Inhibition of Rabbit Heart Carbonyl Reductase by Fatty Acids

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The inhibition of rabbit heart carbonyl reductase (RCHR) by fatty acids was examined using 4-benzoylpyridine (4BP) as a substrate. The inhibitory potency of saturated fatty acids increased with elongation in the carbon chain from caprylic acid to myristic acid, but decreased with further elongation. Myristic acid with 14 carbon atoms most strongly inhibited RCHR. All of the unsaturated fatty acids tested strongly inhibited RCHR; the cis-isomers were more potent inhibitors than the corresponding trans-isomers. The methyl esters and alcohols, which lack a carbonyl group, derived from fatty acids did not exert a significant inhibitory effect on RCHR. These results indicate that the existence of a proper length of carbon chain, double bond(s), and a carbonyl group in a fatty acid molecule is important for RCHR inhibition. We also propose the possibility that myristic acid at low concentrations inhibits the reduction of 4BP by interacting with a binding site other than the coenzyme- and substrate-binding sites of RCHR.

Key words rabbit heart carbonyl reductase; fatty acid; enzyme inhibition; fatty acid analog; inhibition mechanism

Carbonyl reductase [EC 1.1.1.184] is an enzyme responsible for the reduction of endogenous and xenobiotic carbonyl compounds to the corresponding alcohol metabolites. Many carbonyl reductases have been purified from various tissues of mammalian species; we recently purified a new carbonyl reductase from the cytosolic fraction of rabbit heart. The rabbit heart carbonyl reductase (RCHR) is a tetrameric protein composed of 4 identical-size subunits. RCHR effectively reduced not only xenobiotic aldehydes and ketones, but also mandelionic acid, and mandelonic acid under physiological pH conditions. However, our preliminary study has found that RCHR, unlike the pig lung carbonyl reductase, is not activated by the fatty acids. In this paper, we provide evidence for the inhibition of RCHR by fatty acids, and attempt to elucidate the inhibition mechanism.

MATERIALS AND METHODS

Materials 4-Benzyloxydride (4BP) was purchased from Wako Pure Chemicals (Osaka, Japan). Fatty acids and their analogs used were obtained from the following sources: caprylic acid, capric acid, myristic acid, palmitic acid, and stearic acid from Tokyo Kasei (Tokyo, Japan); lauric acid from Nacalai Tesque (Kyoto, Japan); oleic acid, elaidic acid, linoleic acid, α-linolenic acid, γ-linolenic acid, lauryl alcohol, myristyl alcohol, oleyl alcohol, lauric acid methyl ester, myristic acid methyl ester, and oleic acid methyl ester from Sigma (St. Louis, MO, U.S.A.); linoleic acid from Wako Pure Chemicals. NADPH was purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of reagent grade.

Purification of RCHR RCHR was purified to homogeneity from the cytosolic fraction of rabbit heart using 4BP as a substrate according to our previous procedures.

Enzyme Assay The enzyme activity was assayed spectrophotometrically by monitoring NADPH oxidation at 340 nm. 4BP was used as a substrate. The reaction mixture, in a total volume of 0.7 ml, consisted of 100 mM sodium potassium phosphate buffer (pH 6.0), NADPH at 0.25 mM or at various concentrations, the substrate at 1.0 mM or at various concentrations, and the purified enzyme (RCHR). Fatty acids and their analogs dissolved in methanol were added to the reaction mixture at final concentrations of 20 and 50 μM; the concentration of methanol in the reaction mixture was 2.0% (v/v). The concentrations of fatty acids were lower than the critical micellar concentrations (cmc) for all the fatty acids tested. The reaction was initiated by adding the purified enzyme, and the decrease in absorbance at 340 nm was monitored with a Shimadzu UV-240 spectrophotometer. One unit of the enzyme activity was defined as the amount causing a decrease in absorbance at 340 nm corresponding to the oxidation of 1 μmol of NADPH/min at 30°C. Protein concentrations were determined with bovine serum albumin as the standard by the method of Lowry et al.

