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Cytochrome P450 system proteins reside in different regions of the endoplasmic reticulum

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Cytochrome P450 (P450) function is dependent on the ability of these enzymes to successfully interact with their redox partners, NADPH-cytochrome P450 reductase (CPR) and cytochrome b_5, in the endoplasmic reticulum (ER). Because the ER is heterogeneous in lipid composition, membrane microdomains with different characteristics are formed. Ordered microdomains are more tightly packed, and enriched in saturated fatty acids, sphingomyelin and cholesterol, whereas disordered regions contain higher levels of unsaturated fatty acids. The goal of the present study was to determine whether the P450 system proteins localize to different regions of the ER. The localization of CYP1A2, CYP2B4 and CYP2E1 within the ER was determined by partial membrane solubilization with Brij 98, centrifugation on a discontinuous sucrose gradient and immune blotting of the gradient fractions to identify ordered and disordered microdomains. CYP1A2 resided almost entirely in the ordered regions of the ER with CPR also localized predominantly to this region. CYP2B4 was equally distributed between the ordered and disordered domains. In contrast, CYP2E1 localized to the disordered membrane regions. Removal of cholesterol (an important constituent of ordered domains) led to the relocation of CYP1A2, CYP2B4 and CPR to the disordered regions. Interestingly, CYP1A1 and CYP1A2 localized to different membrane microdomains, despite their high degree of sequence similarity. These data demonstrate that P450 system enzymes are organized in specific membrane regions, and their localization can be affected by depletion of membrane cholesterol. The differential localization of different P450 in specific membrane regions may provide a novel mechanism for modulating P450 function.

Key words: Brij 98, cytochrome P450, endoplasmic reticulum, lipid microdomain.

INTRODUCTION

Cytochromes P450 (P450) comprise a superfamily of haem-containing proteins, found in most organisms and catalyse oxidative reactions of both endogenous and exogenous compounds, with P450 enzymes from families CYP1, CYP2 and CYP3 being primarily involved in the metabolism of drugs [1]. Unlike bacterial P450 that are generally expressed as soluble enzymes, the mammalian forms responsible for drug and foreign compound metabolism are membrane-bound proteins that are widely expressed in the endoplasmic reticulum (ER) [2]. Even though the N-terminal region of P450 is known to be responsible for its insertion into the membrane, evidence suggests that amino acids in the catalytic region are also involved in membrane localization and orientation of the protein [3,4].

For functional reactions to occur, P450 enzymes must interact with other integral membrane proteins, particularly NADPH-cytochrome P450 reductase (CPR) and cytochrome b_5, which serve as electron donors [5,6]. Besides the interaction of P450 with its redox partners, many studies have shown that P450 can form complexes with other P450 enzymes in the membrane. In many cases, these complexes alter P450 function. Interactions between CYP1A2–CYP2B4, CYP1A2–CYP2E1, CYP2C9–CYP2D6, CYP2C9–CYP3A4, CYP3A4–CYP3A4 and CYP1A2–CYP1A2 have been reported [7–12]. Although a few studies have shown that these interactions occur in microsomes [13] and living cells [14–16], most of these studies were done using lipid reconstituted systems (RCS), which contain a single class of lipids that generate homogenous lipid membranes. However, biological membranes are composed of numerous types of lipids and the diversity of lipid species provides heterogeneity to the lipid bilayer. These phospholipids are not randomly distributed in the membrane, but associate in a manner to generate membrane regions with different characteristics. Some regions contain phospholipids that are enriched in unsaturated fatty acids and are referred to as liquid-disordered domains. Other regions, known as liquid-ordered domains, have higher concentrations of saturated fatty acids, are enriched in cholesterol and sphingomyelin and are more tightly packed [17]. These ordered domains are commonly referred to as lipid rafts [18], and because they are not readily solubilized by non-ionic detergent, they are also referred to as detergent-resistant membranes (DRMs).

Phospholipids are pivotal components in the formation of the lipid bilayer, and are also essential for normal P450 function [19]. According to studies using mixtures of different phospholipids, alterations in membrane lipid composition were shown to affect P450 behaviour. For example, several groups showed that addition of other phospholipids, such as phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylserine (PS) into phosphatidylcholine (PC) vesicles stimulated P450 catalytic activity. These effects were attributed to the negative charges

Abbreviations: DRM, detergent-resistant membrane; ER, endoplasmic reticulum; MβCD, methyl-β-cyclodextrin; CPR, NADPH-cytochrome P450 reductase; GPI, glycosylphosphatidylinositol; P450, cytochrome P450; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; RCS, reconstituted systems.

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on PA, PI and PS. Although the mechanism for modulation of catalysis is not known, it has been demonstrated that the negatively charged phospholipids increase the P450 insertion rate into model membranes and stability of the enzymes by causing conformational changes of P450 [20–23]. These effects may be mediated by a direct interaction of fatty acid chains of the phospholipids with the N-terminus or other membrane-binding regions of the P450 molecule. As ordered domains contain different phospholipid compositions, such as lower levels of PC and higher concentrations of PS, when compared with disordered regions [24], localization of P450 could also be a significant factor affecting P450 function and their interaction with other proteins. The heterogeneity of the membranes not only might affect P450 activities directly, but also might affect their activities indirectly by either segregating or concentrating the P450 and their electron-donating partners in specific membrane regions.

