Proteomic and bioinformatics analysis of membrane lipid domains after Brij 98 solubilization of
uninduced and phenobarbital-induced rat liver microsomes: Defining the membrane localization of the
P450 enzyme system

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Nonstandard Abbreviations: CID - collision-induced dissociation; CYP or P450 – cytochrome P450; ER – endoplasmic reticulum; IAA – Iodoacetamide; HCD - high-energy collision dissociation IPA – ingenuity pathway analysis; LC-MS – liquid chromatography-mass spectrometry; PB – phenobarbital-induced; TCEP - Tris(2-carboxyethyl)phosphine; TMT – tandem mass tags; UGT – UDP glucuronosyl transferase.

Abstract

The proteomes of ordered and disordered lipid microdomains in rat liver microsomes from control and phenobarbital (PB)-treated rats were determined after solubilization with Brij 98 and analyzed by Tandem Mass Tag (TMT)-LC-MS. This allowed characterization of the liver microsomal proteome and the effects of phenobarbital-mediated induction, focusing on quantification of the relative levels of the drug metabolizing enzymes. The microsomal proteome from control rats was represented by 333 (23%) proteins from ordered lipid microdomains, 517 (36%) proteins from disordered lipid domains, and 587 (41%) proteins that uniformly distributed between lipid microdomains. Most enzymes related to drug metabolism were mainly localized in disordered lipid microdomains. However, CYP1A2, multiple forms of CYP2D, and several forms of UDP glucuronosyltransferases (UGT1A1 & UGT1A6) localized to ordered lipid microdomains. Other drug metabolizing enzymes, including several forms of cytochromes P450, were uniformly distributed between the ordered and disordered regions. The redox partners, NADPH-cytochrome P450 reductase and cytochrome b₅, localized to disordered microdomains. PB induction resulted in only modest changes in protein localization. Less than five proteins were variably associated with the ordered and disordered membrane microdomains in PB and control microsomes. PB-induction was associated with fewer proteins localizing in the disordered membranes, and more being uniformly distributed or localized to ordered domains. Ingenuity Pathway Analysis (IPA) was used to ascertain the effect of PB on cellular pathways, resulting in attenuation of pathways related to energy storage/utilization and overall cellular signaling and an increase in those related to degradative pathways.

Significance Statement

This work identifies the lipid microdomain localization of the proteome from control and phenobarbital-induced rat liver microsomes. Thus, it provides an initial framework to understand how lipid/protein segregation influences protein-protein interactions in a tissue extract commonly used for

studies in drug metabolism and uses bioinformatics to elucidate the effects of phenobarbital induction on cellular pathways.

Introduction

Biological membranes comprise a diverse set of lipids and proteins. Lipid heterogeneity is a hallmark of the cellular and organelle lipid boundaries. In the plasma membrane, heterogeneity is displayed by the coalescence of lipids into ordered microdomains enriched in cholesterol, ethanolamine-containing glycerophospholipids, glycosphingolipids, and straight-chain fatty acyl moieties that facilitate dense packing. These domains, known as lipid rafts, exist in a surrounding disordered milieu containing phospholipids with a preponderance of polyunsaturated fatty acyl moieties (Pike, 2003). Specific proteins preferentially segregate into these lipid rafts in the plasma membrane and are implicated in a number of cellular processes (Roper et al., 2000) (Knorr et al., 2009) (Sankarshanan et al., 2007; Otahal et al., 2010). The interplay of proteins and lipids in driving the formation of ordered domains in the membranes is a complex dynamic (Hemler, 2005; Parton and Simons, 2007; McMahon et al., 2009).

The existence of lipid microdomains is not limited to the plasma membrane but also has been observed in the membranes of internal organelles (Browman et al., 2006; Ouweneel et al., 2020). In particular, lipid microdomains have been identified in the endoplasmic reticulum where they play roles in protein folding and degradation (Sarnataro et al., 2004; Campana et al., 2006); protein/lipid trafficking (Hayashi and Su, 2010; Boslem et al., 2013); and calcium signaling (Hayashi and Fujimoto, 2010). Because the ER has significantly lower levels of cholesterol and sphingomyelin, the ordered microdomains in this organelle are less pronounced than in the plasma membrane and have not been as well characterized.

The cytochromes P450 (CYP or P450) represent a superfamily of enzymes expressed in both vertebrate and invertebrate species that are involved in the metabolism of a wide variety of hydrophobic compounds (Nelson, 2003). The mammalian P450 enzyme members from the CYP1, CYP2, and CYP3 families play critical roles in the metabolism of pharmaceutical compounds. Each individual member

has a wide-ranging substrate specificity that is unique but overlapping with those of the other P450 forms from these families.

The activity of the P450 enzyme system within a given mammalian species is characterized by a high amount of interindividual variability that can partly be attributed to genetic polymorphisms and inducibility. The promoter regions of many P450 genes contain binding sequences for liver specific transcription receptors (Chen et al., 2012). One of the most studied inducers of P450 is the CAR agonist, phenobarbital (PB) (Waxman and Azaroff, 1992). Activation of CAR by PB results in dramatic induction of a range of enzymes including CYP2B and CYP3A. This effect is accompanied by significant changes in membrane lipid composition (Ariyoshi and Takabatake, 1972; Davison and Wills, 1974).

The conventional way of identifying ordered lipid microdomains in biological membranes utilizes their resistance to detergent solubilization. Originally researchers used the non-polar, Triton X-100 to solubilize the membrane, and the ordered microdomains were isolated on a discontinuous sucrose gradient after ultracentrifugation (Brown and London, 1997; London and Brown, 2000). Subsequently, other detergents have been used for this purpose (Riske et al., 2017). One of the limitations of many of the detergents used to isolate ordered lipid microdomains is that the microdomains are only resistant to solubilization at 4°C. This fact has led to the suggestion that the microdomains are not relevant at physiologic temperatures. However, ordered microdomains have also been resistant to solubilization by a number of detergents at 37°C (Schuck et al., 2003; Casadei et al., 2014; Riske et al., 2017).

We have used one of these detergents, Brij 98, in studies of ordered lipid microdomains because of its usefulness at physiologic temperatures (Knorr et al., 2009). Using this detergent, our lab has shown that the different forms of drug-metabolizing P450s selectively segregate into ordered and disordered microdomains in rabbit liver microsomes (Park et al., 2014; Brignac-Huber et al., 2016). Similar to ordered domains in the plasma membrane, the P450 ordered microdomains are also dependent on cholesterol (Brignac-Huber et al., 2011; Brignac-Huber et al., 2013).

In this study, we used Brij 98 solubilization and quantitative, discovery-based Proteomics using tandem mass tags (TMT) and liquid chromatography mass spectrometry (LC-MS) to determine the lipid microdomain distribution of the proteome in rat liver microsomes with emphasis on drug-metabolizing enzymes. Because of the profound effects of PB treatment on protein expression and lipid biosynthesis, we examined the microsomal proteomes from both uninduced and PB-treated rats in order to determine if protein and lipid variability influence the microdomain localization of proteins. Furthermore, we used bioinformatics (IPA) to determine the influence of PB-mediated induction on cellular pathways.

