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Coordinate Up-regulation of CYP1A1 and Heme Oxygenase-1 (HO-1) Expression and Modulation of δ-Aminolevulinic Acid Synthase and Tryptophan Pyrrolase Activities in Pyridine-Treated Rats

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ABSTRACT. To determine the changes in heme metabolism associated with induction of cytochrome P450 expression by pyridine, we compared the time course of CYP1A expression with the time course of (i) expression of heme oxygenase-1 (HO-1) (EC 1.14.99.3), (ii) activity of δ-aminolevulinic acid synthetase (ALAS) (EC 2.3.1.37), and (iii) heme saturation of tryptophan pyrrolase (TPO) (EC 1.13.11.11) in tissues of rats administered a single 100 or 150 mg/kg i.p. dose of pyridine. Both mRNA and protein of HO-1 and CYP1A1 were induced in the liver, kidney, and lung, with the induction of HO-1 mRNA preceding and paralleling that of CYP1A1 mRNA in the liver and lung but not kidney. Induction of CYP1A1 mRNA expression peaked within 9–12 hr and returned to control levels by 24 hr in all tissues examined, whereas induction of HO-1 mRNA expression was sustained for 48 hr in the lung and liver. In contrast to the transient up-regulation of CYP1A1 mRNA, increased microsomal CYP1A1 protein was sustained in all three tissues. Similar to the induction of HO-1 expression, lipid peroxidation was stimulated by pyridine treatment in the kidney, lung, and liver, but with the stimulation being more persistent in the liver and lung than in the kidney. Increased hepatic CYP1A1 or CYP1A2 activity was preceded by increased activities of HO-1 and ALAS. Pyridine treatment negatively modulated heme saturation of hepatic TPO. The findings indicate that pyridine stimulates the synthesis, utilization, and degradation of heme in a coordinate manner, and suggest that these alterations in heme metabolism may contribute to CYP1A1 induction by pyridine. BIOCHEM PHARMACOL **58**;4:723–734, 1999.

KEY WORDS. pyridine; CYP1A1; HO-1; heme metabolism; δ-aminolevulinic acid synthase; tryptophan pyrrolase; lipid peroxidation

Pyridine is a high volume industrial chemical and a major constituent of tobacco smoke [1, 2]. Among the biological effects of the compound is the induction of multiple forms of cytochrome P450, including CYP1A/2 [3, 4], CYP2B1/2 [5], and CYP2E1 [6]. Pyridine is also a structural moiety in many environmental agents that also induce CYP, such as the major tobacco constituent nicotine [7] and the toxic, food-borne heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine [8].

We observed in preliminary studies that hepatic microsomes from pyridine-treated rats contained higher levels of TBARS‖ than microsomes from untreated rats [9]. TBARS are products of oxidative stress reactions and peroxidative tissue damage. There is prior evidence that pyridine stimulates oxidative stress *in vivo* [10, 11]. Oxidative stress and TBARS formation in tissues can be stimulated by free heme [12, 13], which is also essential for functional cytochrome P450 [14]. Heme also has been implicated in the transcriptional activation of one of the forms of P450 induced by pyridine [15]. Accordingly, concomitant induction of functional cytochrome P450 and increased TBARS in microsomes by pyridine suggested increased heme demands and subsequent alterations in tissue metabolism of the pigment. It was, therefore, of interest to examine the relationship between heme metabolism and cytochrome P450 induction.

One measure of tissue heme metabolism is degradation of the compound to bilirubin, an enzymatic reaction in which heme oxygenase is rate-limiting [16]. Two forms of the

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‖ *Abbreviations*: AhR, aryl hydrocarbon receptor; ALA, aminolevulinic acid; ALAS, δ-aminolevulinic acid synthase; EROD, ethoxyresorufin *O*-deethylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HO-1, heme oxygenase-1; MDA, malondialdehyde; MROD, methoxyresorufin *O*-deethylase; TBARS, thiobarbituric acid-reactive substances; and TPO, tryptophan pyrrolase.

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heme-catabolizing enzyme have been identified, HO-1 and HO-2, but only HO-1 is regulated transcriptionally by several agents, including the substrate heme [17]. Commonly, with very few exceptions, HO-1 activity and cytochrome P450 activities are related inversely [17], a relationship that can be rationalized on the basis of the essentiality of intact heme for functional cytochrome P450.

Another measure of tissue heme metabolism is tissue concentration of the free pigment. Free heme concentration, at least in the liver, commonly is estimated indirectly by the degree of heme saturation of the cytosolic heme-dependent enzyme TPO or 2,3-dioxygenase [18–20]. In this estimation, the enzyme, which exists in the liver partially as the apoprotein, is assayed in the absence and in the presence of added heme and the heme saturation of the enzyme is expressed as a percentage of the activity in the absence of exogenous heme with respect to that in the presence of added heme; under these conditions, heme saturation of the enzyme is related inversely to tissue free heme concentration [18–21]. The free heme concentration has been assigned to the so-called regulatory heme pool because it serves such regulatory functions as feedback repression of the rate-limiting enzyme in heme synthesis, ALAS, and transcriptional activation of the *HO-1* gene [18, 19]. These regulatory functions may be related to homeostasis directed at minimizing tissue levels of excessive free heme and its consequent deleterious effects, including increased oxidative stress [12, 13, 18, 19]. Bilirubin, a major product of HO-1-catalyzed heme degradation [16, 17], is an antioxidant [12, 13, 22] and has been shown to be an aryl hydrocarbon receptor-dependent transcriptional activator of the *CYP1A1* gene [23]. Increased catalytic functions of newly synthesized cytochromes P450, as well as other hemoproteins, reflect increased heme utilization and, thus, provide an additional measure of tissue heme metabolism.

We originally observed a concomitant increase in microsomal CYP1A activities and lipid peroxides in the liver 12 hr following pyridine exposure. Therefore, it was of interest to compare these changes in hepatic and extrahepatic tissues over a time course following pyridine administration and to relate them to the changes in heme metabolism. The interest in examining CYP1A1 activities and lipid peroxidation in relationship to heme stems from the fact that both activities require heme and are related inversely in microsomes [24]. Furthermore, there is a dearth of information on the relationship of heme metabolism to the regulation of CYP expression. Although pyridine induces diverse forms of CYP, we focused on CYP1A1 and CYP1A2 in the current study because induction of the two related isozymes by pyridine has been described well [3, 4, 25], is a subject of current research activities in our laboratory, and is more pronounced than that of other P450 isozymes following a single low (< 200 mg/kg) dose of the compound. Our results show that pyridine administration leads to increased ALAS activity and increased HO-1 activity, suggesting enhancement of heme synthesis and degradation, respectively, by the compound; these effects were concomitant with CYP1A1 induction. Our findings that HO-1 expression preceded CYP1A1 expression, coupled with the reported ability of bilirubin, the major product of HO-1-catalyzed heme oxidation, to up-regulate the expression of CYP1A1 directly, suggest that altered heme metabolism, specifically enhanced synthesis and degradation, may contribute to the induction of CYP1A1 expression by pyridine.

MATERIALS AND METHODS

Animals and Pretreatment

Male Sprague–Dawley rats (from Taconic Farms) were housed under standard conditions at our American Association for the Accreditation of Laboratory Animal Care-approved facility for at least 1 week prior to experiments, and weighed 200–250 g at the time of experiments. The animals were treated between 8:00 and 9:00 a.m. either with a single i.p. dose (100 or 150 mg/kg) of pyridine in saline or with saline only (1 mL/kg, untreated controls), and were killed in groups of three to four either immediately (zero-hour treated controls) or at 3, 6, 9, 12, 24, or 48 hr after pyridine treatment. Organs were perfused *in situ* via the ventricles with ice-cold, 10 mM potassium phosphate-buffered 1.15% (w/v) KCl to minimize hemoglobin contamination. Prior to excision, 1- to 2-mg pieces of each organ were removed and snap-frozen in liquid nitrogen for the isolation of total RNA or for the determination of contents of TBARS, and the remaining organ was excised and stored at −80° for the isolation of washed microsomes by differential centrifugation. Prior to differential centrifugation, aliquots of the total liver homogenate were saved for the determination of TPO and ALAS activities.

