0026-895X/07/7206-1419–1424$20.00
MOLECULAR PHARMACOLOGY
Vol. 72, No. 6
40238/3282526
Mol Pharmacol 72:1419–1424, 2007
Printed in U.S.A.

MOL PHARM

MOLECULAR PHARMACOLOGY

ACCELERATED COMMUNICATION

In Vivo Oxidative Damage in Rats Is Associated with Barbiturate Response but Not Other Cytochrome P450 Inducers[S]

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Received July 20, 2007; accepted September 26, 2007

ABSTRACT

Previously published studies have shown that cytochrome P450 (P450) enzyme systems can produce reactive oxygen species and suggest roles of P450s in oxidative stress. However, most of the studies have been done in vitro, and the potential link between P450 induction and in vivo oxidative damage has not been rigorously explored with validated biomarkers. Male Sprague-Dawley rats were pretreated with typical P450 inducers (β-naphthoflavone, phenobarbital (PB), Aroclor 1254, isoniazid, pregnenolone 16α-carbonitrile, and clofibrate) or the general P450 inhibitor 1-aminobenztriazole; induction of P4501A, -2B, -2E, -3A, and -4A subfamily enzymes was confirmed by immunoblotting and the suppression of P450 by 1-aminobenztriazole using spectral analysis. PB and Aroclor 1254 significantly enhanced malondialdehyde and H_2O_2 generation and NADPH oxidation in vitro and significantly enhanced formation in vivo, in both liver and plasma. Some of the other treatments changed in vitro parameters but none did in vivo. The PB-mediated increases in liver and plasma F_2-isoprostanes could be ablated by 1-aminobenztriazole, implicating the PB-induced P450(s) in the F_2-isoprostane elevation. The markers of in vivo oxidative stress were influenced mainly by PB and Aroclor 1254, indicative of an oxidative damage response only to barbiturate-type induction and probably related to 2B subfamily enzymes. These studies define the contribution of P450s to oxidative stress in vivo, in that the phenomenon is relatively restricted and most P450s do not contribute substantially.

The etiologies of a variety of diseases, including atherosclerosis, neurodegeneration, and cancer, among others, have been proposed to involve oxidative damage (Valko et al., 2007). Oxygen-centered free radicals are a major source of oxidative injury and lead to damage of lipids, proteins, and DNA. Numerous sources of oxygen radicals have been proposed, including leakage in the mitochondrial respiratory chain, NADPH oxidase, and myeloperoxidase, as well as the cyclooxygenases and lipoxygenases (Halliwell and Gutteridge, 1990). In addition, cytochrome P450 (P450) enzymes have been suggested to be significant contributors after demonstration of imperfect coupling of NADPH oxidation with substrate oxygenation and the production of superoxide and H_2O_2 in microsomal reactions, the latter of which was reported 50 years ago (Gillette et al., 1957; Nordblom and Coon, 1977). Since then, many articles have been published on the roles of P450s in the phenomenon of oxidative damage, even in the absence of substrates that would promote redox cycling, and the effects of P450 inducers on oxidative damage (e.g., Shiba and Shimamoto, 1999; Strolin-Benedetti et al., 1999; Zangar et al., 2004). The literature includes work on P450s in the gene subfamilies 1A (Liu et al., 2001; Dalton et

This work was supported by U.S. Public Health Service grants R37-CA090426 (to F.P.G.), P50-GM15431 (to J.D.M.), P01-CA77839 (to J.D.M.), R01-DK48831 (to J.D.M.), P01-ES13125 (to J.D.M.), T32-ES007028 (to J.D.B., F.P.G.), and P30-ES000267 (to F.P.G., J.D.M).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.040238.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: P450, cytochrome P450; 1-ABT, 1-aminobenztriazole; F_2-IsoP, F_2-isoprostane; βNF, β-naphthoflavone; CLOF, clofibrate; INH, isoniazid; Aroclor, Aroclor 1254 (a commercial mixture of polychlorinated biphenyls); PCN, pregnenolone 16α-carbonitrile; PB, phenobarbital.

al., 2002), 2B (Tong et al., 2003; Imaoka et al., 2004), 2E (Persson et al., 1990; Dupont et al., 2000; Robertson et al., 2001; Caro and Cederbaum, 2004; Bai and Cederbaum, 2006), 3A (Robertson et al., 2001), and 4A (Robertson et al., 2001). Of these, P450 2E1 has received considerable attention after in vitro inhibition experiments that indicated that it may have a major role in microsomal lipid peroxidation (Ekström and Ingelman-Sundberg, 1989). A role for P450 4A enzymes, induced via the peroxisome proliferator-activated receptor α response pathway, has also been suggested (Robertson et al., 2001).

