Among the human liver cytochrome P450 (P450) enzymes, CYP3A4 is present in the highest concentration and responsible for the metabolism of various kinds of drugs. For this reason, it is considered an important isozyme for the investigation of drug interactions via P450. In a previous study, we reported that mexazolam, a benzodiazepine antianxiety agent, was metabolized by CYP3A4 (Ono et al., 1993). Using mexazolam in a male and the inhibition rat liver microsomes was investigated and compared with that by HMG-CoA reductase inhibitors in human liver microsomes reported previously. The metabolism of mexazolam in male and female rat liver microsomes was inhibited by all the HMG-CoA reductase inhibitors examined except pravastatin (acid). The Kᵣ values in female rats were lower than those in male rats, demonstrating the presence of a sex difference in the inhibition potency of HMG-CoA reductase inhibitors toward mexazolam. Using anti-cytochrome P450 (P450) antisera, the main P450 isozyme responsible for the metabolism of mexazolam was identified as CYP3A in female rats and CYP2C11 in male rats. Based on these results, we speculate that the sex difference in the inhibition potency of HMG-CoA reductase inhibitors for mexazolam observed in rats is caused by their different inhibition potencies against CYP2C11 and CYP3A isoforms. For mexazolam metabolism, the results obtained in female rats, rather than those in male rats, seem to be a much better reflection of the results in humans. Since species and sex differences were observed in P450 isoforms in the present study, our results show that establishing appropriate experimental conditions, in particular with respect to the P450 isoforms responsible for the drug metabolism in question, is indispensable for the investigation of drug interactions using rats as a model animal for humans.

1 Abbreviations used are: P450, cytochrome P450; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors [simvastatin (lactone), simvastatin acid, fluvastatin, atorvastatin, cerivastatin, pravastatin lactone, and pravastatin (acid)]; Dex, dexamethasone; HPLC, high-performance liquid chromatography; Clᵣᵣ, intrinsic metabolic clearance.
In the present study, taking into account the sex difference in the drug interactions observed between simvastatin and itraconazole, the inhibition of mexazolam metabolism by HMG-CoA reductase inhibitors (Fig. 1) was investigated in an in vitro system using female and male rat liver microsomes, and the results were compared with those obtained in humans to identify an appropriate animal model for the study of drug interaction via CYP3A4 inhibition. In addition, the inhibition of mexazolam metabolism by HMG-CoA reductase inhibitors and itraconazole was also investigated under the condition in which the content of P450 isozymes was changed, using liver microsomes of rats treated with dexamethasone, which is known to induce CYP3A in rats (Ghosal et al., 1996a).

Materials and Methods

Chemicals and Reagents. Pravastatin (acid), pravastatin lactone, simvastatin (lactone), simvastatin acid (Na salt), fluvastatin (acid), atorvastatin (acid), cerivastatin (acid), itraconazole, mexazolam, and a metabolite of mexazolam (M-1; Fig. 2) used in the present study were synthesized at Sankyo Co., Ltd. (Tokyo, Japan). Anti-rat P450 antisera preparations (anti-rat CYP2C11 prepared from goat serum and anti-rat CYP3A2 prepared from rabbit serum) were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals and reagents used were commercially available and of reagent grade.

Preparation of Rat Liver Microsomes. Rat liver microsomes were prepared from male and female Sprague-Dawley rats (Japan SLC, Hamamatsu,
An ethanol solution of mexazolam was added to each reaction mixture to make units glucose-6-phosphate dehydrogenase, and 10 mM MgCl2 was added to rat liver microsomes (0.2 mg of protein/ml) in a total volume of 0.5 ml. The reaction was stopped by vortex mixing to stop the reaction and centrifugation at 19,000 g for 2 min. The supernatants obtained after centrifugation were also prepared in the same manner as described above from livers of female and male rats, 24 h after a single intraperitoneal administration of dexamethasone (100 mg/kg, corn oil) according to standard methods. To each isolated liver sample was added mexazolam (20 μM) and phosphate buffer containing potassium chloride at a concentration of 1.15%, and the resulting mixture was homogenized and then centrifuged at 9,000g for 20 min. The supernatant was centrifuged again at 105,000 g for 1 h, and phosphate buffer containing glycerol was added to the obtained pellet to prepare a microsomal suspension. Liver microsomes were also prepared in the same manner as described above from livers of female and male rats, 24 h after a single intraperitoneal administration of dexamethasone (100 mg/kg, corn oil solution) (Dex-treated rats).

