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Role of nuclear factor-erythroid 2-related factor 2 (Nrf2) in the transcriptional regulation of brain ABC transporters during acute acetaminophen (APAP) intoxication in mice

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ABSTRACT

Changes in expression of liver ABC transporters have been described during acute APAP intoxication. However, the effect of APAP on brain ABC transporters is poorly understood. The aim of this study was to evaluate the effect of APAP on brain ABC transporters expression and the role of the oxidative stress sensor Nrf2. Male C57BL/6J mice were administered APAP (400 mg/kg) for analysis of brain mRNA and protein expression of Mrp1-6, Bcrp and P-gp. The results show induction of P-gp, Mrp2 and Mrp4 proteins, with no changes in Bcrp, Mrp1 or Mrp5-6. The protein values were accompanied by corresponding changes in mRNA levels. Additionally, brain Nrf2 nuclear translocation and expression of two Nrf2 target genes, NAD(P)H:quinone oxidoreductase 1 (Nqo1) and Hemoxygenase 1 (Ho-1), was evaluated at 6, 12 and 24 h after APAP treatment. Nrf2 nuclear content increased by 58% at 12 h after APAP along with significant increments in mRNA and protein expression of Nqo1 and Ho-1. Furthermore, APAP treated Nrf2 knockout mice did not increase mRNA or protein expression of Mrp2 and Mrp4 as observed in wildtypes. In contrast, P-gp induction by APAP was observed in both genotypes. In conclusion, acute APAP intoxication induces protein expression of brain P-gp, Mrp2 and Mrp4. This study also suggests that brain changes in Mrp2 and Mrp4 expression may be due to in situ Nrf2 activation by APAP, while P-gp induction is independent of Nrf2 function. The functional consequences of these changes in brain ABC transporters by APAP deserve further attention.

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1. Introduction

Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP) is one of the most common used over the counter analgesic/antipyretic drugs. It is a safe drug when administered within the therapeutic range, but it is well known that overdoses of APAP produce hepatotoxicity [1]. Overdose of acetaminophen is the primary cause of acute liver failure (ALF) in the United States [2]. ALF develops hepatic encephalopathy (HE) along the clinical course of the disease. The pathophysiology of the HE is multifactorial, among them an excess of blood ammonia concentration is one of the most important factor in its genesis [3]. This increment is due to an arrest in the urea cycle produced by the extensive hepatocellular damage. In this way, indirectly, APAP intoxication could produce brain alterations secondary to the presence of ALF [4]. However there are only few papers that described a direct effect of APAP per se on the CNS in the absence of ALF (*e.g.* glutathione perturbations and oxidative stress), however, there are no clinical reports indicating that APAP produces damage to neuronal tissue independent of ammonia accumulation [5–7].

Abbreviations: ALT, alanine aminotransferase; P-gp, *P*-glycoprotein; APAP, acetaminophen; R123, Rhodamine 123; Mrp, multidrug resistance-associated protein; ANOVA, analysis of variance; Ho-1, heme oxygenase; Keap1, Kelch-like ECH-associated protein; NAPQI, *N*-acetyl-*p*-benzoquinone imine; Nqo1, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; TBP, TATA-binding protein; WT, wild type; KO, Knock-out; JNK, c-Jun N-terminal kinase; HIF-1α, Hypoxia-inducible factors; CAR, constitutive androstane receptor.

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Administration of therapeutic doses APAP are mainly metabolized by glucuronidation and sulfation which produce APAP glucuronide (APAP-Glu) and APAP sulfate (APAP-Sulf), respectively [8]. Less than 5% of the dose is handled by the CYP450 family of enzymes to form *N*-acetyl-*p*-benzoquinone imine (NAPQI). This toxic metabolite is detoxified by glutathione conjugation in the liver, catalysed by the GST enzymes, which is then excreted in urine and bile as cysteine and mercapturic acid conjugates. During APAP overdose, these two main metabolic routes (glucuronidation and sulfation) are saturated and the excess of the drug is oxidized by CYP450. Excess NAPQI generated depletes GSH and further exerts its toxic effect by causing oxidative stress and by binding to cellular macromolecules [9].

The antioxidant response element (ARE) was initially identified by Rushmore et al. [10,11] as a *cis*-acting enhancer sequence located in the promoter region of numerous genes involved in detoxification and cytoprotection [12]. Several of the genes known to be regulated in neural tissue are c-glutamyl cysteine ligase catalytic subunit, NAD(P)H: quinone oxidoreductase 1 (NQO1), heme-oxygenase 1 (HO-1), and certain glutathione S-transferase family members. The putative transcription factor that drives ARE-mediated gene expression is nuclear factor erythroid 2-related factor 2 (Nrf2) [13]. Under basal conditions, this transcription factor is inactive and restricted to the cytosol in association with Kelch-like ECH-associated protein 1 (Keap1) [14]. During oxidative stress, SH residues in Keap1 are oxidized, producing disruption of the complex, which then signals the translocation of Nrf2 to the nucleus to bind to ARE and induce the expression of several cytoprotective proteins.

The ATP-Binding Cassette (ABC) proteins are a family of ATP dependent efflux pumps, whose activity decrease intracellular accumulation of a wide range of endogenous and xenobiotic compounds [15,16]. The main ABC transporters associated with drug transport are *P*-glycoprotin (P-gp), breast cancer related protein (Bcrp) and multidrug resistance associated proteins (Mrps). They are expressed in the membrane of mature epithelial cells of many organs, including liver, kidney and intestinal tract. Transporters are also located in specialized barriers that protect extremely sensitive organs, such as the blood brain barrier (BBB), hematotesticular barrier and placenta. Their function contributes to preventing accumulation of toxic drugs or potentially dangerous endogenous compounds in these tissue sanctuaries [17,18].

The BBB is formed by brain capillaries closely associated with perivascular astrocytic endfeet, pericytes and microglia [19,20]. The BBB is not only an anatomical barrier; but it is also a dynamic tissue that expresses multiple transporters, receptors and enzymes. This complex structure is responsible for two main functions. First, it impedes the free diffusion of chemicals between brain fluids and blood and second, provides transport processes for essential nutrients, ions and metabolic waste products [21].

The ABC transporters are located mainly in brain endothelial cells. While, P-gp, Bcrp, Mrp1, Mrp2, Mrp4, Mrp5 and Mrp6 are expressed in the apical membrane. Mrp4 is the only known basolaterally expressed transporter in brain endothelium [22]. Astrocytes express Mrp1, Mrp3, Mrp4 and Mrp6 and a minor quantity of P-gp. It has been demonstrated that in different brain diseases, such as epilepsy, depression and Parkinson, the expression of some of these transporters is modified with ensuing consequences on CNS permeability to compounds that are substrates for these transporters [23]. In a minor quantity, ABC transporters are also expressed in astrocytes, microglia, neurons, and oligodendrocytes. Their function there is also to impede drug penetration into these cells, thus creating a secondary biochemical barrier to permeability in brain parenchyma [24,25].

Further, it has been described that APAP can cross the BBB at therapeutic doses [26], and also at toxic doses in animals [27–29] and humans [28,30]. Expression and function of CYP2E1, one of the CYPs isoforms involved in APAP oxidation, has also been demonstrated in brain tissue [31–33]. Previous works have demonstrated that toxic doses of APAP produce oxidative stress in different regions of rat and mice brains [5,6]. Also mitochondrial swelling, decreases in Na^+/K^+ ATPase function and mitochondrial membrane potential have been described [7].

