Mechanism-Based Inactivation of Cytochromes P450 2B1 and P450 2B6 by *n*-Propylxanthate

Ute M. Kent,[†] Stanislav Yanev,[‡] and Paul F. Hollenberg*[,†]

Department of Pharmacology, The University of Michigan, Ann Arbor, Michigan 48109, and Department of Drug Toxicology, Institute of Physiology, Bulgarian Academy of Sciences, Bulgaria

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n-Propylxanthate (nPX) inactivated the 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) O-deethylation activity of purified, reconstituted rat hepatic P450 2B1 or human P450 2B6 in a mechanism-based manner. The inactivation followed pseudo-first-order kinetics and was entirely dependent on both NADPH and nPX. The maximal rate constant for inactivation of P450 2B1 at 30 °C was 0.2 min^{-1}. The apparent K_I was 44 μM, and the half-time for inactivation was 4.1 min. Purified, reconstituted human P450 2B6 was also inactivated by nPX with a K_I of 12 μM. The $k_{inactivation}$ for P450 2B6 was 0.06 min^{-1}, and the $t_{1/2}$ was 11 min. Incubations of P450 2B1 with nPX and NADPH for 20 min resulted in a 75% loss in enzymatic activity and a concurrent 25% loss of the enzyme's ability to form a reduced CO complex. Little loss in the absolute spectrum of nPX-inactivated P450 2B1 was observed. With P450 2B6, an 83% loss in enzymatic activity and a 12% loss in the CO-reduced spectra were observed. The extrapolated partition ratio for nPX with P450 2B1 was 32. P450 2B1 could be protected from inactivation by nPX by adding an alternate substrate to the reaction mixture. Removal of unbound nPX by dialysis did not reverse the inactivation. The alternate oxidant iodosobenzene was able to partially restore enzymatic activity to nPX-inactivated P450 2B1 samples. A stoichiometry for labeling of 1.2:1 for binding of radiolabeled nPX metabolite to P450 2B1 was seen. These results indicated that nPX inactivated P450 2B1 and P450 2B6 in a mechanism-based manner. P450 2B1 was inactivated primarily by a nPX reactive intermediate that bound to the apoprotein.

Introduction

Liver microsomal cytochromes P450 are involved in the metabolism of many drugs and carcinogens. P450 enzymes catalyze the metabolism of a wide variety of structurally distinct substrates (*1*), and particular P450 isoforms are also able to metabolize a number of different substrates (*1*−*3*). The catalytic mechanism appears to be common to all P450s and involves a two-electron reduction of molecular oxygen to form a reactive oxygen intermediate and water (*1*).

The three-dimensional structure of mammalian cytochromes has not been determined. Current information about the critical active site amino acid residues involved in substrate binding and catalysis has come primarily from site-directed mutagenesis studies or from observations with naturally occurring mutants (*4*, *5*). Additional insight into the active site structure has been gained from examining the crystal structures of a number of bacterial P450s (*6*−*9*). Mechanism-based inactivators that undergo catalytic conversion to reactive intermediates that covalently bind to amino acid side chains have been used to identify peptides within the active site and in some instances have also been used to locate key amino acid residues that are involved in substrate metabolism. Particularly successful were studies with the 2B rat and rabbit enzymes using acetylenic compounds such as 2-ethynylnaphthalene (*10*), 9-ethynylphenanthrene (*11*), or secobarbital (*12*). Relatively little is known about the physiological role of the human 2B homologue, P450 2B6, although some studies suggest that P450 2B6 was expressed at elevated levels in breast cancer (*13*). Studies by Osborne et al. also suggested that an increase in the level of C16 α-hydroxylation of 17β-estradiol metabolism in breast tissue may be a biomarker of breast cancer risk (*14*). P450 2B6 comprises only about 0.2% of the total P450s in human liver microsomes and may not be expressed in all human livers (*15*, *16*). However, a recent study indicated that P450 2B6 expression could be induced by phenobarbital in all the human livers that were screened with a polyclonal anti-2B6 antibody (*17*). The wide variability in the reported detection levels may be due to the low abundance of this isoform combined with a lack of available detection reagents such as specific antibodies, substrates, or inactivators. Recently, a monoclonal antibody to cDNA-expressed P450 2B6 has been developed (*18*). With the use of recombinant vaccinia or baculovirus P450 2B6 expression systems, it has been shown that P450 2B6 can metabolize a number of different substrates such as nicotine (*19*), aminochrysene and 3-methoxy-4-aminoazobenzene (*20*), tamoxifen (*21*), 7-benzyloxyresorufin (*22*), 7-ethoxycoumarin (*23*), testosterone (*24*), RP73401 (*25*), and phenanthrene and diazepam (*18*). Roberts et al. also demonstrated that P450 2B6 expressed in AHH-1[1] lymphoblastoid cells was inactivated by 9-ethynylphenanthrene (*26*).

