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Organization of NADPH-Cytochrome P450 Reductase and CYP1A2 in the Endoplasmic Reticulum—Microdomain Localization Affects Monooxygenase Function

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ABSTRACT

Cytochrome P450 is part of an electron transport chain found in the endoplasmic reticulum (ER), with its catalytic function requiring interactions with NADPH-cytochrome P450 reductase (CPR). The goals of this study were to examine how the P450 system proteins are organized in the membrane and to determine whether they are distributed in detergent-resistant lipid microdomains (DRM). Isolated liver microsomes from untreated rabbits were treated with 1% Brij 98, and DRMs were isolated via sucrose gradient centrifugation. Lipid analysis showed that DRM fractions were enriched in cholesterol and sphingomyelin, similar to that found with plasma membrane DRMs. Approximately 73% of CYP1A2 and 68% of CPR resided in DRM fractions, compared with only 33% of total ER proteins. These DRMs were found to be cholesterol-dependent: CPR and CYP1A2 migrated to the more dense regions of the sucrose gradient after cholesterol depletion. CYP1A2 function was studied in three purified lipid vesicles consisting of 1) phosphatidylcholine (V-PC), 2) lipids with a composition similar to ER lipids (V-ER), and 3) lipids with a composition similar to the DRM fractions (V-DRM). Each system showed similar substrate binding characteristics. However, when the association between CPR and CYP1A2 was measured, V-ER and V-DRM liposomes produced lower apparent K_m values compared with V-PC without any significant change in V_{max}. These findings suggest that CYP1A2 and CPR reside in ER-DRMs and that the unique lipid components of these domains enhance CYP1A2 substrate metabolism through greater efficiency in CPR-CYP1A2 binding.

Introduction

Cytochromes P450 (P450) constitute a family of heme-containing enzymes that are important in oxidative metabolism of a multitude of endogenous and exogenous compounds (Nelson, 2003). P450s catalyze these reactions by interacting with their redox partner, NADPH-cytochrome P450 reductase (CPR) in a 1:1 molar ratio (Miwa et al., 1979). During substrate metabolism, electrons are transferred from NADPH to CPR, which can then transfer electrons to the P450 (Gigon et al., 1969). Although a 1:1 molar complex between CPR and P450 is needed for metabolism, the concentration of P450 enzymes greatly outnumber the level of CPR, approximately 20:1 in liver microsomes (Peterson et al., 1976). The subsaturating levels of CPR create a situation in which a single CPR molecule must supply electrons to a number of P450 enzymes, rendering metabolically silent those P450s that are unable to complex with CPR. Such a system must be highly organized to maintain efficient substrate metabolism, and one potential means of organization is through the lipid bilayer.

The P450s, along with their redox partners, are embedded in the endoplasmic reticulum (ER) membrane (Peterson et al., 1976), and it has been well established that phospholipid is a required component of an active P450 system (Strobel et al., 1970). Most in vitro studies for the reconstitution of P450 activities use dilauroylphosphatidylcholine as the lipid milieu, but other lipids have been used for these systems, including phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid (Ingelman-Sundberg et al., 1981; Kim et al., 2003; Cho et al., 2008; Reed et al., 2008). The alteration of phospholipid components of reconstituted systems (RCS) can lead to variations in the rate of substrate metabolism, P450 incorporation into the membrane, and stability of the enzyme (Blanck et al., 1984; Ingelman-Sundberg et al., 1996; Reed et al., 2006; Jang et al., 2010). Such differences attributable to lipid composition prompt questions as to how the

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ABBREVIATIONS: P450, cytochrome P450; CPR, NADPH-cytochrome P450 reductase; ER, endoplasmic reticulum; PC, phosphatidylcholine; RCS, reconstituted systems; DRM, detergent-resistant membrane; 7-ER, 7-ethoxyresorufin; 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; MβC, methyl-β-cyclodextrin; V-PC, phosphatidylcholine vesicles; V-ER, ER lipid vesicles; V-DRM, DRM lipid vesicles; EROD, ethoxyresorufin *O*-deethylase.

P450 system is organized in the ER lipid bilayer. Members of our laboratory have initiated studies to analyze and characterize the lipid environment of ER and to determine whether the P450 system resides in discrete lipid microdomains, which may influence CPR-P450 and P450-P450 interactions.

Early structural perceptions of the of lipid bilayer were established by the fluid mosaic model (Singer and Nicolson, 1972), which described the bulk of the phospholipids as being organized discontinuously, a small fraction of the lipid specifically interacting with integral proteins. Studies with the plasma and Golgi membranes have greatly enhanced our views on the organization of the lipid membrane, which has been proven to play a fundamental role in protein-protein and protein-lipid interactions (Brown and London, 1998). Sphingolipids and sterols create a liquid-ordered phase of the membrane as a result of their high melting temperatures, and these domains are involved in the sorting, transmembrane signaling, and transporting of lipids and proteins (Brown and London, 1998). These ordered lipid phases prevent the domains from being solubilized by nonionic detergents (Brown and London, 1998), imparting the term detergent-resistant membranes (DRMs). Such domains were initially characterized by their low density and insolubility in ice-cold 1% Triton X-100 (Brown and London, 2000), but more recently, a number of other nonionic detergents have been used including Brij 98 (Drevot et al., 2002).

Relative to the plasma membrane, the roles of lipid microdomains in the structure of the ER membrane and the function of ER-resident proteins has not been fully investigated. This is probably because there are relatively low levels of sphingolipids and cholesterol at the ER membrane (Glaumann and Dallner, 1968). These lipids are two components of the classic DRM located in the plasma membrane (Pike, 2004). Lipid microdomains in the ER that are analogous to those in the plasma membrane have been described (Bae et al., 2004; Pielsticker et al., 2005; Browman et al., 2006; Hayashi and Fujimoto, 2010). Given the specificity of lipid effects on P450 activity (discussed above), it is possible that the function of these enzymes may be affected by lipid microdomain formation in the ER (Bösterling et al., 1979). In this article, we demonstrate the existence of lipid microdomains in the ER and the presence of the P450 system within these regions.

