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The effect of Nrf2 knockout on the constitutive expression of drug metabolizing enzymes and transporters in C57Bl/6 mice livers

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ABSTRACT

Previous reports have proposed a cross-talk between the nuclear factor erythroid-2 p45-related factor-2 (Nrf2)/antioxidant response element (ARE) and the aryl hydrocarbon receptor (AhR)/xenobiotic response element (XRE) signaling pathways. Therefore, the aim of the current study was to examine the level of phase I, phase II drug metabolizing enzymes (DMEs), and phase III transporters and their related transcription factors in the Nrf2 knockout model. Our results showed that phase II DMEs that are under the control of Nrf2 typified by NAD(P)H: quinone oxidoreductase 1 (Nqo1), and glutathione *S*-transferase (Gst) were significantly lower at the mRNA, protein, and catalytic activity levels in the livers of Nrf2 knockout mice compared to wild type. Furthermore, phase I cytochrome P450s (CYPs), Cyp1, and Cyp2b10 at mRNA, protein, and catalytic activity levels were significantly lower in the livers of Nrf2 knockout mice. Interestingly, our results showed that the transcription factors AhR, constitutive androstane receptor (CAR), and pregnane X receptor (PXR) at mRNA, and protein expression levels were significantly lower in the livers of Nrf2 knockout mice compared to wild type. Importantly, phase III drug transporters mRNA levels of the multiple drug resistance associated proteins (Mrp2 and Mrp3), and solute carrier organic anion transporters (Slco1a6 and Slco2b1) were significantly lower in the liver of Nrf2 knockout mice. Co-activators, Ncoa1, Ncoa2, and Ncoa3 mRNA levels were not altered while co-repressors, Ncor1 and Ncor2 were significantly lower in the livers of Nrf2 knockout mice. In conclusion, knockout of Nrf2 causes disruption to the coordination of phase I, phase II drug DMEs, and phase III drug transporters through altering the transcription factors controlling them.

1. Introduction

Humans and higher organisms are simultaneously exposed to different natural and synthetic xenobiotics through the intake of air, water, and food. Therefore, their ability to withstand toxic chemical insults and oxidative stress through a wide array of enzymatic defense systems has become a necessity for survival (Chanas et al., 2002). The biotransformation and detoxification processes are two sequential reactions that involve phase I and phase II drug metabolizing enzymes, respectively. In phase I reactions, xenobiotics are mainly oxidized by cytochrome P450s (CYPs) to become more polar metabolites. Consequently, phase II reactions, facilitated by key enzymes such as NAD(P)H: quinone oxidoreductase-1 (Nqo1), glutathione-*S*-transferases (Gsts), and UDP-glucuronosyltransferases (UGTs) convert reactive phase I metabolites into more hydrophilic secondary metabolites (Nebert and Duffy, 1997).

It is well documented that different families of CYPs participate in the oxidative metabolism of endo- and xenobiotic substrates (Ramana and Kohli, 1998). Importantly, only the mammalian CYP1, 2, and 3 families are known to be involved in the metabolism of xenobiotics through different signaling pathways (Ramana and Kohli, 1998). In general, transcriptional activation of most CYPs occurs through three main nuclear receptor mechanisms: the aryl hydrocarbon receptor (AhR) for CYP1 family, the constitutive androstane receptor (CAR) for the CYP2 family; and the pregnane X receptor (PXR) for the CYP3 family (Ramana and Kohli, 1998). Similarly, the phase II metabolizing enzymes are regulated through one of the most versatile mechanisms that involve the antioxidant response element (ARE). This element was first discovered because of its vital role in regulating the inducible levels of rat GSTA2, and NQO1 gene expressions (Rushmore et al., 1991). Moreover, studies conducted to elucidate the entity of this response element revealed that ARE responds to Michael reaction acceptors, hydroquinones, catechols, isothiocyanates, peroxides, and trivalent arsenicals

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(Dinkova-Kostova et al., 2001). Further studies on the ARE demonstrated that the basic-region leucine-zipper (bZIP) factor, nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) protein, in combination with other small musculoaponeurotic fibrosarcoma (MAF) proteins, mediate the transcription of genes containing the ARE sequence in their enhancer region. In unstressed conditions, Nrf2 resides in the cytoplasm bound to the actin-associated Kelch-like ECH associating protein 1 (Keap1) (Ma et al., 2004). In response to oxidative stress, the Nrf2-Keap1 interaction becomes less stable causing the former to dissociate and translocate to the nucleus (Ma et al., 2004). Upon translocation to the nucleus, Nrf2 dimerizes with the small MAF proteins, thereafter binds to and activate the ARE (Ma et al., 2004).

Recent studies have demonstrated a direct correlation between the absence of Nrf2 and the increased incidences of carcinogenesis. By far, extensive studies have demonstrated that Nrf2 knockout mice have lower phase II enzymatic activity and phase III transporters as compared to wild type mice (McMahon et al., 2001; Shen and Kong, 2009). However, several studies have also shown that phase I biotransforming enzymes might be indirectly under the control of Nrf2 (Shin et al., 2007). Therefore, we hypothesize that Nrf2 knockout mice will possess relatively lower levels of phase I biotransforming enzymes, possibly due to both lowering Nrf2 and the transcription factors controlling them. Therefore, the objectives of the current study were to determine the constitutive expression of different CYPs, phase II enzymes, phase III transporters, related transcription factors, in addition to different co-activators and co-repressors in both wild type and Nrf2 knockout mice livers.

2. Materials and methods

2.1. Animals and treatment

Nrf2$^{+/+}$ (wild type) and Nrf2$^{-/-}$ (knockout) (ICR background) mice (Itoh et al., 1997) were produced from breeding colonies in National Institute of Environmental Health Sciences (NIEHS). Mice were provided food (modified AIN-76A) and water *ad libitum*. Male mice (5–7 week old) were used for all experiments. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Animals were euthanized under isoflurane anesthesia. All animals were allowed free access to food and water. Liver tissues from Nrf2 knockout and wild type mice were excised, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.2. Chemicals and reagents

TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-capacity cDNA reverse transcription kit, and SYBR Green SuperMix were purchased from Applied Biosystems (Foster City, CA). Real time-PCR primers were synthesized by Integrated DNA Technologies Inc. (San Diego, CA) according to previously published sequences. Chemiluminescence Western blotting detection reagents were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Nrf2 primary antibody, and anti-mouse IgG peroxidase secondary antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). Nqo1 rabbit polyclonal primary antibody was generously provided by Dr. David Ross (University of Colorado, Denver, CO). Gsta1 goat polyclonal primary antibody was purchased from Oxford Biomedical Research (Oxford, MI). PXR rabbit polyclonal antibody was purchased from (Novus Biologicals, CO). CAR rabbit polyclonal primary antibody was purchased from (Abcam, MA). Cyp1a mouse polyclonal primary antibody, Cyp2b1 mouse polyclonal primary antibody, AhR rabbit polyclonal primary antibody, actin rabbit polyclonal primary antibody and anti-goat and anti-rabbit IgG peroxidase secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).

