Loss of Nrf2 in Mice Evokes a Congenital Intrahepatic Shunt That Alters Hepatic Oxygen and Protein Expression Gradients and Toxicity

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ABSTRACT

The transcription factor Nrf2 (Nfe2l2 nuclear factor, erythroid 2-like 2) regulates gene expression directly, controlling pharmacological and toxicological responses. These processes may also be influenced by the structure of the hepatic vasculature, which distributes blood flow through compartmentalized microenvironments to maintain organismal stability. Castings of the hepatic portal vasculature of albino C57BL/6J but not ICR Nrf2−/− mice revealed a congenital intrahepatic shunt that was present in two thirds of Nrf2−/− mice. This shunt directly connected the portal vein to the inferior vena cava and displayed characteristics of a patent ductus venosus. Immunohistochemistry revealed that Nrf2−/− mice with an intrahepatic shunt manifest changes to hepatic oxygen and protein expression gradients when compared with wild-type (WT) and non-shunted Nrf2−/− mice. Centrilobular hypoxia found in WT and Nrf2−/− mice without shunts was reduced in Nrf2−/− livers with a shunt. Hepatic protein expression of phosphoenolpyruvate carboxykinase (Pepck), normally confined to the periportal zone, exhibited both periportal and centrilobular zonal expression in livers from Nrf2−/− mice without shunts was reduced in Nrf2−/− livers with a shunt. Centrilobular hypoxia found in WT and Nrf2−/− mice without shunts was diminished in shunted Nrf2−/− livers compared with WT and Nrf2−/− livers without shunts. The intrahepatic shunt in Nrf2−/− mice was further found to diminish acetaminophen hepatotoxicity compared with WT and Nrf2−/− non-shunted mice following a 6 h challenge with 250 mg/kg acetaminophen. The presence of an intrahepatic shunt influences several physiological and pathophysiological properties of Nrf2−/− mice through changes in blood flow, hepatic oxygenation, and protein expression that extent beyond loss of canonical transactivation of Nrf2 target genes.

Key words: Nrf2; intrahepatic shunt; portacaval shunt; liver zonation; acetaminophen toxicity
The transcription factor Nrf2 is known to influence several processes in the liver such as xenobiotic metabolism (Chan et al., 2001; Enomoto et al., 2001; Itoh et al., 1997), intermediary metabolism (Chartoumpekis and Kensler 2013; Shin et al., 2007), bile production (Weerachayaphorn et al., 2012), regeneration (Beyer et al., 2008; Kohler et al., 2013; Wakabayashi et al., 2010), and hepatocarcinogenesis (Guichard et al., 2012). Enhanced Nrf2 signaling can protect against liver injury produced by various hepatotoxins (Liu et al., 2013). Mechanistic studies have shown that Nrf2 enhances mRNA transcription of its target genes following heterodimerization with small avian muscleaponeurotic fibrosarcoma oncogene homolog proteins and binding to DNA at the upstream enhancer sequences known as the antioxidant response element (ARE) (Taguchi et al., 2011). Rates of Nrf2 signaling are controlled through cytoplasmic sequestration and marking by polyubiquitination for proteasomal degradation mediated by the Kelch-like ECH-associated protein 1 (Keap1)-Cullin 3 complex, as well as other post-translational modifications (Itoh et al., 2010). Oxidative and electrophilic stresses, as well as chemopreventive agents, lead to modification of cysteine residues present in Keap1 to disrupt further ubiquitination of Nrf2. De novo synthesis of Nrf2 then enhances pathway signaling via translocation and nuclear accumulation.

