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Misregulation of Membrane Trafficking Processes in Human Nonalcoholic Steatohepatitis

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Abstract

Nonalcoholic steatohepatitis (NASH) remodels the expression and function of genes and proteins that are critical for drug disposition. This study sought to determine whether disruption of membrane protein trafficking pathways in human NASH contributes to altered localization of multidrug resistance-associated protein 2 (MRP2). A comprehensive immunoblot analysis assessed the phosphorylation, membrane translocation, and expression of transporter membrane insertion regulators, including several protein kinases (PK), radixin, MARCKS, and Rab11. Radixin exhibited a decreased phosphorylation and total expression, while Rab11 had an increased membrane localization. PKC δ , PKC α , and PKA had increased membrane activation, whereas PKC ϵ had a decreased phosphorylation and membrane expression. Radixin dephosphorylation may activate MRP2 membrane retrieval in NASH; however, the activation of Rab11/PKC δ and PKA/PKC α suggest an activation of membrane insertion pathways as well. Overall these data suggest an altered regulation of protein trafficking in human NASH, although other processes may be involved in the regulation of MRP2 localization.

Keywords

MRP2; biliary elimination; NASH

Introduction

Nonalcoholic steatohepatitis (NASH) remodels absorption, distribution, metabolism, and elimination (ADME) gene and protein expression (12, 16, 22). The canalicular efflux transporter, multidrug resistance-associated protein 2 (MRP2), is upregulated in NASH, yet is mislocalized away from the plasma membrane (16). These changes are observed in methionine-and-choline deficient (MCD) rodents, where it correlates to increased systemic retention, and shift in toxicity profile, of Mrp2 substrates ezetimibe, methotrexate, pemetrexed, and morphine glucuronide (9, 10, 14, 18). Morphine glucuronide and

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acetaminophen glucuronide disposition are disrupted similarly in NASH patients, suggesting that these changes in Mrp2 regulation are of translational value (3, 11). While the toxicologic implications of Mrp2/MRP2 mislocalization are clear, the mechanism by which this occurs in NASH is not evident.

Membrane trafficking of MRP2 has been studied extensively *in vitro* using acute stimuli. Oxidative stressors and cholestatic compounds, such as lipopolysaccharide, estradiol₁₇ β D-glucuronide, glutathione (GSH) depletion, and taurochenodeoxycholate activate signal transduction pathways that retrieve MRP2 from the canalicular membrane (25, 29, 31, 32). Conversely, GSH restoration and the choleretic stimuli (taurochenodeoxycholate and tauroursodeoxycholic acid) can reverse MRP2 retrieval and/or stimulate membrane insertion (1, 30, 33, 35, 37). Although these studies have been conducted in acute settings, MRP2 mislocalization may be regulated by these pathways in human disease. MRP2 mislocalization is a hallmark of certain types of cholestasis, and has been shown in human liver to dissociate from f-actin crosslinking proteins (20, 21). Oxidative stress and bile acid dysregulation play large roles in the pathogenesis of NASH: antioxidant response pathways are upregulated, overall glutathione and GSH:GSSG levels are decreased, and there is a shift in the composition of bile acids (15, 23). These hallmarks of NASH could contribute to MRP2 mislocalization by perturbing these same pathways implicated in acute *in vitro* settings.

One mechanism of MRP2 membrane retrieval involves protein phosphatase 1 (PP1)-mediated dephosphorylation of radixin, which anchors MRP2 to the f-actin cytoskeleton, and subsequent dissociation of radixin from MRP2 (34). PP1 is generally thought to be stimulated by protein kinase Cs (PKC), including PKC δ and/or PKC ϵ (34). Activation of PKA increases the interaction between radixin and Mrp2; the balance of activated PKC and PKA can influence Mrp2 trafficking (33, 34). PKC ϵ phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS), is also implicated in the retrieval process (31). Membrane insertion of MRP2 is mediated through Rab11, an endosomal protein involved in intracellular trafficking of transporters (4, 28). This process may involve PKCs that have other anticholestatic effects, such as PKC δ , or the PKA/PKC α pathway (27, 37). PKA/PKC α has also been shown to be involved in membrane retrieval of MRP2, so there may be more crosstalk between pathways than is currently appreciated (6).

The purpose of this study was to determine the role of transporter trafficking pathways that have been implicated in the complex regulation of MRP2 localization, in human NASH. This was accomplished by measuring the activation and expression of these mediators in human NASH liver. We determined previously in the MCD model of NASH, that membrane protein retrieval related to radixin phosphorylation is activated, while membrane insertion processes mediated by Rab11 and PKC α are impaired, all of which is consistent with observed Mrp2 mislocalization (8). The study described herein identifies which trafficking pathways, if any, are activated in human NASH, and clarifies whether the MCD model shares mechanistic congruity.

