Rat Liver Cholesterol 7α-Hydroxylase
MODULATION OF ENZYME ACTIVITY BY CHANGES IN PHOSPHORYLATION STATE*

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Four lines of evidence presented here suggest that the activity of cholesterol 7α-hydroxylase in rat liver is modulated by changes in its phosphorylation state. 1) Livers were homogenized and microsomes were isolated and washed in the presence of either 50 mM NaCl or 50 mM NaF, the latter an inhibitor of phosphoprotein phosphatases. The 7α-hydroxylase activity of microsomes prepared with NaF was 80% greater than that of microsomes prepared with NaCl. 2) Incubation of 10,000 x g supernatants from rat liver for 20 min at 37 °C in the absence of 50 mM KF decreased the activity of microsomal cholesterol 7α-hydroxylase by 52%. No significant change was seen in the presence of KF. 3) 7α-Hydroxylase activity fell by 40% when microsomes were incubated with bacterial alkaline phosphatase compared to incubation of microsomes with phosphatase that was inhibited by phosphate and EDTA. 4) 7α-Hydroxylase activity increased by 22% when phosphatase-treated microsomes were incubated for 40 min at 37 °C with 1 mM MgATP, 50 μM cAMP, and 200 units of cAMP-dependent protein kinase.

Cholesterol 7α-hydroxylase is a microsomal mixed function oxidase that includes cytochrome P-450 as one of its subunits and catalyzes the NADPH-dependent hydroxylation of cholesterol. It is considered the rate-limiting enzyme in the conversion of cholesterol to bile acids (1). The activity of the enzyme undergoes a diurnal variation that is related to feeding patterns (1, 2). In addition, the enzyme has an unusually rapid half-life of 2 to 4 h (1). Cholesterol 7α-hydroxylase is chronically influenced by hormones, drugs, and dietary components (1). Its activity is increased by glucocorticoids (3), thyroid hormone (1), 17β-estradiol (4), cholestyramine and phenobarbital treatment (1, 2, 5-7), biliary diversion (8), and lymphatic drainage (8). The activity of the enzyme is decreased by fasting (1), ethanol feeding (9), and bile acid administration (1). Furthermore, rat pups exposed to a high cholesterol, high fat diet during lactation had increased levels of 7α-hydroxylase (10).

There is also evidence for more rapid regulation of cholesterol 7α-hydroxylase. For example, administration of mevalonate to rats rapidly increased 7α-hydroxylase activity and bile acid synthesis (11). Studies in this laboratory demonstrated that 1 mM dibutyryl cAMP stimulates bile acid synthesis by about 80% in freshly isolated rat hepatocytes (12). A potential, yet largely unexplored, mechanism for the rapid control of 7α-hydroxylase is the reversible phosphorylation of the enzyme. A large body of evidence from this and other laboratories has suggested that another microsomal enzyme, HMG-CoA reductase, is regulated by this mechanism (13-19).

The present studies are modeled on the earlier work on HMG-CoA reductase and provide preliminary evidence that the activity of cholesterol 7α-hydroxylase can be altered by changes in its phosphorylation state. A brief account of this work has been presented (20).

MATERIALS AND METHODS

Chemicals—Dithiothreitol, glutathione, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, ATP, cAMP, cAMP-dependent protein kinase (rabbit muscle), Tween 80, β-mercaptoethanol, alkaline phosphatase (type III, bacterial), Coomassie blue G 250, cholesterol, and rhodamine B were purchased from Sigma. 7α-Hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol were from Steraloids, Inc. (Wilton, NH). Thin layer chromatography plates (0.25 mm, Silica Gel G) were from E. Merck. AG1-X8 resin (formate form) was from Bio-Rad. Betafluor was from National Diagnostica. Mevalonolactone and Aquasol were obtained from New England Nuclear. Sources for other materials used in these studies have been previously described (13).

Animals—Male Sprague-Dawley rats (Charles River Breeding Laboratories) had continuous access to water and rat chow and were exposed to a reversed lighting pattern (light, 4 p.m. to 4 a.m.; dark, 4 a.m. to 4 p.m.) for at least 7 days before use. For microsome isolation, rats were sacrificed between 9 and 10 a.m.

Microsome Isolation—Preparation of 10,000 x g supernatant and isolation of endoplasmic reticulum by ultracentrifugation were carried out as previously described (21) with the following exceptions. Livers were homogenized in buffer containing 225 mM sucrose, 25 mM Tris (pH 7.8), 5 mM glutathione, and either 50 mM NaCl or 50 mM NaF. Microsomes were washed once in buffer containing 100 mM sucrose, 40 mM potassium phosphate (pH 7.2), 30 mM EDTA, 20 mM dithiothreitol, and either 50 mM KCl or 50 mM KF (KCl or KF suspension buffer) and were resuspended in the same buffer prior to assay. This buffer composition was chosen because of its prior use in studies of in vitro modulation of microsomal HMG-CoA reductase; when included in the 7α-hydroxylase assay, this buffer yielded higher enzyme activity than the buffer used by Bjorkhem and Danielsson (22).

