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Induction of P450 Monooxygenases in the German Cockroach, *Blattella germanica* L.

Danielle Brown, Li Zhang, Zhimou Wen, and Jeffrey G. Scott*

The cytochrome P450 monooxygenases are an important metabolic system whose level of activity can be influenced by several dietary constituents. We examined the effects of six known P450 inducers on the levels of total cytochromes P450, cytochrome b_5, and six monooxygenase activities in adult German cockroaches. In addition, the levels of CYP6L1 and CYP9E2 mRNA were also investigated. Phenobarbital treatment resulted in increases in total cytochromes P450 and metabolism of three resorufin analogues, but not CYP6L1 nor CYP9E2 mRNA. There was no significant effect of the other five inducers on any of the monooxygenase parameters we measured. In comparison with other insects, the German cockroach seems unusually refractory to most inducing agents. Arch. Insect Biochem. Physiol. 53:119–124, 2003.

KEYWORDS: phenobarbital; P450 induction; monooxygenase activity

INTRODUCTION

The cytochrome P450 monooxygenases are an important metabolic system involved in the detoxification/activation of xenobiotics (as well as in the anabolism and catabolism of endogenous compounds including hormones and pheromones). P450 monooxygenases metabolize a large number of substrates because of the presence of numerous P450s in each species (~90 P450s in *Drosophila melanogaster*, Adams et al., 2000; Tijet et al., 2001; and ~111 in *Anopheles gambiae*, Ranson et al., 2002) and the broad substrate specificity of some P450s (Rendic and Di Carlo, 1997). Expression of P450s can often be influenced by diet with several well-known P450 inducers that have been described (Lewis, 1996).

Among the approximately 30 species of cockroaches that are considered pests, the German cockroach, *Blattella germanica* is probably the most important (Ross and Cochran, 1975). Infestations are frequently associated with human sensitization to cockroach allergens and the development of allergic respiratory diseases, especially bronchial asthma (Arlian, 2002; Brenner, 1995). The P450s of German cockroaches have been examined in numerous studies on insecticide metabolism and have been characterized between different life stages (Valles et al., 1994) and strains (Scharf et al., 1998a,b). Although DDT, dieldrin (Kahn and Matsumura, 1972), pentamethylbenzene (Scharf et al., 1998b), and phenobarbital (Valles et al., 1994) have been examined as monooxygense inducers in German cockroaches, there has been no examination of the comparative effects of commonly known inducers in this species. Recently, CYP6L1 and CYP9E2 from German cockroach were cloned and sequenced. CYP6L1 was shown to be specifically expressed in the reproductive tissues of adult males (Wen and Scott, 2001) while CYP9E2 expres-

Department of Entomology, Cornell University, Ithaca, New York

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*Correspondence to: Dr. Jeffrey G. Scott, Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853-0901.
E-mail: JGS5@CORNELL.EDU

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sion was not limited to a specific sex, tissue, or life stage (Wen et al., 2001). However, the effect of inducers on expression of these individual P450s has not been investigated.

Herein, we examined the effect of six different P450 inducers on the German cockroach. We examined the levels of total P450s, cytochrome b_5, and six monooxygenase activities. The levels of CYP6L1 and CYP9E2 mRNA were also examined to determine if their expression could be correlated with any of the monooxygenase activities.

MATERIALS AND METHODS

Chemicals

Methoxyresorufin, acetone, ethanol, ethoxyresorufin, resorufin, benzyloxyresorufin, phenobarbital, 3-methylcholanthrene, and beta-naphthoflavone were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). Pentamethylbenzene and 7-ethoxycoumarin were obtained from Aldrich Chemical Company (Milwaukee, WI). Dr. C.F. Wilkinson provided the 7-methoxycoumarin, 7-propoxycoumarin, and 7-hydroxycoumarin.

