MODULATION OF RAT CYTOCHROME P-450 BY AN INVESTIGATIONAL HIV PROTEASE INHIBITOR

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ABSTRACT:

Previous studies in vitro have revealed that L-754,394, an HIV protease inhibitor, is a potent suicide inhibitor of cytochrome P-450 enzymes. The present report examines the effect of chronic treatment of L-754,394 on hepatic cytochrome P-450s in adult male rats. L-754,394 was administered orally once a day for 7 days and resulted in significant changes in marker activities. An unusual parabolic (ascending, then descending) profile was observed for testosterone 2β-hydroxylase and 2α,6β-hydroxytestosterone activities during the 7-day treatment with 20 mg/kg L-754,394. These activities, which were elevated 2-fold on day 2, returned to basal levels by day 8. In contrast, testosterone 2α-/6α-(CYP2C11-catalyzed) hydroxylase activities showed an opposite parabolic (descending, then ascending) profile during the same period, reducing to 40% of control activities on day 4, followed by a rebounding trend. Immunoquantitation of CYP 3A1/2 and 2C11 showed that the expressed protein levels were in parallel with the associated activities. Furthermore, mRNA levels of CYP 3A2 and CYP2C11 showed the same trends as the protein expression of the respective isozymes. These observations show that L-754,394 perturbs the relative abundance of P-450 isoforms in rat liver by affecting the regulation at a pretranslational step. This may further involve a disturbance of hormonal homeostasis. Although serum levels of testosterone did not show a marked change during treatment, thyroxine and triiodothyronine markedly decreased on days 2 and 4, and subsequently increased to basal levels.

Materials and Methods

Chemicals. L-754,394 was synthesized at Merck Research Laboratories (West Point, PA). Hydroxylated metabolites of testosterone (2α, 2β, 6β, 7α, 16α, and 16β-hydroxytestosterone) were obtained from Steraloids (Wilton, NH). Testosterone, tolbutamide, thyroxine (T₄), and triiodothyronine (T₃) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Animal Treatments and Microsome Preparation. Adult male Sprague-Dawley rats (Harlan-Sprague-Dawley, Inc., Frederick, MD) were 10 to 12 weeks old at the beginning of treatment and weighed 230 to 250 g. The sulfate salt of L-754,394, dissolved in 0.05 M citric acid, was administered p.o. with 1% methyl cellulose in drinking water. Male Sprague-Dawley rats (Harlan-Sprague-Dawley, Inc., Frederick, MD) were 10 to 12 weeks old at the beginning of treatment and weighed 230 to 250 g. The sulfate salt of L-754,394, dissolved in 0.05 M citric acid, was administered p.o. with 1% methyl cellulose in drinking water.

Immunoinhibition Study. Immunoinhibition of testosterone 2α- and 16α-hydroxylase activities by anti-CYP 2C11 goat antiserum (Gentest Corp., USA) was used for immunoblotting studies. For inhibition studies, preliminary experiments revealed that polyclonal anti-rat CYP 2C11 and 3A1/2 goat antisera, both obtained from Gentest Corp. (Woburn, MA), were used for immunoblotting studies. For inhibition studies, preliminary experiments revealed that polyclonal anti-rat CYP 2C11 and 3A1/2 goat antisera, both obtained from Gentest Corp. (Woburn, MA), were used for immunoblotting studies.

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**Effect of L-754,394 on Testosterone Hydroxylase Activities.**

Testosterone metabolism has been widely accepted as a marker to measure P-450 isoform activities. Testosterone is hydroxylated at the 2β/6β and 2α/16α positions mainly by CYP 3A1/2 and CYP 2C11, respectively, and these activities were measured in rat liver microsomes during the treatment with L-754,394. Testosterone 2β- and 6β-hydroxylase activities were elevated approximately 2-fold by treatment with 20 mg/kg L-754,394; both activities reached maximum values on day 2, declining thereafter to basal levels by day 8 (Fig. 2, top). In contrast, 2α- and 16α-hydroxylase activities of testosterone showed a marked decrease to 40% of control values on days 2 and 4 during the same treatment (Fig. 2, bottom). The reduced activities, however, returned to basal levels afterward and exceeded control values by 20 to 40% on day 8. Notably, as shown in Fig. 2, the changes in CYP3A and 2C11 activities mirrored each other over the course of treatment. Lower doses of L-754,394 (1 and 5 mg/kg) had little or no effect on these hydroxylase activities (data not shown). Additionally, CYP2A1-mediated 7α- and CYP2B1/2-mediated 16β-hydroxylation activities of testosterone showed increasing, then decreasing, trends with 20 mg/kg L-754,394 (Fig. 3); the activities increased to 200% of control values on day 6, followed by a decrease to control levels on day 8.