Alkyl xanthates (R-OCS_2) have been identified as potential mechanism-based inactivators of P450 2B1 in

* To whom correspondence should be addressed: Department of Pharmacology, Medical Science Research Bldg. III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0632. E-mail: phollen@umich.edu.
[†] The University of Michigan.
[‡] Bulgarian Academy of Sciences.

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rat liver microsomes. The microsomal metabolism of ethylxanthate required O_2 and NADPH and could be blocked by CO, metyraprone, and SKF-525A (*27*). Ethylxanthate was found to inhibit CCl_4 and pentobarbital metabolism as well as to protect rats from nitrosodiethylamine-induced liver carcinogenesis (*28*, *29*).

In this study, *n*-propylxanthate (nPX, $CH_3CH_2CH_2OCS_2$) was found to inactivate the major phenobarbital inducible rat liver P450 2B1 and the human 2B homologue, P450 2B6, by a classical mechanism-based mechanism (*30*). The loss of enzymatic activity of P450 2B1 was primarily due to the binding of a nPX reactive intermediate to the apoprotein.

Experimental Procedures

Materials. Dilauroyl-L-α-phosphatidylcholine (DLPC), NADPH, and catalase were purchased form Sigma Chemical Co. (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was obtained from Molecular Probes, Inc. (Eugene, OR), and 7-hydroxy-4-(trifluoromethyl)coumarin (HFC) was from Enzyme Systems Products (Livermore, CA). HPLC-grade methanol and ethyl acetate were purchased from Mallinckrodt (Chesterfield, MO). BCA reagent and Slide-A-Lyzer cassettes were from Pierce (Rockford, IL). Ultima Gold liquid scintillation cocktail was obtained from Packard (Meridien, CT). Centricon-30 micro concentrators were obtained from Amicon (Danver, MA). [^{14}C]-Propanol was purchased from American Radionuclear (St. Louis, MO). *n*-Propylxanthate and [^{14}C]-*n*-propylxanthate were synthesized as previously described (specific activity of 0.4 mCi/mmol, radiochemical purity of 80%) (*31*). Purified P450 2B6 was a kind gift from I. Hanna (Vanderbilt University, Nashville, TN).