However, the literature is very contradictory regarding the roles of these P450s; e.g., articles have been published with conclusions about 1A subfamily P450s enhancing or attenuating oxidative damage or having no effect (Liu et al., 2001; Dalton et al., 2002). The inhibitor 1-aminobenztriazole (1-ABT) did not block ethanol-induced oxidative stress associated with liver injury (Isayama et al., 2003). Two major deficiencies of the research in this area are that 1) a variety of biomarkers of damage are used in the various studies and 2) very few studies have been done in vivo (Strolin-Benedetti et al., 1999; Liu et al., 2001; Twaroski et al., 2001; Tong et al., 2003).

A number of methods of estimating oxidative damage have been proposed and include the quantification of oxidation products of lipids, proteins, and DNA (Kadiiska et al., 2005). While generally reliable as markers of oxidation in vitro, the vast majority are unreliable in vivo. Recent studies suggest, however, that quantification of F_2-isoprostanes (F_2-IsoPs), prostaglandin-like compounds resulting from the free radical-catalyzed peroxidation of arachidonate, are the most reliable, particularly in in vivo settings (e.g., Biomarkers of Oxidative Stress Study, Kadiiska et al., 2005). F_2-IsoPs are only one of a myriad of products of lipid peroxidation, but any reactive oxygen or nitrogen species capable of abstracting a bis-allylic hydrogen atom from a fatty acid will lead to a mixture of F_2-IsoPs and other products.

In this work, we treated rats with the major established P450 inducers under standard protocols, in the absence of obvious tissue injury, and measured isoprostane production in vivo and several in vitro parameters, the former being interpreted as the most relevant. Our results indicate that only barbiturate-type induction produced oxidative damage and the induction of other P450s (subfamilies 1A, 2E, 3A, and 4A) did not.

Materials and Methods

Chemicals. ABT, β-naphthoflavone (βNF), clofibrate (CLOF), isoniazid (INH), methyl cellulose, and phenobarbital (PB) were purchased from Sigma Chemical Co. (St. Louis, MO); Aroclor 1254 (Aroclor) was obtained from Analabs (North Haven, CT); pregnenolone 16α-carbonitrile (PCN) was a gift from P. O'Connell (from the former Upjohn Company, Kalamazoo, MI). All other reagents and solvents were obtained from general commercial suppliers. All chemicals were used without further purification.

Animals. Animals used in this study were male Sprague-Dawley rats weighing 200 to 225 g each (Charles River Breeding Laboratory, Wilmington, DE). All experimental procedures involving the use of experimental animals were performed in accordance with Guiding Principles in the Care and Use of Laboratory Animals, the National Research Council Guide, and the Office of Research, Vanderbilt University Medical Center. The animals were fed a commercial solid diet and water ad libitum. Lighting was maintained on a 12-h light/dark cycle (lights on from 6:00 AM to 6:00 PM); the ambient temperature was maintained between 21 and 24°C. After 7 days of adaptation to standard laboratory conditions, rats were randomly allocated into groups (eight rats per test group) and received either ABT (50 mg/kg, 1.0 ml, once daily for 1 day, oral gavage, in 0.5% methyl cellulose solution); Aroclor 1254 (300 mg/kg i.p., once 3 days before killing, in corn oil); βNF (40 mg/kg i.p., once daily for 3 days, in corn oil); CLOF (200 mg/kg i.p., once daily for 3 days, in corn oil); PCN (40 mg/kg i.p., once daily for 3 days, in corn oil); PB [continuously for 10 days, as 0.1% solution (sodium salt) in drinking water]; INH (continuously for 10 days, 0.1% solution in drinking water), or a combination of PB with ABT (Ortiz de Montellano and Mathews, 1981; Guengerich et al., 1982a; Meschter et al., 1994). Each test group was compared with its individual control group; the control groups received only the vehicle.