Cholesterol 7α Hydroxylase Assay—Cholesterol 7α-hydroxylase was measured by a modification of the method of Bjorkhem and Danielsson (22). Incubations, conducted in triplicate in KCl or KF suspension buffer, contained 0.5 to 0.8 mg of protein from washed microsomes, 1 mM NaDTP, 10 mM glucose-6-phosphate, and 0.15 unit of glucose-6-phosphate dehydrogenase in a final assay volume of 0.5 ml. β-Mercaptoethanol (10 mM) was included to minimize non-enzymatic oxidation of cholesterol (23).

Washed microsomes in buffer with mercaptoethanol were in-

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Modulation of Rat Liver Cholesterol 7α-Hydroxylase

Evidence from four series of experiments suggests that altering the phosphorylation state of cholesterol 7α-hydroxylase in vitro results in a change in its catalytic activity. The strategy for these studies relied on fluoride to inhibit endogenous phosphoprotein phosphatases. In addition, E. coli alkaline phosphatase, which is not inhibited by fluoride, was utilized to remove phosphate covalently bound to protein (26). Comparison studies with phosphatase were performed on HMG-CoA reductase, already known to be modulated by phosphorylation. Finally, cyclic AMP-dependent protein kinase was used to restore phosphate to the enzyme.

In the first experiments, rat livers were homogenized in buffer containing 50 mM NaF or 50 mM NaCl. Microsomes were isolated, washed in the continuous presence of the same anions, and assayed for 7α-hydroxylase activity. Data shown are means and standard errors of the mean from three experiments.

<table>
<thead>
<tr>
<th>Addition</th>
<th>+NADP</th>
<th>-NADP</th>
<th>Net F/Cl</th>
<th>CPM/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>89 ± 31</td>
<td>21 ± 12</td>
<td>68 ± 22</td>
<td>1.8 ± 0.13*</td>
</tr>
<tr>
<td>NaF</td>
<td>150 ± 55</td>
<td>29 ± 18</td>
<td>120 ± 39</td>
<td>0.05 for F/Cl ≤ 1.0</td>
</tr>
</tbody>
</table>

*P < 0.05 for F/Cl ≤ 1.0.

C. D. Goodwin, unpublished observations.
TABLE II
Effect of alkaline phosphatase treatment on the activity of cholesterol 7α-hydroxylase and HMG-CoA reductase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Net enzyme activity</th>
<th>Relative activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol 7α-hydroxylase</td>
<td>Inactivated</td>
<td>120 ± 19</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Phosphatase</td>
<td>76 ± 6.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60 ± 5.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HMG-CoA reductase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Inactivated</td>
<td>0.030</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Phosphatase</td>
<td>0.053</td>
<td>180</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as counts per min/min/mg; means and standard errors of the mean from four experiments.

<sup>b</sup> P < 0.05.

<sup>c</sup> P < 0.01.

<sup>d</sup> Expressed as nanomoles/min/mg; data from one experiment.

TABLE III
Effect of protein kinase treatment on cholesterol 7α-hydroxylase activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Net activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP, MgATP</td>
<td>35 ± 1.5</td>
<td>100</td>
</tr>
<tr>
<td>cAMP, MgATP, and protein kinase</td>
<td>43 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>122 ± 5.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.02.

MGATP resulted in a slight decrease in enzyme activity that could be blocked by fluoride. A greater inhibition by MgATP was seen when crude cytosol or a cytosolic factor was incubated with the microsomes; under these conditions, fluoride had little effect. These workers also described a cytosolic activator that was stable to boiling and, therefore, unlikely to be a protein kinase. It is unclear whether the observations made by Kwok and his co-workers (28, 29) are the consequence of phosphorylation and dephosphorylation or of some other mechanism. Further comparison of the work of Kwok with the present investigation is difficult because of many differences in experimental approach, e.g., animal feeding, buffer composition, conditions for microsome isolation, and the details of the enzyme assay. Furthermore, none of the preincubation experiments conducted by Kwok et al. (28, 29) utilized intact 10,000 × g liver supernatants, which were used in the present studies, but rather employed reconstituted mixtures of microsomes and intact or fractionated cytosol.

The physiological importance of phosphorylation as a potential means of regulation of 7α-hydroxylase is emphasized by recent work of Scallen and his co-workers (30, 31). They found that the administration of mevalonolactone or cholesterol to rats caused a sharp inhibition of HMG-CoA reductase. This inhibition could be reversed in vitro by phosphatase, but only at very early times after the exposure to mevalonolactone or cholesterol. It is also known that mevalonolactone rapidly increases 7α-hydroxylase activity (11). It is tempting to propose that HMG-CoA reductase and cholesterol 7α-hydroxylase, each rate limiting for a major pathway in cholesterol metabolism, are regulated coordinately, but in opposite directions, by phosphorylation and dephosphorylation. This mechanism may mediate rapid hormonal effects on cholesterol metabolism. It is also possible that the activity of other mixed function oxidases, which, like cholesterol 7α-hydroxylase, also contain a cytochrome P-450 component, may be regulated by phosphorylation.

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REFERENCES
Modulation of Rat Liver Cholesterol 7α-Hydroxylase

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