In addition, our own work has previously described changes in gene and protein expression of multiple ABC transporters during acute toxic and sub-toxic APAP administration in liver and intestine [34–38]. Rats treated with APAP showed an increased expression of liver P-gp, Mrp2 and Mrp3 [34,37]. Similar changes were described in mice where induction of Mrp2, Mrp3 and Mrp4 was observed [36,38]. Most importantly, similar changes in liver ABC protein expression were reported in patients intoxicated with APAP. Human liver specimens from APAP overdosed cases showed upregulation of MRP1 and MRP4, at both the mRNA and protein level in sinusoidal membrane, while P-GP and BCRP proteins were induced in the canalicular domain of hepatocytes [35]. This effect of APAP on ABC transporters is not limited to the liver. Subtoxic doses of APAP also increases the expression of P-gp in intestine of rats and also *in vitro*, in a human intestinal cell line [39]. There is convincing documentation that Nrf2 is activated during APAP intoxication, and that Nrf2 activation is in part responsible for the induction of Mrp3 and Mrp4 in livers of APAP treated mice [40]. However, the effect of hepatotoxic doses of APAP on brain ABC transporters has received very limited attention.

Therefore, the aim of this study was to evaluate the effect of APAP on brain ABC transporters expression. Additionally, we examined the role of the transcription factor and the oxidative stress sensor Nrf2 thereon. The data indicated that toxic doses of APAP induce, selectively, brain P-gp, Mrp2 and Mrp4. Our results also demonstrate that activation of the Nrf2-ARE pathway plays an important role in the induction of brain Mrp2 and Mrp4, but not P-pg during APAP intoxication.

2. Materials and methods

2.1. Chemicals

APAP was purchased from Sigma-Aldrich (St Louis, MO). All other chemicals and reagents were of the highest grade available (≥99.0%). Antibodies for Mrp1 and Mrp6 were provided by George Scheffer (Vrije Universiteit Medical Center, Amsterdam, Netherlands). All other antibodies (primary and secondary) were purchase from different vendors as listed in Table 1.

2.2. Animals

Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Nrf2-null and wildtype (C57BL/6J background) mice were kindly provided by Dr. Angela Slitt from the University of Rhode Island. Mice used for comparisons between genotypes were age matched (9 to 11 weeks of age). All mice were housed in a 12-h dark/light cycle in a temperature and humidity controlled environment. They were fed laboratory rodent diet (Harlan Teklad 2018, Madison, WI) and water *ad libitum*. All animal studies were performed in accordance with National Institute of Health standards and the Guide for the Care and Use of Laboratory Animals. This work was approved by the University of Connecticut's Institutional Animal Care and Use Committee [IACUC protocol number A12-050]. Following an overnight fast, mice were treated with APAP (400 mg/kg, i.p.) in saline or vehicle. Animals were sacrificed by decapitation at 24 h after APAP treatment, unless otherwise specified. Blood was collected and brains and livers were excised, rinsed in ice-cold saline solution and snap-frozen in liquid nitrogen. Blood was centrifuged

Table 1
Antibodies and conditions used in WB.

Protein	Primary antibody	Primary dilution	MW (Kd)	Secondary antibody	Secondary dilution
P-gp	C219. Abcam. (Ab 3364)	1/1000 (1%NFDM)	170	Anti-Mouse. Sigma-Aldrich (A4416)	1/2000
Bcrp	BXP-53	1/10000 (1%NFDM)	75	Anti-Rat. Sigma-Aldrich (A9037)	1/2000
Mrp1	MRPr1. VUMC⊕	1/1000 (1%NFDM)	190	Anti-Rat. Sigma-Aldrich (A9037)	1/2000
Mrp2	M_2III-5. VUMC⊕	1/750 (1%NFDM)	190–200	Anti-Mouse. Sigma-Aldrich (A4416)	1/2000
Mrp3	M_3II-2. VUMC⊕	1/750 (1%NFDM)	180–190	Anti-Rat. Sigma-Aldrich (A9037)	1/2000
Mrp4	M_4I-10. VUMC⊕	1/1000 (1%NFDM)	160–170	Anti-Rat. Sigma-Aldrich (A9037)	1/2000
Mrp5	M_5I-10. VUMC⊕	1/750 (1%NFDM)	160–180	Anti-Rat. Sigma-Aldrich (A9037)	1/2000
Mrp6	M_6II-68. VUMC⊕	1/1500 (1%NFDM)	165	Anti-Rat. Sigma-Aldrich (A9037)	1/2000
Nqo1	Abcam (ab2346)	1/1000 (2%NFDM)	32	Anti-Goat. Sigma-Aldrich (A8919)	1/10,000
Ho1	Stressgen Bioreagents SPA-895	1/2000 (1%NFDM)	33	Anti-Rabbit. Sigma-Aldrich (A4914)	1/2000
Nrf2	Cell signaling (8882s)	1/1000 (5%BSA)	110	Anti-Rat. Sigma-Aldrich (A9037)	1/2000
TBP	Abcam (ab51841)	1/1000 (2%NFDM)	38	Anti-Mouse. Sigma-Aldrich (A4416)	1/3000
β-Actin	Abcam (ab 8227)	1/7000	42	Anti-Rabbit. Sigma-Aldrich (A4914)	1/2000

⊕ Gently gift of Dr George Scheffer. VU Medical Center, Amsterdam, The Netherlands.

(1200 × *g* for 15 min) to obtain plasma. All samples were stored at −80 °C until analysis.

2.3. Isolation of total brain membranes

Brain total membranes were isolated as previously described by Vannucci et al. [41] with minor modifications. Briefly, brains were placed in 4 volumes of Tris EDTA sucrose (TES) [20 mM Tris, 1 mM EDTA, and 255 mM sucrose (pH 7.4)] buffer containing protease inhibitors (Roche complete EDTA free inhibitor cocktail supplemented with 1 mM of phenylmethylsulfonyl fluoride, Roche, Indianapolis, IN) Samples were homogenized using a dounce homogenizer by 10 up–down strokes. The homogenates were centrifuged at 1000 × *g* for 30 min at 4 °C. The supernatant was transferred into a clear centrifuge tube and centrifuged at 105,000 × *g* for 90 min at 4 °C. Membrane pellets were resuspended in TES supplemented with protease inhibitors and stored at −80 °C until use.

2.4. Preparation of cytosolic and nuclear extracts for Western blotting

Brain samples were homogenized in cold hypotonic buffer [10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonylfluoride (PMSF)]. The lysates were incubated for 20 min on ice, vortexed and then centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatant comprising of cytosolic proteins was used as cytosolic fraction. The resulting pellets were resuspended in hypertonic buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. Samples were vortexed and centrifuged at 1000 × *g* for 10 min. The resulting supernatant consisting of nuclear proteins was used as nuclear fraction. Samples were stored at −80 °C until use.

2.5. Western blot analysis

For Western blot analysis of ABC transporters, total brain membrane proteins (50 or 70 μg, depending on the transporter) were electrophoretically resolved using 8% polyacrylamide gels and transferred onto PVDF-Plus membrane (Micron Separations, Westboro, MA). For Nrf2 detection, nuclear proteins (50 μg), and for Nrf2 target gene (Nqo1) cytosolic proteins (50 μg) were resolved using 10% polyacrylamide gels. The specific primary antibodies used for each protein are shown in Table 1. β-Actin was used as a loading control. Blots were then incubated with horseradish peroxidase (HRP) conjugated specific secondary antibodies (Table 1). Protein-antibody complexes were detected using a chemiluminescent kit (Thermo Scientific, IL) followed by exposure to X-ray film. Densitometric analysis of the immunoreactive bands was performed using NIH ImageJ software. Protein concentration in membrane preparations was measured by the method of Lowry [42] using bovine serum albumin as standard.