Materials and Methods

Materials. NADPH-cytochrome P450 reductase antibody was purchased from Stressgen (Ann Arbor, MI). Cytochrome P450 1A1/1A2 antibody was purchased from Abcam (Cambridge, MA). TLC Silica Gel 60 F_{254} was purchased from EMD Chemicals Inc. (Darmstadt, Germany). Silica gel H with 7.5% magnesium acetate was purchased from Uniplate (Newark, DE). Cholesterol assay kit and phosphorous assay kit were purchased from Biovision (Mountain View, CA). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). 7-Ethoxyresorufin (7-ER), 7-ethoxy-4-trifluoromethylcoumarin (7-EFC), methyl-β-cyclodextrin, water-soluble cholesterol, and Brij98 were purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit and Slide-a-Lyzer dialysis cassettes were purchased from Thermo Fisher Scientific (Rockford, IL). Protease cocktail inhibitor was purchased from Roche (Indianapolis, IN). A 5-μm syringe filter was purchased from Osmonics (Greenville, SC). YM-30 filters were purchased from Millipore (Billerica, MA).

Enzyme Source. Rabbit NADPH cytochrome P450 reductase was expressed from a recombinant plasmid containing the wild-type cDNA insert in a vector using the T7 promoter (provided by Lucy Waskell, University of Michigan, Ann Arbor, MI) as described previously (Kelley et al., 2005). CYP1A2 was isolated and purified from β-naphthoflavone-treated rabbit liver microsomes as described previously (Coon et al., 1978). P450 levels were determined by measuring the absorbance of the carbon monoxy ferrous complex at 450 nm (Omura and Sato, 1964).

Preparation of Rabbit Liver Microsomes. Microsomes from untreated rabbit liver were prepared by differential centrifugation (Sequeira et al., 1994), and protein concentration was determined using the BCA assay.

Brij 98 Solubilization and Isolation of Detergent-Resistant Membranes by Sucrose Gradient Centrifugation. A 10% stock solution of Brij 98 was made in lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, and protease cocktail inhibitors, pH 7.4). Sucrose solutions were prepared in a buffer of 50 mM HEPES and 150 mM NaCl, pH 7.4. In preliminary studies, a range of Brij 98 concentrations from 0 to 2% was tested. In subsequent analyses, 1% Brij 98 was used because it is a standard detergent concentration for these types of studies. Microsomal samples (2 mg/ml) were treated with a final concentration of 1% (v/v) Brij 98 in a total volume of 1 ml at 37°C for 5 min. DRMs were isolated as described previously (Pielsticker et al., 2005). In brief, solubilized samples were then combined with an equal volume of 80% sucrose and placed at the bottom of a centrifuge tube. A discontinuous gradient was laid on top consisting of 6 ml of 38% sucrose and 3 ml of 5% sucrose. Samples were centrifuged for 19 h at 210,000*g* at 4°C. Eleven 1-ml fractions were removed from the top of the gradient, and the pellet was rehomogenized in 1 ml of lysis buffer. The distribution of proteins in the gradient was analyzed by Western blotting. DRMs float to the 5/38% interface (fractions 2–5) on the gradient.

Western Blot Analysis. Each fraction from the sucrose gradient was probed for CYP1A2 (1:1000 diluted antibody) and CPR (1:4000 diluted antibody). Treated blots were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

Extraction and Isolation of Lipids. Lipid extraction of the samples was performed by a method described previously (Bligh and Dyer, 1959). Fractions 2 to 5 were combined as the DRM fractions and fractions 6 to 11 as the non-DRM fractions. Pooled samples were dialyzed overnight against 20 mM $NaPO_4$, pH 7.0, to eliminate the sucrose. Lipids were extracted and major phospholipid classes were separated using thin-layer chromatography as described elsewhere (Marcheselli and Bazan, 1990). Phosphate content was assayed according to a phosphate colorimetric assay kit (Biovision). A cholesterol assay kit (Biovision) was used to measure cholesterol content of each fraction.

Effects of Cholesterol on the Microsomal DRMs. To determine whether cholesterol was important in stabilizing the DRM framework, microsomes were depleted of cholesterol by incubating microsomes with methyl-β-cyclodextrin (MβC). In brief, a stock solution of 100 mM MβC was dissolved in buffer (50 mM HEPES and 150 mM NaCl, pH 7.4) immediately before use. MβC (25 mM) was added to 2 mg/ml microsomes and incubated for 30 min at 37°C. Samples were then centrifuged for 20 min at 4°C and 20,000*g*. Pellets were resuspended in 1 ml of lysis buffer. The samples were then treated with Brij 98, and DRMs were isolated by sucrose gradient centrifugation. CYP1A2 and CPR gradient location were analyzed with Western blotting (as described under *Western Blot Analysis*) to determine whether the DRMs were disrupted by detergent treatment after cholesterol depletion.

The role of cholesterol in initiating the formation of the DRMs was then demonstrated by adding cholesterol back to the depleted microsomes by using a water-soluble complex of MβC/cholesterol (1:1). In brief, the water-soluble form of cholesterol (1.5 mM) was added to the samples obtained after cholesterol depletion and incubated for 30 min at 37°C. The samples were then treated with Brij 98 and analyzed by sucrose gradient centrifugation as described under *Brij 98 Solubilization and Isolation of Detergent-Resistant Membranes by Sucrose Gradient Centrifugation*. The cholesterol content, P450 con-

centration, NADPH-cytochrome *c* reductase activity, and metabolism of 7-ER and 7-EFC were determined in all of the samples obtained after cholesterol depletion and "repletion" and compared with samples not treated with MβC.