Induction

Adult male CSMA (insecticide susceptible strain) cockroaches were isolated and held without food or water for 24 h (to maximize their subsequent intake of inducers). Each set of 25–30 cockroaches was treated with a single inducer at approximately the maximum sublethal concentration. Phenobarbital (PB), ethanol (EtOH), and acetone (AC) were added to 50 ml of distilled water to a final concentration of 0.5, 30, and 10%, respectively. These solutions were placed in 50 ml Erhlenmeyer flasks and a dental wick was added to allow access to the solution. Pentamethylbenzene (PMB), beta-naphthoflavone (BNF), and 3-methylcholanthrene (3MC) were added to 0.5 g finely powdered Purina dog food to a final concentration of 10, 10, and 5% respectively. Treated food was paired with 50 ml untreated distilled water and treated water was paired with 0.5 g untreated powdered dog food. Controls were fed 50 ml untreated distilled water and 0.5 g untreated powdered dog food. Cockroaches were fed the inducers ad libitum for 5 days (at 27°C) and then used for prepartion of microsomes. There was <10% mortality for all treatments and controls

Preparation of Microsomes

Cockroach abdomens (25–30) from each treatment group were homogenized in 10 ml of homogenization medium (0.1 M sodium phosphate buffer pH 7.5, containing 10% glycerol, 1.0 mM EDTA, 0.1 mM DTT, 1.0 mM PMSF, and 1.0 mM PTU; Lee and Scott, 1989) for 1 min with a motor-strator type biohomogenizer and then filtered through two layers of cheesecloth. The samples were then centrifuged at 10,000*g* at 4°C for 20 min with a Beckman model J21C centrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was filtered through two layers of cheesecloth and then centrifuged at 100,000*g* at 4°C for 1 h in a Beckman L8-70M ultracentrifuge (Beckman Instruments). The pellet was removed and suspended in 0.75 ml resuspension buffer (0.1M sodium phosphate buffer, pH 7.5, 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 1.0 mM PMSF; Lee and Scott, 1989) for storage at –80°C.

Microsomal Assays

Total protein concentration was determined using the method of Bradford (1976) with bovine serum albumin as the standard. Total cytochromes P450 and cytochrome b_5 were determined using the method of Omura and Sato (1964) using a Beckman DU-640 spectrophotometer (Beckman Instruments).

O-dealkylation of methoxyresorufin (MROD), ethoxyresorufin (EROD), and benzyloxyresorufin (BROD) was measured using a CytoFluor® Series 4000 fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham MA) with excitation filter 530/25 and emission filter 580/40 at 32°C. Microsomal protein (0.1 mg) in 200 µl of resuspension buffer and 1,796 µl of reaction buffer (0.1 M potassium phosphate buffer, 0.1 mM EDTA,

5 mM $MgCl_2$, pH 7.8, warmed to 32°C) and 4 µl of 1 mM substrate (methoxy, ethoxy, or benzyloxyresorufin) were mixed together. Aliquots (200 µl) of each sample were loaded into six wells. Either 10 µl of NADPH (1.0 mM) or buffer (control) were added to three wells each. A standard curve was prepared by incremental addition of resorufin to 200 µl of water (0 to 150 pmol resorufin per well). The reaction rates were monitored continuously for 10 min. *O*-dealkylation of methoxycoumarin (MCOD), ethoxycoumarin (ECOD), and propoxycoumarin (PrCOD) were measured using the same methods except that the standard curve was made using hydroxycoumarin (0–0.5 µM), the reaction buffer (pH 7.8) consisted of 50 mM Tris-Base, 150 mM potassium chloride, 1 mM EDTA, and the excitation and emission filters were 360/40 and 460/40 nm, respectively. All experiments were replicated a minimum of three times. Results were analyzed using a paired 2-tailed *t*-test.

RNA Extraction

Total RNA was extracted using standard methods (Sambrook et al., 1989) with some modifications. Briefly, the abdomen of a male German cockroach was amputated, homogenized on ice in 0.4 ml of cold guanidine thiocyanate buffer (4 M, pH 7.0, containing 25 mM sodium citrate, 0.5% N-lauroyl-sarcosine and 0.1 M beta-mercaptoethanol), then incubated with 0.5 ml phenol-chloroform (1:1) at 65°C for 15 min with occasional inversion. The mixture was put on ice for 10 min and then centrifuged for 10 min. The supernatant was removed, mixed with 0.4 ml of 4.0 M lithium chloride, chilled at –70°C for 30 min and centrifuged for 20 min (15,000*g*) at 4°C. The pellet was dissolved in 0.2 ml of TENS buffer (0.1 M Tris buffer, pH 7.4, 50 mM sodium chloride, 0.1 M EDTA, 0.2% SDS) containing 2 µl proteinase K (>400 U/µl) (Gibco/BRL), incubated at 37°C for 0.5–1.5 h and extracted twice with an equal volume of phenol-chloroform. RNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of absolute ethanol. The pellet was dissolved in water and stored at –70°C for further use.

