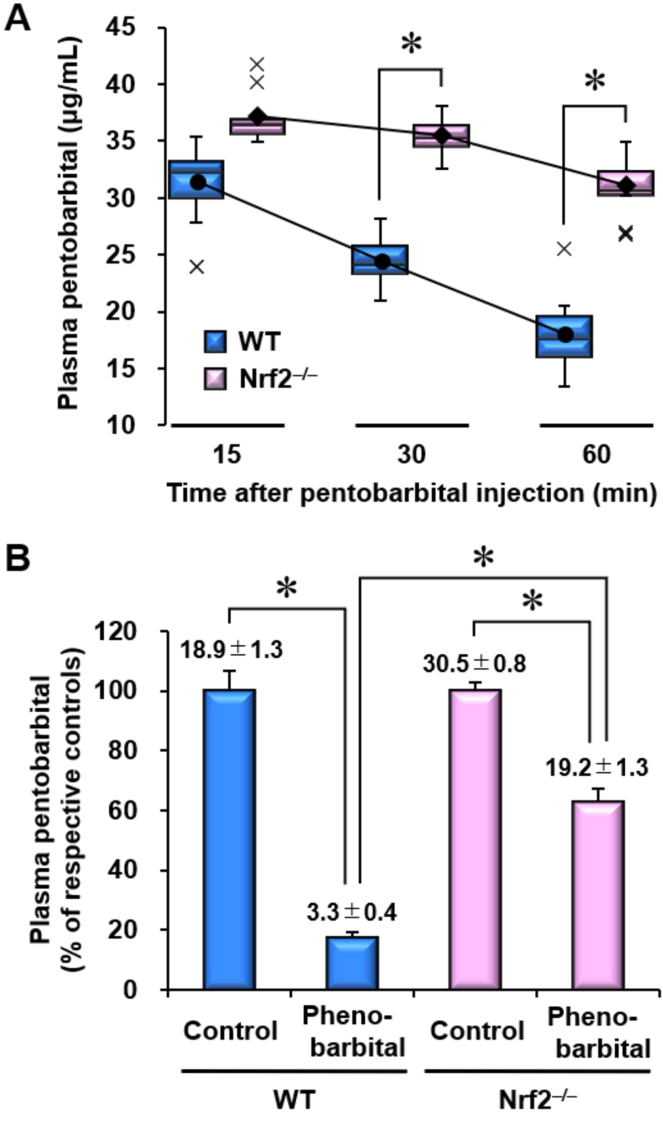
calculated from Lineweaver–Burk plots.

