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Sustained intrahepatic glutathione depletion causes proteasomal degradation of multidrug resistance-associated protein 2 in rat liver

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ABSTRACT

Multidrug resistance-associated protein 2 (MRP2) is a member of a family of efflux transporters that are involved in biliary excretion of organic anions from hepatocytes. Disrupted canalicular localization and decreased protein expression of MRP2 have been observed in patients with chronic cholestatic disorder and hepatic failure without a change in its mRNA expression. We have previously demonstrated that post-transcriptional regulation of the rapid retrieval of rat MRP2 from the canalicular membrane to the intracelluar compartment occurs under conditions of acute (~30 min) oxidative stress. However, it is unclear whether MRP2 expression is decreased during its sustained internalization during chronic oxidative stress. The present study employed buthionine sulfoximine (BSO) to induce chronic oxidative stress in the livers of Sprague–Dawley rats and then examined the protein expression and localization of MRP2. Canalicular MRP2 localization was altered by BSO treatment for 2 h without changing the hepatic protein expression of MRP2. While the 8 h after exposure to BSO, hepatic MRP2 protein expression was decreased, and the canalicular localization of MRP2 was disrupted without changing the mRNA expression of MRP2. The BSO-induced reduction in MRP2 protein expression was suppressed by pretreatment with N-benzyloxycarbonyl (Cbz)-Leu-Leu-leucinal (MG-132), a proteasomal inhibitor. Furthermore, the modification of MRP2 by small ubiquitin-relatedmodifier 1 (SUMO-1) was impaired in BSO-treated rat liver,while that by ubiquitin (Ub) and MRP2 was enhanced. Taken together, the results of this study suggest the sustained periods of low GSH content coupled with altered modification of MRP2 by Ub/SUMO-1 were accompanied by proteasomal degradation of MRP2.

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1. Introduction

Cholephilic compounds are excreted into the bile by distinct ATP-dependent primary active transporters located at the canalicular membrane. Of these, multidrug resistance-associated protein 2 (rat: rMRP2 and human: hMRP2) is of paramount importance. In humans, biliary excretion of organic anions (e.g., bilirubin glucuronides, reduced glutathione (GSH), and GSH conjugates) is mediated by MRP2, a conjugate export pump encoded by the MRP2 gene [1].

Several disorders are associated with deficient canalicular localization/expression of MRP2. For example, Dubin–Johnson syndrome (DJS) is a heredity disease characterized by conjugated hyperbilirubinemia. The deficient hepatobiliary transport of anionic conjugates results from the absence of a functional MRP2 in the canalicular membrane of hepatocytes. A number of mutations in the hMRP2 gene have been identified in patients with DJS. Certain DJS mutations (e.g., missence mutations R768W and I1173F; deletion mutation R1392, M1393) have been reported to cause defects in canalicular sorting and rapid proteasomal degradation of the hMRP2 protein [2–4]. In addition to the inherited abnormality of canalicular hMRP2 expression, it was reported that canalicular hMRP2 localization and overall cellular expression of hMRP2 protein was decreased without changing its mRNA level in patients with chronic liver failure (primary biliary cirrhosis and hepatitis C virus infection) [5,6] Notably, the presence of oxidative stress markers was correlated with chronic cholestatic disorder in these liver disease patients [7].

We have previously demonstrated that rMRP2 internalization from the canalicular membrane to the intracellular compartment occurs within a few minutes after ethacrynic acid (EA) treatment, tert-butyl hydroperoxide (t-BHP) treatment, or within 2 h after lipopolysaccharide (LPS) treatment [8–11]. Similar phenomenon was also confirmed for hMRP2 using liver slices [12]. All these events seem to be triggered by a