Nrf2-disrupted mice have been used extensively to study the etiopathogenesis and prevention of numerous diseases including cancer (Jaramillo and Zhang, 2013), diabetes (Chartoumpekis and Kensler, 2013), nonalcoholic steatosis (Chowdhry et al., 2010), neurodegeneration, and chronic obstructive pulmonary disease (Kensler et al., 2007). Modulation of Nrf2 signaling has recently been found to affect liver size and growth. The liver to body weight ratio of Nrf2−/− mice is reduced when compared with wild-type (Beyer et al., 2008), whereas mice harboring hepatocyte-specific disruption of Keap1 display enhanced liver to body weight ratios (Wakabayashi et al., 2014). Morphological observations in Nrf2−/− mice have also indicated that bile ducts within the liver exhibit reduced micro-branching compared with wild-type and enhanced micro-branching with disruption of Keap1 (Wakabayashi et al., 2014). Interestingly, experiments utilizing partial heptectomy to model liver regeneration have shown reduced liver regeneration in mice with either deficient or enhanced Nrf2 signaling, suggesting further complexity of Nrf2 signaling in liver growth (Beyer et al., 2008; Kohler et al., 2013; Wakabayashi et al., 2010). Nrf2-dependent mechanisms of liver growth are further complicated by the recent report that portal vein branch ligation of mice with genetic or pharmacological activation of Nrf2 signaling have enhanced hepatocyte proliferation and hypertrophy (Shirasaki et al., 2014).

Nrf2 deficiency has recently been shown to reduce vascular density and endothelial sprouting through actions on Notch signaling in the retina (Wei et al., 2013). We hypothesized that disruption of Nrf2 signaling in the hepatic microenvironment would influence vascularization and physiological changes to the hepatic architecture could alter toxicological responses. Vascular corrosion casting of Nrf2−/− mice through the hepatic portal vein revealed a portacaval shunt was present. A recent report has found that widely used wild-type C57BL/6J mice can display portacaval shunts irregularly (Cudalbu et al., 2013), perhaps due to its intensive inbreeding, but with an occurrence far less than we find in the Nrf2−/− mouse. The congenital intrahepatic shunt in Nrf2−/− mice influences hepatocyte oxygenation, gene expression, and sensitivity to hepatotoxins in manners that may supersede the canonical effects of the Nrf2 transcription factor on xenobiotic and endobiotic metabolism through direct regulation of target gene expression. Further, Nrf2 disruption in the C57BL/6J mouse, a strain which is widely used in toxicological studies, may lead to a population subset with altered pharmacological and toxicological responses.

**MATERIALS AND METHODS**

**Animals.** Experiments were conducted in albino C57BL/6J mice that were either 10 days or 8–12 weeks old. The derivation of Nrf2−/− mice has been described previously (Itoh et al., 1997). Adult Nrf2−/− mice in the ICR/CD-1 background were also tested (Itoh et al., 1997). The Institutional Animal Care and Use Committee of the University of Pittsburgh approved all animal and surgical procedures.

Vascular corrosion casting. Vascular corrosion casting of the liver was performed with Mercox II (Ladd Research Industries, Wills-ton, VT). Mice were anesthetized using isoflurane (Piramal Healthcare, Andhra Pradesh, India). The abdominal skin was grasped using a blunt pair of forceps and transverse and longitudinal incisions were made into the abdomen. Using 7–0 silk ligature the pyloric vein was ligated. The portal vein was carefully surrounded by 7–0 silk ligature to allow a 22-gauge IV catheter for 8–12 week-old mice to be tightly secured. After catheter insertion, the inferior vena cava and right atrium were cut and 10 ml of sterile saline was flushed through the liver to remove all blood from the hepatic architecture. Liver casting was performed with 5 ml of Mercox II resin, which was prepared with 5% benzoyl peroxide as a curing agent. The portal vein and vena cava were ligated above and below the liver and the casted liver was incubated overnight at 37 °C to allow the resin to cure. Following complete curing of the liver, corrosion of the liver was performed by immersing the liver in 15% KOH for 5 days. Following complete degradation of the liver tissue, the vascular cast was photographed.

