Abnormal Cholesterol Uptake, Storage, and Synthesis in the Livers of 2-Acetylaminofluorene-fed Rats

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SUMMARY

Rat livers were tested for both rate of cholesterol synthesis and uptake of a p.o. dose of cholesterol-14C after rats were fed 0.06% 2-acetylaminofluorene (AAF) for either 1 or 2 weeks. For the 2 days prior to the assay, all rats also received 5% cholesterol in the diet.

In the control group, the high-cholesterol diet caused almost complete inhibition of cholesterol synthesis, but after AAF treatment the levels of cholesterol synthesis were markedly higher, with considerable variation between individual rats. Uptake of cholesterol-14C given p.o. was significantly lower in the AAF group and was also variable, but there was no correlation with sterol synthesis, indicating that impairment of cholesterol uptake is not the critical factor in loss of control. However, liver cholesterol levels in the AAF-fed rats were significantly lower than those in control animals, and there was a highly significant inverse correlation between liver cholesterol levels and sterol synthesis. Therefore, the lack of regulation of cholesterol synthesis induced by AAF appears to be a result of a defect in intracellular storage of cholesterol rather than defective uptake.

INTRODUCTION

Loss of control of cholesterol synthesis in response to cholesterol feeding is a consistent feature of both primary and transplantable hepatomas (23) and also of livers from animals treated with a wide variety of different carcinogens (7, 8, 11–13, 21, 22). It has been suggested by Sabine et al. (18–20) that this is due to the inability of the normal metabolic regulators to enter the tumor cell. Harry et al. (6) confirmed this hypothesis by demonstrating that the entry of cholesterol-14C (given p.o.) into transplantable hepatomas 7794A and 7787 is very much slower than the rate of entry into the host liver. Similar results were reported for hepatoma 7794A by Horton (7) and were extended to hepatomas 7800 and 9618A by S. Goldfarb (unpublished observations).

Rats treated with the hepatocarcinogen ethionine completely lack inhibition of cholesterol synthesis by dietary cholesterol and, in their livers, uptake of cholesterol-14C is significantly slower than in those of normal rats. However, this cholesterol uptake is approximately 20-fold greater than in the transplantable hepatomas (7), so it is possible that, despite the statistical significance of the difference between normal and ethionine-treated rats, it may not be sufficient to cause the complete lack of inhibition of cholesterol synthesis that occurs.

Not all carcinogens cause such a complete loss of cholesterol control. Rats fed AAF for 1 to 2 weeks exhibit a remarkable degree of variation, from almost normal to almost complete loss of control of cholesterol synthesis, in response to cholesterol feeding (9, 11). The study of cholesterol metabolism in these AAF-fed rats may prove extremely useful in elucidating mechanisms of regulation in normal animals and also abnormalities in this regulation.

We therefore decided to take advantage of this variation by measuring cholesterol uptake in AAF-fed rats to determine whether it is in fact abnormal and, if so, whether the rate of cholesterol uptake does bear any relation to the actual degree of loss of control of cholesterol synthesis for each individual animal.

MATERIALS AND METHODS

Animals and Diets. Male Sprague-Dawley rats (Sprague-Dawley Farm, Madison, Wis.) were used. Rats used in Experiment A weighed 200 to 230 g at the beginning of AAF feeding, and those in Experiment B weighed 270 to 320 g. All rats were subjected to a 12-hr-day, 12-hr-night regime (lights on at 6 a.m. and off at 6 p.m.). They were fed a powdered commercial laboratory ration (Wayne Lab Blox; Allied Mills, Inc., Chicago, III.) beginning 2 weeks before commencement of AAF feeding. This diet was supplemented, where indicated, with 5% cholesterol (General Biochemicals, Inc., Chagrin Falls, Ohio) and/or 0.06% AAF (Mann Research Laboratories, New York, N. Y.).