Electrophoresis and Western Blot Analysis

SDS–PAGE and western blot analysis of microsomes were performed as described previously [26], using a monoclonal antibody against rat CYP1A1, which also recognizes CYP1A2 [27], for the detection of CYP1A1 and CYP1A2, and a polyclonal antibody against rat HO-1 for the detection of HO-1. Immunoreactive bands on the membranes were detected with a horseradish peroxidase-conjugated secondary antibody by enhanced chemiluminescence in the case of CYP1A1 and CYP1A2 or colorimetrically with an alkaline phosphatase-conjugated secondary antibody system in the case of HO-1. The resulting bands on the films or membranes were quantified by densitometry using a Bio Image IQ scanner equipped with a Unix-based data station. Each densitometric (area under the curve) value was linear with respect to the protein concentration analyzed.

Northern Blot Analysis

Northern blotting was performed as described previously [7, 28], with slight modifications. Briefly, 20 μg of total RNA was fractionated on a denaturing formaldehyde/agarose (1%) gel and transferred to a Zeta-Probe nylon membrane

according to the recommendation of the manufacturer. Following UV irradiation, the fixed filters were incubated at 45° for 1 hr in hybridization buffer [3 × 0.15 M NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer, pH 7.4 (3x SSPE), containing 40% (v/v) deionized formamide, 7% (w/v) SDS, and 200 μg/mL of denatured salmon sperm DNA]. The filters were incubated subsequently with hybridization buffer in the presence of ^{32}P-labeled CYP1A1 and HO-1 probes (specific activity: 1–2 × 10^9 cpm/μg) for 20 hr. After hybridization, the filters were washed with a solution of 0.15 M NaCl, 15 mM sodium citrate, pH 7.4 (1x SSC), containing 0.1% SDS, at room temperature for 30 min, and then three times (15 min each) with 0.1x SSC containing 0.1% SDS, at 60°, and the blots were exposed to X-ray film with an intensifying screen at −70°. The membranes were subsequently stripped [29], and hybridization, washing, and autoradiography were repeated using the CYP1A2 probe. The membranes were stripped a second time, probed again with the GAPDH probe, and autoradiographed. Autoradiographic bands corresponding to CYP1A1, CYP1A2, HO-1, or GAPDH transcripts were quantified by densitometry using a Bio Image IQ scanner.

The rat CYP1A1- and mouse CYP1A2-specific probes were prepared by random priming of the inserts from plasmid p210 [30] and plasmid pmP3450–3′, respectively, using [α-^{32}P]ATP, as was the human GAPDH probe. The HO-1 cDNA probe was prepared by random priming with [α-^{32}P]ATP as described previously [31].

Enzymatic Assays

Microsomal O-dealkylation of methoxyresorufin or ethoxyresorufin was assayed fluorometrically as described by Pohl and Fouts [32], at a substrate and protein concentration of 5 μM and 50 μg/mL, respectively. Microsomal heme oxygenase activity was assayed spectrophotometrically as described previously [30]. TPO activity was assayed colorimetrically in the whole homogenate fraction with and without added hematin as described previously [21]. ALAS activity was assayed colorimetrically in the whole homogenate as described by Marver *et al.* [33].

Other Analyses

Tissue concentrations of TBARS were determined in whole homogenate fractions fluorometrically as described by Ohkawa *et al.* [34]. Protein was determined by the method of Lowry *et al.* [35]. Total RNA was isolated as described by Chomczynski and Sacchi [36]. Total tissue heme concentration was determined in whole homogenates by the pyridine hemochrome method [37]. Differences between treated animals and controls (zero-time treated animals) in the densitometric data from northern or western blot analysis or in catalytic activities were analyzed statistically by Student's unpaired *t*-test, with the level of significance set at $P < 0.05$. Analysis of differences between treatment groups was performed using Duncan's multiple range test ($P < 0.05$).

Materials

NADPH, $NADP^+$, hemin (equine), human serum albumin (fatty acid-free), 2-thiobarbituric acid, 1,1,3,3-tetraethoxypropane (malondialdehyde), L-tryptophan, kynurenine, δ-aminolevulinic acid, succinic acid, pyridoxal phosphate, ATP, coenzyme A, succinic thiokinase, 2,4-pentanedione, dimethyl aminobenzaldehyde, and pyridine were obtained from the Sigma Chemical Co. Pyridine contained no detectable impurities based on analysis by gas chromatography-electron impact mass spectrometry as described previously [38]. The following materials were obtained from the commercial sources indicated in parentheses: β-naphthoflavone (Aldrich Chemicals); ethoxyresorufin (Pierce Chemical Co.); methoxyresorufin and resorufin (Molecular Probes); alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Biosource International); rabbit anti-rat hepatic HO-1 polyclonal antibody (StressGen Biotechnologies Corp.); alkaline phosphatase substrate (Kirkegaard & Perry Laboratories); plasmid pmP3450–3′ (American Type Culture Collection); plasmid p210 (provided by Dr. John Fagan, Maharishi International University); human GAPDH cDNA (provided by Dr. Suzie Chen, Rutgers University); enhanced chemiluminescence western blotting analysis system (Amersham Life Science); Zeta-Probe nylon membranes (Bio-Rad Laboratories); nitrocellulose membranes (Schleicher & Schuell); [α-^{32}P]ATP, 3000 Ci/mmol (Dupont-NEN); and Kodak Biomax MR and X-Omat AR imaging films (Fisher Scientific Co.). All other reagents and supplies were of the highest grade of purity.

RESULTS

Effect of Pyridine Administration on Tissue HO-1 mRNA Levels

HO-1 mRNA was expressed constitutively in the kidney, lung, and liver, and was up-regulated to varying extents and with different time courses in each organ following pyridine treatment. In the kidney, the induction had a 6-hr onset and peaked (at 10-fold of the control level) 9 hr following pyridine treatment; thereafter, the transcript level decreased rapidly towards the control level by 12 hr (Fig. 1, A and B). In the lung, in contrast to the kidney, HO-1 mRNA level increased gradually following pyridine treatment, reached a maximum (3-fold of the control) at 12 hr, and decreased to 2-fold of the control level at 24 hr, a level that was maintained for the remaining duration of the experiment (Fig. 1, A and C). In the liver, up-regulation of the transcript was biphasic, with a rapid initial 5.5-fold increase relative to the control at 3 hr, which persisted for 6 hr (Fig. 1, A and D). This was followed by a transient decrease towards the control level at 12 hr, and a subsequent rebound increase at 24 hr to a level (6- to 7-fold of the control) that was sustained for the remaining duration of the study (Fig. 1, A and D). Based on the time course of pyridine-induced HO-1 expression in the three tissues, the

