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# Role of Heme in Cytochrome P450 Transcription and Function in Mice Treated with Lead Acetate

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## SUMMARY

Genetic and acquired heme deficiencies are associated with impaired cytochrome P450 (P450) function in experimental animals and in humans. The hypothetical explanations have been either a decreased supply of heme for saturation of apo-P450 or a requirement of heme for P450 gene transcription. We investigated the effect of heme deficiency on P450 function, mRNA, and transcription in C57BL/6 mice treated with lead acetate (75 mg of $Pb^{2+}$/kg intraperitoneally). Lead caused an increase in δ-aminolevulinic acid levels in plasma (>30-fold) and a decrease in the heme saturation of hepatic tryptophan-2,3-dioxygenase (15 ± 4% versus 33 ± 6% of heme saturation in controls; $p < 0.001$), which is consistent with an effective inhibition of heme synthesis and depletion of the free heme pool. P450-dependent activities (7-ethoxycoumarin *O*-deethylation and *O*-dealkylation of alkoxyresorufins) decreased progressively after lead injection to 56–69% of control levels within 20 hr. This effect was partially counteracted by injection of hematin (4 mg/kg intraperitoneally) to 73–93% of control activities ($p < 0.01$ for 7-ethoxycoumarin *O*-deethylation and $p < 0.05$ for *O*-dealkylation of alkoxyresorufins). The mRNA levels of the P450 Cyp3a11, measured by semiquantitative reverse transcription-polymerase chain reaction under the same experimental conditions, also decreased after lead injection to 45% of control levels. This decrease was accounted for by inhibition of *Cyp3a11* gene transcription, as demonstrated by run-off experiments in liver nuclei isolated 12 hr after lead injection. Hematin did not restore the mRNA levels or the transcriptional activity of *Cyp3a11* in nuclei as well as *in vivo*. We conclude that the decrease of P450 in lead poisoning is a consequence of two different mechanisms: (a) a mechanism unrelated to heme, in which lead decreases P450 transcription; and (b) a mechanism dependent on heme, in which lead inhibits heme synthesis, and this results in a decreased heme saturation of P450 and/or apo-P450 content.

P450 is the collective term for a large family of heme-containing proteins that play an important role in the oxidative metabolism of numerous endogenous and foreign compounds (1). Because P450 is the major hemoprotein in the liver (2), inhibition of heme biosynthesis may interfere with the biogenesis of functional P450. Indeed, P450 activity is impaired in several disorders of heme synthesis. For example, in patients with genetic partial defects in enzymes of heme synthesis, as in acute porphyrias, the capacity to metabolize antipyrine is decreased (3, 4). Similarly, in acquired heme deficiency, as in lead poisoning, P450 function is impaired (5).

The mechanism by which impaired heme synthesis decreases P450 function remains incompletely understood. Two different hypotheses have been proposed: First, the decrease in P450 function could be due to insufficient heme supply and consequent incomplete saturation of P450 apoprotein. Under normal conditions, hemoprotein formation is not limited by heme because when the precursor heme pool is depleted, a feedback mechanism of regulation stimulates the rate of heme synthesis (6). However, when heme synthesis is partially blocked, heme may become limiting for P450 formation, resulting in decreased P450 function. Alternatively, the decrease in P450 function could be a consequence of decreased P450 synthesis. Heme plays a regulatory role in the biogenesis of several hemoproteins by exerting effects at different steps, such as transcription, translation, transport, and assembly (7). Recently, a model for the involvement of heme in the transcriptional regulation of hepatic P450 was proposed. In this model, a nuclear regulatory heme pool acts as a positive modulator in the gene transcription of P450s induced by phenobarbital (8).

The aim of the current work was to study the regulation of constitutive P450 activity in a situation of defective heme biosynthesis and to test these two alternative hypotheses. To

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**ABBREVIATIONS:** P450, or CYP, cytochrome P450; Cyp, mouse cytochrome P450; ALA, δ-aminolevulinic acid; TDO, tryptophan-2,3-dioxygenase; ECOD, 7-ethoxycoumarin-*O*-deethylase; EROD, ethoxyresorufin-*O*-deethylase, PROD, pentoxyresorufin-*O*-dependentilase; RT, reverse transcription; PCR, polymerase chain reaction; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

do this, we investigated the effects of lead acetate on several P450 functions and on the mRNA concentration and gene transcription of a mouse P450 isoenzyme (Cyp3a11). Lead inhibits heme synthesis at different steps of the pathway, the most profound effect being on δ-aminolevulinate dehydratase (9).

The results obtained demonstrate that contrary to the hypotheses mentioned above, the decrease in P450 function observed in lead-poisoned animals seems to be a consequence of two different mechanisms: a mechanism unrelated to heme, in which lead decreases P450 transcription and accordingly reduces P450 synthesis and activity, and a mechanism dependent on heme, in which lead inhibits heme synthesis, and this results in a decreased heme saturation of P450 and/or apo-P450 content.

## Materials and Methods

**Animals and treatments.** C57BL/6 male mice (14–16 weeks old) were purchased from Biological Research Laboratories (Füllinsdorf, Switzerland) and maintained on a 12-hr light/dark cycle in a controlled environment animal facility. Animals had free access to standard rodent chow and tap water.

Lead acetate was dissolved in water and injected at a dose of 75 mg of $Pb^{2+}$/kg intraperitoneally. This dose of lead was selected from experiments like the one shown in Fig. 1. A dose-dependent effect of lead on P450 activity and heme synthesis inhibition (reflected by increased plasma ALA) was observed. The dose of 75 mg/kg was chosen to produce maximal effects. Heme was freshly dissolved in 1% sodium carbonate to obtain the more stable hydroxylated form, called hematin. pH was adjusted to 8 with 1 M HCl, and after dilution with isotonic saline to a final concentration of 0.4 mg/ml, the hematin solution was passed through a 0.22-μm filter and injected immediately at 4 mg/kg intraperitoneally. After treatment, mice were anesthetized with sodium pentobarbital, and blood was taken by heart puncture. Livers were perfused *in situ* with cold isotonic saline and were either frozen in liquid nitrogen for subsequent biochemical analysis and RNA preparation or kept fresh on ice for isolation of nuclei.