Purification of P450 and Reductase. P450 2B1 and reductase were purified from microsomes isolated from livers of fasted male Long Evans rats (175−190 g, Harlan Sprague-Dawley, Indianapolis, IN) given 0.1% phenobarbital in their drinking water for 12 days according to the proceedure of Saito and Strobel (*32*) and Strobel and Dignam (*33*), respectively. Alternatively, reductase was purified after expression in *Escherichia coli* as previously described (*34*). P450 2B6 was expressed in *E. coli* MV1304 cells.[2] Briefly, cultures were grown in TB-peptone medium containing 17 mM KH_2PO_4, 72 mM K_2HPO_4, 12 g of tryptone, 24 g of yeast extract, 2 g of peptone, 4 mL of glycerol, and 100 mg/L ampicillin. Expression was induced with 1 mM isopropyl β-*O*-thiogalactopyranoside, and the cultures were grown at 130 rpm in the above medium supplemented with 0.5 mM δ-aminolevulinic acid, 1 mM thiamine, and 1 mg/L chloramphenicol for 3 days at 25 °C. Cultures that exhibited a CO-reduced spectrum were pooled, and the cell pellet was collected by centrifugation. Cells from 4 L were suspended in 75 mM Tris-acetate (pH 8.0), 0.25 M sucrose, 0.25 mM EDTA, and 12 mg of lysozyme in a final volume of 400 mL, and the mixture was rocked on ice for 60 min. Spheroblasts were collected by centrifugation and resuspended in 200 mL of 50 mM Tris-acetate (pH 8.0), 6 mM magnesium acetate, 0.5 M NaCl, 10 mM β-mercaptoethanol, and 20% glycerol. Cells were lysed by sonication, and the membranes were collected by centrifuging the lysate for 1 h at 4 °C at 90000*g*. The membranes were suspended in 200 mL of 50 mM Tris-acetate (pH 8.0), 0.5 M NaCl, 1% Triton N101 (v/v), 0.5% cholate (w/v), 10 mM β-mercaptoethanol, and 40% glycerol (v/v) (solubilization buffer) and solubilized for 5 h at 4 °C. The clarified supernatant was adjusted to 5 mM with imidazole and applied to a Ni-NTA agarose column (Qiagen, Valencia, CA) equilibrated with solubilization buffer containing 5 mM imidazole. The column was washed to remove Triton N101 with Tris-acetate (pH 8.0), 0.25% cholate, 0.7 M NaCl, β-mercaptoethanol, 15 mM imidazole, and 40% glycerol. P450 2B6 was eluted with 100 mM potassium phosphate buffer (pH 7.4) containing 0.7 M NaCl, 0.5 M imidazole, and 40% glycerol. The fractions that contained P450 2B6 were pooled and dialyzed against two 1 L changes of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol and stored at −80 °C.

Enzyme Activity Assays and Inactivation. Purified P450 2B1 and reductase were reconstituted with lipid for 1 h at 4 °C. Incubation mixtures contained 0.5 μM P450 2B1 or 0.67 μM P450 2B6, 1 μM reductase, 200 μg/mL DLPC, 110 units/mL catalase, and nPX or H_2O in 50 mM potassium phosphate buffer (pH 7.4). After equilibration of the reaction mixture at 30 °C for 3 min, the reactions were initiated by adding NADPH to a final concentration of 1.2 mM (primary reaction). The 7-EFC O-deethylation activity was measured spectrofluorometrically as described by Buters et al. (*35*). At the indicated times, duplicate 10 μL samples (5 pmol of P450 2B1) of the primary reaction mixture were removed and mixed with 990 μL of a secondary reaction mixture containing 0.2 mM NADPH, 100 μM 7-EFC, and 40 μg/mL BSA in 50 mM potassium phosphate buffer (pH 7.4), and incubated at 30 °C for 5 min. For P450 2B6, duplicate 12 μL samples (8 pmol of P450 2B6) of the primary reaction mixture were mixed with 988 μL of the secondary reaction mixture and incubated for 10 min at 30 °C. Enzyme activity was stopped by adding ice-cold acetonitrile to a final concentration of 25%. The fluorescence of the samples was measured directly at room temperature on an SLM-Aminco model SPF-500 C spectrofluorometer with excitation at 410 nm and emission at 510 nm. Enzyme activity was calculated from a standard curve using HFC.

Substrate Protection. Substrate protection from nPX-dependent inactivation of P450 2B1 was assayed by including 25 μM nPX together with 7-EFC at molar ratios of 1:0, 1:1, and 1:2 in the primary reaction mixture. At the indicated times, duplicate 10 μL aliquots were removed and assayed for remaining activity as described above.

Partition Ratio. To estimate the partition coefficient, samples were incubated in the presence of 0.01−1 mM nPX for 20 min to ensure the assay had proceeded to completion. Duplicate aliquots were removed and assayed for 7-EFC activity as described above.

Irreversibility of Inactivation of P450 2B1 by nPX. Cytochrome P450 2B1 (0.5 nmol) was reconstituted and inactivated with 1.4 mM nPX in a total volume of 138 μL as described above. Control samples were incubated with nPX but without NADPH. After 10 min at 30 °C, the samples (0.13 mL) were dialyzed overnight at 4 °C against 2 × 500 mL of 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. The dialyzed samples were reconstituted with 10 μg of lipid for 30 min on ice. Some samples also received fresh reductase. Enzymatic activity was assayed with 7-EFC as described above.