Blood and tissue samples were harvested after rats were deeply anesthetized with i.p. administration of sodium pentobarbital (100 mg/kg) 12 h after the last administration of each substance. Blood was obtained via caudal artery puncture. To obtain plasma, whole blood samples were centrifuged in tubes containing sodium EDTA (7.2 mg/5 ml of blood) at 1000*g* for 15 min. Plasma samples were immediately stored at −70°C. Right sublobes R2 (located at the caudal side) of livers, pieces of right kidneys, and brains were used for in vivo assays. The remainder of the livers, kidneys, and brains were used for in vitro assays. Tissues were immediately frozen and stored at −70°C until processing.

Microsomes were prepared from rat liver, kidney, and brain samples as described previously (Guengerich, 2001) and stored at −70°C. Protein concentrations were estimated using a bicinchoninic acid method (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Assay of F_2-IsoPs. Plasma, liver, kidney, and brain levels of F_2-IsoPs were determined using a gas chromatography-mass spectrometry method as described previously (Morrow and Roberts, 1999).

In Vitro Assays. Concentrations of total P450 were determined as described by Omura and Sato (1964). NADPH-cytochrome *c* reduction was measured as described previously (Phillips and Langdon, 1962). NADPH oxidation reactions were initiated with the addition of 200 μM NADPH to microsomal samples and the absorbance change at 340 nm was monitored (Yun et al., 2005). H_2O_2 formation was measured using a protocol adapted from Hildebrandt et al. (1978). Malondialdehyde, a product of lipid peroxidation, was determined using a thiobarbituric acid assay, with absorbance measurements at 535 nm (Ernster and Nordenbrand, 1967). Chlorzoxazone 6-hydroxylation (Peter et al., 1990) was used as a marker for induction of P450 2E1 activity.

Immunoelectrophoretic blotting assays were done to estimate P450 induction using polyclonal antibodies raised against P450s 1A2, 2B1, 2E1, and 3A1 in this laboratory and have been described previously (Guengerich et al., 1982a,b). Anti-P450 4A1 was a generous gift from J. Capdevila (Vanderbilt University). Known amounts of purified P450s were used on each gel (0.05–1.0 pmol of P450), and the results were quantified using densitometry. Because of the immunochemical cross-reactivity, we designate the induction as that for a subfamily, except in the case of P450 2E1 (only one subfamily member).

Statistical Analysis. Data were analyzed by analysis of variance (one way analysis of variance) followed by multiple comparisons using Kolmogorov-Smirnov's test for normality, Student's *t* test for comparison of two groups, Dunnett's test for comparison of groups against control groups, and Student-Newman-Keul's test for comparison of all groups pair wise. SPSS version 13 software for Windows (SPSS, Chicago, IL) was used for all steps of the analysis. Results in graphs are expressed as means ± S.E.M. Values of $p < 0.05$ were considered to be significant.

Results

Induction of P450s. A classic set of P450 inducers was used, based upon literature precedent. As expected, PB, Aroclor, PCN, and CLOF increased total liver weight and the concentration of P450 per milligram of protein (Supplemental Material). As expected from previous work (Guengerich et al., 1982a,b) immunochemical analyses indicated that P450 1A was induced only by βNF and Aroclor, P450 2B only by PB and Aroclor, P450 2E1 only by INH and PB, P450 3A only by PCN and PB, and P450 4A1 only by CLOF (Supplemental Fig. S1). Induction of P450 2E1 by PB had not been observed in another study (Thomas et al., 1987) but was confirmed by assays of liver microsomal chlorzoxazone 6-hydroxylation (Supplemental Fig. S2).

In the kidney, P450 1A was induced by βNF and Aroclor, P450 2B by Aroclor (but not PB), P450 2E1 by INH (but only marginally by PB), P450 3A by PCN, and P450 4A by CLOF (Supplemental Fig. S3). Slight increases in kidney NADPH-cytochrome P450 reductase were produced with Aroclor, PB, and INH (Supplemental Fig. S4). Several of the P450s could be detected in the brain, at low levels, but we did not find significant induction by any of the treatments (Supplemental Fig. S5).