Lipid Vesicle Preparation. Except for the details described below, vesicular lipid reconstituted systems containing 5 μM CYP1A2 and various concentrations of CPR were prepared at a 500:1 total lipid/P450 ratio as described previously (Reed et al., 2008). Three different lipid compositions were prepared in our RCS. The first vesicle system was prepared with 100% phosphatidylcholine (V-PC). The other two RCS were prepared with lipids to mimic the total microsomal composition (V-ER lipids) and the lipid composition of the DRM fractions (V-DRM) (Table 1). All lipids except for cholesterol (added as described below) were dissolved in chloroform and dried under a stream of N_2 at room temperature until chloroform was completely removed (~1 h). Lipid was rehydrated with 50 mM HEPES, pH 7.5, containing 10% sodium glycocholate and bath-sonicated until it clarified. The solubilized lipid solution was then added to the purified enzymes (to achieve final P450 concentrations of 5 μM) in four equal aliquots of 12.5 μl. N_2 was layered on top of the P450/lipid solution between each addition of the lipid solution before mixing the sample by inversion. This step was used to prevent oxidation of lipids. The mixture was then incubated at 4°C for 1 h before adding 125 mg of Bio-Beads SM-2 to remove the detergent. The vesicles were rocked for 2 h at 4°C, and the vesicles were subsequently separated from the Bio-Beads by drawing up the sample with a 26.5 gauge needle on a 1-ml syringe. The beads were then washed twice with 100 μl of reaction buffer (50 mM HEPES, 15 mM $MgCl_2$, and 5 mM EDTA, pH 7.5) to recover any residual vesicle volume. These washes were added to the original volume extracted from the beads, and the entire sample was filtered through a 5-μm syringe filter. At this point, some vesicles required the addition of cholesterol, which was added as described previously using water-soluble cholesterol (Niu and Litman, 2002). In brief, water-soluble cholesterol was dissolved in H_2O for a final cholesterol concentration of 4 μg/μl. Water-soluble cholesterol was added to obtain a final cholesterol content of 31.25 nmol (5%) in the V-ER lipids and 144 nmol (23%) in the V-DRM lipids. Samples were rocked at room temperature for 2 h, after which they were filtered using an YM-30 filter at 1300*g* for 15 min. This centrifugation allows for the MβC molecule to flow through the filter, leaving the vesicle system above the filter. The vesicle preparation was removed, and the filter was washed twice with 75 μl of reaction buffer. Measurement of the ferrous-CO P450 complex (Omura and Sato, 1964) was used to determine P450 recovery after vesicle preparation. The vesicle preparations were diluted to the desired concentrations with assay buffer and other components before measuring P450 enzymatic activity. Protein incorporation into the lipid vesicles was verified on a size exclusion column. Lipid composition was verified using TLC and cholesterol assay.

Enzymatic Assays. The metabolism of 7-ER was monitored by the change in fluorescence caused by the formation of the product 7-hydroxyresorufin (Lubet et al., 1985). The assays contained 0.05 μM CYP1A2, various concentrations of CPR as indicated in Fig. 6, and 4 μM 7-ER (dissolved in DMSO; final organic concentration was <1%) in reaction buffer. The samples were incubated at 37°C for 1 min before the addition of 0.4 mM NADPH to initiate the reaction. The time course for product formation was measured by fluorescence in real time (excitation, 535 nm; emission, 585 nm). The initial rates were calculated from the linear portions of the fluorescence versus time plots.

7-EFC dealkylation metabolism was also monitored by fluorescence change. The reactions contained 0.15 μM CYP1A2, various concentrations of CPR, and 4 μM 7-EFC (dissolved in DMSO; final organic concentration was <1%). The reaction was again initiated with 0.4 mM NADPH and monitored for 10 min at 37°C. The rate of product formation was determined in real time (excitation, 410 nm; emission, 510 nm). Product formation rates were calculated from standard curves from 7-hydroxy-4-trifluoromethylcoumarin (Hanna et al., 2000). The reaction rates were plotted as a function of CPR concentration, and the apparent K_m (K_m^{app}) and V_{max} values were determined using DynaFit (Kuzmic, 1996), which accounts for the tight binding of CPR with P450s.

Spectral Binding Titrations. Binding constants (K_s) of CYP1A2 for 7-ER and 7-EFC were estimated as described previously (Hosea et al., 2000). In short, vesicle systems were diluted to 0.25 μM CYP1A2 in 100 mM potassium phosphate buffer, pH 7.4, and aliquoted (1 ml) into two glass cuvettes. After an initial baseline (350–500 nm), substrate was added in aliquots for final concentrations ranging from 0.005 to 0.5 μM. The vehicle solvent (DMSO) was added to the reference cuvette each time, and the final organic concentration was ≤1%. The difference in absorbance at 390 nm and 420 nm was plotted against the substrate concentrations. The results were analyzed with a nonlinear regression.

TABLE 1

Lipid composition (mol%) of synthetic reconstituted systems replicating ER lipids

Lipid vesicles were prepared using purified lipids to approximate the lipid composition found in the ER and DRM. CPR and CYP1A2 were incorporated into these vesicles to assess the effect of lipid composition on substrate metabolism and CPR-CYP1A2 binding affinity.

Vesicle Type	PC	PE	SM	PI	PS	PA	Cholesterol
PC	100						
ER lipids	60	20	4	10	1	1	5
DRM lipids	42	18	12	1.5	2	1.5	23

PE, phosphatidylethanolamine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid.

Results

Recent studies have characterized "lipid raft-like" domains at the ER membrane (Pielsticker et al., 2005; Browman et al., 2006; Hayashi and Fujimoto, 2010), and a number of researchers have suggested that specific ER lipids may form organized domains affecting P450 function (Stier and Sackmann, 1973; Kim et al., 2007; Jang et al., 2010). These studies raise the following questions: 1) do the enzymes of the P450 system reside in organized raft-like domains? and 2) does P450 localization in these membrane regions affect P450 function? To address these questions, we attempted to isolate these organized domains from microsomal samples and determine whether the components of the P450 system were localized to these regions. Highly organized lipid domains display different solubility characteristics from disordered domains when treated with nonionic detergents (Brown and London, 2000). Treatment with Triton X-100 at 4°C followed by separation through sucrose gradient centrifugation has been widely used to isolate detergent-resistant domains (Brown and London, 1998). The low temperature is believed to stabilize the membrane phase behavior (Brown and London, 2000), but this protocol has led to criticism that these conditions are not reflective of lipid domain arrangement at physiological temperatures. In contrast, Brij 98, a polyoxyethylene ether detergent, has recently been used in combination with sucrose gradient centrifugation to isolate DRMs at 37°C (Drevot et al., 2002; Pike, 2004).

Isolation of ER-DRMs. In this study, the potential for segregation of CPR and CYP1A2 in ER-DRMs after differential solubilization with various Brij 98 concentrations was examined. After Brij 98 treatment, the samples were applied to discontinuous sucrose gradients. Because of their high lipid/protein ratio, detergent-resistant membranes float to the interface between the 5 and 38% sucrose layers (Pielsticker et al., 2005). Without detergent treatment, both CPR and

CYP1A2 samples remained at the bottom of the sucrose gradient; however, the buoyancy of these proteins was affected by the addition of 0.125% Brij 98 (Fig. 1). At this detergent concentration, both proteins began to migrate to the lighter fractions with significant quantities being found near the 5/38% interface (fractions 2–5). The solubility profiles of CYP1A2 (Fig. 1A) and CPR (Fig. 1B) were similar with Brij 98 concentrations ranging from 0.25 to 1%. When the Brij 98 concentration was elevated to 2%, the proteins no longer accumulated near the 5/38% interface. Therefore, we selected 1% Brij 98 as the concentration for subsequent experiments because it produced consistent effects on protein localization. In addition, 1% Brij 98 was the detergent concentration used in previous lipid domain studies (Brown and London, 1998). Immune blots showed accumulation of CYP1A2 and CPR in the DRM fractions after treatment of the membranes with 1% Brij 98. Densitometric analysis of the blots showed that approximately 73% of CYP1A2 (Fig. 1C) and 68% of CPR (Fig. 1D) were found to reside in the DRM fractions. In contrast, only 33% of the total protein was located in the buoyant DRM fractions.