2.6. Histopathology

Liver samples were fixed in 10% neutral buffered zinc formalin prior to processing and paraffin embedding. Liver sections (5 μm) were stained with hematoxylin and eosin (H&E). Liver tissue sections were examined for histopathological changes by a board-certified veterinary pathologist (Dr. Michael Goedken). The slices were evaluated for the severity of degenerative and necrotic changes in the centrilobular regions. Tissues were given a score reflective of the severity of injury from 0 (no injury) to 4 (more than 50% of hepatocytes affected) as previously described [38,43].

2.7. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total brain RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm and its integrity was

Table 2
Primer sequences used in qPCR.

Protein	Gen	Primers
P-gp	Abcb1a	F: 5′-CGC CCT CGT CAG ACA GCC TCA CAT TT-3′
		R: 5′-GTG CTC CTT GAC CTT GCC GTT CTG AA-3′
	Abcb1b	F: 5′-AAG CCA GTA TTC TGC CAA GCA T-3′
		R: 5′-CAT CAG CAA CAG CAG TCT GGA G-3′
Bcrp	Abcg2	F: 5′-TCG GAA CTC AGT TTA TCC GTG GCA-3′
		R: 5′-GCA TTC GCT GTG CTT GAG TCC AAA-3′
Mrp1	Abcc1	F: 5′-AGG TCG TAC GGG AGC TGG GAA ATC AT-3′
		R: 5′-CCA GGG CCA TCC AGA CTT CTT CAT CA-3′
Mrp2	Abcc2	F: 5′-TCC AGG ACC AAG AGA TTT GC-3′
		R: 5′-TCT GTG AGT GCA AGA GAC AGG T-3′
Mrp3	Abcc3	F: 5′-AGT GGC TGT GAT AGT CTT GCT GAT A-3′
		R: 5′-CCG TTC AGG ATC TCG CTC AT-3′
Mrp4	Abcc4	F: 5′-ACC TCT GCT CGC GCG TGT TCT-3′
		R: 5′-CCA GTA CCG TTG AAG CTC CTC TCC-3′
Mrp5	Abcc5	F: 5′-ACA GCC GCT ATG GAC ACA GAG ACA GA-3′
		R: 5′-AGG CGA AGT TTC AGC AGG ACA GGA TG-3′
Mrp6	Abcc6	F: 5′-GCC ACA GGA TTG ACA GCA GAA GAG GA-3′
		R: 5′-GCA GAC AGC CAA GGA GCC CAA AGA CC-3′
Nqo1	Nqo1	F: 5′-TTT AGG GTC GTC TTG GCA AC-3′
		R: 5′-GTC TTC TCT GAA TGG GCC AG-3′
Nrf2	Nfe212	F: 5′-TCT ATG TGC CTC CAA AGG-3′
		R: 5′-CTC AGC ATG ATG GAC TTG GA-3′
β-Actin	β-Actin	R: 5′-GCA GAC AGC CAA GGA GCC CAA AGA CC-3′
		F: 5′-GCA ACG AGC GGT TCC G-3′

evaluated by agarose gel electrophoresis. RNA (1 μg) was reverse transcribed to cDNA using an M-MLV RT kit (Invitrogen, Carlsbad, CA). mRNA expression of ABC transporters and Nrf2 target genes were quantified using specific primers (Table 2) by the ΔΔCT method and normalized to the housekeeping gene β-Actin. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA). Amplification was performed using an Applied Biosystems 7500 Fast Real-Time PCR System. Amplification was carried out in a 20 μl reaction volume containing diluted cDNA, Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 μM of each primer.

2.8. Analytical determinations

2.8.1. Alanine aminotransferase (ALT) assay

Plasma ALT activity was determined as a biomarker of hepatocellular injury. Infinity ALT Liquid Stable Reagent (Thermo Fisher Scientific Inc., Waltham, MA) was used to determine ALT activity. Samples were analyzed using a Bio-Tek Power Wave X Spectrophotometer.

2.8.2. Quantitation of APAP by HPLC

Total brain homogenate (100 μl) was deproteinized with the addition of $HClO_4$ 0.2 N (1/20 w/v) and centrifuged at 10,000 × *g* for 10 min at 4 °C. An equal volume of mobile phase (12.5% methanol:1% acetic acid in water) was added to serum or brain supernatant and centrifuged at 10,000 × *g* for 10 min at 4 °C. Fifty microliters of collected supernatants were injected onto the HPLC for analysis. The concentration of APAP was calculated using an APAP standard curve. The retention time of APAP was determined by comparison with an authentic standard (McNeil-PPC, Inc., Fort Washington, PA). Quantification was based on integrated peak areas. Data are expressed as μg/ml and ng/mg of protein of APAP for serum and brain, respectively.

Samples were analyzed using a Beckman System Gold HPLC system (Beckman Coulter, Fullerton, CA) equipped with a 128-nm solvent module and a 166-nm detector. APAP and its metabolites were resolved using a ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 μm column and eluted using a mobile phase composed of 12.5% HPLC grade methanol, 1% acetic acid and 86.5% water run isocratically at a flow rate of 1.2 ml/min. The elution of metabolites was monitored at a wavelength of 254 nm.

2.9. Statistical analysis

Results are expressed as means ± standard error (SE). Data were analyzed using the Student's *t*-test or ANOVA followed by Bonferroni's *post hoc* test. Although Student's *t*-test was used to compare means of two groups, ANOVA was used to compare the means of more than two treatment groups that are normally distributed with a common variance. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Effect of APAP on the expression of brain ABC proteins and mRNAs

The expression of the principal brain ABC transporters was studied 24 h after APAP administration in wild type mice (Fig. 1). Panel A shows a significant increment in mRNA expression of P-gp (79%), Mrp2 (154%) and Mrp4 (85%) with APAP treatment when compared to WT-vehicle treated group. No significant changes in Bcrp; Mrp1; Mrp3; Mrp5; and Mrp6 expression were detected. Similar to mRNA, protein expression (shown in panel B) also present a significant increase for P-gp, Mrp2 and Mrp4 by 195, 293 and 38% after APAP, respectively, when compared to WT-vehicle treated group. Protein expression of Bcrp; Mrp1; Mrp5; and Mrp6 showed no changes with APAP treatment. Mrp3 protein was not detectable in either group.

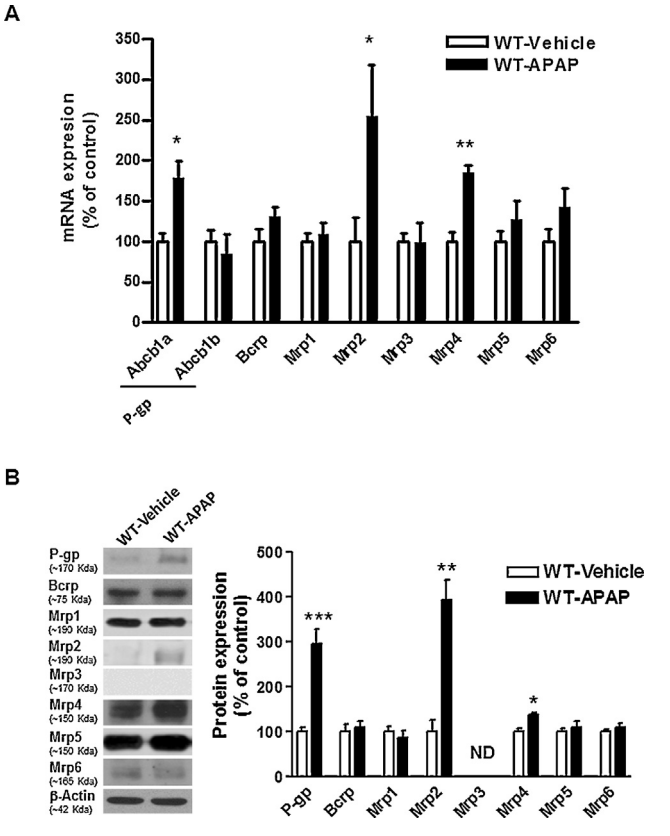


Fig. 1. *Effect of APAP on brain ABC mRNA and protein expression.* (Panel A) Western blot analysis of ABC transporters in total brain membrane fractions. Equal protein (50 or 70 μg, depending on the transporter) was loaded into each well. β-Actin was used as loading control. Densitometric analysis of blots is presented as percentage of control and expressed as mean ± SE ($n = 5$ mice/group. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). ND: non-detectable. (Panel B) Brain mRNA levels of ABC transporters were measured by qPCR. Data are presented as percentage of control and expressed as mean ± SE ($n = 5$ mice/group; ** $p < 0.01$; * $p < 0.05$ between groups).