Micro-computed tomography. To quantify and observe the portal vasculature in mice with normal or disrupted Nrf2 signaling, ex vivo liver micro-computed tomography (μ-CT) was performed. Mice were prepared for casting as above. Liver casting was performed with 3 ml radio-opaque Microfil, which was prepared with 10% of the supplied curing agent and equal amounts of Microfil and the supplied diluent. Perfusion was stopped when back pressure was felt. The portal vein and vena cava were ligated above and below the liver and the casted liver was incubated overnight at 4 °C to allow the compound to cure. Following complete curing of the liver, the liver was fixed with 4% paraformaldehyde overnight at 4 °C. The liver was stored in 70% ethanol following paraformaldehyde treatment until μ-CT studies were performed. Liver vasculature was quantified using a μ-CT system (Siemens Medical Solutions, Hoffman Estates, IL) at the Children’s Hospital of Pittsburgh, Rangos Research Center Animal Imaging Core.

Trypan blue perfusion of the liver. Presence or absence of an intrahepatic shunt was also determined by cannulation of the portal vein with a 22-gauge IV catheter for 8–12-week-old mice and a 25-gauge needle for 10-day-old mice, opening of the inferior vena cava and flushing the liver with saline as described above. A 0.4% solution of trypan blue (Life Technologies, Grand Island, NY) in phosphate buffered saline (PBS) without Ca2+/Mg2+ was then perfused into the portal vein. The liver was then excised and photographed.
Circulating electrolyte and bile acid determination. Circulating levels of sodium, potassium, and ionized calcium were determined using fresh blood and the i-STAT system (Abbott, Princeton, NJ). Total bile acids present in plasma were determined using a commercially available total bile acid assay kit (Diazyme, Poway, CA).

Anesthesia recovery time. Mice were administered intraperitoneal injections of 100 mg/kg ketamine (Henry Schein Animal Health, Dublin, OH) and 5 mg/kg xylazine (Henry Schein Animal Health, Dublin, OH) then placed on their back and timed until movement was seen. Once anesthesia was reached, ocular lubricant was applied to the eyes to prevent drying.

Hepatic hypoxia determination. Mice were injected intraperitoneally with 250 μl of pimonidazole HCl (Hypoxyprobe-1 Omni kit, Hypoxyprobe, Burlington, MA) dissolved in sterile PBS without Ca2+/Mg2+ to obtain a final concentration of 60 mg/kg body weight. After 2.5 h, mice were anesthetized using isoflurane and the portal vasculature was flushed with saline as described above. Improper blanching of the liver was used as an indication of an intrahepatic shunt. Livers were fixed in 4% paraformaldehyde for 16 h at 4 °C.

Immunohistochemistry. Immunohistochemistry was performed with 5% goat serum (Vector Laboratories, Burlingame, CA) in PBS without Ca2+/Mg2+ with 0.05% Tween 20 (EMD Millipore Corporation, Billerica, MA) for blocking and co-incubation with antibodies. Heat induced epitope retrieval was performed at 90 °C for 20 min in 10mM Tris, 1mM EDTA, 0.05% Tween-20 pH 9.0 followed by cooling for 20 min. The primary antibodies rabbit anti-Hypoxyprobe (1/100), rabbit anti-Pepck (I-17) (Santa Cruz, Dallas, TX) (1/250), and rabbit anti-Cyp2e1 (EMD Millipore Corporation, Billerica, MA) (1/250) were incubated for 1 h at 25 °C and goat anti-rabbit horseradish peroxidase secondary (Bio-Rad, Hercules, CA) (1/200) incubated for 20 min at 25 °C. Hypoxia liver zonation was visualized by transformation of light intensity values by ImageJ (U. S. National Institutes of Health, Bethesda, MD) and the Interactive 3D Surface Plot v2.33 plugin (Internationale Medieninformatik, Berlin, Germany).

Acetaminophen challenge. Mice fasted for 16 h were challenged with an intraperitoneal injection of 250 mg/kg body weight of acetaminophen; plasma alanine aminotransferase (ALT) levels were measured 6 h later as described previously (Wakabayashi et al., 2014). Aspartate transaminase (AST), lactate dehydrogenase, and alkaline phosphatase were measured by the Johns Hopkins Phenotyping and Pathology Core (Baltimore, MD). Following blood sampling, trypan blue perfusion of the portal vein was utilized for intrahepatic shunt determination as described above.