Metabolism of Mexazolam in Rat Liver Microsomes. An NADPH-generating system containing 2.5 mM NADP, 25 mM glucose 6-phosphate, 2 units glucose-6-phosphate dehydrogenase, and 10 mM MgCl2 was added to rat liver microsomes (0.2 mg of protein/ml) in a total volume of 0.2 ml, and the resulting mixtures were preincubated at 37°C for 3 min then 2 μl of an ethanol solution of mexazolam was added to each reaction mixture to make concentrations of 5 to 100 μM. After incubation at 37°C for 2 min, 0.4 ml of methanol was added followed by vortex mixing to stop the reaction and centrifugation at 19,000g for 3 min. The amount of mexazolam metabolite (M-1; Fig. 2) in the supernatants was analyzed by high-performance liquid chromatography (HPLC). For the HPLC operating conditions, a Deversil (M-1; Fig. 2) in the supernatants was analyzed by high-performance liquid chromatography (HPLC). For the HPLC operating conditions, a Deversil ODS-UG-5 column (250 × 4.6 mm; Nomura Chemical Co., Ltd., Aichi, Japan) was used.

Inhibition of Mexazolam Metabolism by Anti-P450 Antisera. To identify the enzymes responsible for the metabolism of mexazolam, inhibition experiments using anti-rat P450 antisera were performed. Anti-rat P450 antisera or control sera (0–0.25 mg of IgG; 25 μl) were added to rat liver microsomes (pooled from five male rats or five female rats, 10 mg of protein/ml, 10 μl) separately, and the resulting mixtures were preincubated at room temperature for 30 min. Then, as described above for the metabolism of mexazolam in rat liver microsomes, an NADPH-generating system was added to each mixture, and the resulting solutions were preincubated at 37°C for 3 min. Subsequently, an ethanolic solution of mexazolam was added (final concentration, 20 μM mexazolam) and incubated at 37°C for 2 min (0.2 μg of protein/ml; volume of reaction mixture, 0.5 ml). The reaction was stopped by the addition of 1 ml of methanol and the amount of metabolite (M-1) formed in the supernatants obtained after centrifugation was determined by HPLC.

Inhibition of Mexazolam Metabolism by HMG-CoA Reductase Inhibitors and Itraconazole. To 0.2 ml of each of male and female rat liver microsomal preparations (0.2 mg of protein/ml), 2 μl of an ethanol solution of each of the HMG-CoA reductase inhibitors [pravastatin (acid), pravastatin lactone, simvastatin acid, fluvastatin (acid), atorvastatin (acid), and cerivastatin (acid), 20 to 200 μM; and simvastatin (lactone), 2 to 20 μM] was added separately. The resulting mixtures were preincubated at 37°C for 3 min, and an ethanol solution of mexazolam (2 μl) was added to each microsomal prepa-

**Fig. 4.** Effects of anti-P450 antisera on the formation of M-1 from mexazolam in Dex-treated male (a) and female (b) rat liver microsomes.

**Table 1.** Kinetics of mexazolam metabolite formation in rat liver microsomes.

<table>
<thead>
<tr>
<th></th>
<th>Control Rat</th>
<th>Dex-Treated Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (μM)</td>
<td>Vmax (nmol/min/mg protein)</td>
</tr>
<tr>
<td>Male</td>
<td>10.7 ± 0.4</td>
<td>3.23 ± 0.04</td>
</tr>
<tr>
<td>Female</td>
<td>10.3 ± 0.8</td>
<td>0.29 ± 0.01</td>
</tr>
</tbody>
</table>

|                  | Km (μM)     | Vmax (nmol/min/mg protein) | Clint (ml/min/mg) |
| Male             | 10.6 ± 0.7  | 8.66 ± 0.17     | 1.22  |
| Female           | 11.7 ± 0.5  | 11.2 ± 0.1      | 1.04  |
FIG. 6. Dixon plots for the inhibition of M-1 formation from mexazolam in male (a) and female (b) rat liver microsomes by HMG-CoA reductase inhibitors.

Mexazolam (5–50 μM) was incubated for 2 min at 37°C with rat liver microsomes (one male rat or one female rat, 0.2 mg of protein/ml) in the presence or absence of HMG-CoA reductase inhibitors [pravastatin (acid), 50–400 μM; simvastatin (lactone), 5–20 μM; pravastatin lactone, simvastatin acid, fluvastatin (acid), atorvastatin (acid), and cerivastatin (acid), 20–200 μM]; ○, 5 μM mexazolam; ◊, 10 μM mexazolam; □, 20 μM mexazolam; ▲, 50 μM mexazolam. $V_0$, M-1 formation rate (nanomoles per minute per milligram of protein).
ration to give a final concentration of 10 to 50 μM. The amount of metabolite, M-1, formed in each reaction mixture was determined in the same manner as described above for the metabolism of mexazolam in rat liver microsomes. Inhibition by itraconazole was also examined in the same manner as mentioned above by adding 2 μL of a dimethylacetamide solution of itraconazole (0.1–1 μM) to each of the male and female rat liver microsomal preparations, separately.