Effects of Induction on in Vitro Liver and Kidney Microsomal Parameters. Several classic parameters were measured. NADPH-P450 reductase (measured as NADPH-cytochrome *c* reduction) was induced in liver by treatment with Aroclor, PB, and CLOF but not by βNF or INH (Supplemental Fig. S6). The rate of NADPH oxidation (in the absence of added substrates) was increased by treatment with PB, Aroclor, or CLOF. H_2O_2 production showed the same pattern. Malondialdehyde production was increased by treatment with PB, Aroclor, CLOF, and INH. Treatment with 1-ABT, which depleted P450. significantly decreased the rates of microsomal NADPH oxidation and formation of H_2O_2 and malondialdehyde to a greater extent than the small decrease in NADPH-cytochrome *c* reduction. In assays with kidney microsomes, Aroclor, PB, and INH yielded increases in rates of NADPH oxidation and H_2O_2 and malondialdehyde production, and CLOF also produced increases in these latter two parameters (Supplemental Fig. S4).

Tissue Isoprostanes. Liver F_2-isoPs were increased by treatment with PB or Aroclor but not by any of the other P450 inducers (Fig. 1A). These increases were also significant when F_2-isoPs were expressed on a liver weight basis and are greater than the increases in liver weight (Supplemental Fig. S6A) are considered. Kidney F_2-isoPs were not significantly increased by any of the treatments (Fig. 1B). Brain isoprostanes were very slightly increased by treatment with Aroclor or PCN (Fig. 1C). The P450 inhibitor 1-ABT, which depleted ~ 75% of the P450 in liver (Supplemental Fig. S6B) significantly decreased F_2-isoP levels in liver and kidney (Fig. 1, A and B) although not to a major extent; no change in F_2-isoP levels was found in brain (Fig. 1C).

Plasma Isoprostanes. Plasma F_2-isoPs showed the same pattern as liver (Fig. 1A), with the only major increases seen after treatment with PB or Aroclor (Fig. 2). 1-ABT treatment did not alter the level.

Attenuation of the PB Effect with 1-ABT. The liver (Fig. 1A) and plasma (Fig. 2) studies showed a consistent barbiturate effect, in that Aroclor is known to induce the

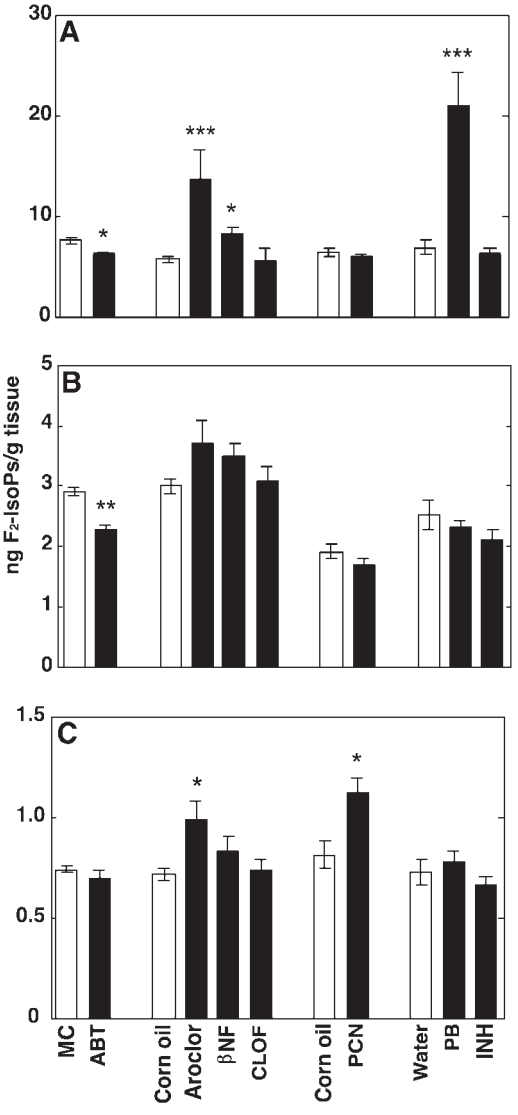

Fig. 1. Measurements of tissue F_2-isoPs. All values are presented as means ± S.E.M. (n = 8), with statistical significance indicated relative to the appropriate vehicle control (within each group, indicated by the open bar in each set) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). A, liver F_2-isoPs; B, kidney F_2-isoPs; C, brain F_2-isoPs. MC, methyl cellulose. The PCN experiment was done with a separate set of animals.

