20β-Hydroxysteroid Dehydrogenase Catalyzes Ketone-Reduction of Acetohexamide, an Oral Antidiabetic Drug, in Liver Microsomes of Adult Male Rats

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ABSTRACT

We examined the catalytic properties and physiological function of an enzyme responsible for the ketone-reduction of acetohexamide, an oral antidiabetic drug, in liver microsomes of adult male rats. Progesterone, 17α-hydroxyprogesterone, cortisol and cortisone, which have a ketone group at 20-position of C21-steroids, were potent inhibitors for ketone-reduction of acetohexamide in liver microsomes of adult male rats. Progesterone was also found to inhibit competitively the ketone-reduction of acetohexamide, suggesting that the ketone-reduction of acetohexamide and progesterone is catalyzed by the same enzyme. When progesterone was used as a substrate, 20β-hydroxysteroid dehydrogenase present in liver microsomes of adult rats, such as acetohexamide reductase, exhibited a male-specific and androgen-dependent activity. Furthermore, a significant correlation was observed between the activities of 20β-hydroxysteroid dehydrogenase and acetohexamide reductase in liver microsomes of individual male rats at various ages. Based on all results, we conclude that 20β-hydroxysteroid dehydrogenase catalyzes the ketone-reduction of acetohexamide in liver microsomes of adult male rats.

Acetohexamide, 4-acetyl-N-(cyclohexylcarbamoyl)benzene-sulfonamide, is an oral antidiabetic drug that has a ketone group within its chemical structure. This drug is mainly reduced to the corresponding alcohol metabolite, (−)-hydroxyacetohexamide, through enzymatic system in humans and animals (McMahon et al., 1965; Imamura et al., 1988). We have demonstrated that the activity of acetohexamide-reducing enzyme (acetohexamide reductase) in liver microsomes of adult rats is much higher in the males than in the females, and is regulated by androgens (Imamura et al., 1987, 1993). Our previous paper (Imamura et al., 1993) has also shown that even in male rats, the activity of the microsomal acetohexamide reductase is not detectable until 4 wk of age after birth, although it markedly increases during pubertal period to reach the adult level. It is most likely that a male-specific and androgen-dependent enzyme, which is responsible for biosynthesis or metabolism of endogenous carboxyl compounds, has the ability to reduce acetohexamide.

20β-HSD (EC 1.1.1.53) is known to catalyze the stereoselective reduction of C21-steroids with a ketone group at 20-position such as progesterone (4-pregnene-3,20-dione) and 17α-hydroxyprogesterone (4-pregnene-17α-ol-3,20-dione). For example, 4-pregnene-17α,20β-diol-3-one (17α,20β-dihydroxy-4-pregnen-3-one) is produced from 17α-hydroxyprogesterone by 20β-HSD (Nakajin et al., 1988, 1989). Interestingly, 4-pregnene-17α,20β-diol-3-one has been identified as maturation-inducing hormone in oocytes of several fish species (Nagahama and Adachi, 1985; Young et al., 1986; Nagahama, 1997), and the detailed molecular mechanisms of oocyte maturation-inducing hormone by this steroid have been proposed (Nagahama, 1997). These reports demonstrate that in fish species, 20β-HSD plays an important role in the induction of oocyte maturation. Furthermore, 20β-hydroxy-C21-steroids have been found in various organs of mammalian species (Tanaka et al., 1992). However, the physiological function of 20β-HSD present in various organs of mammalian species is poorly understood. Recently, 20β-HSD present in liver microsomes of adult rats, as well as acetohexamide reductase present in liver microsomes of adult rats described above, has been reported to exhibit a male-specific activity (Apanovitch et al., 1992; Apanovitch and Walz, 1996). In our study, we describe evidence that the ketone-reduction of acetohexamide in liver microsomes of adult male rats is catalyzed by 20β-HSD.

Materials and Methods

Materials. Acetohexamide was a gift from Shionogi Co. (Osaka, Japan). Hydroxyacetohexamide was synthesized from acetohexamide according to the method of Girgis-Takla and Chronoes (1979). Proges-

ABBREVIATIONS: 20α- and 20β-HSD, 20α-and 20β-hydroxysteroid dehydrogenase; CYP, cytochrome P450; ANOVA, analysis of variance; HPLC, high-performance liquid chromatography.
terone (4-pregnen-3,20-dione), 4-pregnen-20α-ol-3-one and 4-pregnen-20α-ol-3-one were purchased from Sigma Chemical Co. (St. Louis, MO). Testosterone propionate was obtained from Nacalai Tesque (Kyoto, Japan). Other steroids, which were used as inhibitors, were purchased from Sigma. NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co. (Tokyo, Japan). S-Warfarin was obtained from Daicichi Pure Chemicals Co. (Tokyo, Japan). All other chemicals were of reagent grade.