Calculation of inhibition constant (K_i) values from Dixon plots was performed by the simultaneous fitting of the data to the following equation using the WinNonlin program.

**HMG-CoA reductase inhibitors (competitive inhibition):**

\[
\frac{1}{V_o} = \frac{1}{V_{max}} + \frac{K_i}{S} + \frac{1}{V_{max}} \times \frac{I}{K_m}
\]

Itraconazole (noncompetitive inhibition):

\[
\frac{1}{V_o} = \frac{1}{V_{max}} + \frac{I}{K_m} + \frac{S}{V_{max}}
\]

where S and I represent the concentration of the substrate (mexazolam) and each inhibitor (HMG-CoA reductase inhibitors or itraconazole), respectively.

**Results**

**Effects of Anti-P450 Antisera on Mexazolam Metabolism in Rat Liver Microsomes.** The mexazolam metabolism in male rat liver microsomes was inhibited by about 80% after the addition of anti-CYP2C11 antiserum (0.25 mg as IgG), whereas it was scarcely affected by the addition of anti-CYP3A2 antiserum (Fig. 3a). On the other hand, the mexazolam metabolism in female rat liver microsomes was inhibited by about 80% after the addition of anti-CYP3A2 antiserum (0.25 mg as IgG) but was scarcely affected by the addition of anti-CYP2C11 antiserum (Fig. 3b). In the Dex-treated rats, however, the mexazolam metabolism in liver microsomes was inhibited by about 80% in male rats and by about 90% in female rats, after the addition of anti-CYP3A2 antiserum (0.25 mg as IgG). In addition, following the addition of anti-CYP2C11 antiserum (0.25 mg as IgG), the metabolism in male and female rats was also inhibited by about 40% and by about 50%, respectively (Fig. 4, a and b).

**Evaluation of Kinetic Parameters for Mexazolam Metabolism in Rat Liver Microsomes.** The concentration dependence of mexazolam metabolism in control and Dex-treated rat liver microsomes was examined by Eadie-Hofstee plots in Fig. 5. As can be seen, saturation was reached in the formation of mexazolam metabolite, M-1. The calculated kinetic parameters of K_m and V_max are summarized in Table 1. In the control rats, the calculated K_m values in female and male rats were almost identical, but the V_max value and the CLint in male rats were about 11 times higher than those in female rats. In the Dex-treated rats as well, the calculated K_m values in the female and male rats were almost identical, and no difference was observed between the control and Dex-treated rats. The V_max value in the Dex-treated female rats was about 1.3 times higher than that in the Dex-treated male rats, and the V_max values in the Dex-treated female and male rats were higher than those in the control female and male rats by about 39- and 2.7-fold, respectively. The intrinsic clearance values in Dex-treated female and male rats were also higher than those obtained in the control female and male rats by about 37- and 4.0-fold, respectively.

**Effects of HMG-CoA Reductase Inhibitors on Mexazolam Metabolism in Rat Liver Microsomes.** The K_i values of various HMG-CoA reductase inhibitors for mexazolam metabolism in female and male rat liver microsomes were obtained from Dixon plots (Fig. 6, a and b) and summarized in Table 2. Mexazolam metabolism in female rat liver microsomes was not inhibited by pravastatin (acid) but was inhibited competitively by other HMG-CoA reductase inhibitors (Fig. 6b). The order of inhibition potency of these HMG-CoA reductase inhibitors observed, in decreasing order, was simvastatin (lactone), pravastatin (lactone), atorvastatin (acid), cerivastatin (acid), fluvastatin (acid), and simvastatin acid. On the other hand, mexazolam metabolism in male rat liver microsomes was inhibited by all the HMG-CoA reductase inhibitors except simvastatin acid and pravastatin (acid) (Fig. 6a). The order of inhibition potency in decreasing order was simvastatin (lactone), atorvastatin (acid), cerivastatin (acid), fluvastatin (acid), and pravastatin (lactone), demonstrating that the order of inhibition potency differed between female and male rats. Furthermore, the HMG-CoA reductase inhibitors were less potent as inhibitors of mexazolam metabolism in male rat liver microsomes than in female rat liver microsomes (Table 2). In the Dex-treated female and male rat liver microsomes, mexazolam metabolism was inhibited competitively by simvastatin (lactone), pravastatin (lactone), and simvastatin acid but was scarcely affected by pravastatin (acid) (Fig. 7, a and b).