**Assays.** Microsomal samples were prepared by differential centrifugation (10). EROD and PROD were measured according to the method of Burke *et al.* (11), using an end point fluorometric determination of the product instead of monitoring the time course of the reaction. Microsomes (5–20 μg) were preincubated in 75 mM potassium phosphate buffer containing 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, and 5 μM substrate for 3 min; NADPH (0.5 mM final concentration) was added; and the reaction was allowed to proceed at 37° for 10 min. Methanol (2.5 volumes) was added to stop the reaction, and the mixture was centrifuged at 13,000 rpm for 5 min. The fluorescence of the product and identically handled resorufin standards were measured with a Perkin-Elmer LC50B Luminescence-fluorometer (Perkin-Elmer Cetus, Norwalk, CT). ECOD was measured according to the method of Greenlee and Poland (12). Microsomal protein content was determined according to the method of Lowry *et al.* (13) using bovine serum albumin as standard.

The plasma levels of ALA were measured with high performance liquid chromatography (14) as an indicator of the inhibitory effect of lead on the heme synthesis pathway. To estimate changes in the cytosolic free heme pool, we measured the heme saturation of hepatic TDO according to the following procedure. Livers were homogenized (1:3 w/v) in 0.2 M potassium phosphate buffer (pH 7.0), and 50 μl of the 9000 × *g* supernatant was incubated with 2.5 mM tryptophan without hematin (holoenzyme activity) or with 2 μM hematin (total activity) in 0.6 ml of the final volume at 37° for 1 hr. The kynurenine formed was measured as described previously (15), and the enzyme activity was expressed as μmol of kynurenine/g of wet weight/hr. The percentage of heme-saturated enzyme was calculated as (holoenzyme activity/total activity) × 100.

**RNA purification and cDNA synthesis.** Total RNA was purified according to the guanidinium thiocyanate procedure as described by Chomczynski and Sacchi (16) with the addition of a lithium chloride extraction step to remove glycogen (17). RNA was resuspended in diethyl pyrocarbonate-treated $H_2O$, and its concentration was quantified by UV absorbance at 260 nm.

RNA (1 μg) was reverse-transcribed in a total volume of 30 μl containing 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 200 μM concentration of each deoxynucleotide triphosphate, 300 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Baltimore, MD), 30 U of ribonuclease inhibitor (RNasin, Promega, Madison, WI), and 2 μM oligo[$dT_{14}$(A/C/G)] primer. The mixture was incubated at 37° for 60 min, and the reaction was stopped by being heated at 95° for 5 min.

**Semiquantitative PCR.** Primers for Cyp3a11 cDNA (18) were designed to be highly specific for this P450 isoform, avoiding sequence homology with other members of the mouse P450 3a subfamily. The Cyp3a11 forward primer (sense strand) was 553–572 nucleotides (5′-TGAGGCAGAAGGCAAAGAAA-3′), and the Cyp3a11 reverse primer (antisense strand) was 1124–1143 nucleotides (5′-GGTATTCCATCTCCATCACA-3′). For mouse β-actin cDNA (19), the forward primer was 420–439 nucleotides (5′-GCCAACCGTGAAAAGATGAC-3′), and the reverse primer was 859–878 nucleotides (5′-GAAGGAAGGCTGGAAAAGAG-3′).

Diluted cDNA (3 μl) was amplified in a 30 μl of final volume containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris·HCl, pH 9, 50 μM concentration of each deoxynucleotide triphosphate, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer), and 0.2 μM concentration of each primer. The appropriate cDNA dilutions were empirically determined for each gene to ensure that signals were derived only from the exponential phase of the amplification (20). PCR was performed with a DNA Thermal Cycler (Perkin-Elmer) programmed for a initial

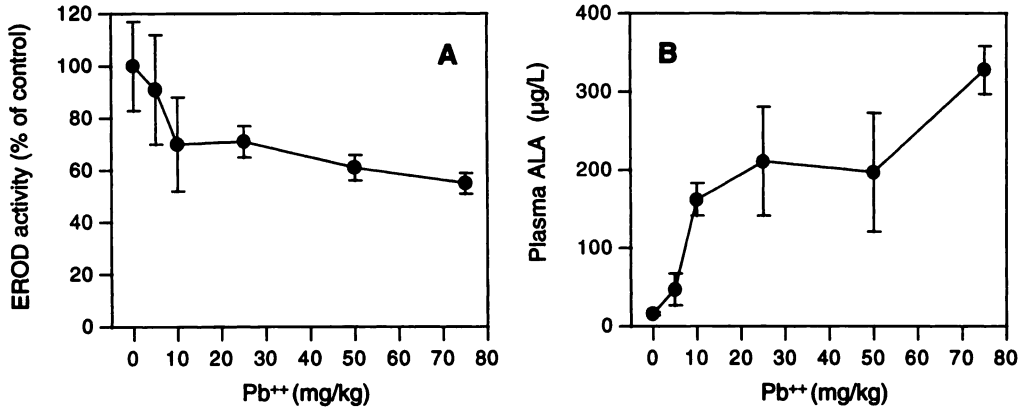


**Fig. 1.** Dose-dependent effect of lead on P450 activity (A) and heme synthesis inhibition (B). Lead was injected at 0, 5, 10, 25, 50, and 75 mg/kg, and after 20 hr P450 activity EROD was determined in liver microsomes (A), and ALA concentration was measured in plasma (B). Control EROD activity was 107 ± 19 pmol of resorufin/mg/min. Results are expressed as mean ± standard deviation of three experiments.

denaturation of 4 min at 94°, followed by 25 cycles of 45 min at 94°, 45 min at 55°, and 1 min at 72°, and a final extension of 5 min at 72°.

For quantitative analysis, 0.1 μCi of [α-$^{32}$P]dATP (3000 Ci/mmol, Amersham) was included in the PCR reaction to label the product. Aliquots (20 μl) of the PCR reaction were subjected to electrophoresis on 1.2% agarose gel; after visualization of the products by ethidium bromide staining, the gel was dried and exposed to phosphor storage screens for ≤1 hr. After the screens were scanned with a PhosphorImager, the intensity of the bands was quantified with the use of ImageQuant software (Molecular Dynamics, Sunnyvale, CA). These results were plotted on a logarithm/logarithm scale against the corresponding dilution factor of the cDNA. The PCR reaction was considered to be exponential if 2-fold amplification was detected. Normalization of PCR results were done by analysis of β-actin from the same cDNA dilution series.

