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INDUCTION OF LIVER CYTOCHROME P450 2B1 BY β-IONONE IN SPRAGUE DAWLEY RATS[1]

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Induction of liver cytochrome P450 2B1 by β-ionone was investigated in male and female Sprague Dawley rats. Administration of β-ionone subcutaneously 72 and 48 hr before sacrificing the animals not only significantly induced the liver microsomal activity of pentoxyresorufin O-dealkylase, but also clearly increased in the level of cytochrome P450 2B1 protein. The induction of cytochrome P450 2B1 by β-ionone was much greater in male rats than in female rats. A slot blot analysis showed that the mRNA level was increased from 6 hr after treatment with β-ionone in male rats and from 12 hr after treatment in female rats. Taken together, the present results indicate for the first time that the induction of cytochrome P450 2B1 by β-ionone might be regulated by the accumulation of mRNAs.

β-Ionone (4-[2,6,6-trimethyl-1-cyclohexen-1-yl]-3-buten-2-one) was isolated from the volatile oil of numerous plants including *Boronia megastigma* (Rutaceae) and ginger flowers and is a basic nucleus structure of retinoic acid, β-carotene and vitamin A (1, 2). Parke and Rahman originally investigated the effects of some terpenoids including β-ionone on hepatic drug-metabolizing enzymes (2). The authors found that pretreatment with β-ionone resulted in the increase in the activity of biphenyl 4-hydroxylase and the content of cytochrome P450 (P450), and that hexobarbital sleeping times were decreased by half the normal. Without any further characterization of inductive effects of β-ionone on P450 enzymes, it has been introduced as a P450 inducer in many studies on cocaine-induced hepatotoxicity in which metabolic activation by P450 is required. When β-ionone was injected subcutaneously 72 and 48 hr prior to cocaine administration, cocaine-induced hepatotoxicity was greatly potentiated in mice as measured by the elevation of SGPT activity (3). In addition, β-ionone has been observed to produce the most striking increases in cocaine hepatotoxicity. The SGPT activity

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resulting from cocaine administration was dramatically increased by the pretreatment with β-ionone, and this potentiation was much greater than phenobarbital pretreatment (4). Most recently, exposure to β-ionone for 7 consecutive days was found to induce not only P450 1A1/2 and P450 2B1/2 proteins, but also the enzyme activities of ethoxyresorufin O-deethylase (EROD), pentoxyresorufin O-dealkylase (PROD) and cocaine N-demethylase, which is the first step reaction in cocaine bioactivation to hepatotoxic metabolites, in female B6C3F1 mice (5). However, no informations on the effects of β-ionone on P450 induction are available in rats, which are one of the most useful animal models in studying drug metabolism and chemical-induced toxicity.

The primary objective of the present study was to determine the inductive effect of β-ionone on liver microsomal P450 2B1 enzyme, and to characterize the regulatory mechanism of induction by β-ionone in Sprague Dawley rats. Moreover, sex difference in P450 induction by β-ionone was also studied to accumulate informations on inductive effects of β-ionone in rats. β-Ionone was administered subcutaneously 72 and 48 hr before sacrificing animals, as has been used to determine the role of metabolic activation in cocaine-induced hepatotoxicity in which β-ionone was a model inducer of P450 responsible for the activation of cocaine to its hepatotoxic metabolites (3, 4).

MATERIALS AND METHODS

Animals: Specific pathogen-free male and female Sprague Dawley rats were obtained from the animal breeding laboratory at this institute. The rats received at 4 - 5 weeks of age were acclimated for at least 1 week. Upon arrival, rats were randomized and housed four per cage. All animals were maintained on gamma-irradiated Jeil Lab Chow (Taejon, Korea) and UV-irradiated tap water *ad libitum*. Rats at 6 - 8 weeks old were used in these studies. The animal quarters were strictly maintained at 23 ± 3°C and 40 - 60% relative humidity. A 12-hr light /dark cycle was used with an intensity of 150 - 300 Lux.

Materials: β-Ionone, pentoxyresorufin, resorufin, NADPH, glucose 6-phosphate, nitrocellulose filters, guanidinium thiocyanate, 2-mercaptoethanol and formaldehyde were purchased from Sigma Chemical Company (St. Louis, MO). [^{32}P]ATP was purchased from Amersham (Arlington Heights, IL). Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody, alkaline phosphatase substrate kit, and a kit for protein determination were purchased from Bio-Rad Laboratory (Richmond, CA). All other chemicals used were of reagent grade commercially available.

