Activation of Human Liver 3α-Hydroxysteroid Dehydrogenase by Clofibrate Derivatives

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ABSTRACT

The NADP⁺-dependent dehydrogenase activity of a predominant isoenzyme of human liver 3α-hydroxysteroid dehydrogenase was activated by antihyperlipidemic drugs, such as bezafibrate, clofibrate and clofibrate acid, and fenofibrate and fenofibrate acid (active metabolites of clofibrate and fenofibrate, respectively). The optimal pH of the activation by the drugs was about 7.5, and the concentrations giving maximum stimulation (1.8-fold) were 100, 50, 400 and 50 μM for bezafibrate, clofibrate, clofibrate acid and fenofibrate acid, respectively. Clofibrate and fenofibrate acted as weak inhibitors, and the clofibrate acid derivatives that lack the chloro group, methyl group on the α-carbon or carboxyl group greatly decreased the stimulatory effects. The activation by the drugs increased both Km and kcat (turnover number) values for the coenzyme and substrates. Kinetic analysis with respect to NADP⁺ showed that bezafibrate, clofibrate, clofibric acid and fenofibric acid were non-essential activators showing dissociation constants of 32, 6, 103 and 11 μM, respectively. The combined activators experiments with one of the above drugs and sulfobromophthalein, a known activator specific for this enzyme, and comparison of their effects on the activities of mutant enzymes (with Met replacing Lys-270 or Arg-276) indicated that sulfobromophthalein and the drugs bind to an identical site on the enzyme. These results suggest that the long-term therapy with the antihyperlipidemic drugs influences the metabolism of steroid hormones, bile acids and several ketone-containing drugs mediated by the enzyme.

3αHSD distributes in mammalian tissues, and is involved in the biosynthesis and inactivation of steroid molecules and the regulation of steroid hormone action (Penning et al., 1996). For example, the enzyme has been reported to work in concert with 5α-reductase to regulate the intracellular concentration of 5α-dihydrotestosterone, 5α-androstan-3α,17β-diol and 3α-hydroxy-5α-dihydroprogesterone that play key roles in prostate growth (Lombardo et al., 1992), parturition (Mahendroo et al., 1996) and modulation of the activity of γ-aminobutyric acid receptor (Majewska et al., 1986; Martini et al., 1993). The liver 3αHSD inactivates circulating steroid hormones and plays a role in the bile acid synthesis (Tomkins, 1956; Usui and Okuda, 1986). The outstanding feature of mammalian hepatic 3αHSD is its broad specificity for prostaglandins, drug ketones and trans-dihydrodiols of aromatic hydrocarbons, as well as the steroids (Wörner and Oesch, 1984; Penning et al., 1986; Hara et al., 1988; Ohara et al., 1994, 1995), and the enzyme has the ability to bind bile acids (Stolz et al., 1987). These findings have suggested that this enzyme also acts as prostaglandin oxidoreductase, carbonyl reductase, dihydriodiol dehydrogenase and bile acid-binding protein in the tissue.

In human liver, 3αHSD with dihydriodiol dehydrogenase activity exists in multiple forms (Hara et al., 1990, 1996; Takikawa et al., 1992), and at least four types of cDNAs for the enzyme have been cloned from human liver cDNA library (Qin et al., 1993; Stolz et al., 1993; Deyashiki et al., 1994). The 3αHSD isoforms reveal 83 to 98% sequence identities, belong to the AKR superfamily, and have been systematically named as AKR 1C1, 1C2, 1C3 and 1C4 (Jez et al., 1997). AKR 1C1, the cDNA for which was originally cloned as that encoding hepatic bile acid-binding protein (Stolz et al., 1993), is identified with human liver dihydriodiol dehydrogenase isoform 1 that exhibits high 20α-hydroxysteroid dehydrogenase and very low 3αHSD activities (Hara et al., 1990, 1996). AKR 1C2 has been shown to be dihydriodiol dehydrogenase isoform 2 and bile acid-binding protein of human liver (Hara et al., 1996), and AKR 1C4 is identical with hepatic dihydriodiol dehydrogenase isoform 4 (Deyashiki et al., 1995) and chlorderone reductase (Winters et al., 1990). Although recombinant AKR 1C3 possesses low 3αHSD activity for limited 3α-hydroxy-
steroids (Khanna et al., 1995), its protein has not been identified in human tissues. Purification of the human liver 3aHSD shows that its predominant form is the AKR 1C4, which has high activity for various 3a-hydroxysteroids including bile acids, in contrast to low activity of AKR 1C1 and 1C2 for some androgens and progestins (Hara et al., 1990; Deyashiki et al., 1992)

