



A Novel Decrease of Mrp3 Protein in Liver of β -Thalassemic Mouse

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Authors' contributions

This work was carried out in collaboration between all authors. Author MQ performed experiments, analyzed results and wrote the first draft of the manuscript. Authors LF and AI performed treatments and provide liver samples. Author CB designed the study, analyzed the data and revised the first draft of the manuscript. Author CT designed the study and analyzed the results. All authors read and approved the final manuscript.

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ABSTRACT

Background: Hepatocytes have a fundamental system of efflux proteins that protect cells from toxic insults. Unconjugated bilirubin at higher concentration is toxic to cells and its intracellular accumulation is limited by the induction of efflux proteins such as Mrp3. *In vivo* studies showed an induction of hepatic Mrp3 expression in response to non-hemolytic hyperbilirubinemia as a compensatory mechanism to reduce UCB toxicity.

Study Design: In the present study, we analyzed the hepatic Mrp3 expression profile during

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hemolytic hyperbilirubinemia. We used β -thalassemic mouse and WT rodents treated with phenylhydrazine as an animal model of chronic and acute hemolysis, respectively.

Results: Unexpectedly, Mrp3 protein was 75% down-regulated in β -thalassemic mouse although Mrp3 mRNA was normal. Mrp3 mRNA was significantly induced in PHZ treated animals while again; Mrp3 protein was 60% down-regulated.

Conclusion: For the first time we observed a clear down-regulation for hepatic Mrp3 protein that linked to hemolysis, not to bilirubin. We hypothesize that a similar decrease for hepatic Mrp3 proteins is occur in hemolytic patients such as β -thalassemia.

Keywords: Hyperbilirubinemia; Mrp3, β -thalassemia; phenylhydrazine.

1. INTRODUCTION

The hepatocytes have a fundamental system of efflux proteins to protect them from toxic insults [1]. Unconjugated bilirubin (UCB) is the metabolite that is produced mostly in spleen, and in hepatocytes it is conjugated with glucuronic acid by the activity of UGT1A1 enzyme and exported to the bile [2]. In cases of hyperbilirubinemia, the high serum level of UCB is toxic to cells and its accumulation in tissues was suggested to be limited by active transport through the basolateral membrane, backing UCB to blood serum [3]. The multidrug resistance proteins (Mrps) can transport a large number of xeno- and endo-biotic compounds [4]. MRP3 transporter is expressed in the liver, at the basolateral membrane of both intrahepatic bile duct epithelial cells (cholangiocytes) and hepatocytes [5] indicating a physiological function of this transporter. As described by others, Mrp3 expression is induced *in vivo* under non-hemolytic hyperbilirubinemia, during bile duct ligations [6–9] or in the absence of UGT1A1 activity [10]. This up-regulation was suggested as a compensatory mechanism in which endogenously conjugated and unconjugated bilirubin is exported from hepatocytes back to bloodstream for renal excretion [6–10]. The aim of the present study was to characterize the expression profile of Mrp3 *in vivo* in response to hemolytic hyperbilirubinemia.

2. MATERIALS AND METHODS

2.1 Animals and Phenylhydrazine Treatment

The study plan was approved by the Local Committee for Care and Use of Laboratory. Animals were received human care. We choose a model of chronic hemolytic hyperbilirubinemia (β -Thalassemic mouse) [11] and two models of induced acute hemolytic hyperbilirubinemia, in which wild type mouse and wild type rat

were treated with phenylhydrazine (PHZ, 20 mg/kg/day for two days) as we described previously (PHZ-mouse and PHZ-rat, respectively) [12] (n = 6 / each group).