**Purification of mouse liver nuclei.** Livers (1–2 g) were homogenized in ~10 volumes of 0.3 M sucrose in buffer A (60 mM KCl, 25 mM NaCl, 0.15 mM spermin, 0.5 mM spermidin, 14 mM β-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 15 mM HEPES, pH 7.5) with a Dounce homogenizer (5–10 strokes).

The homogenates were filtered through three layers of cheese cloth, layered over a 10-ml cushion of 30% sucrose in buffer A, and spun for 10 min at 2500 rpm in the HB4 rotor of a Sorvall centrifuge (Newtown, CT) at 4°. The crude nuclei were resuspended in 3.5 ml of 2 M sucrose in buffer B (the same as buffer A, except for the use of 0.1 mM EGTA and 0.1 mM EDTA), layered over 2 M sucrose in buffer B, and sedimented at 29,000 rpm in a Kontron TST41.14 rotor at 4° for 1 hr. The clean nuclei were resuspended in nuclei storage buffer (20 mM Tris·HCl, pH 8, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM phenylmethylsulfonyl fluoride, 40% v/v glycerol) at a concentration of 50 × $10^6$ nuclei/150 μl and stored at −70° (21).

**Nuclear run-off transcription assay.** Nuclei were mixed with 0.5 volume of 3× reaction buffer [75 mM KCl; 5 mM $MgCl_2$; 1 mM DTT; 0.25 mM EDTA; 20 mM Tris·HCl, pH 8; 1.5 mM concentration each of ATP, GTP, and CTP; and 135 μM digoxigenin-11-UTP (Boehringer-Mannheim Biochemicals, Indianapolis, IN)] and 120 U of RNase inhibitor (RNasin, Promega) and incubated at 30° for 40 min. To the run-off mixture, 1 mM $CaCl_2$ and 100 U of RNase-free DNase I (Promega) were added, and incubation was continued for 10 min. Proteinase K (Boehringer-Mannheim) was added to a final concentration of 100 μg/ml in sodium dodecyl sulfate/EDTA/Tris buffer (0.5% sodium dodecyl sulfate, 5 mM EDTA, 10 mM Tris·HCl, pH 7.4) with 200 μg/ml yeast tRNA, and the mixture was incubated for 30 min at 42°. In the lysate, genomic DNA was broken by shearing forces using a 25-gauge needle. Digoxigenin-labeled RNA was extracted according to the guanidinium thiocyanate procedure described above (16, 17). Samples with an equivalent labeling yield were used for hybridization against cDNA probes immobilized on nylon membranes.

The Cyp3a11 and β-actin cDNAs were amplified by PCR as described above except that *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used instead of *Taq* DNA polymerase to ensure high fidelity during primer extension. PCR products were gel purified and cloned in pCR-Script Direct SK(+) vector (Stratagene).

cDNA inserts (2 μg) were boiled for 10 min in 0.4 M NaOH/10 mM EDTA, chilled on ice, and blotted on positively charged nylon membranes (NYTRAN; Schleider & Schuell, Dassel, Germany) under slow vacuum with a slot-blot apparatus (BioRad, Hercules, CA). The membrane was UV irradiated in a UV-Stratalinker (Stratagene) and baked at 80° for 1 hr. Hybridization, washes, and detection of the chemiluminescent signal were performed as described previously (22).

## Results

**Effect of lead on heme synthesis and the free heme pool.** It is well known that in human and experimental lead poisoning, there is a marked accumulation of ALA and porphyrins that is in large part due to the inhibition of ALA dehydratase, the second enzyme of the heme pathway (9). To follow the effect of a lead dose of 75 mg/kg intraperitoneally, in C57BL/6 mice we measured the accumulation of ALA in plasma. The results in Fig. 2A show that after a single acute injection of lead acetate, plasma ALA increased by >30-fold, suggesting a strong inhibition of heme synthesis at the level of ALA dehydratase.

We also investigated the effect of lead on the free heme pool by measuring the heme saturation of hepatic TDO. This cytosolic hemoprotein is a very sensitive marker for assessment of subtle changes in the cytosolic free heme concentration (15, 23, 24). TDO exists in two forms: heme-free apoenzyme and heme-containing holoenzyme. Inhibition of heme synthesis and increased degradation or utilization of heme decrease the saturation of TDO (15, 23, 24). The results in Fig. 2B demonstrate a significant negative effect of lead on the heme saturation of hepatic TDO. The enzyme saturation was 15±4% versus 33±6% in controls ($p < 0.001$) at 18 hr

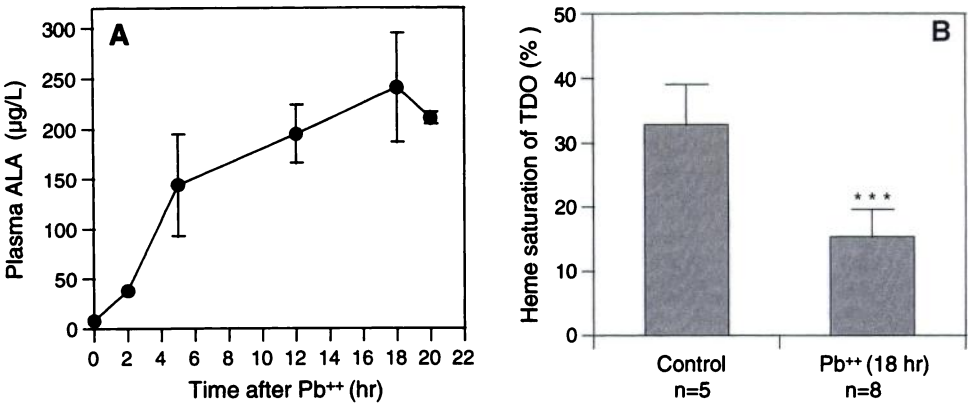


**Fig. 2.** Effect of lead treatment on heme synthesis and the free heme pool. A, Effect of lead on plasma ALA levels. Mice were injected with lead acetate (75 mg of $Pb^{2+}$/kg intraperitoneally), and blood samples were taken at the indicated times. ALA concentration was determined in plasma as described in Materials and Methods. Results are expressed as mean ± standard deviation of three or four experiments. B, Effect of lead on the heme saturation of hepatic TDO. TDO activity was determined with fresh liver cytosols without hematin supplement (holoenzyme activity) and with saturating hematin (total activity). The percentage of heme-saturated enzyme was calculated as the fraction (holoenzyme activity/total activity) × 100. Control holoenzyme activity was 2.17 ± 0.67 μmol of kynurenine/g of wet weight/hr. Results are expressed as mean ± standard deviation. ***, $p < 0.005$ according to Student's *t* test.

after lead injection. Total TDO activity (holoenzyme plus apoenzyme) was not affected by lead. This indicates that under our experimental conditions, lead inhibits the heme pathway and consequently depletes the free heme pool.