From pharmaceutical and pharmacological points of view, our previous studies focused to elucidate the effects of drugs on the activities of the human liver 3aHSD isoforms involved in the metabolism of endogenous and xenobiotic compounds, and showed the inhibition of AKR 1C2 and 1C4 by anti-inflammatory drugs (Deyashiki et al., 1992), and the activation of AKR 1C4 by sulfobromophthalain (Matsuura et al., 1996).

We have found that of the four 3aHSD isoforms AKR 1C4 was activated by antihyperlipidemic clofibrate derivatives that are therapeutically administered for a long period, and show the specificity in terms of the structure of activating molecule and binding site of the enzyme, and the effect on the kinetic properties.

**Methods**

**Chemicals.** Probucol, pravastatin, sodium bezafibrate and clinofibrate were gifts from Otsuka Pharmaceutical Co., Sankyou Pharmaceutical Co., Kissui Pharmaceutical Co., and Sumitomo Pharmaceutical Co., Japan, respectively. Fenofibrate and steroids were purchased from Sigma Chemicals (St. Louis, MO). Clofibric acid (4-chlorophenoxyisobutyric acid) and its derivatives were obtained from Ardrich Chemicals (Milwaukee, WI) and Tokyo Kasei Organic Chemicals (Tokyo, Japan). Fenofibric acid [4-4'-chlorobenzoyl]phenoxynisobutyric acid] was prepared by alkaline hydrolysis of fenofibrate and recrystallized from ethyl acetate. Benzene dihydrodiol (trans-1,2-dihydroxy-1,2-dihyrobenzene) was synthesized as described by Platt and Oesch (1977). S(+)-Indan-1-ol was obtained from Fluka Chemie AB (Buchs, Switzerland); NADP \(^+\) was from Oriental Yeast (Tokyo, Japan) and clofibrate and sulfobromophthalein were from Nakalai Tesque (Kyoto, Japan).

**Enzymes.** AKR 1C1, 1C2 and 1C4 were purified from human liver by the method of Hara et al. (1990). Recombinant AKR 1C4 and its mutant enzymes (K270M and R276M) expressed in *Escherichia coli* were purified as described (Deyashiki et al., 1995; Matsuura et al., 1997). Because the hepatic and recombinant AKR 1C4 preparations showed the same activation by the clofibrate derivatives, the recombinant enzyme was used in this study. AKR 1C3 was expressed in *E. coli* from its cDNA (Khanna et al., 1995), and purified to homogeneity (specific activity was 1.1 U/mg of protein). It showed higher \(K_m\) values and lower activity for some 3a-hydroxysteroids than did AKR 1C4 (Matsuura K. and Hara A., unpublished observations).