2.2 RNA Extraction, Reverse Transcriptase and Real-time PCR

Total RNA from liver tissues of different models was isolated using the TRI Reagent (Sigma, USA) following the manufacturer's instructions. The cDNA was obtained from 1 μ g of purified MMLV-derived reverse transcriptase system kit (iScript™cDNA Synthesis Kit, Bio Rad laboratories, Hercules, CA, USA) following the manufacturer's instructions. Reverse transcriptase was performed in a thermal cycler at 25°C for 5 min, 45°C for 30 min, and 85°C for 5 min. Real Time quantitative PCR (qRT-PCR) was performed according to the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) protocol. PCR amplification was carried out in 25 μ L reaction volume containing 25 ng of cDNA, 1 \times iQ SYBR Green Supermix (100mM KCl; 40mM Tris-HCl; pH: 8.4; 0.4mM each dNTP; 40 U/mL iTaq DNA polymerase; 6mM MgCl₂; SYBR Green I; 20 mM fluorescein; and stabilizers) (Bio-Rad) and 250 nM of gene specific primers. Primer sequences designed using Beacon Designer 4.02 software (PREMIER Biosoft International, Palo Alto, CA, USA) and listed in (Table 1). Cycling parameters were determined and the results were analyzed by using the comparative Ct method as the means of relative quantification, normalized to two references genes and expressed as $2^{-\Delta\Delta CT}$. Melting curve analysis was performed to assess product specificity.

2.3 Western Blot Analysis

Liver tissue were thawed and homogenized with 150 μ l of 10 mM Hepes (pH 7.4), containing 0.2 mM CaCl₂ and 0.25M sucrose, using a tight Dounce–Potter device and proteins

Table 1. List of primers sequence

Animals	Target gene	Accession number ^a	Forward primers 5' - 3'	Reverse primers 5' - 3'
Mouse	Mrp3	NM_029600	GGTTATCCAGGCTCAAGAC	GCCGTGGAAGGAATAGAAC
	β -actin ^b	NM_007393	CCTTCTTGGGTATGGAATCCTGTG	CAGCACTGTGTTGGCATAGAGG
	18S ^b	NR_003278	TCCGATAACGAACGAGAC	CTAAGGGCATCACAGACC
Rat	Mrp3	NM_080581	TGTGGGTCTTTCCGTGTC	GCCTCAGTCTCCGTCTTAG
	β -actin ^b	NM_031144.2	GGTGTGATGGTGGGTATG	CAATGCCGTGTTCAATGG
	18S ^b	X01117	CGTCTGCCCTATCAACTTTCG	GCCTGCTGCCTTCCTTGG

^a Accession numbers of target genes obtained from NCBI database^b Housekeeping genes used for normalizing the expression of target genes

concentration were assessed using the bicinchoninic acid reaction [13]. Proteins were separated by SDS-PAGE in 10% acrylamide gel. At the end of electrophoresis the gel was obtained and blotted onto a nitrocellulose membrane (PVDF) with a wet blotting system (Sigma, St. Louis, MO, USA). The membrane was washed three times by T-TBS (0.2% Tween 20; 20mM Tris; and 500mM NaCl; pH 7.5), and blocked with the 4% milk-T-TBS solution for 1 hour at room temperature. Membranes were incubated with first antibodies anti-Mrp3 (C-18, santa cruz biotechnology, USA) and anti-actin (A2066, Sigma Aldrich). Next day, the membrane was washed and incubated with anti-rabbit HRP-conjugated (Dacko laboratories, Italy) for 1 hour at room temperature. The peroxidase reaction was obtained by exposure of the membrane in the ECL-Plus Western Blotting detection system solutions (Amersham-PharmaciaBiotech, UK). The optical density of the target proteins were analyzed by Scion Image program, normalized to α -actin and represented as protein relative expression.