Stoichiometry and Specificity of Binding. The stoichiometry of binding was determined by extensively dialyzing 500 μL samples containing 2 nmol of P450 2B1 reconstituted with reductase and lipid as described above that had been incubated with 0.4 mM radiolabeled [^{14}C]nPX, with or without NADPH for 10 min at 30 °C. Aliquots were removed to assess the extent of inactivation of P450 2B1 based on the residual 7-EFC O-deethylation activity and to determine the amount of heme loss by reduced CO difference spectroscopy prior to dialysis (*36*). Samples were dialyzed in Slide-A-Lyzer cassettes against 4 × 500 mL of 50 mM potassium phosphate (pH 7.4) containing 20% glycerol, 10 mM sodium cholate, and 0.1 mM EDTA. Aliquots were removed, and the amount of radioactivity remaining after dialysis was measured by liquid scintillation counting. Cytochrome P450 2B1 recovery was determined spectrophotometrically by measuring the reduced CO difference spectra, and protein recovery was quantified using the BCA reagent (*37*). The stoichiometry of binding was calculated after subtracting

[1] Abbreviations: reductase, NADPH−cytochrome P450 reductase; DLPC, dilauroyl-L-α-phosphatidylcholine; nPX, *n*-propylxanthate; BSA, bovine serum albumin; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; AHH-1, AHH-1 human lymphoblastoid cell line.

[2] I. Hanna et al., manuscript in preparation.

Table 1. Effect of nPX on the 7-EFC O-Deethylation Activity of Purified P450 2B1 and Cytochrome P450 Content[a]

	% activity remaining		% P450 remaining	
primary reaction	0 min	20 min	0 min	20 min
P450 2B1				
− nPX + NADPH	100	82 ± 4	100	88 ± 6
+ nPX − NADPH	92 ± 1	80 ± 6		88 ± 2
+ nPX + NADPH	91 ± 1	24 ± 2		75 ± 2
	% activity remaining		% P450 remaining	
primary reaction	0 min	30 min	0 min	30 min
P450 2B6				
− nPX + NADPH	100	94 ± 2	100	100
+ nPX − NADPH	103 ± 2	102 ± 2		102 ± 2
+ nPX + NADPH		17 ± 1		88 ± 1

[a] Assay conditions were as described in Experimental Procedures. The values shown represent the averages from two separate experiments.

the background counts from dialyzed samples incubated with nPX in the absence of NADPH.

Spectrophotometric Quantitation of P450 2B1 and P450 2B6. At the times indicated, 200 μL aliquots of the primary reaction incubation were removed and diluted with 800 μL of ice-cold 50 mM potassium phosphate (pH 7.4), containing 40% glycerol and 0.6% Tergitol NP-40. The sample was gently bubbled with CO for 90 s, and the spectrum was recorded from 400 to 500 nm on a DW2 UV/Vis spectrophotometer (SLM Aminco, Urbana, IL) equipped with an OLIS spectroscopy operating system (On-Line Instrument Systems, Inc., Bogart, GA). Dithionite was added, and the reduced carbonyl spectrum was recorded (*36*). For absolute spectral determinations, P450 2B1 and reductase were reconstituted with lipid at a 1:1 ratio. The final concentrations were 1 μM P450 2B1, 1 μM reductase, 200 μg/mL DLPC, and 110 units/mL catalase in 50 mM potassium phosphate (pH 7.4). The reference contained catalase and lipid in 50 mM potassium phosphate (pH 7.4). Spectra were recorded by scanning from 375 to 500 nm.

Iodosobenzene-Supported Activity. P450 2B1 and reductase were reconstituted as described for the 7-EFC activity assays above and incubated with 50 μM nPX and 0.5 mM NADPH for 10 min at 30 °C. Aliquots were removed and diluted into 990 μL of a secondary reaction mixture containing 100 μM 7-EFC and 50 mM potassium phosphate (pH 7.4). Either NADPH at a final concentration of 0.2 mM or iodosobenzene at a final concentration of 0.8 mM was added, and the fluorescence emission at 510 nm was recorded over the course of 99 s. The initial linear slope between 0 and 10 s was used to calculate the percent activity remaining of the nPX-inactivated samples as compared to control samples incubated with vehicle alone.