RESULTS

Inhibition by Saturated Fatty Acids The inhibition of RCHR by saturated fatty acids was investigated at the concentrations of 20 and 50 μM. Caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, and stearic acid were used as saturated fatty acids. As shown in Table 1, the inhibitory potency of saturated fatty acids increased with elongation in the carbon chain from caprylic acid to myristic acid, but decreased with further elongation. Myristic acid with 14 carbon atoms most strongly inhibited RCHR, while stearic acid with 18 carbon atoms was a poor inhibitor.

Inhibition by Unsaturated Fatty Acids Table 2 summarizes the inhibition of RCHR by unsaturated fatty acids. Although all unsaturated fatty acids examined, as well as stearic acid, have 18 carbon atoms, they strongly inhibited RCHR at the concentrations of 20 and 50 μM. The cis-isomer...
mers, oleic acid and linoleic acid, were found to be more potent inhibitors of RHCR than the corresponding *cis*-isomers, elaidic acid and linolelaic acid; γ-linolenic acid was slightly potent compared to α-linolenic acid.

**Kinetics of Inhibition by Myristic Acid**  
The inhibition of RHCR by myristic acid was kinetically analyzed using Lineweaver–Burk plots. As shown in Fig. 1, myristic acid at low concentrations (2.5 and 5.0 μM) inhibited the reduction of 4BP catalyzed by RHCR uncompetitively with respect to both 4BP and NADPH. The inhibition constant, *K*_ _i_, was determined from replots of the intercepts of double-reciprocal plots with the inhibitor at low concentrations. When 4BP and NADPH were the varied substrates, the *K*_ _i_ values were 1.0 and 5.1 μM, respectively. However, kinetics of the inhibition of RHCR by myristic acid at high concentrations was complicated, since the regression line in the presence of this acid at a concentration of 10.0 μM was not parallel to the control line (Fig. 1).

**DISCUSSION**

We demonstrated in this study that the inhibition of RHCR by saturated fatty acids increases with elongation in the carbon chain from caprylic acid to myristic acid, but decreases with further elongation. These results indicate that a hydrophobic region exists in RHCR, and suggest that the hydrophobic region has a proper length corresponding to the carbon chain of myristic acid. Stearic acid with 18 carbon atoms was a poor inhibitor of RHCR, while unsaturated fatty acids with 18 carbon atoms were found to inhibit it much more strongly than stearic acid. The carbon chain of unsaturated fatty acids is thought to be more condensed in length than that of saturated fatty acids with the corresponding carbon atoms.25) Thus, unsaturated fatty acids with 18 carbon atoms may be well fitted to the hydrophobic region of their binding site on RHCR.

It has been reported that in unsaturated fatty acids the *cis*-isomers are more potent for several enzymes than the corresponding *trans*-isomers.23–26) For example, Kido *et al.*25) showed that the *cis*-isomer, oleic acid, is a more potent inhibitor of chymase than the corresponding *trans*-isomer, elaidic acid. This is because the steric structure of *cis*-unsaturated fatty acids is different from that of the *trans*-isomers. The present study also provides evidence that the *cis*-unsaturated fatty acids with 18 carbon atoms, oleic acid and linoleic acid, more potently inhibit RHCR than the corresponding *trans*-isomers, elaidic acid and linolelaic acid. These *cis*-unsaturated fatty acids may more tightly interact with their binding site on RHCR than the corresponding *trans*-isomers. Furthermore, γ-linolenic acid was found to be only a slightly potent inhibitor of RHCR compared to α-linolenic acid. Liang and Liao23) demonstrated that a similar result was observed for the inhibition of rat liver 5 α-reductase by α- and γ-linolenic acids. It is interesting that the inhibition of RHCR by unsaturated fatty acids is altered by a change in the position of the double bond.