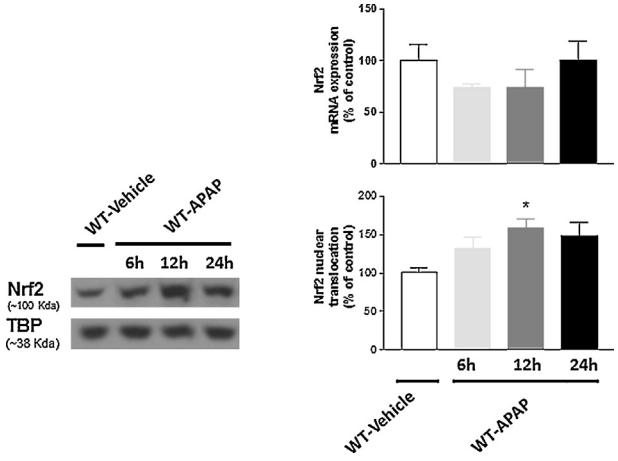


Fig. 2. *Time-dependent effect of APAP on brain Nrf2 gene expression and its nuclear localization.* Brain Nrf2 mRNA levels were measured by qPCR. Data are presented as percentage of control and expressed as means ± SE ($n = 5$ mice/group). Nrf2 protein nuclear concentration was determined by Western blotting. Equal protein (50 μg) was loaded into each lane. TATA-binding protein (TBP) was used as loading control. Densitometric analysis of blot is presented as percentage of control and expressed as mean ± SE ($n = 5$ mice/group. * $p < 0.05$ vs WT-Vehicle).

3.2. Effect of APAP on time-dependent nuclear translocation and expression of Nrf2 and its target gene Nqo1 in the brain

To evaluate the potential role of Nrf2 in the changes in brain ABC transporters expression by APAP, we studied the nuclear translocation of this transcription factor and the temporal expression of two known Nrf2 target genes. Fig. 2 shows that there are no changes in brain mRNA expression for Nrf2 at any of the time points examined. However, nuclear content of Nrf2 is significantly increased at 12 h after APAP administration. This result is indicative of Nrf2 activation in brain by APAP. Further supporting this, we observed significant increments in mRNA expression of Ho-1 at 6 h (40%) after APAP administration (Fig. 3A). Similarly, Ho-1 protein expression increased at 12 and 24 h by 20 and 80%, respectively. Nqo1 mRNA and protein expression levels were also significantly increased 24 h after APAP by 40 and 82%, respectively (panel B). Collectively, these results indicative that APAP induces a Nrf2 response in brain.

3.3. Time course of P-gp, Mrp2 and Mrp4 mRNA expression

To study the relationship between ABC brain transporters expression and Nrf2 function in APAP treated mice, we evaluated the gene expression of ABC transporters in brain at different time points (6, 12 and 24 h) after APAP administration. The data are shown in Fig. 4. The time course for P-gp expression demonstrates a significant increase in mRNA levels at 6 h, which remained significantly elevated throughout the 24 h time course. In comparison, Mrp2 mRNA expression was increased significantly at 12 and 24 h, but not at 6 h. Lastly, Mrp4 gene expression was only elevated at 24 h. This time profile suggests that the changes in P-gp gene and protein expression by APAP may not be mediated by Nrf2, since P-gp induction is detected prior to the enhanced Nrf2 nuclear accumulation observed in Fig. 2.

3.4. Characterization of liver damage and brain APAP concentration in wildtype and Nrf2 knockout mice

To further determine if Nrf2 mediates the changes in expression of ABC brain transporters by APAP, their gene and protein expression profiles were analyzed in Nrf2 knockout mice. First,

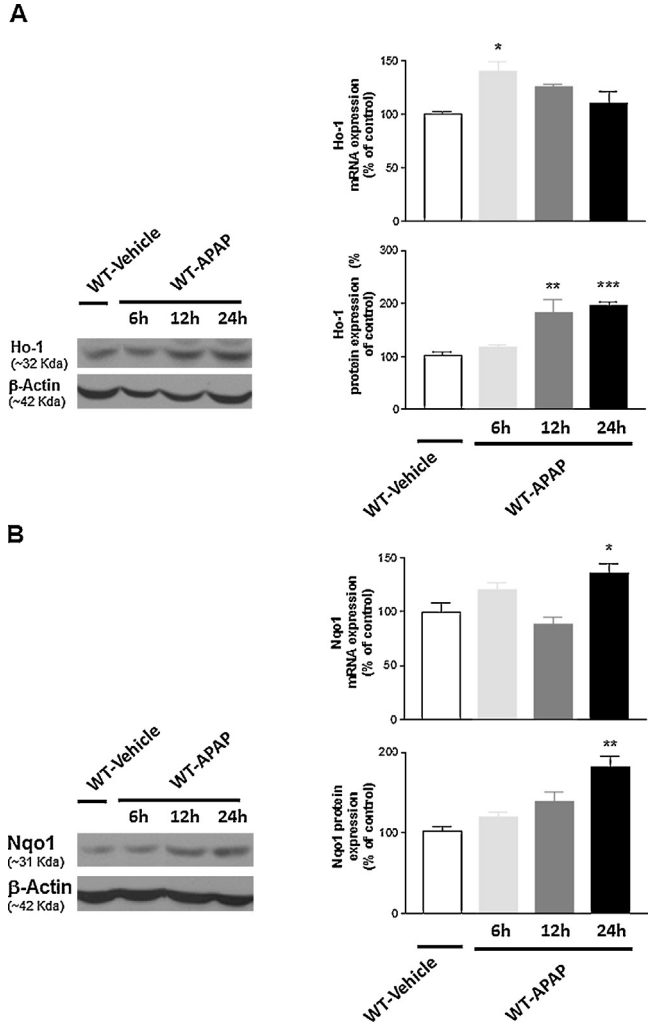


Fig. 3. *Time-dependent effect of APAP on the expression of the Nrf2 target genes Ho-1 and Nqo1.* Brain Ho-1 (Panel A) and Nqo1 (Panel B) mRNA and protein levels were measured by qPCR and Western blotting, respectively. mRNA data are presented as percentage of control and expressed as mean ± SE. For western blotting, equal cytosolic protein (50 μg) was loaded into each lane. β-Actin was used as loading control. Densitometric analysis of blots is presented as percentage of control values and expressed as mean ± SE. ($n = 5$ mice/group. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ vs WT-Vehicle).