**Effect of lead on P450 function.** Initial experiments were carried out to determine the effect of lead on several mouse hepatic P450 activities; in confirmation of previous reports in rats, we found a time-dependent decrease in P450 function (Fig. 3). We measured the effect of lead on three different P450 activities: EROD and PROD, which are specific to the major methylcholantrene-inducible P450 isoenzymes (Cyp1a1/2), and a phenobarbital-inducible P450 isoenzyme (Cyp2b10), respectively (11), and ECOD, widely used as a measure of mono-oxygenase activity involving several P450 isoenzymes (25). The results indicate that the three activities measured decrease progressively with a similar profile to 50–60% of control levels within 24 hr after lead injection (Fig. 3).

**Effect of lead on P450 mRNA levels and gene transcription.** To determine whether the decrease in P450 activity was due to a lower heme saturation of P450 apoprotein or to a decrease in P450 synthesis, we studied the effect of lead on P450 mRNA. We focused on the P450 isoenzyme Cyp3a11 because it can be detected well in uninduced mouse liver (18). The substrate specificity of this mouse isoenzyme has not been fully characterized.

To overcome the detection problem in samples with a lower level of expression and the problem of cross-detection of closely related isoforms of the 3a subfamily (Cyp3a13 and Cyp3a16) (26, 27), we evaluated the relative changes in Cyp3a11 mRNA levels by semiquantitative RT-PCR. In parallel, we analyzed β-actin as an internal control. We found that the amplification of β-actin mRNA by semiquantitative RT-PCR depends only on the input RNA and not on the dose or time-point of lead administration. A critical parameter of this method is defining conditions under which the cDNA is the only limiting factor of the reaction. In such a situation, the yield of the PCR product is proportional to the input cDNA (20, 28). We determined that in control samples, Cyp3a11 and β-actin amplification was in the exponential phase of the reaction when the input cDNA was diluted by >200-fold and >400-fold, respectively, and was amplified for 25 cycles.

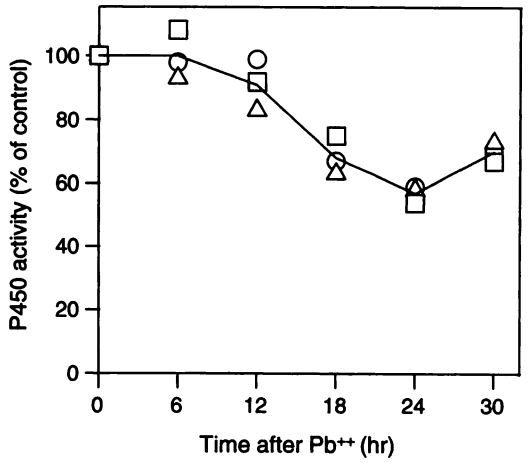


**Fig. 3.** Time course of the effect of lead on P450 function. Mice were injected with lead acetate, and three different P450-associated activities were measured in hepatic microsomes at the indicated times: △, EROD (control value, 81 pmol of resorufin/mg/min); ○, PROD (control value, 21 pmol of resorufin/mg/min); and □, ECOD (control value, 2027 pmol of 7-hydroxycoumarin/mg/min). Results represent the average values of two independent experiments.

Once the optimal conditions for semiquantitative RT-PCR were established, we measured the time-dependent effect of lead on Cyp3a11 mRNA concentration. Similar to the effect of lead on P450 activity, the mRNA levels of Cyp3a11 decreased progressively up to 30–40% of control level after 18–24 hr (Fig. 4A), and this effect was dose dependent with a 33% decrease in Cyp3a11 mRNA levels at a lead dose of 10 mg/kg (Fig. 4B).

To further investigate whether the decrease in P450 mRNA levels was a consequence of inhibited gene transcription or lower mRNA stability, we performed run-off transcription assays with nuclei from livers of mice treated with lead. These experiments showed that the transcriptional activity of *Cyp3a11* is strongly inhibited 12 hr after lead (Fig. 5), when Cyp3a11 mRNA began to decrease.

The decrease in *Cyp3a11* transcription and mRNA preceded the decline in P450 activity, suggesting that this decrease in P450 activity is due to an inhibition in P450 synthesis.

**Effect of exogenous heme in the decrease of P450 caused by lead.** We treated lead-poisoned mice with hematin to investigate whether the decrease in *Cyp3a11* expression was a consequence of the depletion of the regulatory free heme pool or a direct toxic effect of lead.

Heme decreases the activity of ALAS, the first enzyme of the heme pathway, by repressing its synthesis and inhibiting its translocation into mitochondria (6). Therefore, an effective dose of exogenous hematin should reduce the activity of this enzyme and consequently decrease the plasma concentration of its product, ALA.

Hematin was injected at a dose of 4 mg/kg intraperitoneally 5 hr after lead, and animals were killed 13–15 hr later. The plasma levels of ALA were reduced by 40% in lead-poisoned mice [119 ± 28 μg/liter (four experiments) versus 193 ± 30 μg/liter (five experiments) in controls; Fig. 6], suggesting an effective inhibition of exogenous heme on ALAS function.

Hematin was also able to partially restore P450 activity in lead-treated mice. The three P450 activities measured increased significantly by 20–25% (Fig. 7). However, hematin did not have the same effect on Cyp3a11 mRNA levels. With a dose of heme that was able to reduce plasma ALA levels and partially restore P450 activity, there was no change in Cyp3a11 mRNA concentration (Fig. 8). Similarly, the inhibitory effect of lead on *Cyp3a11* gene transcription was not reversed by hematin treatment (Fig. 9).