2.3. RNA extraction and cDNA synthesis

Total RNA from the frozen livers was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. Thereafter, first-strand cDNA synthesis was performed by using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.5 μg of total RNA from each sample was added to a mix of 2.0 μl 10X RT buffer, 0.8 μl 25X dNTP mix (100 mM), 2.0 μl 10X RT random primers, 1.0 μl MultiScribe™ reverse transcriptase, and 3.2 μl nuclease-free water. The final reaction mix was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 s, and finally cooled to 4 °C.

2.4. Quantification by real time-PCR

Quantitative analysis of specific mRNA expression was performed by real time-PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). 25 μL reaction mix contained 0.1 μL of 10 μM forward primer and 0.1 μL of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μL of SYBR Green Universal Mastermix, 11.05 μL of nuclease-free water, and 1.25 μL of cDNA sample. The primers used in the current study were chosen from previously published studies and are listed in Table 1. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and anneal/extension at 60 °C for 1 min. Melting curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

2.5. Real time-PCR data analysis

The real time-PCR data were analyzed using the relative gene expression i.e. ($\Delta\Delta C_T$) method as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen (2001). In brief, the primers used in the current study were tested to avoid primer dimers, self-priming formation, or unspecific amplification. To ensure the quality of the measurements, each plate included, for each gene, a negative control and a positive control. For each sample, a threshold cycle (C_T) was calculated based on the time (measured by the number of PCR cycles) at which the reporter fluorescent emission increased beyond a threshold level (based on the background fluorescence of the system). The triplicate measurements for each sample were averaged to give an average C_T value for each group, after removing of outliers (Oscar Aparicio et al., 2005). The samples were diluted in such a manner that the C_T value was observed between 15 and 30 cycles. Results were expressed using the comparative C_T method as described in User Bulletin 2 (Applied Biosystems). Briefly, the ΔC_T values were calculated in every sample for each gene of interest as follows: $C_{T\ \text{gene of interest}} - C_{T\ \text{reporter gene}}$, with β-actin as the reporter gene. Calculation of relative changes in the expression level of one specific gene ($\Delta\Delta C_T$) was performed by subtraction of ΔC_T of wild type

Table 1
Primers sequences used for real-time PCR reactions.

Gene	Forward primer	Reverse primer
AhR	5′-CGG CTT CTT GCA AAA CAC AGT-3′	5′-GTA AAT GCT CTC GTC CTT CTT CAT-3′
B-actin	5′-TAT TGG CAA CGA GCG GTT CC-3′	5′-GGC ATA GAG GTC TTT ACG GAT GTC-3′
CAR	5′-TCA ACA CGT TTA TGG TGC AA-3′	5′-CTG CGT CCT CGA TCT TGT AG-3′
Cyp1a1	5′-GGT TAA CCA TGA CCG GGA ACT-3′	5′-TGC CCA AAC CAA AGA GAG TGA-3′
Cyp1a2	5′-TGG AGC TGG CTT TGA CAC AG-3′	5′-CGT TAG GCC ATG TCA CAA GTA GC-3′
Cyp1b1	5′-AAT GAG GAG TTC GGG CGC ACA-3′	5′-GGC GTG TGG AAT GGT GAC AGG-3′
Cyp2b10	5′-GGG AAC CTC TTG CAG ATG-3′	5′-CCC AGG TGC ACT GTG AA-3′
Gsta1	5′-CCC CTT TCC CTC TGC TGA AG-3′	5′-TGC AGC TTC ACT GAA TCT TGA AAG-3′
Mrp2	5′-GTG TGG ATT CCC TTG GGC TTT-3′	5′-CAC AAC GAA CAC CTG CTT GG-3′
Mrp3	5′-AGT CTT CGG GAG TGC TCA TCA-3′	5′-AGG ATT TGT GTC AAG ATT CTC CG-3′
Ncoa1	5′-GAC CCT GCA AAC CCA GAC TC-3′	5′-CGT GGA TTT CTC TTG CTC CAT T-3′
Ncoa2	5′-ATG AGT GGG ATG GGA GAA AAC A-3′	5′-GCT GGT CGG GAC ATT CCT T-3′
Ncoa3	5′-AGT GGA CTA GGC GAA AGC TCT-3′	5′-GTT GTC GAT GTC GCT GAG ATT T-3′
Ncor1	5′-CCA ACC ACA AAA CCA GCA GAA-3′	5′-CAC CGC ACT CCT CAT GGT C-3′
Ncor2	5′-GCA GAG AAA CCC GCA TTC TTT-3′	5′-GCG TGT GGG GAA GTC TTG A-3′
Nrf2	5′-CGA GAT ATA CGC AGG AGA GGT AAG A-3′	5′-GCT CGA CAA TGT TCT CCA GCT-3′
Nqo1	5′-GGA AGC TGC AGA CCT GGT GA-3′	5′-CCT TTC AGA ATG GCT GGC A-3′
PXR	5′-AAG AAG CAG ACT CTG CCT TGG A-3′	5′-GTG GTA GCC ATT GGC CTT GT-3′
Slco1a6	5′-ACA GGG TCA GGT GCT TTG C-3′	5′-ATC ACC AAA AGG TTA CCC ATC TC-3′
Slco2b1	5′-CTC AGG ACT CAC ATC AGG ATG C-3′	5′-CTC TTG AGG TAG CCA GAG ATC A-3′

mice livers from the ΔC_T of the Nrf2 knockout mice livers. The values and ranges given in different figures were determined as follows: $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T \pm$ S.E., where S.E. is the standard error of the mean of the $\Delta\Delta C_T$ value (User Bulletin 2, Applied Biosystems).

2.6. Microsomal and cytosolic protein preparation and Western blot analysis

Microsomal protein was prepared from the liver tissue as described previously (Barakat et al., 2001). Briefly, livers were washed in ice-cold KCl (1.15% w/v), cut into pieces, and homogenized separately in cold sucrose solution (1 g of tissue in 5 mL of 0.25 M sucrose). Microsomal and cytosolic proteins from homogenized tissues were separated by differential ultracentrifugation. The final pellet, microsomes, was reconstituted in cold sucrose, and supernatant, cytosol, were stored at −80 °C. Liver microsomal and cytosolic protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951). Western blot analysis was performed using a previously described method (Zordoky et al., 2010). Briefly, 20 μg of liver microsomal or cytosolic proteins from each group was separated by 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE), and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (TBS), 5% skim milk, 2% bovine serum albumin, and 0.5% Tween-20. After blocking, the blots were incubated with the following primaries: primary polyclonal mouse anti-human Nrf2, primary polyclonal rabbit anti-mouse AhR, primary polyclonal mouse anti-rat Cyp1a, primary polyclonal rabbit anti-rat Cyp1b1, primary polyclonal mouse anti-mouse Cyp2b10, primary polyclonal goat anti-mouse Nqo1, primary polyclonal goat anti-rat Gsta1, primary polyclonal rabbit anti-mouse CAR, primary polyclonal rabbit anti-mouse PXR, primary polyclonal rabbit anti-mouse actin, or primary polyclonal goat anti-mouse Gapdh for 2 h at room temperature. Incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for Nqo1, AhR, and Cyp1b1, CAR, PXR, actin or goat anti-mouse IgG secondary antibody for Nrf2, Cyp1a, and Cyp2b10, or rabbit anti-goat IgG secondary antibody for Gsta1 and Gapdh was carried out for another 2 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ). The intensity of the protein bands were quantified, relative to the signals obtained for actin, using ImageJ software (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.).

