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Interactions of phospholipase D and cytochrome P450 protein stability

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Abstract

Previous studies have suggested a relationship between cytochrome P450 (P450) 3A (CYP3A) conformation and the phospholipid composition of the associated membrane. In this study, we utilized a novel microsomal incubation system that mimics many of the characteristics of CYP3A degradation pathway that have been observed in vivo and in cultured cells to study the effects of phospholipid composition on protein stability. We found that addition of phosphatidylcholine-specific phospholipase D (PLD) stabilized CYP3A in this system, but that phosphatidylinositol-specific phospholipase C (PLC) was without effect. Addition of phosphatidic acid also stabilized CYP3A protein in the microsomes. The use of 1,10-phenanthroline (phenanthroline), an inhibitor of PLD activity, decreased CYP3A stability in incubated microsomes. Similarly, 6-h treatment of primary cultures of rat hepatocytes with phenanthroline resulted in nearly complete loss of CYP3A protein. Treatment of rats with nicardipine or dimethylsulfoxide (DMSO), which have been shown to affect CYP3A stability, altered the phospholipid composition of hepatic microsomes. It did not appear, though, that the changes in phospholipid composition that resulted from these in vivo treatments accounted for the change in CYP3A stability observed in hepatic microsomes from these animals.

Keywords: Cytochrome P450; CYP3A; Microsomes; Liver; Phospholipase D; Phospholipids

1. Introduction

Cytochrome P450 (P450) 3A (CYP3A) metabolizes approximately half of all therapeutic drugs as well as a variety of hydrophobic endogenous compounds, such as testosterone [1]. In humans, this enzyme is expressed at high levels in the liver and intestine where it can be a key factor in first-pass metabolism and systemic clearance of drugs. As such, drug-mediated induction or suppression of CYP3A is an important factor in drug–drug interactions. CYP3A has an unusually short half-life for a protein in the endoplasmic reticulum, although it can be stabilized by substrates [2]. Dimethylsulfoxide (DMSO) also stabilizes CYP3A protein, albeit by a mechanism that appears to be distinct from substrate-mediated stabilization [3]. Certain suicide substrates of CYP3A have been reported to accelerate CYP3A degradation in a process that may be associated with ubiquitination and proteasomal degradation [4,5].

Nicardipine is an efficacious inducer of CYP3A, such that treatment of rats with this anti-hypertensive agent results in hepatic microsomal CYP3A levels several fold greater than observed with the prototypical CYP3A inducer, dexamethasone [6]. We recently demonstrated that when incubated for 2 h at 37 °C, hepatic microsomes from nicardine-treated rats formed high-molecular mass (HMM) complexes of CYP3A and ubiquitin [7]. This reaction was atypical of the classical ubiquitination reaction in that these HMM complexes were formed in the absence of ATP, monoubiquitin, cytosol or the ubiquitin-activating enzyme E1. The formation of the HMM CYP3A complexes appeared to be an early event in protein degradation since proteolytic loss of these HMM complexes was observed in the presence of cytosol. Since formation of the HMM complexes and cytosolic-dependent degradation was inhibited by CYP3A substrates, this in vitro process appears to provide a mechanistic model of the molecular processes that contribute to CYP3A's short half-life and substrate-mediated stabilization. The formation of HMM CYP3A was observed in hepatic microsomes from rats treated with other inducers of CYP3A, and may be associated with oxidative stress that results from uncoupling of

Abbreviations: CYP3A, cytochrome P450 3A; DMSO, dimethylsulfoxide; HMM, high-molecular mass; P450, cytochrome P450; phenanthroline, 1,10-phenanthroline; PLC, phospholipase C; PLD, phospholipase D

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the P450 catalytic cycle [8]. Even so, the amount of HMM CYP3A formed was greater in microsomes from nicardipine-treated rats, and the reason why this agent is particularly effective at inducing the formation of HMM CYP3A is currently unknown.

In primary cultures of rat hepatocytes, treatment with hemin induced the formation of HMM CYP3A bands that were very similar to those that form in the incubated microsomes [9]. This result suggests that living cells also form these HMM CYP3A aggregates prior to degradation of this protein. Interestingly, in extracts from cultured cells, these HMM aggregates are concentrated in a dense, poorly soluble pellet that has not traditionally been analyzed in the study of P450 degradation. Therefore, past studies on P450 degradation may have mistaken early loss of P450 protein from the microsomal fraction as rapid degradation rather than aggregate formation and what is likely to be a slower degradative process.

While the catalytic activity of other purified P450s is not dependent upon the phospholipid side chain, CYP3A requires reconstitution with phospholipids that contain unsaturated fatty acids [10]. These phospholipid effects on CYP3A catalytic activity are believed to result from alterations in protein conformation [10]. This concept is consistent with other studies indicating that CYP3A4 exists in multiple conformations [11,12]. Substrate-mediated stabilization of CYP3A is also believed to be associated with conformational changes resulting from substrate binding [2]. Therefore, based on these previous studies, we speculated that phospholipase-mediated changes in the membrane environment could affect CYP3A conformation and thereby alter protein stability.

In this study, we demonstrate that addition of 1, 10-phenanthroline (phenanthroline), which inhibits Zn^{2+}-dependent enzymes, such as phospholipase D (PLD), or addition of PLD have opposing effects on the formation of HMM CYP3A in incubated microsomes. Similarly, phenanthroline treatment of primary cultured rat hepatocytes results in a rapid loss of CYP3A protein, suggesting that PLD may be an important regulator of CYP3A protein stability in living cells. Together, these results suggest that CYP3A stability may be dependent upon the activity of microsomal enzymes that regulate the phospholipid environment. However, our results do not support the conclusion that DMSO or nicardipine treatment effects on CYP3A stability are mediated by changes in the phospholipid composition of the endoplasmic reticulum.