Materials and Methods

Materials

Phenobarbital (PB) was purchased from Mallinckrodt (St. Louis, MO). Brij 98 was purchased from Acros Organics (Distributed by Thermo Fisher (Fair Lawn, NJ)). The 100 x protease/phosphatase inhibitor cocktail used in the preparation of microsomes was purchased from Cell Signaling Technology (Danvers, MA). The protease inhibitor used in the Brij 98-mediated solubilization of microsomes was the "Complete" EDTA-free tablet from Sigma (St. Louis, MO). The primary antibodies used were as follows: Anti-CYP1A1 from Everest Biotech (Oxfordshire, UK) (EB11171) used at 1:500; Both Anti-CYP3A (PM40) and anti-CYP2E1 (PA26) were from Oxford Biomedical (Rochester Hills, MI) and were used at 1:1000 and 1:4000, respectively; Anti-CYP2D6 (MBS821765) from MyBioSource (San Diego, CA) was used at 1:1000. Protein standards used for westerns were purified rabbit CYP1A2 ((Backes et al., 1998); purified, recombinant rabbit CYP2E1 (Cheng et al., 2004); purified N-truncated, recombinant CYP3A4 (Moutinho et al., 2012); and CYP2D6 human supersomes (Gentest-Corning). All other chemicals of reagent quality that were used were purchased from Sigma.

Methods

Preparation of Liver Microsomes: All animals used in this study were treated according to IACUC restrictions to minimize suffering. Six male Sprague Dawley rats were used in the study. Three rats were administered sodium phenobarbital by intraperitoneal injection as described previously (Cawley et al., 2001) (once daily for three days at a dose of 80 mg/kg in 0.9% saline). Three control rats were treated with an equivalent volume of the saline solution. The day following the last injection, the animals were sacrificed by decapitation and microsomes were isolated by differential centrifugation (Sequeira et al., 1994) with the following modifications. The liver was diced and washed three times in a solution of ice-cold 0.25M sucrose (pH 7.0). Following the final wash, the liver pieces were suspended in a homogenization buffer of 10 mM HEPES (pH 7.5) and 0.25 M sucrose containing 100

μ M PMSF and a 100x phosphatase/protease inhibitor cocktail. The 0.25 M sucrose was added at a volume that was 4 times the weight (in g) of the collected liver. The liver was homogenized with 6 passes in a 50 mL Potter-Elvehjem homogenizer. The homogenized liver was filtered through cheesecloth and centrifuged at 25,000 x g for 10 min. The resulting supernatant was carefully decanted and was centrifuged for 1 hr at 136,000 x g. The resulting pellet from 14 g of starting liver weight was resuspended in 20 ml of 0.15 M KCl and centrifuged at 136,000 x g for 1 hr. The final microsomal pellet was resuspended in 10 mM HEPES (pH 7.5) in a volume that was 0.6 ml/starting liver weight (g). The final microsomal suspensions were all diluted to 15 mg/ml in protein using the bicinchoninic acid assay method (Pierce-Thermo) to assay protein levels and were then stored at -80°C.

Solubilization of microsomes with Brij 98: Liver microsomes were solubilized using Brij 98 as described previously (Brignac-Huber et al., 2011; Park et al., 2014) with the following modifications. A solution of 10% Brij 98 was prepared by preheating solubilization buffer (containing 0.05 M HEPES-NaOH (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, and a Complete EDTA-free, protease inhibitor tablet) at 40-50°C. The pre-warmed solubilization buffer (0.9 ml) was then added to 0.1 ml of Brij 98 and vigorously vortexed until the detergent crystals were completely dissolved. The microsomal protein was solubilized at a concentration of 2 mg/ml in 1% Brij 98. Specifically, the Brij 98 solution was diluted in solubilization buffer and microsomes were added subsequently so that the Brij concentration was reduced as much as possible before exposing the microsomes to detergent. The microsomes were then solubilized by subjecting the samples to a brief pulse (the minimal amount of time to agitate and mix the sample) with a vortex at half-maximal setting followed by incubation at 37°C for 5 min. Afterwards, the samples were placed on ice, and 2 ml of ice-cold solubilization buffer was added to stabilize centrifuge tubes (7 ml) during ultracentrifugation at 100,000 x g for 1 hour. After centrifugation, supernatants were immediately separated from the pellets. The pellets were then resuspended in 3 ml of solubilization buffer with protease inhibitors by five passes in a 5 ml Potter-Elvehjem homogenizer on ice. Control samples were treated the same except that they were not treated with Brij 98, and the pellets were directly resuspended in their corresponding supernatants after centrifugation. Samples

from each rat microsomal sample were then stored at -80°C as Brij-treated supernatants and pellets and controls that were not subjected to Brij 98.

In the quantitative proteomic analysis, disordered proteins were defined as those that were depleted in the pellet relative to the supernatant after Brij 98 treatment (Pellet/Supernatant < 1; p-value < 0.05), and proteins from ordered microdomains were defined as those with a Pellet/Supernatant ratio > 1 (p-value < 0.05). Proteins with pellet/supernatant values that were not significantly different from 1 were taken to be proteins that were equally distributed between ordered and disordered domains and were designated as “uniform”.

Western blot analysis: Aliquots (0.15 ml) of the Brij-treated supernatants and pellets, and controls that were not subjected to Brij 98 were diluted with 4 x loading buffer (containing 5% BME) for western blotting analysis (see below). Four micrograms of protein from each sample were loaded on a 10 % polyacrylamide bis-Tris gel and were run at 150 v for 1.25 hr. The gels were transferred to nitrocellulose using the Power Blotter system (Pierce-Thermo). Blots were blocked for 1 hour in PBS containing 0.05% Tween 20 (pH 7.4) and 2% bovine serum albumin. The blots were then incubated in primary antibody by gentle rocking overnight at 4°C. The following day, the blots were washed in PBS with 0.05% Tween 20 and then incubated in anti-IgG-peroxidase conjugates (Sigma) for relevant species at 1:4000 dilutions for 1 hour. After 1 hour at room temperature, blots were washed and visualized by chemiluminescence using the Super signal West Pico kit (Pierce-Thermo Scientific). The primary antibodies used were as follows: CYP 1A - 19936-1-AP from Protein Tech (Rosemont, IL) at 1:1000 ; CYP2E1 – PA26 from Oxford (Riviera Beach, FL) at 1:4000; CYP2D - MBS821765 from Mybiosource (San Diego, CA) at 1:1000; and CYP3A – PM40 from Oxford at 1:1000.