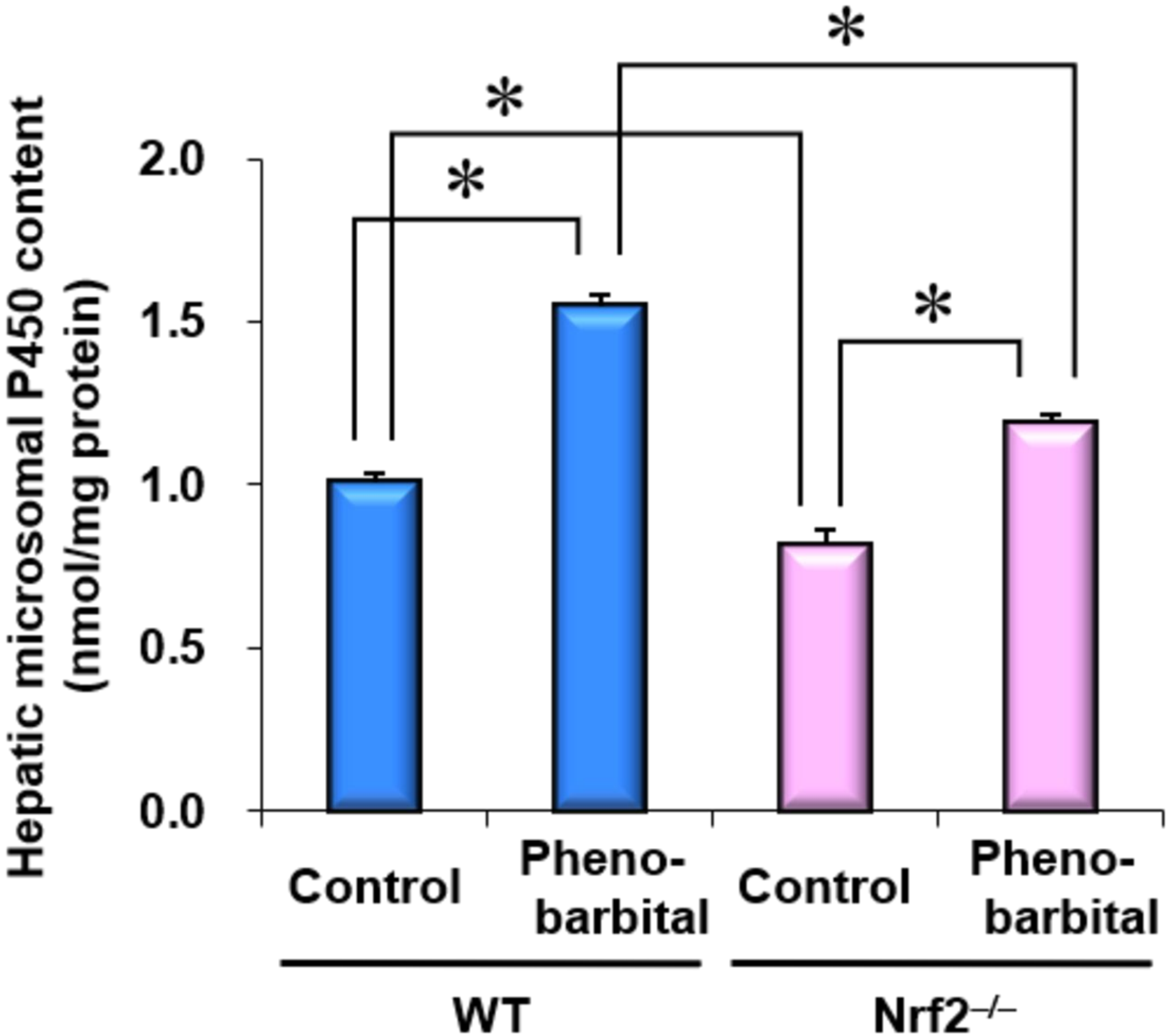
Fig. 8. Proposed model for the decline in P450 gene expression and activity by Nrf2 depletion. Increase in ROS down-regulates P450 gene expression through a negative feedback mechanism, thereby decreasing P450 protein expression and activities in $Nrf2^{-/-}$ mice, which have suppressed ROS elimination ability due to loss of antioxidant function, to maintain drug concentrations in the blood.