2. Materials and methods

2.1. Materials

The anti-peptide antibody specific for CYP3A23 has been described previously [6,13]. A monoclonal antibody raised against ubiquitin was obtained from Santa Cruz Biotechnology. Secondary antibodies conjugated to horseradish peroxidase were obtained from Jackson ImmunoResearch. SuperSignal West Pico chemiluminescent reagent was obtained from Pierce. Other reagents, including phosphatidylcholine-specific PLD (from *S. chromofuscus*) and phosphatidylinositol-specific phospholipase C (PLC; from *Bacillus cerus*), were from Sigma Chemicals.

2.2. Microsome incubation reactions

Hepatic microsomes were isolated from 7-week-old male Sprague–Dawley rats treated with 100 mg nicardipine/kg per day for 7 days, as described previously [6]. Microsome aliquots were stored at −80 °C for 1 year or more without previous thawing and refreezing. Incubations were undertaken at 37 °C using 75 μg of microsomal protein, 50 mM Tris, pH 7.5, 25 mM sucrose, 3 μM $ZnCl_2$, 0.154 mM KCl, 2 mM $CaCl_2$ in a total volume of 50 μl. Reactions were terminated by addition of 50 μl of SDS–PAGE loading buffer (62.5 mM Tris, pH 6.8, 1% SDS, 11% glycerol, 370 μM bromophenol blue, 0.5% 2-mercaptoethanol) and heating to 98 °C for 5 min.

2.3. Primary cultured rat hepatocytes

Primary cultures of hepatocytes were isolated and cultured from 250 to 300 g male Sprague–Dawley rats as described previously [14]. Cells were cultured without treatment for 3 days prior to the initiation of a 30-h treatment with CYP3A inducing drug, 2 mM phenobarbital. For the final 6 h of incubation, cells were co-treated with 200 μM deferoxamine or phenanthroline. These treatments did not alter cell viability, as determined by lactate dehydrogenase release into the culture medium, which was measured as described previously [14]. Microsomes and total RNA were prepared as described previously [14]. Protein levels were determined using the bicinchoninic acid method [15].

2.4. Western and Northern blot analyses

Western blot analyses were undertaken as described previously [7]. In brief, microsome samples were reduced and denatured in SDS–PAGE loading buffer (62.5 mM Tris, pH 6.8, 1% SDS, 11% glycerol, 370 μM bromophenol blue, 5% 2-mercaptoethanol) and heating to 98 °C for 5 min. Protein was then fractionated on a two-tiered SDS–PAGE gel that contained a 3.5% acrylamide loading gel and a 7.5% acrylamide separating gel, and then transferred to nitrocellulose. Blots were probed with antibodies raised against CYP3A23 or ubiquitin at dilutions of 1:60,000 or 1:750, respectively. Blots were then probed using 1:5000 dilution of secondary antibody conjugated to horseradish peroxidase. Protein levels were determined using SuperSignal chemiluminescent reagents and were imaged using a Lumi-Imager F1 (Roche Diagnostics).

2.5. Phospholipid analyses

Seven-week-old male Sprague–Dawley rats were treated with 100 mg nicardipine/kg per day for 7 days, mixed in the food [6] or with daily intraperitoneal injections of 1 ml DMSO per rat for 3 days, as previously described [6,16]. Hepatic microsomes were prepared as described [17] and stored at −80 °C. Phospholipid extraction and analyses were undertaken within 2 months thereafter. Cell lipids were extracted using chloroform/methanol (2:1, v/v) and individual phospholipids and lysophospholipid classes were separated by two-dimensional thin-layer chromatography as previously described [18]. Briefly, samples were separated on silica gel 60G plates using chloroform/methanol/ammonium hydroxide (65:35:5.5, v/v/v) as the first developing solvent and using chloroform/methanol/88% formic acid/water (55:28:5:1, v/v/v/v) as the second developing solvent. Following chromatography, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, sphingomyelin and cardiolipin were scraped from the plate into tubes containing monoheptadecanoin as an internal standard. Samples were methylated in the presence of 6% HCl at 75 °C for 16 h. Following transmethylation, fatty acid methyl esters were further purified on silica gel plates using toluene prior to quantification by capillary gas chromatography [19]. Total lipid phosphorus was assayed as previously described [20].

2.6. Statistics

Data were first analyzed using a one-way ANOVA and, when appropriate, followed by a Duncan's test. A probability value of <0.05 was used for all analyses.

3. Results

3.1. Effects of phospholipases C and D on CYP3A stability in incubated microsomes

Initial studies were conducted to determine if PLD or PLC altered the amount of HMM CYP3A formed in the incubated microsomes. In these studies, varying concentrations of one phospholipase were added at the start of the 2-h incubations and effects on CYP3A were then examined by immunoblotting. Increasing concentrations of PLD blocked the formation of the HMM CYP3A and prevented the loss of the 55 kDa CYP3A band (Fig. 1). In contrast, equivalent units of PLC had no effect on the reaction rate (data not shown). These data demonstrate that PLD-mediated phospholipid metabolism can affect the rate at which CYP3A forms HMM complexes in the incubated microsomes.

Mammalian PLD converts phosphatidylcholine to phosphatidic acid. Therefore, a study was conducted to determine if phosphatidic acid stabilized microsomal CYP3A. These studies demonstrated that addition of synthetic phosphatidic acid to the incubation mixture partially blocked the loss of the 55 kDa CYP3A protein (Fig. 2). This result is consistent with phosphatidic acid being a factor in the mechanism by which PLD stabilized CYP3A in incubated microsomes.

Studies were undertaken to determine if constitutive PLD activity in microsomes might alter the formation of HMM CYP3A. For these studies, we wished to determine the effect of Zn^{2+}, an essential cofactor of PLD, on the formation of HMM CYP3A. However, because enzymatic reactions can be generally dependent upon cation concentrations, the effects of other divalent cations on the formation of HMM CYP3 were first examined. Therefore, initial studies were conducted in the presence or absence of 2 mM concentrations of $MgCl_2$ or $CaCl_2$. These studies indicated that Mg^{2+} had no effect on the formation of HMM CYP3A bands after 2 h of incubation (data not shown). In contrast, Ca^{2+} accelerated the formation of HMM CYP3A bands (Fig. 3B). Therefore, it appears that divalent cations do not necessarily have an affect on the CYP3A aggregation reaction, although they may. Since Ca^{2+} affected the overall reaction rate, further microsomal reactions described in this manuscript were conducted in the present of $CaCl_2$ (but not $MgCl_2$), unless noted otherwise.