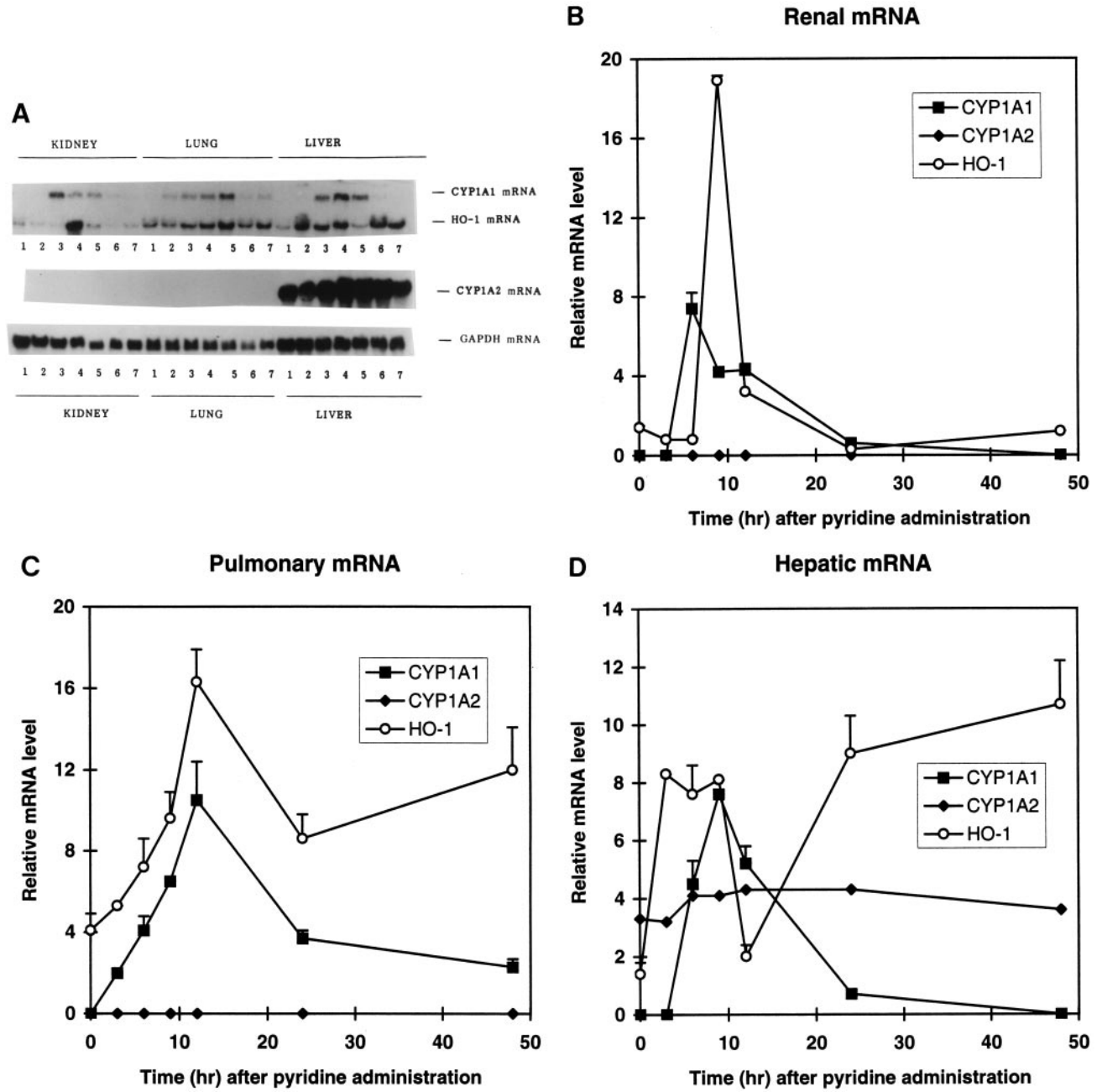

FIG. 1. Time course of CYP1A mRNA and HO-1 mRNA expression in rat kidney, lung, and liver following a single 150 mg/kg i.p. dose of pyridine. (A) Animals were treated with 150 mg/kg of pyridine and killed either immediately (lane 1, zero-hour treated control) or at 3 hr (lane 2), 6 hr (lane 3), 9 hr (lane 4), 12 hr (lane 5), 24 hr (lane 6), or 48 hr (lane 7). Total RNA from each tissue was analyzed by northern blotting, using a cDNA probe specific for CYP1A1, CYP1A2, HO-1, or GAPDH as described in Materials and Methods. (B–D) Densitometric analysis of northern blots of kidney (B), lung (C), or liver (D) RNA from experiments similar to those described in Fig. 1A. Relative RNA level is the abundance of the CYP1A or HO-1 transcript relative to that of GAPDH. Each point represents the average (± range) of determinations in 2 rats from 2 separate experiments.

induction can be characterized as transient in the kidney, relatively sustained in the lung, and persistent in the liver.

Effect of Pyridine Administration on Tissue CYP1A mRNA Levels

CYP1A1 mRNA was undetectable in the kidney, lung, or liver of control rats at the level of total RNA that we analyzed, but was up-regulated following pyridine treatment to varying magnitudes and with different time courses in each of the three tissues. In the kidney, up-regulation of the transcript was characterized by a 3-hr lag, with a maximum and near-control level attained at 6 and 24 hr, respectively (Fig. 1, A and B). In the lung, CYP1A1 mRNA up-regulation did not exhibit a lag, reached a maximum at 12 hr, and then decreased to a minimum level at 24 hr (Fig. 1,

A and C). In the liver, up-regulation of the transcript was characterized by a 3-hr lag and a maximum at 9 hr, with near-control level attained at 48 hr (Fig. 1, A and D). Only in the lung was the elevated CYP1A1 transcript sustained at the termination of the experiment at 48 hr (Fig. 1C). CYP1A2 mRNA, in contrast to CYP1A1 mRNA, was present only in the liver in untreated animals, and pyridine treatment did not affect the level of the transcript significantly either in the liver, as previously reported [3], or in extrahepatic tissues (Fig. 1, A–D).

Effect of Pyridine Treatment on Microsomal CYP1A Levels

Microsomal CYP1A1 protein was detectable in the lung and kidney but not the liver of untreated rats, and was increased by pyridine treatment in all three organs, but to varying magnitudes and with varying kinetics. In the kidney, the induction was characterized by a 3-hr lag and a 9.2-fold maximum at 12 hr, which was sustained for the subsequent 12 hr of the experiment (Figs. 2A and 3A). CYP1A1 induction in the lung, in contrast to that in the kidney, exhibited no lag and was rapid, reaching 6- and 7-fold of the control level at 6 and 24 hr, respectively (Figs. 2B and 3B). In the liver, the protein was not detected prior to 6 hr following pyridine administration, and its level at 12 and 24 hr was 10- and 20-fold, respectively, of the level observed at 6 hr (Figs. 2C and 3C). Similar to CYP1A2 mRNA expression, CYP1A2 protein was detectable only in the liver but was induced 3-fold, 24 hr following pyridine administration, in contrast to the insignificant up-regulation of the mRNA (compare Figs. 1D and 3C). Parallelism was observed between the increase in CYP1A1 protein and the expression of its mRNA only for the first 12 hr, after which the increase in protein but not mRNA was sustained in all three tissues.

Effect of Pyridine Administration on Catalytic Activities of Hepatic Microsomal CYP1A

Liver microsomal EROD and MROD activities, which are catalyzed preferentially by CYP1A1 and CYP1A2, respectively, at the substrate concentrations used in the current study [39], were comparable in untreated rats and were induced gradually and equally during the initial 12-hr period of pyridine administration (Fig. 4). Thereafter, EROD activity was induced to a greater extent than MROD activity, with EROD and MROD activity reaching 8.8- and 4.3-fold of their respective controls at 24 hr (Fig. 4). A comparison of the level of the CYP1A2 immunoreactive protein with the level of its activity showed that the magnitude of induction of MROD activity (4-fold) approximated that of the protein (3-fold). On the other hand, a comparison of the level of the CYP1A1 protein with the level of its activity at 24 hr relative to the 6-hr point, the earliest time point at which the protein was measurable, showed that the magnitude of induction of EROD activity