Results

Inactivation of P450 2B1 by nPX. After incubation of reconstituted P450 2B1 with 50 μM nPX and NADPH for 20 min, only 24% of the enzyme's 7-EFC O-deethylation activity remained (Table 1). However, these samples retained most of their ability to form a reduced CO complex. Control incubations without nPX or with nPX but without NADPH did not lead to a substantial loss in either enzymatic activity or the ability of P450 2B1 to bind CO. Similarly, 83% of the absolute absorbance spectrum at 417 nm was retained after incubating for 20 min with nPX and NADPH (data not shown). When P450 2B6 was incubated with 50 μM nPX and NADPH for 30 min, an 83% loss in the 7-EFC O-deethylation activity was observed. With the same samples, 88% of the reduced CO complex was formed compared to control samples incubated without nPX. Samples that were incubated either without nPX or without NADPH did not show reduced activity or diminished CO binding spectra.

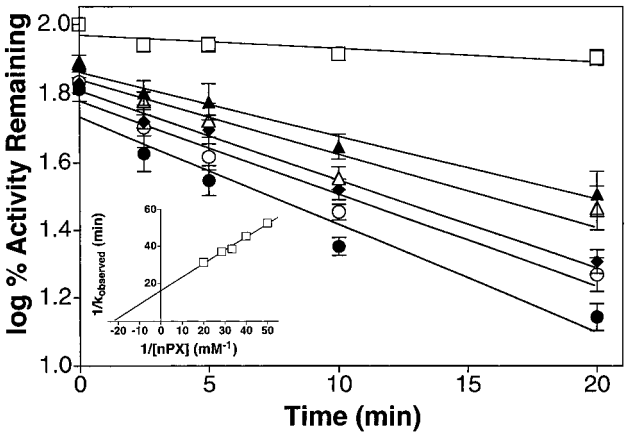

Figure 1. Time- and concentration-dependent inactivation of the P450 2B1 7-EFC O-deethylation activity by nPX. Aliquots were removed from the primary reaction mixture and assayed for residual activity as described in Experimental Procedures. Each point shown represents the mean and standard deviation from three separate experiments. For some points, the standard deviation was smaller than the size of the symbol. The concentrations of nPX were (□) 0, (▲) 20, (△) 25, (○) 30, (◆) 35, and (●) 50 μM. The inset shows the double-reciprocal plot of the rates of inactivation as a function of nPX concentration.

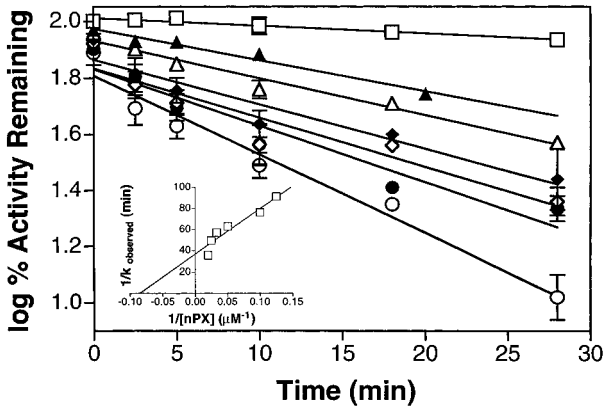

Figure 2. Time- and concentration-dependent inactivation of the P450 2B6 7-EFC O-deethylation activity by nPX. Aliquots were removed from the primary reaction mixture and assayed for residual activity as described in Experimental Procedures. The data shown represent the mean and standard deviation from duplicate samples of three separate experiments. For some points, the standard deviation was smaller than the size of the symbol. The concentrations of nPX were (□) 0, (▲) 8, (△) 10, (◆) 20, (◇) 30, (●) 40, and (○) 50 μM. The inset shows the double-reciprocal plot of the rates of inactivation as a function of nPX concentration.