2.7. Microsomal incubation and measuring EROD, MROD and PROD catalytic activities

Liver microsomes (1 mg protein/mL) were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH 7.4) at 37 °C in a shaking water bath (50 rpm). A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. The concentrations of substrate were 2 μm for 7-ethoxyresorufin (EROD) and 7-methoxyresorufin (MROD) and 10 μm for 7-pentoxyresorufin (PROD). After incubation at 37 °C (5 min for EROD, and 10 min for MROD and PROD assays), the reaction was stopped by adding 0.5 mL of cold methanol. The amount of resorufin formed in the resulting supernatant was measured using the Baxter 96-well fluorescence plate reader using excitation and emission wavelengths of 545 and 575 nm, respectively. Formation of resorufin was linear with incubation time and protein amount. Enzymatic activities were expressed as picomole of resorufin formed per minute and per milligram of microsomal proteins.

2.8. Determination of Nqo1 enzymatic activity

The Nqo1 activity was determined by the continuous spectrophotometric assay to quantitate the reduction of its substrate, 2,6-dichlorophenolindophenol (DCPIP) as described previously (Korashy and El-Kadi, 2006; Preusch et al., 1991). Briefly, 20 μg of cytosolic protein was incubated with 1 mL of the assay buffer [40 μM DCPIP, 0.2 mM NADPH, 25 mM Tris–HCl, pH 7.8, 0.1% (v/v) Tween 20, and 0.7 mg/mL bovine serum albumin, 0 or 30 μM dicoumarol]. The rate of DCPIP reduction was monitored over 90 s at 600 nm with an extinction coefficient (ε) of 2.1 mM^{-1} cm^{-1}. The Nqo1 activity was calculated as the decrease in absorbance per min per mg of total protein of the sample which quantitates the dicoumarol-inhibitable reduction of DCPIP.

2.9. Determination of GST activity

GST activity was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the method of Habig et al. (1974). Briefly, 20 μg of cytosolic or micro-

somal protein were incubated with 1 mM CDNB, 1 mM lower glutathione in 0.1 M potassium phosphate buffer, pH 6.5 at 25 °C in a total volume of 1 mL. GST activity was measured as the amount of CDNB conjugate formed by recording the absorbance at 340 nm for 1.5 min with an extinction coefficient of 9600 M^{-1}/cm^{-1}. The enzyme activity was expressed as nmol/min/mg protein.

2.10. Statistical analysis

Data are presented as mean ± standard error of the mean. Wild type and knockout measurements were compared using student *t*-test. A result was considered statistically significant where $P < 0.05$.

3. Results

3.1. The effect of Nrf2 knockout on Nrf2 mRNA and protein expression level

In order to confirm the knockout of Nrf2, we measured its mRNA and protein expression levels in the livers of Nrf2 knockout mice and their wild type animals. Our results showed the Nrf2 mRNA levels were completely abolished in the livers of Nrf2 knockout mice (Fig. 1A). Moreover, to confirm the knockout of the Nrf2 at the protein level we measured its expression in the cytosolic fraction of the livers using Western blot analysis. Similar to the results obtained at the mRNA level, Nrf2 protein was decreased by approximately 100% in the livers of Nrf2 knockout mice compared to wild type (Fig. 1B).

3.2. The effect of Nrf2 knockout on Nqo1 and Gsta1 mRNA levels

To further confirm that the knockout of Nrf2 is in fact causing a decrease in the ARE-regulated genes typified by Nqo1 and Gsta1, total RNA was extracted from livers of both wild type and Nrf2 knockout mice. Thereafter, we determined the gene expression of Nqo1 and Gsta1 in wild type and Nrf2 knockout mice livers. Our results showed that Nqo1 and Gsta1 mRNA levels were significantly lower in the livers of Nrf2 knockout mice by 93% and 99%, respectively, compared to wild type (Fig. 2A and B).

3.3. The effect of Nrf2 knockout on Nqo1 and Gsta1 protein expression levels

The fact that mRNA levels of Nqo1 and Gsta1 were lower in the livers of Nrf2 knockout mice compared to those of wild type prompted us to investigate their protein expression levels. For this purpose, cytosolic and microsomal protein was prepared from the livers of wild type and Nrf2 knockout mice. Thereafter, Nqo1 and Gsta1 protein expression levels were determined using Western blot analysis relative to Gapdh. Our results demonstrated that Nqo1 and Gsta1 protein expression levels were decreased in the livers of Nrf2 knockout mice by 73% and 87%, respectively, compared to wild type (Fig. 3). Similar to the results of mRNA expression levels, Gsta1 protein expression was more affected by Nrf2 knockout than Nqo1.

3.4. The effect of Nrf2 knockout on Nqo1 and Gst activity

In an attempt to examine whether the observed decrease in Nqo1 and Gsta1 mRNA, and protein expression levels can be translated to their catalytic activity levels we measured cytosolic Nqo1, and cytosolic and microsomal Gst activities. Our results showed that the constitutive Nqo1 activity in the livers of wild type mice is 176 nmol/min/mg protein. On the other hand in the livers of Nrf2 knockout mice, Nqo1 activity was significantly lower by 95%

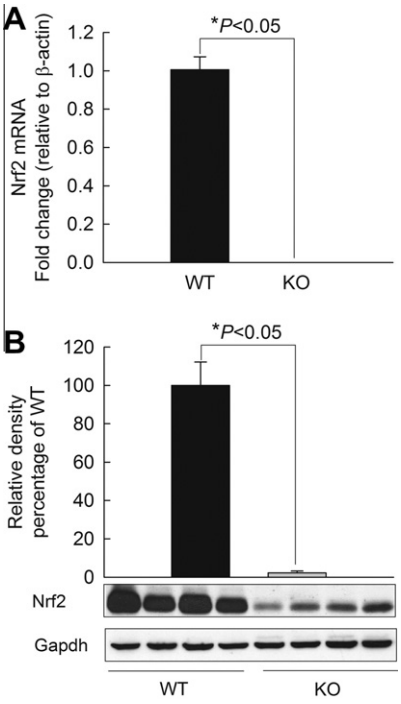

Fig. 1. Effect of Nrf2 knockout on Nrf2 mRNA and protein expression levels. (A) Total RNA was isolated from livers of untreated Nrf2 wild type (WT) and untreated Nrf2 knockout (KO) mice and gene expression of Nrf2 was determined by real time-PCR. Results are presented as mean ± SEM ($n = 6$). $^*P < 0.05$ compared to WT. (B) Liver cytosolic protein was isolated from the livers of WT and KO mice. 20 μg of cytosolic protein were separated on a 10% SDS–PAGE. Nrf2 protein was detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to Gapdh signals (mean ± SEM, $n = 4$), and the results are expressed as percentage of the WT values taken as 100%. $^*P < 0.05$ compared to WT.