Previously, our lab has examined the potential for domain formation in the ER. These experiments demonstrated that: (i) ordered domains in the ER have similar lipid compositions to those found in the plasma membrane; and (ii) CPR and CYP1A2 selectively localize in the ordered membranes, instead of being randomly distributed throughout the ER [25]. In a follow-up study, both proteins were also shown to distribute into ordered regions when reconstituted into membranes with compositions similar to those found in the ER [26]. These studies raised a question related to whether other P450 are similarly located in the ordered regions of the ER. Therefore, we compared the localization of CYP2B4 and CYP2E1 in the ER membrane to that of CYP1A2 and CPR. In the present study, we demonstrate that different P450 enzymes reside in different membrane regions, with CYP1A2 congregating predominantly in the ordered regions [25], CYP2B4 localizing in both ordered and disordered regions and CYP2E1 segregating into the disordered membranes. The data indicate that components of the P450 system are not randomly distributed throughout the ER, but are organized in specific microdomains.

EXPERIMENTAL

Materials

Primary antibodies against CPR were obtained from Stressgen. Anti-CYP2B4 and anti-CYP2E1 were purchased from Oxford Biomedical Research. The antibody for CYP1A2 was provided by Dr Kristopher Krausz from the National Institutes of Health. Amplex Red cholesterol assay kit was purchased from Invitrogen. BCA protein assay kit and phenobarbital were purchased from Fisher Scientific. Methyl-β-cyclodextrin (MβCD), Brij 98, pyrazole and all other reagents were obtained from Sigma–Aldrich.

Induction of P450 and isolation of microsomes

Male New Zealand white rabbits were treated with either phenobarbital (80 mg/kg) or pyrazole (200 mg/kg) for induction of CYP2B4 and CYP2E1, respectively. Phenobarbital was administered by daily intraperitoneal injections for 3 days, and pyrazole was administered as a single intraperitoneal injection. Rabbits were killed 24 h after the final injection, the livers removed and placed into ice-cold 0.25 M sucrose. Microsomes were then prepared by differential centrifugation [27] and the final pellets were resuspended in 10 mM potassium phosphate buffer (pH 7.25). Protein concentration was determined using the BCA assay and the samples were stored as aliquots at $-80\,^{\circ}$C.

Microsomal membrane solubilization by Brij 98

Detergent solubilization of microsomes was performed as described previously [25]. Briefly, Brij 98 was diluted to a 10 % stock solution using membrane solubilization buffer [50 mM Hepes, pH 7.25, containing 150 mM NaCl, 5 mM EDTA and a protease inhibitor cocktail (Roche)]. The Brij 98 solution was added to the microsomal samples (2 mg/ml) to a final concentration of 1 % Brij 98. Membrane solubilization buffer was added to each sample bringing the total volume to 1 ml. Samples were then incubated in a water bath at 37 °C for 5 min prior to either direct centrifugation or sucrose gradient centrifugation as described below.

Isolation of DRMs by discontinuous sucrose gradient centrifugation

Solubilized samples were mixed with 1 ml of 80 % sucrose solution. A 6 ml layer of 38 % sucrose was carefully placed on top followed by a 3 ml layer of 5 % sucrose. The samples were centrifuged for 19 h in a SW41 rotor at 210 000 ***g*** at 4 °C. After centrifugation, 1 ml aliquots was taken from the top and placed in 11 separate tubes. Solubilization buffer (1 ml) was added to the empty tube to re-homogenize pellets. Samples were then analysed by immune blotting. The DRMs were designated as fractions 2–5 at the 5 %/38 % sucrose interface as described previously [25].

Isolation of DRMs alternatively by pelleting

For a more rapid separation of soluble proteins from those in DRMs, microsomes were treated, as described above, with 1 % Brij 98 at 37 °C for 5 min. Subsequently, membrane solubilization buffer was added to obtain a final volume of 3 ml. The samples were then centrifuged using a SW55Ti rotor at 290 000 ***g*** for 15 min. The supernatants were saved and pellets were resuspended in 3 ml of membrane solubilization buffer. The supernatants and pellets were then analysed by immune blotting.

Immune blot analysis

Samples were subjected to SDS/PAGE (10 % gels) and transferred on to nitrocellulose membranes (Bio-Rad). Membranes were probed using mouse anti-CYP1A1/1A2, mouse anti-rat CYP2B1, mouse anti-rat CYP2E1 or rabbit anti-rat CPR antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse secondary antibodies were used at 1:4000 dilution. Treated blots were visualized using Supersignal West Pico chemiluminescent substrate (Thermo Fisher Scientific). Densitometry was performed with Quantity One (Bio-Rad) or Image Lab software (Bio-Rad).

Microsomal cholesterol depletion with MβCD treatment

Cholesterol depletion was performed as described previously with some modifications [25]. A 100 mM stock solution of MβCD was prepared in buffer (50 mM Hepes and 150 mM NaCl, pH 7.4). Microsomes (2 mg/ml) were incubated with or without 50 mM MβCD at 37 °C for 30 min. Then, samples were centrifuged and the resulting cholesterol-depleted microsomal pellets were resuspended in 1 ml membrane solubilization buffer as described previously [25]. To confirm cholesterol depletion, 20 μl of the resuspended pellet was diluted 10-fold and the cholesterol content of each sample was measured using the Amplex Red Cholesterol Assay Kit (Invitrogen). The samples were then treated with