RESULTS

Nrf2−/− Mice Have Altered Portal Vasculature

To determine whether hepatic microvasculature was altered between mice of Nrf2−/− and WT genotypes, morphometric analyses were conducted initially using vascular corrosion casting in adult mice. Blood from the portal vein normally flows to each of the liver lobes and through the sinusoidal capillaries, which then combine to form hepatic veins that allow blood to exit the liver and flow into the inferior vena cava as can be seen in WT mice following vascular corrosion casting (Fig. 1A). However, in the Nrf2−/− mice, the vascular corrosion casting revealed that resin perfused into the hepatic vasculature through the portal vein quickly entered into the inferior vena cava and bypassed the liver lobules (Fig. 1A). The penetrance of this phenotype was incomplete; we have observed that 67% (32/48) of livers from Nrf2−/− mice manifested this shunt. Interestingly, perfusion studies demonstrated that 6% (2/34) of WT mice also bypassed the liver.

To further investigate the hepatic vasculature of Nrf2−/− mice, the radio-opaque polymer Microfil was used for a more complete determination of the venous shunt by μ-CT imaging. The viscosity of the Microfil utilized for casting prevented the resin from flowing into the sinusoidal capillaries and could only reach the inferior vena cava if a large shunt were present in the liver. μ-CT imaging showed that Nrf2−/− mice had an intrahepatic shunt that connected the portal vein directly to the inferior vena cava, which caused the resin to bypass the liver sinusoids (Fig. 1B). Casting of Nrf2−/− livers that did not flow directly to the inferior vena cava was found to be visually similar to WT with full perfusion of the liver vasculature (Fig. 1C). The intrahepatic shunts that were observed in Nrf2−/− mice with vascular corro-
sion casting and μ-CT were visually similar. The portal vein was connected directly to the inferior vena cava in all Nrf2−/− liver samples with intrahepatic shunts. Nrf2−/− mice with shunts displayed characteristic phenotypes such as a non-smooth liver that was a brighter shade of red compared with WT and non-shunted Nrf2−/− mice. The diameter of the hepatic artery was also visually larger in Nrf2−/− mice with shunts.

To investigate if the intrahepatic shunt seen in Nrf2−/− mice was an acquired or congenital defect, 10-day old Nrf2−/− mice were perfused with trypan blue through the portal vein. Perfusion of trypan blue into the livers of young Nrf2−/− mice revealed the shunt was present at an early age and suggested a congenital developmental defect of the liver (Fig. 1D). Further perfusion studies with 2-day old Nrf2−/− mice revealed the portacaval shunt was present in three of five mice tested (data not shown). The intrahepatic shunt in Nrf2−/− mice displayed the characteristics of a patent ductus venosus.

To further investigate whether loss of Nrf2 caused abnormal development of the hepatic vasculature, Nrf2−/− mice in the ICR background were studied. Perfusion studies revealed that loss of Nrf2 in the ICR background did not lead to the presence of an intrahepatic shunt in any of the eight mice studied (data not shown).

**Phenotypic Examination of Nrf2−/− Mice with an Intrahepatic Shunt**

To more fully characterize the phenotype of Nrf2−/− mice with an intrahepatic shunt, liver to body weight ratios and liver histology were examined. Nrf2−/− mice with an intrahepatic shunt had liver to body weight ratios (4.4 ± 0.1%) that were significantly reduced when compared with Nrf2+/− non-shunted mice (5.0 ± 0.2%) (mean ± SE, n = 6–8). Liver histology from Nrf2−/− mice with an intrahepatic shunt revealed dysplasia in the area surrounding the portal triad, which was not seen in WT or Nrf2−/− mice without shunts (Fig. 2).