**Animals and treatments.** Male Fischer-344 (Fischer) rats at 4, 6, 7, 9, 12 and 15 wk of age and female Fischer rats at 9 wk of age were purchased from Japan SLC (Shizuoka, Japan). Testectomy of male rats was performed at 6 wk of age. The testectomized rats were raised up to 9 wk of age under controlled lighting, temperature and humidity. Testosterone propionate (10 mg/kg body weight) dissolved in 0.5 ml of corn oil was injected s.c. to the testectomized rats once every day for 7 days before they were killed by decapitation, and corn oil alone was given to control animals.

**Preparation of liver microsomes.** Male and female rats were killed by decapitation. After perfusion with ice-cold 1.15% KCl solution, the livers were immediately removed and homogenized in a Potter-Elvehjem homogenizer with 3 volumes of 10 mM phosphate buffer containing 1.15% KCl (pH 7.4). All subsequent procedures were performed at 3 to 5°C. The homogenates were centrifuged at 10,000 × g for 20 min and the resulting supernatants were centrifuged at 105,000 × g for 60 min to obtain the microsomal pellets. The microsomal pellets were suspended in 10 mM phosphate buffer containing 1.15% KCl (pH 7.4) and were recentrifuged at 105,000 × g for 60 min. The microsomal pellets obtained were used for the assay of enzyme activity.

**Assay of acetohexamide reductase activity.** The assay of acetohexamide reductase activity was conducted in an NADPH-generating system consisting of acetohexamide (1.0 mM), NADP (0.25 mM), glucose-6-phosphate (6.25 mM), glucose-6-phosphate dehydrogenase (0.25 U), MgCl₂ (6.25 mM), enzyme (microsomal suspension) and 100 mM phosphate buffer (pH 7.4) in a final volume of 2.0 ml. The reaction mixture was incubated at 37°C for 10 min and the reaction was stopped by adding 0.5 ml of 1.0 N HCl to the mixture. The reduction product was determined by HPLC (Takagi et al., 1979).

**Assay of 20α- and 20β-HSD activities.** The assay of 20α- and 20β-HSD activities was conducted in an NADPH-generating system consisting of progesterone (0.1 mM), NADP (0.25 mM), glucose-6-phosphate (6.25 mM), glucose-6-phosphate dehydrogenase (0.25 U), MgCl₂ (6.25 mM), enzyme (microsomal suspension) and 100 mM phosphate buffer (pH 7.4) in a final volume of 2.0 ml. The reaction mixture was incubated at 37°C for 10 min and the reaction was stopped by adding 0.5 ml of 1.0 N HCl to the mixture. The reduction products were determined by HPLC according to a slightly modified method of Swinney et al. (1987). The reaction mixture was extracted with 5.0 ml of benzene-chloroform mixture (5:1, v/v). The organic layer (4.0 ml) was removed and evaporated in vacuo. The residue was dissolved in acetonitrile (0.2 ml) and subjected to HPLC. HPLC was carried out using a Hitachi 655A-11 HPLC apparatus equipped with a ODS column and a Hitachi 620-41 UV monitor (244 nm). Mixture of water-acetonitrile-methanol-tetrahydrofuran (44:28:17:11, v/v) was used as a mobile phase at a flow rate of 0.6 ml/min. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

**Statistical analysis.** Student’s t test was used to analyze differences between two groups. ANOVA was used to analyze differences among more than two groups, and the significance of difference between two means in these groups was evaluated using Duncan’s multiple range test. P ≤ 0.05 was considered to be significant.

**Results**

**Inhibitory effects of various steroids on acetohexamide reductase activity.** Table 1 summarizes the inhibitory effects of various steroids on acetohexamide reductase activity in liver microsomes of adult male rats at 9 wk of age. Of these steroids tested, only C₂₃-steroids with a ketone group at 20-position such as progesterone, 17α-hydroxyprogesterone, cortisone (4-pregnen-17α,21-diol-3,11,20-trione) and cortisol (4-pregnen-11β, 17α, 21-triol-3, 20-dione) potently inhibited the enzyme activity. Furthermore, the inhibitory effect of progesterone on the hepatic microsomal acetohexamide reductase activity was kinetically examined. As shown in figure 1, progesterone was a competitive inhibitor of the enzyme with respect to the substrate (acetohexamide).