Initial tests examining the effects of $ZnCl_2$ on the aggregation reactions were ambiguous. Depending upon the presence or absence of other divalent cations, addition of 3 μM Zn^{2+} either had no effect or appeared to decrease the formation of CYP3A aggregates (data not shown). We speculated that since only very low levels of this cation are normally present physiologically, that trace levels of Zn^{2+} contamination in various reagents or samples could mask any effects of added Zn^{2+}. Therefore, we used phenanthroline as a relatively specific chelator of Zn^{2+} that has previously been demonstrated to inhibit both soluble and membrane-bound forms of PLD [21–23]. Addition of 100 μM phenanthroline increased the loss of the 55 kDa CYP3A band in the presence of 2 mM Ca^{2+} (Fig. 3A, compare the 2 h controls and the "Phen" samples). Addition of 2 mM EGTA, which preferentially chelates Ca^{2+} but is a very weak chelator of Zn^{2+}, had the opposite effect of phenanthroline. Based on our evidence that Ca^{2+} accelerates CYP3A aggregation, when equimolar concentrations of calcium and EGTA are present, EGTA's effect appears to be primarily due to Ca^{2+} chelation. Indeed, when Ca^{2+} was omitted from the reaction buffer, EGTA addition had the opposite effect, such that it now had a similar effect as phenanthroline addition (Fig. 3B). Although phenanthroline can also chelate iron, other studies have demonstrated that iron chelation suppresses formation of HMM CYP3A in incubated microsomes [8], an effect opposite to what is observed with phenanthroline treatment. Overall, these results combined with

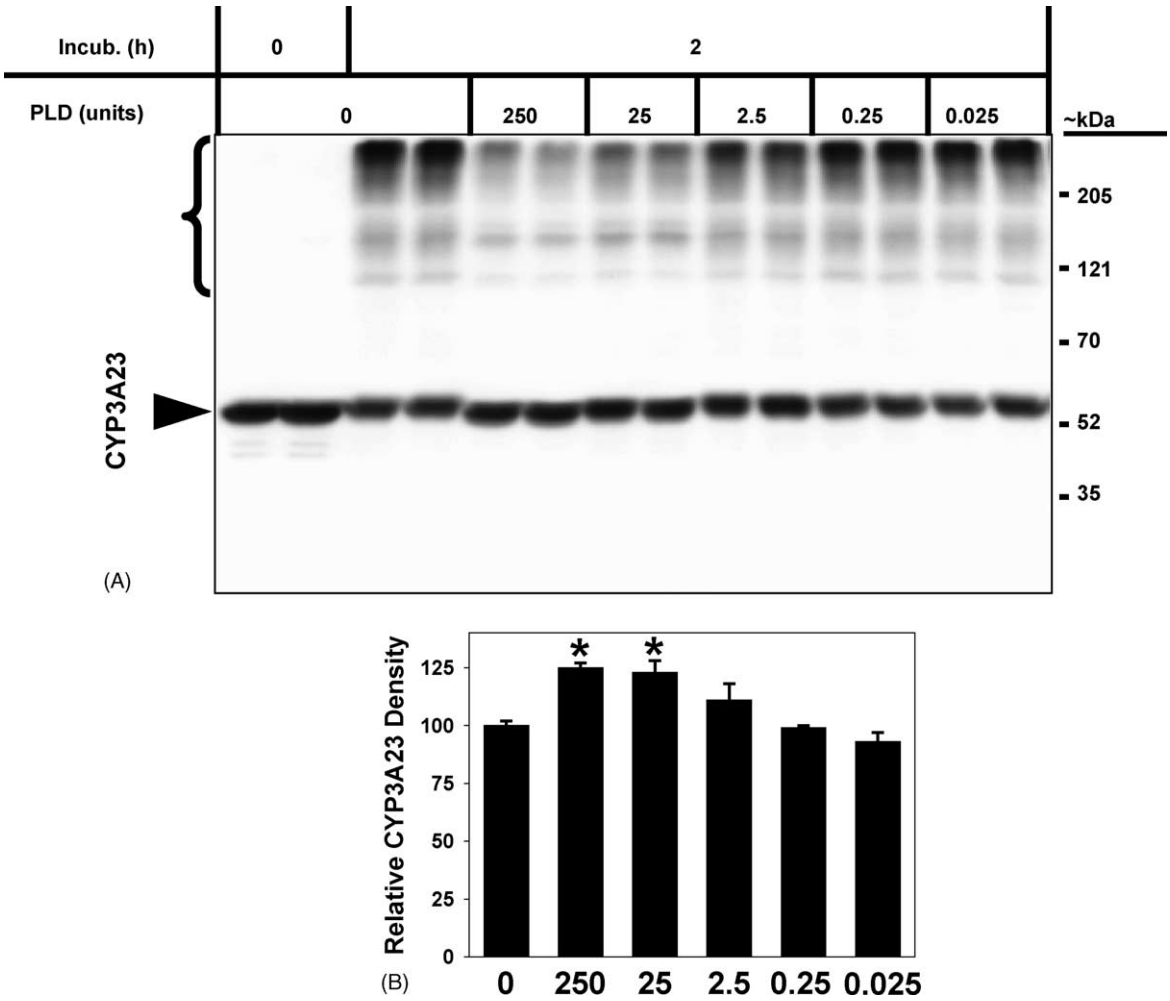

Fig. 1. Phospholipase D (PLD) stabilizes CYP3A23 protein in incubated microsomes. Aliquots of a single microsome sample, which was prepared from the pooled livers of nine rats that had been treated with nicardipine, were not incubated (0 h), or were incubated for 2 h in the presence of varying amounts of PLD. Samples were then analyzed by immunoblot for CYP3A23, as described in Section 2. (A) Representative immunoblot analysis. The ~55 kDa CYP3A band and the HMM CYP3A bands are indicated by an arrowhead and a bracket, respectively. Each lane represents a separate sample or aliquot. (B) Summarized data from four separate analyses conducted on 2 days. Columns and crossbars represent the mean and S.E., respectively, of the 55 kDa CYP3A band density from four separate samples analyzed on 2 days. *Statistically significant ($P < 0.05$) from sample that lacked added PLD.

previous data, which demonstrated that Fe^{2+} chelation by deferoxamine has the opposite effect of phenanthroline [8], are consistent with the concept that a microsomal Zn^{2+}-dependent enzyme, such as PLD, has a role in CYP3A23 protein degradation.

