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METABOLISM OF DIPHENYLOXAZOLE (PPO) BY MOUSE LIVER MICROSOMES

Elroy T. Cantrell, Mary Abreu-Greenberg, Jerry Guyden, and David L. Busbee

Departments of Basic Health Sciences and Biology
North Texas State University
Denton, Texas 76203

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Abstract

2,5-Diphenyloxazole (PPO) is an inducer and inhibitor of aryl hydrocarbon hydroxylase. We report that PPO is itself metabolized to an alkali-extractable metabolite with intense fluorescence. The fluorescence spectra of excitation and emission indicate peaks at 345 nm and 510 nm, respectively. The reaction is linear with respect to time and enzyme concentration. NADPH is required for activity and the reaction is inhibited by carbon monoxide and 7,8-benzoflavone but not by SKF-525A or hexobarbital. The intensity of fluorescence produced is similar to that of benzo(a)pyrene. PPO may be a useful model compound in studies of drug metabolism by the mixed function oxidase.

The observations (1-3) that diphenyloxazole (PPO) is both an effective inducer and inhibitor of aryl hydrocarbon hydroxylase led us to study this compound for possible metabolism by the microsomal enzyme complex. The present report shows that PPO is metabolized by the cytochrome P_1-450 enzyme complex and that the principal metabolite of PPO formed by mouse microsomes can be detected by a method similar to that used by Nebert and Gelboin (4) for 3-hydroxybenzo(a)pyrene.

Methods

Materials: Benzo(a)pyrene (BP), 1,2-benzanthracene(BA), hexobarbital and NADPH were purchased from Sigma Chemical Co.; 2,5-diphenyloxazole was purchased from Fisher Chemical Co.; and 7,8-benzoflavone was obtained from Aldrich. SKF-525A was a gift from Smith, Kline, and French. Phenobarbital was obtained locally. Male C57BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Procedures: Groups of five mice were treated with BA, PPO, or PB (as described in the figure legends) prior to sacrifice by cervical dislocation. Hepatic microsomes were prepared from a 10% liver homogenate in TMS buffer (50 mM Tris, pH 7.5, containing 3 mM $MgCl_2$ and 200 mM sucrose). Microsomes

were also prepared from five control mice. The microsomes were diluted in TMS buffer to a protein concentration of approximately 0.5 mg/ml. Dilutions were made from these stocks.

Metabolism of BP and PPO was determined as follows. Duplicate 1.0 ml aliquots of microsomes were incubated with shaking at 37° with substrate in the presence of 1.0 mg of NADPH under conditions indicated for the various experiments. The enzyme reaction was stopped by the addition of 3.0 ml of 10% acetone in hexane, and the tubes were vortex-mixed for 20 seconds. After centrifuging to separate the phases, the upper, organic phase was transferred to 1.0 ml of 1N NaOH in a 13 x 100 mm culture tube, vortex-mixed for 15 seconds, and centrifuged to separate the phases. The fluorescence of the lower aqueous phase was determined in an Aminco-Bowman spectrophotofluorometer. The wavelength maxima for excitation and emission of 3-hydroxybenzo(a)pyrene (BPOH) were 396 nm and 522 nm, respectively. The maxima for the PPO metabolite were 345 nm and 510 nm.

Results and Discussion

The excitation and emission spectra of the metabolite of PPO is shown in Figure 1a. Under the conditions of the study, the peak of excitation in 1N NaOH was at 345 nm, and the peak of emission was at 510 nm. A shoulder may be seen on the emission peak at approximately 420 nm. After neutralizing the NaOH with an equal volume of 1N HCl, the fluorescence peaks were shifted to 330 nm for excitation and 400 nm for emission (Figure 1b). In hexane, the peak of excitation was further shifted to 312 nm, and a double emission peak appeared at 355 nm and 365 nm.

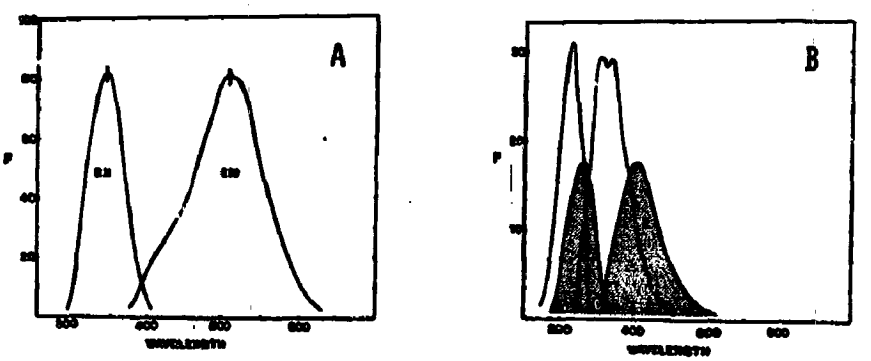

FIG. 1
Excitation and emission spectra of PPO metabolite. (a) An aliquot of the metabolite was extracted as described in Methods into 1.0 ml of 1N NaOH and the excitation spectrum recorded with the emission wavelength set at the emission peak. The emission spectrum was recorded with the excitation wavelength set at the excitation peak. (b) An equal volume of 1N HCl was added to the above cuvette and the excitation-emission spectra determined as indicated by the shaded peaks. Subsequent extraction into hexane produced spectra indicated by the unshaded areas.