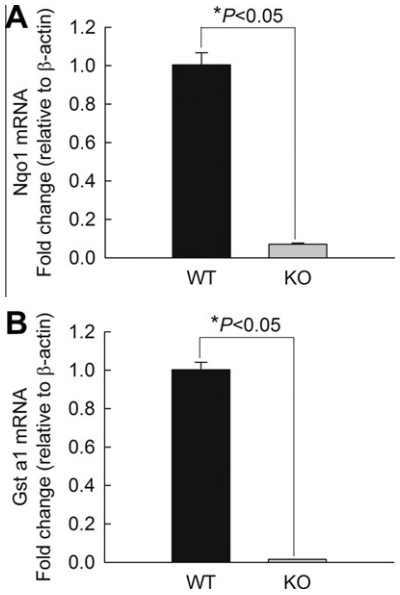

Fig. 2. Effect of Nrf2 knockout on Nqo1 and Gsta1 mRNA levels. Total RNA was isolated from livers of Nrf2 wild type (WT) and Nrf2 knockout (KO) mice and gene expression of Nqo1 (A) and Gsta1 (B) was determined by real time-PCR. Results are presented as mean ± SEM ($n = 6$). $^*P < 0.05$ compared to WT.

(Fig. 4A). The constitutive cytosolic and microsomal Gst activities in the livers of wild type mice were 1900 and 600 μmol/min/mg protein, respectively (Fig. 4B and C). Interestingly, cytosolic and

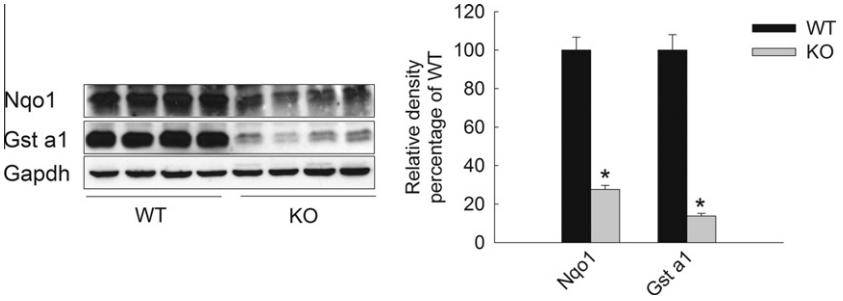

Fig. 3. Effect of Nrf2 knockout on Nqo1 and Gsta1 protein expression levels. Liver cytosolic protein was isolated from the livers of Nrf2 wild type (WT) and Nrf2 knockout (KO) mice. 20 μg cytosolic protein for Nqo1, and Gsta1 were separated on a 10% SDS–PAGE. Nqo1 and Gsta1 proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to Gapdh signals (mean ± SEM, $n = 4$), and the results are expressed as percentage of the WT values taken as 100%. $^{*}P < 0.05$ compared to WT.

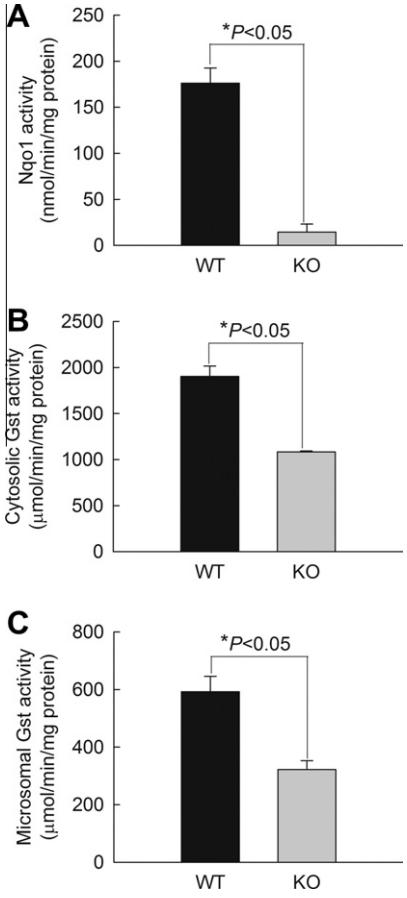

Fig. 4. Effect of Nrf2 knockout on Nqo1 and Gst activity. Liver cytosolic and microsomal proteins were isolated from the livers of Nrf2 wild type (WT) and Nrf2 knockout (KO) mice. Nqo1 enzyme activity (A) was determined spectrophotometrically in liver cytosol using DCPIP as substrate, and dicoumarol as specific Nqo1 inhibitor. Gst activity (B and C) was determined spectrophotometricaly using CDNB as a substrate as described under materials and methods. Values are presented as mean ± SEM ($n = 4$). $^{*}P < 0.05$ compared to WT.

microsomal Gst activities were significantly lower in the livers of Nrf2 knockout mice by 43% and 46%, respectively, compared to wild type (Fig. 4B and C).

3.5. The effect of Nrf2 knockout on the Cyp1a1, Cyp1a2, 1b1, and Cyp2b10 mRNA levels

To examine whether Nrf2 knockout alters the expression of Cyp1 family which are under the control of AhR, total RNA was extracted from livers of both wild type and Nrf2 knockout mice. Thereafter, we determined the gene expression of Cyp1a1, Cyp1a2, and Cyp1b1 in the livers of wild type and Nrf2 knockout mice using reverse transcription followed by real time-PCR. Our results showed that, Cyp1a1, Cyp1a2, and Cyp1b1 mRNA levels were significantly lower in the livers of Nrf2 knockout mice by 88%, 70%, and 50%, respectively, compared to wild type (Fig. 5A–C).

To further examine whether other members of phase I DMEs such as Cyp2b10 is altered in Nrf2 knockout mice, total RNA was extracted from livers of both wild type and Nrf2 knockout mice. Thereafter, we determined the gene expression of Cyp2b10, a prototypical gene regulated by CAR and PXR in the livers of wild type and Nrf2 knockout mice. Our results demonstrated that Cyp2b10 mRNA level was significantly lower by approximately 100% in Nrf2 knockout mice livers compared to wild type (Fig. 5D).

3.6. The effect of Nrf2 knockout on Cyp1a1/2, Cyp1b1, and Cyp2b10 protein expression levels

In an attempt to correlate the effect of Nrf2 knockout on the mRNA levels of Cyp1a, Cyp1b1, and Cyp2b10 to their protein expression levels we investigated the protein expression levels of these enzymes in the livers of wild type and knockout mice. For this purpose, microsomal protein was prepared from the livers of wild type and Nrf2 knockout mice. Thereafter, Cyp1a, Cyp1b1, and Cyp2b10 protein levels were determined using Western blot analysis. Our results demonstrated that Cyp1a and Cyp1b1 protein expression levels were decreased in the livers of Nrf2 knockout mice by 56% and 50%, respectively, compared to wild type (Fig. 6). Moreover, Cyp2b10 protein expression level was also decreased in the livers of Nrf2 knockout mice by 45%, compared to wild type (Fig. 6).

3.7. The effect of Nrf2 knockout on EROD, MROD, and PRDO catalytic activities

To examine whether the observed effects of Nrf2 knockout on Cyp1a, and Cyp2b10 mRNA and protein expression levels is further reflected at the catalytic activity levels, we measured the catalytic activity of Cyp1a1, Cyp1b1, Cyp1a2, and Cyp2b10 using EROD, MROD, and PROD, respectively. Our results showed that EROD, MROD, and PROD catalytic activities in wild type mice livers were 59, 180, and 5 pmols/min/mg protein, respectively (Fig. 7A–C). On the other hand, EROD, MROD, and PROD activities were decreased in the livers of Nrf2 knockout mice by 50%, 70%, and 75%, respectively, compared to wild type (Fig. 7A–C).