2.4 Statistical Analysis

Data are reported as mean \pm SD of 6 animals / each group. Differences between means were assessed by the two-tailed Student "t test". * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

3. RESULTS

Results showed a clear decrease of hepatic Mrp3 proteins (about 60%, $P = 0.001$) in β -thalassemic mouse compared to WT controls, while no significant change was observed at mRNA level (Fig. 1A). The antibodies allowed the identification of two bands of Mrp3 protein that were described by others [5,14]. To determine whether other members of Mrps family maybe modulated to compensate for Mrp3 protein down-regulation in β -thalassemic mouse, we examined the level of expression of selected Mrp1 and Mrp2 that are located at basolateral and apical

membrane of hepatocytes, respectively. Both transporters have the same predicted topological structure of Mrp3 and shared a wide spectrum of related substrates with Mrp3 [4,15]. Mrp1 and Mrp2 mRNA and proteins were not changed in β -thalassemic mouse compared to WT controls (data not shown). To confirm Mrp3 protein down-regulation we induced hemolysis in mice and rats by PHZ. Hepatic Mrp3 mRNA expression was 2 folds higher in PHZ-mouse ($P = 0.05$) and PHZ-rat ($P = 0.01$) compared to controls, while Mrp3 protein levels showed a decrease of 70% and 60% in PHZ-mouse and PHZ-rat ($P = 0.01$), respectively (Figs. 1B and 1C).

4. DISCUSSION

Impairment of the bilirubin conjugation or exportation via canalicular route leads to the higher induction of Mrp3, indicating that it functions in the export of bilirubin from hepatocytes to blood stream for renal excretion [6-10]. In the present study, we asked whether Mrp3 will also be up-regulated *in vivo* in response to higher level of bilirubin induced by hemolysis. Total serum bilirubin was 6 folds and 4 folds higher in β -thalassemic mouse and PHZ-treated rodents, respectively (data not shown).

Unexpectedly, we observed a clear decrease of Mrp3 protein in β -thalassemic mouse, although the mRNA expression was not significantly changed (Fig. 1A). Once we analyzed the Mrp3 expression in PHZ treated rodents, we detected the same decrease of hepatic Mrp3 protein although the Mrp3 mRNA was induced (Figs. 1B and 1C). This indicates that Mrp3 protein down-regulation is transcriptionally independent. It is worth to note, that hemolysis is the major difference between our models and the previous non-hemolytic models where Mrp3 was induced by bilirubin [6-10]. This suggests that hepatic Mrp3 protein down-regulation is linked to hemolysis (or related metabolites) and not to bilirubin. Hemolytic disorders are associated with iron overload in liver, which responsible for the

generation of oxidative stress that leads to protein damage [16,17]. However, Mrp3 protein decrease appears to be Mrp3 specific since the protein expression of the closely related transporters (Mrp1 and Mrp2) was not affected by hemolysis (data not shown). Further studies are needed to identify the cause(s) of specific Mrp3 protein downregulation under hemolysis.

Using the same β -thalassemic mouse model, another study showed a down-regulation of Mrp6 at the transcriptional level [18]. As a results, authors suggested complicated syndromes

(Pseudoxanthoma-elasticum-like syndrome) that implies the presence of abnormal circulating molecules due to alteration in Mrp6 expression profile that will affects the transporter activity toward its biological substrates. Mrp3 Substrates include endogenous compounds (estradiol-17 β -glucuronide, leukotriene C4, monovalent bile salts such as cholate and glycocholate), and several drugs, such as acetaminophen glucuronide and fexofenadine [19]. Weather the hepatic Mrp3 protein down-regulation under hemolysis affects the transporter activity toward its substrates is still unknown.

