ABSTRACT:

Rat liver microsomal testosterone (250 μM) hydroxylation and immunoreactive CYP3A protein were compared after administration of the antiglucocorticoid RU 486 (50 mg/kg −1·day−1 for 4 days) and the hypocholesterolaemic drug SR-12813 (150 mg/kg −1·day−1 for 4 days). Markers of CYP3A-mediated enzyme activity (testosterone 15β-, 6β-, and 2β-hydroxylation) were increased after administration of both drugs. Testosterone 6β-hydroxylation was increased 5-fold by RU 486 and 9-fold by SR-12813. Administration of dexamethasone alone at 150 mg·kg−1·day−1 or in combination with RU 486 induced testosterone 6β-hydroxylation 15- to 20-fold. The lack of antagonistic effect of RU 486 on dexamethasone-mediated CYP3A induction strengthens support for the hypothesis that the “classical glucocorticoid receptor” does not play a part in this process. The induction of CYP3A enzymes by the bisphosphonate SR-12813 suggests the existence of a new class of compounds with CYP3A inducing properties.

Materials and Methods

Materials. All solvents were of HPLC grade obtained from BDH (Poole, UK) and Rathburn Chemicals, Ltd. (Walkerburn, Scotland) and were vacuum filtered through 45 μm filters (Millipore, Ireland) before use. Testosterone, β-mercaptoethanol, glycerol, Pyronin Y, sodium dodecyl sulfate, and dexamethasone were purchased from Sigma Chemical Co. (Poole, Dorset, UK). RU 486 and SR-12813 were gifts from Roussel Uclaf (Paris) and Symphar Pharmaceuticals, respectively. Hydroxytestosterone metabolites were purchased from Steraloids Inc. (Wilton, NH). Polyclonal anti-CYP3A antibodies for Western immunoblotting were gifts donated by Dr. Colin Henderson, University of Dundee.

Animals. Female Sprague-Dawley rats (250–300 g) were housed under conditions of 12 hr light-12 hr darkness cycle for at least 1 week before use and fed CRM diet (Special Diet Services, Witham, Essex, UK).

Administration of drugs. RU 486 (50 mg·kg−1·day−1) for 4 days) and SR-12813 (150 mg·kg−1·day−1 for 4 days) were dissolved in olive oil (1 ml·kg−1) before ip administration. Dexamethasone was dissolved in 0.9% (w/v) saline and administered at 150 mg·kg−1·day−1 for 4 days. After overnight fasting, animals were killed on the fifth day and liver microsomes prepared immediately.

Preparation of liver microsomes. After cervical dislocation, livers were placed in washing buffer. After removal of nonhepatic tissue, the liver was placed in homogenization buffer (4 × weight of the washing buffer). The liver was scissor-minced and homogenized with 7 strokes of a motor-driven plastic pestle in a glass vessel (Elvejhem-Potter). The homogenate was then centrifuged at 15,000g for 20 min. The supernatant was decanted and centrifuged at 180,000 g for 60 min. The pellet was washed in homogenizing buffer and centrifuged at 180,000 g (30 min.). The washed pellet was resuspended in storage buffer at a concentration of 20–60 mg·ml−1 and stored at −15°C. Protein content was determined according to the method of Lowry et al. (12).

Testosterone hydroxylation in liver microsomes. The assay methodology was based on that described by Sonderfan (10). Micromolar incubations were performed in 10-ml screw-top, round bottomed glass tubes (BDH Ltd., Poole, UK). All glassware was silanised using Repelcote (BDH). Testosterone (25 μl of a 10 mM solution in methanol) was added in the test tube and was followed by testosterone metabolism buffer (TMB, 50 mM phosphate buffer, pH 7.4 containing 5 mM MgCl2) and microsomal protein (1 mg) to make a final volume of 750 μl. The tubes were then shaken in a water bath (37°C) for 5 min before the reaction volume was made up to a total of 1ml by addition of freshly prepared NADPH generating system (0.8 mg NADP, 1.6 mg isocitric acid, 1
and distilled water (35% v/v), and acetonitrile (1.5% v/v). The mobile phase flow rate was 1ml·min⁻¹ with a gradient program of 25% B during 0–10 min; 50.5%–70% B during 18.5–25 min; 70–100% B during 25–35 min; 100% B during 35–50% min; 100–25% B during 50–60 min, and 25% B during 60–70 min. The flow rate typically generated a back pressure of 2500 psi.