Further studies were conducted with mixed gender adult Nrf2−/− mice to determine whether the portacaval shunt altered the levels of circulating bile acids and electrolytes in the blood and if anesthesia timing was altered. Total bile acids were significantly elevated in Nrf2−/− mice with shunts when compared with mice without shunts (34.9 ± 3.4 and 12.8 ± 1.5 μmol/l, respectively) (mean ± SE, n = 3). Circulating sodium, potassium and ionized calcium levels were found to be within the normal range for shunted and non-shunted Nrf2−/− mice. To explore whether the intrahepatic shunt found in Nrf2−/− mice altered the sleeping duration of anesthesia, Nrf2−/− mice were challenged with 100 mg/kg ketamine and 5 mg/kg xylazine and recovery time was monitored. Nrf2−/− mice without shunts remained under anesthesia and without movement for 66.7 ± 3.3 min, whereas Nrf2−/− mice with intrahepatic shunts had a significantly delayed recovery time of 83.7 ± 2.0 min (mean ± SE, n = 3).

**Hepatic Zonation of Oxygen and Protein Expression Is Altered in Nrf2−/− Mice with an Intrahepatic Shunt**

Zonal oxygen gradients are present in the liver with areas of high oxygenation surrounding the portal triad and low oxygenation surrounding the central vein. Pimonidazole was used to probe whether changes to hypoxic liver zonation were present in Nrf2−/− mice that possessed an intrahepatic shunt. Immunohistochemistry of WT and Nrf2−/− livers that did not contain shunts had visually similar staining for hypoxic regions, which prototypically surround the centrilobular area (Fig. 3). Nrf2−/− livers with a portacaval shunt displayed much less centrilobular hypoxia and oxygen zonation (Fig. 3). Oxygen tension affects the expression of multiple hepatic genes. To determine whether hepatic protein expression was also altered in a shunt-dependent, but Nrf2-independent manner, two marker enzymes prototypically found in either the periportal or pericentral regions were studied. The distribution of phosphoenolpyruvate carboxykinase (Pepck), an enzyme involved in gluconeogenesis which is usually confined to the periportal region, was greatly expanded and found to be expressed in the centrilobular region as well in Nrf2−/− mice with intrahepatic shunts (Fig. 4A). WT and Nrf2−/− mice without intrahepatic shunts displayed a similar, classical pattern of Pepck expression (Fig. 4A). Cytochrome P450 2E1 (Cyp2e1) expression, which is usually confined to the centrilobular region, had a reduced area of expression in Nrf2−/− mice with intrahepatic shunts when compared with WT and Nrf2−/− mice without shunts (Fig. 4B). Thus, the intrahepatic shunt in Nrf2−/− mice altered the distribution of hepatic oxygenation and the gradients of expression of two marker enzymes, Pepck and Cyp2e1.

**Acetaminophen Toxicity Is Altered in Nrf2−/− Mice with an Intrahepatic Shunt**

Altered liver vasculature could affect toxicological responses. Acetaminophen was used to acutely challenge WT and Nrf2−/− mice with and without intrahepatic shunts to determine whether initial manifestations of hepatotoxicity were altered as a function of the shunting of portal blood flow. Intrahepatic shunts were characterized by portal perfusion of trypan blue following blood collections. Nrf2−/− mice without intrahepatic shunts had 34% lower plasma ALT levels than WT 6 h after a challenge with 250 mg/kg acetaminophen (Fig. 5). Nrf2−/− mice with intrahepatic shunts had plasma ALT levels that were more than 90% lower than WT or Nrf2−/− mice without portacaval shunts. Other circulating biomarkers of damage were also measured, such as AST and lactate dehydrogenase, and a similar trend of protection was found in Nrf2−/− mice with an intrahepatic shunt. AST levels were elevated in WT (20,840 ± 6,931 U/L) and Nrf2−/− mice without intrahepatic shunts (26,913 ± 7,904 U/L) when compared with Nrf2−/− mice with shunts (567 ± 199 U/L). Lactate dehydrogenase levels were found to be elevated in WT (13,807 ± 2,177 U/L) and Nrf2−/− mice without intrahepatic shunts (7,693 ± 2,034 U/L) when compared with Nrf2−/− mice with shunts (1,163 ± 465 U/L) (mean ± SE, n = 3). Interestingly, alkaline phosphatase levels did not follow the trend and were found to be elevated in Nrf2−/− mice with shunts (126.7 ± 2.0 U/L) when compared with WT (72.7 ± 15.3 U/L) and Nrf2−/− mice without intrahepatic shunts (103.3 ± 2.3 U/L). The presence of an intrahepatic shunt in Nrf2−/− mice was highly protective following 6 h of acetaminophen treatment. The two WT mice identified with portacaval shunts were also much less sensitive to APAP hepatotoxicity in this short-term assay as well.