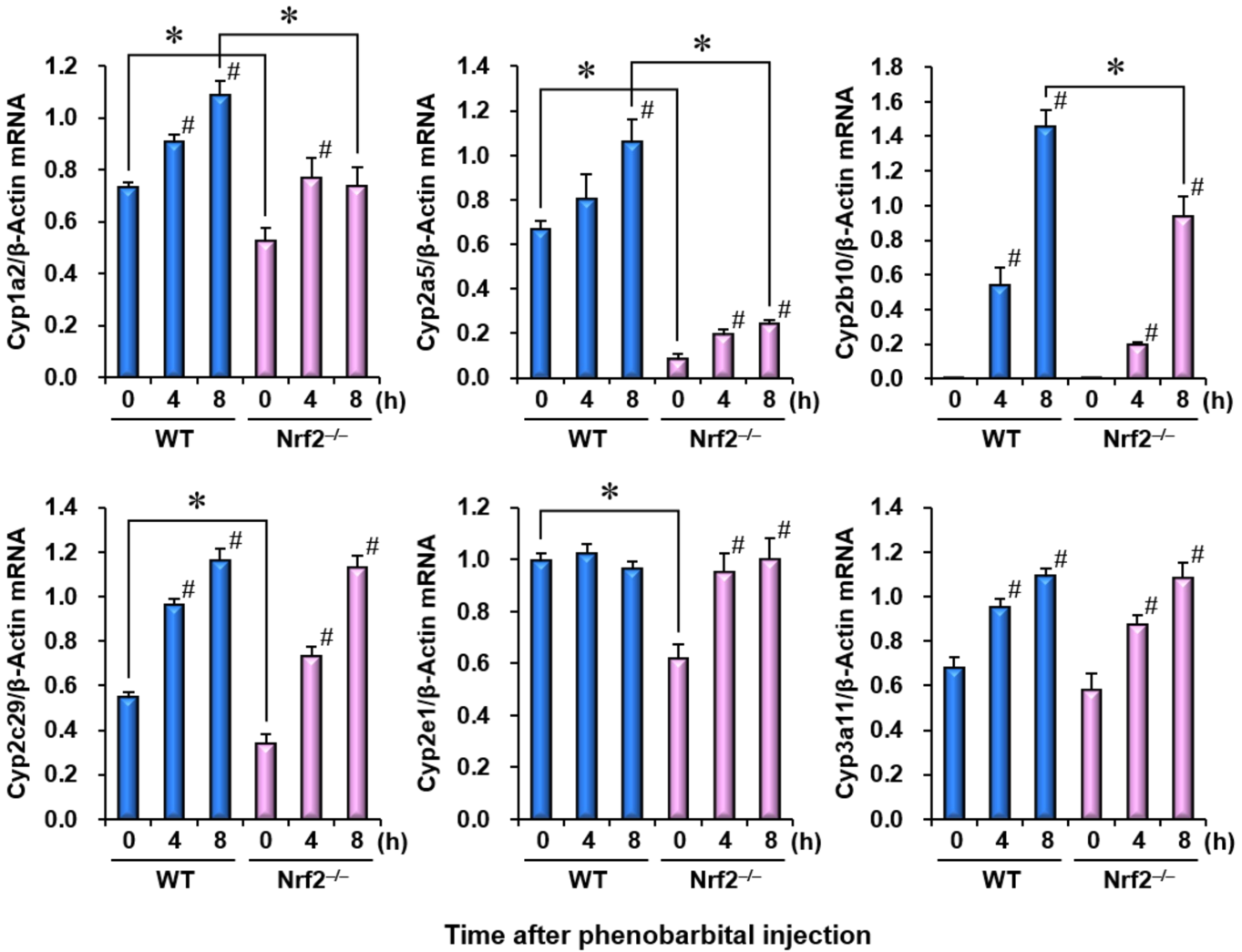
Footnotes

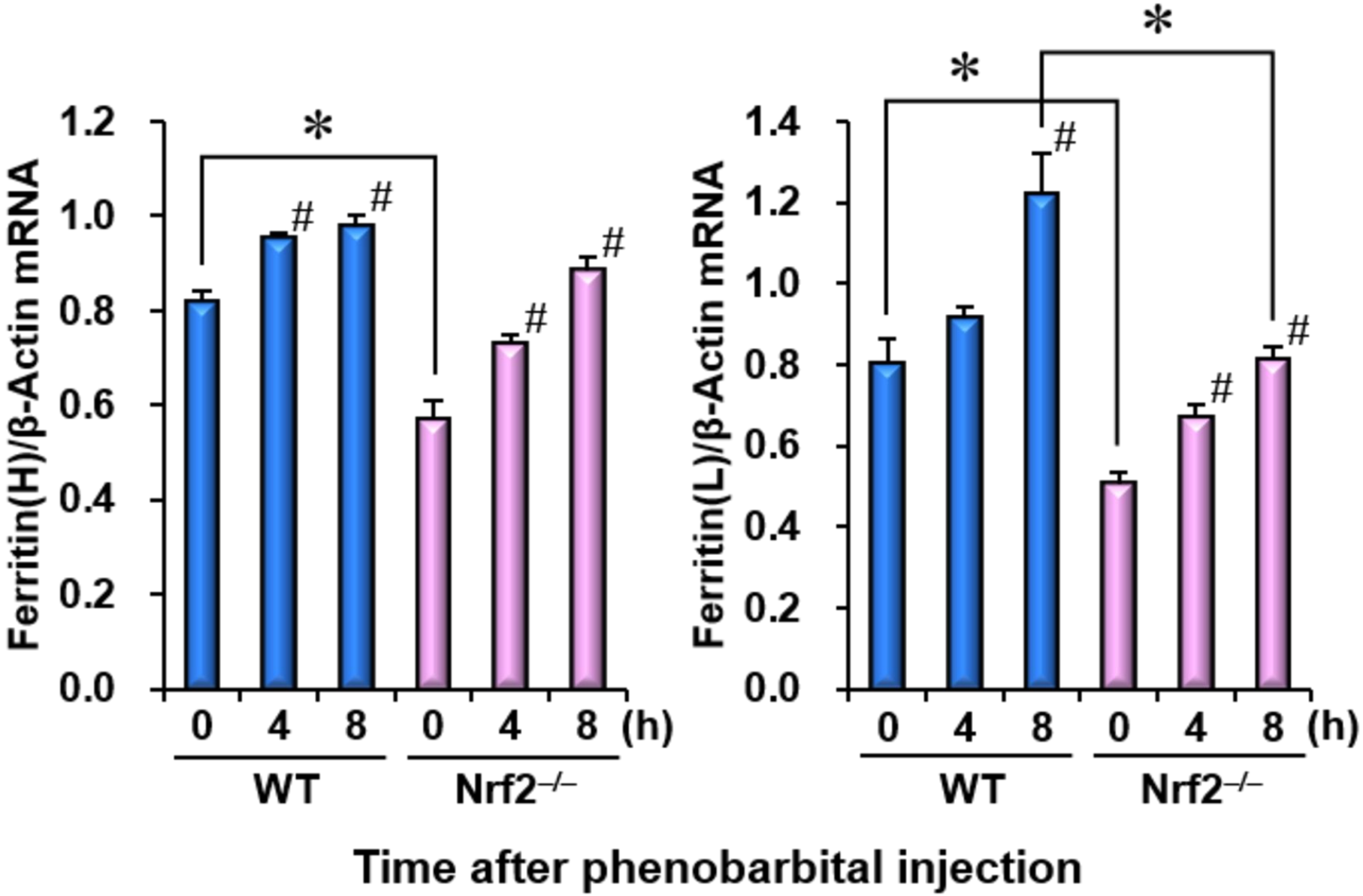
This work was supported by JSPS KAKENHI [18K08115] (to T.A.); and Takeda Science Foundation [2015061858] (to T.A.).