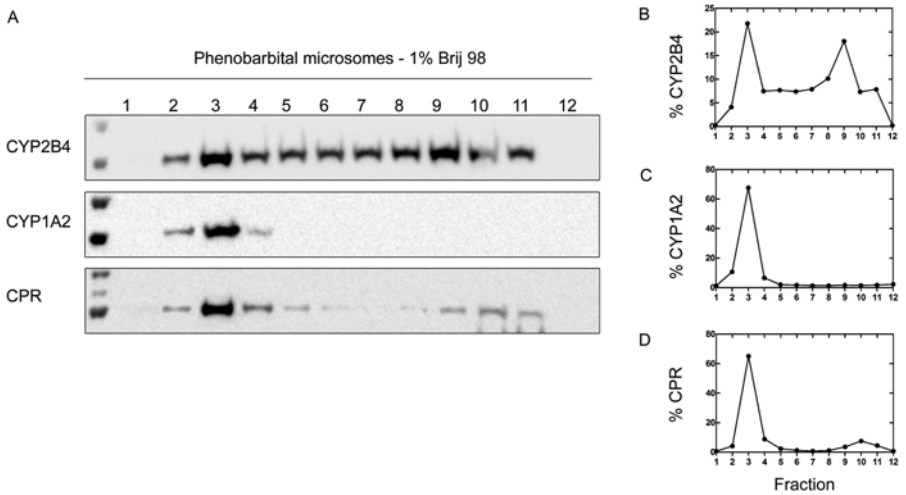

Figure 1 Distribution of CYP2B4 between ordered and disordered membrane regions

Microsomes from phenobarbital-treated rabbit liver (2 mg/ml) were incubated with 1 % Brij 98 at 37 °C for 5 min, followed by sucrose gradient centrifugation. Eleven fractions (1 ml each) were collected from the top, and samples from each fraction were subjected to SDS/PAGE. The localization of CYP2B4, CYP1A2 and CPR was shown by immune blotting (**A**). Relative distribution (%) of proteins was measured by densitometry (**B**–**D**). The results are representative of at least three independent experiments.

1 % Brij 98 and subjected to discontinuous sucrose gradient centrifugation.

RESULTS

Microdomain localization of CYP2B4 in the ER of phenobarbital-treated rabbits

As noted from our previous studies, CPR and CYP1A2 were found to localize in 'ordered' regions of the ER that contain higher concentrations of cholesterol and sphingomyelin. This raised the question of whether all P450 proteins similarly localize into these microdomains, and led to the current experiments examining the localization of CYP2B4 and CYP2E1. To examine microdomain localization of CYP2B4 proteins, liver microsomes isolated from phenobarbital-treated rabbits were subjected to detergent treatment with 1 % Brij 98, followed by discontinuous sucrose gradient centrifugation. After centrifugation, 1 ml fractions were collected from the top of the tube, and each fraction was analysed for cholesterol and protein content. Because of their high lipid/protein ratio, membranes containing ordered domains float within the low-density sucrose fractions [28]. Fractions between 2 and 5 possessed the highest cholesterol/protein ratios (Supplementary Figure S1A) as reported previously for ER from untreated rabbits [25]. These fractions correspond to the detergent-resistant (ordered) membrane regions. To analyse protein localization, each fraction was subjected to SDS/PAGE and visualized by immune blotting. The majority of CYP1A2 and CPR proteins were found in the low-density sucrose fractions, which is consistent with our previous results from microsomes isolated from untreated rabbits showing their localization in the ordered DRM membranes [25]. However, unlike the distribution of CYP1A2 and CPR, CYP2B4 appeared to reside in both the ordered and disordered microdomains (Figure 1). These differences appeared to be a characteristic of the proteins, as the relative composition of lipid classes (PC, phosphatidylethanolamine, PI, PS, PA, sphingomyelin and cholesterol) were not affected by phenobarbital treatment (L. M. Brignac-Huber, J. R. Reed and W. L. Backes, unpublished work and [25]). Although it has been reported that amino acid motifs and post-translational modifications, such as glycosylphosphatidylinositol (GPI) anchor and palmitoylation are involved in ordered domain localization [29,30], it has not been examined with P450. Therefore, further study is required to identify the factors responsible for localization of P450 in ordered domains.

Distribution and differential detergent solubility of microsomal proteins from pyrazole-treated rabbits

Next, CYP2E1 localization was examined. Since the CYP2E1 expression level is low in untreated and phenobarbital-treated rabbits, pyrazole was used to enhance CYP2E1 levels. The increased expression level of CYP2E1 was confirmed by immune blot (data not shown). As described for phenobarbital-treated rabbits, ordered domains were isolated by detergent treatment of microsomes from pyrazole-treated rabbits followed by sucrose gradient centrifugation. Microsomes from pyrazole-treated rabbits had a similar cholesterol/protein ratio to microsomes from phenobarbital-treated rabbits (Supplementary Figure S1B). The CYP2E1 composition from each fraction was analysed by immune blotting and a dramatic difference was observed in the distribution of this P450. CYP2E1 was exclusively found in high-density sucrose fractions indicative of their presence in the detergent-soluble 'disordered' domains, whereas CYP1A2 and CPR were mainly localized in the ordered domains (fractions 2–5, Figure 2).