**Enzyme assay.** Dehydrogenase activities of human liver and recombinant 3aHSDs were assayed spectrophotometrically or fluorometrically by recording the production of NADPH as described (Matsuura et al., 1996). The standard assay for the activity was performed in 2.0 ml of 0.1 M potassium phosphate, pH 7.4, containing 0.25 mM NADP\(^+\), 2 mM S-indan-1-ol and enzyme. The activities of the K270M and R276M mutant enzymes were determined with 0.5 mM and 5 mM NADP\(^+\), respectively, in the above reaction mixture because of their high \(K_m\) values for the coenzyme (Matsuura et al., 1997). One unit of the enzyme activity was defined as the amount catalyzing the formation of 1 nmol NADPH/min at 25°C. The stoichiometry of the production of NADPH and oxidized product (1-indanone) was examined by measuring the amount of 1-indanone in the reaction mixture by high-performance liquid chromatography. The reaction was started in the absence or presence of 50 \(\mu\)M fenofibric acid as the activator by the addition of the enzyme (50 \(\mu\)g), incubated for 5 min with monitoring the production of NADPH at 340 nm and then terminated by the addition 4 ml of ice-cold methanol containing benzaldehyde as the internal standard. After centrifugation at 10,000 \(\times\) g for 10 min, 50 \(\mu\)l of the supernatant were subjected to reversed-phase high-performance liquid chromatography (Japan Spectroscopic Co. Ltd., Hachioji, Japan) using a Nova-Pack C18 column (3.9 \(\times\) 15 mm, Waters, Milford, MA) with a 4 to 40% (\(\nu/\nu\)) linear gradient of methanol/25 mM potassium phosphate buffer, pH 5.6, at a flow rate of 0.8 ml/min and at 25°C. S-Indan-1-ol and 1-indanone were detected at 270 and 250 nm, respectively, and the respective retention times are 22.2 and 23.9 min. The concentrations of the substrate and product were determined by their peak areas.

The drugs and their derivatives with free carboxyl group were dissolved in 0.1 M NaOH, and then neutralized with 0.1 M HCl. Probucol, fenofibrate and clofibrate were dissolved in methanol, and added to the reaction mixture to give a final methanol concentration of 2.5%. The drugs and their derivatives were added to the reaction mixture just before the reaction was started by the addition of the enzyme. The pH dependency of the enzyme activity was determined with 0.1 M potassium phosphate buffers (pH 6.5–10.0) which were prepared by mixing solutions of H\(_2\)PO\(_4\) and K\(_2\)PO\(_4\) in a separate experiment to test the reversibility of the activation, the activating drugs-(50 or 200 \(\mu\)M) enzyme mixture was diluted 10-fold with 10 mM potassium phosphate buffer, pH 7.4, or dialyzed against the buffer containing 20% glycerol at 4°C and then the activity of the preparation was assayed.

Lineweaver-Burk analyses were performed in the presence of five different substrate concentrations with saturating NADP\(^+\) concentration of 0.25 mM, and the kinetic data were analyzed as described (Matsuura et al., 1996). The kinetic studies in the presence of inhibitors were carried out in a similar manner, and the inhibition constant (\(K_i\)) was determined as described by Cornish-Bowden (1995). Initial velocity analyses for determination of the dissociation constant (\(K_\alpha\)), \(\alpha\) and \(\beta\) values for the activating drug were carried out in the presence of its six different concentrations ranging from 0.2 \(\times\) \(K_\alpha\) to 5 or 15 \(\times\) \(K_\alpha\) values. The kinetic values were calculated from secondary replots of 1/\(\Delta\) slope or 1/\(\Delta\) intercept versus 1/drug (Segel, 1975). The \(\Delta\) slope values and \(\Delta\) intercept values were obtained from individual Lineweaver-Burk plots. The values of the enzyme activity and kinetic constants were expressed as means \(\pm\) S.E. of at least three determinations.

**Results**

**Effect of antihyperlipidemic drugs and related substances on 3aHSD activity.** The kinetic properties of human liver 3aHSD isoforms have been determined at a physiological pH of 7.4, and S-indan-1-ol has been employed as one of their good model substrates (Hara et al., 1990; Deyashiki et al., 1992, 1995). The standard 3aHSD assay was used to investigate the effects of various concentrations of several antihyperlipidemic drugs on the activities of the enzymes. The results of this experiment are present in figure 1 and table 1. Although probucol, pravastatin, fenofibrate and clofibrate were inhibitory to or had no effect on the activities of all the enzymes, bezafibrate and clinofibrate stimulated the activity of AKR 1C4 by 1.8- and 2.0-fold, respectively. Because clofibrate and fenofibrate administered are rapidly hydrolyzed to their active metabolites, clofibric acid and fenofibric acid, respectively (Thorp, 1962; Elsom et al., 1976), the effects of the active metabolites were examined. They also activated only the activity of AKR 1C4 by about 2.4-fold. Although the enzyme activity was assayed by monitoring the production of NADPH, the stoichiometry of the enzyme reaction was confirmed by measuring the oxidation product, 1-indanone, of S-indan-1-ol. The rates of NADPH and 1-indanone...
produced in the reaction mixture without activator were 5.5 ± 0.1 and 5.9 ± 0.6 nmol/min respectively, and the respective rates in the presence of 50 μM fenofibrate acid were 13.1 ± 0.3 and 13.0 ± 1.1 nmol/min. These activating drugs brought about a biphasic activity vs. concentration curve; at low concentrations they stimulated the activity, but stimulation percentages decreased at their higher concentrations.