Discovery-based Proteomics using Tandem Mass Tags (TMT) and Liquid Chromatography Mass Spectrometry (LC-MS): Aliquots (0.9 ml) of each Brij 98-treated supernatant and pellet and the corresponding controls (described above) were diluted with 10% SDS to a final concentration of 1% SDS. Samples were prepared for discovery-based quantitative proteomic analysis by sonication until

homogenous. The protein concentration was determined using BCA protein assay kit (Pierce, Thermo Scientific) using an 8 point standard curve. Based on the protein concentration, 100 µg of each protein sample was prepared for trypsin digestion by reducing the cysteines with Tris(2-carboxyethyl)phosphine (TCEP) followed by alkylation with Iodoacetamide (IAA). After chloroform-methanol precipitation, 100 µg of protein per sample was digested with 2 µg sequencing grade trypsin (Pierce-Thermo Scientific) overnight at 37°C. The digested product was labeled using 2-TMT 10-plex Reagent sets (Pierce-Thermo Scientific), utilizing the 131 isobaric tag as a pooled internal control, according to the manufacturer's protocol.

An equal amount of each TMT-labelled sample was pooled together in a single tube and SepPak purified (Waters, Ireland) using acidic reverse phase conditions to remove unreacted TMT and quenched-TMT molecules. After drying to completion, an off-line fractionation step was employed to reduce the complexity of the sample. The sample was brought up in 10 mM ammonium hydroxide, pH 10. This mixture was subjected to basic pH reverse phase chromatography (Dionex U3000, Thermo Fisher) as described previously (Harman et al., 2018). Briefly, 48 – 200 µl fractions were combined into 12 “super fractions” in a checkerboard fashion. The 12 “super fractions” were then run on a Dionex U3000 nano-flow system coupled to a Thermo Fisher Fusion Orbitrap mass spectrometer. Each fraction was subjected to a 95-min chromatographic method employing a gradient from 2-25% ACN in 0.1% formic acid (FA) (ACN/FA) over the course of 65 min, a gradient to 50% ACN/FA for an additional 10 min, a step to 90% ACN/FA for 5 min, and a 15-min re-equilibration into 2% ACN/FA. Chromatography was carried out in a “trap-and-load” format using an EASY-Spray source (Thermo); trap column C18 PepMap 100, 5 µm, 100 Å and the separation column was an EASY-Spray PepMap RSLC C18 2 µm, 100Å, 75 µm x 25 cm (Thermo Fisher Dionex, Sunnyvale, CA). The entire run was at a flow rate of 0.3 µl/min. Electrospray was achieved at 1.8 kV.

TMT data acquisition utilized an MS3 approach for data collection, as previously described (Yue and Guidry, 2019). Survey scans (MS1) were performed in the Orbitrap utilizing a resolution of

120,000. Data-dependent MS2 scans were performed in the linear ion trap using a collision-induced dissociation (CID) of 25%. Reporter ions were fragmented using high-energy collision dissociation (HCD) of 65% and detected in the Orbitrap using a resolution of 50,000 (MS3). This was repeated for a total of four technical replicates. The 4 runs of 12 “super fractions” were merged and searched using the SEQUEST HT node of Proteome Discoverer 2.4 (Thermo). The Protein FASTA database was *Rattus norvegicus*, SwissProt tax ID=10116, version 2017-10-25 and contained 9616 sequences. Static modifications included TMT reagents on lysine and N-terminus (+229.163), carbamidomethyl on cysteines (+57.021), dynamic phosphorylation of Serine, Threonine and Tyrosine (+79.966Da), and dynamic modification of oxidation of methionine (+15.9949). Parent ion tolerance was 10 ppm, fragment mass tolerance was 0.6 Da for MS2 scans, and the maximum number of missed cleavages was set to 2. Only high scoring peptides were considered utilizing a false discovery rate (FDR) of < 1%, and only one unique high-scoring peptide was required for inclusion of a given identified protein in our results. Multiplexed TMT 10-plex experiments were quantified using “Controls-On-Average” using the 131 reporter ion (pooled control) in Proteome Discoverer 2.4. The mass spectrometry proteomics data was deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022460 and 10.6019/PXD022460 (Perez-Riverol et al., 2019). *Ingenuity Pathway Analyses*: Following completion of data acquisition and analysis in Proteome Discoverer 2.4, bioinformatic analyses were performed using Qiagen’s Ingenuity Pathway Analysis (IPA) software to analyze quantitative pairwise mass spectrometry data; specifically PB control/Saline control. One initial ‘Core Analysis’ was performed in IPA for the comparison (see Results for detail). In our bioinformatics analyses, a z-score threshold of $\geq \pm 2$ was applied, which represents a $p < 0.05$ metric for the non-randomness of directionality of a given dataset. Top Canonical Pathways were reported in both tabular and heat map imagery. Mass Spec IPA Analysis content information: Report Date: 2019-11-22, Report ID: 18557437, Content Version: 49309495 (Release Date: 2019-08-30). SOMA IPA Analysis content information: Report Date: 2019-11-22, Report ID: 18557437, Content Version: 49309495 (Release Date: 2019-08-30).

Results

A. Effects of Phenobarbital Treatment

Proteomics of microsomes from saline- and PB-treated rats: Our proteomic data, derived from TMT LC-MS analysis and shown in the ProteomeXchange Consortium repository (see Materials and Methods) detected 1435 and 1349 proteins in liver microsomes from Saline- and PB-treated rats, respectively. The data indicate the relative levels in the saline and PB controls (cntl) that were not treated with Brij 98 in addition to the supernatants (sup) and pellets (pel), resulting from Brij 98 treatment followed by ultracentrifugation. The data also show the measurements of statistical significance of the compared values. Proteins that were significantly enriched in the supernatant relative to the pellet are considered to reside in “disordered” domains; whereas, those significantly enriched in the pellet are described as residents of “ordered” or detergent-resistant microdomains. Proteins that did not have statistically different levels in the supernatants and pellets were considered equally distributed between the domains and were designated as “uniform”.

Supplementary Table S1 lists the proteins that showed significant changes in expression after treatment with PB. Eighty-eight proteins had elevated expression, and 405 proteins had decreased expression upon PB treatment (p-value less than 0.05). In addition, 108 proteins were detected only in PB microsomes; whereas, 197 other proteins were detected only in microsomes from saline-treated rats. Figure 1 is a volcano plot showing the effects of PB on enzyme expression relative to the statistical significance of the comparisons. The expression levels of many more enzymes were inhibited than were stimulated by PB treatment. The majority of the enzymes showing PB-related changes in expression experienced only modest (but statistically significant) levels of alteration (<2-fold). However, PB did dramatically induce (> 4-fold) a limited number of enzymes.