**Effects of Itraconazole on Mexazolam Metabolism in Rat Liver Microsomes.** The formation of mexazolam metabolite in control female rat liver microsomes was considered to be inhibited by the dimethylacetamide used to dissolve the itraconazole, and the amount of metabolite formed was less than the detection limit. The mexazolam metabolism in male rat liver microsomes was not inhibited by itraconazole (Fig. 8a). On the other hand, in the Dex-treated female and male rat liver microsomes, mexazolam metabolism was noncompetitively inhibited by itraconazole, with K_i values of 0.693 μM in female rats and 0.877 μM in male rats (Fig. 8, b and c).

**Discussion**

We have previously reported that mexazolam is metabolized mainly by CYP3A4 in humans (Ono et al., 1993) and that this metabolism by human liver microsomes is inhibited by a variety of HMG-CoA reductase inhibitors (Ishigami et al., 2001a). In the present study, we investigated the inhibition of mexazolam metabolism in male and female rat liver microsomes in vitro by HMG-CoA reductase inhibitors and compared the results with those previously re-
ported in humans to identify an appropriate animal model for the study of drug interaction via CYP3A inhibition. Experiments using anti-rat P450 antisera demonstrated that the P450 isozymes responsible for mexazolam metabolism in rat liver microsomes differ between female and male rats. In the male rat liver microsomes, CYP2C11 was the major contributor to mexazolam metabolism, whereas in the female rat liver microsomes, the CYP3A isoforms were the main contributors (Fig. 3).

In mature female rat liver, CYP3A2 and CYP3A1 are reported to be expressed at extremely low levels (Cooper et al., 1993). Therefore, the significantly lower $V_{\text{max}}$ value for mexazolam metabolism in female rats than that in male rats may be due to the low expression level of the CYP3A isoforms responsible for mexazolam metabolism in female rats. On the other hand, CYP2C11, the male rat-specific P450 isozyme is expressed in the liver to a greater extent than the CYP3A isoforms, leading to a much higher level of mexazolam metabolism in

**Fig. 7.** Dixon plots for the inhibition of M-1 formation from mexazolam in Dex-treated male (a) and Dex-treated female (b) rat liver microsomes by simvastatin (lactone), simvastatin acid, pravastatin lactone, and pravastatin (acid). Mexazolam (5–50 μM) was incubated for 2 min at 37°C with pooled rat liver microsomes (5 Dex-treated male rats or 5 Dex-treated female rats; 0.2 mg of protein/ml) in the presence or absence of simvastatin acid, pravastatin lactone, and pravastatin (acid) (20–200 μM); and simvastatin (lactone) (5–20 μM); □, 5 μM mexazolam; ▲, 10 μM mexazolam; □, 20 μM mexazolam; ▲, 50 μM mexazolam. $V_{\text{max}}$, M-1 formation rate (nanomoles per minute per milligram of protein).
Mexazolam (5–50 μM) was incubated for 2 min at 37°C with pooled rat liver microsomes (5 male rats, 5 Dex-treated male rats, or 5 Dex-treated female rats; 0.2 mg of protein/ml) in the presence or absence of itraconazole (0.1–1 μM). □, 5 μM mexazolam; △, 10 μM mexazolam; □, 20 μM mexazolam; △, 50 μM mexazolam. V_{O2}, M-1 formation rate (nanomoles per minute per milligram of protein).

CYP2C11 isozyme also appeared to participate in mexazolam metabolism in Dex-treated rats (Fig. 4). It has been reported that CYP3A1, CYP3A2, and CYP2C11 are induced by dexamethasone in female rats, whereas in male rats, CYP3A1 and CYP3A2 are induced, but CYP2C11 is reduced by dexamethasone (Ghosal et al., 1996a). These findings are consistent with the results obtained in the present study.

Mexazolam metabolism in male rats was not inhibited by itraconazole, whereas in the Dex-treated rats, mexazolam metabolism was inhibited by itraconazole in both female and male rats. Furthermore, we previously found that itraconazole did not inhibit simvastatin metabolism in male rats but inhibited it in female rats (Ishigami et al., 2001b), which was consistent with the results obtained in the present study. Taking into account that the midazolam metabolism mediated by CYP3A2 in male rats (Ghosal et al., 1996b) was inhibited by itraconazole (Yamano et al., 1999), the sex difference in the inhibition by itraconazole, like simvastatin acid, may be due to the considerably higher inhibition potency of itraconazole against CYP3A than CYP2C11.

In conclusion, since species and sex differences are observed in P450 isozymes, appropriate experimental conditions that take into account P450 isozymes responsible for the drug metabolism in question will be indispensable for the investigation of drug interactions using rats as a model animal for humans.

References