The concentrations of bezafibrate, clinofibrate, clofibrate acid and fenofibrate acid at which maximum stimulation was achieved were 100, 50, 400 and 50 μM, respectively. These drugs quickly activated when they were added to the reaction mixture without the activator, and the activation was decreased to the control level by 10-fold diluting or dialyzing the enzyme-drug mixture against a buffer without the drugs, and brought about biphasic activity vs. concentration curve; at concentrations above 1 mM. It was noted that pivalic acid did not activate the enzyme activity and inhibited at its concentration above pH 9.5 (fig. 2).

Because the stimulatory drugs are phenoxypropionic acid derivatives, the effects of various concentrations of the other phenoxypropionic acid derivatives on the activity of AKR 1C4 were examined. Most of the derivatives activated the enzyme and brought about biphasic activity vs. concentration curves, similar to those of the drugs, and the Smax (apparent maximum stimulation percentage) and SC50 (apparent concentration giving ½ Smax) values for the derivatives are present in table 2. Comparing to the values for clofibrate acid, removing a methyl group (i.e., 2-(4-chlorophenoxy)-propionic acid) and two methyl groups (i.e., 4-chlorophenoxyacetic acid) from the α-carbon of clofibrate acid molecule increased SC50 and decreased Smax values, which resulted in large decrease in activation efficiency (Smax/SC50). Removing the chloro group (i.e., 2-phenoxypropionic acid and phenoxyacetic acid) from the corresponding chlorinated derivatives also decreased the activation efficiency. As clofibrate, ethyl ester of clofibrate acid, showed only weak inhibitory effect on the activity of AKR 1C4, 2-phenoxypropenol that has an alcohol group instead of the carboxyl group of 2-phenoxypropionic acid did not activate the enzyme activity and inhibited at its concentrations above 1 mM. It was noted that pivalic acid...
3-Phenoxypropionic acid

6

2-Phenoxypropionic acid 100

ity on NADP

2-(4-Chlorophenoxy)propionic acid competitive with respect to compared. The inhibition patterns by fenofibric acid were all

bi mechanism in which coenzyme binds first to the enzyme reaction of AKR 1C4 has been shown to follow an Ordered bi

tions of clofibric acid, fenofibric acid, bezafibrate and clinofibrate respectively. Because the

from the clofibric acid molecule, had no effect on the enzyme activity, as the concentration of clofibric acid was increased

exclusively to increase both

in figure 3. It is apparent from the results shown in A that at

clofibric acid on the kinetic properties of the enzyme is shown

activation phase of the activity vs. activator concentration curve.

3-Phenoxypropionic acid and 2-phenoxypropanol inhibited the activity at concentra-

ations above 3 and 1 mM, respectively.

(trimethylacetic acid), which lacks the 4-chlorophenoy ring from the clofibric acid molecule, had no effect on the enzyme activity up to 5 mM concentrations.

In contrast to the activation of AKR 1C4 by clofibrate, bezafibrate and the active metabolites of clofibrate and fenofibrate, the other human liver isoforms were inhibited by these drugs. The IC50 (apparent concentration giving 50% inhibition) values of the drugs, which showed significant inhibition for the respective isoforms, are shown in table 1. Because fenofibric acid gave relatively low IC50 values for the three isoforms, its inhibition patterns and constants were compared. The inhibition patterns by fenofibric acid were all competitive with respect to S-indan-1-ol, and the K1 values were 9.6 ± 1.6, 15 ± 3 and 1.7 ± 0.4 µM for AKR 1C1, 1C2 and 1C3, respectively.