Characterization of lipid in DRMs. Having demonstrated the presence of CYP1A2 and CPR in DRM fractions using a typical Brij 98 treatment protocol, the next step was to determine whether the lipid composition of these fractions possessed classic detergent-resistant membrane characteristics. As mentioned previously, the classic detergent-resistant membranes found within the plasma membrane and other intracellular organelles are enriched in cholesterol and sphingolipids. Upon analysis of each fraction, cholesterol was found to be enriched in the DRM fractions (Fig. 2). The lipid composition of the total microsomal membrane, DRM, and non-DRM fractions was then analyzed by thin-layer chromatography. Analysis of the DRM fractions illustrated a significant enrichment of sphingomyelin (Fig. 3), which made up approximately 12% of the lipid of these fractions, in contrast to 4% in the total microsomal membrane and less than 1% in the non-DRM fractions. There were significantly lower levels of phosphatidylcholine and phosphatidylinositol in the DRM fractions compared with the total microsomal membrane. Cholesterol accounted for approximately 23% of the DRM fraction lipids. The level of this component is high in contrast to the 5 and 2% composition in the total and non-DRM fractions, respectively. The lipid-to-protein ratio was roughly 3.7 times higher in the DRM fraction compared with the non-DRM fractions (data not shown). It is noteworthy that this specific lipid composition is similar to that of the DRMs found in the plasma membrane and other intracellular organelles (Brown and London, 2000; Pike, 2004; Hayashi and Fujimoto, 2010). It should be noted that although cholesterol content is highest in fraction 3 of the sucrose gradient (Fig. 2), the highest amounts of CYP1A2 and CPR are present in fraction 4 (Fig. 1). These results demonstrate the heterogeneity in DRM fractions and that not all cholesterol-containing DRMs contain CYP1A2 and CPR. Nonetheless, there are significant quantities of cholesterol in the fractions exhibiting enrichment of CPR and CYP1A2.

Cholesterol Dependence of DRM Structure. Some DRMs require less cholesterol than others to maintain their integrity (Pike, 2004); one way to examine the role cholesterol plays in DRM formation is to deplete cholesterol before

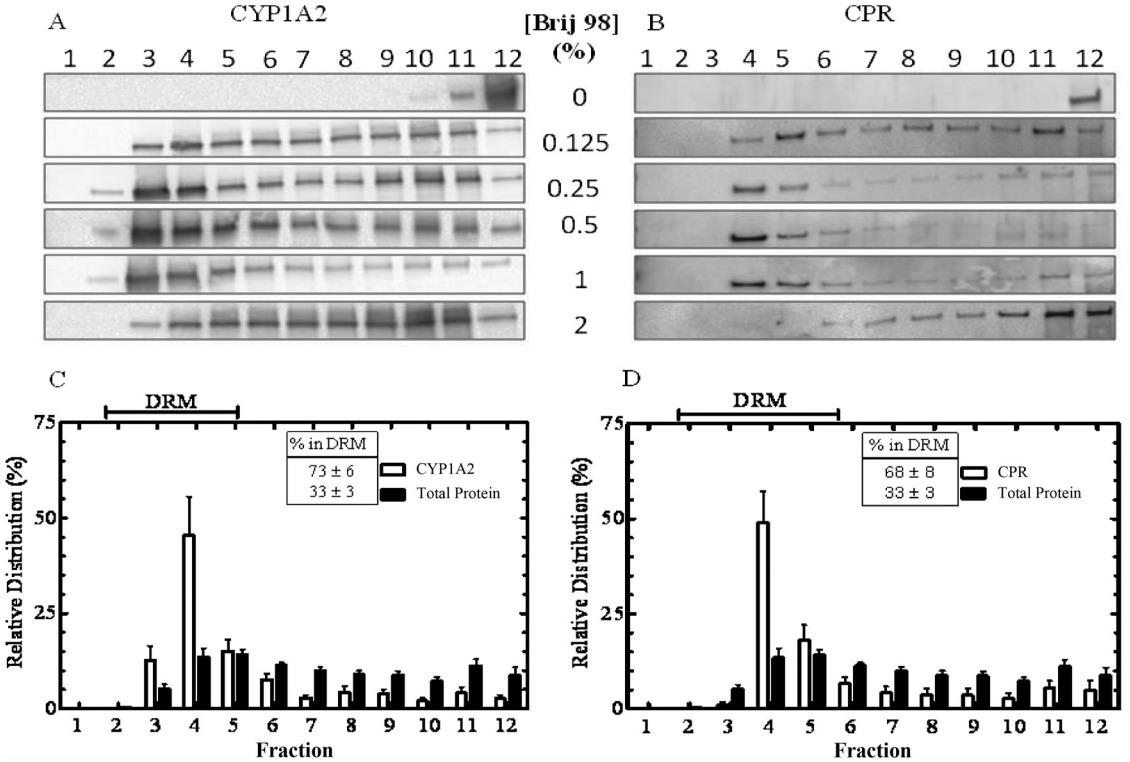


Fig. 1. Association of P450s with DRMs. Microsomes (2 mg protein/ml) were solubilized with increasing concentrations of Brij 98 for 5 min at 37°C, and DRMs were isolated via a sucrose gradient as described under *Materials and Methods* (top). One-milliliter fractions were removed from the top of the gradient and were analyzed by Western blot, probing for CYP1A2 (A) and CPR (B). Densitometry analysis for CYP1A2 (C) and CPR (D) was performed on the blots from 1% Brij 98 solubilization to determine the percentage composition of the proteins in each fraction. Fractions 2 to 5 are designated as DRM fractions. Total protein content is represented in the black bars as determined by BCA assay. (n = 8, ± S.E.M.).

detergent treatment and sucrose density centrifugation. MβC is commonly used for this purpose (Pike, 2004). MβC treatment leads to depletion of cholesterol from the DRMs, making them more susceptible to detergent solubilization. Consequently, proteins in these cholesterol-depleted regions tend be solubilized upon detergent treatment and do not float to the DRM fractions but instead migrate to less buoyant regions of the sucrose gradient. Cholesterol levels, protein content, and substrate metabolism were measured in microsomes treated with 25 mM MβC and compared with untreated microsomes. The results of this comparison are shown in Fig. 4. MβC treatment alone (without subsequent Brij 98 solubilization) had no significant effect on protein content or any of the microsomal activities examined. However, 25 mM MβC treatment depleted cholesterol levels to approximately 35% of control values.

