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Interaction of ergotamine with liver Cytochrome P450 3A in rats

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ABSTRACT

This study was conducted to investigate the effect of the ergot alkaloid, ergotamine (ET), on the induction of CYP3A and the interaction *in vivo* and *in vitro* with ET. Sprague-Dawley rats were treated intraperitoneally for 4 days as follows: control (injecting with 0.5 ml of only corn oil); dexamethasone treatment (injecting with 100 mg/kg of dexamethasone in corn oil); and ergotamine treatment (injecting with 100 mg/kg of ergotamine in corn oil). Liver tissues were collected from each group (n = 5, total of 30 rats) and liver microsomes were prepared. Cytochrome CYP3A activity was evaluated using ET and its isomer as substrates in medium containing liver microsomes and NADPH at 37˚C for 30 min. HPLC was used to measure the disappearance of the substrate and the appearance of the metabolites. Liver microsomes from rats pretreated with dexamethasone were five times more (P < 0.01) active than microsomes from the control animals in the biotransformation of ET (32.1 and 7.0 nM/min/mg protein, respectively; SE = 4.83) or ET-isomer (21.6 and 4.7 nM/min/mg protein, respectively; SE = 1.7079) into its corresponding ET metabolites. The ergotamine treatment produced no increase (P > 0.05) in activity of CYP3A when compared to the control group (5.2 vs 7.0 nM ET/min/mg protein; SE = 4.83) or ET isomer (1.5 vs 4.7 nM ET isomer/min/mg protein; SE = 1.70). When ketoconazole was used as specific inhibitor of CYP3A, ergotamine metabolisms were inhibited in a dose dependent fashion reaching a maximum at an inhibitor to substrate ratio of greater than one and LD50 at 0.5 nM of ketoconazole/mg protein. The data presented in this study suggest that although the ergot alkaloids ergotamine and its isomer are ideal substrates for the isozyme CYP3A, these compounds have no effect on the induction of CYP3A after 4 days of treatment.

Keywords: Microsomes; Liver; Rat

1. INTRODUCTION

Cytochrome P450 (CYP) enzyme systems play a key role in the biotransformation of many endogenous and exogenous compounds including both toxins and drugs [1-4]. The CYP enzyme family consists of a large number of proteins with different substrate specificities and catalytic properties which are membrane-bound, mostly localized to the endoplasmic reticulum and in mitochondrial inner membranes. CYP1-3 families are active in the metabolism of xenobiotics with CYP3A subfamilies being the most important in drug metabolism [5]. The metabolism of ergot alkaloids, such as bromocriptine, ergotamine and other structurally similar ergot derivatives is mediated mainly by CYP3A4 [6]. Moochhala *et al.* [7] reported that bromocriptine interferes with P450-dependent oxidative metabolism of xenobiotics. Later it was demonstrated that cytochrome P450 3A exhibits a particularly high affinity for ergopeptides. The CYP3A family expression patterns differ based on maturity. CYP3A4 is mainly expressed in adult liver, CYP3A5 in extrahepatic tissues, and CYP3A7 in fetal liver [8]. Activation or inhibition of the induction process of such enzyme systems can have severe consequences. Witkamp *et al.* [9] reported that tiamulin, a semi-synthetic antibiotic frequently used in agricultural animals, strongly inhibited the hydroxylation rate of testosterone at the 6 beta-position via the formation of a cytochrome P450 3A4 metabolic intermediate complex in both microsomes and hepatocytes.

The induction of CYP3A4 is mainly regulated by the novel orphan receptor or pregnane X receptor (PXR) [10, 11], but other receptors including the constitutively active receptor (CAR) and, indirectly, the glucocorticoid receptor (GR) are involved [12,13]. Dexamethasone, one of the glucocorticoids, influences several aspects of CYP3A induction. However, most of these effects are not dependent on GR binding to CYP genes, but rather on complex protein-protein interplay between GR and various other receptors [14]. Information on how or if ET fits

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into the induction processes of rat CYP3A is limited or not available. Therefore, this report was designed to study the effects of administering ET in vivo on the induction of CYP3A family using dexamethasone as a specific CYP3A inducer for similarity and to evaluate the in vitro interaction of ET with CYP3A using ketoconazole as specific inhibitor. Ergotamine and similar ergot alkaloids are extremely susceptible to photolytic and air oxidation, hydration and isomer formation at the C-8 position of the ergolene ring. Therefore, ergotamine isomer was included in this study.

2. MATERIALS AND METHODS

All the chemicals and reagents used in these experiments were of the highest quality available and were purchased from Sigma Chemical Co. (St. Louis. Mo) unless stated otherwise.

Sprague-Dawley rats (n = 30 rats; BW ~250 g) were allowed ab libitum access to water and chow. Rats were randomly assigned to treatment and treated intraperitoneally for 4 consecutive days with one of the following treatments: 1) Control treatment (n = 5 rats, injections of 0.5 ml of corn oil); 2) DXM treatment (n = 20 rats, injections of 100 mg/kg dexamethasone in 0.5 ml of corn oil); and 3) ET treatment (n = 5 rats, injections of 100 mg/kg Ergotamine in 0.5 ml of corn oil). Approximately 24 h after the last injection (except in DXM treatment), each rat was anesthetized with chloroform, decapitated, and liver harvested. Livers were stored at –20˚C until microsomes were prepared. In the DXM treatment, five rats were removed each day for time dependence evaluation and livers were harvested in the same manner.