**DISCUSSION**

The liver has a unique dual blood supply. The portal vein supplies 80% of blood flow to the liver, with the hepatic artery accounting for the remaining 20%. Reduction of portal blood flow to the liver has been previously shown to activate the hepatic arterial buffer response, which leads to enhancement of hepatic arterial blood flow to the liver (Lautt, 1985). The presence of an intrahepatic shunt that bypasses portal blood flow from the liver therefore may enhance hepatic arterial blood flow to the liver. We reasoned that an enhancement of highly oxygenated blood flow to the liver by the hepatic artery would increase oxygen tension in the centrilobular regions and reduce the area of the
naturally hypoxic zones. Experiments revealed that the zonal oxygen gradient in the liver was in fact disrupted in Nrf2−/− mice without (middle) and with an intrahepatic shunt (right) following intraperitoneal administration of pimonidazole. Transformation of light intensity values was performed to more easily visualize liver zonation (bottom) where light blue regions are highly oxygenated and white areas have low oxygenation. Scale bar, 500 μm. Representative images are shown from three biological replicates.

The alteration of portal blood circulation in Nrf2−/− mice with portacaval shunts was also found to increase circulating levels of total bile acids. Normally, the majority of bile secreted from the gallbladder into the intestines is recycled through enterohepatic circulation via portal blood flow back to the liver. The presence of a portacaval shunt can cause portal blood flow to enter systemic circulation and lead to enhanced levels of bile acids in the bloodstream. The physiological alteration of blood flow to the liver in Nrf2−/− mice was further studied using immunohistochemistry to determine whether changes to zonal hepatic protein expression were also present. Pepck is important for regulation of gluconeogenesis and its expression has been shown to be influenced by oxygen tension, which normally confines expression to the periporal region (Bratke et al., 1999). The area of Pepck protein expression in Nrf2−/− mice with an intrahepatic shunt displayed consistent staining in both the periporal and centrilobular zones unlike WT and Nrf2−/− mice without a shunt, which displayed clear zonal expression patterns. An enhancement of Pepck has been noted in previous studies with C57BL/6J Nrf2−/− mice in response to high fat diet (Char-toumpekis et al., 2011), but a functional ARE was not identified in the promoter region of this gene. Some Cyp450 family members have been shown to be influenced by oxygen tension as well (Maier et al., 1994). Cyp2e1 mediates the bioactivation of hepatotoxins such as alcohol and acetaminophen. Like Pepck, it is not a direct transcriptional target of Nrf2. Expression of Cyp2e1, which is normally confined to the centrilobular region, was also found to be altered. Expression of Cyp2e1 occupied a smaller area surrounding the central vein in Nrf2−/− mice with an intrahepatic shunt compared with WT and Nrf2−/− mice without a shunt. These results suggest that other genes normally confined to the periporal or centrilobular regions may also have shunt-dependent changes in protein expression when examined in Nrf2−/− livers. The transcription factor Nrf2 has been previously found to influence toxicity through changes to proteins involved in the functionalization, conjugation, and elimination of xenobiotics such as acetaminophen. The intrahepatic shunt present in Nrf2−/− mice may alter xenobiotic metabolism to either supplement or oppose the effects of the constitutive differences in gene expression resulting from a lack of Nrf2 signaling. Acetaminophen toxicity is dependent on the production of the reactive metabolite N-acetyl-p-benzoquinone imine (Kaplowitz, 2004), formed by Cyp2e1 and other cytochrome P450s. Decreased conjugation of acetaminophen and dammed levels of glutathione, as seen in livers of Nrf2-disrupted mice, are thought to impede acetaminophen detoxication and clearance