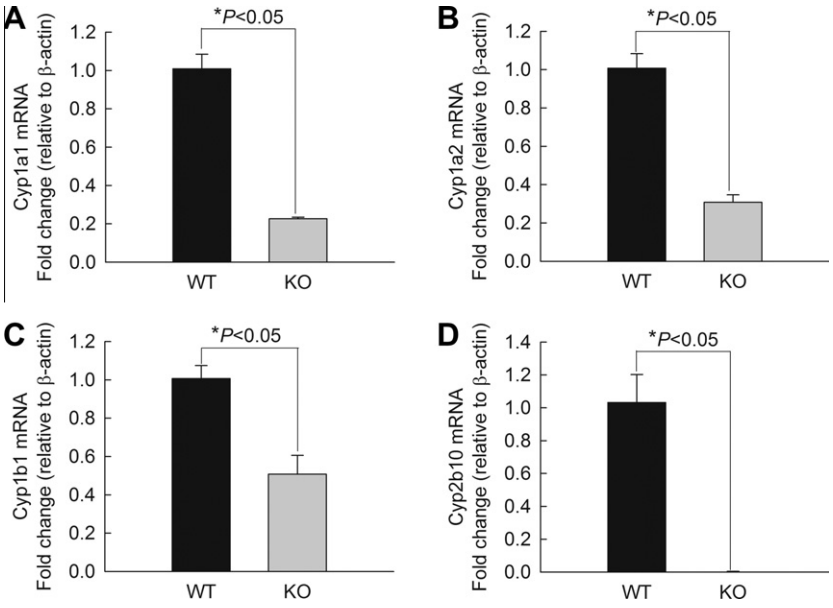

Fig. 5. Effect of Nrf2 knockout on Cyp1a1, 1a2, 1b1, and 2b10 mRNA levels. Total RNA was isolated from livers of untreated Nrf2 wild type (WT) and Nrf2 knockout knockout (KO) mice and gene expression of Cyp1a1 (A), Cyp1a2 (B), Cyp1b1 (C), and Cyp2b10 (D), was determined by real time-PCR. Results are presented as mean ± SEM ($n = 4$). $^{*}P < 0.05$ compared to WT.

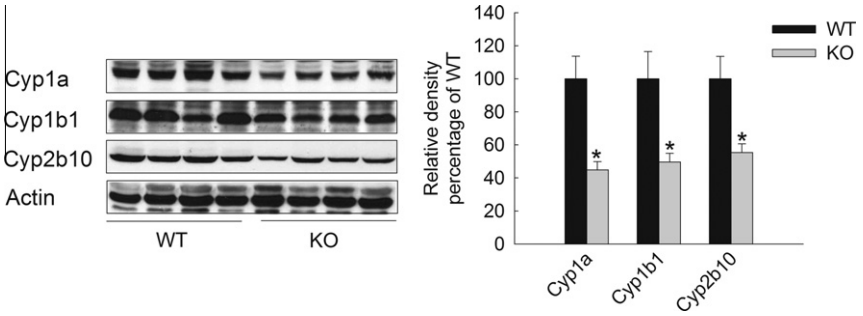

Fig. 6. Effect of Nrf2 knockout on Cyp1a1/2, Cyp1b1, and Cyp2b10 protein expression levels. Liver microsomal protein was isolated from the livers of wild type (WT) and knockout (KO) animals. 20 μg of microsomal protein for Cyp1a1/2 and Cyp1b1, and Cyp2b10 were separated on a 10% SDS–PAGE. Cyp1a1/2, Cyp1b1, and Cyp2b10 proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to Actin signals (mean ± SEM, $n = 4$), and the results are expressed as percentage of the WT values taken as 100%. $^{*}P < 0.05$ compared to WT.

3.8. The effect of Nrf2 knockout on the transcription factors: AhR, CAR, PXR mRNA levels

To determine the effect of Nrf2 knockout on the transcription factors regulating Cyp1 and Cyp2 families, namely AhR, CAR, and PXR, respectively, total RNA was extracted from livers of both wild type and Nrf2 knockout mice. Thereafter, the expression of different genes was measured using reverse transcription followed by real time-PCR as described under materials and methods. Our results demonstrated that AhR, CAR, and PXR mRNA levels were significantly lower in the livers of Nrf2 knockout mice by 60%, 84%, and 73%, respectively, compared to wild type (Fig. 8A–C).

3.9. The effect of Nrf2 knockout on AhR, CAR, and PXR protein expression levels

The fact that mRNA levels of AhR, CAR, and PXR were lower in the livers of Nrf2 knockout mice compared to those of wild type prompted us to investigate the protein expression levels of these genes. For this purpose, cytosolic protein was prepared from the livers of wild type and Nrf2 knockout mice. Thereafter, AhR, CAR, and PXR protein levels were determined using Western blot analysis relative to Gapdh. Our results demonstrated that AhR protein expression level was decreased by 40% in the livers of Nrf2 knockout mice compared to wild type (Fig. 9). Similarly, CAR and PXR protein expression levels were decreased in the livers of Nrf2 knockout mice by 80% and 35%, respectively, compared to wild type (Fig. 9).

3.10. The effect of Nrf2 knockout on phase III multiple drug resistance associated proteins (Mrp2 and Mrp3), and solute carrier organic anion transporters (Slco1a6 and Slco2b1)

To further evaluate the effect of Nrf2 knockout on the phase III transporters, total RNA was extracted from livers of both wild type and Nrf2 knockout mice. Thereafter, we determined the gene expression of Mrp2, Mrp3, Slco1a6, and Slco2b1. Our results demonstrated that Mrp2, Mrp3, Slco1a6, and Slco2b1 were significantly lower in the livers of Nrf2 knockout mice by 68%, 96%, 90%, and 22%, respectively (Fig. 10).

3.11. The effect of Nrf2 knockout on co-activators Ncoa1, Ncoa2, and Ncoa3 mRNA levels

The decrease in phases I and II drug metabolizing enzymes, and phase III transporters with the concomitant decrease in their con-

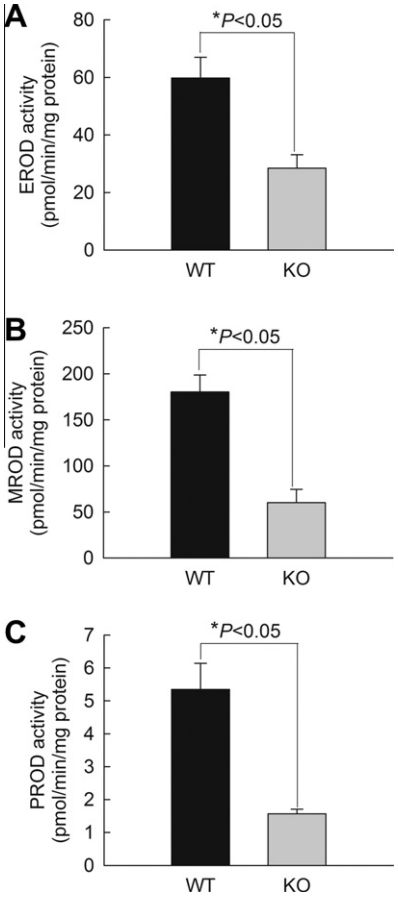

Fig. 7. Effect of Nrf2 knockout on EROD, MROD, and PROD catalytic activities. Liver microsomal protein was isolated from the livers of wild type (WT) and knockout (KO) animals. EROD activity (A) MROD activity (B), PROD activity (C) was measured using 7-ethoxyresorufin, 7-methoxyresorufin, and 7-pentoxyresorufin as a substrates, respectively. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice cold methanol. Values are presented as mean ± SEM ($n = 4$). $^*P < 0.05$ compared to WT.