**Sex-related difference and hormonal regulation of 20α- and 20β-HSD activities.** 20β-HSD activity in liver microsomes of adult male rats was compared with that in liver microsomes of adult female rats. When progesterone was used as a substrate, the hepatic microsomal 20β-HSD activity was detected in male rats, but was not in female rats (fig. 2A). Acetohexamide reductase activity in liver microsomes of adult rats also exhibited a similar sex-related difference (fig. 2B). However, the activity of 20α-HSD in liver microsomes of male rats was much lower than that of 20β-HSD in liver microsomes of male rats, and there was no sex-related difference in the hepatic microsomal 20α-HSD activity (data not shown). We further examined the regulation mechanism of 20β-HSD activity in liver microsomes of male rats. As shown in figure 3A, testectomy markedly decreased the hepatic microsomal 20β-HSD activity. However, the decreased enzyme activity was restored by repeated treatment with testosterone propionate. A similar androgen-dependent decrease and restoration was observed in the hepatic microsomal acetohexamide reductase activity (fig. 3B).

**Age-related alteration of 20β-HSD activity.** Figure 4 shows age-related alteration of 20β-HSD activity in liver microsomes of male rats. The enzyme activity markedly increased during pubertal period (6–7 wk of age) to reach the adult level. To establish whether 20β-HSD can catalyze the

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Inhibition (%)</th>
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<tr>
<td>5α-Androstan-3,17-dione</td>
<td>9.9 ± 6.3</td>
</tr>
<tr>
<td>5α-Androstan-3,17-dione</td>
<td>20.3 ± 7.6</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>9.4 ± 5.3</td>
</tr>
<tr>
<td>(5α-Androstan-17β-ol-3-one)</td>
<td>17.1 ± 2.8</td>
</tr>
<tr>
<td>(5β-Dihydrotestosterone)</td>
<td>21.9 ± 3.4</td>
</tr>
<tr>
<td>(4-Androsten-17β-ol-3-one)</td>
<td>10.5 ± 5.8</td>
</tr>
<tr>
<td>Androsterone</td>
<td>19.6 ± 3.8</td>
</tr>
<tr>
<td>(5α-Androstan-17β-ol-3-one)</td>
<td>69.2 ± 1.8</td>
</tr>
<tr>
<td>(5α-Androstan-17β-ol-3-one)</td>
<td>92.3 ± 3.2</td>
</tr>
<tr>
<td>Cortisone</td>
<td>91.3 ± 1.4</td>
</tr>
<tr>
<td>(4-Pregnen-17α,21-diol-3,11,20-trione)</td>
<td>91.6 ± 0.4</td>
</tr>
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*The concentrations of substrate and inhibitors were 1.0 and 0.1 mM, respectively. Each value represents the mean ± S.D. of three experiments.*

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ketone-reduction of acetohexamide in liver microsomes of male rats, the relationship between the hepatic microsomal 20β-HSD and acetohexamide reductase activities was examined in individual male rats at 4, 6, 7, 9, 12 and 15 wk of age. A significant regression line ($r = 0.989$, $P < .001$) was obtained from the plots of 20β-HSD activity vs. acetohexamide reductase activity (fig. 5).

**Discussion**

Our study has demonstrated that C21-steroids with a ketone group at 20-position such as progesterone, 17α-hydroxyprogesterone, cortisone and cortisol are potent inhibitors toward ketone-reduction of acetohexamide in liver microsomes of male rats. Furthermore, progesterone was found to inhibit competitively the ketone-reduction of acetohexamide in liver microsomes of male rats. These results suggest that the ketone-reduction of acetohexamide and progesterone in liver microsomes of male rats is catalyzed by the same enzyme, and that either 20α- or 20β-HSD catalyzes these ketone-reductions.