from the animal (Chan et al., 2001; Enomoto et al., 2001). Indeed, plasma clearance of acetaminophen is retarded in Nrf2−/− mice compared with WT in mice challenged with a low (50 mg/kg) intravenous dose (Reisman et al., 2009). The presence of an intrahepatic shunt in Nrf2−/− mice would also be expected to alter normal first-pass metabolism of acetaminophen and extend its half-life. We found that Nrf2−/− mice with an intrahepatic shunt had much lower plasma ALT levels and were protected from the initial (6 h) hepatotoxicity of 250 mg/kg acetaminophen when compared with WT and Nrf2−/− mice without a shunt. Altered clearance and diminished bioactivation by Cyp2e1 may contribute to the paradoxical protection seen in the Nrf2−/− mice. Previous reports have noted that Nrf2−/− mice are either more sensitive or have similar sensitivity as WT to acetaminophen challenge when using higher doses and lethality as the primary endpoint (Chan et al., 2001; Enomoto et al., 2001; Liu et al., 2013; Reisman et al., 2009). Comparisons between these studies are challenging, as different genetic strains, acetaminophen doses, vehicles, fasting or ad libitum feeding, and other parameters affecting outcomes have been used. Our results reflect changes in the damage of acetaminophen under fasted conditions within a relatively short time frame and therefore may differ from previously published studies with longer times to outcomes and higher doses of acetaminophen. Altered rates of metabolism between Nrf2−/− mice with and without shunts were also found to occur when mice were challenged with anesthesia. Nrf2−/− mice with intrahepatic shunts had delayed recovery times when admin-
FIG. 4. (A) Immunohistochemistry detection of Pepck in WT (left) and Nrf2−/− livers without (middle) and with intrahepatic shunts (right). (B) Immunohistochemistry detection of Cyp2e1 in WT (left) and Nrf2−/− livers without (middle) and with intrahepatic shunts (right). Scale bar, 500 μm. Representative images are shown from three biological replicates.

FIG. 5. (A) Plasma ALT levels in fasted WT (left) and Nrf2−/− mice without (middle) and with intrahepatic shunts (right) were determined following acetaminophen challenge (250 mg/kg) for 6 h. *p < 0.05, ANOVA. Values are mean ± SE (n = 5 or more). (B) Representative livers are shown following portal trypan blue perfusion of acetaminophen-challenged mice to determine whether an intrahepatic shunt was present.

The C57BL/6J mouse strain has been recently noted to display portacaval shunts intermittently (Cudalbu et al., 2013), as observed in our WT controls, but at a frequency far less than in the Nrf2−/− mouse. Our finding that loss of Nrf2 in the genetic background of ICR mice does not recapitulate the intrahepatic shunt phenotype suggests that functional Nrf2 signaling is but one factor in hepatic vascular development and has a more dominant effect in the C57BL/6J mouse strain, which is more predisposed to develop intrahepatic shunts. Although this is the first complete characterization of a defect to liver vasculature in the Nrf2−/− mouse, other models employing disruption of transcription factor pathways similarly evoke a patent ductus venosus. Mice deficient in Ahr and Arnt, also in the C57BL/6J background, display a patent ductus venosus in all animals tested, which unlike WT mice (Lahvis et al., 2000; Walisser et al., 2004), fails to close after post-natal day two (Lahvis et al., 2005). Hypomorphic alleles of the Ahr chaperone aryl hydrocarbon receptor interacting protein also exhibited the phenotype in 83% of mice tested (Lin et al., 2008). Further studies discovered that Ahr-dependent ductus venosus patency was dependent upon repression of Ahr signaling in endothelial or hematopoietic cells through a conditional mouse model (Walisser et al., 2005). Diminished expression of the Ahr signaling pathway has also been noted in purebred Irish wolfhounds with similar congenital intrahepatic shunts, which is thought to arise from the cumulative effects of many genes (van Steenbeek et al., 2009, 2013).