Concerning enzymes involved in drug metabolism, CYP2B1, CYP2B2, CYP2C6, CYP2C55, CYP2G1, and CYP3A showed over 50% higher expression in PB-treated microsomes (Table S1). Of these proteins, CYP2B1, CYP2C55, CYP2G1, and CYP3A9 all showed 50-fold or greater expression in the microsomes from PB-treated rats (actually, CYP2C55 and CYP3A9 were not observed in the saline group of proteins). CYP4A12 was observed in saline microsomes but not in PB microsomes, and CYP2E1 had about 60% lower expression after PB treatment. The expression levels of NADPH-cytochrome P450 reductase (+1.7-fold), epoxide hydrolase (+2.1-fold), UDP glucuronosyl transferases (UGT) 1A1 (+ 1.43-fold), UGT1A7 (not observed in uninduced microsomes), UGT2B2 (+ 1.16-fold), UGT2B1 (+ 2.8-fold), and flavin monooxygenase 5 (+1.79-fold), also were elevated after PB treatment (Table S1). P450s that were expressed at lower levels after PB treatment were CYP1A2 (- 45%), CYP2B3 (- 40%), CYP2C13 (-30%), CYP2E1 (-60%), CYP2J3 (- 22%), CYP4A10 (- 40%), CYP4F6 (- 30%), and CYP4V2 (- 18%).

In the case of CYP3A9, the protein is expressed in a sexually dimorphic pattern, having hepatic mRNA levels as much as 28-fold higher in female rats (Anakk et al., 2003). CYP3A9 levels are elevated by growth hormone and estrogen treatment (Wang and Strobel, 1997; Robertson et al., 1998). Interestingly, CYP3A9 mRNA is induced by treatment with phenobarbital (Mahnke et al., 1997), which can explain why detectable CYP3A9 levels were observed in the PB-treated group.

Another P450 enzyme of interest was CYP2E1, which was decreased by about 60% after phenobarbital treatment in the current study (Supplemental Table 1). Literature reports on the effect of phenobarbital on hepatic CYP2E1 are mixed, with some reports showing induction (Caron et al., 2005), and others not reporting any significant induction (Anzenbacher and Anzenbacherova, 2001; Kim et al., 2001; Ejiri et al., 2005). Part of this discrepancy may be due to the focus on phenobarbital as a CYP2B inducer, and many studies have not reported on the ability of the agent to affect CYP2E1. Regardless, the results of the present study suggest only a small effect of phenobarbital on CYP2E1.

Ingenuity Pathway Analysis (IPA) to determine the effects of PB treatment on cellular pathways:

A direct comparative IPA of the proteomic data for the PB control (not treated with Brij 98) and saline control samples indicates the cellular pathways influenced by PB treatment (Figure 2). Pathways with Z-scores having absolute values greater than 2.0 as indicated by orange or blue shading corresponding to the extent to which the pathway is up- or down-regulated, respectively, relative to saline control microsomes. Pathways with p-values less than 0.05 ($-\log(p\text{-values}) > 1.3$) indicate that the number of pathway proteins showing significant PB-related changes in expression are statistically greater than one would expect from random sampling. The pathways down-regulated by PB are mainly related to energy storage/utilization (e.g. glycolysis I, ketogenesis, oxidative phosphorylation, fatty acid β -oxidation, and gluconeogenesis) and cellular signaling in general (e.g. sirtuin signaling, insulin secretion signaling, RhoA signaling, xenobiotic metabolism related to AHR signaling, actin signaling, PPAR α /RXR α activation, VEGF signaling, paxillin signaling, necroptosis signaling, AMPK signaling, signaling by Rho family GTPases, estrogen receptor signaling, and renin-angiotensin signaling). Most of the upregulated pathways following PB treatment are degradative in nature (e.g. nicotine II and III, melatonin I, acetone I, and bupropion), related in large part to altered expression of participating P450 enzymes. Estrogen biosynthesis was also slightly upregulated by PB.

B. Microdomain Localization of Microsomal Proteins

Brij 98 solubilization of microsomes: Brij 98-mediated solubilization of liver microsomes from saline- and PB-treated rats at 37°C was performed as described in Materials and Methods. In a previous study, we compared the conventional method of isolating ordered lipid microdomains by flotation on a discontinuous sucrose gradient after ultracentrifugation to one in which the Brij 98-insoluble (detergent resistant) membranes were directly pelleted by ultracentrifugation in HEPES solubilization buffer (Park et al., 2014). The results showed the latter method was comparable to the conventional method in the assignment of multiple P450s in different lipid microdomains. Figure 3 shows western blotting results comparing the distribution of P450s in lipid microdomains after Brij 98 treatment of rat liver microsomes.

As observed with rabbit liver microsomes (Park et al., 2014), CYP1A2 and CYP2E1 distributed mainly to ordered and disordered domains, respectively in rat liver microsomes. In addition, our findings show for the first time that CYP2D forms tend to reside primarily in ordered microdomains; whereas, CYP3A forms partition largely into disordered lipid microdomains.

The TMT LC-MS allowed us to categorize the lipid microdomain localization of over 1300 proteins in rat liver microsomes. As a separate means of validating the TMT LC-MS analyses, the relative levels of four P450s in the Brij 98-treated supernatants and pellets were compared to the relative levels as determined from western blot densitometry (Table 1). Both techniques result in identical assignments with respect to membrane localization for the different forms of P450 although the ratios of pellets to supernatants varied with the different applications. The western blotting values were measured with respect to the proportionality of the band densitometry and not from ratios of actual abundances (as would be determined by reference to a standard curve), so this might explain the quantitative discrepancies in the pellet/supernatant values for the two methods.

With respect to the entire collection of liver microsomal proteins, Fig. 4 shows the percent distributions from control and PB-induced microsomes into ordered, disordered, and uniformly distributed groups. In control microsomes, there were roughly equal proportions of “uniform” and “disordered” proteins ($\approx 41\%$ and 36% , respectively) and approximately half as many proteins in the ordered lipid microdomain group ($\approx 23\%$). PB induction was associated with an increased proportion of proteins in the uniform group ($\approx 45\%$) at the expense of those in the disordered group ($\approx 29\%$), and 113 proteins followed this trend. The proportion of proteins in ordered lipid microdomains was roughly unchanged after PB induction.

When examining the localization of specific proteins, PB did not lead to major changes in relocalization. Although PB caused moderate changes in distribution of some proteins (e.g. ordered to uniform, or disordered to uniform and *vice versa*), very few proteins completely shifted from ordered to disordered localization or *vice versa*. Only starch-binding domain-containing protein 1 (SWISS-

PROT/TrEMBL identifier: Q5FVN1), glycogen phosphorylase (P09811), and apolipoprotein F (Q5M889) were assigned to disordered domains in saline microsomes and ordered in PB microsomes, whereas, only dynamin-like 120 kDa protein (Q2TA68) was localized in the ordered microdomains of control microsomes but in the disordered domain of PB microsomes. Although there was a dearth of examples of proteins showing a dramatic change from ordered to disordered localization and vice versa, there was a clear trend for an increase in protein distribution to more ordered localization after PB treatment. For instance, 51 proteins classified as uniformly distributed in saline microsomes were categorized as ordered in PB-treated microsomes; whereas, the reciprocal change (ordered in saline to uniform in PB-treated) was represented by only 29 proteins. Similarly, 113 proteins changed from localization in disordered membrane in saline microsomes to uniformly distributed in PB microsomes; whereas, only 29 proteins showed the opposite trend (uniform to disordered). Thus, the overall effect of PB induction was the protein distribution shifted to a higher degree of residence in an ordered lipid environment.