Abbreviations: ARC, apoptosis repressor with caspase recruitment domain; BSA, bovine serum albumin; Bsep/BSEP, bile salt export pump; BSO, buthionine-sulfoximine; CD26, cluster of differentiation 26; CrM, crude membrane; DJS, Dubin-Johnson syndrome; EA, ethacrynic acid; GADPH, glyceraldehyde-3-phosphate dehydrogenase; γ-GCS, γ-glutamyl cysteine synthetase; GSH, glutathione; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; MRP2, multidrug resistance-associated protein 2; PFIC, progressive familial intrahepatic cholestasis; PDZK1, PSD95/Dlg/ZO1-containing 1; PKC, protein kinase C; SUMO, small ubiquitin-like modifier; t-BHP, tert-butyl hydroperoxide; TTBS, Tween 20/Tris-buffered saline; Ub, ubiquitin; UBA2, ubiquitin-activating enzyme 2; Ubc9, ubiquitin-conjugating enzyme 9

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decrease of GSH in the hepatocytes. Based on our line of previous experiments using isolated rat hepatocyte culture model, we proposed that GSH decrease causes intracellular Ca^{2+} elevation, NO release and finally leads to PKC activation [9]. Then, interaction of rMRP2 with radixin, a cytoskeltal linker protein connecting rMRP2 to the filamentous actin, was decreased as a result of dephosphorylation of radixin [22]. We also demonstrated that once decreased rMRP2 surface expression induced by t-BHP was recovered to the control level by the replenishment of intracellular GSH within an hour [10]. On the other hand, only limited information is available on the fate of rMRP2 under sustained GSH decrease condition; i.e. stably pooled in the intracellular compartment to be recycled back to the cell surface or directed to degradation pathway.

Ubiquitination is a regulated post-translational modification that conjugates ubiquitin (Ub) to the lysine residues of target proteins, thereby determining their intracellular fate. The canonical role of ubiquitination is to mediate the proteasomal degradation of proteins that carry a single Ub molecule or a polymeric chain of Ub molecules on a specific lysine residue. Several proteins (transglutaminase 2 and apoptosis repressor with caspase recruitment domain (ARC)) are known to be degraded by ubiquitination during oxidative stress [14]. Recently, experiments employing the yeast-two hybrid screening demonstrated that small ubiquitin-like modifier (SUMO)-related enzymes and molecules, including ubiquitin-activating enzyme 2 (UBA2) and ubiquitin-conjugating enzyme 9 (Ubc9), interacted with the linker regions of rat and human MRP2 [15]. Moreover, modification of rMRP2 by SUMO-1 was confirmed (an isoform of the SUMO family member), and rMRP2 protein expression was decreased in Ubc9 knock-down rat hepatoma cells [15]. Although the fundamental role of SUMO has not yet been completely elucidated, it has been established that SUMO competes with Ub for modification of the same lysine residue in target proteins. In this manner, SUMO protects proteins from the Ub/proteasome-dependent degradation pathway [16–18].

The current study was designed to evaluate the hypothesis that SUMO-1/Ub-mediated modifications of rMRP2 are key factors that regulate the fate of rMRP2 during sustained GSH decreased condition. To examine this possibility, Sprague–Dawley rats were treated with buthionine-sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase (γ-GCS). γ-GCS is a rate-limiting enzyme in the synthesis of GSH and as such, its inhibition can cause long-lasting GSH depletion for at least 8 h, which is longer than our previous experimental condition. The results of this study indicate that rMRP2 protein is internalized at early phase as reported previously and subsequently degraded at later phase in a proteasome-dependent manner if GSH decease is sustained for a while (8 h). Ubiquitination and SUMOylation of rMRP2 may have a role in determining its fate after internalization.