Effect on kinetic properties of AKR 1C4. The interactions of clofibric acid, fenofibric acid, bezafibrate and clonofibrate with AKR 1C4 were analyzed kinetically. Because the reaction of AKR 1C4 has been shown to follow an Ordered bi mechanism in which coenzyme binds first to the enzyme (Deyashiki et al., 1995), the dependency of the enzyme activity on NADP+ was studied at different fixed concentrations of drug in the presence of saturating concentration of S-indan-1-ol. A typical Lineweaver-Burk analysis of the effects of clofibric acid on the kinetic properties of the enzyme is shown in figure 3. It is apparent from the results shown in A that at low concentration (0.0-0.2 mM) the effect of clofibric acid is exclusively to increase both Vmax and Km values. Interestingly, as the concentration of clofibric acid was increased from 0.4 to 1.5 mM (fig. 3B), a different pattern was observed; in this case, the slopes increased with increasing drug concentration but without altering the Km for the coenzyme. Similar kinetic patterns were obtained when the effects of fenofibric acid, bezafibrate and clonofibrate on the enzyme activity were examined by Lineweaver-Burk analysis. At the low concentrations of the four drugs (<200 µM for clofibrate, <20 µM for fenofibric acid, <40 µM for bezafibrate and <20 µM clofibrate), the replots of the reciprocal of change in slope or intercept of the respective primary reciprocal plot data vs. 1/(drug) were linear, as the representative replot for clofibrate-induced activation is shown in the inset of figure 3A. The results are consistent with a nonessential activation system (fig. 4), in which the kinetic constant can be determined (Segel, 1975). The values of K1 (dissociation constant for the activator), α and β calculated from the replots with the four drugs are summarized in table 3.

When the kinetic effect of clofibrate acid on AKR 1C4 was also examined as a function of S-indan-1-ol concentration (fig. 3C), the reciprocal plots of 1/V and 1/S gave complicated patterns of straight lines, which showed that the drug acted as a partial activator at its low concentrations and as a competitive inhibitor at its high concentrations. Therefore, the kinetic constants for several substrates were determined at the low concentrations near the respective Km values of clofibrate acid and bezafibrate, and compared. The effects of the two drugs on the constants were essentially the same (table 4). The addition of the drugs led to increases in Km and kcat values for the substrates, compared with those reported in the absence of the activator (Deyashiki et al., 1995). Although the increases in the two kinetic values on the drug-induced activation ranged from 1.2- to 3.2-fold depend on the substrates, the catalytic efficiency (kcat/Km) of the enzyme slightly increased or remained unchanged.

Binding site for activating drugs. Sulfobromophthalein has been shown to be a nonessential activator specific for AKR 1C4 (Matsuura et al., 1996) and to inhibit the mutant K270M and R276M enzymes (Matsuura et al., 1997). This drug is a more potent activator than the antihyperlipidemic drugs (table 3). To gain insight regarding to the binding site(s) for sulfobromophthalein and the antihyperlipidemic drugs, we first examined the combined effects of each antihyperlipidemic drug on the stimulatory effect by sulfobromophthalein (fig. 5). The antihyperlipidemic drugs were inhibitory to the simulation by sulfobromophthalein, which suggests that these activators compete for a site on the enzyme. Second, the effects of the antihyperlipidemic drugs on the mutant enzymes, K270M and R276M, were compared