The time-dependent effects of phenanthroline on CYP3A stability in the incubated microsomes were also examined. These studies demonstrated that in microsomal samples incubated for 30 min, there was a corresponding increase in the HMM CYP3A and a greater loss of the 55 kDa CYP3A band in the samples treated with phenanthroline (Fig. 4). The amount of HMM CYP3A detected in the phenanthroline-treated microsomes decreased with longer incubations. In the images shown, only the separating portion of the SDS–PAGE gel is shown. We also undertook analyses of the CYP3A protein that remained in the stacking gel using immunoblot analysis. Although the stacking gel is soft and sticky and produces immunoblots with irregular background, it was clear from these analyses that the HMM CYP3A continued to increase with time in the incubates that contained phenanthroline. However, the HMM complexes were retained in the stacking gel, which was not transferred in the blots shown in Fig. 3 or Fig. 4 (data not shown). This result is consistent with our previous observation that the HMM CYP3A complexes are either not degraded or are very slowly degraded in the incubated microsomes [7].

3.2. *Effects of phenanthroline treatment on CYP3A stability in primary cultured hepatocytes*

In order to determine if PLD might have a role in stabilizing CYP3A in living cells, we examined the effects of phenanthroline in primary cultured rat hepatocytes. These hepatocytes were first treated for 24 h with phenobarbital, which transcriptionally induces CYP3A but does

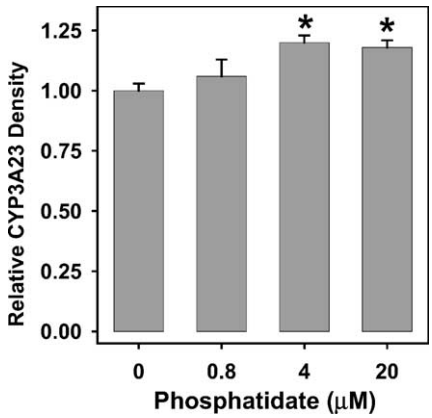

Fig. 2. Phosphatidic acid (PA) stabilizes CYP3A23 protein in incubated microsomes. Aliquots of a single microsome sample, which was prepared from the pooled livers of nine rats that had been treated with nicardipine, were incubated for 2 h in the presence of increasing concentrations of dioleoyl phosphatidic acid. Samples were then analyzed by immunoblot for CYP3A23, as described in Section 2. Columns and crossbars represent the mean and S.E., respectively, of the 55 kDa CYP3A band density from four separate samples analyzed on 2 days. *Statistically significant ($P < 0.05$) from 2-h sample.

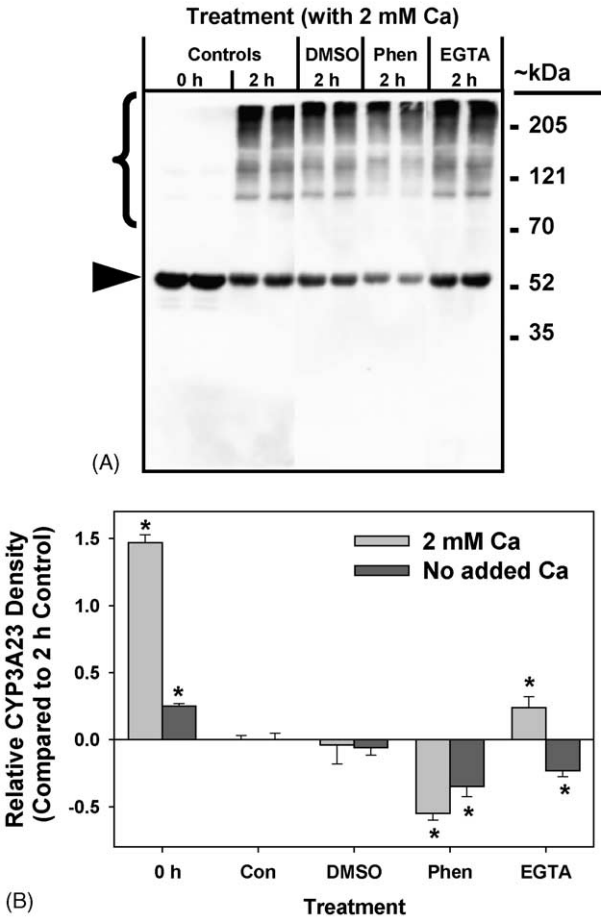

Fig. 3. Phenanthroline treatment increases the loss of the 55 kDa CYP3A band in incubated microsomes. Aliquots of a single microsome sample, which was prepared from the pooled livers of nine rats that had been treated with nicardipine, were incubated for 0 h, or for 2 h in the presence or absence of 2 mM $CaCl_2$, 0.1% DMSO, 100 μM phenanthroline (Phen), or 2 mM EGTA. (A) Representative immunoblot analysis of CYP3A23. Samples in this immunoblot were incubated in the presence of 2 mM $CaCl_2$. The ~55 kDa CYP3A band and the HMM CYP3A bands are indicated by an arrowhead and a bracket, respectively. Each lane is representative of a separate analysis. (B) Summarized effects of DMSO, phenanthroline, and EGTA data from four separate analyses conducted on 2 separate days. These samples were incubated in either 2 mM $CaCl_2$ or with no added Ca. Columns and crossbars represent the mean and S.E., respectively, of the 55 kDa CYP3A band density. *Statistically significant ($P < 0.05$) from 2-h sample.

