**In Vivo** Oxidative Damage in Rats is Associated with Barbiturate Response but Not Other Cytochrome P450 Inducers

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ABBREVIATIONS: 1-ABT, 1-aminobenztriazole; Aroclor, Aroclor 1254 (a commercial mixture of polychlorinated biphenyls); ®NF, ®-naphthoflavone (5,6-benzoflavone); CLOF, clofibrate; F2-isoP, F2-isoprostane; INH, isoniazid; P450, cytochrome P450; PB, phenobarbital; PCN, pregnenolone 16α-carbonitrile.

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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 the typical P450 inducers β-naphthoflavone, phenobarbital (PB), Aroclor 1254, isoniazid, pregnenolone 16α-carbonitrile, and clofibrate or the general P450 inhibitor 1-aminobenztriazole; induction of P450 1A, 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 significantly enhanced malondialdehyde and H2O2 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 F2-isoprostanes could be ablated by 1-aminobenztriazole, implicating the PB-induced P450(s) in the F2-isoprostane elevation. The markers of in vivo oxidative stress were influenced mainly by PB and Aroclor, 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.
A variety of diseases have been proposed to involve oxidative damage in their etiology, including atherosclerosis, neurodegeneration, and cancer, among others (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 cycloxygenases and lipoxygenases (Halliwell and Gutteridge, 1990). In addition, cytochrome P450 (P450) enzymes have been suggested to be significant contributors following demonstration of imperfect coupling of NADPH oxidation with substrate oxygenation and the production of superoxide and H₂O₂ 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 (Dalton et al., 2002; Liu et al., 2001), 2B (Imaoka et al., 2004; Tong et al., 2003), 2E (Bai and Cederbaum, 2006; Caro and Cederbaum, 2004; Dupont et al., 2000; Persson et al., 1990; Robertson et al., 2001), 3A (Robertson et al., 2001), and 4A (Robertson et al., 2001). Of these, P450 2E1 has received considerable attention, following 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 proliferation activator receptor a response pathway, has also been suggested (Robertson et al., 2001).
However, the literature is very contradictory regarding the roles of these P450s, e.g. papers have been published with conclusions about 1A subfamily P450s enhancing or attenuating oxidative damage or having no effect (Dalton et al., 2002; Liu et al., 2001). 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 (i) a variety of biomarkers of damage are used in the various studies and (ii) very few studies have been done in vivo (Liu et al., 2001; Strolin-Benedetti et al., 1999; Tong et al., 2003; Twaroski et al., 2001).

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 F₂-isoprostanes (F₂-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). F₂-IsoPs are only one of the 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 F₂-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, with 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, 4A) did not.
Materials and Methods

Chemicals. ABT, β-naphthoflavone (βNF), clofibrate (CLOF), isoniazid (INH), methylcellulose, and phenobarbital (PB) were purchased from Sigma Chemical Company (St. Louis, MO); Aroclor 1254 (Aroclor) was obtained from Analabs (North Haven, CT); pregnenolone 16α-carbonitrile (PCN) was obtained (gratis, 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 Spraque-Dawley rats weighing 200-225 g each, from 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 seven 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% methylcellulose solution); Aroclor 1254 (300 mg/kg, once 3 days before killing, i.p., in corn oil); βNF (40 mg/kg, once daily for 3 days, i.p., in corn oil); CLOF (200 mg/kg, once daily for 3 days, i.p., in corn oil); PCN (40 mg/kg, once daily for 3 days, i.p., 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 (Guengerich et al., 1982a; Meschter et al., 1994; Ortiz de Montellano and Mathews, 1981).
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 $10^3 \times g$ 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 (Guengerich, 2001) and stored at -70 °C. Protein concentrations were estimated using a bicinchoninic acid method (Pierce-Fisher, Rockford, IL) with bovine serum albumin as a standard.