As described above, lauric acid, myristic acid, and oleic acid were potent inhibitors of RHCR. However, the methyl esters and alcohols, which lack a carboxyl group, derived from these fatty acids did not exert a significant inhibitory effect on RHCR (data not shown). These results clearly indicate that a carboxyl group plays an important role in the inhibition of RHCR by fatty acids. The pH of the carboxyl

### Table 1. Inhibitory Effects of Saturated Fatty Acids on RHCR

<table>
<thead>
<tr>
<th>Saturated fatty acid</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td></td>
<td>20 μM</td>
</tr>
<tr>
<td>Caprylic acid (C8)</td>
<td>9.1±2.8</td>
</tr>
<tr>
<td>Capric acid (C10)</td>
<td>35.9±1.5</td>
</tr>
<tr>
<td>Lauric acid (C12)</td>
<td>42.2±1.5</td>
</tr>
<tr>
<td>Myristic acid (C14)</td>
<td>52.2±4.4</td>
</tr>
<tr>
<td>Palmitic acid (C16)</td>
<td>30.9±1.9</td>
</tr>
<tr>
<td>Stearic acid (C18)</td>
<td>13.6±2.8</td>
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</tbody>
</table>

The number of carbon atoms is shown in parentheses. The values are the means±S.D. of 3 experiments.

### Table 2. Inhibitory Effects of Unsaturated Fatty Acids on RHCR

<table>
<thead>
<tr>
<th>Unsaturated fatty acid</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 μM</td>
</tr>
<tr>
<td>Oleic acid (C18:1, <em>cis</em>-9)</td>
<td>50.3±0.7</td>
</tr>
<tr>
<td>Elaidic acid (C18:1, <em>trans</em>-9)</td>
<td>42.7±2.1</td>
</tr>
<tr>
<td>Linoleic acid (C18:2, <em>cis</em>-9, 12)</td>
<td>47.0±5.7</td>
</tr>
<tr>
<td>Linolelaic acid (C18:2, <em>trans</em>-9, 12)</td>
<td>40.9±1.5</td>
</tr>
<tr>
<td>α-Linolenic acid (C18:3, <em>cis</em>-9, 12, 15)</td>
<td>49.3±1.6</td>
</tr>
<tr>
<td>γ-Linolenic acid (C18:3, <em>cis</em>-6, 9, 12)</td>
<td>66.9±1.2</td>
</tr>
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</table>

The number of carbon atoms and the number and position of double bond(s) are shown in parentheses. The values are the means±S.D. of 3 experiments.

Fig. 1. Lineweaver–Burk Plots for the Inhibition of RHCR by Myristic Acid

(A) The concentration of NADPH was 0.25 mM. (B) The concentration of 4BP was 1.0 mM. The concentrations of myristic acid were 0 μM (○), 2.5 μM (■), 5.0 μM (●), and 10.0 μM (▲).
group on fatty acids is known to be about 4.8. Therefore, this group is mostly ionized at pH 6.0 in this study. The negatively charged carboxyl group on fatty acids may interact with arginine or lysine residue located in their binding site on RHCR.

Our previous paper reported that the reduction of 4BP catalyzed by RHCR follows an ordered Bi Bi mechanism, in which NADPH binds to the enzyme first and NADP leaves the enzyme last. In the present study, we have shown that myristic acid at low concentrations inhibits the reduction of 4BP catalyzed by RHCR uncompetitively with respect to both 4BP and NADPH. This inhibition pattern, based on the ordered Bi Bi mechanism in the enzyme reaction described above, led us to conclude that myristic acid at low concentrations selectively binds to the enzyme-NADPH-4BP ternary complex. We propose the possibility that myristic acid at low concentrations inhibits the reduction of 4BP by interacting with a binding site other than the coenzyme- and substrate-binding sites of RHCR.

In conclusion, this study has demonstrated that the existence of a proper length of carbon chain, double bond(s), and a carboxyl group in a fatty acid molecule is important for the inhibition of RHCR, and that myristic acid at low concentrations interacts with a binding site other than the coenzyme- and substrate-binding sites of RHCR. It should be noted that fatty acids, especially naturally occurring unsaturated fatty acids, can strongly inhibit RHCR, even though the physiological significance of such inhibition is not yet clear. Further studies are in progress to elucidate the detailed mechanism and physiological significance of the inhibition of RHCR by fatty acids.

REFERENCES