The HPLC system (LKB, Pharmacia) used a variable wavelength UV monitor at 254 nm (8) and a Waters Novapack C18 3.9 × 150mm, 4μm column with guard column. The retention times of the hydroxysterosterone metabolite standards and testosterone were 6β = 9.6 min, 15α = 10.5 min, 7α = 11.6 min, 16α = 16.5 min, 16β = 18.7 min, 11β = 20.8 min (internal standard), 2α = 21.6 min, 2β = 22.5 min, androstenedione = 27.4, and testosterone = 29.5 min.

Western blotting. Heparic microsomal proteins were resolved by SDS-PAGE with vertical mini-gel electrophoresis equipment (Life Technologies, Ltd., Paisley, Scotland) using an adaptation of a previously described method (15). Samples of liver microsomal protein (10 μg per lane) were denatured in 10 μl loading buffer (4 ml distilled water, 1 ml 0.5M Tris-HCl pH 6.8, 0.8 ml glycerol, 1.6ml 10% w/v SDS, 0.4 ml β-mercaptoethanol, 0.05 ml 0.05% w/v Pyronin Y) and were separated on a 10% w/v resolving gel. Proteins were transferred from the polyacrylamide gel to the nitrocellulose sheets by an electrophoretic method (16). Rabbit anti-rat CYP3A polyclonal antibody (not cross-reactive with other rat protein) was a gift from Dr. Colin Henderson, University of Dundee. CYP3A approtein was detected by secondary conjugation to the primary antibody by a horseradish peroxidase-linked sheep anti-rabbit antibody using diaminobenzidine as substrate.

Statistical analysis. Testosterone hydroxylation activity was linear with respect to protein (0–2 mg) and time (0–10 min). Raw data was transformed into logarithms due to nonconstant variance. Statistical analysis was carried out using Dunnett’s t test (where multiple treatments are compared with saline control). Differences with a p value equal or less than 0.05 were taken to be significant.

Results

Testosterone metabolism. The effect of drug administration on testosterone hydroxylation activities is shown in table 1. RU 486 increased testosterone 2β-hydroxylation (p < 0.01) and 6β-hydroxylation (p < 0.01) approximately 5-fold (table 1). Testosterone 15β-hydroxylation also increased after RU 486 administration (203 pmol/mg-min in liver microsomes from rats treated with RU 486 compared with undetectable activity in hepatic microsomes from treated animals). Dexamethasone increased testosterone 6β-and 2β-hydroxylation activities 15- to 20-fold, as did administration of dexamethasone in combination with RU 486. SR-12813 increased testosterone 6β-hydroxylation 9-fold (p < 0.01) and also increased hydroxylation activity at the 15β (p < 0.01) and 2β (p < 0.01) positions.

Western blot analysis using an anti-CYP3A antibody. To confirm the induction of CYP3A by RU 486 and SR-12813 CYP3A enzyme protein levels were determined by Western blotting (figs. 2 and 3). In fig. 2 the absence of signal in lanes 1 and 2 (saline treatment) correlates with the very low constitutive level of dexamethasone only. Administration of RU 486 induced immunoreactive CYP3A protein as shown in lanes 3 and 4. The greater intensity of immunoreactive staining in lanes 7 and 8 confirms the induction of CYP3A enzyme levels by SR-12813. In both figs. 2 and 3, the levels of enzyme induction by dexamethasone over control are similar (15- to 20-fold). Inter-individual variation in immunoprecipitable CYP3A protein (and testosterone 6β-hydroxylation) increases with the level of induction, as exemplified by the
differences in specific activities (2858 to 4336 pmol mg⁻¹ min⁻¹) reported in figs. 1 and 2 after dexamethasone treatment and the large SD in specific activities after dexamethasone RU 486 treatment in table 1.

