Organization of NADPH-Cytochrome P450 Reductase and CYP1A2 in the Endoplasmic Reticulum – Microdomain localization affects monooxygenase function

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Abbreviations – Cytochrome P450 (P450), NADPH-cytochrome P450 reductase (CPR), detergent-resistant microdomains (DRMs), Phosphatidylcholine vesicles (V-PC), ER lipids vesicles (V-ER), DRM lipid vesicles (V-DRM), dilauroylphosphatidylcholine (DLPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA), phosphatidylinositol (PI), sphingomyelin (SM), cholesterol (chol) reconstituted systems (RCS), liquid-ordered (lo), 7-ethoxyresorufin (7-ER), 7-ethoxy-4-trifluoromethylcoumarin (7-EFC), methyl-β-cyclodextrin (MβC), apparent dissociation constant (Ks), apparent Michaelis constant for the CPR-CYP1A2 interaction (Km_app), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoserine (POPS), endoplasmic reticulum (ER)
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 if 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 as compared to 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 were measured, V-ER and V-DRM liposomes produced lower apparent $K_{m}$ values compared to 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 more efficient CPR-CYP1A2 binding.
Introduction

Cytochrome P450 (P450) is 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, about 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 making the P450s not able to complex with CPR metabolically silent. 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 (DLPC) as the lipid milieu, but other lipids have been used for these systems including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid (Reed et al., 2008; Cho et al., 2008; Kim et al., 2003; Ingelman-Sundberg et al., 1981). 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 (Ingelman-Sundberg et al., 1996; Reed et al., 2006; Blanck et al., 1984; Jang et al., 2010). Such differences
due to lipid composition impose questions as to how the P450 system is organized in the ER lipid bilayer. Our lab has initiated studies to analyze and characterize the lipid environment of ER and to determine if 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 with 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 (lo) phase of the membrane due to their high melting temperatures and these domains have implicated roles in lipid and protein sorting, transmembrane signaling proteins through GPI-anchored proteins and secretory and endocytic pathways (Brown and London, 1998). These ordered lipid phases prevent the domains from being solubilized by non-ionic detergents (Brown and London, 1998), lending to the term detergent-resistant membranes (DRMs). Such domains were initially characterized by their low density and insolubility in cold 1% Triton X-100 (Brown and London, 2000), but more recently, a number of other non-ionic 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 due to the fact that there are relatively low levels of sphingolipids and cholesterol at the ER membrane (Glaumann and Dallner, 1968). These lipids are two components of the classical DRM located in the plasma membrane (Pike, 2004). Recently, groups have described lipid
microdomains in the ER that are analogous to those in the plasma membrane (Bae et al., 2004; Browman et al., 2006; Pielsticker et al., 2005; 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. (Bosterling et al., 1979) In this paper, 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 F254 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-cyclodextran, water-soluble cholesterol, and Brij98 were purchased from Sigma-Aldrich (St. Louis, MO). BCA protein assay kit and slide-a-lyzer dialysis cassettes were purchased from Thermo Scientific (Rockford, IL). Protease cocktail inhibitor was purchased from Roche (Indianapolis, IN). 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) as previously described (Kelley et al., 2005). CYP1A2 was isolated and purified from β-napthoflavone-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 bicinchoninic acid (BCA) assay (Thermo Scientific-Pierce).
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 (50mM HEPES, 150mM NaCl, 5mM EDTA, protease cocktail inhibitors, pH 7.4). Sucrose solutions were prepared in a buffer of 50mM HEPES, 150mM NaCl, pH 7.4. In preliminary studies, a range of Brij 98 concentrations from 0 - 2% was tested. In subsequent analyses 1% Brij 98 was used as it is a standard detergent concentration for these types of studies. Microsomal samples (2mg/ml) were treated with a final concentration of 1% Brij98 (v/v) in a total volume of 1 ml at 37°C for 5 minutes. DRMs were isolated as previously described (Pielsticker et al., 2005). Briefly, 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 hours at 210,000 x g at 4°C. Eleven one ml fractions were removed from the top of the gradient and the pellet was re-homogenized 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.

Extraction and Isolation of Lipids. Lipid extraction of the samples was performed by a previously described method (Bligh and Dyer, 1959). Fractions 2-5 were combined as the DRM fractions and 6-11 as the non-DRM fractions. Pooled samples were dialyzed overnight against 20mM NaPO₄, 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). Phosphorous content was assayed according to a phospholipid assay kit.
(Biovision). Cholesterol content of each fraction was measured by a cholesterol assay kit (Biovision).