Microsomes were then treated with MβC and then with 1% Brij 98 to partially solubilize the membranes. The samples were applied to a sucrose gradient, and CYP1A2 (Fig. 5A) and CPR (Fig. 5B) localization was detected by immune blot analysis. There was a significant shift of CYP1A2 and CPR into the more dense regions of the gradient after treatment with MβC. Whereas intact DRM fractions contained more than 73% of microsomal CYP1A2 and 68% of CPR, only 6% of CYP1A2 and 2.5% of CPR was detected in DRM fractions after MβC treatment and detergent solubilization. After cholesterol depletion and Brij 98 treatment, 7-ER and 7-EFC activities were approximately 62 and 45% lower, respectively, than microsomes that were treated only with Brij 98 (Fig. 5C). It is noteworthy that when the cholesterol-depleted microsomes were reconstituted with cholesterol by treatment with the MβC-cholesterol complex, both CYP1A2 and CPR migrated back into the DRM fractions of the sucrose gradient. Cholesterol repletion also allowed for partial recovery of activity in the microsomes for both substrates tested. Collectively, these results demonstrate that cholesterol is an important structural component of the DRM and affects the catalytic efficiency of microsomal substrate metabolism.

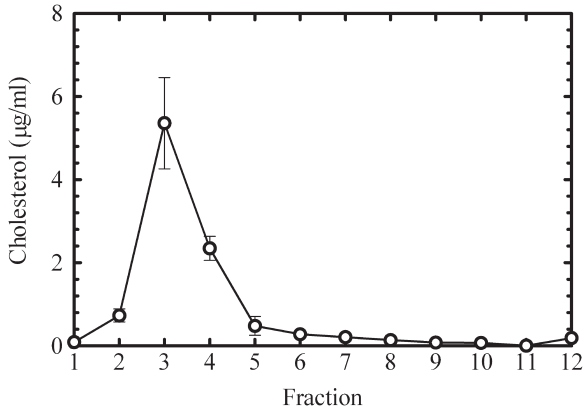


Fig. 2. Enrichment of cholesterol in DRM fractions. After solubilization and isolation of DRMs as described under *Materials and Methods*, cholesterol from each fraction was measured according to the cholesterol assay kit (Biovision). ($n = 5 \pm$ S.E.M.).

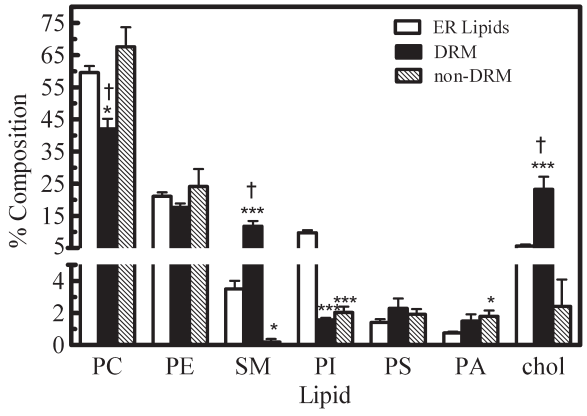


Fig. 3. Lipid profile of ER lipids, DRM, and non-DRM fractions. After solubilization and sucrose gradient centrifugation, lipids from DRM and non-DRM fractions were pooled and extracted. The lipid profiles of total microsomal membrane, DRM fractions, and non-DRM fractions were quantified using phosphorus assay and cholesterol assay kits (Biovision). (*, $p < 0.05$; ***, $p < 0.0001$ statistical comparison of DRM and non-DRM to total) (†, $p < 0.0001$, statistical comparison of DRM with non-DRM).

The Effect of Lipid Composition on Substrate Binding to CYP1A2. To determine whether lipid composition affected substrate binding, spectral substrate binding was examined in three different vesicle systems. The first system consisted of 100% PC vesicles (V-PC), because this is a standard system for many P450 studies and is the most common phospholipid in the ER membrane. The second system (V-ER lipids) mimicked the lipid composition that was found in the total microsomal membrane. Finally, "detergent-resistant membrane" vesicles (i.e., V-DRM) were prepared with the lipid composition found in the DRM fractions as described in Table 1. All reconstituted systems were prepared by the glycocholate–Bio-Beads method (Reed et al., 2006, 2008), a method used to ensure integration of the proteins into the membrane.

Both substrates examined, 7-ER and 7-EFC, produced typical type I spectral changes (data not shown). Only minor effects on both the maximal spectral change and the apparent K_s were observed (Table 2). These results demonstrate that alterations in the membrane have only minor effects on the ability of substrate to interact with this P450 enzyme.