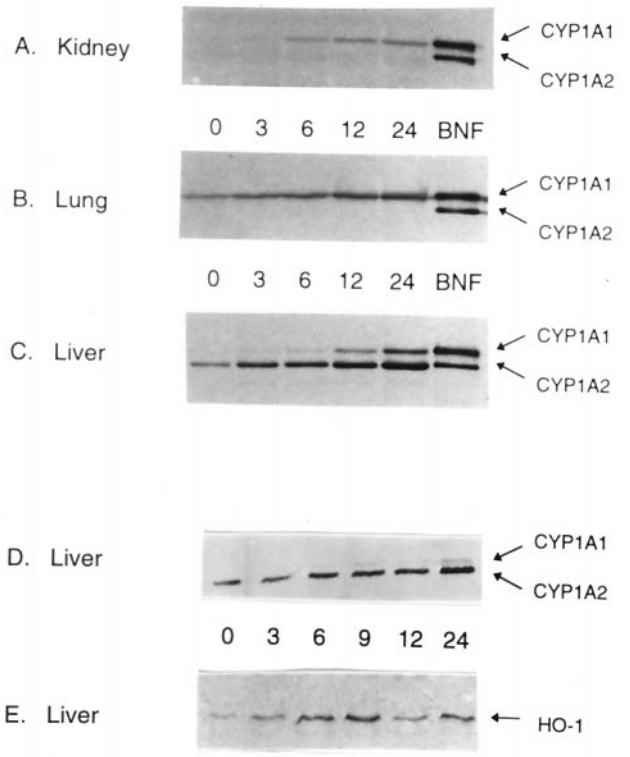

FIG. 2. Time course of kidney, lung, and liver microsomal CYP1A and liver microsomal HO-1 induction in pyridine-treated rats. (A–C) Rats were either administered a single 150 mg/kg i.p. dose of pyridine and then killed in groups of three immediately (0) or 3 hr (3), 6 hr (6), 12 hr (12), or 24 hr (24) after treatment, or administered a daily 40 mg/kg i.p. dose of β-naphthoflavone for 2 consecutive days and killed 24 hr after the second dose (BNF). Microsomal protein from pyridine-treated rats (100 μg for kidney and lung or 10 μg for liver) or from β-naphthoflavone-treated rats (1.5 μg for liver only) was analyzed for CYP1A1 and CYP1A2 by western blotting as described in Materials and Methods. The immunoprecipitin bands were detected by enhanced chemiluminescence. (D and E) Rats were treated with 100 mg/kg pyridine and killed in groups of three either immediately (0) or at 3 hr (3), 6 hr (6), 9 hr (9), 12 hr (12), or 24 hr (24) after treatment, and 20 μg of liver microsomal protein was analyzed by western blotting for CYP1A1 and CYP1A2 (panel D) or for HO-1 (panel E). The immunoprecipitin bands were detected by staining with an alkaline phosphatase system.

(4-fold) was significantly lower than that of CYP1A1 (20-fold).

Effect of Pyridine Administration on the Level and Activity of Hepatic Microsomal HO-1

Hepatic microsomal HO-1 was induced at all time points following pyridine treatment, with the time course of induction of the protein paralleling that of its activity (Fig. 5). There was a transient decrease at 12 hr followed by a rebound increase in both the activity and protein level (Fig. 5); this biphasic increase also was observed for the HO-1 transcript (Fig. 1D). We did not analyze pulmonary or renal microsomal HO-1 protein, HO-1 catalytic activity, or CYP1A activities because of the limited availability of

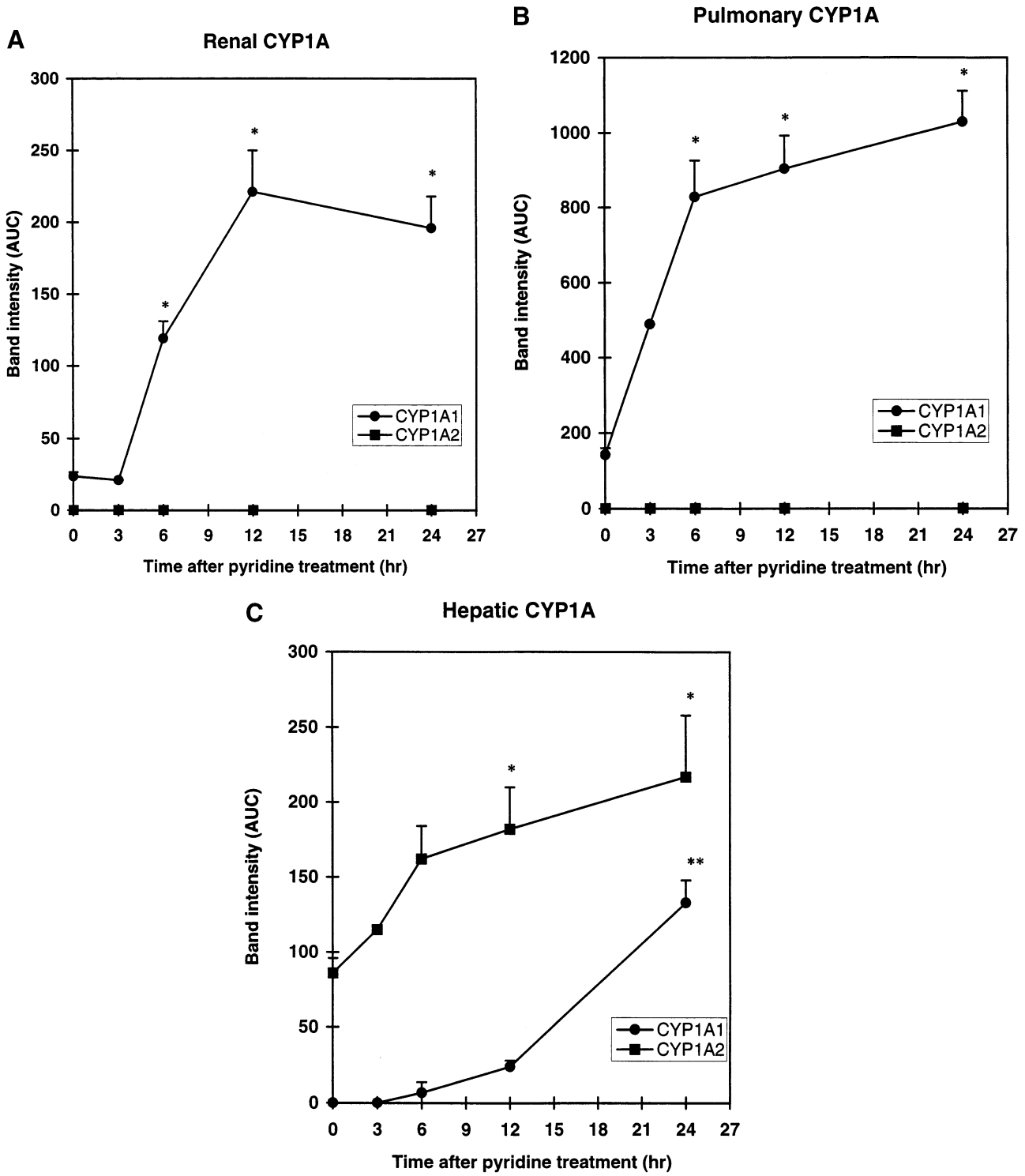

FIG. 3. Densitometric estimation of CYP1A from the western blot analysis of kidney (A), lung (B), and liver (C) microsomes from pyridine-treated rats. The analyses were on blots from experiments similar to and including those described in Fig. 2. Each data point represents the mean (± SD) of determinations in 3–4 rats, except those without error bars, which represent the average of determinations in 2 rats. Key: (*) significantly different from zero-time treated controls ($P < 0.05$), and (**) significantly different from zero-time treated controls or 6-hr treated animals ($P < 0.05$).

microsomal protein. However, we expect the level of the HO-1 protein and activity in the lung and kidney to parallel that of the respective HO-1 mRNA, based on the parallelism observed in the liver among the mRNA level, protein level, and activity.

Effect of Pyridine Administration on Tissue Levels of Lipid Peroxide

TBARS were present in tissues of zero-time treated animals at concentrations (nmol MDA equivalent/g tissue) of

18.2 ± 3.4, 14.4 ± 4.0, and 12.1 ± 2.0 in the kidney, lung, and liver, respectively. These concentrations were comparable in corresponding tissues from untreated animals (data not shown) and were elevated with time following pyridine treatment. The elevation in the kidney was rapid, with the peak level (2-fold of the control) attained 6 hr following pyridine administration, and was transient, with the control level re-established by 12 hr following pyridine administration (Fig. 6). The level in the lung peaked (at 1.5-fold of the control) 3 hr following pyridine treatment, but unlike that in the kidney, the elevation was sustained for the 24-hr duration of the analysis (Fig. 6). In the liver, in contrast to the kidney and lung, peak TBARS formation (2.3-fold of the control) was not attained until 12 hr following pyridine administration, after which the level slowly declined, but without attaining the control level by 24 hr. Based on the above time–course data, tissue differences in pyridine-induced formation of TBARS could be characterized as transient in the kidney and relatively sustained in the liver and lung.