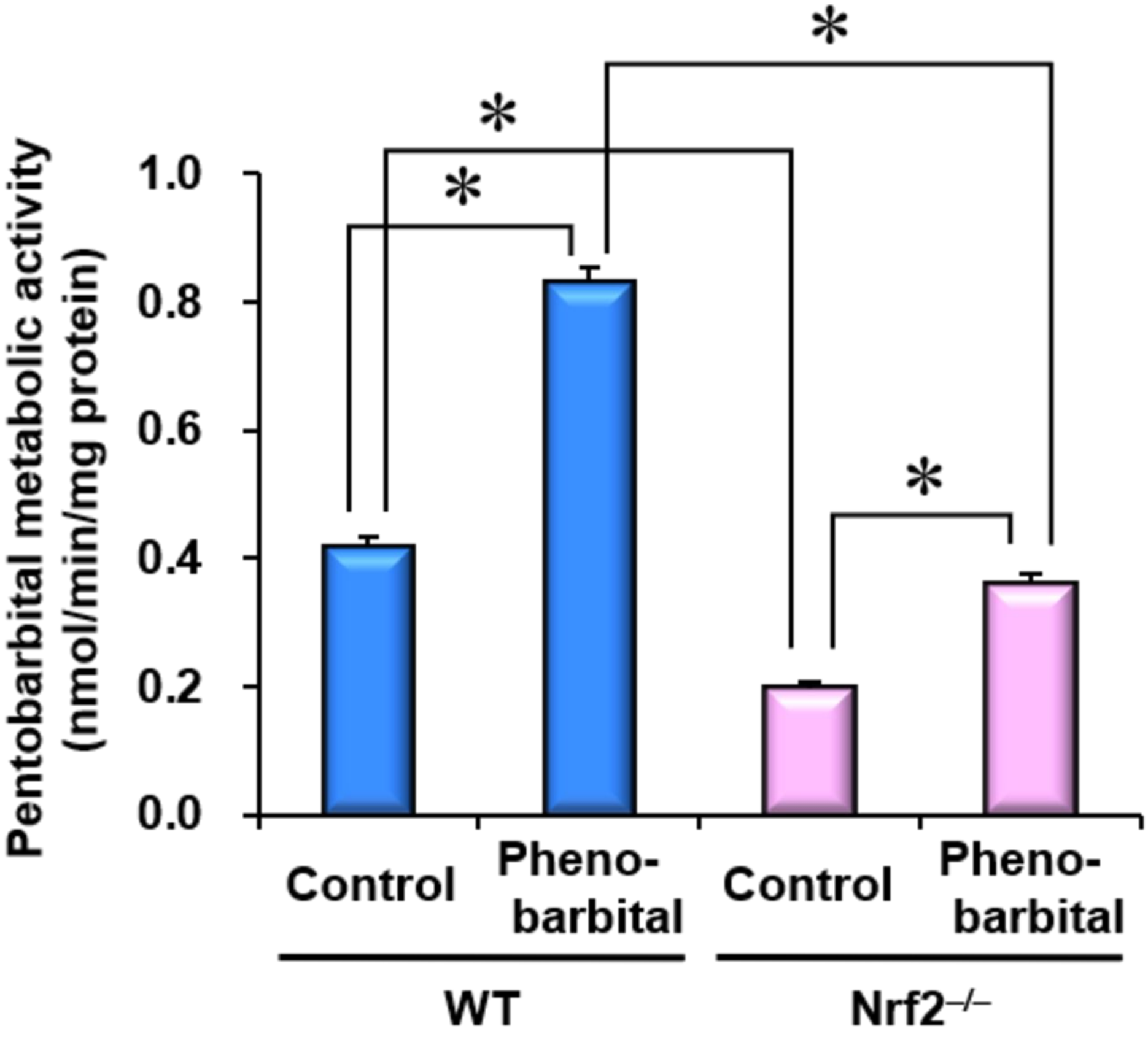

Sleeping time (min)
400
300
200
100
0
*
*
*
Control
Pheno-barbital
Control
Pheno-barbital
WT
Nrf2−/−


A
Plasma pentobarbital (μg/mL)
45
40
35
30
25
20
15
10
*
*
WT
Nrf2−/−
15
30
60
Time after pentobarbital injection (min)
B
Plasma pentobarbital
(% of respective controls)
120
100
80
60
40
20
0
*
*
*
18.9 ± 1.3
3.3 ± 0.4
30.5 ± 0.8
19.2 ± 1.3
Control
Pheno-
barbital
Control
Pheno-
barbital
WT
Nrf2−/−