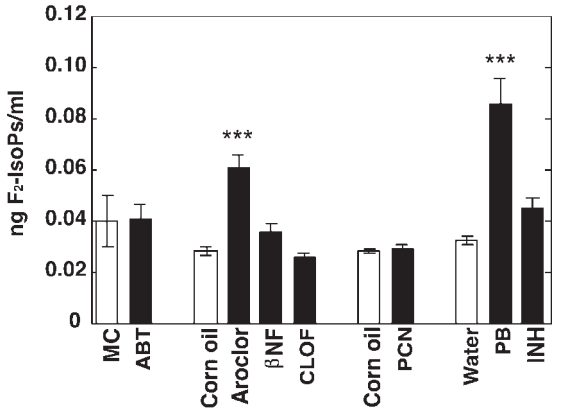

Fig. 2. Measurements of plasma F_2-isoPs. All values are presented as means ± S.E.M. (n = 8), with statistical significance indicated relative to the appropriate vehicle control (within each group, indicated by the open bar in each set) (***, $p < 0.001$). MC, methyl cellulose. The PCN experiment was done with a separate set of animals.

same enzymes that PB does, plus others (Guengerich et al., 1982a). However, the barbiturate effect might not necessarily be attributable to P450 induction because of the very pleiotropic effect of PB. A combination of PB and 1-ABT attenuated the level of total liver P450 and the levels of F_2-isoPs in both liver and plasma (Fig. 3), indicative of the role of P450 in the enhanced F_2-isoP production.

Discussion

The production of H_2O_2 in liver microsomes was first reported in 1957 (Gillette et al., 1957), and over the years, the number of reports associating P450s with the generation of reactive oxygen species has grown considerably. However, very few of these articles involve in vivo measurements; accordingly, we analyzed the effects of classic P450 induction protocols on a validated marker of in vivo oxidative injury and lipid peroxidation, F_2-IsoP production (Kadiiska et al., 2005), for the first time.