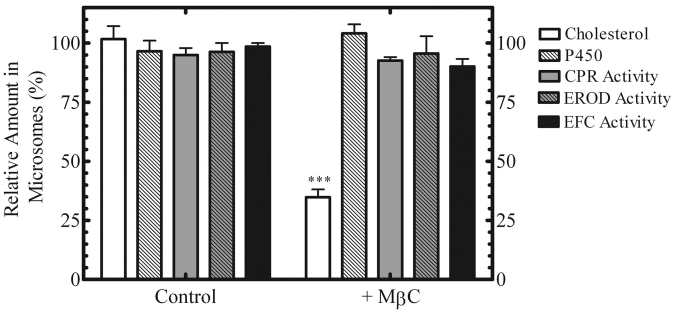


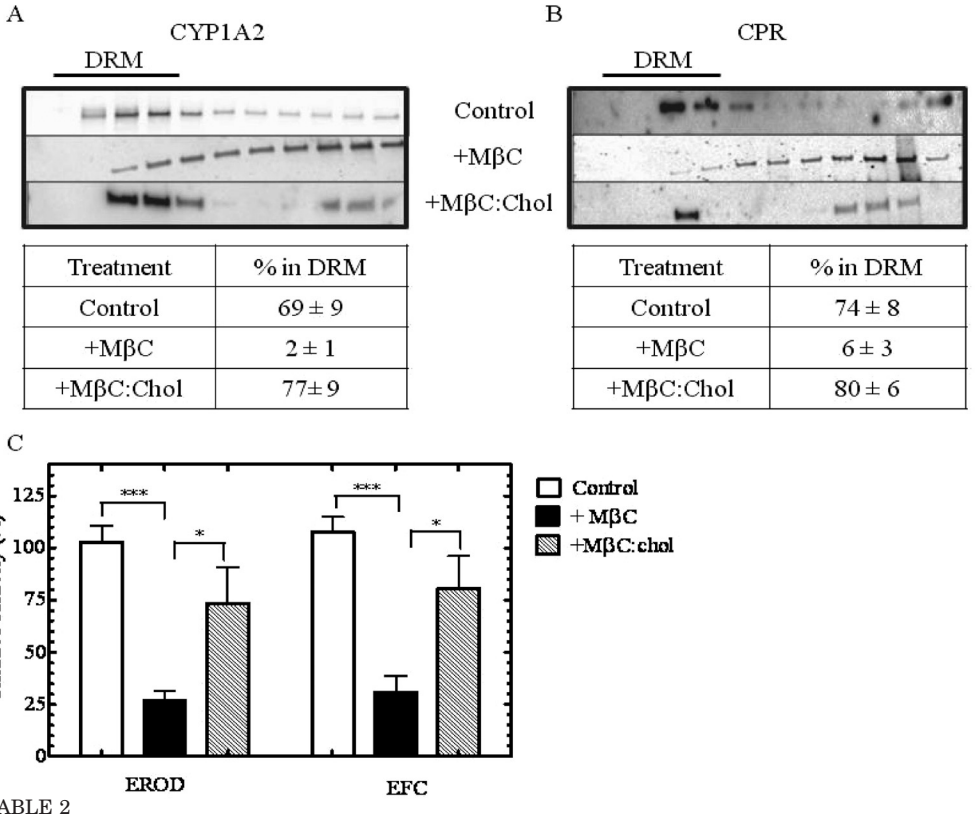
Fig. 4. Effect of MβC treatment on microsomal cholesterol, protein, and activity. Microsomes were treated with and without 25 mM MβC for 30 min at 37°C. Samples were centrifuged and pellets were rehomogenized as stated under *Materials and Methods*. Cholesterol content was assayed as described previously. P450 content was determined using the absorbance at 450 nm of P450-CO complex. NADPH-cytochrome *c* reductase was used to estimate CPR activity, and 7-ER and 7-EFC metabolism was measured using standard assays. Results for MβC-treated samples were normalized to samples that were not treated with MβC. The 100% values were 1.1 μg/ml for cholesterol, 1.1 μM for P450 content, 64.3 nmol of product/min for CPR, 20.7 pmol of resorufin/min/pmol of P450 for 7-ER, 221 pmol of HFC/min/pmol of P450. These data represent the mean ± S.E.M for three determinations; significant differences from control samples are represented by ***, $p \leq 0.001$.

Lipid Composition of Purified Lipid Vesicles Affects CYP1A2 Substrate Metabolism and CPR-CYP1A2 Binding. Optimal activity of the purified P450 monooxygenase system has been shown to be dependent on phospholipid (Strobel et al., 1970), which has led to the reconstitution of purified P450 proteins and CPR into dilauroylphosphatidylcholine and other PC-containing systems. The goal of these experiments was to determine whether the specific lipid content of the total microsomal membrane (V-ER) and DRM fractions (V-DRM) affected CYP1A2 substrate metabolism and CPR-CYP1A2 binding characteristics. Substrate metabolism for both 7-ER and 7-EFC was examined in the same vesicle systems as described above, V- PC, V-ER lipids, and V-DRM vesicles (Table 1).

To assess the potential for the lipid environment to influence P450 function, the effects on CYP1A2-mediated 7-ER and 7-EFC activities were examined. Compared with bovine PC vesicles, the V_{max} values for 7-ER (Fig. 6A, Table 3) and 7-EFC (Fig. 6B, Table 3) in V-ER lipids and V-DRM were not significantly different. However, there was a large and significant effect on the K_m^{app} for CPR. The additional lipid components in the V-ER and V-DRM produced a considerably decreased K_m^{app} for CPR. Compared with PC vesicles, the K_m^{app} values for CPR were decreased 21- and 29-fold in the V-DRM when 7-ER and 7-EFC, respectively, were used as substrates. It is noteworthy that when using 7-ER as a substrate in the presence of "ER lipids," a 9-fold decrease in the K_m^{app} was observed; however, 7-EFC was decreased by only 3-fold under similar membrane conditions. These results suggest that there is a differential sensitivity of CPR binding to CYP1A2 depending on the substrate present. These results clearly demonstrate that the lipid components found in detergent-resistant membranes stimulate CYP1A2 activities, primarily by increasing the efficiency of the CPR-CYP1A2 complex.

Discussion

Several studies have illustrated that different phospholipid species affect the P450 monooxygenase system (Ahn et al., 1998; Kim et al., 2003, 2007; Jang et al., 2010). Most studies focused only on the purified lipid system, titrating in different lipid components and analyzing the effects on P450 function. A majority of the aforementioned studies look at binary and ternary lipid systems examining the effects of lipid mixtures at relative ratios that are not physiologically relevant. The present study used a different approach—to



Treatment	% in DRM
Control	69 ± 9
+MβC	2 ± 1
+MβC:Chol	77± 9

Treatment	% in DRM
Control	74 ± 8
+MβC	6 ± 3
+MβC:Chol	80 ± 6

Fig. 5. Effects of cholesterol on the microsomal DRMs. Microsomes were treated with 25 mM MβC for 30 min at 37°C (cholesterol depletion) or with MβC and then subsequently with 1.5 mM MβC-cholesterol (MβC:Chol) for 30 min at 37°C (cholesterol repletion). Control samples were not treated with MβC or MβC-cholesterol. All samples were then treated with 1% Brij 98 as described previously. CYP1A2 (A) and CPR (B) gradient location was analyzed by Western blot, and relative distribution was quantified by densitometry analysis. After the treatments, metabolism of 7-ER and 7-EFC was measured (C). Activity was normalized to samples that were not depleted of cholesterol. The 100% activity values represent the normalized activity for the samples not treated with MβC (20.2 pmol of product/min/pmol of P450 for 7-ER and 231.5 pmol of product/min/pmol of P450 for 7-EFC). These data represent the mean ± S.E.M for three determinations; significant differences in activity are represented by ***, $p \leq 0.001$.