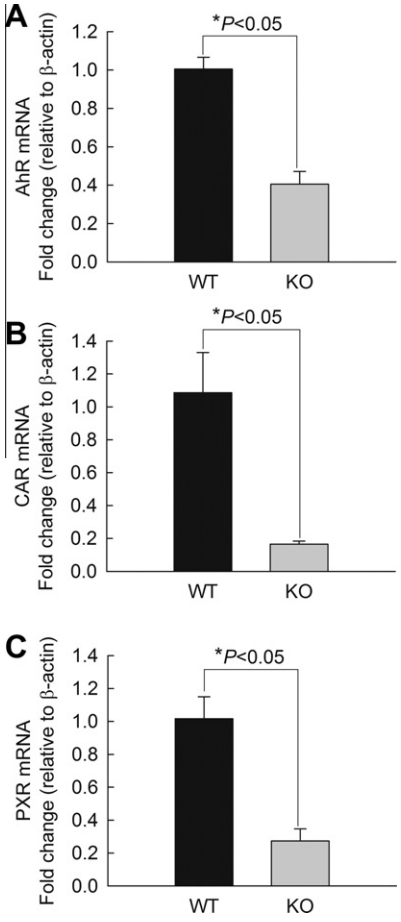

Fig. 8. Effect of Nrf2 knockout on the transcription factors: AhR, CAR, and PXR mRNA levels. Total RNA was isolated from livers of Nrf2 wild type (WT) and Nrf2 knockout (KO) mice and gene expression of AhR (A), CAR (B), and PXR (C) were determined by real time-PCR. Results are presented as mean ± SEM ($n = 4$). $^*P < 0.05$ compared to WT.

trolling transcription factors prompted us to investigate whether or not there is a similar effect on the co-activators that are known to be associated with these transcription factors. For this reason, total RNA was extracted from livers of both wild type and Nrf2 knockout mice. Thereafter, the expression of Ncoa1 (Src-1), Ncoa2 (Src-2), and Ncoa3 (Src-3) in the livers of wild type and Nrf2 knockout mice was determined using reverse transcription followed by real time-PCR. Our results demonstrated that Ncoa1, Ncoa2, and Ncoa3 mRNA levels were not significantly altered in the livers of Nrf2 knockout mice compared to wild type (Fig. 11).

3.12. The effect of Nrf2 knockout on co-repressors Ncor1 and Ncor2 mRNA levels

In an attempt to further investigate the role of the co-repressors associated with the transcription factors regulating phase I and II drug metabolizing enzymes, and phase III transporters, total RNA was extracted from livers of both wild type and Nrf2 knockout mice. Thereafter, the expression of Ncor1 (RIP-13) and Ncor2 (SMRT) in the livers of wild type and Nrf2 knockout mice was determined using reverse transcription followed by real time-PCR. Our results demonstrated that Ncor1 and Ncor2 mRNA levels were significantly lower in the livers of Nrf2 knockout mice compared to wild type by 45% and 37%, respectively (Fig. 12).

4. Discussion

The present work provides the first demonstration that livers of Nrf2 knockout mice have lower expression of phase I, phase II drug metabolizing enzymes, and phase III drug transporters that is related to the decrease of their transcription factors.

Previous preclinical and clinical research has proposed that modulation of the body's drug metabolizing enzymes could provide an effective strategy for cancer prevention. So far, the induction of phase II drug metabolizing enzymes such as Nqo1 and Gsts, which has been termed the antioxidant response, is currently serving as a central strategy in cancer prevention (Thimmulappa et al., 2002). This so called antioxidant response is part of the cellular defense system arsenal and is mainly mediated through the Nrf2/ARE pathway (Nioi et al., 2003). The induction of phase II drug metabolizing enzymes through this pathway is believed to contribute to the prevention of DNA adducts formation (Thimmulappa et al., 2008). Intriguingly, the decrease in phase II drug metabolizing enzymes has been merely attributed to the decrease in Nrf2 (Jana and Mandlekar, 2009). For example, it has been shown that Nrf2 knockout mice have lower basal and inducible mRNA levels of Nqo1 and Gsts when compared to wild type mice (McWalter et al., 2004). Similarly, mice lacking Nrf2 has been shown to exhibit lower GSH levels making them more susceptible to acetaminophen-mediated liver injury than wild type mice (Reisman et al., 2009). Our results showed that both Nqo1 and Gsta1 mRNA

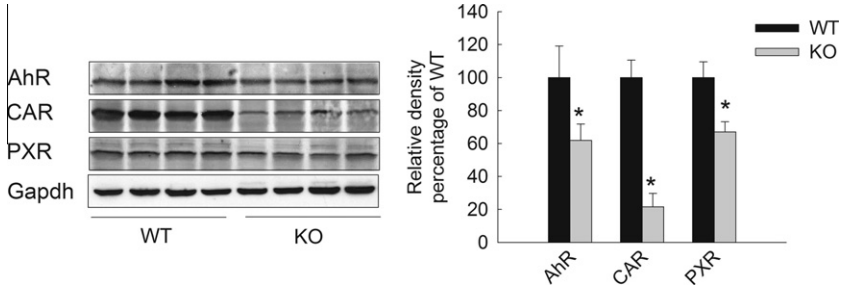

Fig. 9. Effect of Nrf2 knockout on AhR, CAR, and PXR protein expression levels. Liver cytosolic protein was isolated from the Nrf2 wild type (WT) and Nrf2 knockout (KO) mice. 20 μg cytosolic protein were separated on a 10% SDS–PAGE. AhR, CAR, and PXR proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of protein normalized to Gapdh (mean ± SEM, $n = 4$), and the results are expressed as percentage of the WT values taken as 100%. $^{*}P < 0.05$ compared to WT.