Because disordered domains are less tightly packed than ordered domains, we hypothesized that proteins segregating primarily in disordered domains would be more easily released from the microsomal membrane with lower concentrations of Brij 98. Therefore, the solubilities of CYP1A2, CPR and CYP2E1 were compared using various concentrations of Brij 98 (0 %, 0.125 %, 0.25 % and 1 %) in microsomes from pyrazole-treated rabbits. As expected, CYP2E1 was partially solubilized by 0.125 % Brij 98 and was completely solubilized at 1 % Brij (Figure 3). In contrast, both CYP1A2 and CPR showed solubility profiles that differed from CYP2E1 – in the Brij 98 concentration range from 0.25 % to 1 % there was little solubilization of these proteins from their membrane environment. These data

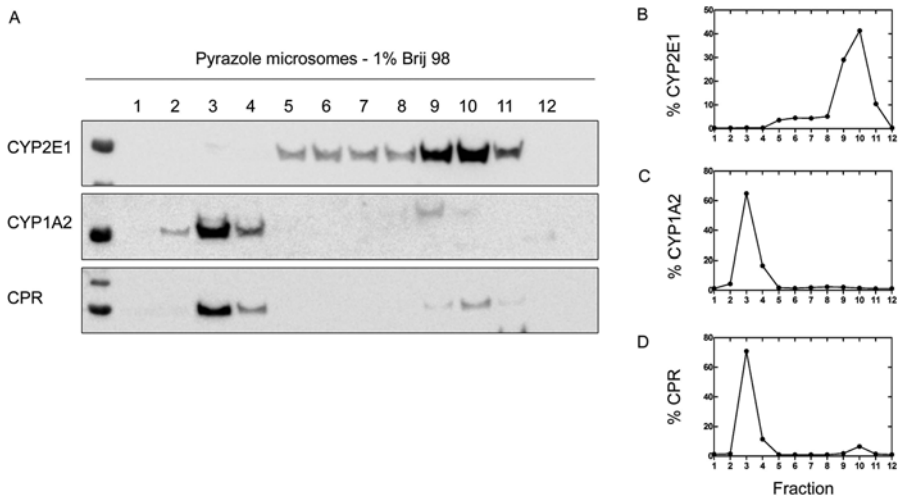

Figure 2 Distribution of CYP2E1 between ordered and disordered membrane regions

Microsomes from pyrazole-treated rabbit liver (2 mg/ml) were separated by sucrose gradient centrifugation after being solubilized by 1 % Brij 98 at 37 °C for 5 min. Protein distribution of collected fractions was analysed by immune blotting (**A**). Relative distribution (%) of proteins was measured by densitometry (**B**–**D**).

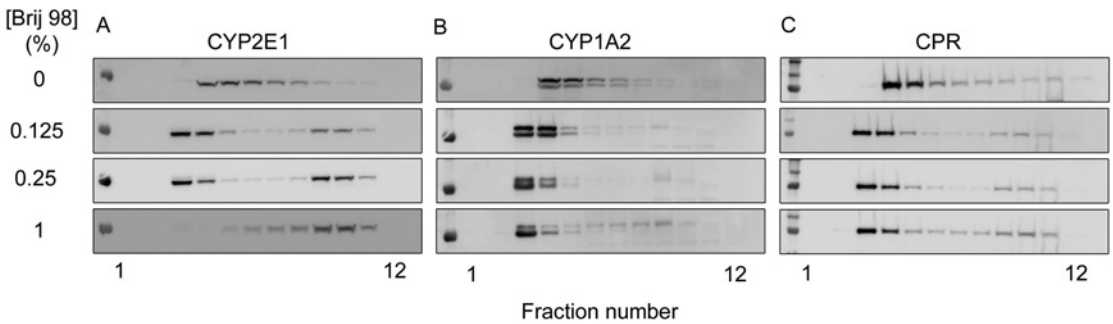

Figure 3 Differential solubility of DRM- and non-DRM-localized proteins by various concentrations of Brij 98

Microsomes from pyrazole-treated rabbits (2 mg/ml) were solubilized with different concentrations (0 %, 0.125 %, 0.25 % and 1 %) of Brij 98 for 5 min and DRMs were isolated via sucrose gradient centrifugation. Samples from each fraction were analysed by immune blotting, probing for CYP2E1 (**A**), CYP1A2 (**B**) and CPR (**C**).

demonstrate that different P450 system enzymes localize in different membrane microdomains.

Confirmation of different localization of P450 by pelleting

Although high-speed centrifugation with sucrose gradients make insoluble fractions float to the interface between 5 % and 38 % sucrose layers, pelleting (high-speed centrifugation without sucrose) pulls down insoluble membrane fractions to the bottom of centrifuge tubes [28]. Using this method, the supernatant contains the solubilized proteins. This method has the advantage of more rapidly separating the ordered and disordered regions (15 min compared with 19 h). In order to validate this methodology, 1 % Brij 98-treated samples were separated by ultracentrifugation and the results compared with the sucrose gradient method. After the ultracentrifugation of microsomal samples, Brij 98 treatment caused CYP2B4 and CPR to distribute into both pellet and supernatant, whereas most of CYP1A2 remained in the pellet (Figure 4). In contrast, CYP2E1 was completely solubilized by Brij 98 and was found only in the supernatant of the samples (Figure 4). These results are consistent with the previous experiments utilizing the discontinuous sucrose gradient, and confirmed that different P450 localize into distinct microdomains.