**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). Briefly, a stock solution of 100mM MβC was dissolved in buffer (50 mM HEPES, 150 mM NaCl, pH 7.4) immediately before use. MβC (25 mM) was added to 2 mg/ml of microsomes and incubated for 30 minutes at 37°C. Samples were then centrifuged for 20 minutes at 4°C and 20,000 x 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 above) in order to determine if 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). Briefly, 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 above. The cholesterol content, P450 concentration, NADPH-cytochrome c reductase activity, and metabolism of 7-ER and 7-EFC were determined in all of the samples obtained following cholesterol depletion and “repletion” and compared to 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 previously described (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 N₂ at room temperature until chloroform was completely removed (~1 hour). 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. N₂ 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 hour before adding 125 mg of Biobeads SM-2 to remove the detergent. The vesicles were rocked for 2 hr at 4°C and the vesicles were subsequently separated from the Biobeads by drawing up the sample with a 26.5 gauge needle on a 1 ml syringe. The beads were then washed twice with 100µl of Reaction Buffer (50mM HEPES, 15mM MgCl₂ 5mM 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 previously described using water-soluble cholesterol (Niu and Litman, 2002). Briefly, water-soluble cholesterol was dissolved in H₂O for a final cholesterol concentration of 4µg/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 hours upon which they were filtered using an
YM-30 filter at 1300 x g for 15 minutes. 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, the indicated concentrations of CPR, and 4 µM 7-ER (dissolved in DMSO; final organic concentration was < 1%) in Reaction Buffer. The samples were incubated at 37°C for one minute 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 4µM 7EFC (dissolved in DMSO; final organic concentration was < 1%). The reaction was again initiated with 0.4 mM NADPH and monitored for 10 minutes 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 (Kᵢ) of CYP1A2 for 7-ER and 7-EFC were estimated as previously described (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 µM- 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 of 390 nm and 420 nm was plotted against the substrate concentrations. The results were analyzed with a nonlinear regression.
Results

Recent studies have characterized “lipid raft-like” domains at the ER membrane (Pielsticker et al., 2005; Hayashi and Fujimoto, 2010; Browman et al., 2006) 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 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? In order to address these questions, we attempted to isolate these organized domains from microsomal samples, and determine if 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 non-ionic 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 member of the polyoxyethylene ether detergents, has been recently utilized 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 following differential solubilization with various Brij 98 concentrations was examined. After Brij 98 treatment, the samples were applied to discontinuous sucrose gradients. Due to 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 (Figure 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 (Figure 1A) and CPR (Figure 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.

Additionally, 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 (Figure 1C) and 68% of CPR (Figure 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 if the lipid composition of these fractions possessed classical detergent-resistant membrane characteristics. As previously mentioned, the classical 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 (Figure 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 (Figure 3), which comprised about 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 (PC) and phosphatidylinositol (PI) in the DRM fractions when compared to the total microsomal membrane. Cholesterol comprised 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 when compared to the non-DRM fractions (data not shown). Interestingly, this specific lipid composition is similar to that of the DRMs found in the plasma membrane and other intracellular organelles (Hayashi and Fujimoto, 2010; Brown and London, 2000; Pike, 2004). It should be noted that while cholesterol content is highest in fraction 3 of the sucrose gradient (Figure 2), the highest amounts of CYP1A2 and CPR are present in fraction 4 (Figure 1). These results demonstrate the heterogeneity in DRM fractions and that all cholesterol-containing DRMs do not 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) and one such way to examine the role cholesterol plays in DRM formation is to deplete cholesterol prior to detergent treatment and sucrose density centrifugation. Methyl-β-cyclodextrin (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 figure 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 about 35% of control values.

Microsomes were then treated with MβC, and then treated 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 following MβC treatment and detergent solubilization. After cholesterol depletion and Brij 98 treatment, 7-ethoxyresorufin (7-ER) and 7-ethoxytrifluorocoumarin (7-EFC) activities were approximately 62% and 45% lower, respectively, than microsomes that were only treated with Brij 98 (Fig 5C). Interestingly, 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 also affects the catalytic efficiency of microsomal substrate metabolism.

The Effect of Lipid Composition on Substrate Binding to CYP1A2. In order to determine if 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), as 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. Lastly, “detergent-resistant membrane” vesicles (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-Biobeads method (Reed et al., 2006; Reed et al., 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 (not shown). Only minor effects on both the maximal spectral change and the apparent Kᵢ 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.