![Fig. 3. Lineweaver-Burk analysis of the dual effects of clofibrate acid on the dehydrogenase activity of AKR 1C4.](image)
identities of 83 to 97%, but in contrast with activation of AKR 1C4 the antihyperlipidemic drugs and sulfobromophthalein had no effect on or were inhibitory to the other isoforms. Recent crystallographic studies of the AKR family proteins such as aldose reductase (Harrison et al., 1994) and rat liver 3αHSD (Bennett et al., 1996; Jez et al., 1996) have shown that competitive inhibitors with free carboxylic acid bind in a specific anionic site delineated by the C4N of the nicotinamide coenzyme and the side chains of Tyr and His residues at the enzyme active site. Because the two residues in the anion binding site are conserved in the four human AKR 1C isoforms, the competitive inhibition of AKR 1C1, 1C2 and 1C3 by fenofibric acid suggests that the drug binds in the anion binding sites of the enzymes. By contrast, the activation of AKR 1C4 by the antihyperlipidemic drugs suggests the existence of the activator-binding site distinct from the anion binding site. It has been reported that aldose reductase also has another anion binding site for its activators such as citrate, cacadylate and phosphate, in addition to the anion binding site at the enzyme active site (Harrison et al., 1994).

The kinetic activation mechanisms of the antihyperlipidemic drugs for AKR 1C4 are the same as that of sulfobromophthalein (Matsuura et al., 1996), and the experiment with combined activators and the effects on the mutant enzymes indicate that the antihyperlipidemic drugs and sulfobromophthalein bind to the identical activator site of the enzyme. Our previous kinetic analyses of the K270M and R276M showed large increases in the affinity for NADP⁺ and kcat value without significant change in kinetic constants of the NAD⁺-linked reaction, which suggests the roles of the two basic residues in the binding to the 2'-phosphate of NADP⁺ (Matsuura et al., 1997), as shown by crystallographic studies of the AKR family proteins such as aldose reductase (Harrison et al., 1994) and rat liver 3αHSD (Bennett et al., 1996). Sulfobromophthalein, which activates the NADP⁺-linked activity of wild-type AKR 1C4 but not the NAD⁺-linked activity, inhibits the NADP⁺-linked activity of the mutant enzymes, and has been thought to interact with the two basic residues through the sulfonic group of this activator (Matsuura et al., 1997). The antihyperlipidemic drugs, similarly to sulfobromophthalein, increased the Kₘ values for NADP⁺, and the importance of the existence of a carboxyl group in the activator molecule demonstrated by our study supports the interaction of the negatively charged groups of these drugs with one or both of the two basic residues. Thus, the activator site is probably composed of the residue(s) required for binding NADP⁺, but is not the same as the coenzyme binding site, because the kinetic analysis of the activation by the drugs indicated the mechanism involving the production of the ternary enzyme-NADP⁺-activator complex (fig. 3). Such drug binding would cause perturbation of the interaction between the 2'-phosphate group of NADP⁺ and the two basic residues, which results in rapid release of the products and, hence, the observed increase in the kcat value.

There are two differences between the stimulatory effects of sulfobromophthalein and the antihyperlipidemic drugs on AKR 1C4. First, the Kₘ, α and β values for the drugs are different. As discussed above, one of the structural requirements for the activator is the hydrophobic part of the molecule, and the difference in the kinetic constants may be caused by slightly different enzyme conformations induced by the binding of the structurally distinct other parts of the

with that of sulfobromophthalein (table 5). These drugs, similarly to sulfobromophthalein, did not activate and inhibited the activities of the two mutant enzymes.