not alter CYP3A protein stability [24,25]. Cells were subsequently co-treated with phenanthroline and phenobarbital for 6 h. Previous studies in primary rat hepatocytes have indicated that, based on the normal half-life of CYP3A protein in this system, that the 6-h time point is sufficient to detect changes in CYP3A protein stability [3] while minimizing the possibility of non-specific effects on cells that are more likely to occur with long-term xenobiotic treatment. Since phenanthroline can also chelate free iron and thereby decrease oxidative stress in cells, we also treated cells with the deferoxamine, which preferentially chelates free iron. Treatment of the primary hepatocytes with deferoxamine did not alter CYP3A protein or mRNA levels (Fig. 5). In contrast, 6-h treatment with phenanthroline almost completely eliminated CYP3A protein levels (Fig. 5A and B). CYP3A mRNA levels were also decreased ~12% by phenanthroline treatment (Fig. 5A and C). This decrease may potentially reflect the effects of phenanthroline on Zn-dependent transcription factors, such as the glucocorticoid receptor. However, since the decrease in CYP3A mRNA levels are much less than the decrease in CYP3A protein levels, it appears that the decrease in protein levels was primarily the result of post-transcriptional mechanisms.

3.3. Changes in hepatic microsomal phospholipid composition after in vivo treatment with nicardipine or DMSO

The results obtained with incubated microsomes and cultured hepatocytes suggested that drugs could alter CYP3A stability by inducing changes in phospholipid composition of the endoplasmic reticulum. In order to test this hypothesis, we examined the levels of major phospholipid fractions in hepatic microsomes from rats treated with DMSO or nicardine, both of which are known to alter CYP3A protein stability [3,7]. Total phospholipid recovery per milligram of microsomal protein was not significantly altered between samples (mean ± S.E. for nanomoles of phosphate/milligram of protein were—control: 624 ± 30; DMSO treatment: 537 ± 18; and nicardipine treatment: 590 ± 49; $n = 4$). However, on a molar basis, nicardipine treatment significantly increased phosphatidylinositol and phosphatidic acid levels by 38 and 117%, respectively, compared to levels in samples from control animals (Fig. 6). In contrast, DMSO treatment had no effect on any of the phospholipid fractions.