Northern Blotting

Northern blot analysis of CYP6L1 and CYP9E2 mRNA expression was performed as described by Sambrook et al. (1989). Briefly, 10 µg of total RNA was fractionated on 1% denaturing formaldehyde agarose gel. After washing in distilled water for about 3–4 h with several changes, the RNA was transferred to a GeneScreen Plus® hybridization transfer membrane (NEN™ Life Science Products, Inc.). Equal loading was monitored by comparing the density of the 18S ribosomal RNA (rRNA) band (Savonet et al., 1997; Spiess and Ivell, 1998) on the membrane after transfer. CYP6L1 and CYP9E2 cDNA probes were amplified by PCR as described previously (Wen et al., 2001; Wen and Scott, 2001), labeled with [α-^{32}P] dCTP using the RadPrime labeling system (GIBCO/BRL), and used individually as the hybridization probes. The membrane was hybridized to the probe in QuickHyb solution (Stratagene) at 68°C. Washing was done at high stringency (i.e., two 15 min washes with 2 × SSC + 0.1% SDS at room temperature, followed by a 30 min wash with 0.2 × SSC + 0.1% SDS at 60°C). The membrane was air dried and the samples were quantified using a phosphorimager (Molecular Dynamics). All Northern analyses were repeated at least three times using independent preparations of RNA.

RESULTS AND DISCUSSION

The results of treating adult male cockroaches with six inducers on total P450, cytochrome b_5, and three monooxygenase activities are shown in Figure 1. Of the six inducers tested, only PB produced consistent induction (Fig. 1). PB treatment resulted in significant induction of total P450 content (4-fold), MROD (4-fold), EROD (14-fold), and BROD (4-fold). PB did not significantly change the levels of cytochrome b_5 (Fig. 1). These results are in general agreement with those of Valles and Yu (1996) who found PB induced MROD and EROD activities, although they reared adults with constant exposure to PB, which makes exact comparisons between our studies impossible. There was