Effect of Pyridine Administration on Hepatic TPO Activity

To assess whether the above observed increase in lipid peroxidation and HO-1 up-regulation was related to increased hepatic heme concentration, we determined the total heme concentrations in the animals but observed no effect of pyridine on the hepatic content of the pigment at any of the time points examined (data not shown). Therefore, we determined the effect of pyridine treatment on the size of the free heme pool by measuring the heme saturation of hepatic TPO, a commonly used measure of the size of the regulatory heme pool [18, 19].

Figure 7 shows that TPO activity was increased slightly (1.3-fold) 3 hr following pyridine administration, but decreased gradually thereafter to 38% of the control activity at 24 hr. Added heme stimulated the activity 1.5-, 1.9-, 2.3-, 1.9-, and 3.2-fold at 0, 3, 6, 12, and 24 hr, respectively (Fig. 7), with a resulting heme saturation of the enzyme of 66, 53.1, 43.6, 53.8, and 31.4% at 0, 3, 6, 12, and 24 hr, respectively (Fig. 7, insert). The data, thus, suggest that pyridine treatment did not increase but rather decreased the size of the free heme pool.

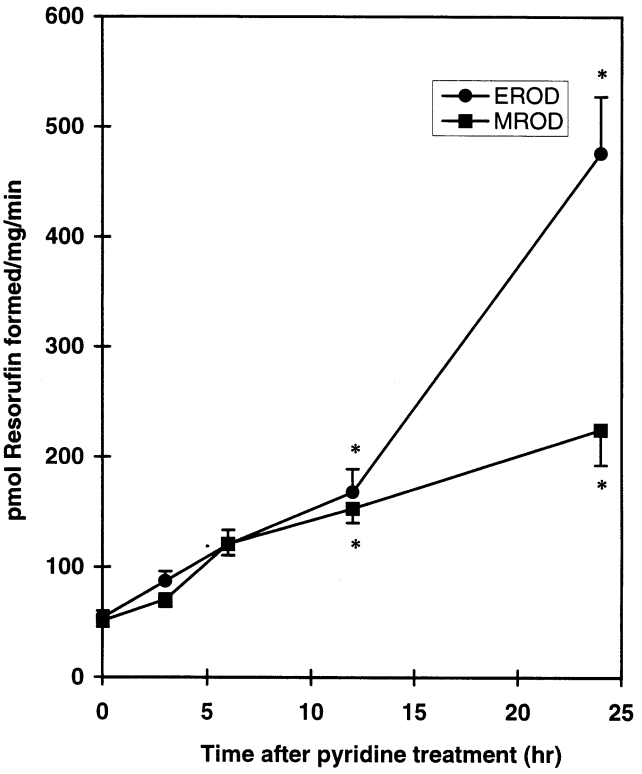

FIG. 4. Time course of hepatic microsomal EROD activity or MROD activity in pyridine-treated rats. Rats were treated with a single 150 mg/kg i.p. dose of pyridine and killed at the indicated time points as described in the legends to the preceding figures. Each data point is the average of determinations in 2 rats (where no error bars are indicated) or the mean (± SD) of determinations in 3–4 rats. Key: (*) significantly different from corresponding zero-time treated controls ($P < 0.05$).

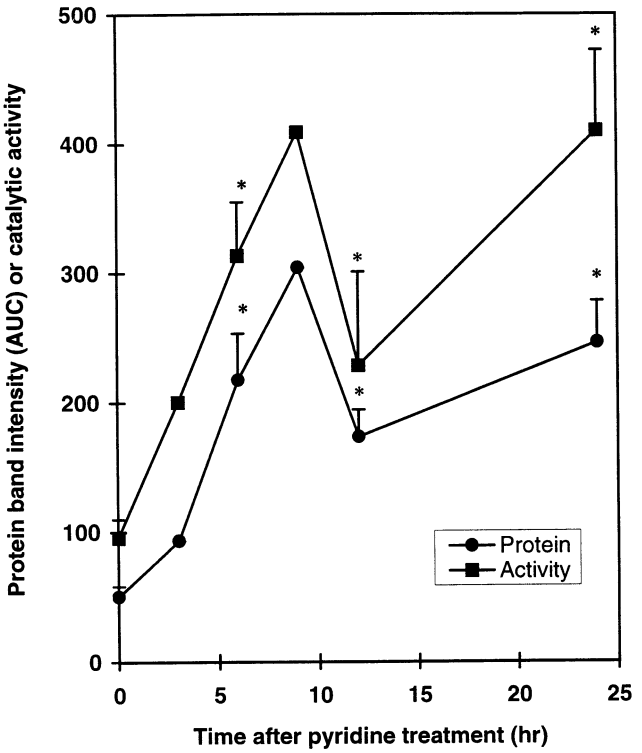

FIG. 5. Time course of hepatic microsomal HO-1 protein abundance and catalytic activity in pyridine-treated rats. Rats were treated with a single 100 mg/kg dose of pyridine and killed at the time points indicated. HO-1 protein abundance, quantified from western blots of liver microsomes similar to that shown in Fig. 2E, is expressed in arbitrary densitometric (area under the curve) units. Catalytic activity is expressed as picomoles of bilirubin formed per milligram of microsomal protein per minute × 10^{-1}. Each data point represents either the average of determinations in 1 or 2 rats (where no error bars are indicated) or the mean (± SD) of determinations in 3 rats. Key: (*) significantly different from zero-time treated controls ($P < 0.05$).

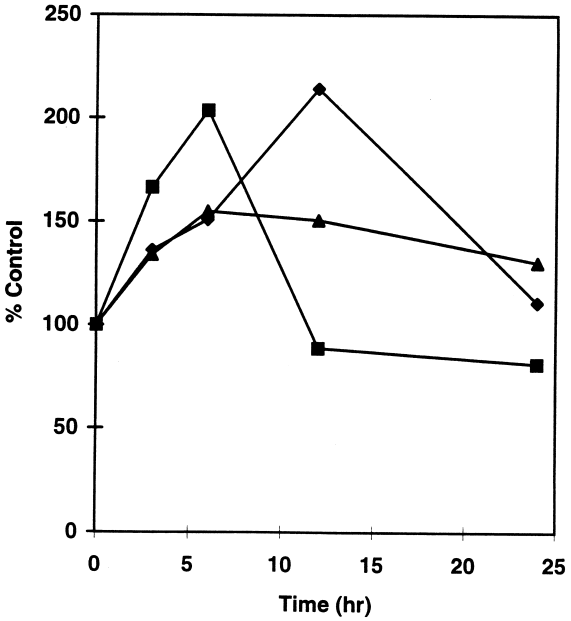

FIG. 6. Time course of tissue peroxide levels in pyridine-treated rats. Rats were treated i.p. with a single 100 mg/kg dose of pyridine and killed in groups of 3 at the time points indicated. Control (100%) peroxide levels (expressed as nanomoles of thiobarbituric acid-reactive substance equivalents per gram of tissue) determined in the animals immediately following pyridine treatment (zero-time controls) were 18.2 ± 3.4, 14.4 ± 4.0, and 12.1 ± 2.0 for lung, kidney, and liver, respectively. Each data point represents the mean of determinations from 3 rats. The assay was repeated at least once with similar results.

Effect of Pyridine Administration on Hepatic ALAS Activity

Since the preceding results indicated that pyridine treatment led to a decrease in saturation of the free heme pool, it was relevant to assess whether the decrease was associated with impaired heme synthesis by determining the activity of ALAS, the rate-limiting enzyme in heme synthesis [18, 19]. As shown in Fig. 8, hepatic ALAS activity was increased nearly 2.5-fold 3 hr following pyridine administration, and then declined slightly at 6 hr but rebounded to 2.7-fold of the control level at 12 hr, and remained at that level for the remaining duration of the experiment. The activity (nmol ALA formed/g liver/hr) in zero-time treated rats (5.1 ± 1.1) was comparable to that in untreated (saline-treated control) rats killed along with the treated rats at 0 hr (4.8 ± 0.6), 3 hr (5.0), 6 hr (5.1), 12 hr (4.9 ± 0.0.8), or 24 hr (5.2 ± 0.8), indicating that pyridine *per se* rather than other factors effected the enhanced ALAS activity.