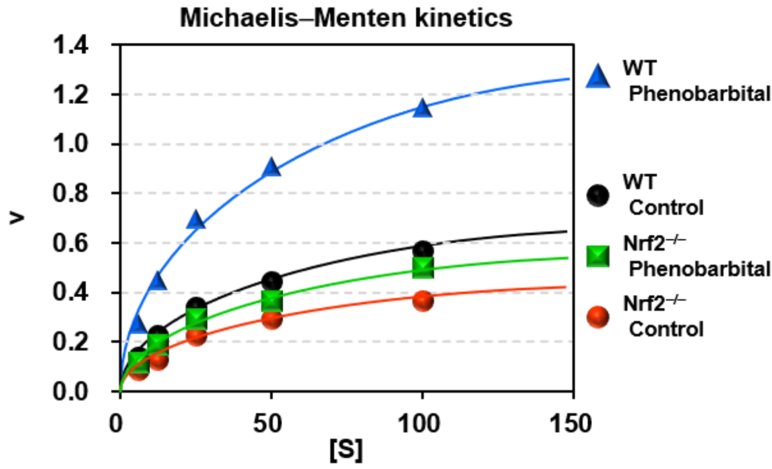

Hepatic microsomal P450 content
(nmol/mg protein)
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Control
Pheno-
barbital
Control
Pheno-
barbital
WT
Nrf2−/−


Cyp1a2/β-Actin mRNA
Cyp2a5/β-Actin mRNA
Cyp2b10/β-Actin mRNA
Cyp2c29/β-Actin mRNA
Cyp2e1/β-Actin mRNA
Cyp3a11/β-Actin mRNA
0 4 8 0 4 8 (h)
WT
Nrf2−/−
Time after phenobarbital injection

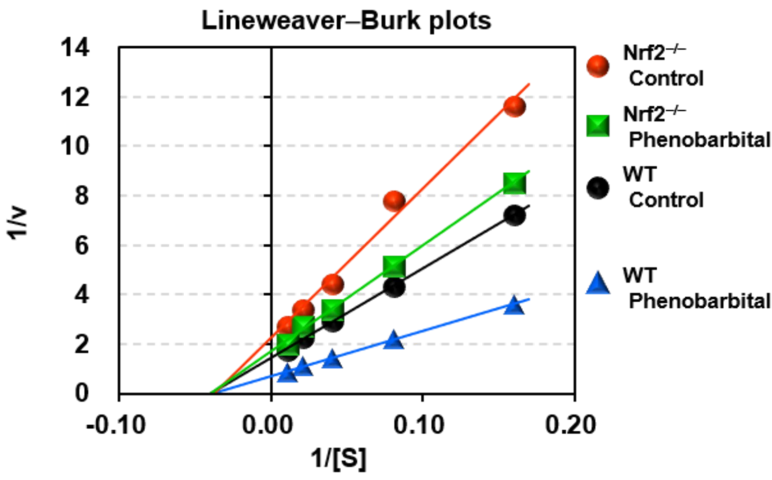

Ferritin(H)/β-Actin mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
#
#
#
0
4
8
0
4
8
(h)
WT
Nrf2−/−
Ferritin(L)/β-Actin mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
#
#
#
0
4
8
0
4
8
(h)
WT
Nrf2−/−
Time after phenobarbital injection


Pentobarbital metabolic activity
(nmol/min/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
Control
Pheno-
barbital
Control
Pheno-
barbital
WT
Nrf2−/−