Materials and Methods

Sample Acquisition

Frozen and formalin-fixed, paraffin-embedded (FFPE) adult human liver tissue was obtained from the Liver Tissue Cell Distribution System that is coordinated through the University of Minnesota, Virginia Commonwealth University, and the University of Pittsburgh), funded by NIH Contract # HHSN276201200017C. All samples were scored and categorized by a medical pathologist within the Liver Tissue Cell Distribution System. Scoring was performed according to the NAFLD activity score system developed by Kleiner et al. (19) followed by confirmation via histological examination at the University of Arizona. Donor information, including age and gender, was published previously (12). The samples were diagnosed as either normal (n=20), steatotic (n=12), NASH with fatty liver (NASH fatty, n=11), and NASH without fatty liver (NASH not fatty/cirrhosis, n=11). Those samples exhibiting 10% fatty infiltration of hepatocytes were staged as steatotic. Samples were diagnosed as NASH (fatty) when 5% fatty infiltration of hepatocytes occurred with significant inflammation and fibrosis. NASH (not fatty) samples were diagnosed based on a reduction in fatty deposits within the hepatocytes to 5% with more marked inflammation and fibrotic branching. In a previous transcriptomic analysis, a pairwise comparison analysis of the gene expression at the 0.01 level of significance was performed to compare NASH fatty versus NASH not fatty, which were determined to have lack of mechanistic differences despite histological differentiation (22). Because of this comparison and to provide a direct comparison to the MCD model previously published, NASH (fatty) and NASH (not fatty) groups were combined into one NASH group for the Western blots, and steatosis was not included in the analyses.

Protein Preparations

Whole-cell lysate preparations were prepared from 300 mg of tissue homogenized in NP-40 buffer [20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25 ml] at 4°C. Homogenized tissue was then agitated at 4°C for 2 hours and centrifuged at 10,000g for 30 min, and the supernatant was transferred to a clean collection tube. For crude membrane samples, liver was homogenized with 10× buffer [100mM Tris-HCl pH 7.4 with 1 Protease Inhibitor Cocktail tablet/25mL (Roche)], using a dounce homogenizer. Homogenate was centrifuged at 100,000g for 60 min, and the pellet was re-suspended in buffer via sonication. Protein concentrations were determined using the Pierce BCA Protein Quantitation Assay (Thermo Fisher Scientific) per the manufacturer's recommendations.

Immunohistochemistry

Immunohistochemical staining for MRP2 was performed on formalin-fixed, paraffin-embedded samples. Liver sections were deparaffinized in xylene and rehydrated in ethanol, followed by antigen retrieval with Tris-EDTA buffer pH 9.0. Endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide in methanol for 20 minutes. MRP2 staining was performed by overnight incubation (4°C) with anti-MRP2, M8316 (Sigma Aldrich, St. Louis, MO) at a dilution of 1:250, followed by the MACH3 staining kit (Biocare Medical,

Concord, CA) per the manufacturer's protocol. All slides were imaged with a Leica DM4000B microscope and a DFC450 camera (Leica Microsystems, Buffalo Grove, IL).

Western Blot

Fifty micrograms of whole-cell lysate or crude membrane preparation was reduced and denatured in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) and 5% 2-mercaptoethanol at 80°C for 5min. Samples were separated on 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The following antibodies were used to detect each protein: MARCKS (sc-6455), PKA α / β / γ cat (sc-365615), phospho-PKC α (Ser657) (sc-12356) and ERK2 (sc-154) from Santa Cruz Biotechnology, Inc. (Dallas, TX), phospho-MARCKS (Ser152/156) (2741), phospho-PKA C (Thr197) (4781), PKC α (2056), PKC δ (2058), phospho-PKC δ (Thr505) (9374), PKC ϵ (2683), and Rab11 (3539) from Cell Signaling Technology (Danvers, MA), phospho-Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558) (AB3832) from Millipore (Billerica, MA), phospho-PKC ϵ (Ser729) (ab63387) and pan-Cadherin (ab16505) from abcam (Cambridge, UK), and Radixin (MAB2735) from Abnova (Taipei, Taiwan). Pan-Cadherin and ERK2 were used as loading control for blots from membrane and whole cell preparations, respectively. Images were captured using the Image Laboratory Imaging System (Bio-Rad Laboratories) and relative protein quantities were determined using ImageJ software (National Institutes of Health, Bethesda). Data reported are mean $\pm$ S.D., graphed in GraphPad Prism 5.0 (La Jolla, CA), with significance (*) defined to be $p < 0.05$, ** as $p < 0.01$, and *** as $p < 0.001$ in a Student's t-test.