Table 2 compares our results and those of other studies that have identified protein markers from ordered microdomains with two types of Triton detergents. Because of our interest in the P450 system, the table includes many markers from ordered microdomains identified in the ER where most P450s are localized. As shown in previous studies in which multiple detergents are compared by the same laboratory using an identical method for membrane solubilization with each detergent, the ordered microdomains isolated with the various detergent have different collections of proteins (Schuck et al., 2003; Kim et al., 2004; Hayashi and Su, 2010). For instance, the sigma 1 receptor was in ordered lipid microdomains when isolated by Triton X-100 and Triton X-114 solubilization but not when isolated by Tween 20, CHAPS, or Brij 35 (Hayashi and Su, 2010). Although this putative marker was enriched in the disordered fraction after Brij 98 treatment (as it was with solubilization by Brij 35), the sigma intracellular receptor 2 was dramatically partitioned to the ordered microdomains in the current study (Pellet:Supernatant = 40:1 to 50:1).

Without additional steps to purify liver microsomes, those derived directly by differential centrifugation invariably contain admixtures from other cellular membranes besides the endoplasmic reticulum (Galeva and Altermann, 2002; Peng et al., 2012). Because discovery-based LC-MS is extremely sensitive, proteins present at quantitatively negligible levels can be detected. Using our TMT LC-MS data in the proteome repository and NCBI Gene Ontology-defined cellular components, excellent markers for Brij 98 ordered lipid microdomains in membranes from various cellular compartments were identified. Some of these include the nucleolar protein 16 (Q1RP77) for nuclear membrane; phosphate carrier protein (P16036) for mitochondria; translocon-associated protein subunit delta (Q07984) for endoplasmic reticulum; UDP-N-acetylglucosamine transporter (Q6AXR5) for Golgi; and band 3 anion transport protein (P23562-1) for plasma membrane.

Brij 98-mediated solubilization of P450 Monooxygenase system and other enzymes related to drug metabolism: The TMT LC-MS detected a large number of P450s that affords a detailed categorization of membrane domain localization for the liver representatives of this superfamily (Table 3). Similar to our previous results with rabbit liver microsomes, rat liver CYP1A2 localized to ordered lipid microdomains. The only other P450s to localize preferentially in ordered lipid microdomains as detected by TMT LC-MS are 5 members of the CYP2D family (although CYP2D4 is evenly distributed in the two domains in PB-induced microsomes). Most of the P450s show a preference for disordered regions of the microsomes. Only CYP2C7, CYP2C13, and the three CYP4A members show even distribution in both control and PB-induced microsomes. The CYP4As tended to have pellet/supernatant values > 1, and this trend was observed in preliminary experiments. Thus, it is possible that the members from the CYP4A subfamily also preferentially partition into the ordered lipid microdomains. When comparing microsomes from saline- and PB-treated rats, no P450s were found in ordered lipid microdomains in one type of microsomes and in disordered regions in the other. When a difference was observed, the P450 typically localized in disordered lipid regions in control and was uniformly distributed in PB microsomes (Table 3).

With regard to other proteins that participate in the P450 reaction, NADPH-cytochrome P450 reductase and cytochrome b5 preferentially partition to the disordered region. Other enzymes involved in drug metabolism also show variable localization. The three members of flavin monooxygenases fractionate mainly to disordered regions, and UGTs show variable localization in the same manner as P450s. UGT1A1 and UGT1A6 are enriched in ordered microdomains. UGT2B15 and UGT2B2 are partitioned preferentially to disordered regions, and UGT1A5, UGT1A7, UGT2B17, and UGT2B37 are evenly distributed between the two domains.

Discussion

Comparison to other proteomic studies: This study has used quantitative mass spectrometry analysis through TMT and LC-MS to determine the proteomes of disordered and ordered membrane lipid microdomains after Brij 98 solubilization of liver microsomes from control and PB-treated rats. Previous studies have examined the proteomes of liver microsomes from control and PB-treated rats (Galeva and Altermann, 2002) (Wu et al., 2011) (Klepeisz et al., 2013). Similarly, other studies have used bioinformatics to ascertain related cellular pathways in the liver (Peng et al., 2012) and the effects of PB on cellular pathways (Klepeisz et al., 2013). Our analysis identified over 1350 proteins in control and PB-induced microsomes. All of the previous proteomic studies were qualitative or semi-quantitative in distinguishing the effects of PB by using gel localization of protein bands to ascertain proteomic differences in control and PB samples. Thus, of all these studies, ours is the only one to quantify the relative levels of proteins in PB and control samples using TMT-LC-MS. A comparison of these studies demonstrates the advancement in techniques and capability for proteomic analysis over the last two decades.

Although there have been numerous mass spectrometry (MS) studies that have analyzed lipid raft proteomes (reviewed in Foster, L.J. 2007), our study is atypical in using a direct pelleting method to

isolate the DRMs. This modification allowed for a direct comparison of detergent-resistant and detergent-soluble proteomes, which in turn facilitated quantitation of the relative protein levels. The previous proteomic studies have almost invariably isolated DRMs by flotation on a discontinuous sucrose gradient. Thus, the protein constituents from ordered lipid microdomains have been analyzed solely and specifically by MS in the earlier proteomic studies. Conversely, our study using quantitative TMT LC-MS to group domain proteins by their relative abundance in each fraction, allowed us to also distinguish proteins from disordered lipid regions and those that uniformly distribute between ordered and disordered lipid microdomains. To our knowledge, this distinction has not been determined in previous studies of membrane lipid microdomains.

Lipid microdomain distribution of drug-metabolizing enzymes: The main interest of our lab relates to the membrane organization of the drug metabolizing enzymes. As a first step in elucidating the potential biological role(s) of lipid microdomain localization of P450s in drug metabolism, this discovery-based proteomic study provided a comprehensive assignment of the different liver P450s to lipid microdomains in microsomes with over thirty P450s being detected in control and PB-treated rat microsomes (Table 2). Representative P450s are found in each of the three categories of membrane proteins that we have defined here (ordered lipid microdomains, disordered lipid microdomains, and uniformly distributed). Most of the P450s were associated with the disordered region. Only CYP1A2 and the CYP2D members predominantly localized to the ordered lipid microdomains. With respect to other drug metabolizing enzymes, UDP-glucuronosyltransferases 1A1 and 1A6 also partitioned to the ordered domains. Flavin monooxygenases, NADPH-cytochrome P450 reductase, and cytochrome b₅ were mainly localized in disordered lipid regions of the membrane.

Effects of PB-induction on the localization of proteins to lipid microdomains: Because PB treatment has been shown to lower the proportions of both cholesterol and sphingomyelin in the rat endoplasmic reticulum (Davison and Wills, 1974), it was thought there might be a noticeable effect on protein localization. In terms of the distribution of total proteins, the relative proportions are not

dramatically different in PB and control microsomes. Only four proteins (starch-binding domain containing protein (Q5FVN1), glycogen phosphorylase (P09811), apolipoprotein F (Q5M889), and dynamin-like protein (Q2TA68)) changed from predominantly ordered to disordered microdomains or vice versa. Surprisingly, three of these proteins (apolipoprotein F, starch binding protein, and glycogen phosphorylase) are classified as molecular components of cytoplasm. Each of these “cytosolic” proteins were identified as being localized to disordered membrane in saline microsomes and to ordered lipid microdomains in PB microsomes.