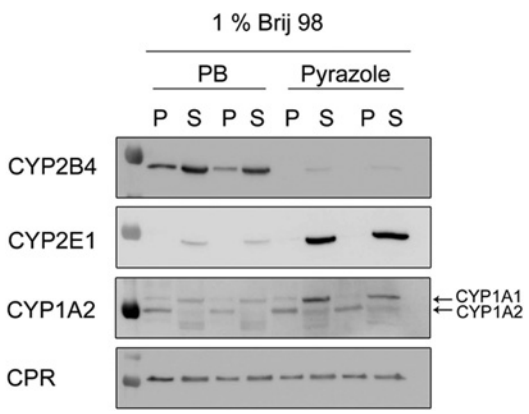

Figure 4 Isolation of DRM and non-DRM proteins by high-speed centrifugation

Liver microsomes from phenobarbital- or pyrazole-treated rabbits were solubilized by 1 % Brij 98 and then diluted 3-fold with 50 mM Hepes, 150 mM NaCl and 5 mM EDTA with protease inhibitor cocktail (Roche), pH 7.4. Diluted microsomes were separated by centrifugation using an SW55Ti rotor at 290 000***g*** for 15 min into supernatant (S) and pellet (P). The supernatant was saved and the precipitate was resuspended in same volume as the supernatant. P450 and CPR were visualized by immune blotting.

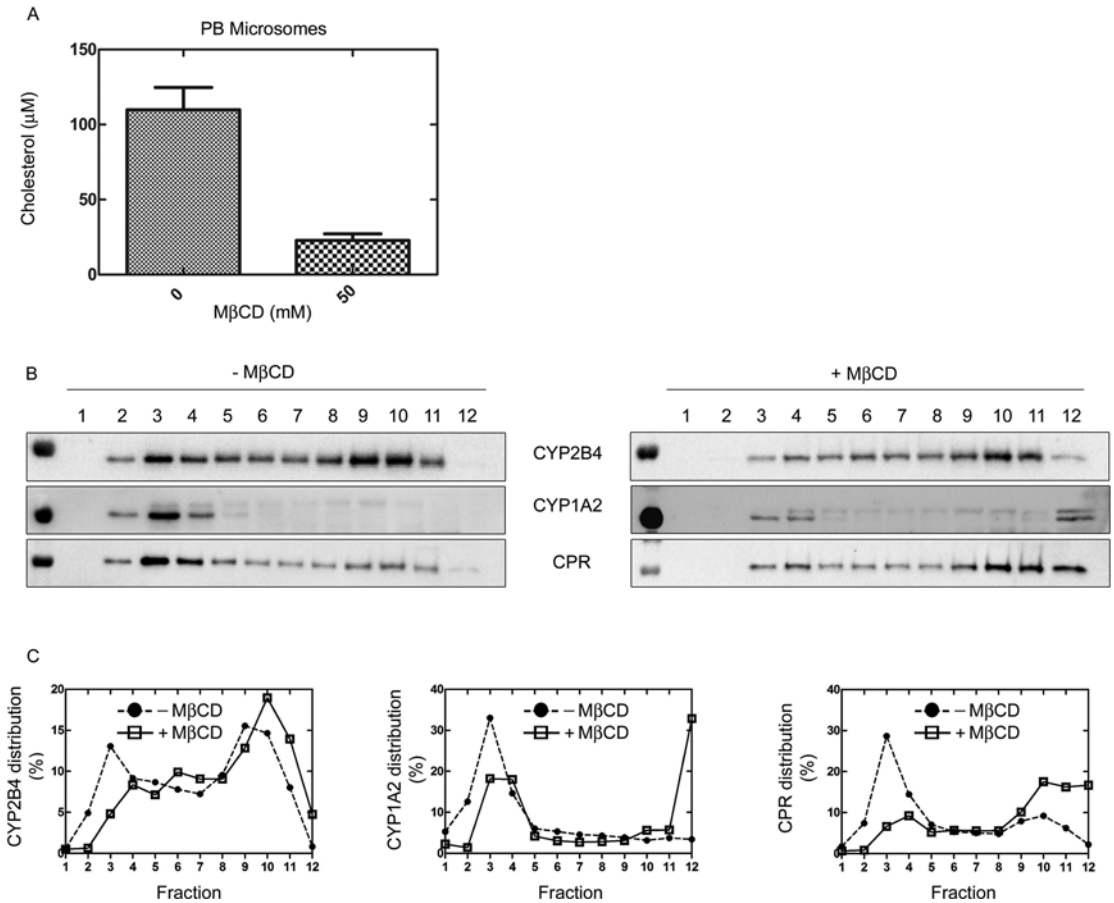

Figure 5 Effect of cholesterol depletion on P450 localization in microsomes from phenobarbital-treated rabbits

Microsomes (2 mg/ml) were incubated in the presence or absence of 50 mM MβCD at 37 °C for 30 min. Microsomal samples were centrifuged at 20 000 ***g*** for 20 min and the pellet was resuspended in solubilization buffer. (**A**) Cholesterol contents were assayed by using Amplex Red Cholesterol assay kit. Resuspended microsomes were treated with 1 % Brij 98 at 37 °C for 5 min and subjected to sucrose gradient centrifugation. Protein composition of each fraction was analysed by immune blotting (**B**) and percentages of protein composition were estimated by densitometric analysis (**C**).

We obtained a particularly interesting result when examining CYP1A protein distribution (Figure 4). Both CYP1A1 and CYP1A2 are expressed in rabbit liver [31], and can be seen in the Figure with the upper and lower bands representing CYP1A1 and CYP1A2, respectively. Despite their high level of sequence similarity, these proteins showed contrasting results after microsomal treatment with Brij 98. Although CYP1A2 proteins localized in the ordered membrane regions (found in the pellet after centrifugation), a significant portion of CYP1A1 was found in the disordered regions (i.e. the supernatant) (Figure 4), suggesting that subtle differences in the protein sequence can influence P450 localization.