Michaelis-Menten kinetics was used to evaluate the interaction of CYP3A with ET and the linearity of the Lineweaver-Burk plot of 1/V versus 1/S was further examined by measuring enzyme activity at various concentrations (2.0 to 20.0 µM) of the enzyme substrate, ET, in the presence and absence of kentozole (3.0 and 5.0 µM).

Liver microsomes were prepared as reported by Moubarak and Rosenkrans [15]. Briefly, liver tissue (3 - 5 g) were diced with scissors and then washed with 150 mM sodium chloride buffer. The diced tissue was ground (1 g tissue/10 ml of buffer (250 mM sucrose, 100 mM Tris-HCl, 1 mM EDTA, pH 7.4)) with ice-cold medium using a precooled blender for 10 to 20 sec and further homogenized using a Polter-Elvehjem (5×). The homogenate was sequentially centrifuged at 800 g for 10 min, at 13,500 g for 20 min collecting the supernatant and then at 105,000 g for 60 min collecting the pellet which contained the microsomal fraction. The pellet (microsomal fraction) was resuspended in buffer containing 100 mM sodium phosphate and 20% v/v glycerol. The protein concentration after resuspension was approximately 40 mg/ml. Aliquots of microsomal suspensions were stored at –20˚C and were used within 20 to 30 days. Protein concentration was determined by either the method of Lowry [16] using serum albumin as standard or by BCA Protein Assay (Pierce Chemical kit no. 23225).

Ergotamine (ET) and its isomer (ET_iso) were used as substrates to assay presumptive CYP3A4 activity in vitro. Our standard assay methods for the metabolism of ET were analyzed in 330 µl of assay medium, 100 µl of cofactor generating system (NADPH), 20 µl of ET (final concentration was 4 µg/ml of fully isomerized ET), and 50 µl microsomal protein (final concentration was 0.4 mg/ml). The assay medium (pH 7.4) consisted of 100 mM potassium phosphate, 0.1 mM EDTA, and 5.0 mM MgCl2. The NADPH generating system consisted of assay medium with 10 mM NADP+, 10 mM D-glucose-6-phosphate, and 2.0 U/ml of Glucose-6-phosphate dehydrogenase. The reactions were initiated by adding the NADPH generating system and were terminated after 30 min by adding 100 µl of 94% acetonitrile and 6% glacial acetic acid. After the stop solution was added the mixture was centrifuged at 12,000 g for 4 min. Supernatant (150 µl) from each reaction was examined for the disappearance of ET and ET_iso and the appearance of their metabolites (M1, M2 from ET and M1_iso and M2_iso from ET_iso) using the HPLC method described by Moubarak and Rosenkrans [15].

Induction data for the dexamethasone treated rats were analyzed using one-way ANOVA with day as the independent variables and concentration of remaining substrate or metabolite as the dependent variables. Ergotamine and dexamethasone induction data after 4 days of treatment were analyzed using one-way ANOVA with treatment as the independent variable and concentration of remaining substrate or metabolites as the dependent variables. The kinetics data of kentoconazole inhibition was interpreted using the double reciprocal plot method of 1/S vs 1/V.

3. RESULTS AND DISCUSSION

Data in **Figure 1** represents the disappearance of ET and its isomer after incubation with liver microsomes from rats that had been treated sequentially for 1, 2, 3, or 4 days with 100 mg/kg of dexamethasone, a specific inducer of CYP3A. Dexamethasone induced CYP3A activity in rats to a significant ($P < 0.01$) level only after the third and the fourth days of treatments. These results indicate that the mechanism of DXM induction of rat liver microsomal CYP3A was time dependent, and it took at least three to four successive days of treatments to reach the maximum level of activity. Liver microsomes from rats pretreated with dexamethasone were five times more ($P < 0.01$) active than microsomes from

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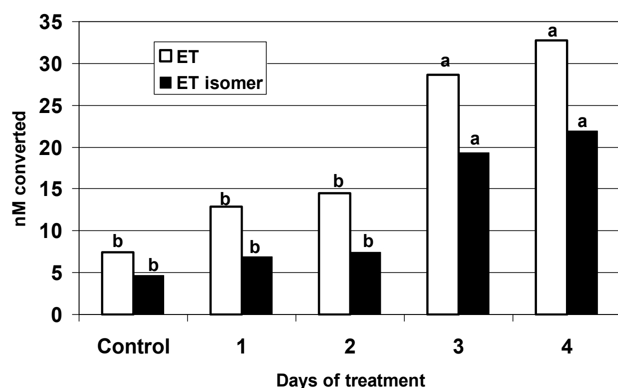

Figure 1. The effects of intraperitoneal injections of dexamethasone (DXM) on the induction of rat liver CYP3A activity over four consecutive days of injection. Comparisons were made within substrate (ETor ET isomer). Bars containing the same letter were not different significantly (P > 0.05).

the control animals in the biotransformation of ET (32.1 and 7.0 nM/min/mg protein, respectively; SE = 4.83) or ET-isomer (21.6 and 4.7 nM/min/mg protein, respectively SE = 1.7079) into its corresponding ET metabolites.