Dwarki *et al.* (29) and Bhat and Padmanaban (30) reported that heme at a low concentration (0.25 mg/kg) was able to counteract the inhibitory effect of cobalt on P450 induction by phenobarbital. However, when the heme concentration was increased to >1 mg/kg, this counteracting potential was lost. We therefore investigated whether lower hematin concentrations (1 and 0.25 mg/kg) were able to restore the decrease of *Cyp3a11* gene transcription and mRNA after lead injection. We could not find a concentration-dependent effect of heme in our experimental model (data not shown). Venkateswar and Padmanaban (8) and Bhat and Padmanaban (29) also reported that the addition of heme *in vitro* ($10^{-5}$ to $10^{-7}$ M) to

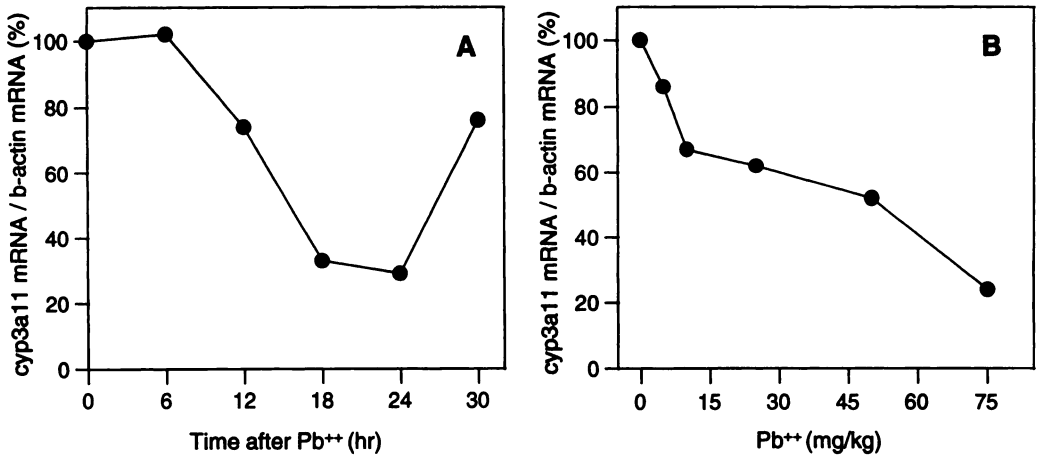


**Fig. 4.** Time course (A) and dose-dependent (B) effect of lead on Cyp3a11 mRNA levels. Total RNA was isolated from livers of lead-treated mice and subjected to RT. The resulting cDNAs were serially diluted and amplified for 25 cycles using primer sets specific for Cyp3a11 and β-actin. PCR products were quantified and confirmed in the exponential phase of the reaction as described in Materials and Methods. The Cyp3a11 values were normalized with the corresponding β-actin values. For time course experiments (A), lead was injected at a dose of 75 mg/kg, and animals were killed at the indicated times. For dose-response experiments (B), lead was injected at several doses, and mice were killed after 20 hr. Results represent the average values of two independent experiments.

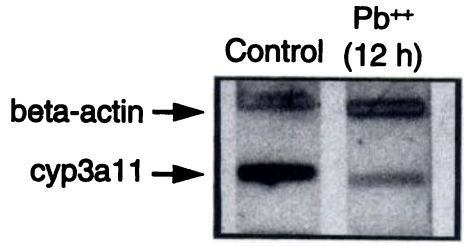


**Fig. 5.** Effect of lead on Cyp3a11 gene transcription. Mice were treated with lead acetate for 12 hr, and liver nuclei were isolated. Newly synthesized digoxigenin-labeled RNA was isolated and hybridized to nylon filter-bound cDNA for Cyp3a11 and β-actin.

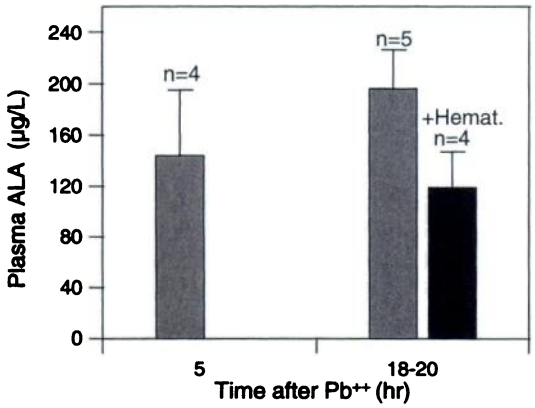


**Fig. 6.** Effect of hematin injection on plasma levels of ALA in lead-treated mice. Mice were injected with lead acetate, and 5 hr later hematin was administrated at a dose of 4 mg/kg intraperitoneally. Blood samples were taken at the indicated times, and plasma ALA concentration was determined. Results are expressed as mean ± standard deviation (number of animals).

nuclei isolated from livers of heme-depleted rats restored P450 gene transcription. However, the addition of hematin ($5 \times 10^{-6}$ to $2 \times 10^{-7}$ M) to nuclei isolated from lead-poisoned mice did not restore the transcriptional elongation rate of *Cyp3a11* (Fig. 9 and data not shown).

Therefore, under our experimental conditions, exogenous

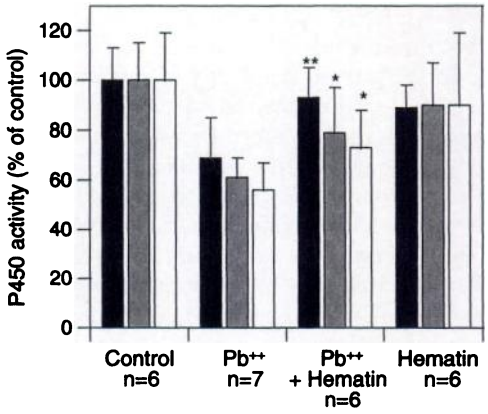


**Fig. 7.** Hematin partially restores P450 activity in lead-treated mice. Animals were injected with lead acetate, and 5 hr later hematin was administered. P450-associated activities were determined in liver microsomes 20 hr after lead injection (15 hr after hematin). *Filled bars*, ECOD (control value, 2225 ± 289 pmol of 7-hydroxy-coumarin/mg/min); *shaded bars*, EROD (control value, 89 ± 13 pmol of resorufin/mg/min); *open bars*, PROD (control value, 23 ± 4 pmol of resorufin/mg/min). Results are expressed as mean ± standard deviation of four independent experiments (number of animals) **, $p < 0.01$; *, $p < 0.05$; statistical significance of lead+hematin-treated versus lead-treated mice according to Student's *t* test.

heme can partially restore P450 activity but not P450 transcription or mRNA levels.