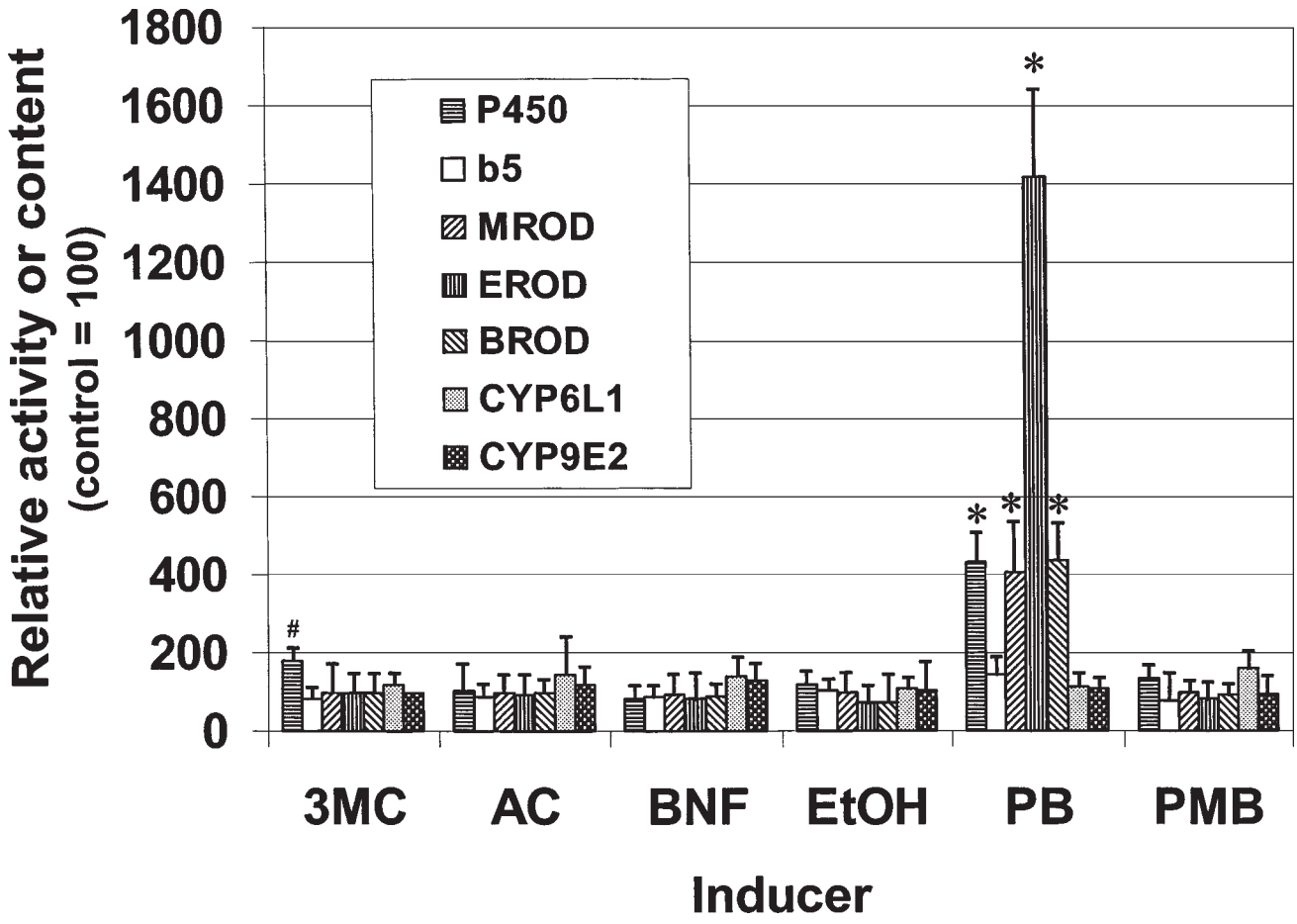

Fig. 1. Effect of treatment with 3-methylcholanthrene (3MC), acetone (AC), beta-naphthoflavone (BNF), ethanol (EtOH), phenobarbital (PB), or pentamethyl benzene (PMB) on total cytochromes P450, cytochrome b_5, three monooxygenase activities and mRNA levels of CYP6L1 and CYP9E2 in adult male German cockroaches. Assays significantly greater than controls are indicated by *($P \leq 0.01$) or #($P \leq 0.1$). Bars represent the SEM.

no induction noted for PMB-treated cockroaches, which agrees with Scharf et al. (1998b) who found no increase in total P450 levels following PMB treatment. Thus, PMB seems to be an inducer of total P450s in Southern armyworm (Brattsten and Wilkinson, 1973), but not in cockroaches or house flies (Scott et al., 1996). Although acetone is an inducer of P450s in mammals (Honkakoski et al., 1988), it did not cause induction in any of the parameters we measured, which is consistent with results from house flies (Scott et al., 1996). No induction was observed in the ethanol-treated cockroaches, even though this is an inducer in mammals (Fuhr, 2000; Honkakoski et al., 1988; Ueng et al., 1993) and was found to slightly induce total P450s in house fly (Scott et al., 1996). BNF induces monooxygenase activities in *Drosophila melanogaster* (Amichot et al., 1998; Hallstrom and Grafstrom, 1981), however, no induction was observed in cockroaches or in house flies (Scott et al., 1996). Cockroaches treated with 3MC showed a slight increase in total P450 levels (1.8-fold), but no increase was detected in cytochrome b_5, MROD, EROD, or BROD activities (Fig. 1). The increase noted for P450 levels following 3MC treatment contrasts with studies on house flies (Scott and Lee, 1993; Yu and Terriere, 1973) and Southern armyworms (Chang et al., 1983) where no induction was observed. Recently, 3-MC was suggested to induce P450 monooxygenases in Colorado potato

beetle larvae (Yoon et al., 2002). There was no MCOD, ECOD, or PrCOD activity detected in any of the control or treated cockroaches (data not shown).

The levels of CYP6L1 and CYP9E2 mRNA were investigated in control and induced cockroaches. Even though expression of CYP9E2 is very general and CYP6L1 is very specific (i.e., sex, life stage, and tissue specific), there was no significant induction of either of these mRNAs following treatment with any of the inducers (Fig. 1). Thus, neither of these P450s appears to be involved in the increased MROD, EROD, nor BROD activities seen following PB treatment.

From our studies, it appears that PB was the only compound that produced induction of both total P450 and three monooxygenase activities. These results contrast with those of house flies and *Drosophila* where several inducers were identified. Given that both German cockroaches and house flies are omnivorous it does not appear that this aspect of an insect's life history is intimately tied to their ability to be induced by xenobiotics.

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