TABLE 2
Effect of lipid composition on spectral binding of 7-ER and 7-EFC to CYP1A2
Apparent changes in substrate binding affinity for CYP1A2 were determined by spectral titrations in each vesicle system. Both substrates exhibited a type I response typical of a low-to-high spin conversion. (n = 3 ± S.E.M.)

Vesicles	7-ER		7-EFC	
	K_s	ΔAbs_{max}	K_s	ΔAbs_{max}
	μM		μM	
PC	0.058 ± 00.006	0.011 ± 0.0003	0.044 ± 0.006	0.009 ± 0.0003
ER lipids	0.071 ± 00.008	0.008 ± 0.0002	0.050 ± 0.01	0.008 ± 0.0006
DRM lipids	0.075 ± 0.005	0.01 ± 0.0002	0.053 ± 0.01	0.009 ± 0.0007

characterize the lipid compositions of ER-DRMs in rabbit microsomal tissue and to investigate the localization of P450 system components within these domains by detergent solubilization and sucrose gradient centrifugation. The study corroborates previous findings indicating that DRMs exist in the ER membrane (Pielsticker et al., 2005; Browman et al., 2006; Hayashi and Fujimoto, 2010) and demonstrates that CYP1A2 and CPR reside primarily in these domains. We then tested the effects of lipid composition on CYP1A2 function by comparing its activity in lipid vesicles that were representative of the total ER and ordered (detergent-resistant) lipid domains to that in standard phosphatidylcholine vesicles. This is the first investigation to examine the effects of these specific lipid compositions on P450 activity and its interaction with CPR with lipids at physiologically relevant concentrations.

Plasma membrane DRMs have been described as heterogeneous domains typically enriched in sterols and sphingomyelin (Brown and London, 1998; Pike, 2004). In agreement with other reports, the current study illustrated that the overall ER lipid composition was low in cholesterol and sphingomyelin (Glaumann and Dallner, 1968), which may explain the small number of investigations attempting to characterize ER-DRMs. However, by using a standard technique to isolate these domains from rabbit liver microsomes with Brij 98 at 37°C (Drevot et al., 2002), detergent-resistant regions were found to be enriched in cholesterol and sphingomyelin. There were major differences in the lipid composition of the total ER membrane compared with the lipid composition in the DRM fractions. These results demonstrate lipid domain formation within the ER bilayer. Lipid analysis demonstrated that ER microdomains could be isolated at physiological temperatures and had a composition similar to DRMs found in the plasma membrane and other intracellular organelles (Brown and London, 2000; Gkantiragas et al., 2001; Pielsticker et al., 2005).

CYP1A2 and CPR were found to be enriched in the DRM fractions after Brij 98 solubilization, in contrast to the total microsomal protein. Our data are consistent with a previous large proteomic study identifying 39 proteins including CYP1A2 and CPR in ER microdomains (Bae et al., 2004). It is noteworthy that our results are in contrast to those found by Hayashi and Fujimoto (2010), who reported that CPR did not reside in DRMs. This discrepancy can be explained by the difference in the tissue source. Their experiments were done using Chinese hamster ovary cells. The ER of these cells has different lipid and protein compositions, which could significantly affect the localization of individual proteins. Our study further investigated the nature of the CYP1A2- and CPR-containing DRMs by demonstrating that the domains were cholesterol-dependent. Cholesterol sequestration by MβC rendered the DRMs sensitive to detergent treatment, which led to the solubilization of CYP1A2 and CPR. Analogous to the plasma membrane, the cholesterol component seems necessary for the structural integrity of these domains. These results are similar to those of other studies in which MβC-mediated cholesterol depletion led to solubilization of proteins residing in ER-DRMs (Browman et al., 2006; Hayashi and Fujimoto, 2010). It is noteworthy that when MβC-depleted microsomes were reconstituted with cholesterol, both CYP1A2 and CPR migrated to the DRM fractions, and catalytic activities were restored.

Using the lipid analysis of the ER microsomal tissue and DRM fractions, the effects of these specific lipid environments on CYP1A2 activity in purified reconstituted systems were examined. Previous research has shown that P450 activity is influenced by the presence of anionic phospholipids (Ingelman-Sundberg et al., 1981; Kim et al., 2003, 2007; Jang et al., 2010); however, the lipid effects described in our study are distinct from the previously described effects of anionic phospholipids. For instance, Ahn et al. (1998) demonstrated that CYP1A2 catalysis of 7-ethoxycoumarin was increased with increasing concentrations of anionic phospholipids (e.g., phosphatidic acid, phosphatidylinositol, and phosphatidylserine). This group reported up to a 3-fold increase in CYP1A2 activity, but it should also be noted that they monitored the peroxidative activity of CYP1A2 in the absence of CPR (Ahn et al., 1998). Thus, the anionic phospholipids affected the catalytic potential of the CYP1A2 and not its affinity for the CPR. In contrast, our data implying that cholesterol and sphingomyelin have a role in the formation of DRMs showed that these lipids had no effect on the catalytic potential of CYP1A2 (as evidenced by the V_{max} of our CPR titration curves). Instead, our results showed that increasing cholesterol and sphingomyelin contents were associated with greater apparent binding affinity (lower K_m^{app}) between the