DISCUSSION

An objective of the present study was to compare the changes in the induction of cytochrome P450 expression with changes in the reactions related to heme metabolism in rats following pyridine administration. The reactions of heme metabolism that we examined were those of synthesis (specifically ALAS activity), degradation (as estimated by HO-1 expression and activity), and utilization (as measured by CYP1A expression and activities). Our findings, collectively, support the conclusion that pyridine treatment stimulates the synthesis, utilization, and degradation of heme.

Our evidence for increased heme synthesis stimulated by pyridine is the sustained increase in hepatic ALAS activity following administration of the compound. Interestingly, the increased ALAS activity did not appear to result in tissue heme accumulation at any time point during the experiment. Furthermore, pyridine treatment caused a decrease rather than an increase in heme saturation of hepatic TPO, a commonly used estimate of the level of free heme concentration in the liver [18, 19]. In addition, the apo-CYP1A1 induced by pyridine may have been saturated only partially with heme, based on the observation that immunoreactive CYP1A1 was induced 20-fold compared with only a mere 4-fold increase in its catalytic activity at 24 hr relative to the 6-hr point, the earliest time point that

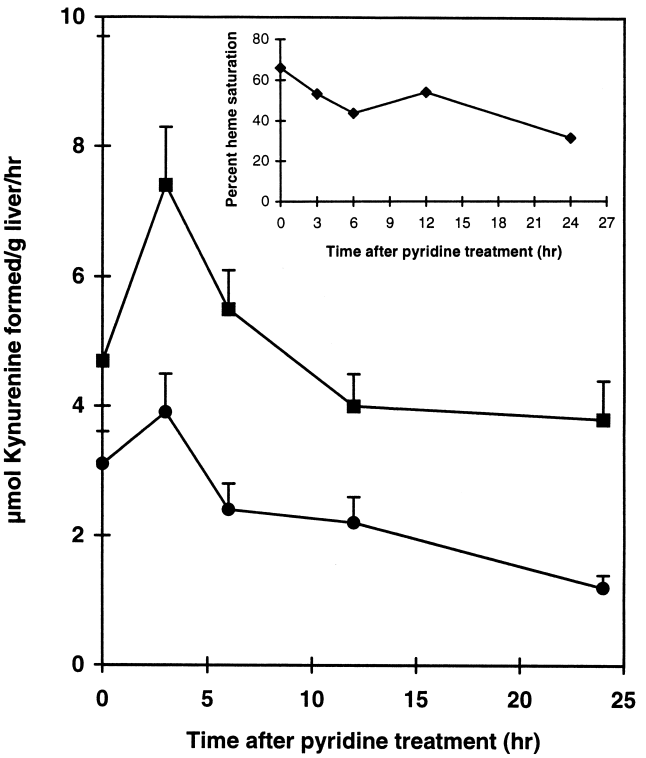

FIG. 7. Time course of hepatic TPO activity and percent heme saturation of the enzyme in pyridine-treated rats. Pyridine treatment was as described in Fig. 6. The assay was performed in the whole liver homogenate of rats in the absence (—●— or presence (—■—) of added heme (2 μM, final concentration). Each data point is the mean (± SD) of determinations in 3 rats from 2 separate experiments. Tryptophan pyrrolase activity in untreated control rats was 2.4, 2.0, 2.3, 2.9, and 2.8 μmol kynurenine formed/g wet liver at 0, 3, 6, 12, and 24 hr following initiation of the experiment. Insert: Kinetics of heme saturation of the tryptophan pyrrolase. The degree of heme saturation of the enzyme at each time point is expressed as the ratio of the activity without added heme to that in the presence of added heme (× 10^2).

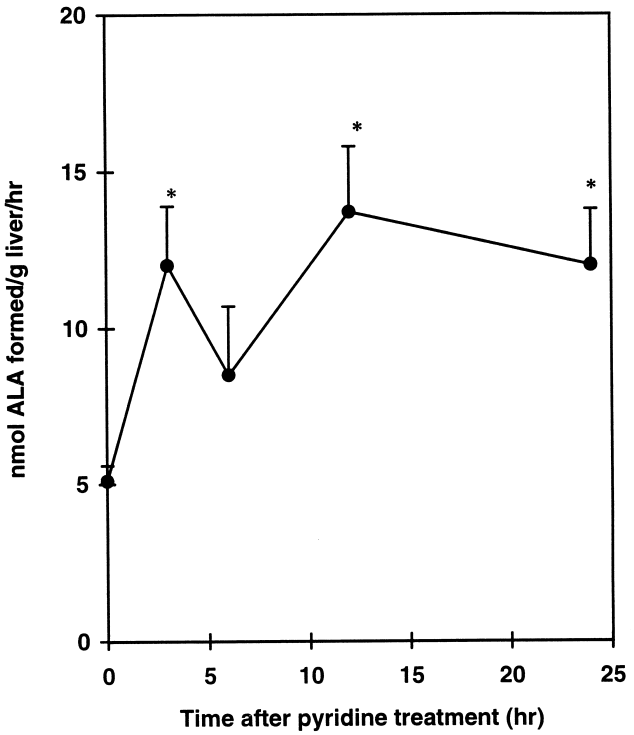

FIG. 8. Time course of hepatic ALAS activity in pyridine-treated rats. Animals were treated as described in Fig. 2. Each value is the mean (± SD) of determinations in 3 rats. ALAS activity 0, 3, 6, 12, and 24 hr following initiation of experiments in control rats not treated with pyridine was 5.3, 4.6, 4.5. 4.3, and 5.1 pmol ALA formed/g liver/hr, respectively. Key: (*) significantly different from zero-time treated controls ($P < 0.05$).

CYP1A1 was measurable. We speculate that the lack of accumulation of heme despite the increased ALAS activity resulted from increased utilization of the iron protoporphyrin at a rate that exceeded that of its synthesis. Persistent and exaggerated increase in total heme utilization that is not balanced by synthesis would be expected to decrease free heme concentration, with consequent derepression and sustained increase in ALAS activity. Other potential causes of the lack of heme accumulation despite the suggested increase in synthesis of the iron protoporphyrin include enhanced catabolism (as indicated by the observed up-regulation of HO-1), and increased loss as a result of increased participation of the pigment in reactions (such as lipid peroxidation) that lead to its degradation. In addition, pyridine forms complexes with heme iron [37], and such complexes could have contributed to the heme depletion in tissues of the treated animals. However, we did not observe evidence of the hemichromes or hemochromes characteristic of such complexes in tissue homogenates from pyridine-treated animals [38]. Alternatively, the increased ALAS activity following pyridine treatment could have resulted from inhibition of heme synthesis by either pyridine or its metabolites at steps downstream from ALA synthesis. This possibility, however, is unlikely, given the need for heme synthesis by the multiple P450 forms induced by pyridine.

Our evidence for pyridine-induced increase in heme utilization includes the induction of functional CYP1A1 and CYP1A2. The induction by pyridine of other functional forms of P450 isozymes [5, 6] provides additional evidence of pyridine-induced increase in hepatic heme utilization. Our data on the time course of pyridine-induced expression of hepatic CYP1A mRNA and protein agree with the findings of Kim *et al.* [3] and extend the findings to the lung and kidney, extrahepatic tissues in which the time course of pyridine-induced CYP1A1 expression has hitherto not been examined. However, parallelism between CYP1A1 and its mRNA was observed by other investigators [3] for 24 hr following pyridine exposure, whereas we observed the parallelism for only 12 hr, after which the induced protein but not mRNA was sustained in each of the three tissues examined. The difference in the data from the two studies underscores, perhaps, the significance of the dose of pyridine and the age of animals in CYP induction by the compound. We have also shown that the route of exposure and concomitant exposure to other agents are important determinants of CYP1A1 induction by pyridine [38, 40].