To assure that the alterations of enzyme activities observed during treatment indeed reflect those catalyzed by specific isoforms (CYP3A and CYP2C11), an immunoinhibition study was conducted. The specific contribution of CYP3A and CYP2C11 isoforms in their corresponding testosterone activities was confirmed as indicated by the near complete (>95%) inhibition of each activity with the respective

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**Fig. 1. Chemical structure of HIV protease inhibitor L-754,394.**

Woburn, MA) and that of 2β- and 6β-hydroxylase activities by anti-CYP 3A1 rabbit antibody (Human Biologics, Inc., Phoenix, AZ) were estimated as described herein after a 30-min preincubation at room temperature. A sufficient amount of anti-CYP 2C11 antisem (60 μg/ml microsomal protein) or anti-CYP 3A1 antisem (10 mg/nmol P-450) for maximum inhibition, which had been determined in preliminary experiments, was added to the microsomes and the reaction was initiated by the addition of an NADPH-generating-system as described herein. The same amount of control goat serum or preimmune rabbit IgG was added to control samples.

**Gel Electrophoresis and Western Immunoblotting.** SDS electrophoresis was conducted in 10% polyacrylamide gels according to the procedure of Laemmli (1970). Proteins were transferred onto a nitrocellulose membrane after electrophoresis (Towbin et al., 1979). The proteins of CYP 3A1/2 and CYP 2C11 were probed by goat antisem (Genest Corp., Woburn, MA) directed against the corresponding isomor based on autoradiography (Molecular Dynamics, Sunnyvale, CA) to quantify the intensities of the bands.

**Hormone Measurements.** Total serum estradiol, testosterone, T<sub>α</sub>, and T<sub>β</sub> were separately measured by solid phase radioimmunoassay using commercially available kits (Diagnostic Products Corporation, Los Angeles, CA).

**Statistics.** Differences between means from control (untreated) and treated microsomal studies were detected using Student's t test with results presented as mean ± S.D.
The integrity of the sample preparation for the respective isoform. The expression of the respective isoform. mRNA encoding CYP 3A1/2 and CYP 2C11 (Fig. 5). Although there were no detectable CYP 3A protein (Fig. 4). The changes in the CYP3A and CYP2C11 catalytic activities during treatment, therefore, strongly suggested an alteration in their respective P-450 isoform contents. Due to the lack of antibodies for CYP 2A1 and 2B1/2 at the time of this study, it was not possible to measure the contents of the corresponding isozymes.

Immunoquantitation of CYP3A1/2 and CYP2C11. To examine whether altered protein levels were the cause for the changes in the catalytic activities observed during treatment, immunoquantitation experiments using antibodies for CYP 3A or CYP 2C11 were conducted. As shown in Fig. 2, the protein levels for CYP 3A and CYP 2C11 were consistent with the respective catalytic activities. The content of CYP 3A showed an upward (to 180% of control) or downward (to basal level) alteration with a 20-mg/kg dose of L-754,394 (Fig. 2). For CYP 2C11, the same dose of L-754,394 decreased the CYP 2C11 content (to 50% of control) during the first 4 days, followed by a rebound to exceed the basal level on day 8 (Fig. 2).

Slot-Blot Analysis for mRNA Levels Encoding CYP3A2 and CYP2C11. In addition, slot-blot analyses were carried out to determine the effect of chronic treatment with L-754,394 on the level of mRNA encoding CYP 3A2 and CYP 2C11 (Fig. 5). Although there was a large interindividual variation among animals, mRNA levels for CYP3A2 and CYP2C11 showed the same trends as the protein expression of the respective isoform. The integrity of the sample preparation was not a factor for the observed variation as shown by the relatively consistent mRNA levels of α-tubulin, a constitutively expressed gene. These results suggest that the observed alteration is due to an effect at the pretranslational level.

Serum Levels of Steroid and Thyroid Hormones. To examine the mechanism responsible for the effect of L-754,394 on P-450 regulation, we have measured serum levels of steroid hormones (estradiol and testosterone) and thyroid hormones (T4 and T3) in the rats treated with L-754,394 (Table 1). Estradiol was not detectable before and after treatment and no discernible change was observed for testosterone. In contrast, thyroid hormones showed a marked decrease on days 2 and 4, followed by a return to basal levels on day 8.

Discussion

It has been demonstrated that the half-lives of rat P-450 de novo synthetase are relatively short (12–20 h) (Shiraki and Guengerich, 1984). Therefore, any chemical reagent, such as L-754,394, which directly affects P-450 synthesis and/or degradation, may alter the steady-state level. Chronic dosing of L-754,394 indeed resulted in an immediate response in the regulation of major P-450 isozymes. Interestingly, although in vitro, both CYP 2C11 and CYP 3A were destroyed by L-754,394, chronic dosing resulted in a striking and surprising response that was isoform-dependent; mirror-image profiles were observed for the catalytic activities of the two predominantly male isozymes, CYP 3A and CYP 2C11 (Fig. 2). These distinct catalytic profiles were confirmed by Western immunoblotting (Fig. 2) and immunoinhibition (Fig. 4) studies. Furthermore, slot-blot mRNA analysis using cDNA oligo-probes specific for the constitutive CYP 3A2 and CYP 2C11 revealed that L-754,394 is likely acting at a pretranslational step in P-450 regulation resulting in mRNA profiles (Fig. 5) paralleling the respective isoform protein contents and catalytic activities (Fig. 2).