The inactivation of P450 2B1 by nPX was time- and concentration-dependent (Figure 1). Pseudo-first-order inactivation kinetics were observed at 30 °C with nPX concentrations ranging from 20 to 50 μM. The kinetic constants describing the inactivation of P450 2B1 with nPX were determined from the inset of Figure 1. The maximal rate of inactivation at saturation ($k_{inactivation}$) was 0.2 min^{-1}; the concentration required for half-maximal inactivation (K_I) was 44 μM, and the time required for half of the enzyme to become inactivated was 4.1 min. No effect on the rates of inactivation was observed when 10 mM GSH or 1 mM DTT was added to the primary incubation mixtures (data not shown).

Figure 2 shows that the inactivation of P450 2B6 by nPX was also time- and concentration-dependent (Figure

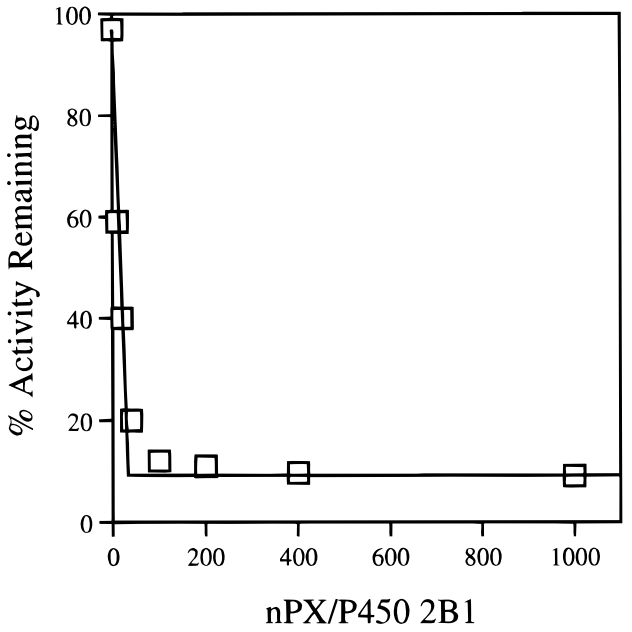

Figure 3. Loss of P450 2B1 activity as a function of the ratio of nPX to 2B1. P450 2B1 was incubated with different concentrations of nPX as described in Experimental Procedures. The extrapolated partition ratio was determined from the intercept of the linear regression line from the lower ratios and the straight line obtained from the higher ratios.

Table 2. Irreversibility of P450 2B1 Inactivation by nPX[a]

	% activity remaining	
sample	control	inactivated
before dialysis	100	10 ± 2
after dialysis	100	11 ± 1
after dialysis with fresh reductase	100	14 ± 1

[a] Assay conditions were as described in Experimental Procedures. The data shown represent the average of two separate experiments.

2). The kinetic constants were determined from the inset of Figure 2. The K_I was 12 μM; the $k_{inactivation}$ was 0.06 min^{-1}, and the $t_{1/2}$ was 11 min.

Partition Ratio. The number of molecules of nPX metabolized per molecule of 2B1 inactivated (partition ratio) was estimated from Figure 3. P450 2B1 was incubated with different concentrations of nPX, and the inactivation reaction was allowed to go to completion. The percent activity remaining was plotted as a function of the molar ratio of nPX to 2B1. The turnover number (partition ratio + 1) was extrapolated from the intercept between the linear regression line obtained from the lower nPX to 2B1 ratios with the straight line derived from the higher nPX to P450 2B1 ratios (*30*). With this method, the partition ratio was found to be 32.

Irreversibility of 2B1 Inactivation by nPX. Control samples or samples where P450 2B1 was inactivated with nPX were dialyzed extensively and tested for activity. Table 2 shows that the removal of free nPX by dialysis did not lead to a recovery of the 7-EFC activity of nPX-inactivated samples. No additional activity was regained when fresh reductase was added to the dialyzed samples.

Substrate Protection. Incubations of P450 2B1 with nPX together with an alternate substrate in the primary reaction mixture slowed the rate at which nPX inactivated P450 2B1 (Figure 4). Virtually no inactivation was seen when the alternate substrate concentration was twice that of nPX.