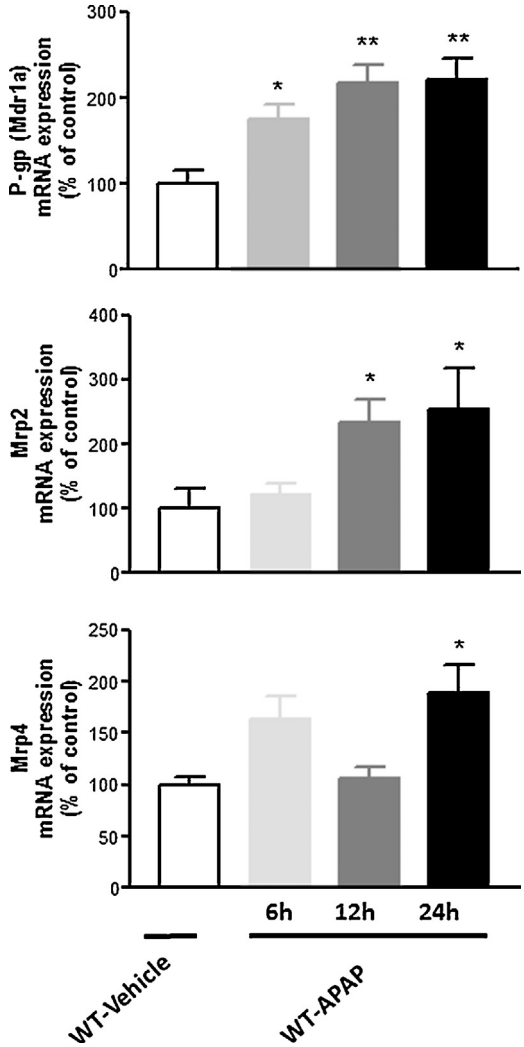
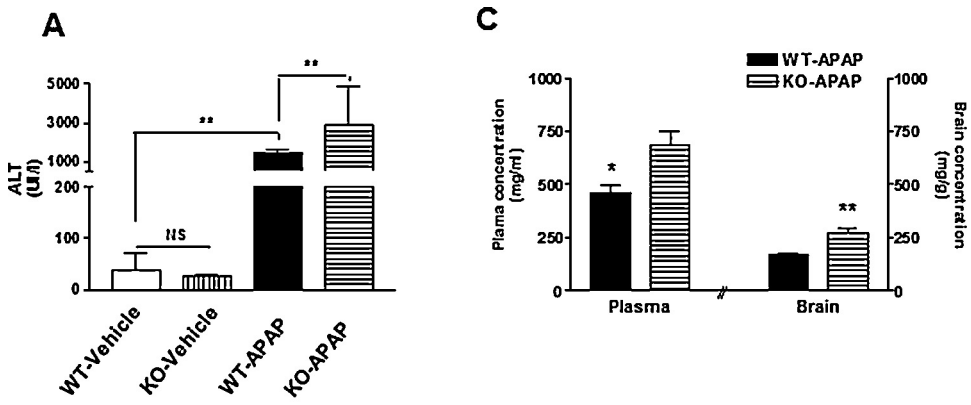


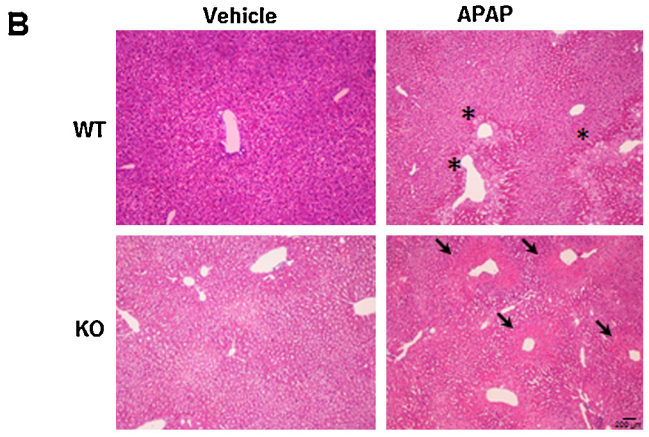
Fig. 4. *Time-dependent effect of APAP on P-gp, Mrp2 and Mrp4 protein expression.* Protein expression of these three brain ABC transporters was determined by Western blotting. Equal protein (50 μg) was loaded into each. β-Actin was used as loading control. Densitometric analysis of western blots is presented as percentage of control values and expressed as mean ± SE ($n = 5$ mice/group. ** $p < 0.01$; * $p < 0.05$ vs WT-Vehicle).

we confirmed the higher susceptibility of null mice to APAP hepatotoxicity [40,44–46]. Fig. 5A shows plasma ALT activity at 24 h after APAP for both genotypes. As expected, this biochemical marker of hepatocyte necrosis was significantly increased in both APAP groups, with significantly greater ALT values in the knockouts. Histology images in Fig. 5B show characteristic centrilobular hepatic necrosis in both wildtype and Nrf2 knockout mice, with greater injury in knockouts. Lastly, we analyzed the APAP concentration in both blood and brain. Fig. 5C shows that plasma APAP concentration at 2 h is significantly higher in Nrf2 knockout mice. Similarly, APAP brain concentration in knockout mice is greater than in wildtypes. These data suggest that any differences in ABC transporter expression between gentopytes cannot be attributed to lower penetration of APAP into brain of Nrf2 knockout mice.

3.5. Expression of Nrf2 and its target gene Nqo1 in brain of wildtype and Nrf2 knockout mice after APAP treatment

To investigate whether the changes in brain Nqo1 gene expression by APAP are Nrf2 dependent, mRNA levels were analyzed in wildtype and Nrf2 knockout mice treated with APAP (400 mg/kg; 24 h after i.p. injection) by qRT-PCR. As noted previously in Fig. 3B, APAP treatment produced a significant increase in brain Nqo1 mRNA levels in Nrf2 wildtype mice (Fig. 6B). In contrast, mRNA values in APAP treated knockout mice were no different than knockout and wild vehicle control groups. As expected, Nrf2 mRNA expression in both groups of Nrf2 knockout mice was 10–15% of what is observed in wildtypes (Fig. 6A).





Group	Histopathologic grade				
	0	1	2	3	4
WT-Vehicle (n=5)	5/5	0/5	0/5	0/5	0/5
WT-APAP (n=9)	0/9	5/9	4/9	0/9	0/9
KO-Vehicle (n=5)	4/5	1/5	0/5	0/5	0/5
KO-APAP (n=9)	0/9	2/9	3/9	4/9	0/9

Fig. 5. *Analysis of liver damage, and plasma and brain APAP concentrations in wildtype and Nrf2 knockout mice.* (Panel A) Plasma ALT values (IU/l) in control and APAP groups. Data are expressed as mean ± SE (n = 10 mice/group; ** $p < 0.01$). (Panel B) Liver histopathology examination and analysis. Hematoxylin and eosin stained liver sections were examined and scored for degree of damage (degeneration and necrosis) as described in Section 2. Representative tissue sections from each treatment group (n = 5) are shown. Asterisks highlight areas of centrilobular degeneration while arrows highlight areas of necrosis. (Panel C) APAP concentration in plasma and brain of wildtype and Nrf2 knockout mice 2 h after treatment. Samples were analyzed by HPLC and data are expressed as mean ± SE (n = 5 mice/group; * $p < 0.05$; ** $p < 0.01$).

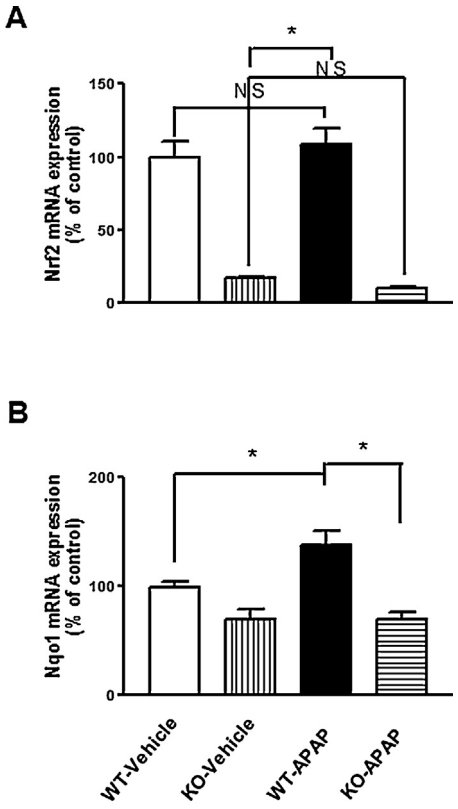


Fig. 6. *mRNA expression of Nrf2 and its target gene Nqo1 in wildtype and Nrf2 knockout mice.* Brain mRNA levels of Nrf2 (Panel A) and Nqo1 (Panel B) were measured by qPCR. Data are presented as percentage of control and expressed as means ± SE. (n = 5 mice/group. * $p < 0.05$).