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 (DLPC) and other PC-containing systems. The goal of these experiments was to determine if 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 were 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 effect on CYP1A2-mediated 7-ER and 7-EFC activities were examined. When compared to bovine PC vesicles, the Vₘₐₓ for 7-ER (Figure 6A, Table 3) and 7-EFC (Figure 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. When compared to 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. Interestingly, 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 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; Kim et al., 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 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 (Browman et al., 2006; Pielsticker et al., 2005; 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 utilizing a standard technique to isolate these domains from rabbit liver microsomes with Brij 98 at 37°C (Drevot et
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 when compared to 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 following 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). Interestingly, our results are in contrast to those found by Hayashi and Fujimoto, who reported that CPR did not reside in DRMs (Hayashi, 2010 2320 /id). This discrepancy can be explained by the difference in the tissue source. These 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 appears necessary for the structural integrity of these domains. These results are similar to 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). Interestingly, when MβC-depleted microsomes were
reconstituted with cholesterol, both CYP1A2 and CPR migrated to the DRM fractions and catalytic activities were restored.

Utilizing 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., 2007; Jang et al., 2010; Kim et al., 2003); however, the lipid effects described in our study are distinct from the previously described effects of anionic phospholipids. For instance, Ahn et al. demonstrated that CYP1A2 catalysis of 7-ethoxycoumarin was increased with increasing concentrations of anionic phospholipids (e.g. PA, PI, and PS) (Ahn et al., 1998). This group reported up to a 3-fold increase in CYP1A2 activity. However, it should also be noted that the previous study 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 implicating the role of cholesterol and sphingomyelin in the formation of DRMs showed that these lipids had no effect on the catalytic potential of CYP1A2 (as evidenced by the \( V_{\text{max}} \) of our CPR titration curves). Instead, our results showed that increasing cholesterol and sphingomyelin contents were associated with greater apparent binding affinity (lower \( K_{\text{m,app}} \)) between the P450 and CPR using both 7-ER and 7-EFC as substrates. Thus, it appears that CPR and CYP1A2 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 \( K_{\text{m,app}} \) for the CPR-CYP1A2
complex (Fig. 6), corresponding data was 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 found (Ingelman-Sundberg et al., 1981) CYP2B4 activity increased in lipid vesicles containing PS. This effect was attributed to more efficient interaction between CPR and CYP2B4. More recently, Das and Sligar illustrated that CPR redox potential became more negative in nanodiscs comprised of 50% 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) / 50% 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoserine (POPS) when compared to 100% POPC (Das and Sligar, 2009). 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 demonstrated that CYP2B1 induced the formation of anionic lipid-rich microdomains particularly with PA (Kim et al., 2007). This lipid milieu allowed for increased binding of
CYP2B1 to the membranes that 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.

There are several conclusions that can be made 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 that 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 appear to modulate P450 function. Reconstituted systems having similar phospholipid content to that found in the ER membrane cause alterations in CYP1A2 metabolic activities. Although the $V_{\text{max}}$ values obtained from the CPR titration curves were not significantly changed, there was a substantial decrease in the $K_m^{\text{app}}$ for CPR when compared to phosphatidylcholine alone. Interestingly, elevation of sphingomyelin and cholesterol to levels seen in detergent resistant microdomains caused a further decrease in the $K_m^{\text{app}}$ for CPR. All told, the $K_m^{\text{app}}$ for CPR in reconstituted systems having phospholipid content similar to that found in DRMs is between 21-29 times smaller than that found in PC vesicles, suggesting that the unique lipid content of these vesicles substantially increase 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.
Authorship Contribution

Participated in Research Design: Brignac-Huber, Reed, Backes
Conducted Experiments: Brignac-Huber
Performed Data Analysis: Brignac-Huber, Reed, Backes
Wrote or contributed to the writing of the manuscript: Brignac-Huber, Reed, Backes
Other: Backes acquired funding for the research
Reference List


Footnotes

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Legends for Figures

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

Figure 2. Enrichment of Cholesterol in DRM fractions. After solubilization and isolation of DRMs as described in Material and Methods, cholesterol from each fraction was measured according to the cholesterol assay kit (Biovision). (n=5 ± S.E.M.)

Figure 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 a phosphorous assay and cholesterol assay kits (Biovision). (*, p< 0.05, ***, p < 0.0001 statistical comparison of DRM and non-DRM to total) († p < .0001, statistical comparison of DRM to non-DRM).

Figure 4. Effect of MβC Treatment on Microsomal Cholesterol, Protein and Activity. Microsomes were treated with and without 25 mM MβC for 30 minutes at 37°C. Samples were
centrifuged and pellets were re-homogenized as stated in Material 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 product/min for CPR, 20.7 pmol resorufin/min/pmol P450 for 7-ER, 221 pmol HFC/min/pmol P450. These data represent the mean ± S.E.M for 3 determinations, where significant differences from control samples are represented by ***, p ≤ 0.001.