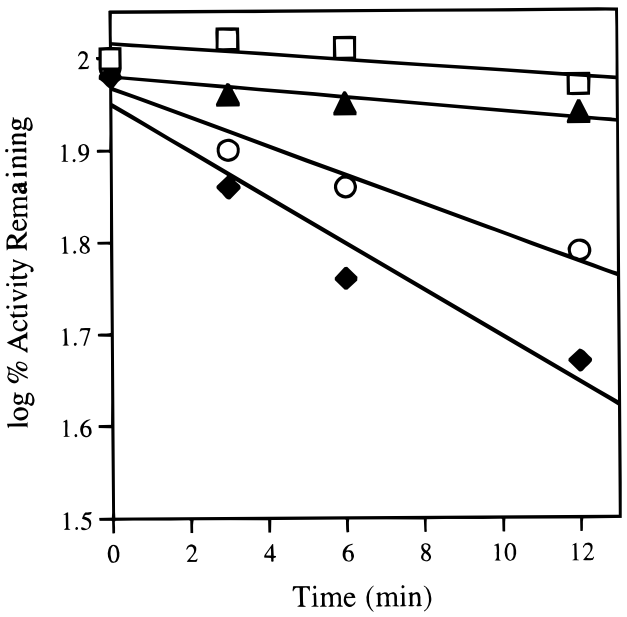

Figure 4. Substrate protection against P450 2B1 inactivation by nPX. Samples were removed from the primary incubation mixture at the indicated time points and assayed for remaining activity as described in Experimental Procedures. The data shown are representative of two separate experiments. The primary reaction mixtures contained molar rations of nPX to 7-EFC of (□) 0:0, (◆) 1:0, (○) 1:1, and (▲) 1:2.

Table 3. Iodosobenzene-Supported Activity of nPX-Inactivated P450 2B1[a]

	% activity	
compd supporting activity	control	inactivated
NADPH	100	7.8 ± 0.2
iodosobenzene	105 ± 15	59 ± 10

[a] Assay conditions were as described in Experimental Procedures. The data shown represent the average of two separate experiments.

Stoichiometry of Labeling. The stoichiometry of binding of a radiolabeled nPX metabolite to P250 2B1 was determined. Control samples incubated only with [^{14}C]nPX and inactivated samples incubated with [^{14}C]-nPX and NADPH were dialyzed extensively until no more counts were detected in the dialysis buffer. Sample aliquots were assayed for P450 recovery by reduced CO spectroscopy and for radioactivity by liquid scintillation counting. Background counts from control samples incubated without NADPH (344 and 471 dpm/nmol of P450 2B1) were subtracted from the counts obtained for the inactivated samples (1124 and 1353 dpm/nmol of P450 2B1). Determinations from two separate experiments resulted in an average binding stoichiometry of 1.2 mol of metabolite bound per mole of inactivated P450 2B1.

Iodosobenzene-Supported Activity. The alternate oxidant iodosobenzene was used to test if substrate binding by the nPX-treated P450 2B1 samples was compromised. In all cases, the rates of 7-EFC metabolism supported by iodosobenzene were linear only for the first 10−15 s. The rates observed during the first 10 s were used to calculate the data shown in Table 3. When NADPH was used to support the 7-EFC metabolism, only 7% of the O-deethylation activity in samples that had been inactivated with nPX was seen compared to control samples. When iodosobenzene was used as the alternate oxidant, 59% of the control O-deethylation activity was observed.

Discussion

This report describes the mechanism-based inactivation of rat P450 2B1 and human P450 2B6 by *n*-propylxanthate. nPX was chosen for these studies because it is structurally distinct from the mechanism-based inactivators that have previously been used to label the active site of P450 2B1 (*10*−*12*). While both isoforms were inactivated with nPX, little effect on either the P450 2B1 or P450 2B6 reduced CO spectrum was observed. The data in Table 1 suggested that the inactivation by nPX was not due to the destruction of the heme moiety but rather to a modification of the apoprotein. Additional evidence that the heme moiety was not affected came from analysis of butanone extracts of P450 2B1 that was inactivated with ^{14}C-labeled nPX. No counts were observed in the heme-containing fraction of these samples (data not shown). In addition, our data show that inactivation by nPX required a catalytic step since both NADPH and nPX were necessary to inactivate P450 2B1 and P450 2B6.