The mechanisms by which pyridine up-regulates CYP1A1 expression and the role of unchanged pyridine therein remain to be established. Ongoing studies, using *in vitro* gel mobility shift assays, show that pyridine is a poor agonist for the AhR,* suggesting that CYP1A1 induction by pyridine may not involve activation of the AhR by this compound [41]. The disparity between the level of CYP1A1 and that of its mRNA observed 12 hr after pyridine treatment suggests that protein stabilization or increased translation contributed to the sustained elevation of the protein. The significant induction of hepatic microsomal CYP1A2 by pyridine, in contrast to the lack of effect of the compound on the CYP1A2 mRNA level, suggests that enhanced translation or protein stabilization may account for CYP1A2 induction by the compound.

Similar to our previous observation in rat lung microsomes [4], the immunoreactive level of hepatic microsomal CYP1A1 protein in the present study was induced by pyridine to a greater extent (20-fold) than EROD activity (4-fold) at 24 hr relative to the 6-hr point. We interpret this disparity between CYP1A1 activity and its protein level as evidence that over 50% of the total CYP1A1 protein induced by pyridine lacked catalytic activity. We speculate that the catalytically nonfunctional fraction of CYP1A1 resulted from accumulation of newly synthesized heme-deficient apo-CYP1A1. *In vivo* formation of metabolite complexes between pyridine and CYP1A1 heme, similar to those reported for other compounds [42], could have contributed to the catalytic deficit. However, this possibility is unlikely because pyridine appears not to be a substrate for CYP1A1 [6], and we did not detect metabolite–cytochrome P450 complexes in tissues from pyridine-treated rats [4]. The catalytic deficit appears to be peculiar

* Iba MM and Fung J, unpublished observations.

to CYP1A1 because CYP1A2 ([4], and this study), CYP2E1 [4], and CYP2B1/2 [5] are induced by pyridine to magnitudes that are comparable with their catalytic activities.

The evidence for a pyridine-induced increase in heme degradation is our observed up-regulation of HO-1 expression. The parallelism observed between the time course of HO-1 mRNA expression and that of its protein level or catalytic activity following pyridine treatment suggests that expression of the enzyme by pyridine is regulated transcriptionally, as reported for other agents known to regulate expression of the enzyme [17]. The factor(s) directly responsible for the transcriptional activation of the HO-1 gene in the present study is unknown but may include pyridine itself. Heme, a known transcriptional activator of the gene [17], is unlikely to have contributed because, as discussed above, we observed no evidence that the pigment accumulated to levels sufficient to trigger the induction. Peroxides and other intermediates of oxidative stress reactions also are known to up-regulate HO-1 expression [43]. The peroxides were elevated by pyridine treatment in the current study, and the elevation preceded HO-1 expression in the tissues examined. Accordingly, these products of lipid peroxidation, or, even more likely, the reactive oxygen species that initiated their formation, may have been responsible for HO-1 up-regulation following pyridine treatment. Pyridine metabolites also may have contributed to HO-1 induction in the liver, based on the reported ability of *N*-oxides, which are major metabolites of pyridine and pyridine derivatives, to induce lipid peroxidation [44]. The role of pyridine metabolites in hepatic HO-1 induction also is suggested by the observed second surge in the induction of hepatic HO-1 expression.

Among the salient findings of the current study is the coordinate expression of HO-1 and CYP1A1 in the lung and liver, a finding that contrasts with the more commonly observed inverse relationship between cytochromes P450 and HO-1 activities [17, 45]. Pyridine, thus, becomes one of only a few agents capable of the coordinate induction of the two enzymes. The other agents reported to induce cytochrome P450 as well as HO-1 include 3,3′-dichlorobenzidine [28], arsenite [46], low doses of lipopolysaccharide [47], and ethanol [48]. Only in the kidney did CYP1A1 expression precede HO-1 expression, and it was exclusively inverse to HO-1 expression. This apparent tissue specificity in the coordinate expression of CYP1A1 and HO-1 is attributable to tissue-specific differences in such factors as pyridine metabolism, regulation of heme metabolism, and responses to oxidative stress. While it remains to be established whether the coordinate induction of CYP1A1 and HO-1 by pyridine represents independent or related events, the time course of induction of the two enzymes in the current study strongly suggests that the induction of both enzymes may be related, at least in the liver and perhaps the lung. In the liver, HO-1 induction preceded and paralleled CYP1A1 induction immediately following pyridine administration; only late in the treatment was HO-1 expression inverse to CYP1A1 expression. Hepatic CYP1A1 expression not only was preceded by HO-1 expression, but was sustained at the early peak of HO-1 expression. Therefore, it is not unreasonable to speculate that HO-1 expression and activity contributed to CYP1A1 expression. The speculated involvement of HO-1 activity in CYP1A1 induction deserves further investigation and is supported by several, albeit indirect, experimental observations, one of which is the reported transcriptional activation of the *CYP1A1* gene by bilirubin [23], a major product of HO-1 activity. Furthermore, CYP1A1 is up-regulated markedly in congenitally hyperbilirubinemic Gunn rats in comparison with their normal counterparts [49]. The possibility also exists that the up-regulation of HO-1 and CYP1A1 are independent and perhaps unrelated events. CYP1A1 up-regulation could result from reactive oxygen species-mediated release of a preformed endogenous inducer such as bilirubin or oxidized tryptophan derivatives, which are known inducers of the enzyme [50, 51]. Under these circumstances, the time course of tissue CYP1A1 induction would coordinate to tissue levels of the endogenous inducer(s) rather than HO-1 induction. These possibilities are being examined.

In summary, we have shown that pyridine treatment induces the up-regulation of HO-1 expression concomitantly with the expression of CYP1A1, an effect that was most prominent in the liver and lung. The data also show that pyridine treatment stimulates ALAS activity, which suggests increased heme synthesis. Nevertheless, pyridine treatment did not cause heme accumulation, due, perhaps, to increased utilization of the pigment. Obviously, more definitive experiments will be necessary to determine the effect of pyridine on the balance between heme synthesis and utilization. Based on the observed time course of HO-1 and CYP1A1 induction, our observation in ongoing studies that pyridine *per se* may not activate the *CYP1A1* gene, and the reported ability of bilirubin to activate the *CYP1A1* gene [23], CYP1A1 induction by pyridine could be influenced significantly by the changes in heme metabolism induced by the compound.

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References

1. International Agency for Research on Cancer, IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Tobacco Smoking. *IARC Monographs*, Vol. 38. IARC, Lyon, France, 1986.
2. ATSDR, *Toxicological Profile for Pyridine*, TP-91/24. U.S. Department of Health and Human Services, PHS, Agency for Toxic Substances and Disease Registry, Atlanta, GA, 1992.
3. Kim SG, Reddy S, States JC and Novak RF, Pyridine effects