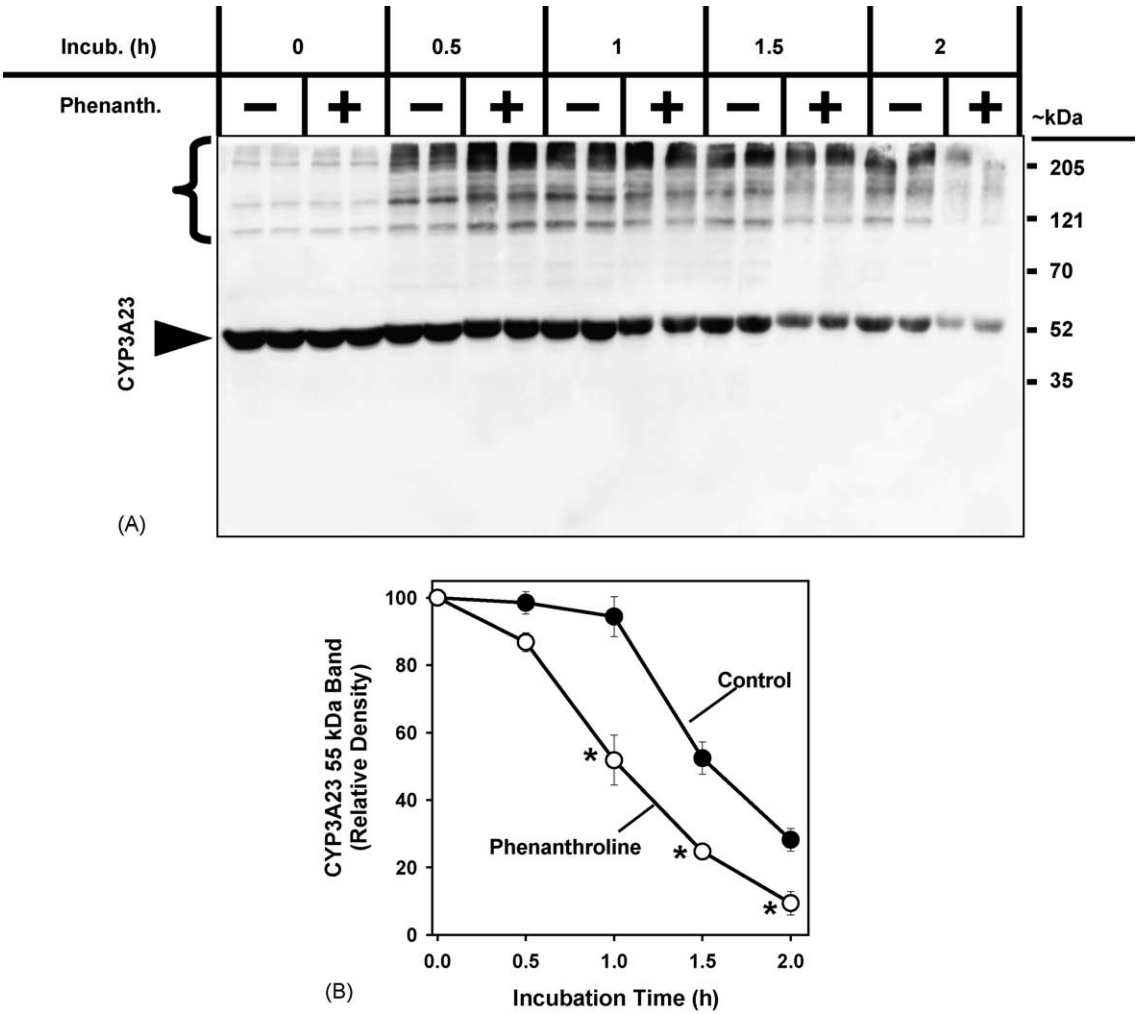

Fig. 4. Time course of the effects of phenanthroline treatment. Aliquots of a single microsome sample, which was prepared from the pooled livers of nine rats that had been treated with nicardipine, were incubated for 0 h, or for 2 h in the presence or absence of 100 μM phenanthroline. (A) Representative immunoblot analysis of CYP3A23. Each lane is representative of a separate analysis. The ~55 kDa CYP3A band and the HMM CYP3A bands are indicated by an arrowhead and a bracket, respectively. (B) Summarized data from four separate analyses conducted on 2 days. Data points and crossbars represent the mean and S.E., respectively. *Statistically significant ($P < 0.05$) from the control sample at the same time point.

Since reconstitution studies with CYP3A have suggested that the level of unsaturated fatty acids in the phospholipid side chains may be more important than total levels of phospholipids [10], we undertook a detailed analysis of the fatty acid components of individual phospholipid groups from hepatic microsomes isolated from rats that had been treated with nicardine or DMSO. Results for each fatty acid component are presented as a molar percentage of the total amount of the phospholipid fraction. As such, these analyses are designed to detect changes in the proportions of a fatty acid moiety in a particular phospholipid fraction that is independent of any changes in the total levels of that fraction. We report on levels of fatty acid moieties that accounted for more than 2% (on a molar percentage basis) of the total fatty acid composition, since moieties that were at least this abundant could be accurately measured. Only modest significant changes (17% or less) were observed in the major phosphatidylinositol side chains of C18:0 (stearoyl) and C20:4$n-6$ (arachidonoyl) (Fig. 7), suggesting that the increase in total phosphatidylinositol observed in nicardipine-treated rats (Fig. 6) was primarily the result of a general increase in this phospholipid fraction. Overall, DMSO treatment had only minor, albeit statistically significant, effects on the phosphatidylinositol composition (Fig. 7).

In the case of phosphatidylcholine, the C16:0 (palmitoyl), C18:0, or C22:6$n-6$ fatty acid moieties from nicardipine-treated rats were significantly decreased 26% or increased by 41 or 26%, respectively. However, DMSO treatment had no effect on the phosphatidylcholine composition. Nicardipine treatment also had modest but statistically significant effects on a variety of fatty acid components of phosphatidylethanolamine and phosphatidylserine (Fig. 7). The only significant effect that DMSO treatment had on these fractions was a 25% increase in C18:2$n-6$ moiety in the phosphatidylethanolamine fraction.

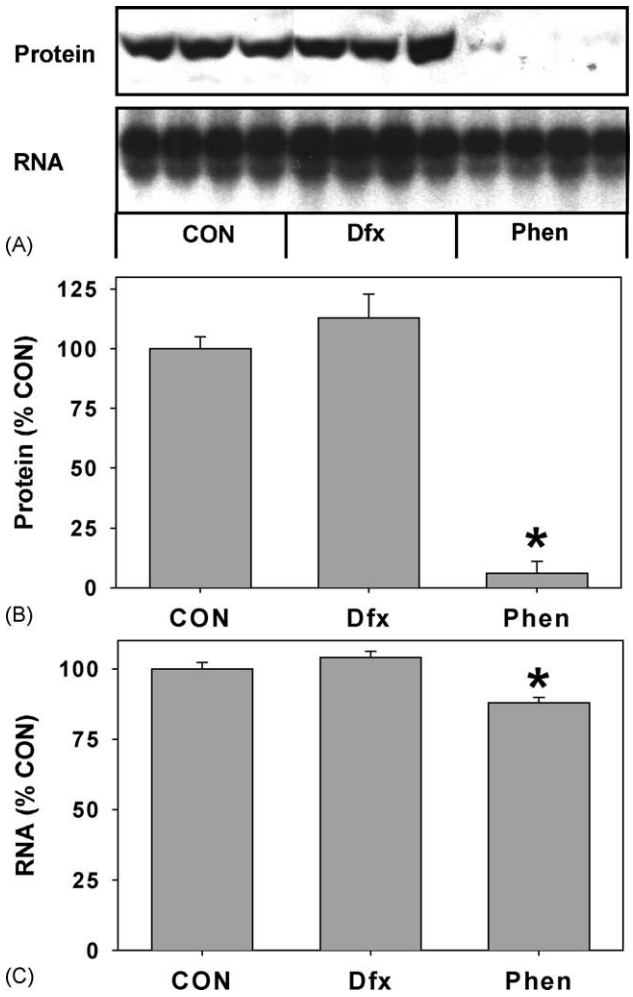

Fig. 5. Zinc chelation decreases CYP3A protein levels in primary cultures of rat hepatocytes. After 3 days of culture without xenobiotic treatment, CYP3A expression was induced by 24-h treatment with 2 mM phenobarbital. Cells were then co-treated with 2 mM phenobarbital and either 100 μM deferoxamine (Dfx) or phenanthroline (Phen) for 6 h. Microsomal protein and total RNA was then isolated and analyzed by immunoblot or Northern blot analyses, respectively, as described in Section 2. For the protein samples, each lane represents a unique microsomal sample that was prepared by pooling cells from three dishes of hepatocytes. For the RNA samples, each lane represents a unique sample that was prepared from a single dish. (A) Immunoblot and Northern blot analyses of CYP3A protein and mRNA, respectively. (B) Graph showing mean and S.E. of the CYP3A immunoblot band densities shown in panel A. (C) Graph showing mean and S.E. of the CYP3A Northern blot band densities shown in panel A. *Statistically significant ($P < 0.05$) from the control data.