CYP2B4 localization was altered by cholesterol depletion

Cholesterol and sphingomyelin are known as important constituents leading to the formation of ordered microdomains. Therefore, having demonstrated that CYP2B4 can reside in these ordered regions, we tested whether depletion of cholesterol would disturb the distribution of DRM-localized proteins in a manner similar to that observed with CYP1A2 and CPR [25]. For cholesterol depletion, microsomes from phenobarbital-treated rabbits were treated with MβCD that selectively extracts cholesterol from membranes via a binding pocket on the molecule [32]. After incubation at 37 °C for 30 min, MβCD-treated microsomes were centrifuged to remove extracted cholesterol. Cholesterol contents of resuspended microsomes after MβCD treatment and centrifugation were decreased by almost 80 % (Figure 5A). Next, the microsomes were solubilized by 1 % Brij 98 and applied to a sucrose gradient and centrifuged. Protein compositions of each fraction were then analysed by immune blot. The results showed that proteins in DRM fractions were shifted to fractions of higher sucrose density after MβCD treatment (Figures 5B and 5C), consistent with disruption of the ordered domains that contain CYP2B4, CPR and CYP1A2. Interestingly, the susceptibility to detergent solubilization after MβCD pre-treatment differed among the proteins. In Table 1, averages of relative distribution (%) of proteins with or without cholesterol depletion were calculated from sucrose gradient fractions 2–5. For example, with microsomes from phenobarbital-treated animals, disruption of the DRM caused the release of more than 60 % of the CPR; however, only approximately 45 % of the DRM-localized CYP1A2, and 40 % of CYP2B4 were released by MβCD/detergent treatment (Table 1). These results suggest that at least some of the P450 located in the ordered domains may not be co-localized in the same ordered regions of the membrane.

Non-DRM localization of CYP2E1 is independent of cholesterol depletion

As reported in Figure 4, the distribution of CPR, CYP1A2 and CYP2B4 enzymes in ordered fractions was affected by cholesterol depletion from the ER membrane. Conversely, because CYP2E1 appeared to reside in the less ordered membrane regions, its

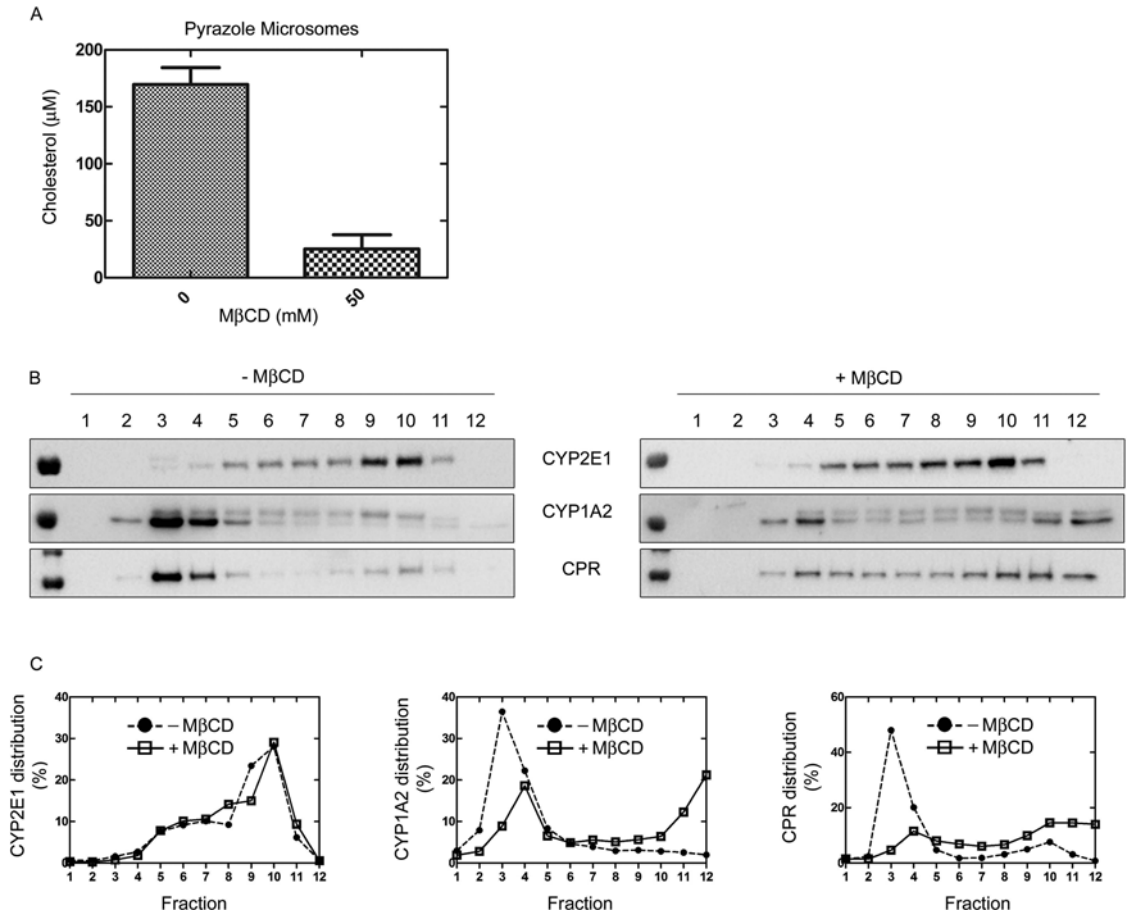

Figure 6 Effect of cholesterol depletion on P450 distribution in microsomes from pyrazole-treated rabbits

Microsomes were treated with or without 50 mM MβCD at 37 °C for 30 min. Resuspended microsomes, following centrifugation at 20 000 ***g*** for 20 min were used to determine cholesterol contents (**A**) and subjected to sucrose gradient centrifugation after 1 % Brij 98 treatment at 37 °C for 5 min. Protein concentrations in each fraction were analysed by immune blotting (**B**) and densitometry (**C**).