Discussion

The antiglucocorticoid RU 486 and the hypocholesterolaemic drug SR-12813 increase hepatic levels of CYP3A enzymes in the female rat. Evidence from previous CYP3A induction studies on the effects of antiglucocorticoids (17), and hypocholesterolaemic drugs (9) suggests these drugs increase CYP3A levels via separate mechanisms.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lane No.</th>
<th>Testosterone 6β-hydroxylase activity pmoles mg protein⁻¹ min⁻¹</th>
</tr>
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<tbody>
<tr>
<td>Saline (N = 7)</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Olive oil (N = 3)</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SR-12813 (N = 4)</td>
<td></td>
<td>483 ± 117**</td>
</tr>
<tr>
<td>RU 486 (N = 4)</td>
<td></td>
<td>203 ± 58**</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td></td>
<td>663 ± 157**</td>
</tr>
<tr>
<td>(N = 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td></td>
<td>737 ± 461**</td>
</tr>
<tr>
<td>+ RU 486 (N = 4)</td>
<td></td>
<td>15β  6β  2β  16α  16β</td>
</tr>
<tr>
<td>Saline (N = 7)</td>
<td></td>
<td>0 227 ± 76 70 ± 79 136 ± 74 101 ± 59</td>
</tr>
<tr>
<td>Olive oil (N = 3)</td>
<td></td>
<td>0 198 ± 28 0 171 ± 3 89 ± 19</td>
</tr>
<tr>
<td>SR-12813 (N = 4)</td>
<td></td>
<td>1889 ± 330**</td>
</tr>
<tr>
<td>RU 486 (N = 4)</td>
<td></td>
<td>909 ± 304**</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td></td>
<td>3301 ± 703**</td>
</tr>
<tr>
<td>(N = 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td></td>
<td>3663 ± 1646**</td>
</tr>
<tr>
<td>+ RU 486 (N = 4)</td>
<td></td>
<td>1358 ± 129**</td>
</tr>
</tbody>
</table>

Activities (pmol/mg/min) for each sample of hepatic microsomes were made in duplicate from each liver (N = no. animals per group). Values represent the means ± SD. Statistical analysis comparing multiple treatments against saline control was made using one-way ANOVA followed by Dunnett’s t test (*p < 0.05, **p < 0.01).

Fig. 2. Effect of RU 486 on induction of hepatic microsomal CYP3A enzymes by dexamethasone as shown by immunoblot analysis with a polyclonal anti-CYP3A antibody.

Immunoreactive protein and testosterone hydroxylation activity was determined in hepatic microsomes from 8 individual rats (2 per treatment group). For immunoblot analysis microsomal protein (10 μg) was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose, as described in Materials and Methods. Testosterone hydroxylation activity was also determined in liver microsomes from the same batch, as described in Materials and Methods.

Differences in specific activities (2858 to 4336 pmol·mg⁻¹·min⁻¹) reported in figs. 1 and 2 after dexamethasone treatment and the large SD in specific activities after dexamethasone + RU 486 treatment in table 1.

Fig. 3. Effect of SR-12813 on hepatic microsomal CYP3A levels as shown by immunoblot analysis with polyclonal anti-CYP3A antibody.

Immunoreactive protein and testosterone hydroxylation activity was determined in hepatic microsomes from 8 individual rats (2 per treatment group). For immunoblot analysis microsomal protein (10 μg) was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose, as described in Materials and Methods. Testosterone hydroxylation activity was also determined in liver microsomes from the same batch, as described in Materials and Methods.