2. Materials and methods

2.1. Chemicals and reagents

BSO, *o*-phthalaldehyde, 2-mercaptoethanol, and trifluoroacetic acid were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). N-benzyloxycarbonyl (Cbz)-Leu-Leu-leucinal (MG-132) was obtained from Calbiochem (Darmstadt, Germany), and 3-fluorotyrosine was obtained from Tokyo Kasei Chemical Industry Co., Ltd. (Tokyo, Japan). Rabbit anti-MRP2 antiserum was raised against the 12-amino acid sequence at the carboxyl terminus of rat rMRP2 [19]. Mouse monoclonal antibody against MRP2 (M_2III6) was obtained from Chemicon International, Inc. (Temecula, CA). Monoclonal mouse antibody (FK2) against mono- and poly-ubiquitinated conjugates was obtained from Enzo Life Sciences, Inc. (Plymouth, PA). Purified mouse anti-rat cluster of differentiation 26 (CD26; OX-61) and mouse monoclonal anti-β-actin antibodies were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Rabbit polyclonal anti-SUMO-1 and SUMO-2/3 antibodies and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals and solvents were of analytical grade.

2.2. Animals

Male Sprague–Dawley rats (Japan SLC Inc., Shizuoka, Japan), 6 to 7 weeks of age and weighing 170 to 220 g, were used for all experiments. The animals were treated humanely in accordance with the "Guide for the Care and Use of Laboratory Animals" issued by the National Institutes of Health (Bethesda, MD). In addition, all protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

2.3. Experimental model

The rats were divided into two groups that each consisted of four to five rats. Experimental animals were given an intraperitoneal (i.p.) injection of BSO (1 mg/kg). Control animals were injected with saline instead of BSO. The rats were sacrificed 2, 8, or 24 h after BSO or saline injection, and the livers were harvested. In some experiments, the proteasome inhibitor MG-132 (0.5 mg/kg i.p.) was injected 1 h before BSO or saline treatment.

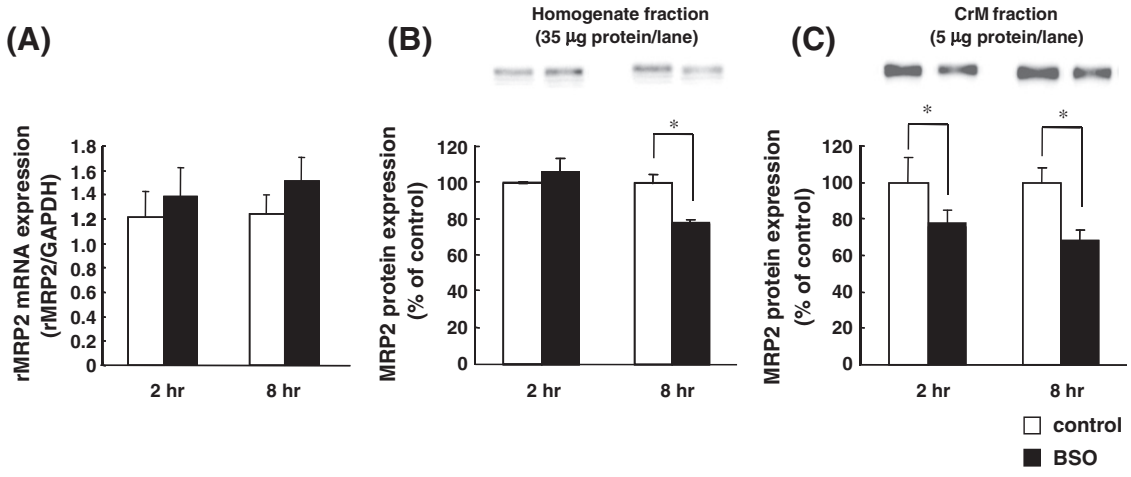

Fig. 1. Effect of BSO treatment on hepatic rMRP2 protein and mRNA expression. Rats were given BSO (1 mg/kg body weight) or saline by i.p. injection. (A) rMRP2 mRNA expression levels were measured by real-time RT-RCR. rMRP2 mRNA levels were normalized to GAPDH mRNA levels. (B, C) Crude liver homogenates (B) and CrM fractions (C) were subjected to immunoblot analysis with anti-MRP2 antiserum. The relative densities of the rMRP2 bands are shown. The band densities are expressed as a percentage of the corresponding saline-treated control value. Results are given as the mean ± S.D. (n = 4 independent rat livers for each condition.) *$p<0.05$ compared with control.

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