## Discussion

Lead strongly inhibits ALA dehydratase, leading to a marked inhibition of heme synthesis. On the other hand, acute lead administration results in a decrease in P450 function. Therefore, it was tempting to suggest a direct relationship between the heme deficiency caused by lead and the decrease in P450 function (9). In this study, we investigated the mechanism by which lead decreases P450 function, and our data demonstrate that the decrease in P450 activity is in part due to an inhibition of P450 gene transcription that is unrelated to heme.

Heme has been reported to regulate the transcriptional

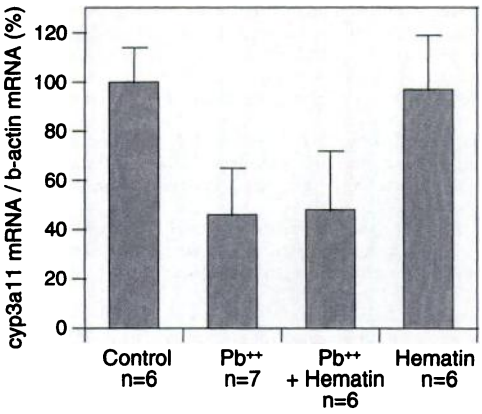


**Fig. 8.** Effect of hematin on Cyp3a11 mRNA levels in lead-treated mice. Animals were treated as stated in the legend to Fig. 7. Liver total RNA was isolated and subjected to RT. The resulting cDNAs were serially diluted and amplified for 25 cycles using primer sets specific for Cyp3a11 and β-actin. PCR products were quantified and confirmed in the exponential phase of the reaction, and Cyp3a11 values were normalized with the corresponding β-actin values. Results are expressed as mean ± standard deviation of four independent experiments (number of animals).

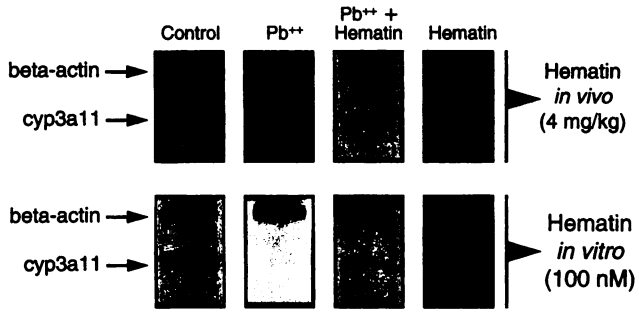


**Fig. 9.** Effect of hematin on Cyp3a11 gene transcription in lead-treated mice. Animals were injected with lead acetate, and 5 hr later hematin was administered. Liver nuclei were isolated 12 hr after lead injection. Newly synthesized digoxigenin-labeled RNA was isolated and hybridized to nylon filter-bound cDNA for Cyp3a11 and β-actin. *Bottom*, hematin (100 nM) was added *in vitro* to the runoff transcription assays instead of *in vivo* injection.

elongation of CYP 2B1/2 during phenobarbital induction (8, 29–32), but other indirect studies did not support such a role on P450 gene transcription (33, 34) and even suggested that heme may negatively affect P450 expression (35, 36). One important factor could be the extent of depletion of the so-called regulatory free heme pool (8). Some inhibitors of heme synthesis and stimulators of heme breakdown, such as cobalt, aminotriazol, and thioacetamide, strongly reduce the heme content in microsomes and in nuclei, whereas other, more-specific inhibitors, such as succinylacetone, have only minor effects on the free heme pool (8). In this study, we demonstrated that lead was able to decrease the heme saturation of TDO from 33% to 15%, suggesting an important depletion in the available free heme. A second issue of controversy is the dose of exogenous heme applied to study the proposed effect on P450 gene transcription. It has been claimed that only low doses (<1 mg/kg) are effective (29, 30). We selected 4 mg/kg because it has been shown in rats to be able to inhibit ALAS and to restore the levels of intermediates of heme synthesis without a substantial induction of heme oxygenase, which would lead to a fast degradation of the exogenous heme incorporated into the cell (37). Furthermore, this dose of hematin was able to stop the accumulation of ALA in lead-poisoned mice, and it partially restored the activity of P450. However, despite these demonstrated effects, heme did not change the transcriptional activity or the mRNA levels of Cyp3a11. Lower doses of hematin were also assayed *in vivo* and *in vitro*, but they did not have a positive effect on *Cyp3a11* gene transcription. All of these results do not support a positive role of heme in the transcriptional activation of P450.

Lead probably decreases P450 gene transcription via a mechanism unrelated to its inhibition of heme synthesis. Indeed, lead has a high potential to interfere and disrupt several of the most important pathways that regulate gene transcription (38): 1) lead binds to calmodulin with higher affinity than that of calcium and results in the activation of calmodulin-dependent reactions; 2) lead stimulates partially purified protein kinase C at concentrations as low as $10^{-15}$ M (this may alter both the specificity and the rate of substrate phosphorylation and, ultimately, a multitude of regulatory processes, including gene expression); and 3) based on the mechanism of inhibition of lead on ALA dehydratase, it is theoretically possible that lead displaces the zinc ion of zinc-finger proteins, altering the conformation of these factors and affecting the regulation of many genes. Further studies are necessary to demonstrate whether any of these mechanisms are directly involved in the inhibition of *Cyp3a11* gene transcription.

The decrease in parallel of different P450 activities and Cyp3a11 mRNA is in concordance with a general effect of lead on P450 isoenzymes. This hypothesis is in agreement with the results of Degawa *et al.* (39, 40), which demonstrate negative effects of lead in the expression and function of other P450 isoforms, such as P450 1A2, and support a mechanism of lead action at the transcriptional level.

Administration of exogenous hematin partially restored P450 function in lead-poisoned mice without a concomitant increase in P450 gene transcription or mRNA concentration. In agreement with our results, Sinclair *et al.* (34) showed that inhibition of heme synthesis with succinylacetone affects both the net formation of immunoreactive P450 apoprotein and the content of functional holocytochrome without a concomitant effect on P450 mRNA. These results suggest that a heme-deficiency state could result in a translational or post-translational negative effect on P450 apoprotein synthesis that can be restored by hematin injection.