Results

MRP2 Mislocalization in NASH

Human liver samples obtained from the Liver Tissue Cell Distribution System were diagnosed as healthy, simple steatosis, NASH (fatty), and the more advanced state, NASH (not fatty). Hematoxylin and eosin-stained representative samples from each diagnostic group are shown in Figure 1. To assess the cellular localization of MRP2 in healthy and NASH livers, immunohistochemistry was employed to specifically stain for MRP2. Shown in Figure 2, MRP2 is localized to the canalicular membrane in healthy liver samples, while the MRP2 staining in the representative NASH (not fatty) sample exhibits a vesicularized staining pattern indicative of some degree of mislocalization.

Active MRP2 Membrane Retrieval and Inactive Membrane Insertion of MRP2 in NASH

To assess MRP2 membrane retrieval and insertion signaling pathway function in NASH, a comprehensive Western blot approach was used to determine the activation of mediators involved in the processes. The activation statuses were assessed using both the phosphorylation, and by comparing total expression in the whole cell and crude membrane fractions of healthy and NASH livers. Radixin, MARCKS, and Rab11 are substrates for protein kinases, and involved in membrane recycling mechanisms for membrane-bound proteins, including MRP2. The prominent protein kinases in these pathways were also measured, and include PKA, PKC α , PKC δ and PKC ϵ .

Radixin was found to have a decreased phosphorylation status in NASH whole cell fractions compared to control, which is likely due to a decreased overall expression (Figure 3A). No change was found in phosphorylated radixin, or total radixin expression in the crude membrane fraction. These data suggest that there is no change in translocation between the membrane and the cytosol for radixin, but the decreased whole cell phosphorylation indicates a decreased overall activation. MARCKS does not exhibit any significant change in phosphorylation or membrane localization in NASH (Figure 3B), while Rab11 has an increased expression at the membrane, with no overall change in expression, indicating a potential translocation-related activation (Figure 3C).

The results of protein kinase analyses are shown in Figures 4 and 5. PKA has a decreased overall expression in NASH livers, yet exhibits an increased phosphorylation at the membrane, which suggests increased kinase activity (Figure 4A). PKC α exhibits no change in phosphorylation or expression, but has a significantly increased membrane expression, suggesting a translocation of PKC α to the membrane (Figure 4B). In NASH, PKC δ was found to have increased membrane and overall phosphorylation, and an increased membrane expression, despite no change in total expression. This indicates activation both by kinase activity and translocation to the membrane (Figure 5A). PKC ϵ also has a decreased whole cell phosphorylation, and a decreased membrane expression, despite having no change in total expression, suggesting decreased activation by kinase and translocation (Figure 5B).

Discussion

Active membrane retrieval of MRP2 is stimulated by a variety of oxidative stress and choleretic stimuli (26, 29, 31, 32). The purpose of this study was to characterize the putative mediators of MRP2 membrane insertion and retrieval pathways described in the literature, to identify whether any of these pathways are perturbed in human NASH. Analyzing mediators of membrane trafficking, such as radixin, Rab11, and MARCKS, in addition to protein kinases allows us to implicate specific pathways, as kinases are involved in several aspects of NASH pathogenesis. PKC δ regulates insulin signaling pathways and lipid metabolism, and PKA is a regulator of PPAR α and glucose tolerance (13, 24, 36, 38). Understanding kinase activation in the context of a larger pathway is necessary for elucidating their possible role in membrane trafficking of MRP2.

The current study saw a total decrease in phosphorylated species of radixin, which correlates with the decrease in total radixin expression in the whole cell fraction of NASH patients. These decreases are consistent with literature descriptions of MRP2 membrane retrieval. PKC δ activation and increased membrane localization of Rab11 suggest an activated Rab11 pathway. Rab proteins are generally involved in vesicle formation, and tethering of proteins to vesicles to facilitate the recycling processes. cAMP and tauroursodeoxycholate-induced MRP2 membrane insertion has been shown to involve Rab11 specifically, over other isoforms (28). Interestingly, Rab11 is also involved in BSEP translocation, suggesting an overlap in transport regulatory mechanisms (40).

Membrane MARCKS was unaltered, suggesting this pathway is unaltered in NASH; however, the decreased PKC ϵ activation indicates it may still play a role in regulation of

MRP2 or other membrane proteins through other pathways not mediated by MARCKS. For instance, PKC ϵ has been shown to directly phosphorylate human MRP2, and is implicated in tauroolithocholate-induced cholestasis (2, 37). Human NASH liver exhibited increased phosphorylation of PKA, and increased membrane localization of PKC α , suggesting an intact anti-cholestatic mechanism. Overall, our results suggest that the radixin-related membrane retrieval pathway may be activated in human NASH; however, pathways targeting MRP2 to the plasma membrane are also activated.