Assay for Liver Cholesterogenesis in Vivo. This assay has been described in detail previously (8). Briefly, rats were given i.p. injections of 10 μmoles of AY-9944 per kg. This drug specifically inhibits the conversion of 7-dehydrocholesterol to cholesterol (2).

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3The abbreviations used are: AAF, 2-acetylaminofluorene (N-2-fluorenylacetamide); AY-9944, trans-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride.
Blood was collected from the tail vein 24 hr later, saponified in 30% KOH, and then extracted with light petroleum (b.p., 38–48°). After evaporation of the light petroleum, the 7-dehydrocholesterol was measured by color development, exactly 2 min after the addition of the Liebermann-Burchard reagent.

**Assay for Serum and Liver Sterol.** Duplicate samples of tissue (300 to 500 mg liver; 0.5 to 1.0 ml of serum) were saponified with 0.5 ml 10% KOH (75° for 2 hr). After the addition of 1.0 ml ethanol, sterols were extracted in 10.0 ml light petroleum (b.p., 38–48°). Aliquots (4 ml) were evaporated to scintillation vials for measurement of radioactivity in 10 ml Scintisol Complete, in a Packard Tri-Carb Model 3375 spectrophotometer. For total-sterol determination, a 2nd 4-ml aliquot was evaporated, and the residue was dissolved in 0.5 ml acetic acid. Liebermann-Burchard reagent was added to each sample, and the mixture was immediately incubated at 37°. Absorbance (625 nm) was measured after exactly 2 min (maximum color development by 7-dehydrocholesterol), and after 20 min (maximum color development by cholesterol) with a Gilford 2400 spectrophotometer.

Samples of the light petroleum extract have been examined by means of silica gel thin-layer chromatography (16) (benzene:ethyl acetate, 9:1) and 10% silver nitrate in silica gel (ether:acetic acid, 99:1). No significant color development occurred at 2 or 20 min for any compounds other than 7-dehydrocholesterol, cholesterol, and cholesteryl esters, which are present due to incomplete hydrolysis but which give color development similar to that of cholesterol (1).

**Experiment A.** One week after arrival in our laboratory, 30 rats were fed the high-cholesterol diet for 2 days, then were assayed for cholesterol synthesis in vivo. A week later, they were tested again on the normal low-cholesterol diet. Of these 30 rats, 22 were then fed 0.06% AAF for 7 days, while the remaining 8 control rats were maintained on the powdered chow. From the 5th to 7th day of AAF feeding, all rats were maintained on the high-cholesterol diet. On the 6th day, all rats were dosed with AY-9944 (7:00 to 7:30 a.m.), and blood samples for 7-dehydrocholesterol determination were collected exactly 24 hr later. The rats were then all returned to the low-cholesterol diet to limit impairment of intestinal cholesterol absorption.

On that same day (Day 7), all rats were dosed with 6 µCi (0.21 µg) cholesterol-7-3H (New England Nuclear, Boston, Mass.) by stomach tube (4:00 to 4:30 p.m.), and were killed exactly 4 hr later, for measurement of liver and serum cholesterol and cholesterol-3H.

**Experiment B.** Twenty-nine rats were tested for sterol synthesis in vivo as before, and 7 were selected as representative controls. The rest of the rats were then fed 0.06% AAF for 2 weeks. All animals were also fed 5% cholesterol from the 12th day of carcinogen feeding.

On the 13th day, the rats were dosed with AY-9944 at 12:00 to 12:30 p.m., and 6 µCi cholesterol-7-3H exactly 20 hr later. They were killed on the 14th day, exactly 24 hr after the AY-9944 treatment. Total cholesterol, 7-dehydro-cholesterol, and cholesterol-3H levels were measured in the liver and serum of all rats.

**RESULTS**

**Experiment A.** Liver sterol synthesis is shown in Table 1. This was measured as the 7-dehydrocholesterol released into the blood in 24 hr in the presence of AY-9944, which specifically inhibits the conversion of 7-dehydrocholesterol to cholesterol (2).