Table 1 Differential detergent solubility of P450 and CPR after cholesterol depletion

Rabbit liver microsomes were treated with MβCD to remove cholesterol from the membranes, or with buffer as a control. After treatment, the samples were treated with 1 % Brij 98 to solubilize the membranes. The amount of protein remaining in the more ordered detergent-resistant membranes was identified as those proteins remaining in fractions 2–5 after discontinuous sucrose gradient centrifugation as described in the Experimental section. These data are the percentage remaining in ordered membrane regions and are expressed as the means ± S.E.M. for three experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ using a paired Student's *t* test.

(a) Phenobarbital-pre-treated microsomes

	No treatment	50 mM MβCD
CYP2B4	32.3 ± 1.8	19.6 ± 1.3*
CYP1A2	84.9 ± 9.2	44.7 ± 3.7*
CPR	66.0 ± 4.3	23.7 ± 1.9**

(b) Pyrazole-pre-treated microsomes

	No treatment	50 mM MβCD
CYP2E1	14.0 ± 1.5	10.6 ± 0.8
CYP1A2	85.6 ± 5.4	50.0 ± 6.7**
CPR	75.1 ± 2.7	17.6 ± 4.1**

distribution should not be altered by MβCD pretreatment. Therefore, microsomes from pyrazole-treated rabbits were subjected to MβCD/detergent and the localization of CPR, CYP1A2 and CYP2E1 were determined after sucrose gradient centrifugation. MβCD treatment decreased cholesterol content by more than 80 % (Figure 6A) and shifted DRM-localized proteins (CYP1A2 and CPR) to later higher-density fractions (Figures 6B and 6C). In contrast, most of CYP2E1 was found in high-density sucrose fractions without MβCD treatment, and this distribution was not affected by the removal of cholesterol by MβCD (Figures 6B and 6C). These results clearly demonstrate that CYP2E1 localization was not affected by removal of cholesterol, which is consistent with it residing in the disordered membrane regions.

Consistent with the results shown with microsomes from phenobarbital-treated rabbits, differences in the release of the proteins that reside in the ordered microdomains (CPR and CYP1A2) were observed after MβCD treatment with microsomes from pyrazole-treated rabbits. Although greater than 75 % of the CPR was released from the ordered membranes, MβCD only led to the release of 40 % of CYP1A2 (Table 1). Again, the results are consistent with the idea that even though multiple proteins can exist within the ordered microdomains, the microdomains are heterogeneous with regard to protein composition.

DISCUSSION

It has been established that the biological lipid bilayer is composed of numerous types of lipids that are heterogeneously distributed

to form ordered and disordered microdomains. The ordered domains in cellular membranes have been reported to play important roles in cellular signalling and transport of lipids and proteins [33–35]. Because of the complexity of cellular membranes and technical limitations related to their isolation, it is difficult to directly visualize ordered domains in biological membranes. However, the organization of microdomains in model membranes has been well characterized and visualized [36,37]. Furthermore, the lipid domains isolated by detergent treatment in living cells showed similar properties to liquid ordered domains from model membranes [38]. Microdomains from cell membranes have mostly been characterized from the plasma membrane where the cholesterol and sphingomyelin compositions range from 20 % to 30 % [24]. In contrast, the cholesterol/phospholipid ratios in other organelles range from 5 % to 20 % [39]. The cholesterol and sphingomyelin compositions of the ER are particularly low – with both being in the 4 %–5 % range [25,40]. The low concentration of cholesterol in the ER raised the question as to whether this organelle contains cholesterol-rich lipid microdomains. However, several groups have shown the potential for the formation of ordered domains within the ER membrane, as well as in other organelles such as the Golgi, mitochondria and late endosomes [25,41–44]. Our laboratory has shown that DRM from the ER are enriched in cholesterol [25], a result that also is borne out from the present study (Supplementary Figure S1).

Even though cholesterol is a key component responsible for the formation/stability of ordered domains found in the plasma membrane [45], some raft marker proteins including GPI-anchored proteins, galectin-4 and flotillins are not affected by cholesterol depletion, whereas other proteins in ordered domains are released from the membrane [46–48]. These findings suggest that there is heterogeneity within the population of ordered domains and the requirement of cholesterol in the formation/stability of ordered domains is different among microdomains [49]. This may explain why CPR distribution was most disturbed by MβCD treatment among proteins in ordered domains in Table 1, and reflect the existence of different types of DRM. In other words, even though some proteins can be found in the same fractions of the discontinuous sucrose gradient, it does not guarantee that those proteins co-localize in the same microdomains.