Although total levels of cardiolipin were not statistically altered (Fig. 6), some of the largest changes in fatty acid composition were observed in this phospholipid fraction (Fig. 7). The C18:0, C18:1$n-9$ (oleoyl) and 22:6$n-3$ (docosahexaenoyl) side chains in cardiolipin from nicardipine-treated rats were significantly increased by 59–98%. In contrast, C18:2$n-6$ and C18:1$n-7$ were both decreased about 50% in the cardiolipin fraction from nicardipine-treated rats, as was 22:6$n-3$ in samples from DMSO-treated rats.

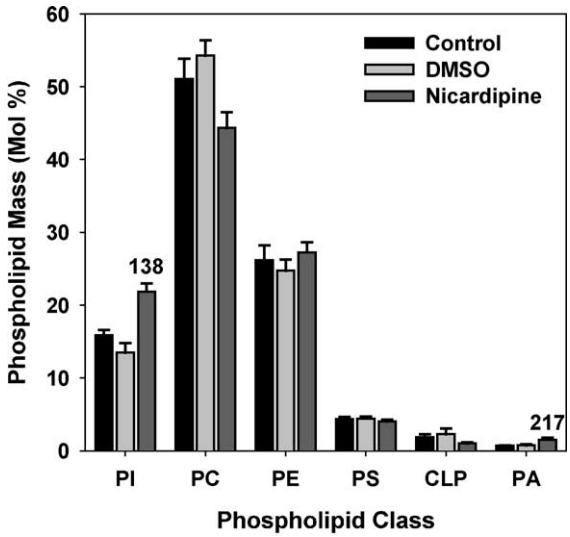

Fig. 6. Effects of in vivo treatment of rats with DMSO or nicardipine on the major phospholipid classes in hepatic microsomes. Total phospholipid levels were extracted, fractionated, and quantified as described in Section 2. Columns and crossbars represent the mean and S.E., respectively, of phospholipid concentrations (as a molar percentage of total phospholipid levels) from four individual animals. Abbreviations are: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; CLP, cardiolipin; PA, phosphatidic acid. A number above a column represents the relative concentration, as a percent of the control value, and is only shown for values that are significantly different ($P < 0.05$) from the control group for that phospholipid class.

For the phosphatidic acid moieties, nicardipine treatment significantly decreased the C18:0 fraction 45% but the C18:1$n-9$ fraction was increased by 68%. As such, the greater than twofold increase observed for total levels of phosphatidic acid (Fig. 6) cannot be accounted for by changes in any particular subfraction on this phospholipid. DMSO had no significant effect on any of the fatty acid moieties.

4. Discussion

Several studies have suggested that CYP3A exists in multiple conformational states and that these states can affect enzymatic activity and protein stability [10–12]. In the current study, we initially examined CYP3A stability in a microsomal incubation system that mimics the many aspects of CYP3A degradation observed in vivo, including the formation of HMM CYP3A complexes and protein stabilization by substrates [7]. Remodeling of the phospholipid environment in response to addition of PLD, but not PLC, alters CYP3A stability in this in vitro system. Addition of phosphatidic acid, the major product of PLD activity, also stabilized microsomal CYP3A. Based on our phospholipid analyses, microsomes from control animals contain the equivalent of about 6 μM phosphatidic acid in each assay. Since addition of 4 and 20 μM phosphatidic acid to the incubated microsomes had a stabilizing effect