A number of physiological and nutritional factors, as well as in vivo treatment with foreign chemicals, have been documented to affect the relative abundance of male-dominant constitutive P-450 isozymes in rats. One of the most important factors that may inversely regulate CYP3A (CYP2A1 and CYP2B1/2) and CYP2C11 appears to be the thyroid hormone. T4 and its potent metabolite, T3, have been demonstrated to suppress the expressions and/or catalytic functions of CYP...
A sufficient amount of anti-CYP 2C11 antiserum (60 μl/mg microsomal protein) or anti-CYP 3A1 antibody (10 μg/nmol P-450) for maximum inhibition was preincubated with microsomes before testosterone metabolism, whereas the same amount of control goat serum or preimmune rabbit IgG was added to control samples.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Testosterone (nM)</th>
<th>Estradiol (pg/dL)</th>
<th>Thyroxine (μg/dL)</th>
<th>Triiodothyronine (ng/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.24 ± 2.30</td>
<td>&lt;0.07</td>
<td>4.70 ± 0.26</td>
<td>68.8 ± 11.9</td>
</tr>
<tr>
<td>Day 2</td>
<td>4.32 ± 4.68</td>
<td>&lt;0.07</td>
<td>3.13 ± 0.44*</td>
<td>49.0 ± 9.3*</td>
</tr>
<tr>
<td>Day 4</td>
<td>2.92 ± 2.31</td>
<td>&lt;0.07</td>
<td>3.80 ± 0.23*</td>
<td>54.3 ± 4.0</td>
</tr>
<tr>
<td>Day 6</td>
<td>4.73 ± 0.46</td>
<td>&lt;0.07</td>
<td>4.78 ± 0.46</td>
<td>71.3 ± 11.8</td>
</tr>
<tr>
<td>Day 8</td>
<td>5.58 ± 5.67</td>
<td>&lt;0.07</td>
<td>4.45 ± 0.68</td>
<td>56.8 ± 6.2</td>
</tr>
</tbody>
</table>

* Data are expressed as mean ± S.D. of four animals.
* Significantly different from control (p < .01).
* Significantly different from control (p < .05).

The changes of thyroid hormone levels were small (~35%), a significant decrease was observed in the serum levels of both T4 and T3 2 and 4 days after the onset of treatment with a 20-mg/kg dose of L-754,394 Table 1). This decrease in thyroid hormone levels may partially relieve its suppressive effect on CYP 3A, resulting in an increase in CYP 3A, as well as a decrease in the expression of CYP 2C11 which could be a consequence of the decreased growth hormone secretion secondary to the reduced thyroid hormone at the pituitary. A positive correlation was reported between an individual CYP2C11 activity and the plasma T4 concentration in cyclophosphamide-treated rats (Kraner et al., 1996). Recently, it was also demonstrated that the reduction of thyroid hormone level in the plasma was responsible for the alterations in CYP2C11 (30% decrease) and 3A2 (30% increase) after the administration of retinol to rats (Badger et al., 1998).

Steroid hormones, such as testosterone and estradiol, are among such factors that play a major role in regulating the gender-specific
expression of P-450 isoforms (Dannan et al., 1986; Waxman, 1988). Circulating testosterone is known to be required for maintaining the full expressions of CYP2C11 and CYP3A in adult male rats (Gustafsson and Stenberg, 1974; Waxman et al., 1985). Thus, decrease of this androgen level results in the suppression of both constitutive, male-predominant CYP 2C11- and 3A-catalyzed metabolism. Unlike the anticancer compounds that caused decreased expression of both isoforms (LeBlanc and Waxman, 1988, 1990), L-754,394 treatment resulted in an increase in CYP 3A and simultaneous decrease in CYP 2C11 expressions. Examination of serum testosterone levels showed no discernable trends and serum estradiol levels remained below the detection limit (Table 1). These observations precluded the possibility that perturbation of these steroids is involved in the P-450 modulation by L-754,394.

In summary, the protein contents of two predominately male P-450 isoforms and their associated catalytic activities were found to be inversely affected by L-754,394 during chronic treatment in rats. Unlike in vitro, in which L-754,394 destroys both CYP 3A and CYP 2C11, chronic treatment affected the isoforms in opposite directions. Analyses of CYP 2C11 and CYP 3A2 mRNA indicated that L-754,394 is acting at a pretranslational step in P-450 regulation. Although this effect likely involves perturbation of the thyroid hormone homeostasis, the mechanism remains to be examined.

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