Our recent study examined these same mediators in the MCD rat model of NASH, and identified distinct differences in MRP2 trafficking pathways (Table 1) (8). There was a perturbation in the radixin pathway, which suggests a conservation in radixin pathway dysregulation between human NASH and the rodent model; except that in humans, the decreased radixin phosphorylation correlates to expression, while rat total radixin levels did not change (8). Unlike in the MCD rats, which had a decreased Rab11 expression, Rab11 in human NASH was found to have an increased membrane localization. MCD rats exhibited decreased PKC α phosphorylation and expression, while human NASH increased membrane translocation but not kinase activity. These differences suggest that MCD rats harbor some degree of impaired membrane insertion mechanisms, human NASH retains active membrane insertion mechanisms.

Besides the present exploration of membrane trafficking, there are other pathways that may influence MRP2 localization. Glycosylation has been identified as an important prerequisite for proper Mrp2 localization and function (7, 39). The human NASH liver samples used in the current study have been reported to have an increased expression of de-glycosylated MRP2 (5, 17). A transcriptomic analysis of the same human NASH samples revealed a downregulation of genes vital to the biosynthesis of N-glycans used in the processing of proteins in the ER (5). Human NASH samples demonstrated a significant loss in the glycosylated forms of several uptake transporters, and of MRP2 (5, 17). These reports strongly suggest that impaired glycosylation processes may contribute to improper localization of MRP2 in human NASH, whereas the current data reveal that several membrane trafficking pathways are dysregulated in human NASH.

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Abbreviations

ADR	adverse drug reaction
MARCKS	myristoylated alanine-rich C-kinase substrate
MCD	methionine and choline deficient
MRP	multidrug resistance-associated protein
NAFLD	nonalcoholic fatty liver disease

NASH	nonalcoholic steatohepatitis
PK	protein kinase
PP1	protein phosphatase 1

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Figure 1. Progression of human NAFLD. Hematoxylin and eosin-stained liver sections of representative healthy, steatosis, NASH (fatty) and a fibrotic, pre-cirrhotic NASH (not fatty) human liver samples. Black arrows indicate microvesicular steatosis, white arrows indicate macrovesicular steatosis, and asterisks indicate regions of fibrotic lesions.

Figure 2. MRP2 Mislocalization in Human NASH. Immunohistochemical staining of MRP2 in healthy and NASH (not fatty) livers indicate proper canalicular staining of MRP2 in healthy samples, and instances of vesicularized staining patterns in NASH samples (black arrows). White arrows indicate proper localization of MRP2 in NASH.

Figure 3. Western Blot Phosphorylation and Translocation Analyses for Kinase Substrates involved in MRP2 Membrane Recycling. Radixin, 80 kDa (A), MARCKS, 80 kDa (B), and Rab11, 25 kDa (C) were analyzed in healthy and NASH livers for whole cell and membrane phosphorylation and total expression (n=7-9). Erk2, 42 kDa and Cadherin, 135 kDa were used as loading controls for whole cell and membrane fractions, respectively. Data represent mean $\pm$ S.D., where * denotes $p < 0.05$, and *** denotes $p < 0.001$ after a Student's t-test.

Figure 4. Western Blot Phosphorylation and Translocation Analyses for the PKA/PKC α Pathway of Mrp2 Membrane Insertion. PKA, 40 kDa (A), and PKC α , 80 kDa (B) were analyzed in healthy and NASH livers for whole cell and membrane phosphorylation and total expression (n=7–9). Erk2, 42 kDa and Cadherin, 135 kDa were used as loading controls for whole cell and membrane fractions, respectively. Data represent mean $\pm$ S.D., where ** denotes p 0.01, and *** denotes p 0.001 after a Student's t-test.

Figure 5. Western Blot Phosphorylation and Translocation Analyses for PKCδ and PKCε. PKCδ, 78 kDa (A), and PKCε, 82 kDa (B) were analyzed in healthy and NASH livers for whole cell and membrane phosphorylation and total expression (n=7–9). Erk2, 42 kDa and Cadherin, 135 kDa were used as loading controls for whole cell and membrane fractions, respectively. Data represent mean ± S.D., where * denotes p 0.05, ** denotes p 0.01, *** denotes p 0.001 after a Student's t-test.

Table 1

Activation Status of Membrane Localization Mediators in Human NASH and the MCD rat model of NASH.

Mediator	Human NASH	MCD Rat ^a
Radixin	Decreased activation and expression	Decreased activation, increased membrane
MARCKS	No change	Decreased activation
Rab11	Increased membrane	Decreased expression
PKA	Decreased activation at membrane	Decreased expression, increased membrane
PKC α	Increased membrane	Decreased activation
PKC δ	Increased activation and membrane	Increased expression, increased membrane
PKC ϵ	Decreased activation and membrane	Increased membrane

^aResults described here were published previously (8)