3.6. Role of Nrf2 in the APAP-induced expression of P-gp, Mrp2 and Mrp4 in the brain

Finally, to confirm the role of Nrf2 in brain ABC transporter regulation, we analyzed the expression of P-gp, Mrp2 and Mrp4 in wildtype and Nrf2 knockout mice receiving APAP. Fig. 7A shows mRNA expression data for P-gp, Mrp2 and Mrp4, while protein expression is shown in Fig. 7B. P-gp expression significantly increased in both wildtype and Nrf2 knockout mice treated with APAP at both the protein and mRNA level. This suggests that P-gp induction in brain by APAP is not mediated by Nrf2. In contrast, gene and protein expression of Mrp2 and Mrp4, which is significantly increased by APAP in wildtype mice, did not change in Nrf2 knockouts. In fact, expression values for both gene and protein in APAP treated null mice is the same as in both groups of vehicle treated mice. This clearly indicates that Nrf2 regulates the changes in expression of brain Mrp2 and Mrp4 by APAP. It is important to also note that Nrf2 null mice treated with vehicle show significantly lower Mrp4 protein expression than the corresponding vehicle wildtypes. This suggests that the basal expression of Mrp4 is controlled, in part, by Nrf2 function.

4. Discussion

Until now, it has been reported that APAP modulates not only the hepatic expression of ABC transporters [34–38], but also that in extrahepatic tissues such as intestine [39] and placenta [47]. However, the effect of toxic doses of APAP on brain ABC transporters has not been extensively studied. Considering that the main isoforms of CYPs involved in APAP bioactivation to its reactive intermediate, CYP2E1 and CYP3A2, are expressed in the brain [48,49] and that APAP can go across the BBB in animals [26–29] and humans [28,30], we hypothesized that toxic doses of APAP can similarly affect the expression of brain ABC transporters. We further hypothesized that such changes in brain transporters expression produced by APAP would be regulated, in part, by Nrf2 given its well documented role as a sentinel of redox homeostasis and its previously demonstrated role in reguating drug transporters under oxidative stress conditions [14,40,50].

Our data demonstrate that a toxic dose of APAP selectively induces brain Mrp2, Mrp4 and P-gp in mice. No changes in protein or mRNA expression of Mrp1, Mrp3; Mrp5; Mrp6 or Bcrp were observed. This work describes for first time the modulation of brain Mrps expression by APAP. In agreement with our results, Slosky et al. recently demonstrated the induction of P-gp protein and function in BBB of rats treated with APAP at 500 mg/kg [51]. However, there are differences in the time profile for induction of this transporter between their results and ours. They observed an early increase in P-gp protein that peaked at 6 h, then returned to basal values by 24 h. In contrast, we report a 3-fold increase in P-gp protein expression by APAP at 24 h. Although we did not evaluate P-gp protein expression at earlier timepoints, mRNA analysis show a significant increase in gene expression at 6 h post APAP administration, suggesting that P-gp protein in mouse brain could also be elevated prior to our 24 h timepoint. This temporal difference in P-gp expression and induction between studies could be due to differences in regulatory events and/or differences in susceptibility to APAP between rats and mice. It is well documented that rats are more resistant to APAP hepatotoxicity than mice and that this resistance is related to differences in APAP toxicokinetics [52,53].

NAPQI, the reactive metabolite of APAP, produces oxidative stress and is normally detoxified by conjugation with GSH. A previous report showed that administration of a high oral dose of APAP (3 g/kg) to rats decreased GSH concentrations in different regions of the brain [6]. Also, administration of a toxic dose of APAP (600 mg/kg) in mice increased the formation of ROS, TBARS (thiobarbituric acid reactive species) and dicloro fluorescein oxidation levels in brain homogenates [54]. Collectively, these results indicate that doses of APAP that are considered to be hepatotoxic also lead to measureable oxidative stress in the brain. Nrf2 is a transcriptional activator that senses redox imbalances and oxidative injury. In the present study we observed increased accumulation of Nrf2 in nucleus, which is indicative of its activation. In support of this, the expression of Nqo1 and Ho-1, two known Nrf2 target genes, were significantly increased in brain of APAP treated mice. Both genes showed different temporal patterns of induction. mRNA and protein expression of Nqo1 were only increased at 24 h after APAP administration, while Ho-1 mRNA levels increased as early as 6 h. Ho-1 protein also exhibited an earlier induction profile. These temporal differences in expression of these two genes could be related to differences in regulatory events. Yeligar et al. showed that ethanol up-regulates of HO-1 gene expression *via* JNK-1, HIF-1α and Nrf2 activation, whereas NQO1 regulation utilizes Nrf2 only [55]. In other words, regulation of the Ho-1 gene in models of oxidative stress involves multiple transcriptional activators, including Nrf2, while under the same conditions, Nqo1 undergoes single regulation by Nrf2.

We also examined the temporal patterns of P-gp, Mrp2 and Mrp4 mRNAs to determine if the timing of induction for each transporter bares correlation with the time of maximal Nrf2 nuclear localization. Our results demonstrated that P-gp is significantly induced at 6 h while Mrp2 and Mrp4 are induced at 12 h, the time of P-gp induction is produced earlier than when Nrf2 translocation to the nucleus is significantly enhanced. This strongly suggests a possible role of this transcription factor in the induction of Mrps but not in P-gp.