The induction of CYP3A gene expression by an intra-cellular species that stereospecifically recognizes glucocorticoids like dexamethasone and anti-glucocorticoids such as pregnenolone 16α-carbonitrile led to the proposal of a “non-classical” mechanism for the involvement of the glucocorticoid receptor in this process (17). The inducing effect of RU 486 on CYP3A expression observed in this study fits in with this hypothesis, especially in the light of evidence that confirms the antagonism by RU 486 of dexamethasone-induction of genes regulated by glucocorticoids (18). The anti-glucocorticoid, pregnenolone 16α-carbonitrile, also induces CYP3A1 gene transcription (19). The observation that RU 486 induces CYP3A mRNA levels...
in rat, human, and rabbit hepatocytes (20, 21) suggests that the mechanism responsible is conserved across mammalian species. The hypothesis that nonsteroidal compounds achieve their inducing effects by increasing the level of circulating endogenous glucocorticoids in the liver was tested by comparing drug effects in vivo and in vitro (22). This study revealed that phenobarbital and phenobarbital-like inducers of CYP3A induce enzyme levels directly on primary cultures of hepatocyte monolayers. A subsequent study showed a dose differentiation of drug effect for induction of CYP2B and CYP3A genes mediated by phenobarbital, clotrimazole, and trans-nonachlor (23). These results implicate multiple mechanisms for induction of these enzymes, which may involve intracellular glucocorticoids (RU 486 antagonizes the phenobarbital-induction of CYP2C6 mRNA in mouse hepatoma cells (24).

Kocarek et al. (9) reported the induction of CYP3A1-mediated gene expression by the HMG-CoA reductase inhibitor lovastatin. Lovastatin induced rat CYP3A1 gene expression directly in primary cultures of rat hepatocytes and potentiates the phenobarbital- and clotrimazole-mediated induction but not dexamethasone-mediated CYP3A1 induction in rat hepatocytes (9), once more indicating that multiple mechanisms are responsible for CYP3A1 induction. Two other “stain” drugs, simvastatin (25) and pravastatin (8, 25), increase the urinary excretion ratio of 6β-hydroxy cortisol/cortisol in man, an in vivo marker of CYP3A activity. Lovastatin also induced CYP3A gene expression in human and rabbit hepatocytes (21). HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis and its activity is reduced by lovastatin (25) via enzyme inhibition at the active site and by SR-12813 (26) via an increased degradation of the enzyme. As SR-12813 is also a potent inducer of CYP3A activity, it seems possible that there may be an association between the control of cholesterol biosynthesis and the induction of CYP3A gene expression. This hypothesis is supported by the fact that either mevalonate, the immediate product of HMG-CoA reductase, or the regulatory oxysterol 25-hydroxycholesterol suppress the lovastatin-mediated induction of CYP3A1 mRNA (9). Oxysterols have an intracellular role in the suppression of genes coding for proteins that maintain balanced cellular cholesterol levels, including HMG-CoA reductase, the LDL receptor protein (27) and most interestingly lanosterol 14a-demethylase coded by the P450 gene CYP51 (28). It is possible that CYP3A gene expression is also negatively regulated by oxysterols. In that case both SR-12813 and lovastatin would induce CYP3A gene expression by removing the suppressive influence of successive products of HMG-CoA reductase from mevalonate onwards to oxysterols (29).

The use of cultured hepatocytes would provide an isolated system to investigate whether phenobarbital but not dexamethasone induction of CYP3A1 expression is potentiated by SR-12813 (as in the case of lovastatin (9)). These studies would provide direct evidence for separate control mechanisms for CYP3A gene expression. Information in this paper shows that the antiglucocorticoid RU 486 also induces CYP3A enzyme levels. This compound is structurally and pharmacologically unrelated to SR-12813 but achieves a similar effect. Furthermore, another antiglucocorticoid, pregnenolone 16α-carbonitrile, is an inducer of CYP3A enzymes in the rat to the same level as the glucocorticoid dexamethasone (17). The existence of multiple mechanisms of control and the involvement of intracellular messengers such as oxysterols may point to an endogenous role for these enzymes in addition to the distinct xenobiotic metabolizing role that these enzymes have.

The results in this paper clearly show that SR-12813, a member of a new class of hypocholesterolaemic drugs (bisphosphonates) induces levels of hepatic CYP3A enzymes. Further research is required to establish whether the CYP3A inducing property of SR-12813 extends to other bisphosphonates and the mechanism responsible for their inducing effect.

Acknowledgments. Thanks to Dr. B. J. Blaauwboer for kindly providing the 15β-hydroxytestosterone. Thanks also to Dr. Sean Ekins for helpful suggestions in preparing this manuscript.

References