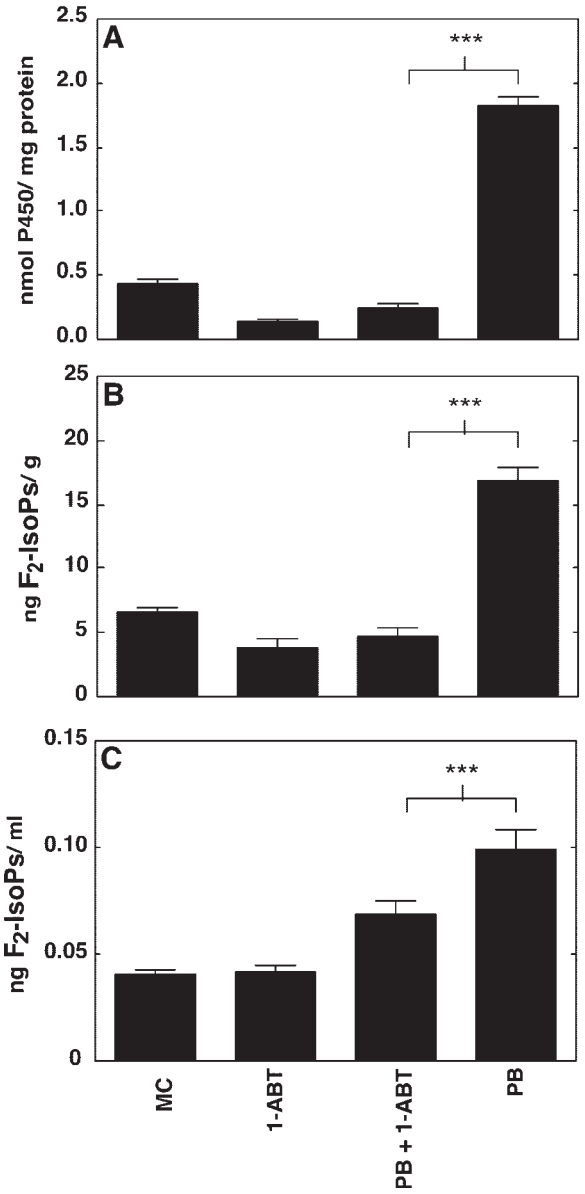

Fig. 3. Attenuation of the barbiturate response by 1-ABT. Rats were treated with PB, 1-ABT, or a mixture of the two (PB for 10 days, followed by a single i.p. injection of 1-ABT in vehicle), with treatment of all rats with the same vehicle (methyl cellulose). A, total microsomal P450; B, liver F_2-isoPs; C, plasma F_2-isoPs. All values are presented as means ± S.E.M. ($n = 6$), with statistical significance indicated (***, $p < 0.001$).

Of the enzyme inducers used here, only PB (and Aroclor, which yields a barbiturate-type response) substantially elevated F_2-IsoP levels in rat liver and plasma (Fig. 1). None of the inducers had a significant effect on F_2-IsoP levels in the extrahepatic tissues analyzed (i.e., kidney and brain) (Fig. 1, B and C). CLOF, INH, and βNF had some effects in some of the in vitro assays, but none of these had much effect on in vivo parameters (Fig. 1), although P450 induction was documented. These results indicate that P450s in the 1A, 2E(1), and 4A subfamilies do not contribute substantially to in vivo oxidative stress.

Barbiturates evoke a complex, pleiotropic response and interpretation of results can be difficult (Elrick et al., 2005).[1] It is noteworthy that markers of oxidative stress were elevated by PB in transcriptomic assays with rat liver (Elrick et al., 2005). PB (and Aroclor) also induces NADPH-P450 reductase, which in principle could be responsible for the in vivo oxidative stress (Supplemental Fig. S6). However, CLOF also induced the reductase but did not elevate F_2-IsoP levels (Fig. 1A). Evidence that P450 induction by PB is responsible for this increase in F_2-IsoPs comes from the results of the experiment with 1-ABT (Fig. 3), in which the PB-induced increase was clearly ablated by this selective P450 inhibitor, which destroys P450 heme as a mechanism-based inactivator (Ortiz de Montellano and Mathews, 1981; Meschter et al., 1994). The results of this experiment also argue against a mechanism for F_2-IsoP accumulation as a result of decreased clearance. Which PB-inducible P450 is responsible for the oxidative stress? The lack of effect of PCN (Figs. 1A and 2) argues for 2B subfamily enzymes as opposed to 3A, although contribution of moderately inducible rat P450s such as 2C6 (Guengerich et al., 1982a) cannot be ruled out.

1-ABT can deplete ~75% of liver P450, with the effect being without apparent toxicity under chronic conditions (e.g., up to 13 weeks) (Meschter et al., 1994). 1-ABT treatment produced only a modest decrease in liver F_2-IsoPs (Fig. 1A) and none in plasma F_2-IsoPs (Fig. 2). These results argue that the constitutive P450s (in male rats) contribute relatively little to generalized oxidative stress.

The literature is replete with studies on the contribution of P450 2E1 in oxidative stress. For instance, in in vitro experiments (with liver microsomes), it is possible to block a substantial fraction of malonaldehyde production with anti-P450 2E1 (Ekström et al., 1989). In addition, many other in vitro experiments have been done using expression in cultured cells (Caro and Cederbaum, 2004; Bai and Cederbaum, 2006). The lack of an effect of INH induction on F_2-IsoP levels suggests that P450 2E1 does not make a strong contribution in vivo [i.e., the inducibility of P450 2E1 by PB—which was not seen in another study (Thomas et al., 1987) but was documented here—cannot be interpreted as evidence that P450 2E1 is responsible for the observed PB effects seen here, in that INH did not elevate F_2-IsoPs; furthermore, Aroclor, which did elevate F_2-IsoPs, did not induce P450 2E1]. However, a caveat to our interpretation