Isolation of liver microsomes: Male and female rats were treated subcutaneously with β-ionone in corn oil at 300, 600 and 1200 mg/kg 72 and 48 hr before sacrificing animals. Livers were homogenized with four volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The liver homogenates were centrifuged at 9,000xg for 10 min at 4°C and the resulting post-mitochondrial S-9 fractions were centrifuged again at 105,000xg for 60 min at 4°C. The microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Aliquots of liver microsomes were stored at -70°C until use. The content of microsomal protein was determined according to the method of Bradford using bovine serum albumin as a standard (6).

Assay of microsomal PRODactivity: PROD activity was determined by the method of Lubet *et al.* with a slight modification (5, 7). The reaction mixture (2 ml) consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mg/ml of bovine serum albumin, 10 μM dicumarol, 5 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 5 μM NADPH, and 2.0 μM pentoxyresorufin. The formation of resorufin was monitored fluorometrically at an extinction maximum of 550 nm and an emission maximum of 585 nm.

Western immunoblotting analysis: Microsomal proteins (10 μg/well) were resolved by 10% SDS-PAGE and were transferred to nitrocelluose filters. The filters were incubated with 2.5% non-fat dry milk for 30 min to block the nonspecific binding, and then were incubated with

rabbit polyclonal antibodies against rat P450 2B1/2, followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. The primary antibody against P450 2B1/2 were prepared as described previously (8). For immunostaining, the nitrocellulose filters were developed with a mixture of 5-bromo-4-chloro 3-indolylphosphate, nitroblue tetrazolium and 0.1 M Tris buffer (1:1:10) under an instruction by the manufacturer.

Slot blot analysis of P450 2B1 mRNAs: β-Ionone at 600 mg/kg were administered subcutaneously for 6, 12, 24 and 48 hr. Total cellular RNAs were isolated, cross-linked on a nitrocellulose filter by UV light, and hybridized with P450 2B1 mRNA-specific 18-mer oligonucleotide probe, which has been introduced previously (9).

Statistics: Dunnett's t-test was used to compare the significance of data obtained and significant values at $P<0.05$ were represented as asterisks.

RESULTS

As shown in Figure 1, the administration of β-ionone clearly induced P450 2B1-specific PROD activity with a dose-dependent manner in liver microsomes of Sprague Dawley rats. The induction by β-ionone was much greater in male rats than was in female rats. The induction of P450 2B1 protein by β-ionone was also confirmed in the Western immunoblotting analysis using P450 2B1-specific polyclonal antibody, as presented in Figure 2. The induction of P450 2B1 protein was also greater in male rats as observed in the assay of PROD activity.

To investigate the mechanism of induction by β-ionone, a time-course effect of β-ionone on the expression of P450 2B1 mRNA was studied in the present study. As shown in Figure 3, a slot blot analysis showed that the amounts of P450 2B1 mRNAs were started to be elevated 6 hr after treatment with β-ionone in male rats and 12 hr after the treatment in female rats. These results indicate that the induction of P450 2B1 by β-ionone may be regulated by the accumulation of mRNAs in Sprague Dawley rats, and that the induction may occur more rapidly in male rats.

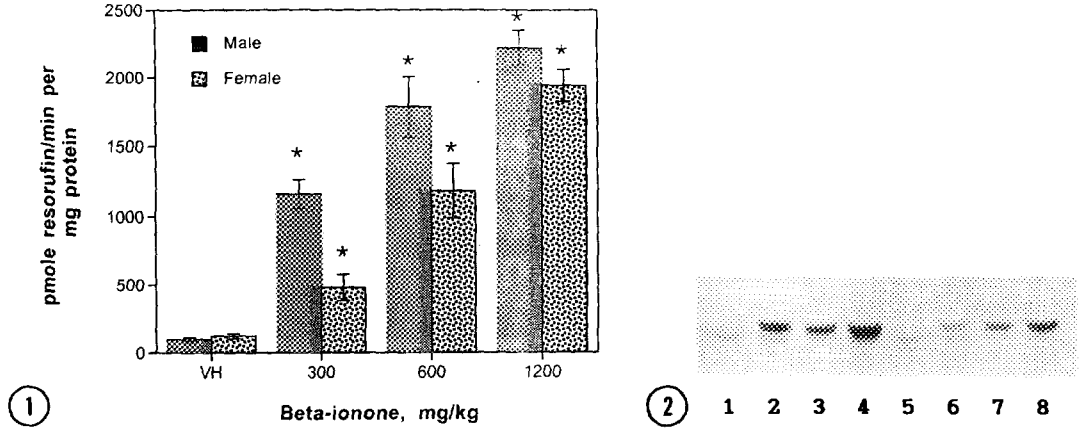

Figure 1. Induction of liver microsomal activity of pentoxyresorufin O-dealkylase by β-ionone. Sprague Dawley rats were administered β-ionone subcutaneously 72 and 48 hr before sacrifice. Each value represents the mean activity ± S.E. of four animals. An asterisk indicates the value significantly different from the vehicle control at $P<0.05$.