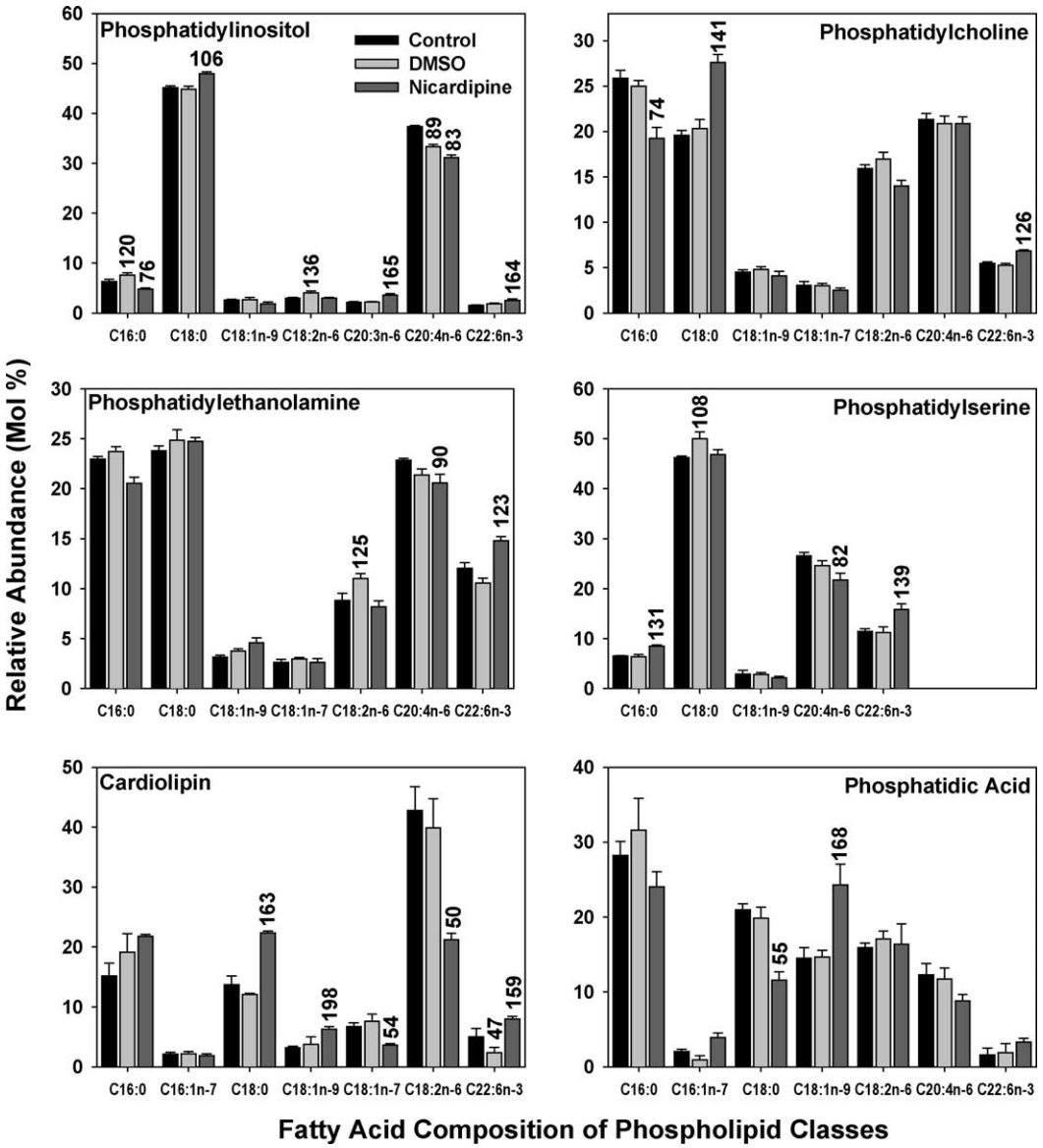

Fig. 7. Effects of in vivo treatment of rats with DMSO or nicardipine on fatty acid moieties of hepatic microsomal phospholipid classes. Total phospholipids were extracted, fractionated, and the levels of the fatty acid side chains determined as described in Section 2. Columns and crossbars represent the mean and S.E., respectively, of samples from four individual animals. A number above a column represents the relative concentration, as a percent of the control value, and is only shown for values that are significantly different ($P < 0.05$) from the control group for the same fatty acid side chain from the same phospholipid class.

on CYP3A protein, physiologically relevant changes in phosphatidic acid could be important in CYP3A stability. Since PLD activity has been reported to be at high levels in microsomes relative to cytosol or crude membrane fractions [26], we suspected that endogenous PLD activity in the microsomes may also be a factor in determining microsomal CYP3A stability. Addition of phenanthroline, a PLD inhibitor, accelerated formation of the HMM CYP3A complexes. Since this effect was opposite to PLD addition, this result was consistent with PLD activity having a stabilizing effect on CYP3A stability. Still, we cannot rule out a role for other Zn^{2+}-dependent enzymes. In order to determine if PLD activity may impact CYP3A protein stability in living cells, we treated primary cultured rat hepatocytes with phenanthroline. Similar to results observed in the incubated microsomes, 6-h treatment with phenanthroline dramatically decreased CYP3A protein levels, suggesting that PLD activity could be an important factor in maintaining a stable CYP3A conformation in cultured cells.

Together, these data suggested a model in which increasing phosphatidic acid content in the microsomes alters CYP3A conformation in such a way as to stabilize this protein. Therefore, we determined if agents that we have previously observed to alter CYP3A protein stability in vivo might alter microsomal phosphatidic acid levels. These studies indicated that nicardipine, but not DMSO, increased phosphatidic acid levels in microsomes.

Although the direct effect of nicardipine on CYP3A is to stabilize the protein, this effect likely results from substrate-mediated stabilization [7] rather than changes in phosphatidic acid levels. In contrast, treatment of rats with high doses of nicardipine for a week or more results in fundamental changes in the hepatic microsomes that predisposes CYP3A to the formation of HMM conjugates [7]. Therefore, based on the results we obtained from the experiments with PLD, we initially hypothesized that extended nicardine treatment might regulate CYP3A stability by decreasing phosphatidic acid levels. Since the opposite effect, an increase in phosphatidic acid level, was observed after in vivo nicardipine treatment, it seems unlikely that changes in phosphatidic acid levels was the primary mechanism by which nicardipine treatment induced the formation of HMM CYP3A conjugates in incubated microsomes. Rather, we have previously found that in vivo nicardipine treatment induces a low level of oxidative stress in isolated microsomes and that this effect is a key factor in stimulating the formation of the HMM CYP3A [8]. Therefore, we conclude that it is more likely that treatment of rats with nicardipine alters CYP3A stability in hepatic microsomes by causing oxidative stress rather than by altering the phospholipid composition.

Interestingly, the increase in phosphatidic acid levels in the microsomes from nicardipine-treated animals may actually be the result of P450 induction. Tests using microsomal and purified P450s have demonstrated that several of these enzymes have significant phosphatidylcholine-specific PLD activity [27]. Of five human and five rat P450 forms examined, CYP3A had the lowest level of PLD activity. However, the reconstituted system used in these studies lacked phospholipids containing unsaturated fatty acids, the presence of which markedly enhances other catalytic activities of this P450 in reconstitution systems [10]. Therefore, it is unclear if the low level of PLD activity by CYP3A was the consequence of the nature of the reconstitution system or an inherent lack of this activity.

Overall, the results of this study provide the first evidence that remodeling of the surrounding phospholipid environment can alter microsomal CYP3A stability under in vitro conditions. Although we found that treatment of animals with drugs, such as nicardipine, can alter phospholipid content in the microsomal fractions, it is less clear if phospholipid remodeling is a factor that affects P450 stability in vivo. Based on our results in the incubated microsomes, the increase in phosphatidic acid in response to nicardipine treatment would be expected to stabilize CYP3A. Nicardipine also had marked effects on the levels of the fatty acids components in cardiolipin. In the absence of other phospholipids, cardiolipin is not sufficient to support CYP3A activity in reconstituted systems [10]. It is unclear, however, whether differences in cardiolipin composition have any effects on CYP3A conformation or stability in a complex phospholipid environment. Although DMSO treatment stabilizes CYP3A in rats and cultured cells [3,16], we found that treatment of rats with this agent had little effect on phospholipid composition, suggesting that DMSO effects are mediated by other mechanisms. Although these studies apparently provide new evidence that drugs and other xenobiotics can alter the phospholipid composition of the endoplasmic reticulum, further research is needed to determine what effects these changes have on P450 function and stability.

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