Several mechanisms could explain the restoration of P450 activity by heme. 1) After a certain time of heme synthesis inhibition, the free heme is depleted and becomes a limiting factor in the synthesis of new P450. Apo-P450 could then be reconstituted by exogenous heme. Partial reconstitution of apo-P450 has been demonstrated after selective heme-stripping of P450 with suicidal inactivators such as allylisopropylacetamide (41). 2) It has also been speculated that heme may be necessary to stabilize the newly formed P450 apoprotein (29). This hypothesis is supported by experiments showing that treatment of rats with allylisopropylacetamide results in both a rapid loss of P450 heme and a shorter half-life of the newly synthesized apo-P450 (29). 3) A third possibility is that heme may be needed for an efficient translation of P450 mRNA. Protein synthesis is regulated by heme in rabbit reticulocyte lysate. This regulation is mediated by a protein

kinase that phosphorylates the eukaryotic initiation factor eIF-2$\alpha$, leading to inhibition of polypeptide chain initiation. Heme is able to inactivate this protein kinase, allowing initiation and protein synthesis (42). This heme-sensitive repressor is expressed not only in reticulocytes but also in other nonerythroid tissues, such as rat liver (43). Whether any of these mechanisms are involved in the regulation of P450 biogenesis by heme remains to be established.

Finally, our results also suggest that the use of chemicals such chemicals as lead acetate, cobalt, and others to study the regulation of P450 by heme has major shortcomings. In addition to inhibiting heme synthesis, these compounds may have nonspecific toxic effects, such as the inhibition of P450 gene transcription caused by lead. Therefore, it would be useful to find an alternative model that is more suitable. In our laboratory, a knock-out mouse with a targeted disruption of porphobilinogen deaminase (the third enzyme of the heme pathway) has been developed. This mouse shows biochemical features of disturbed heme synthesis, such as massive induction of ALA synthase after phenobarbital treatment (44). The role of heme in the regulation of P450 expression is being studied with this genetic model of heme deficiency.

In summary, we demonstrated that the decrease in P450 function observed in lead-treated animals seems to be a consequence of two different mechanisms: a mechanism unrelated to heme, in which lead decreases P450 transcription and accordingly reduces P450 synthesis and activity, and a mechanism dependent on heme, in which lead first inhibits heme synthesis, and this results in decreased heme saturation of apo-P450 or/and a lower apoprotein content.