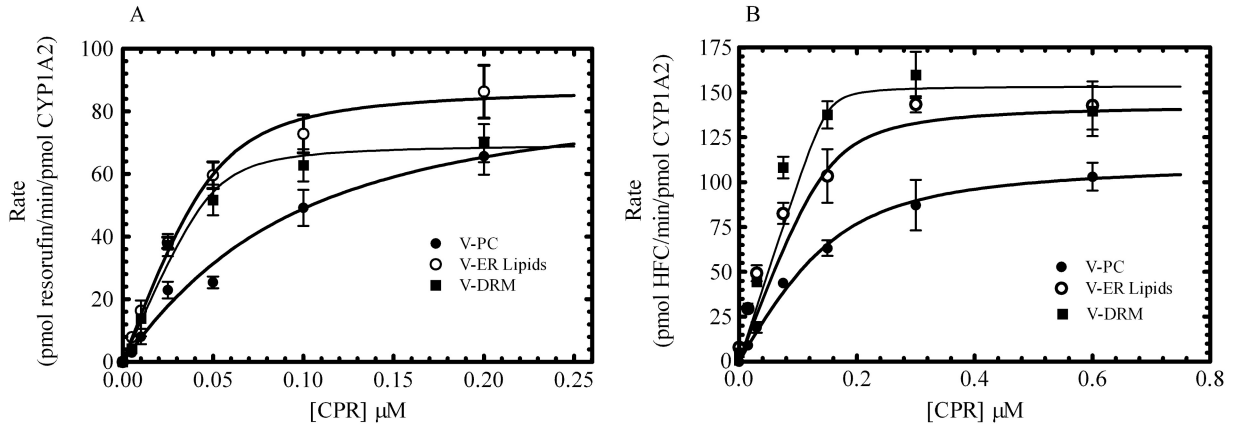


Fig. 6. Effect of lipid composition on CYP1A2-mediated EROD and EFC as a function of CPR concentration. EROD (A) and EFC (B) were determined as a function of CPR concentration in V-PC, V-ER, and V-DRM. Each lipid system for EROD contained 0.05 μM CYP1A2, varying reductase from 0 to 0.2 μM and 2500 μM lipid. Each system for 7-EFC contained 0.15 μM CYP1A2, varying reductase from 0 to 0.6 μM and 2500 μM lipid. The experimental data for each vesicle system (in both A and B) could be effectively fit using a simple model allowing the formation of binary complexes.

P450 and CPR using both 7-ER and 7-EFC as substrates. Thus, CPR and CYP1A2 seem to form a productive complex more efficiently in the DRM environment. This assumption is also corroborated by the decrease in CYP1A2-specific activity after microsomal depletion of cholesterol using MβC (Fig. 5).

Although our data using a single P450 enzyme and CPR support the idea that sphingomyelin and cholesterol lead to an apparent decrease in the K_m^{app} for the CPR-CYP1A2 complex (Fig. 6), corresponding data were not observed after depletion of cholesterol from the ER membranes (Fig. 4). A potential explanation for this apparent discrepancy is that the vesicular systems are simpler than the ER, having fewer proteins and a defined lipid system. The presence of multiple P450s in the ER could alter the CPR dependence on EROD and 7-EFC metabolism, as heteromeric P450-P450 complexes are known (Kelley et al., 2005).

Looking at another isoform of P450, Ingelman-Sundberg et al. (1981) found CYP2B4 activity increased in lipid vesicles containing phosphatidylserine. This effect was attributed to more efficient interaction between CPR and CYP2B4. Das and Sligar (2009) illustrated that CPR redox potential became more negative in nanodiscs composed of 50% 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine/50% 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine compared with 100% 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine. This anionic environment would favor electron transfer from CPR to P450s. Although these studies do show that a negatively charged lipid environment can affect how CPR interacts with P450 enzymes other than CYP1A2, the anionic phospholipid concentrations used by the previous studies are much higher (in some cases up to 30 mol% higher) than that observed physiologically. In contrast, we have observed effects on CYP1A2 metabolism using physiologically relevant anionic phospholipid concentrations. Furthermore, our data show that the highest binding affinity between CPR and CYP1A2 was observed in the V-DRM, which contained the lowest concentration of anionic phospholipids.

More recently, other in vitro studies have suggested that P450 enzymes use anionic phospholipids to induce the formation of lipid domains that influence P450 function. Kim et al. (2007) demonstrated that CYP2B1 induced the formation of anionic lipid-rich microdomains, particularly with PA. This lipid milieu allowed for increased binding of CYP2B1 to the membranes, which in turn led to increased catalytic activity. Another study illustrated similar results with CYP3A4 demonstrating that PE induced the formation of domains enriched in anionic phospholipids. These domains were associated with stimulated CYP3A4 membrane binding and activity (Kim et al., 2003). Although these studies raise interesting questions about the possible influence of P450 enzymes on lipid segregation in the ER, it is debatable whether the proportions of anionic phospholipids used to initiate the formation of these domains in these studies are physiologically relevant.

Several conclusions can be drawn from the current study. First, detergent-resistant lipid microdomains can be found in the endoplasmic reticulum and, similar to that found in the plasma membrane, the ER DRMs are enriched with both sphingomyelin and cholesterol. Second, CYP1A2 and CPR are found to preferentially localize to these resistant membranes, and the removal of cholesterol by treatment with methyl-β-cyclodextrin leads to a significant shift of CYP1A2 and CPR out of the DRM fractions to more dense regions of the gradient. Furthermore, the DRM phospholipids seem to modulate P450 function. Reconstituted systems having phospholipid content similar to that found in the ER membrane cause alterations in CYP1A2 metabolic activities. Although the V_{max} values obtained from the CPR titration curves were not significantly changed, there was a substantial decrease in the K_m^{app} for CPR compared with phosphatidylcholine alone. It is noteworthy that elevation of sphingomyelin and cholesterol to levels seen in detergent-resistant microdomains caused a further decrease in the K_m^{app} for CPR. All told, the K_m^{app} for CPR in reconstituted systems having phospholipid content similar to that found in DRMs is between 21 and 29 times smaller than that found in PC vesicles, suggesting that the unique lipid content of these vesicles substantially increases CYP1A2-dependent metabolism by increasing the efficiency of CPR-CYP1A2 interactions. These results demonstrate that lipid microdomains can have a significant influence on the localization of proteins of the P450 electron transport chain and that residence in these DRMs can have a significant influence on P450 function.

TABLE 3

Kinetic constants of the simulated curves for CPR-CYP1A2 vesicle systems

Apparent K_m (K_m^{app}) and V_{max} values for CYP1A2 metabolism of 7-ER and 7-EFC were determined from the data in Fig. 6, using DynaFit (BioKin). These data represent the mean ± S.E.M. for four determinations.

Vesicles	7-ER		7-EFC	
	K_m^{app}	V_{max}	K_m^{app}	V_{max}
	μM	*pmol/min/pmol 1A2*	*μM*	*pmol/min/pmol A2*
PC	0.07 ± 0.02	102 ± 15	0.043 ± 0.008	130 ± 11
ER lipids	0.0078 ± 0.003*	109 ± 7	0.013 ± 0.008*	161 ± 10
DRM	0.0033 ± 0.001*	86 ± 7	0.0015 ± 0.001***	171 ± 11

* $P \leq 0.05$, significantly different from PC.
*** $P \leq 0.001$, significantly different from PC.

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Authorship Contributions

Participated in research design: Brignac-Huber, Reed, and Backes.
Conducted experiments: Brignac-Huber.
Performed data analysis: Brignac-Huber, Reed, and Backes.
Wrote or contributed to the writing of the manuscript: Brignac-Huber, Reed, and Backes.
Other: Backes acquired funding for the research.

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