Figure 2. Western immunoblotting analysis of P450 2B1 protein. The liver microsomal protein (10 μg/well) prepared from rats administered β-ionone at 300, 600 and 1200 mg/kg were resolved on 10% SDS-PAGE. Lane 1 and 5, vehicle control; lane 2 and 6, β-ionone 300 mg/kg; lane 3 and 7, β-ionone 600 mg/kg; lane 4 and 8, β-ionone 1200 mg/kg. Lane 1 to 4, male rats; lane 5 to 8, female rats.

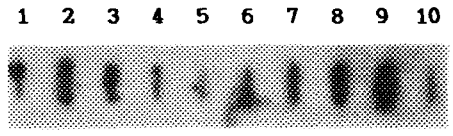

Figure 3. Effects of β-ionone on the expression of P450 2B1 mRNAs in Sprague Dawley rats. A time-course study. Rats were administered 600 mg/kg of β-ionone subcutaneously for 6, 12, 24 and 48 hr. Twenty μg of total cellular RNAs isolated from livers were used for slot blot analysis. Lane 1 and 6, vehicle control; lane 2 and 7, treated for 6 hr; lane 3 and 8, treated for 12 hr; lane 4 and 9, treated for 24 hr; lane 5 and 10, treated for 48 hr. Lane 1 to 5, male rats; lane 6 to 10, female rats.

DISCUSSION

Although β-ionone has been widely used as a model inducer of P450s in studying the possible role of metabolic activation in cocaine-induced hepatotoxicity (3, 4), its inductive effects on P450s have not been extensively investigated. In the present study, the effect of β-ionone on the P450 2B1 induction was investigated in Sprague Dawley rats using the P450 2B1-associated monooxygenase activity (i.e., PROD) and the Western immunoblotting to detect the P450 2B1 protein in the liver microsomes, because the P450 2B1 has been relatively well characterized as one of the most important isozymes to metabolize cocaine to its hepatotoxic metabolites in rats (10). Under the same condition that has been used in previous studies (2, 3), β-ionone induced liver microsomal activity of PROD and proteins dose-dependently in both male and female rats. The sex difference was so obvious in inductive effects of β-ionone that the PROD activity and P450 2B1 protein induced were much greater in male rats compared to those in female rats (Figure 1 and 2). From the slot blot analysis of P450 2B1 mRNAs, we demonstrated for the first time that P450 2B1 induction by β-ionone was closely associated with increased accumulation of P450 2B1 mRNAs in rats, although the mechanism behind mRNA elevation is still uncertain.

At present, although the effects of β-ionone on P450 induction has never been studied in rats, many reports have demonstrated or, at least, implicated the inductive effects of β-ionone in mice and other species. For example, when female B6C3F1 mice were treated with β-ionone for 7 days at 300, 600 and 1200 mg/kg, the liver microsomal activities of P450 2B1 and P450 1A1 (i.e., PROD and EROD, respectively) and the proteins were dose dependently induced (5). In addition, mice treated with β-ionone 72 and 48 hr before sacrifice also clearly showed the induction of P450 2B1 and P450 1A1 enzyme activities and proteins (T.C. Jeong, S.D. Jordan, R.A. Matulka, S.S. Park and M.P. Holsapple, submitted), indicating that the original dosing schedule of β-ionone (i.e., pretreatment with β-ionone 72 and 48 hr before cocaine administration) in previous papers may actually induce P450 enzyme(s) responsible for cocaine bioactivation to hepatotoxic metabolites (3, 4). Moreover, a dietary supplementation of vitamin A, which possesses the β-ionone moiety in its structure, has been reported to induce P450 3A2, as well as the 6β-hydroxylation of progesterone (11). Similarly, the supplementation of high vitamin A diet was found to elicit an increase in microsomal aminopyrine N-demethylase activity in the rabbit and guinea pig (12).

Our present results that β-ionone induced liver microsomal P450 2B1 protein via the accumulation of mRNAs clearly explains the former investigation in which β-ionone was introduced as a P450 inducer in studying the possible role of metabolic activation in cocaine-induced hepatotoxicity and immunosuppression (3 - 5). A study on the inductive effects of β-ionone on other isozymes of P450s is currently underway to further characterize the induction profile of P450s by β-ionone, because there has been an inconsistency of the induction profiles of P450 2B1 with potentiation of cocaine-induced hepatotoxicity by β-ionone and phenobarbital (4). Since β-ionone was neither hepatotoxic nor immunosuppressive at the dose showing P450 induction (5), it will be a useful model compound to induce P450 2B1 in investigating the role of metabolism in chemical-induced toxicity.

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