Figure 5. Effects of Cholesterol on the Microsomal DRMs. Microsomes were treated with 25 mM MβC for 30 minutes at 37°C (cholesterol depletion) or with MβC and then subsequently with 1.5 mM MβC:Chol for 30 minutes at 37°C (cholesterol repletion). Control samples were not treated with either MβC or MβC:cholesterol. All samples were then treated with 1% Brij 98 as previously described. CYP1A2 (A) and CPR (B) gradient location was analyzed by western blot and relative distribution was quantified by densitometry analysis. Following 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 P450 for 7-ER and 231.5 pmol of product/min/pmol P450 for 7-EFC). These data represent the mean ± S.E.M for 3 determinations, where significant differences in activity are represented by ***, p ≤ 0.001.

Figure 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 PC (V-PC), ER lipid vesicles (V-ER), and DRM vesicles (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 7EFC 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 panels A and B) could be effectively fit using a simple model allowing the formation of binary complexes.
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 in order to assess the effect of lipid composition on substrate metabolism and CPR-CYP1A2 binding affinity.

<table>
<thead>
<tr>
<th>Vesicle Type</th>
<th>PC</th>
<th>PE</th>
<th>SM</th>
<th>PI</th>
<th>PS</th>
<th>PA</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ER lipids</td>
<td>60</td>
<td>20</td>
<td>4</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>DRM lipids</td>
<td>42</td>
<td>18</td>
<td>12</td>
<td>1.5</td>
<td>2</td>
<td>1.5</td>
<td>23</td>
</tr>
</tbody>
</table>
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.)

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>7-ER</th>
<th>7-EFC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_s$ (µM)</td>
<td>$\Delta A_{max}$</td>
</tr>
<tr>
<td>PC</td>
<td>0.058 ± 0.006</td>
<td>0.011 ± 0.0003</td>
</tr>
<tr>
<td>ER lipids</td>
<td>0.071 ± 0.008</td>
<td>0.008 ± 0.0002</td>
</tr>
<tr>
<td>DRM lipids</td>
<td>0.075 ± 0.005</td>
<td>0.01 ± 0.0002</td>
</tr>
</tbody>
</table>
Table 3. Kinetic Constants of the Simulated Curves for CPR-CYP1A2 vesicle systems.

Apparent $K_m$ ($K_m^{\text{app}}$) and $V_{\text{max}}$ values for CYP1A2 metabolism of 7ER and 7EFC were determined from the data in Fig.6, using DynaFit (BioKin). These data represent the mean ± S.E.M for 4 determinations, where significant differences from PC are represented by * $p \leq 0.05$, ***, $p \leq 0.001$.

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>7-ER</th>
<th></th>
<th>7-EFC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m^{\text{app}}$ (µM)</td>
<td>$V_{\text{max}}$ (pmol/min/pmol A2)</td>
<td>$K_m^{\text{app}}$ (µM)</td>
<td>$V_{\text{max}}$ (pmol/min/pmol A2)</td>
</tr>
<tr>
<td>PC</td>
<td>0.07 ± 0.02</td>
<td>102 ± 15</td>
<td>0.043 ± 0.008</td>
<td>130 ± 11</td>
</tr>
<tr>
<td>ER lipids</td>
<td>0.0078 ± 0.003*</td>
<td>109 ± 7</td>
<td>0.013 ± 0.008*</td>
<td>161 ± 10</td>
</tr>
<tr>
<td>DRM</td>
<td>0.0033 ± 0.001*</td>
<td>86 ± 7</td>
<td>0.0015 ± 0.001 ***</td>
<td>171 ± 11</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3

The figure shows the percentage composition of various lipids in different cellular compartments. The y-axis represents the percentage composition, ranging from 0% to 75%. The x-axis lists different lipids: PC, PE, SM, PI, PS, PA, and chol (cholesterol).

- **ER Lipids** are represented by open bars.
- **DRM** (Detergent-resistant membrane) are represented by black bars.
- **Non-DRM** are represented by hatched bars.

Significant differences are indicated by symbols: * for p < 0.05, ** for p < 0.01, *** for p < 0.001, and † for other significance levels.

The data suggests that certain lipids have higher concentrations in specific compartments, with notable differences in DRM and non-DRM compared to ER lipids.
Figure 5

A) CYP1A2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% in DRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>69 ± 9</td>
</tr>
<tr>
<td>+MβC</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>+MβC:Chol</td>
<td>77 ± 9</td>
</tr>
</tbody>
</table>

B) CPR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% in DRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>74 ± 8</td>
</tr>
<tr>
<td>+MβC</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>+MβC:Chol</td>
<td>80 ± 6</td>
</tr>
</tbody>
</table>

C) Relative Activity (%)

- Control
- +MβC
- +MβC:Chol

![Graph showing relative activity for EROD and EFC](molpharm.aspetjournals.org)