on expression and molecular regulation of the cytochrome P450IA gene family. *Mol Pharmacol* **40:** 52–57, 1991.
4. Iba MM, Bennett S, Storch A and Thomas PE, Synergistic induction of CYP1A1 and CYP1A2 by acetone in combination with pyridine. *Cancer Lett* **74**: 69–74, 1993.
5. Kim H, Putt D, Reddy S, Hollenberg PF and Novak RF, Enhanced expression of rat hepatic CYP2B1/2B2 and 2E1 by pyridine: Differential induction kinetics and molecular basis of expression. *J Pharmacol Exp Ther* **267:** 927–936, 1993.
6. Kim SG, Williams DE, Schuetz EG, Guzelian PS and Novak RF, Pyridine induction of cytochrome P-450 in the rat: Role of P-450j (alcohol inducible form) in pyridine N-oxidation. *J Pharmacol Exp Ther* **246:** 1175–1182, 1988.
7. Iba MM, Scholl H, Fung J, Thomas PE and Alam J, Induction of pulmonary CYP1A1 by nicotine. *Xenobiotica* **28:** 827–843, 1998.
8. Kleman M, Overvik E, Mason G and Gustafsson J-A, Effects of the food mutagens MeIQx and PhIP on the expression of cytochrome P450IA proteins in various tissues of male and female rats. *Carcinogenesis* **11:** 2185–2189, 1990.
9. Iba MM, Thomas PE and Fung J, Comparative expression of CYP1A and lipid peroxidation and enzymes of heme metabolism in rats exposed to pyridine alone or with acetone. *Fundam Appl Toxicol* **36** (Suppl)**:** 84, 1997.
10. Baxter JH, Hepatic and renal injury with calcium deposits and cirrhosis produced in rats by pyridine. *Am J Pathol* **24:** 503–525, 1948.
11. Pinsky C and Bose R, Pyridine and other coal tar constituents as free radical-generating environmental neurotoxicants. *Mol Cell Biochem* **84:** 217–222, 1988.
12. Ma M and Eaton JW, Oxidative stress genes and proteins: Fire alarms and fire extinguishers. In: *Oxidative Damage and Repair. Chemical, Biological and Medical Aspects* (Ed. Davis KJA), pp. 1–4. Pergamon Press, New York, 1991.
13. Stocker R, Induction of heme oxygenase as a defense against oxidative stress. *Free Radic Res Commun* **9:** 101–112, 1990.
14. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239:** 2370–2378, 1964.
15. Bhat GJ and Padmanaban G, Heme is a positive regulator of cytochrome P450 gene transcription. *Arch Biochem Biophys* **264:** 584–590, 1988.
16. Tenhunen R, Marver HS and Schmid R, The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* **61:** 748–755, 1968.
17. Maines MD, *Heme Oxygenase. Clinical Applications and Functions*. CRC Press, Boca Raton, 1993.
18. Moore MR, McColl KEL, Rimington C and Goldberg A, *Disorders of Porphyrin Metabolism*. Plenum Publishing Co., New York, 1987.
19. Kappas A, Sassa S, Galbraith RA and Nordmann Y, The porphyrias. In: *The Metabolic and Molecular Basis of Inherited Disease* (Eds. Scriver CR, Beaudet AL, Sly WS and Valle D), pp. 2103–2158. McGraw-Hill, New York, 1995.
20. Feigelson M and Greengard O, A microsomal iron-porphyrin activator of rat liver tryptophan pyrrolase. *J Biol Chem* **236:** 153–157, 1961.
21. Badawy AA-B and Evans M, Regulation of rat liver tryptophan pyrrolase by its cofactor haem: Experiments with haematin and 5-aminolaevulinate and comparison with the substrate and hormonal mechanisms. *Biochem J* **150:** 511–520, 1975.
22. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN and Ames BN, Bilirubin is an antioxidant of possible physiological significance. *Science* **235:** 1043–1056, 1987.
23. Sinal CJ and Bend JR, Aryl hydrocarbon receptor-dependent induction of Cyp1a1 by bilirubin in mouse hepatoma Hepa 1c1c7 cells. *Mol Pharmacol* **52:** 590–599, 1997.
24. Lu AYH, Jacobson M, Poyer JL and McCay PB, Lipid peroxidation and the degradation of cytochrome P-450 heme. *Arch Biochem Biophys* **158:** 842–852, 1973.
25. Carlson GP, Comparison of the effects of pyridine and its metabolites on rat liver and kidney. *Toxicol Lett* **85:** 173–178, 1966.
26. Iba MM and Thomas PE, Hepatic activation of dichlorobenzidine: Involvement of cytochrome P-450d. *Carcinogenesis* **2:** 718–725, 1988.
27. Thomas PE, Reik LM, Ryan DE and Levin W, Characterization of nine monoclonal antibodies against rat hepatic cytochrome P-450c. Delineation of at least five spatially distinct epitopes. *J Biol Chem* **259**: 3890–3899, 1984.
28. Iba MM and Alam J, Heme oxygenase-1 mRNA levels, metallothionein mRNA levels, lipid peroxidation and microsomal CYP1A activities in rats treated with 3,3′-dichlorobenzidine and some other inducers of P450. *Redox Rep* **1**: 279–286, 1995.
29. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
30. Fagan JB, Pastewka JV, Chalberg SC, Gozukara E, Guengerich FP and Gelboin HV, Noncoordinate regulation of mRNAs encoding cytochromes $P\text{-}450_{BNF/MC\text{-}B}$ and $P\text{-}450_{ISF/BNF\text{-}G}$. *Arch Biochem Biophys* **244:** 261–272, 1985.
31. Alam J, Shibahara S and Smith A, Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse hepatoma cells. *J Biol Chem* **264**: 6371–6375, 1989.
32. Pohl RJ and Fouts JR, A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal cellular fractions. *Anal Biochem* **107:** 150–155, 1980.
33. Marver HS, Tschudy DP, Perlroth MG and Collins A, δ-Aminolevulinic acid synthetase. I. Studies in liver homogenates. *J Biol Chem* **241:** 2803–2809, 1966.
34. Ohkawa H, Ohishi N and Yagi K, Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* **95:** 351–358, 1979.
35. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1961.
36. Chomczynski P and Sacchi A, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
37. Falk JE, *Porphyrins and Metalloporphyrins*. Elsevier Publishing Co., New York, 1964.
38. Scholl H and Iba MM, Pharmacokinetics of and CYP1A induction by pyridine and acetone in the rat: Interactions and effects of route of exposure. *Xenobiotica* **27:** 265–277, 1997.
39. Yang H-YL, Namkung MJ and Juchau MR, Cytochrome-P-450-dependent biotransformation of a series of phenoxazone ethers in the rat conceptus during early organogenesis: Evidence for multiple P-450 isoenzymes. *Mol Pharmacol* **34:** 67–73, 1988.
40. Scholl H, Fung J, Thomas PE, Alam J and Iba MM, Interactive induction of CYP1A by pyridine and acetone: Dose-response relationships. *Fundam Appl Toxicol* **36** (Suppl)**:** 87, 1997.
41. Okey AB, Riddick DS and Harper PA, Molecular biology of the aromatic hydrocarbon (dioxin) receptor. *Trends Pharmacol Sci* **15:** 226–232, 1994.
42. Watkins PB, Wrighton SA, Schuetz EG, Maurel P and Guzelian PS, Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P-450p in rat hepatocytes *in vivo* and in primary monolayer culture. *J Biol Chem* **261:** 6264–6271, 1986.
43. Kyse SM and Tyrell RM, Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by

UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* **86:** 99–103, 1989.
44. Murray M, Sefton RM, Martini R and Butler AM, Induction of cytochromes P450 2B and 2E1 in rat liver by isomeric picoline *N*-oxides. *Toxicol Lett* **93:** 195–203, 1997.
45. Kikuchi G and Yoshida T, Function and induction of the microsomal heme oxygenase. *Mol Cell Biochem* **53/54:** 163–183, 1993.
46. Albores A, Sinal CJ, Cherian MG and Bend JR, Selective increase of rat lung cytochrome P450 1A1 dependent monooxygenase activity after acute arsenite administration. *Can J Physiol Pharmacol* **73:** 153–158, 1995.
47. Stanley LA, Adams DJ, Lindsay R, Meehan RR, Liao W and Wolf CR, Potentiation and suppression of mouse liver cytochrome P-450 isozymes during acute-phase response induced by bacterial endotoxin. *Eur J Biochem* **174:** 31–36, 1988.
48. Okuno F, Arai M, Sujita K, Eto S and Ishii H, Alterations in hepatic δ-aminolevulinic acid synthetase and heme oxygenase activities after chronic ethanol consumption in rats. *Alcohol* **8:** 449–451, 1991.
49. Kapitulnik J and Gonzalez FJ, Marked endogenous activation of the *CYP1A1* and *CYP1A2* genes in the congenitally jaundiced Gunn rat. *Mol Pharmacol* **43:** 722–725, 1993.
50. Rannug A, Rannug U, Rosenkranz HS, Winqvist L, Westerholm R, Agurell E and Grafstrom AK, Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. *J Biol Chem* **262**: 15422–15427, 1987.
51. Sindhu RK, Risz-Porszasz S, Hankinson O and Kikkawa Y, Induction of cytochrome P4501A1 by photooxidized tryptophan in Hepa 1c1c7 cells. *Biochem Pharmacol* **52**: 1883–1893, 1996.