The discovery-based LC-MS method we used is extremely sensitive. Thus, one could envisage identification of cytosolic proteins in the disordered fraction as residual contaminants of the membranes. However, it seems unlikely that these proteins would be enriched in the detergent-insoluble, ordered membrane fraction. Interestingly, both starch-binding protein and glycogen phosphorylase do participate in an ER membrane-bound complex involved in the breakdown of glycogen (Prats et al., 2018). Given our IPA findings that pathways related to energy storage/utilization are downregulated by PB treatment, glycogen degradation may be initiated in response. In conjunction with this line of reasoning, glycogen phosphorylase was induced almost 25-fold in PB microsomes. In turn, our data would suggest that the formation of the glycogenolytic complex on the ER membrane may involve the localization of the participating proteins to ordered lipid microdomains. Despite the PB-related change in glycogen phosphorylase expression, glycogen degradation was not an upregulated pathway according to IPA (z-score $\approx$ 0). Thus, PB apparently has mixed effects on the expression of proteins involved in this pathway.

Although apolipoprotein F is a soluble, secreted protein that was identified in the ordered microdomains of PB microsomes. It is processed from a precursor proprotein that is membrane-bound as it is translated into the ER lumen (Day et al., 1994). This apolipoprotein has a tendency to bind cholesterol (Lagor et al., 2009). Since this lipid is an important component of ordered, lipid microdomains, the apo F may have been associated, while membrane-bound, with the detergent-

insoluble fraction by virtue of its affinity for cholesterol. The mature form of apo F also is heavily glycosylated. The cellular trafficking of secretory proteins is known to involve localization to lipid rafts (Surma et al., 2012) following N-glycosylation (Pang et al., 2004; Imjeti et al., 2011). Thus, it would not be unexpected for the precursor form of a secretory protein to be associated with ordered lipid microdomains as it is apically sorted. Glycosylation processes may be somewhat compromised in PB microsomes. The expression of a number of enzymes related to this process were downregulated by PB (e.g. mannose-1-phosphate guanyl transferase alpha, UDP-N-acetylglucosamine transferase subunit ALG13 homologue, phosphoglucomutase-1, UDP-N-acetylglucosamine transporter, oligosaccharyltransferase complex subunit OSTC, and probable N-acetyltransferase CML2). Some of these enzymes were not even detected in the PB microsomes. It is not known at what point apo F disassociates from the ER membrane during/after its translation. If its release from the membrane is dependent on the extent of glycosylation involving one of these downregulated enzymes, the apolipoprotein may remain membrane-bound in PB microsomes.

Despite using a modest criterion to distinguish ordered from disordered microdomains in the microsomes (Pellet/Supernatant > 1), the rarity of occurrences in which proteins varied from one localization to the other in PB and saline-treated microsomes indicates a clear distinction between ordered and disordered microdomains. This observation is not meant to imply that PB had no effects on protein localization. There were 226 proteins (16.7%) showing PB-related changes in localization involving the uniformly distributed proteins, and most of these involved redistribution into a more ordered membrane environment. Thus, protein localization in microsomes did not follow the trend expected from the reported changes in membrane lipid composition after extended PB treatment (*i.e.* lower cholesterol and sphingomyelin). Although PB lowers proportions of cholesterol and sphingomyelin after an extended treatment course, we recently compared lipid compositions of saline- and PB-treated rabbit liver microsomes after a three-day treatment (Brignac-Huber, 2011) (Supplemental Figure 1). Under our conditions of treatment, PB has no effect on the lipid composition of liver microsomes. PB treatment is known to cause proliferation of the smooth endoplasmic reticulum

in addition to causing changes in protein expression (Orrenius et al., 1965). PB-related proliferation of smooth ER could provide more ordered sites available for protein localization, and this, in turn, might favor a greater distribution of proteins to the ordered lipid microdomains. Thus, our findings may indicate that the ordered lipid microdomain sites within the membrane are saturated with respect to protein binding in saline microsomes. In considering the possibility that PB microsomes can accommodate a larger fraction of the total protein in ordered lipid microdomains because of an increase in the amount of these microdomains relative to the total protein, one must also take into account the changes in enzyme expression caused by PB. Although PB treatment does induce the expression of many proteins, there were many more proteins showing lower expression in the PB microsomes (Figure 1 and Supplemental Table 1). Thus, protein density within the membrane is probably lowered after PB treatment even without smooth ER proliferation. Future work will determine whether PB may be leading to changes in protein function through their relocalization to the ordered microdomains.

Effects of PB-induction on cellular pathways by IPA: The proteomic data also are amenable to bioinformatics. Ingenuity Pathway Analysis (IPA) was used to evaluate cellular pathways influenced by PB induction. Previous proteomic studies differed in the assessment of PB effects. Initially, it was proposed that PB induced a stress response based on the induction of 78 kDa glucose regulated protein and protein disulfide isomerases A₃ and A₆ (Galeva and Altermann, 2002). Subsequently, mixed changes in glycolysis and increased oxidative stress and proteasomal-mediated protein degradation were proposed based on the observed, PB-related, changes in protein expression (Wu et al., 2011). Finally, in the detailed proteomic study of hepatocytes and non-parenchymal liver cells, PB-induced changes in metabolism, oxidative stress, DNA stress/damage, and estrogen metabolism were proposed. Because our study was quantitative, we were able to calculate the significance of PB-related alterations in specific cellular pathways using IPA. Our results showed a clear diminution in pathways related to energy storage/utilization including glycolysis I, ketogenesis, oxidative phosphorylation, fatty acid β -oxidation, and gluconeogenesis, and also in those related to general cellular signaling such as those involving sirtuin, insulin secretion, RhoA, xenobiotic metabolism via the aryl hydrocarbon

receptor, actin, PPAR α /RXR α activation, VEGF, paxillin, necroptosis, AMPK, Rho family GTPases, estrogen receptor, and renin-angiotensin. The pathways up-regulated by PB treatment were almost entirely related to degradative pathways such as nicotine II/III, melatonin I, superpathway of melatonin degradation, acetone I, and bupropion. However, the pathway for estrogen biosynthesis was slightly upregulated by PB. These results suggest a relatively narrow scope of pathways are significantly upregulated following PB induction. Interestingly, in contrast to the conclusions of previous proteomic studies proposing an oxidative stress response, PB treatment did not correspond to an increase in NRF2-mediated oxidative stress.