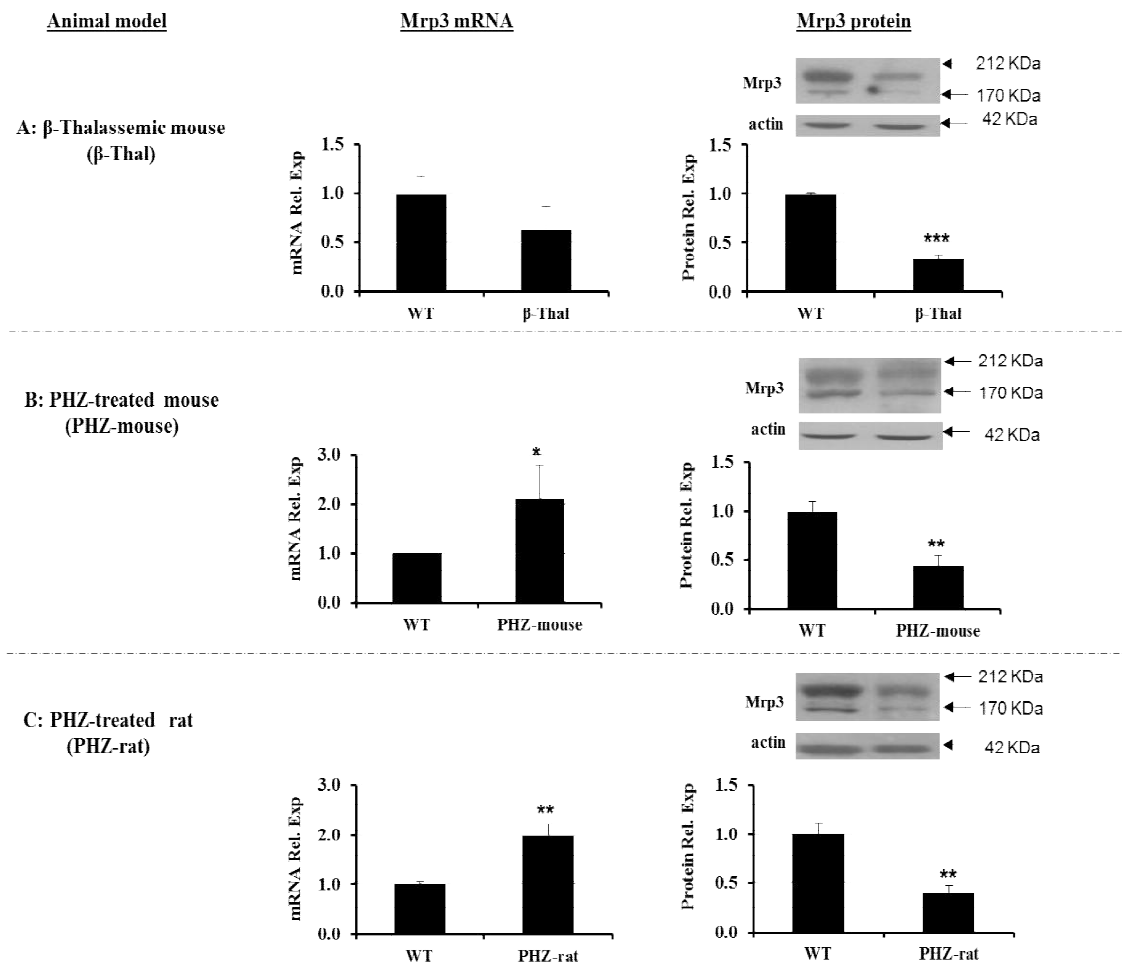


Fig. 1. Hepatic Mrp3 mRNA and protein expression. Left panel: Different animal models. (A) β -thalassemic mouse (β -Thal) vs wild type mouse (WT). (B) PHZ treated mouse (PHZ-mouse) vs wild type mouse (WT). (C) PHZ treated rat (PHZ-rat) vs wild type rat (WT). Middle panel: Mrp3 mRNA expression was analyzed by qRT-PCR. mRNA expression was normalized to housekeeping genes (β -actin and 18S) and represented as relative to WT controls. Right panel: Representative Western blot analysis for Mrp3 and α -actin proteins. The optical density of Mrp3 proteins were normalized to actin and represented as relative expression

5. CONCLUSION

Our present data showed a novel observation of down-regulation of hepatic Mrp3 protein during hemolysis. We hypothesize that a similar decrease might occur in patients of different hemolytic disorders and further studies are needed to discover the effects of this decrease on liver physiology under hemolysis.

CONSENT

It is not applicable.

ETHECAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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