**Discussion**

Our study demonstrates that the antihyperlipidemic clofibrate derivatives, which are structurally different from the known activator, sulfobromophthalein, exert stimulatory effects specifically on AKR 1C4 of human liver 3αHSD isoforms. For the activation by sulfobromophthalein, there has been no direct information about the structurally specific interaction between the molecule and the enzyme, except for its sulfanyl group(s) (Matsuura et al., 1996, 1997). Our results of the comparative study of the efficacies of the antihyperlipidemic drugs and their related compounds provide the following specific structural requisites for the activator. 1) The existence of a negatively charged carboxyl group, together with at least a hydrophobic aromatic ring, in the activator molecule is necessary to interact with the activator site of the enzyme, because the clofibrate acid derivatives lacking the free carboxyl group or the aromatic ring did not activate. Because the pKa values of the carboxyl group of the drugs are about 3, almost all of the drug carboxyl group are ionized at pH 7.5 which was the optimal condition for the activation. 2) The presence of hydrophobic alkyl moieties on the α-carbon in the hydrocarbon chain is also important for the activation, as indicated by the great decrease in the activation efficiency with removing methyl groups on the α-carbon of the clofibrate acid molecule. 3) In addition to the above structural requisites, nonpolar substituents at the 4-position of the aromatic ring in the activator molecules probably contribute to their high affinity to the activator site, as shown in the low Kₘ values for clinofibrate, fenofibric acid and bezafibrate compared with that for clofibrate acid with only a chloro group. Thus, the activator-binding site on the enzyme may be composed of several hydrophobic amino acid residues in addition to the positively charged residues which interact with the negatively charged carboxyl group of the activator molecule.

The human AKR 1C1–1C4 isoforms show high sequence

![Fig. 4. Scheme for nonessential activators. E, Enzyme; A, activator; P, product.](image-url)
activators. Although except for the negatively charged sulfonic and carboxyl groups no structural relationship between sulfobromophthalein and the other drugs as the activator could be elucidated at present, sulfobromophthalein, that is a more effective activator than the other drugs, has a hydrophobic large tetrabromophthalein ring that may contribute to its high $K_a$ and low $k_{cat}$ values. Second, the antihyperlipidemic drugs showed the decrease of the activation percentages at their high concentrations, whereas such a significant decrease has not been observed with sulfobromophthalein (Matsuura et al., 1996). To explain the progression from activation to inhibition as the concentration of the antihyperlipidemic drug in the assay medium is increased, it is helpful to conceive that the drug alternatively binds to another drug binding site other than the activator site of the enzyme. Because the inhibition caused by high concentrations of clo-

### Table 4

<table>
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<tr>
<th>Substrate</th>
<th>$K_a$ (mM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_{cat}/K_a$ (mM$^{-1}$)</th>
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<tr>
<td>Salicylic acid</td>
<td>200</td>
<td>0.1</td>
<td>1.2</td>
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<tr>
<td>Acetamide</td>
<td>20</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>5a-Acetoxy-3,6,17-pregnan-3-ol</td>
<td>50</td>
<td>0.5</td>
<td>0.1</td>
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<tr>
<td>5a-Acetoxy-3,6,17-pregnan-3-one</td>
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<tr>
<td>Phenanthrene</td>
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<td>0.1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
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<td>0.1</td>
</tr>
<tr>
<td>4-Methylbenzene</td>
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<td>0.1</td>
</tr>
<tr>
<td>4-Methylphenol</td>
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<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>4-Methoxyphenol</td>
<td>100</td>
<td>1.0</td>
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</tr>
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| The values determined without drug are taken from Deyashiki et al. (1995).

### Table 5

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>$K_{cat}$ (min$^{-1}$)</th>
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<tr>
<td>Sulfochromophthalein</td>
<td>3</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>Sulfochromophthalein</td>
<td>20</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>500</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>1000</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Clofibric acid</td>
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<tr>
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<tr>
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<td>42 ± 2</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>100000</td>
<td>42 ± 2</td>
</tr>
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</table>
| The values determined without drug are taken from Deyashiki et al. (1995).
fibric acid was apparently competitive with respect to the substrate, this drug binding site is present at or near the substrate binding domain of the enzyme, and is probably the anion binding site at the enzyme active site, as discussed above for the competitive inhibition of the other human AKR 1C isoforms by fenofibric acid. The different effects between sulfobromophthalein and the antihyperlipidemic drugs may be due to their structural difference other than the negatively charged groups, which also suggests the existence of the two distinct binding sites, the activator site and anion binding site, on AKR 1C4. Further direct binding and structural studies are needed to clarify whether the drug binds to the two binding sites simultaneously or alternatively.