**Assay of F₂-IsoPs.** Plasma, liver, kidney, and brain levels of F₂-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 (Omura and Sato, 1964). NADPH-cytochrome c reduction was measured as described (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). H₂O₂ formation was measured using a protocol adapted from Hildebrandt et al. (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; Guengerich et al., 1982b). Anti-P450 4A1 was a generous gift from J. Capdevila (Vanderbilt University). Known amounts of purified P450s were used on each gel (0.05 to 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 ANOVA) followed by multiple comparisons using Kolmogorov–Smirnov’s test for normality, Student’s t-test for comparison of two groups, Dunnet’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 was used for all steps of the analysis. Results in graphs are expressed as means ± SEM. 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 mg protein (Supplemental Material). As expected from previous work (Guengerich et al., 1982a; Guengerich et al., 1982b) immunochemical analyses indicated that P450 1A was only induced by βNF and Aroclor, P450 2B only by PB and Aroclor, P450 2E1 by
INH and PB, P450 3A by PCN and PB, and P450 4A1 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. H₂O₂ 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 H₂O₂ 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 H₂O₂ and malondialdehyde production, and CLOF also produced increases in these latter two parameters (Supplemental Fig. S4).
**Tissue Isoprostanes.** Liver F₂-isoPs were increased by treatment with PB or Aroclor but none of the other P450 inducers (Fig. 1A). These increases were also significant when F₂-isoPs were expressed on a liver weight basis and are greater when the increases in liver weight (Supplemental Fig. S6A) are considered. Kidney F₂-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 F₂-isoP levels in liver and kidney (Figs. 1A, 1B) although not to a major extent; no change in F₂-isoP levels was found in brain (Fig. 1C).

**Plasma Isoprostanes.** Plasma F₂-isoPs showed the same pattern as liver (Fig. 1A), with the only major increases seen following 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 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 pleitropic effect of PB. A combination of PB and 1-ABT attenuated the level of total liver P450 and the levels of F₂-isoPs in both liver and plasma (Fig. 3), indicative of the role of P450 in the enhanced F₂-isoP production.

**Discussion**

The production of H₂O₂ in liver microsomes was first reported in 1957 (Gillette et al., 1957), and over the years the number of papers associating P450s with the generation of reactive oxygen species has grown considerably. However, very few of these papers involve in vivo
measurements, and accordingly we analyzed the effects of classic P450 induction protocols on a validated marker of in vivo oxidative injury and lipid peroxidation, F2-IsoP production (Kadiiska et al., 2005), for the first time.

Of the enzyme inducers used here, only PB (and Aroclor, which yields a barbiturate-type response) substantially elevated F2-IsoP levels in rat liver and plasma (Fig. 1). None of the inducers had a significant effect on F2-IsoP levels in the extrahepatic tissues analyzed, i.e. kidney and brain (Fig. 1B, 1C). 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). It is of interest to note 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 F2-IsoP levels (Fig. 1A). Evidence that P450 induction by PB is responsible for this increase in F2-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 (Meschter et al., 1994; Ortiz de Montellano and Mathews, 1981). The results of this experiment also argue against a mechanism for F2-IsoP accumulation due to decreased clearance. The question of which PB-inducible P450 is responsible for the oxidative stress can be raised. The lack of effect of PCN (Figs. 1A, 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 F2-IsoPs (Fig. 1A) and none in plasma F2-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). Also, many other in vitro experiments have been done using expression in cultured cells (Bai and Cederbaum, 2006; Caro and Cederbaum, 2004). The lack of an effect of INH induction on F2-IsoP levels suggests that P450 2E1 does not make a strong contribution in vivo (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 F2-IsoPs; also Aroclor, which did elevate F2-IsoPs, did not induce P450 2E1). However, a caveat to our interpretation is that INH could also be a P450 2E1 ligand (accounting for some of the induction due to 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). Further, in mice, we obtained preliminary evidence that both liver and urinary F2-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 nor does P450 2e1 gene deletion in mice (Isayama et al., 2003).

One question that can be raised is whether the presence of a substrate for an induced P450 would 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 $\leq 2$-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 sub-toxic 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 chronic 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). CCl₄-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 (−CCl₃).

Another point to make is that even when F₂-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 in comparison to 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 CCl₄ alone raised plasma F₂-IsoP levels by an order of magnitude (Kadiiska et al., 2005; Morrow et al., 1992), and the levels rise ~ 100-fold after CCl₄ 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 \textit{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 examined the contribution of P450 enzymes to endogenous oxidant stress, for the first time. 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.

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References


**Footnotes**

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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 (Blätter et al., 2007).

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.

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 the glutathione depletion by itself is know to raise F$_2$-IsoP levels (Morrow et al., 1998).
**Figure Legends**

**Fig. 1.** Measurements of tissue F₂-isoPs. All values are presented as means ± SEM ($n = 8$), 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 F₂-isoPs; B, kidney F₂-isoPs; C, brain F₂-isoPs. MC: methyl cellulose. The PCN experiment was done with a separate set of animals.

**Fig. 2.** Measurements of plasma F₂-isoPs. All values are presented as means ± SEM ($n = 8$), 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.

**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 ip injection of 1-ABT in vehicle), with treatment of all rats with the same vehicle (methyl cellulose). A, Total microsomal P450; B, liver F₂-isoPs; C, plasma F₂-isoPs. All values are presented as means ± SEM ($n = 6$), with statistical significance indicated ($p < 0.001$, ***).