A

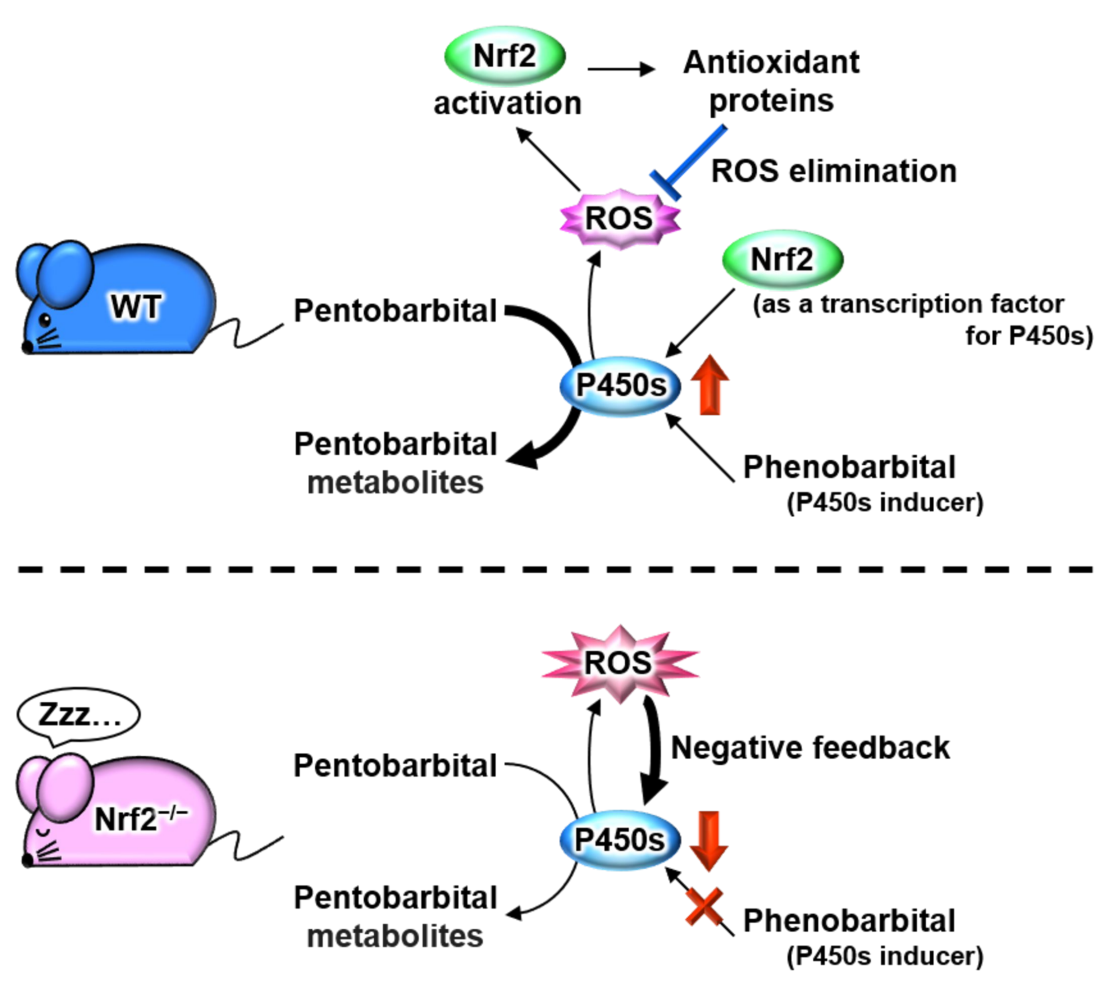


B



C

		Vmax (nmol/min/mg protein)	Km (μM)
WT	Control	0.68 (100%)	24.8
	Phenobarbital	1.40 (204%)	25.6
Nrf2$^{-/-}$	Control	0.44 (100%)	26.6
	Phenobarbital	0.58 (132%)	24.8


Nrf2
activation
Antioxidant
proteins
ROS elimination
ROS
Nrf2
(as a transcription factor
for P450s)
WT
Pentobarbital
P450s
Pentobarbital
metabolites
Phenobarbital
(P450s inducer)
ROS
Zzz…
Negative feedback
Pentobarbital
Nrf2−/−
P450s
Pentobarbital
metabolites
Phenobarbital
(P450s inducer)