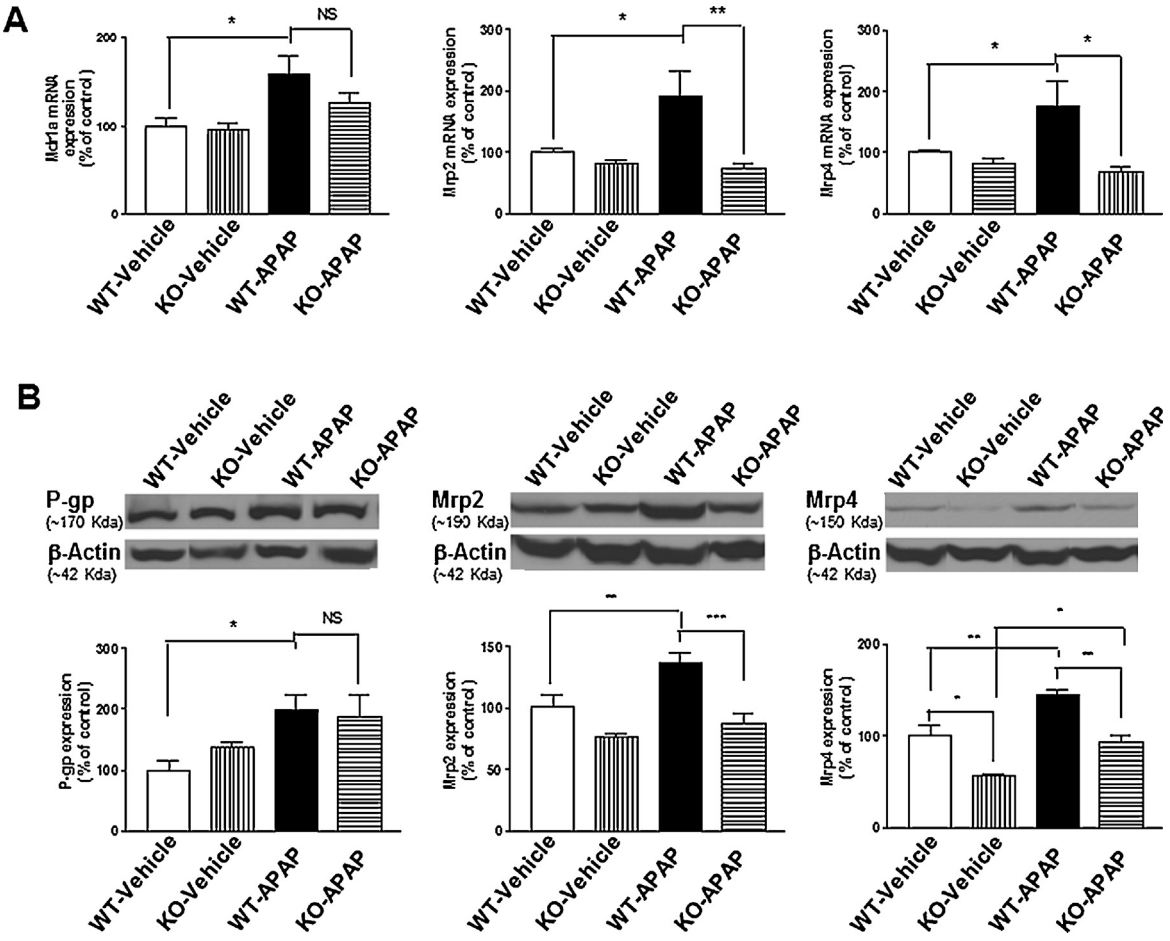


Fig. 7. *Effect of APAP on P-gp, Mrp2 and Mrp4 gene and protein expression in wildtype and Nrf2 knockout mice.* (Panel A) mRNA levels were measured by qPCR. Data are presented as percentage of control and expressed as mean ± SE. (n = 5 mice/group. ** $p < 0.01$; * $p < 0.05$). (Panel B) Protein expression was analyzed by Western blotting. Equal protein (50 or 70 μg, depending on the transporter) was loaded into each well. β-Actin was used as loading control. Densitometric analysis is presented as percentage of control. Data are expressed as mean ± SE (n = 5 mice/group. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

To conclusively determine if Nrf2 participates in the regulation of brain ABC transporters, we repeated the APAP intoxication experiment using Nrf2 knockout mice. As previously reported, hepatotoxicity by APAP is greater in Nrf2 knockouts than wildtype mice. This is evidenced here by plasma ALT and histopathological analysis of tissues [40,44–46]. This enhanced hepatotoxicity is due to altered Phase 1 and 2 metabolism of APAP, particularly lower gene and protein expression as well as catalytic activity of Ugt1a6. Quantitatively, this is considered to be the most important detoxification pathway for APAP [45]. Reduced APAP-glucuronide formation increases in the availability of parent APAP for bioactivation to its reactive metabolite, thus resulting in greater NAPQI formation. In fact, we confirmed here the presence of higher concentrations of APAP in plasma and brain of Nrf2 knockouts in comparison to wildtype mice. This supports the availability of more parent compound for bioactivation to NAPQI in the absence of Nrf2. Finally, we described that in the absence Nrf2, there is no induction of Mrps (protein or mRNA level), while P-gp induction by APAP was not affected by the absence of Nrf2. The results also show that the basal expression of Mrp4 in brain is under Nrf2 regulation. Overall, our results demonstrate that Nrf2 selectively regulates Mrp2 and Mrp4 expression in response to APAP and not P-gp. Our results are in agreement with Slosky et al.'s description of brain P-gp induction in APAP treated rats with CAR activation as the cause of induction [51]. Wang et al. recently showed that the administration of sulforaphane, a Nrf2 ligand found in cruciferous vegetables increases the expression of P-gp, Bcrp and Mrp2 in rats [56]. Furthermore, they also showed that capillaries isolated from Nrf2 knockout mice had activation of p53 and increased P-gp expression with the conclusion that Nrf2 indirectly, through the action of p53, p39 MAPK and NF-κB regulates P-gp, Bcrp and possibly Mrp2. Collectively, these studies illustrate that regulation of ABC transporters in brain, particularly P-gp is a multifactorial process involving different regulatory elements.

In conclusion, our data demonstrate that toxic APAP treatment induces brain P-gp, Mrp2 and Mrp4 expression in a selective manner because the expression of other ABC transporters did not change. Our study has also documente for the first time that activation of the Nrf2-ARE pathway has a permissive role in the changes in gene expression for Mrp2 and Mrp4, but not P-gp in mouse brain during APAP intoxication.

Acknowledgments

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References

[1] James LP, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. Drug Metab Dispos 2003;31:1499–506.