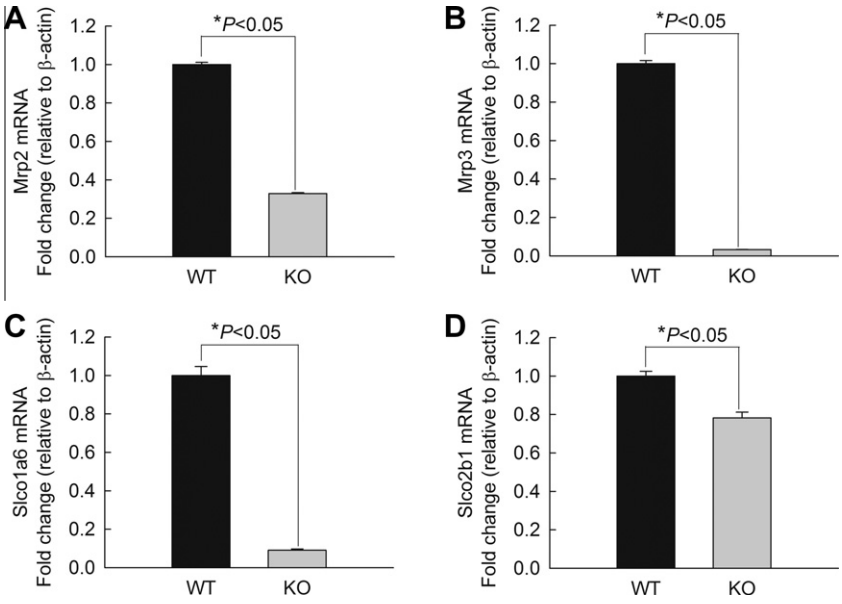

Fig. 10. Effect of Nrf2 knockout on Mrp2, Mrp3, Slco1a6, and Slco2b1 mRNA levels. Total RNA was isolated from livers of untreated Nrf2 wild type (WT) and Nrf2 knockout knockout (KO) mice and gene expression of Mrp2 (A), Mrp3 (B), Slco1a6 (C), and Slco2b1 (D), was determined by real time-PCR. Results are presented as mean ± SEM ($n = 4$). $^{*}P < 0.05$ compared to WT.

expression levels were lower in the livers of Nrf2 knockout mice compared to wild type. However, the effect of Nrf2 knockout was more pronounced at the mRNA level of Gsta1 more than those of Nqo1.

The decrease in Nqo1 and Gsta1 mRNA levels in the liver of Nrf2 knockout mice was also translated to the protein and catalytic activity levels in which Nqo1 and Gsta1 proteins were significantly lower in the livers of Nrf2 knockout animals, confirming the results obtained at the mRNA level. Examining the activities of both enzymes, we measured Nqo1 activity in the cytosolic fraction of the wild type and Nrf2 knockout mice livers. In addition, we measured Gsts activity in the cytosolic and microsomal fractions of these livers. In agreement with our results at the mRNA and protein expression levels, the catalytic activities of Nqo1 and Gsta1 were significantly lower in the livers of Nrf2 knockout mice compared to wild type. Importantly, microsomal Gsts activity was significantly lower in the livers of Nrf2 knockout mice. Previous studies have shown that Gsts can be divided into three classes namely cytosolic, mitochondrial, and microsomal Gsts (Hayes et al., 2005). Microsomal Gsts are also called membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) which regulation in human is thought to be through the ARE (Hayes et al., 2005). In the current study we have seen that both cytosolic and microsomal Gsts activities were decreased in the livers of Nrf2 knockout mice. Thus, these results may suggest that mouse MAPEGs might be regulated in part through ARE similar to humans MAPEGs.

The decreased levels of Nrf2 does not only influence phase II drug metabolizing enzymes but it also affects phase I drug metabolizing enzymes, and their controlling transcription factors. In the current study we have shown that the expression of Cyp1a1, 1a2, 1b1, 2b10 are significantly lower in the livers of Nrf2 knockout mice compared to wild type. For example, the expression of Cyp1a1, Cyp1a2, and Cyp1b1 at mRNA, protein, and catalytic activity levels were significantly lower in the livers of Nrf2 knockout mice compared to wild type. Cyp1a1 mRNA was the most affected enzyme followed by Cyp1a2, and Cyp1b1. The constitutive expression of Cyp1a2 is much higher than Cyp1a1 as the former is a hepatic enzyme while the latter is an extrahepatic enzyme. Moreover, Cyp1b1 was the least affected enzyme by the Nrf2 knockout. In this regard, it has been previously reported that there are at least two regulatory pathways that control rat *CYP1B1* gene expression. The first is the hormonal regulation which maintains the constitutive expression of CYP1B1 in steroidal tissues (Bhattacharyya et al., 1995), and the second is the AhR-dependent pathway which governs the *CYP1B1* gene expression in response to environmental pollutants (Bhattacharyya et al., 1995). Although there is no proof that mouse Cyp1b1 is regulated in a similar fashion to that of rat, one

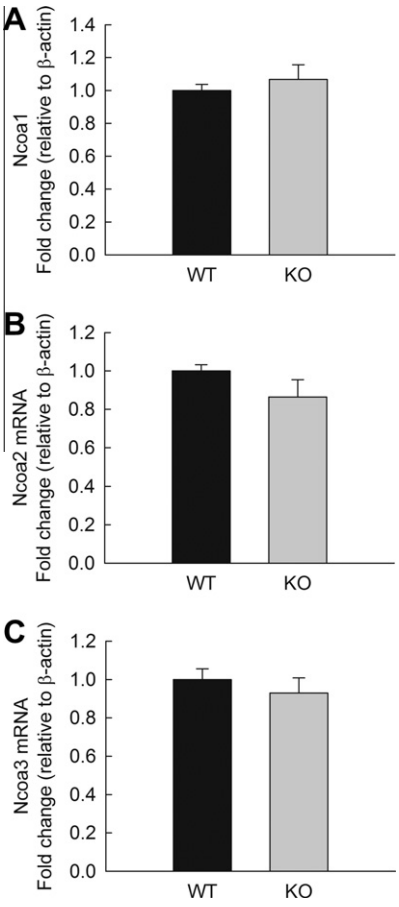

Fig. 11. Effect of Nrf2 knockout on Ncoa1, Ncoa2, and Ncoa3 mRNA levels. Total RNA was isolated from livers of untreated Nrf2 wild type (WT) and Nrf2 knockout knockout (KO) mice and gene expression of Ncoa1 (A), Ncoa2 (B), and Ncoa3 (C) was determined by real time-PCR. Results are presented as mean ± SEM (n = 4).

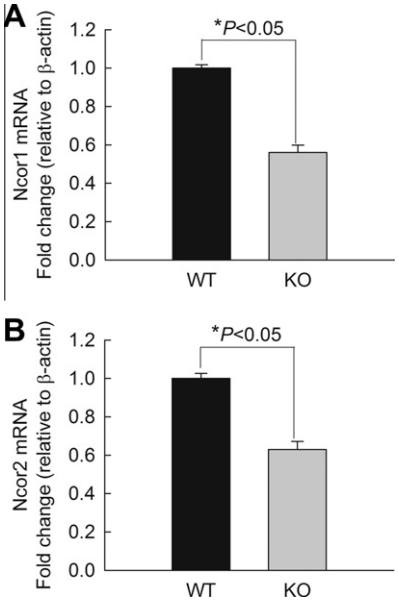

Fig. 12. Effect of Nrf2 knockout on Ncor1 and Ncor2 mRNA levels. Total RNA was isolated from livers of untreated Nrf2 wild type (WT) and Nrf2 knockout knockout (KO) mice and gene expression of Ncor1 (A), and Ncor2 (B) was determined by real time-PCR. Results are presented as mean ± SEM (n = 4). *P < 0.05 compared to WT.

may speculate that the effect of Nrf2 knockout on mouse Cyp1b1 might be mediated through affecting the hormonal pathway. At the catalytic activity levels, EROD, MROD, and PROD catalytic activities were significantly lower in the livers of Nrf2 knockout mice.