Future directions: Future work is needed to assess the effects of membrane domain localization on P450 function and/or distribution within the cell. P450-mediated metabolism is determined by specific protein-protein interactions. Interactions with the redox partners, NADPH-cytochrome P450 reductase and cytochrome b₅, are essential to provide electrons needed for the mixed-function oxygenation of substrates. Furthermore, catalysis by P450s is heavily influenced by protein-protein interactions involving other P450s and membrane-bound enzymes (reviewed in (Reed and Backes, 2016)). It is possible that the sequestration to ordered lipid microdomains will influence the activities of specific P450s by attenuating or potentiating specific protein-protein interactions. We previously provided evidence using purified, reconstituted systems that NADPH-cytochrome P450 reductase can relocate into ordered lipid microdomains in the presence of CYP1A2 in a manner that promotes the interaction of the two proteins (Brignac-Huber et al., 2011; Brignac-Huber et al., 2013). Thus microdomain localization may affect P450 function by allowing the proteins to either segregate between or coalesce into membrane regions.

It is also possible that ordered lipid microdomain localization affects the trafficking of P450s to different cellular membranes. The localization of P450s to cellular membranes other than the ER has been well described (Anandatheerthavarada et al., 1997; Neve and Ingelman-Sundberg, 2008). The mechanism by which this trafficking occurs is not well understood (Szczesna-Skorupa et al., 1995;

Szczesna-Skorupa et al., 2000). The localization of proteins to ordered lipid microdomains has been shown to play a role in apical/basolateral sorting to the plasma membrane (Cao et al., 2012; Surma et al., 2012) and anterograde/retrograde transport within the ER and trans-golgi network (Helms and Zurzolo, 2004; Ouweeneel et al., 2020). Perhaps, the ordered microdomain localization of P450s plays either a role in their retention within the ER or their transport to the membranes of other organelles. Future work will examine whether microdomain localization of P450s influences protein-protein interactions and/or cellular trafficking.

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Figure Legends

Figure 1: Volcano plot showing the effects of PB on enzyme expression.

The fold-changes in enzyme expression in PB- and saline-treated microsomes (taken from the PB control/saline control in the TMT LC-MS proteomic data) were expressed as Log_2 (x-axis) and plotted versus the $-\text{Log}_{10}$ of the measured p-values. The green and red regions indicate the points showing more than 2-fold changes in expression levels (inhibited and stimulated, respectively). The horizontal line indicating the p-value 0.05 serves as the lower border of the green and red shaded regions. The points at the top border of the figure had p-values less than 10^{-15} .

Figure 2: Ingenuity Pathway Analysis showing differences between PB and saline microsomes.

Proteomic data from TMT LC-MS were used for IPA to determine the influence of PB treatment on cellular pathway expression by determining the relative abundances of representative proteins from the pathways in the saline and PB microsomes (PB control/saline control). A Z-score of $|2|$ was used to assess the levels by which PB altered pathway expression. Orange indicates pathway up-regulation by PB; whereas, blue indicates down-regulated by PB. Data were filtered only to include pathways for which the p-values were < 0.05 . The dashed line shows the threshold for p-values less than 0.05 ($-\log(\text{p-values}) > 1.3$).

Figure 3: Western blot analysis of fractions resulting from ultracentrifugation of Brij 98-treated rat liver microsomes. Liver microsomes from three control rats (labelled as 1, 2, and 3 in figure) were treated with Brij 98 and ultracentrifuged; the resulting supernatants (“S” in figure) were drawn off; and the pellets (“P” in figure) were resuspended. The detergent-treated fractions along with the untreated microsomal samples (“M” in figure) were loaded in separated lanes of a 10% polyacrylamide gel (four micrograms of proteins in each lane) and western blots were performed as described in Materials and Methods. The gels were loaded with 0.5 pmol of purified enzyme or baculovirus-expressed CYP supersomes (Gentest, Corning, NY) on the far left of the gel (“Std”) followed by the three sets of

samples described above (from left to right). Panel A: CYP1A2 blot; Panel B: CYP2E1 blot; Panel C: CYP2D blot; and Panel D: CYP3A blot.

Figure 4: Distribution of proteins into lipid membrane domains after solubilization of rat liver microsomes by Brij 98. Rat liver microsomes were treated with Brij 98 and ultracentrifuged. The relative distributions of proteins into ordered and disordered domains, in addition to those that were uniformly distributed, were determined by TMT LC-MS as described in Materials and Methods. Panel A: Protein distributions in liver microsomes from control rats. The fraction in each wedge of the pie represents the number of proteins in that group (disordered, ordered, or uniformly distributed) relative to the total number of proteins (representing the whole pie chart) identified by TMT LC-MS. The percentages of the total number of proteins identified are shown in parentheses. Panel B: Protein distribution in liver microsomes from PB-treated rats.

Table 1. Comparison of Brij 98 solubilization of rat liver microsomes using western blotting and TMT LC-MS. Brij 98 solubilization of three preparations of liver microsomes from saline rats was performed as described in Materials and Methods and in the legend to Figure 2. The relative levels of P450s (CYP1A2, CYP2D, CYP2E1, and CYP3A) in the supernatants and pellets recovered from each of the three samples after treatment with Brij 98 were measured by western blotting and by TMT LC-MS. The average and the standard deviation of the pellet/supernatant ratios for the three samples were determined. The P-values were calculated from a paired t test comparing the respective Brij 98 supernatants and pellets from each microsomal sample.

Cytochrome P450	Western blot Avg Pellet/SUP ± S.D.	Western blot P-Value	TMT LC-MS-MS Avg Pellet/SUP	TMT LC-MS-MS P-Value
CYP1A2	4.08 ± 0.92	0.0051	1.428	0.00002
CYP2E1	0.072 ± 0.032	0.0115	0.148	1 X 10 ⁻¹⁵
CYP2D(1)	4.69 ± 1.41	0.0003	2.834	1 X 10 ⁻¹⁵
CYP3A(2)	0.095 ± 0.032	0.0194	0.198	1 X 10 ⁻¹⁵

Table 2. Comparison of membrane localization for selected proteins in control and PB microsomes that were identified as

DRM proteins in previous studies. Proteins identified as residents of ordered lipid microdomains in previous studies (column 6) were compared with the results from this study using Brij 98 in control (column 4) and PB (column 5) microsomes. Column 2 shows the Uniprot ID. Column 7 shows the detergent used to identify ordered lipid microdomain components in the referenced study.