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**References**

1. Nelson, D. R., T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda, and D. W. Nebert. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12**:1-51 (1993).
2. Meyer, U. A., and H. S. Marver. Chemically induced porphyria: increased microsomal heme turnover after treatment with allylisopropylacetamide. *Science (Washington D. C.)* **171**:64-66 (1971).
3. Mustajoki, P., J.-J. Himberg, O. Tokola, and R. Tenhunen. Rapid normalization of antipyrine oxidation by heme in variegate porphyria. *Clin. Pharmacol. Ther.* **51**:320-324 (1992).
4. Birnie, G. G., K. E. L. McColl, G. G. Thompson, M. R. Moore, A. Goldberg, and M. J. Brodie. Antipyrine metabolism in acute hepatic porphyria in relapse and remission. *Br. J. Clin. Pharmacol.* **23**:358-361 (1987).
5. Alvares, A. P., S. Leigh, J. Cohn, and A. Kappas. Lead and methyl mercury: effects of acute exposure on cytochrome P-450 and the mixed function oxidase system in the liver. *J. Exp. Med.* **135**:1406-1409 (1972).
6. Ades, I. Z. Heme production in animal tissues: the regulation of biogenesis of *delta*-aminolevulinate synthase. *Int. J. Biochem.* **22**:565-578 (1990).
7. Padmanaban, G., V. Venkateswar, and P. N. Rangarajan. Heme as a multifunctional regulator. *Trends Biochem. Sci.* **14**:492-496 (1989).
8. Venkateswar, V., and G. Padmanaban. Involvement of heme in the transcriptional activation of CYPIIB1/B2 gene by phenobarbitone in rat liver: studies with succinylacetone. *Arch. Biochem. Biophys.* **290**:167-172 (1991).
9. Sassa, S. Toxic effects of lead, with particular reference to porphyrin and heme metabolism, in *Heme and Hemoproteins* (F. De Matteis and W. N. Aldridge, eds.). Springer-Verlag, Berlin, 333–371 (1978).
10. Meier, P. J., H. K. Mueller, B. Dick, and U. A. Meyer. Hepatic monooxygenase activities in subjects with a genetic defect in drug oxidation. *Gastroenterology* **85**:682-692 (1983).
11. Burke, M. D., S. Thompson, C. R. Elcombe, J. Halpert, T. Haaparanta, and R. T. Mayer. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P450. *Biochem. Pharmacol.* **34**:3337-3345 (1985).
12. Greenlee, W. F., and A. Poland. An improved assay of 7-ethoxycoumarin-O-deethylase activity: induction of hepatic enzyme activity in C57 BL/6J and DBA/2J mice by phenobarbital, 3-methylcholantrene and 2,3,7,8-tetrachlorobenzo-p-dioxin. *J. Pharmacol. Exp. Ther.* **205**:569-605 (1978).
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275 (1951).
14. Tomokuni, K., M. Ichiba, Y. Hirai, and T. Hasegawa. Optimized liquid-chromatographic method for fluorometric determination of urinary *delta*-aminolevulinic acid in workers exposed to lead. *Clin. Chem.* **33**:1665-1667 (1987).
15. Badawy, A. A.-B., and M. Evans. The regulation of rat liver tryptophan pyrrolase by its cofactor haem: experiments with haematin and 5-aminolevulinate and comparison with the substrate and hormonal mechanisms. *Biochem. J.* **150**:511-520 (1975).
16. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159 (1987).
17. Puissant, C., and L. M. Houdebine. An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Biotechniques* **8**:148-149 (1990).
18. Yanagimoto, T., S. Itoh, D. Muller-Enoch, and T. Kamataki. Mouse liver cytochrome P-450 (P-450IIIAM1): its cDNA cloning and inducibility by dexamethasone. *Biochim. Biophys. Acta* **1130**:329-332 (1992).
19. Tokunaga, K., H. Taniguchi, K. Yoda, M. Shimizu, and S. Sakiyama. Nucleotide sequence of a full-length cDNA for mouse cytoskeletal beta-actin mRNA. *Nucleic Acids Res.* **14**:28-29 (1986).
20. Murphy, L. D., C. E. Herzog, J. B. Rudick, A. T. Fojo, and S. E. Bates. Use of the polymerase chain reaction in the quantitation of *mdr*-1 gene expression. *Biochemistry* **29**:10351-10356 (1990).
21. Schibler, U., O. Hagenbüchle, P. K. Wellauer, and A. C. Pittet. Two promoters of different strengths control the transcription of the mouse *alpha*-amylase gene *amy*-1 in the parotid gland and the liver. *Cell* **33**:501-508 (1983).
22. Merscher, S., R. Hanselmann, C. Welter, and S. Dooley. Nuclear runoff transcription analysis using chemiluminescent detection. *Biotechniques* **16**:1024-1026 (1994).
23. Welch, A. N., and A. A.-B. Badawy. Tryptophan pyrrolase in haem regulation: experiments with administered haematin and the relationship between the haem saturation of tryptophan pyrrolase and the activity of 5-aminolevulinate synthase in rat liver. *Biochem. J.* **192**:403-410 (1980).
24. Badawy, A. A.-B. Tryptophan pyrrolase, the regulatory free haem and hepatic porphyria. *Biochem. J.* **172**:487-494 (1978).
25. Edwards, A. M., M. L. Glistak, C. M. Lucas, and P. A. Wilson. 7-Ethoxycoumarin deethylase activity as a convenient measure of liver drug metabolizing enzymes: regulation in cultured rat hepatocytes. *Biochem. Pharmacol.* **33**:1537-1546 (1984).
26. Yanagimoto, T., S. Itoh, S. Sawada, H. Hashimoto, and T. Kamataki. Molecular cloning and functional expression of a mouse cytochrome P-450 (Cyp3a-13): examination of Cyp3a-13 enzyme to activate aflatoxin B1 (AFB1). *Biochim. Biophys. Acta* **1201**:405-410 (1994).
27. Itoh, S., M. Satoh, Y. Abe, H. Hashimoto, T. Yanagimoto, and T. Kamataki. A novel form of mouse cytochrome P450 3A (Cyp3a-16): its cDNA cloning and expression in fetal liver. *Eur. J. Biochem.* **226**:877-882 (1994).
28. Schilter, B., and C. J. Omiecinski. Regional distribution and expression modulation of cytochrome P-450 and epoxide hydrolase mRNAs in the rat brain. *Mol. Pharmacol.* **44**:990-996 (1993).
29. Dwarki, V. J., V. N. K. Francis, G. J. Bhat, and G. Padmanaban. Regulation of cytochrome P-450 messenger RNA and apoprotein levels by heme. *J. Biol. Chem.* **262**:16958-16962 (1987).
30. Bhat, G. J., and G. Padmanaban. Heme is a positive regulator of cytochrome P-450 gene transcription. *Arch. Biochem. Biophys.* **264**:584-590 (1988).
31. Bhat, G. J., and G. Padmanaban. Heme regulates cytochrome P-450 gene transcription elongation. *Biochem. Biophys. Res. Commun.* **151**:737-742 (1988).
32. Rangarajan, P. N., and G. Padmanaban. Regulation of cytochrome P-450b/e gene expression by a heme- and phenobarbitone-modulated transcription factor. *Proc. Natl. Acad. Sci. USA* **86**:3963-3967 (1989).
33. Srivastava, G., M. Bawden, A. J. Hansen, and B. M. May. Heme may not be a positive regulator of cytochrome P-450 gene expression. *Eur. J. Biochem.* **178**:689-692 (1989).
34. Sinclair, P. R., E. G. Schuetz, W. J. Bement, S. A. Haugen, J. F. Sinclair, B. K. May, D. Li, and P. S. Guzelian. Role of heme in phenobarbital induction of cytochromes P450 and 5-aminolevulinate synthase in cultured rat hepatocytes maintained on an extracellular matrix. *Arch. Biochem. Biophys.* **282**:386-392 (1990).
35. Srivastava, G., A. J. Hansen, M. J. Bawden, and B. K. May. Hemin administration to rats reduces levels of hepatic mRNAs for phenobarbitone-inducible enzymes. *Mol. Pharmacol.* **38**:486-493 (1990).
36. Muller-Eberhard, U., J. L. Eiseman, M. Foidart, and A. P. Alvares. Effect of heme on allylisopropylacetamide-induced changes in heme and drug metabolism in the rhesus monkey (*Macaca Mulatta*). *Biochem. Pharmacol.* **32**:3765-3769 (1983).
37. Tokola, O., I.-B. Linden, and R. Tenhunen. The effects of haem arginate

and haematin upon the allylisopropylacetamide-induced experimental porphyria in rats. *Pharmacol. Toxicol.* **61**:75-78 (1987).
38. Goering, P. L. Lead-protein interactions as a basis for lead toxicity. *Neurotoxicology* **14**:45-60 (1993).
39. Degawa, M., H. Arai, S. Miura, and Y. Hashimoto. Preferential inhibition of hepatic P450 IA2 expression and induction by lead nitrate in the rat. *Carcinogenesis* **14**:1091-1094 (1993).
40. Degawa, M., H. Arai, M. Kubota, and Y. Hashimoto. Ionic lead, a unique metal ion as an inhibitor for cytochrome P450 IA2 (CYP1A2) expression in the rat liver. *Biochem. Biophys. Res. Commun.* **200**:1086-1092 (1994)
41. Farrell, G. C., and M. A. Correia. Structural and functional reconstitution of hepatic cytochrome P-450 *in vivo*: reversal of allylisopropylacetamide-mediated destruction of the hemoprotein by exogenous heme. *J. Biol. Chem.* **255**:10128-10133 (1980).
42. Fagard, R., and I. M. London. Relationship between phosphorylation and activity of heme-regulated eukaryotic initiation factor 2-alfa kinase. *Proc. Natl. Acad. Sci. USA* **78**:866-870 (1981).
43. Mellor, H., K. M. Flowers, S. R. Kimball, and L. S. Jefferson. Cloning and characterization of cDNA encoding rat hemin-sensitive initiation factor-2-alfa (eIF-2-alfa) kinase: evidence for multitissue expression. *J. Biol. Chem.* **269**:10201-10204 (1994).
44. Lindberg, R. L. P., C. Porcher, B. Grandchamp, B. Ledermann, K. Bürki, S. Brandner, A. Aguzzi, and U. A. Meyer. Porphobilinogen deaminase deficiency in mice causes a neuropathy resembling that of human hepatic porphyria. *Nat. Genet.* **12**:195-199 (1996).

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