The antihyperlipidemic drugs are usually indicated in the long-term management of the patients. Doses of these drugs range from 200 mg (the lowest case of bezafibrate) to 1500 mg (the highest case of clofibrate) a day, which result in their peak plasma concentrations from 30 μM (for bezafibrate) to 300 μM (as clofibrate acid for clofibrate) (Abshagen et al., 1979; Cayen 1980; Sumitomo Pharmaceutical Co., personal communication). The distribution of the drugs has been studied in rats, and the hepatic concentration of clofibrate acid after the administration of clofibrate are slightly lower than the plasma concentration (Cayen et al., 1977), but in the case of bezafibrate its concentration in liver is 4-fold higher than that in blood (Nishiyama et al., 1988). The plasma concentrations of the respective drugs are comparable with or superior to their $K_A$ values for the activation of human liver AKR 1C4 that was activated from the concentrations of one-fifth of the $K_A$ values. Therefore, it is possible that these drugs administered elevate the enzyme activity. Although these drugs are less potent activators than sulfobromophthalein, they are indicated in the long-term management of hyperlipidemias, whereas sulfobromophthalein is used only to test liver function. AKR 1C4 is the predominant human liver 3α-HSD isoform with broad specificity for 3α-hydroxysteroids and involved in the metabolism of steroid hormones and bile acids, whereas the other isoforms do not accept bile acids as the substrate and show low catalytic efficiency for bile acids, whereas the other isoforms do not accept bile acids as the substrate and show low catalytic efficiency for bile acids but in the case of bezafibrate its concentration in liver is 4-fold higher than that in blood (Nishiyama et al., 1988). The plasma concentrations of the respective drugs are comparable with or superior to their $K_A$ values for the activation of human liver AKR 1C4 that was activated from the concentrations of one-fifth of the $K_A$ values. Therefore, it is possible that these drugs administered elevate the enzyme activity. Although these drugs are less potent activators than sulfobromophthalein, they are indicated in the long-term management of hyperlipidemias, whereas sulfobromophthalein is used only to test liver function.

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The physiological significance of the inhibition of AKR 1C3 by fenofibric acid is unknown, because the purified enzyme did not oxidize bile acids and showed very low activity for only some 3α-hydroxyxysteroid dehydrogenases in contrast to the earlier report with the crude E. coli extract of the recombinant enzyme (Khanna et al., 1995). These findings suggest that administration of the clofibrate derivative drugs exclusively leads to the elevation of hepatic cholesterol catabolism through the activation of AKR 1C4. The antihyperlipidemic drugs decrease both plasma triglyceride and cholesterol concentrations. One of the mechanisms for the antihypercholesterolemic action of the drug has been shown to be the suppression and inhibition of hydroxymethylglutaryl coenzyme A reductase, and the $IC_{50}$ values of clofibrate acid, clinofibrate and bezafibrate for the reductase have been reported to be 6, 0.47 and 3.1 mM (Kusama et al., 1988), which are much higher than the $K_A$ values for the respective drugs in the activation of human liver AKR 1C4.

We propose that the elevation of hepatic cholesterol catabolism although the activation of this enzyme by the drugs is an additional mechanism of the antihypercholesterolemic action that has not been reported. However, AKR 1C4 also plays a significant role in hepatic reductive metabolism of several drug ketones administered therapeutically (Ohara et al., 1995b), combined administration of the drug ketones and the antihyperlipidemic drugs may influence the pharmacological potency of the drug ketones. In addition, 3αHSD has been shown to be involved in the metabolism of 3α-reduced androgens that are required for both normal and abnormal growth of prostate (Isaacs et al., 1983; Nakha et al., 1995) and parturition (Mahendroo et al., 1996). The antihyperlipidemic drugs are distributed in peripheral tissues, and 3αHSDs with similar properties to AKR 1C4 have been purified from human prostate (Amet et al., 1992; Trapp et al., 1992), and mRNA for hepatic 3αHSD is elevated in murine uterus during late gestation (Mahendroo et al., 1996). If the 3αHSDs in the tissues are identical with hepatic AKR 1C4, the long-term therapy with these antihyperlipidemic drugs would modulate hormone actions in the steroid-target tissues.

References


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