The enzyme reaction was linear with time for at least 15 minutes, and with respect to enzyme protein concentration over at least two orders of magnitude (5-500 µg/ml). The reaction required NADPH (Table I) and was inhibited by carbon monoxide and by 7,8-benzoflavone (alpha-naphthoflavone), both of which are inhibitors of the microsomal mixed function oxidases. The reaction was not inhibited by SKF-525A or hexobarbital.

TABLE I

Effect of Cofactors and Inhibitors on PPO Metabolism by BA-Induced Microsomes

Sample	Fluorescence (345-510 nm)
Control, 0 mins	1.4
Complete Mixture[a]	184.
less microsomes	1.9
less NADPH	0.9
less PPO	1.2
plus 80% CO	15.
plus 7,8-benzoflavone	107.
plus hexobarbital	195.
plus SKF-525A	185.

[a]The complete reaction mixture was incubated 15 mins at 37° and contained 0.8 mg microsomal protein, 1 mg NADPH and 10 µg PPO in 1.1 ml TMS buffer, pH 7.5. Concentrations of each inhibitor were: 7,8-benzoflavone 12.5 µg/ml, hexobarbital 12 µg/ml and SKF-525A 15 µg/ml.

Benzo(a)pyrene hydroxylase is also called aryl hydrocarbon hydroxylase (AHH) because this enzyme complex can metabolize a variety of aromatic hydrocarbons, mainly by aromatic hydroxylation. In view of the findings that 7,8-benzoflavone and carbon monoxide were effective inhibitors of PPO metabolism but hexobarbital and SKF-525A did not inhibit PPO metabolite formation, and that the enzyme reaction was NADPH dependent, we concluded that this reaction is mediated by the cytochrome P_1-450 (P-448) dependent mixed function oxidase (AHH). That the reaction is an aromatic hydroxylation is also supported by the observation of a red shift in the fluorescence spectra at high pH, as is the case with 3-hydroxybenzo(a)pyrene.

The Michaelis constant (Km) for metabolism of PPO was similar to that for metabolism of BP in microsomes obtained from untreated mice (Table II). The observation that BA-treated microsomes had less affinity for PPO than for BP may reflect differences in characteristics of the induced P_1-450 or may be related to experimental variability (5).

Without a standard for reference, the PPO metabolite can only be quantitated as fluorescence produced; therefore, a comparison of "V_{max}" for metabolism of PPO and BP is expressed in relative fluorescence units (F). We otherwise express a unit of AHH as fluorescence equivalent to a picomole of 3-hydroxybenzo(a)pyrene per minute and one picomole per ml produces one fluorescence unit.

TABLE II

Kinetics of BP and PPO Metabolism by Hepatic Microsomes from Control and BA-Induced Mice

		Km (μM)	V_{max} (F/min)
Control	BP	3.8	27
Control	PPO	3.8	16
BA-induced	BP	4.0	44
BA-induced	PPO	6.9	30

One ml aliquots of microsomes containing 95 μg protein were incubated for 15 mins with 1.0 mg NADPH in 1.0 ml of TMS buffer. Varying concentrations of substrate were added at zero time to start the reaction.

PPO was previously reported to be an inducer of AHH in fetal hamster cells (1), mouse prostate cells (2), and rat derived hepatoma and hepatocyte cell lines (3). We have found that AHH is also induced by PPO in cultured human lymphocytes (6). In contrast to the induction of AHH in culture, PPO pretreatment of mice resulted in depressed levels of BP and PPO metabolism by hepatic microsomes (Figure 2). BA and PB were effective inducers of BP and PPO metabolism by mouse microsomes. Cytochromes P_1-450 and P-450 were induced by BA and PB, respectively. No change in levels of cytochromes was seen after PPO treatment.

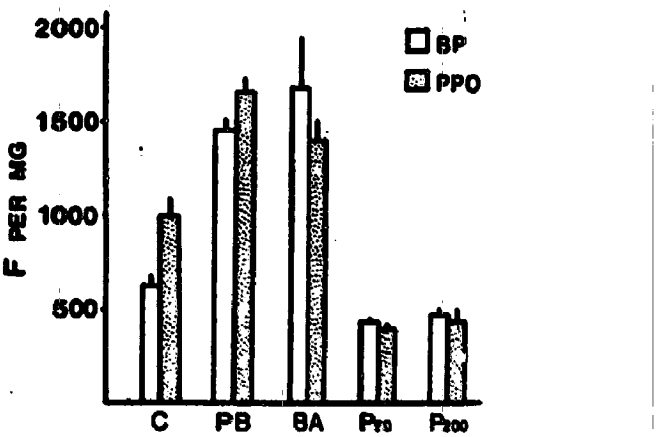

FIG. 2

Effects of inducers and PPO on metabolism of BP and PPO by mouse hepatic microsomes. Groups of five C57BL/6J mice were injected intraperitoneally on four consecutive days prior to sacrifice. Doses were as follows: PB 80 mg/kg, BA 50 mg/kg, PPO 50 mg/kg, and PPO 200 mg/kg. Duplicate 1.0 ml aliquots of microsomes containing approximately 100 μg protein were incubated 15 mins with either BP 10 μg or PPO 10 μg. The vertical lines depict the S.E.M.

Although PPO was not an inducer of AHH *in vivo* it serves as a substrate for the enzyme complex. The affinity for the binding sites was similar to that of BP in control microsomes. but less than that of BP in BA-induced microsomes. The fluorescence intensity of the metabolite of PPO is nearly that of the BP metabolite, 3-hydroxybenzo(a)pyrene. The low toxicity of PPO (7) and its possible lack of carcinogenicity may provide some indication for PPO in studies of chemical carcinogenesis and drug metabolism in model systems.

Acknowledgments

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