Most Cyp enzymes are induced by receptor-mediated mechanisms leading to their increased gene expression. Nuclear receptors that have been involved in the induction of Cyp1, Cyp2, and Cyp3 families are AhR, CAR, and PXR, respectively (Pascussi et al., 2004). Of these, only the AhR is a member of the Per-Arnt-Sim (PAS) family of transcription factors, whereas CAR and PXR are member of the orphan nuclear receptors family (Pascussi et al., 2004). Of importance, the role of these nuclear receptors in the constitutive expression of Cyps is well established (Forman et al., 1998; McLemore et al., 1989; Willson and Kliewer, 2002).

Since all members of the Cyp1 family are largely controlled by the AhR, it was of importance to examine whether the decreased expression of these enzymes in the livers of Nrf2 knockout mice could be due to a decrease in the expression of the transcription factor controlling them. Therefore, we examined the constitutive expression of AhR, at the mRNA and protein expression levels in the livers of Nrf2 knockout mice. In the current study we demonstrated that in the livers of Nrf2 knockout mice the AhR mRNA and protein expression levels are lower than those of the wild type mice. Recent reports provide further evidence for a hypothesized cross-talk between the Nrf2/ARE pathway and the pathway leading to the induction of XRE-controlled genes by the AhR. For example, it was shown that Nrf2 gene transcription is directly modulated by AhR through "XRE-like elements" in the Nrf2 gene promoter (Miao et al., 2005). Inversely, and in agreement with our results, previous studies have shown that Nrf2 regulates the transcription of AhR through an ARE available in its enhancer region (Shin et al., 2007; Yeager et al., 2009). Thus, the cross-talk between Nrf2 and AhR could be summarized by the presence of XRE element in the Nrf2 promoter (Miao et al., 2005), and the presence of ARE element in the AhR promoter (Shin et al., 2007). Therefore, our study demonstrated that the level of AhR is dependent on the expression of Nrf2. Furthermore, via affecting the AhR levels, the knockout of Nrf2 will have consequent effects on AhR down-stream targets typified by Cyp1a1, Cyp1a2, and Cyp1b1.

The fact that AhR and its down-stream targets, Cyp1a1, Cyp1a2, and Cyp1b1, mRNA, protein and catalytic activity levels were lower in the livers of Nrf2 knockout mice compared to wild type prompted us to examine the levels of Cyp2b10 and its controlling transcription factors, CAR and PXR, in the livers of Nrf2 knockout mice. Surprisingly, the levels of Cyp2b10 mRNA and protein levels were significantly lower in the livers of Nrf2 knockout mice. Interestingly, CAR and PXR, the transcription factors that regulate Cyp2b10, mRNA and protein expression levels were significantly lower in the livers of Nrf2 knockout mice. In agreement with our results, it has been previously reported that the level of CAR and PXR are lower in Nrf2 knockout mice (Shen and Kong, 2009). Although, CAR and PXR expression were not completely abolished in the livers of Nrf2 knockout mice, Cyp2b10 mRNA was not detected. The contradiction between the effect of Nrf2 knockdown on CAR and its down-stream target Cyp2b10 could be explained by the fact that there is a functional overlap between CAR and PXR in regulating Cyp2b10 (Chang, 2009; Shaban et al., 2005). In fact, it has been previously reported that CAR and PXR form a heterodimer that bind to a specific sequence in the Cyp2b10 promoter (Honkakoski et al., 1998). Therefore, the observed effect of Nrf2 knockout on Cyp2b10 mRNA levels could have been a summation of the effect on CAR and PXR.

Recent studies have also demonstrated that Nrf2 can potentially influence the levels of hepatic phase III drug transporters (Klaassen and Slitt, 2005). It has been recently postulated that drug transporters can function synergistically with phase II DMEs to facilitate the cellular excretion of conjugated metabolites (Shen and Kong,

2009). These conjugated metabolites could be endogenous/exogenous carcinogens, reactive metabolites, or xenobiotics. In general, transporters can be divided into two categories: ATP-binding cassette transporters superfamily (ABC), and solute carrier superfamily (Sloc) (Kis et al., 2010). The ABC transporters superfamily is further divided to P-glycoprotein (also known as P-GP, MDR1, ABCB1), multi-drug resistance-associated proteins (MRPs) containing 9 members (MRPs1–9), and breast cancer resistance protein (BCRP, ABCG2) (Murakami and Takano, 2008).

In the current study, we have shown that Mrp2 and Mrp3 mRNA levels are significantly lower in the livers of Nrf2 knockout mice compared to wild type. It is not clear however; if these lower Mrp2 and Mrp3 mRNA expression levels are due to the direct effect of Nrf2 knockout or due to the indirect effect of other nuclear receptors such as AhR, CAR, and PXR. However, recent studies implied an interplay mechanism between Nrf2, AhR, CAR, and PXR in the regulation of both Mrp2 and Mrp3. These studies have also demonstrated that Nrf2 regulates Sloc expression (Klaassen and Slitt, 2005). Our results demonstrated that the mRNA expression levels of Slco1a6 and Slco2b1 are significantly lower in the livers of Nrf2 knockout mice compared to wild type. In this context, previous studies have shown that Slco1a6 and Slco2b1 are increased after the administration of CAR and Nrf2 activators (Cheng et al., 2005).

Nuclear receptors such as AhR, CAR, and PXR and more generally transcription factors are essential for gene regulation yet they lack the enzymatic activities necessary for modulating chromatin structure (Gronemeyer et al., 2004). These enzymatic activities are catalyzed by co-receptors that are recruited in response to different signals including ligand–receptor binding signaling cascades (Giguere, 1999). These co-receptors could be further classified to co-activators and co-repressors (Pascussi et al., 2008). Co-activators, such as Ncoa1, Ncoa2, and Ncoa3 (also known as SRC-1, SRC-2, and SRC-3), could be histone acetyltransferases (HATs) or methyltransferases, or they might serve as docking partners for enzymes with such activities, and are accordingly involved in chromatin relaxation which is a prerequisite for recruitment and association of basic transcriptional machinery (Pascussi et al., 2008). On the other hand, co-repressors such as Ncor1 and Ncor2 (also known as RIP-13, and SMRT, respectively) preferentially bind to un-activated receptors including ligand-free, antagonist-bound, and inverse agonist-bound, to recruit various forms of histone deacetylases (HDACs), thus leading to chromatin condensation and repression of gene expression (Pascussi et al., 2008). Our results showed that the co-activators Ncoa1, Ncoa2, and Ncoa3 mRNA levels are not attenuated in the livers of Nrf2 knockout mice compared to wild type, suggesting that these co-activators do not play a role in lowering phases I and II DMEs or phase III drug transporters. On the other hand, the co-repressors Ncor1 and Ncor2 were significantly lower in the livers of Nrf2 knockout mice compared to wild type. Hence, co-activators, and co-repressors appear to be integral parts of the signaling pathways with co-repressors being the most vulnerable to Nrf2 knockout.

In conclusion, the knockdown of Nrf2 will cause a disruption in drug metabolism/transport capacity. With the fact that Nrf2 is a transcription factor of crucial chemopreventive capacity, the whole detoxification system will be malfunctioning due to its loss. Furthermore, Nrf2 knockdown will lower phases I and II drug metabolizing enzymes in addition to phase III drug transporters, probably through lowering the transcription factors controlling their expression.

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Disclosure statement

There is no conflict of interest.

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