[2] Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, et al. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology 2005;42:1364–72.
[3] Mohsenin V. Assessment management of cerebral edema and intracranial hypertension in acute liver failure. J Crit Care 2013;28:783–91.
[4] Bailey B, Amre DK, Gaudreault P. Fulminant hepatic failure secondary to acetaminophen poisoning: a systematic review and meta-analysis of prognostic criteria determining the need for liver transplantation. Crit Care Med 2003;31:299–305.
[5] Micheli L, Fiaschi AI, Cerretani D, Giorgi G. Effect of acetaminophen on glutathione levels in several regions of the rat brain. Curr Ther Res 1993;56:730–6.
[6] Nencini C, Giorgi G, Micheli L. Protective effect of silymarin on oxidative stress in rat brain. Phytomedicine 2007;14:129–35.
[7] Bajt ML, Knight TR, Lemasters JJ, Jaeschke H. Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by *N*-acetyl cysteine. Toxicol Sci 2004;80:343–9.
[8] Bessems JG, Vermeulen NP. Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. Crit Rev Toxicol 2001;31:55–138.
[9] David JP. The molecular toxicology of acetaminophen. Drug Metab Rev 2005;37:581–94.
[10] Rushmore TH, Pickett CB. Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. J Biol Chem 1990;265:14648–53.
[11] Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem 1991;266:11632–39.
[12] Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 2007;47:89–116.
[13] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236:313–22.
[14] Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T, Yamamoto M. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. Genes Cells 2003;8:379–91.
[15] Keppler D, Kamisako T, Leier I, Cui Y, Nies AT, Tsujii H, et al. Localization, substrate specificity, and drug resistance conferred by conjugate export pumps of the MRP family. Adv Enzyme Regul 2000;40:339–49.
[16] Sun J, He ZG, Cheng G, Wang SJ, Hao XH, Zou MJ. Multidrug resistance *P*-glycoprotein: crucial significance in drug disposition and interaction. Med Sci Monit 2004;10:RA5–14.
[17] Fromm MF. Importance of *P*-glycoprotein at blood-tissue barriers. Trends Pharmacol Sci 2004;25:423–9.
[18] Leslie EM, Deeley RG, Cole SP. Multidrug resistance proteins: role of *P*-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol 2005;204:216–37.
[19] Kacem K, Lacombe P, Seylaz J, Bonvento G. Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: a confocal microscopy study. Glia 1998;23:1–10.
[20] Hellstrom M, Gerhardt H, Kalen M, Li X, Eriksson U, Wolburg HBetsholtz C. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol 2001;153:543–53.
[21] Redzic Z. Molecular biology of the blood–brain and the blood–cerebrospinal fluid barriers: similarities and differences. Fluids Barriers CNS 2011;8:3.
[22] Dallas S, Miller DS, Bendayan R. Multidrug resistance-associated proteins: expression and function in the central nervous system. Pharmacol Rev 2006;58:140–61.
[23] Loscher W, Potschka H. Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. Prog Neurobiol 2005;76:22–76.
[24] Ashraf T, Kao A, Bendayan R. Functional expression of drug transporters in glial cells: potential role on drug delivery to the CNS. Adv Pharmacol 2014;71:45–111.
[25] Ashraf T, Kis O, Banerjee N, Bendayan R. Drug transporters at brain barriers: expression and regulation by neurological disorders. Adv Exp Med Biol 2012;763:20–69.
[26] Courad JP, Besse D, Delchambre C, Hanoun N, Hamon M, Eschalier A, et al. Acetaminophen distribution in the rat central nervous system. Life Sci 2001;69:1455–64.
[27] Fischer LJ, Green MD, Harman AW. Levels of acetaminophen and its metabolites in mouse tissues after a toxic dose. J Pharmacol Exp Ther 1981;219:281–6.
[28] Bannwarth B, Netter P, Lapicque F, Gillet P, Pere P, Boccard E, et al. Plasma and cerebrospinal fluid concentrations of paracetamol after a single intravenous dose of propacetamol. Br J Clin Pharmacol 1992;34:79–81.
[29] Massey TE, Walker RM, McElligott TF, Racz WJ. Acetaminophen-induced hypothermia in mice: evidence for a central action of the parent compound. Toxicology 1982;25:187–200.
[30] Anderson BJ, Holford NH, Woollard GA, Chan PL. Paracetamol plasma and cerebrospinal fluid pharmacokinetics in children. Br J Clin Pharmacol 1998;46:237–43.
[31] Upadhya SC, Tirumalai PS, Boyd MR, Mori T, Ravindranath V. Cytochrome P4502E (CYP2E) in brain: constitutive expression, induction by ethanol and localization by fluorescence in situ hybridization. Arch Biochem Biophys 2000;373:23–34.
[32] Howard LA, Miksys S, Hoffmann E, Mash D, Tyndale RF. Brain CYP2E1 is induced by nicotine and ethanol in rat and is higher in smokers and alcoholics. Br J Pharmacol 2003;138:1376–86.
[33] Joshi M, Tyndale RF. Induction and recovery time course of rat brain CYP2E1 after nicotine treatment. Drug Metab Dispos 2006;34:647–52.
[34] Ghanem CI, Gomez PC, Arana MC, Perassolo M, Ruiz ML, Villanueva SS, et al. Effect of acetaminophen on expression and activity of rat liver multidrug resistance-associated protein 2 and *P*-glycoprotein. Biochem Pharmacol 2004;68:791–8.
[35] Barnes SN, Aleksunes LM, Augustine L, Scheffer GL, Goedken MJ, Jakowski AB, et al. Induction of hepatobiliary efflux transporters in acetaminophen-induced acute liver failure cases. Drug Metab Dispos 2007;35:1963–9.
[36] Aleksunes LM, Scheffer GL, Jakowski AB, Pruimboom-Brees IM, Manautou JE. Coordinated expression of multidrug resistance-associated proteins (Mrps) in mouse liver during toxicant-induced injury. Toxicol Sci 2006;89:370–9.
[37] Ghanem CI, Ruiz ML, Villanueva SS, Luquita M, Llesuy S, Catania VA, et al. Effect of repeated administration with subtoxic doses of acetaminophen to rats on enterohepatic recirculation of a subsequent toxic dose. Biochem Pharmacol 2009;77:1621–8.
[38] Aleksunes LM, Slitt AM, Cherrington NJ, Thibodeau MS, Klaassen CD, Manautou JE. Differential expression of mouse hepatic transporter genes in response to acetaminophen and carbon tetrachloride. Toxicol Sci 2005;83:44–52.
[39] Ghanem CI, Arias A, Novak A, Carpini GD, Villanueva S, Blazquez AG, et al. Acetaminophen-induced stimulation of MDR1 expression and activity in rat intestine and in LS 174T human intestinal cell line. Biochem Pharmacol 2011;81:244–50.
[40] Aleksunes LM, Slitt AL, Maher JM, Augustine LM, Goedken MJ, Chan JY, et al. Induction of Mrp3 and Mrp4 transporters during acetaminophen hepatotoxicity is dependent on Nrf2. Toxicol Appl Pharmacol 2008;226:74–83.
[41] Vannucci SJ. Developmental expression of GLUT1 and GLUT3 glucose transporters in rat brain. J Neurochem 1994;62:240–6.
[42] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[43] Manautou JE, Hoivik DJ, Tveit A, Hart SG, Khairallah EA, Cohen SD. Clofibrate pretreatment diminishes acetaminophen's selective covalent binding and hepatotoxicity. Toxicol Appl Pharmacol 1994;129:252–63.
[44] Chan K, Han XD, Kan YW. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc Natl Acad Sci USA 2001;98:4611–6.
[45] Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, et al. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol Sci 2001;59:169–77.
[46] Rohrer PR, Rudraiah S, Goedken MJ, Manautou JE. Is nuclear factor erythroid 2-related factor 2 responsible for sex differences in susceptibility to acetaminophen-induced hepatotoxicity in mice? Drug Metab Dispos 2014;42:1663–74.
[47] Blazquez AG, Briz O, Gonzalez-Sanchez E, Perez MJ, Ghanem CI, Marin JJ. The effect of acetaminophen on the expression of BCRP in trophoblast cells impairs the placental barrier to bile acids during maternal cholestasis. Toxicol Appl Pharmacol 2014;277:77–85.
[48] Ferguson CS, Tyndale RF. Cytochrome P450 enzymes in the brain: emerging evidence of biological significance. Trends Pharmacol Sci 2011;32:708–14.
[49] Miksys S, Tyndale RF. Cytochrome P450-mediated drug metabolism in the brain. J Psychiatry Neurosci 2013;38:152–63.
[50] Adachi T, Nakagawa H, Chung I, Hagiya Y, Hoshijima K, Noguchi N, et al. Nrf2-dependent and -independent induction of ABC transporters ABCC1, ABCC2, and ABCG2 in HepG2 cells under oxidative stress. J Exp Ther Oncol 2007;6:335–48.
[51] Slosky LM, Thompson BJ, Sanchez-Covarrubias L, Zhang Y, Laracuente ML, Vanderah TW, et al. Acetaminophen modulates *P*-glycoprotein functional expression at the blood-brain barrier by a constitutive androstane receptor-dependent mechanism. Mol Pharmacol 2013;84:774–86.
[52] Ioannides C, Steele CM, Parke DV. Species variation in the metabolic activation of paracetamol to toxic intermediates: role of cytochromes p-450 and p-448. Toxicol Lett 1983;16:55–61.
[53] Gregus Z, Madhu C, Klaassen CD. Species variation in toxication and detoxication of acetaminophen in vivo: a comparative study of biliary and urinary excretion of acetaminophen metabolites. J Pharmacol Exp Ther 1988;244:91–9.
[54] da Silva MH, da Rosa EJ, de Carvalho NR, Dobrachinski F, da Rocha JB, Mauriz JL, et al. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. Neurotox Res 2012;21:334–44.
[55] Yeligar SM, Machida K, Kalra VK. Ethanol-induced HO-1 and NQO1 are differentially regulated by HIF-1alpha and Nrf2 to attenuate inflammatory cytokine expression. J Biol Chem 2010;285:35359–73.
[56] Wang X, Campos CR, Peart JC, Smith LK, Boni JL, Cannon RE, et al. Nrf2 upregulates ATP binding cassette transporter expression and activity at the blood-brain and blood-spinal cord barriers. J Neurosci 2014;34:8585–93.