Pseudo-first-order kinetics for inactivation were observed with both enzymes. Although the two P450s share approximately 76% sequence similarity (*38*), a 4-fold difference between the K_I of P450 2B1 (44 μM) and that of P450 2B6 (12 μM) for nPX was observed. The rate of inactivation for P450 2B6 was slower (0.06 min^{-1}) when compared to the $k_{inactivation}$ for P450 2B1 (0.2 min^{-1}). Similar differences between the two enzymes in the K_I and $k_{inactivation}$ were seen when AHH-1 microsomes expressing P450 2B6 were compared to P450 2B1[3] and when the kinetics of inactivation by 2-phenyl-2-piperidinopropane of the purified P450 2B1 and P450 2B6 were compared.[4]

P450 2B1 samples that were incubated with nPX or inactivated with nPX and NADPH were dialyzed extensively to determine if removal of free inactivator would restore enzymatic activity, or if the inactivation was reversible. No regain of enzymatic activity of the inactivated sample compared to the control sample was observed after dialysis. These results suggest that under these conditions the inactivation was irreversible. The inactivation appeared to be due to a modification of the P450 and not the reductase since adding fresh reductase back to the dialyzed samples also did not restore enzymatic activity to the nPX-inactivated P450 2B1. In addition, no loss in enzymatic activity was observed when P450 2B1 was incubated with nPX together with a 2-fold molar excess of an alternate substrate. This observation suggested that nPX inactivated P450 2B1 and that the inactivation was due to binding of a nPX reactive intermediate to the P450 active site. The addition of exogenous nucleophiles such as glutathione (1 or 10 mM) or dithiothreitol (1 mM) to the reaction mixture had no effect on the rates of inactivation (data not shown). These observations further indicate that the inactivation of P450 2B1 by nPX was due to the binding of a nPX reactive intermediate at the active site and not because the intermediate diffused out of the active site and bound elsewhere on the P450 molecule or the reductase. In addition, the 1.2:1 stoichiometry of radiolabeled nPX metabolite to P450 2B1 would also suggest that the binding occurred at the active site and that approximately one molecule of inactivator was bound per molecule of P450 2B1.

Significant enzymatic activity could be restored to nPX-inactivated samples when iodosobenzene was used as an alternate oxidant in place of NADPH and O_2. This observation suggests that the nPX reactive intermediate bound to an amino acid residue(s) critical for a reductase-dependent step. Alternatively, the modification may have altered the binding or dissociation of the substrate and favored oxidation with iodosobenzene. A third possibility is that the nPX modification disrupted a proton transfer step required to generate the ferryl intermediate. Experiments designed to isolate the nPX-labeled peptide and to identify the modified amino acid(s) are in progress.

The reactive intermediate responsible for inactivating P450 2B1 and P450 2B6 has not been identified. Earlier reports from in vivo experiments where rats were injected with radiolabeled xanthates showed that the corresponding alkanes and alkenes appeared in the animal's expired air (*27*). These observations led the investigators to propose a possible reaction mechanism that involved an attack at the α-carbon to generate an alkane radical that, in the presence of H^+ or OH^-, could be converted to the alkane or alkene, respectively (*27*). With the purified enzyme, no corresponding alkane or alkene could be detected in the headspace by gas chromatography (data not shown). However, propionaldehyde was able to inactivate P450 2B1 at millimolar concentrations but could not compete for labeling of the enzyme by nPX. When nPX that was ^{3}H-labeled at the α-carbon was used to inactivate P450 2B1, all the counts were released into the buffer (data not shown). When nPX labeled with ^{14}C at the α-carbon was used, the counts remained with the P450 protein (data not shown). Taken together, these observations suggest that the initial site of oxidation by P450 2B1 is the α-carbon of nPX and that the inactivating species could be a hydroxylated propyl radical. Experiments designed to test this hypothesis are in progress.

In summary, this report demonstrates that nPX is an effective mechanism-based inactivator for both rat P450 2B1 and human P450 2B6. The data indicate that the reactive intermediate generated during the metabolism of nPX bound to the P450 2B1 active site. Experiments designed to identify the amino acid residue(s) involved in the inactivation are in progress.

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