The mechanisms underlying the presence of a patent ductus venosus in Nrf2−/− mice are unclear. As seen in the Ahr knockout mice, the defect is one of vascular development. This portacaval shunt allows venous blood flow to bypass the liver during embryonic development and cause a portion of the maternal umbilical blood to circumvent the liver and directly enter the inferior vena cava. The shunt is observed in young mice with either disruption of Ahr or Nrf2 signaling. It is likely a remnant of an embryonic structure and is not acquired after birth as a consequence of portal hypertension. Interestingly, Nrf2 has been previously shown to exhibit functional pathway crosstalk with the Ahr pathway. Mutual signaling enhancement of Nrf2 through Ahr and vice versa has been described. Nrf2 activation by Ahr has been found to occur due to the presence of a xenobiotic response element in the 5′-untranslated region of the Nrf2 exon (Miao et al., 2005), whereas Nrf2 has been shown to enhance Ahr...
transcription through an active ARE located in the upstream region of the Ahr promoter (Shin et al., 2007). Deficient Nrf2 signaling in mouse embryonic fibroblasts revealed basal transcription and translation of Ahr was reduced. Other studies have found reduced Ahr expression and its downstream targets in the liver of Nrf2−/− mice when compared with WT (Anwar-Mohamed et al., 2011). Whether the shunt found in Nrf2−/− mice is due to the direct reduction of Ahr expression in hepatic endothelial cells is currently unknown. However, such an interaction may explain the incomplete penetrance of Nrf2 for this phenotype.

These results have wide ranging implications for in vivo experiments with Nrf2−/− mice, which have been studied extensively to catalog the array of toxicities and other processes modulated by Nrf2 status. Surgical portosystemic shunting in rats has been previously shown to alter the expression of inflammatory cytokines and anti-inflammatory modulators of the liver as well as the intestines and spleen (Garcia et al., 2011). Processes such as metabolism and liver regeneration may also be altered due to changes in blood flow and hepatic oxygenation. Pharmacokinetic profiles of various drugs and toxins would be expected to be altered in the presence of an intrahepatic shunt. Reduced portal blood flow and metabolic capacity of hepatocytes can alter elimination kinetics to enhance systemic circulation and half-life. For example, oral or intraperitoneal administration of dibenzyolmethane to Nrf2−/− mice was previously associated with increases in pharmacokinetic parameters such as mean plasma concentrations at later time points and terminal half-life when compared with WT (Lin et al., 2011). Interestingly, oral administration of dibenzylmethane to Nrf2−/− mice was shown to have reduced peak and mean concentrations at earlier time points compared with WT. Metabolic changes found in Nrf2−/− mice with intrahepatic shunts could be affected by the increased oxygenation of the centrilobular zone. An expansion of the periportal zone may alter the response of Nrf2−/− mice with an intrahepatic shunt to high fat diet-induced effects, which has been surprisingly found to be protective (Chartoumpekis and Kessler, 2013). The presence of a shunt in Nrf2−/− mice may also influence liver size and regenerative potential. Insertion of a portal vein to inferior vena cava shunt has been shown to affect both homeostatic liver size and the ability of the liver to regenerate after three days in rats (Sato et al., 1997). Structural and developmental changes evoked by Nrf2 confound the direct ARE-dependent gene expression changes conjured to explain the impact of Nrf2 signaling on intermediary and xenobiotic metabolism in murine models.

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