DRM Marker	Uniprot ID	Membrane Component	Domain in Saline	Domain in PB	Reported study	Detergent Used
Erlin-2	B5DEH2	ER	DRM	DRM	(Browman et al., 2006)	Triton X-100
Ribophorin 1	P07153	ER	DRM	DRM	(Poston et al., 2011); (Bae et al., 2004)	Triton X-114; Triton X-100
Oligosaccharyl transferase 48 kDa subunit	Q641Y0	ER	DRM	DRM	(Poston et al., 2011); (Bae et al., 2004)	Triton X-114; Triton X-100
Calreticulin	P18418	ER	DRM	DRM	(Poston et al., 2011)	Triton X-114
Protein disulfide isomerase	P04785	ER	Disordered	Disordered	(Poston et al., 2011)	Triton X-114
NADPH P450 Reductase	P00388	ER	Disordered	Disordered	(Poston et al., 2011)	Triton X-114
sigma 1 receptor	Q9R0C9	ER	Disordered	Disordered	(Hayashi and Fujimoto, 2010)	Triton X-100/-114
Glucose-6-phosphatase	P43428	ER	DRM	Uniform	(Bae et al., 2004)	Triton X-100
Antigen peptide transporter 1	P36370	ER	Uniform	Uniform	(Bae et al., 2004)	Triton X-100
Cytochrome b5	P00173	ER	Disordered	Disordered	(Bae et al., 2004)	Triton X-100
P450 1A2, P450 2A1, P450 2A3, P450 2B3, P450 2E1	P04799 P11711 P20812 P13107 P05182	ER	DRM Disordered Uniform Disordered Disordered	DRM Disordered Disordered Uniform Disordered	(Bae et al., 2004)	Triton X-100
epoxide hydrolase	P07687	ER	Disordered	Disordered	(Bae et al., 2004)	Triton X-100
Fatty aldehyde dehydrogenase	P30839	ER	Uniform	Uniform	(Bae et al., 2004)	Triton X-100
Microsomal glutathione-S-transferase	P08011	ER	DRM	DRM	(Bae et al., 2004)	Triton X-100
UGT-2B1	P09875	ER	Uniform	Uniform	(Bae et al., 2004)	Triton X-100
VDAC1	Q9Z2I0	Mitochondria	DRM	DRM	(Garofalo et al., 2005)	Triton X-100
fission 1 protein	P84817-1	Mitochondria	Disordered	Disordered	(Garofalo et al., 2005)	Triton X-100

mrp1	P43245	Plasma	DRM	DRM	(Goma et al., 2014)	Triton X-100
flotillin 1	Q9WTS8	Plasma	DRM	DRM	(Bickel et al., 1997)	Triton X-100

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Table 3. Summary of domain localization of cytochromes P450 and other drug metabolizing enzymes identified by TMT LC-MS in control and PB microsomes. The domain localization (defined as ordered lipid microdomains, disordered domains, or uniformly distributed between ordered and disordered microdomains) and the effects of PB on expression of rat P450s and other drug-metabolizing enzymes were determined by TMT LC-MS as described in Materials and Methods.

Protein Uniprot ID	Membrane Localization (Saline)	Membrane Localization (PB-Induced)	Effect of PB on Expression
Cytochrome P450 1A2 (P04799)	Ordered	Ordered	Down
Cytochrome P450 20A1 (Q6P7D4)	Disordered	Disordered	Unchanged
Cytochrome P450 2A1 (P11711)	Disordered	Disordered	Up
Cytochrome P450 2A2 (P15149)	Disordered	Uniform	Unchanged
Cytochrome P450 2A3 (P20812)	Uniform	Disordered	Unchanged
Cytochrome P450 2B1 (P00176)	Uniform	Disordered	Up
Cytochrome P450 2B2 (P04167-1)	Disordered	Disordered	Up
Cytochrome P450 2B3 (P13107)	Disordered	Uniform	Down
Cytochrome P450 2C11 (P08683)	Disordered	Disordered	Unchanged
Cytochrome P450 2C12 (P11510)	Disordered	Uniform	Unchanged
Cytochrome P450 2C13 (P20814)	Uniform	Uniform	Down
Cytochrome P450 2C23 (P24470)	Disordered	Disordered	Unchanged
Cytochrome P450 2C55 (P33273)	Not detected	Uniform	Up
Cytochrome P450 2C6 (P05178)	Disordered	Disordered	Up
Cytochrome P450 2C7 (P05179)	Uniform	Uniform	Up
Cytochrome P450 2C70 (P19225)	Disordered	Disordered	Up
Cytochrome P450 2D1 (P10633)	Ordered	Ordered	Unchanged
Cytochrome P450 2D10 (P12939)	Ordered	Ordered	Down
Cytochrome P450 2D26 (P10634)	Ordered	Ordered	Unchanged
Cytochrome P450 2D3 (P12938)	Ordered	Ordered	Unchanged
Cytochrome P450 2D4 (Q64680)	Ordered	Uniform	Unchanged
Cytochrome P450 2E1 (P05182)	Disordered	Disordered	Down
Cytochrome P450 2G1 (P10610)	Disordered	Disordered	Up
Cytochrome P450 2J3 (P51590)	Disordered	Disordered	down
Cytochrome P450 3A1 (P04800)	Disordered	Disordered	Up
Cytochrome P450 3A18 (Q64581)	Uniform	Disordered	Unchanged
Cytochrome P450 3A2 (P05183)	Disordered	Disordered	Up
Cytochrome P450 3A9 (P51538)	Not detected	Disordered	Up
Cytochrome P450 4A10 (P08516)	Uniform	Uniform	Down
Cytochrome P450 4A14 (P20817)	Uniform	Uniform	Unchanged
Cytochrome P450 4A2 (P20816)	Uniform	Uniform	Unchanged
Cytochrome P450 4F1 (P33274)	Disordered	Disordered	Unchanged
Cytochrome P450 4F4 (P51869)	Disordered	Uniform	Unchanged
Cytochrome P450 4F6 (P51871)	Disordered	Uniform	Down
Cytochrome P450 4V2 (A2RRT9)	Disordered	Disordered	Down
NADPH-cytochrome P450 reductase	Disordered	Disordered	Up

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(P00388)			
Cytochrome b5 (P00173)	Disordered	Disordered	Unchanged
Dimethylaniline monooxygenase [N-oxide-forming] 1 (P36365)	Disordered	Disordered	Unchanged
Dimethylaniline monooxygenase [N-oxide-forming] 3 (Q9EQ76)	Disordered	Disordered	Unchanged
Dimethylaniline monooxygenase [N-oxide-forming] 5 (Q8K4C0)	Disordered	Disordered	Up
UDP-glucuronosyltransferase 1A1 (Q64550-1)	Ordered	Ordered	Up
UDP-glucuronosyltransferase 1A5 (Q64638-1)	Uniform	Uniform	Unchanged
UDP-glucuronosyltransferase 1A6 (P08430-1)	Ordered	Ordered	Unchanged
UDP-glucuronosyltransferase 1A7 (Q64633-1)	Not Detected	Uniform	Up
UDP-glucuronosyltransferase 2B1 (P09875)	Uniform	Uniform	Up
UDP-glucuronosyltransferase 2B15 (P36511)	Disordered	Disordered	Unchanged
UDP-glucuronosyltransferase 2B17 (P08542)	Uniform	Uniform	Unchanged
UDP-glucuronosyltransferase 2B2 (P08541)	Disordered	Disordered	Up
UDP-glucuronosyltransferase 2B37 (P19488)	Uniform	Uniform	Unchanged

Figure 1

Analysis: PB cntl / Sal cntl

Figure 2

Figure 3

A

B

■ Disordered ■ Ordered ■ Uniformly distributed

Figure 4