Despite evidence that the ER membrane contains microdomains [28,41] and that membrane lipid composition affects P450 function [20,22,23], little is known about whether P450 system components reside in different regions of the ER membrane and if this localization influences P450 function. Previously, our laboratory isolated ordered domains in rabbit microsomes by Brij 98 solubilization and sucrose gradient centrifugation and showed higher concentrations of cholesterol and sphingomyelin in the ordered domains [25]. The study showed that CYP1A2 and CPR proteins were predominantly localized to ordered regions of the microsomal membrane. In addition, when purified CYP1A2 and CPR were incorporated into RCS, vesicles containing higher levels of cholesterol and sphingomyelin were associated with an increase in CYP1A2-mediated substrate metabolism and a decrease in apparent K_D for CPR in comparison with PC vesicles. The data suggested that increased CYP1A2 activity in the vesicles containing higher levels of cholesterol and sphingomyelin was mediated by increasing the efficiency of interaction of CYP1A2 and CPR [25]. It is currently unknown how much of this change in apparent K_D^{CPR} is due to concentration of the proteins into a smaller surface area (i.e. the ordered microdomains), and how much is a direct effect of the anionic phospholipids on protein activity.

These data led us to investigate the localization of other P450, CYP2B4 and CYP2E1 in the ER. Unlike CYP1A2 and CPR, CYP2B4 was equally distributed between the ordered and disordered domains and CYP2E1 was almost exclusively distributed in disordered domains (Figures 1 and 2). With CPR residing predominantly in the ordered regions, interactions with CYP1A2 and the fraction of CYP2B4 localized in the ordered regions would be facilitated. However, interactions with CYP2E1 might be limited by its segregation from CPR into different domains. CYP2E1 is known to generate significant amounts of hydrogen peroxide due to its high degree of uncoupling. Therefore, segregation of CPR and CYP2E1 in different microdomains might serve the physiological function of limiting the generation of this damaging reactive oxygen species.

The present study is the first to show the differential localization of P450 system components into different membrane regions. It is well established that some signal transduction pathways, such as immune receptor signalling, work efficiently through specific microdomains [29,50]. These microdomains can bring proteins together to enhance their ability to interact, or segregate the proteins into different regions to limit their interaction. In a similar manner, the presence of microdomains in the ER could affect P450 function by influencing the ability of particular P450 enzymes to interact with CPR. Although the ratio of CPR to P450 is variable in different tissues, in most tissues such as the liver the CPR:P450 ratio ranges from 1:5 to 1:30 [12]. The co-localization of CPR and a P450 (e.g. CPR and CYP1A2) would be expected to increase their relative concentrations within the microdomain and enhance their ability to interact. In contrast, segregation of proteins into different microdomains, as shown with CPR and CYP2E1, would be expected to diminish activities. Consequently, for CYP2E1 to function in this system, a mechanism for complex formation is required. Likely possibilities involve: (i) the translocation of CYP2E1 to the ordered lipid domains due to some stimulus such as the presence of substrate, (ii) the transfer of CPR to the disordered domains, or (iii) the interaction of CYP2E1 with the small amount of CPR that already resides in the disordered region, a condition that would limit its activity. Therefore, further study is required to determine if stimuli such as substrate or NADPH induce translocation of P450 and the reductase. Protein localization will be expected not only to affect formation of the functional CPR–P450 complex, but also to influence interactions with other protein partners, such as P450 and cytochrome b_5. Several studies have reported that P450 enzymes are capable of both homomeric [11,14,16] and heteromeric [7–10,12] interactions, which result in altered P450 activities. However, many of these studies were done in RCS containing a single lipid type. The results from the present study showing localization of P450 into different microdomains in the ER can either limit or stimulate these potential interactions. As an example, our laboratory has previously reported that CYP1A2 and CYP2E1 interact in RCS [7]. The segregation of CYP2E1 and CYP1A2 into disordered and ordered domains, respectively, has the potential to significantly limit the ability of these proteins to form CYP1A2–CYP2E1 complexes.

The sensitivity for localization of a protein into ordered or disordered domains is exemplified by the comparison of CYP1A1 and CYP1A2. Even though these proteins showed approximately 80 % amino acid sequence similarity, they produced contrasting results regarding their regional localization, with CYP1A1 residing primarily in the disordered regions and CYP1A2 being found in the ordered microdomains (Figure 4). When comparing the amino acid sequences of these two proteins, most variations were found in early N-terminus and catalytic regions. Unlike conserved subcellular targeting motifs, such as KDEL for ER

localization, very little is known about microdomain-targeting motifs within the subcellular compartments. These data suggest that specific sequence motifs may be responsible for targeting CYP1A proteins into ordered or disordered microdomains. A similar explanation could be provided for the pan-domain distribution of CYP2B4. In this case, a subtle change in the sequence of the putative domain-targeting motif could lead to incomplete localization of the protein into ordered microdomains. Studies to identify the protein sequences responsible for their selective incorporation into membrane microdomains are currently ongoing.

In conclusion, the data shown in the present study demonstrate that different forms of P450 are organized in specific membrane regions of the ER. The differential localization of different P450 may provide a novel mechanism for modulating P450 function. Future study will be required to determine if there are changes in dynamic movement of the proteins with stimuli and under particular physiological or pathological conditions modulating membrane lipid composition.

AUTHOR CONTRIBUTION

Ji Won Park, James Reed and Wayne Backes participated in the research design. Ji Won Park and Lauren Brignac-Huber conducted the experiments. Ji Won Park, James Reed, Lauren Brignac-Huber and Wayne Backes performed the data analysis. Ji Won Park, James Reed and Wayne Backes wrote or contributed to the writing of the manuscript.

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