[1] A literature search yielded only limited studies of the effect of barbiturates on production of reactive oxygen species in cell culture [e.g., a recent study in chicken hepatocytes in which a dye assay was used (Blättler et al., 2007)].

is that INH could also be a P450 2E1 ligand (accounting for some of the induction as a result of protein stabilization) and thus inhibits the enzyme in vivo (Zand et al., 1993). However, the rate of NADPH oxidation in microsomes prepared from INH-treated rats was not inhibited (or enhanced) by INH at concentrations as high as 50 μM (results not shown).[2] Furthermore, in mice, we obtained preliminary evidence that both liver and urinary F_2-isoP levels are not significantly different in homozygous P450 $2e1^{-/-}$ and $2e1^{+/+}$ mice, arguing further against an in vivo role for P450 2E1 (C. Chen, M. Dostalek, F. J. Gonzalez, F. P. Guengerich, K. D. Hardy, J. D. Morrow, unpublished results). These results are also consistent with the reported finding that 1-ABT does not prevent the oxidative stress associated with alcohol-induced liver injury in rats and mice. P450 2e1 gene deletion also does not prevent such oxidative stress (Isayama et al., 2003).

Would the presence of a substrate for an induced P450 elevate the generation of reactive oxygen species? In microsomes or reconstituted P450 systems, the effect of adding a substrate is generally to increase the rate of abortive oxygen reduction, usually ≤2-fold (Nordblom and Coon, 1977). We did not directly address the issue, but in several cases, the inducer is also a substrate and was administered at a subtoxic but relatively high dose. PB is metabolized by enzymes it induces, including P450s, and the sleeping time of rats decreases after a few days of continuous treatment. βNF-inducible rat P450 1A1 uses βNF as a substrate (Vyas et al., 1983), and some of the polychlorinated biphenyls in Aroclor are also substrates for the PB- and βNF-inducible P450s (Kaminsky et al., 1981). CCl_4-induced isoprostane production is exacerbated by treatment of rats with INH or PB (Morrow et al., 1992), but this is a rather extreme case in that the only product is a reactive radical itself ($\cdot CCl_3$).

Furthermore, even when F_2-IsoP production was enhanced by P450 induction (i.e., PB and Aroclor), the extent was 2- to 3-fold. These changes can be considered relatively modest compared with other agents that affect oxidative stress, particularly in that the inducers were administered at saturating doses. For instance, treatment of rats with a high dose of CCl_4 alone raised plasma F_2-IsoP levels by an order of magnitude (Morrow et al., 1992; Kadiiska et al., 2005), and the levels rise ~100-fold after CCl_4 treatment and P450 induction (Morrow et al., 1992). The changes of ~2-fold seen with a very high dose of PB in this work are similar to the effect of cigarette smoking in humans (Morrow et al., 1995). The possibility does exist that some P450s could produce higher local concentrations of reactive oxygen species and isoprostanes in specific cells, without the effect being large enough to detect in the intact tissues, although this prospect remains speculative in the absence of very specific probes of localized oxidative damage that could be used in specific cells in vivo (to correlate with P450 induction). P450s may be uncoupled in vivo but not to the extent that isoprostanes accumulate and general tissue damage is seen.[3]

In summary, our studies have, for the first time, examined the contribution of P450 enzymes to endogenous oxidant stress. Based on our results, we conclude that in vivo oxidative damage is increased with barbiturate response primarily via the subfamily 2B enzymes and not other P450s.

Acknowledgments

We thank M. V. Martin for help with animal protocol approvals and K. Trisler for assistance in preparation of the manuscript.

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[2] The literature indicates a $t_{1/2}$ for plasma isoniazid of 0.4 h irrespective of route of administration (Belanger et al., 1989). Thus, 30 half-lives elapsed before the samples were analyzed, and the residual level of isoniazid should have been very low.

[3] An additional load on the oxidative defense systems could conceivably exacerbate an effect of P450 induction (e.g., glutathione depletion). We focused on the effect of P450 induction per se in that glutathione depletion by itself is known to raise F_2-IsoP levels (Morrow et al., 1998).

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