Ergotamine treatment for 4 consecutive days produced no significant (P > 0.05; 5.2 vs 7.0 nM ET/min/mg protein; SE = 4.83 and 1.5 vs 4.7 nM ET isomer/min/mg protein; SE = 1.70) increase in the CYP3A activity over that of the control animals (**Figure 2**). Moubarak *et al.* [17] showed that treatment of rats with similar ergot alkaloids dihydroergotamine (DHET) or ergonovine (EN) at a concentration of 100 mM did not produce any significant increase in the CYP3A activity over that of control rats. Although it is generally accepted that most compounds that are metabolized by CYP's are to some degree inducers of that CYP's enzyme system [10], data from this study and from a previous study in our laboratory have indicated that all of the ergot alkaloids studied (ergotamine, dihydroergotamine and ergonovine) have very small or no induction effects on CYP3A in rats, yet those alkaloids have been shown to be metabolized by rat CYP3A. Previous studies have demonstrated that both DXM and ET can also inhibit the in vitro metabolism of tacrolimus mediated by the CYP3A subfamily [18,19]. Another study showed that dihydroergocryptine, a dopamine agonist for the treatment of Parkinson's disease, has an inhibitory effect on CYP3A4-mediated testosterone metabolism and additionally could induce CYP3A4 and CYP2E1 mRNA when added at 10 µM to cultured human hepatocytes [20]. Moubarak *et al.* [17] reported that both ergonovine and dihydroergotamine inhibited in vitro CYP3A4 activity in a dose dependent manner when ET was used as a substrate, producing quadratic inhibition curves. One can ask, is it possible for a group of compounds such as the ergot alkaloid group (ET, DHET, EN, etc.) to play a double role? One role is to be involved at the upstream induction of CYP3A and another is to directly interact with the structure of the enzyme inhibiting its catalytic activity. Ergot alkaloids have been demonstrated to affect the cytochrome p450 system, especially isoenzyme CYP3A4, by binding to the isoenzyme as a substrate [15]. The linearity of the Lineweaver-Burk plot of $1/V(V_{max})$, versus 1/S (ET and ET isomer) shows that the interaction between the substrate (ET) and CYP3A in both the presence and absence of ketoconazole (**Figure 3**) follows Michaelis-Menten kinetics. Furthermore, the fact that the slope did not change with the addition of ketoconazole, and the decrease in the velocity of the reaction (V_{max}), indicated an uncompetitive inhibition. Thus ketoconazole interacts with the enzyme-substrate complex.

In this study we have demonstrated that although both ET and ET isomer are ideal substrates for CYP3A, they appear to have no induction effect on rat hepatic CYP3A during the 4 days of treatment when compared to the classic dexamethasone induction. The data from the in vitro interaction among ET, CYP3A and its specific inhibitor (ketoconazole) showed that the binding site for

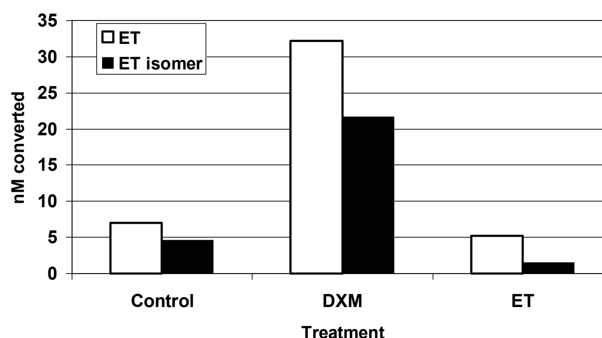

Figure 2. The amount of substrate (ET or ET isomer) remaining after incubation with rat hepatic CYP3A preparation from control (CON), Dexamethsone (DXM) and Ergotamine (ET) treated rats. Comparisons between treatments were made within substrate. Bars containing the same letter were not different significantly (P > 0.05).

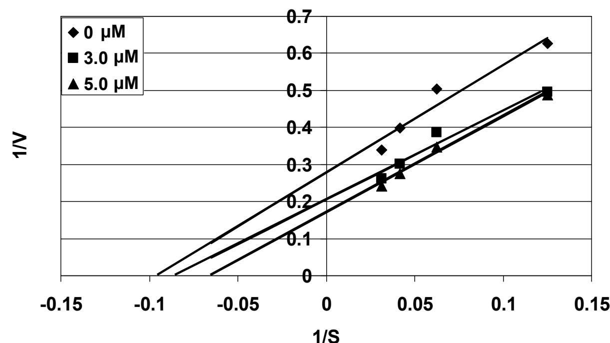

Figure 3. Lineweaver-Burk plots of rat hepatic CYP 3A4 activity in 0, 3, and 5 µM of ke-toconazole.

ET was different from that for ketoconazole in rat hepatic CYP3A.

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