We have so far shown that acetohexamide reductase activity in liver microsomes of adult male rats is much higher than that in liver microsomes of adult female rats (Imamura et al., 1987, 1993). Recently, Apanovitch et al. (1992) have reported that when progesterone is used as a substrate, a male-specific 20β-HSD activity is detected in liver microsomes of adult rats. In our study, we confirmed that there was a significant sex-related difference not only for acetohexamide reductase activity, but also for 20β-HSD activity in liver microsomes of adult rats. However, no significant sex-related difference was observed in the hepatic microsomal 20α-HSD activity. 20β-HSD and acetohexamide reductase present in liver microsomes of adult male rats were also found to be an androgen-dependent enzyme. Furthermore, a significant correlation was observed between the activities of 20β-HSD and acetohexamide reductase activity (fig. 5).

**Fig. 1.** Inhibition of acetohexamide reductase activity in liver microsomes of adult male rats by progesterone. The concentrations of progesterone were 0 μM (○), 20 μM (●), 50 μM (□) and 100 μM (■). Velocity was expressed as nmol/min/mg protein.

**Fig. 2.** Sex-related differences of 20β-HSD (A) and acetohexamide reductase (B) activities in liver microsomes of adult rats. Each bar represents the mean ± S.D. of four rats. N.D., Not detectable. **Significantly different from the males ($P < .001$).

**Fig. 3.** Effect of testectomy and treatment with testosterone propionate on activities of 20β-HSD (A) and acetohexamide reductase (B) in liver microsomes of adult male rats. C, Control; Tx, testectomy; TP, treatment with testosterone propionate. Each bar represents the mean ± S.D. of four to six rats. One-way ANOVA indicated a significant difference among the activities in control and treatments ($P < .001$). *$P < .01$ (Duncan’s multiple range test).

**Fig. 4.** Age-related alteration of 20β-HSD activity in liver microsomes of male rats. Each point represents the mean ± S.D. of four rats.

**Fig. 5.** Relationship between 20β-HSD and acetohexamide reductase activities in liver microsomes of individual male rats at 4 (○), 6 (●), 7 (□), 9 (■), 12 (▲) and 15 (○) wk of age.
hexamide reductase in liver microsomes of male rats at various ages. Based on all results described above, we conclude that 20β-HSD catalyzes the ketone-reduction of acetohexamide in liver microsomes of adult male rats.

In general, drugs with a ketone group such as acetohexamide are reduced to the corresponding alcohol metabolites by carbonyl reductase (EC 1.1.1.184) (Jakoby and Ziegler, 1990). Nakajin et al. (1988) have purified a 20β-HSD from cytosolic fraction of neonatal pig testis. The purified 20β-HSD catalyzed effectively the reduction of 20-ketone group of 17α-HSD. The ketone-reduction of acetohexamide in liver microsomes of adult male rats is much smaller than those of progesterone, the ketone-reduction of acetohexamide in liver microsomes of adult male rats, but metyrapone reduced ketone within its chemical structure, is reduced in liver microsomes of adult male rats probably functions as one of carbonyl reductases that catalyze the ketone-reduction of drugs, even though its primary structure is not yet determined.

Recently, the ketone-reduction of S-warfarin and progesterone in liver microsomes of adult male rats has been demonstrated to be catalyzed by different enzymes (Apanovitch and Walz, 1996). This implies that S-warfarin is not a substrate of 20β-HSD. In our study, we have found that the inhibitory effect of S-warfarin (% inhibition of 14.6 ± 2.9) on the ketone-reduction of acetohexamide is smaller than that of progesterone, 17α-hydroxyprogesterone, cortisone and cortisol, suggesting that S-warfarin-reducing enzyme is distinguished from acetohexamide-reducing enzyme. Our previous paper (Imamura et al., 1997) has also shown that metyrapone, which has a ketone group within its chemical structure, is reduced in liver microsomes of adult male rats, but metyrapone reductase purified partially from liver microsomes of adult male rats has no ability to reduce acetohexamide. It should be noted that these drugs tested, only acetohexamide can be reduced by 20β-HSD. In liver microsomes of adult male rats, a variety of enzymes appears to be involved in the metabolic reduction of exogenous carbonyl compounds including drugs. CYP 2C11, as with 20β-HSD, is known to be a male-specific and androgen-dependent enzyme (Kato and Yamazoe, 1993). However, there is no possibility that 20β-HSD is CYP2C11, because the enzyme reaction in this study is performed under an aerobic condition. Further studies are progress to elucidate the catalytic properties of 20β-HSD present in liver microsomes of adult male rats.

References


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