After 1 week of AAF feeding, the average sterol synthesis of rats on a high-cholesterol diet was significantly higher than that of control rats, indicating a marked loss of regulation of cholesterol synthesis. However, the response in individual AAF-fed rats was extremely variable, from almost normal cholesterol inhibition to almost complete lack of control, as can be seen from the range of values obtained (Table 1).

The uptake of cholesterol-3H by the liver 4 hr after administration of the p.o. dose (Table 2) is expressed in 2 ways. The specific activity of cholesterol in liver relative to serum would be the most satisfactory parameter if a complete and rapid equilibrium existed between all forms of cholesterol in the serum and if an independent equilibrium existed between all forms of cholesterol in the liver. However, this is almost certainly not the case, and since the total liver and serum cholesterol levels differed significantly between control and AAF-treated rats, the simple ratio of cpm in the tissues may be a more accurate indication of comparative cholesterol uptake. It is not valid to consider either liver cpm or liver cholesterol specific activity alone, since there were marked variations in serum cholesterol-3H, indicating variation in the cholesterol uptake from the intestine.

The cholesterol-3H uptake into the AAF-treated rat livers (measured as liver cpm: serum cpm) was significantly lower (p < 0.001) than in the control rat livers. If the loss of control was caused simply by decreased cholesterol uptake, then the individual levels of synthesis should be inversely related to the uptake of cholesterol. However, in these AAF-treated rats, the correlation between cholesterol synthesis and the ratio of cpm in liver and serum was low and not significant (Table 3), so this hypothesis appears to be incorrect. In the same animals there was a high negative correlation (p < 0.001) between total-liver cholesterol and cholesterol synthesis. This is consistent with the results of Harry et al. (6), who found that transplantable hepatomas fail to accumulate cholesterol even after 21 days on a high-cholesterol diet. Although lack of cholesterol storage was assumed to be a result of the slow cholesterol uptake in those hepatomas, there was no correlation between cholesterol-3H uptake and the level of liver cholesterol in these AAF-treated animals. These results suggested that cholesterol storage in rats on a high-cholesterol diet could be more significant than short-term uptake for the control of cholesterol synthesis, so an additional experiment was carried out.

**Experiment B.** In this experiment, rats were maintained...
Cholesterol Uptake, Storage, and Synthesis

Table 1
Effect on sterol synthesis of 1 week of AAF feeding
Sterol synthesis was measured in vivo in all rats at weekly intervals, before and after they were fed AAF, with the use of the assay described in "Materials and Methods." For the first and last assays, the rats were fed 3% cholesterol for 2 days before and during the assay period. AAF feeding was begun immediately after completion of the assay at Week 0.

<table>
<thead>
<tr>
<th>Rats</th>
<th>No. tested</th>
<th>Diet</th>
<th>Sterol synthesis* (µg 7-dehydrocholesterol/ml blood) at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Week -1</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>High cholesterol</td>
<td>23 ± 2* (14-31)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low cholesterol</td>
<td></td>
</tr>
<tr>
<td>AAF-fed</td>
<td>22</td>
<td>High cholesterol</td>
<td>23 ± 1 (11-32)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low cholesterol</td>
<td></td>
</tr>
</tbody>
</table>

* Twenty-four hr after treatment with AY-9944.
* Mean ± S.E.
' Numbers in parentheses, range of values.
* Significantly different from controls (p < 0.001).

Table 2
Cholesterol-3H uptake by livers of rats fed AAF for 1 week
Duplicate samples of serum and liver from each animal (8 controls and 22 fed AAF) were taken 4 hr after treatment with cholesterol-3H. In all samples, both total cholesterol and cholesterol-3H were measured.

<table>
<thead>
<tr>
<th>Rats</th>
<th>cpm/g liver: cpm/ml serum</th>
<th>S.A.* liver cholesterol:S.A. serum cholesterol</th>
<th>Liver cholesterol (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.94 ± 0.22 (4.15-6.03)*</td>
<td>0.79 ± 0.07 (0.64-1.09)</td>
<td>3.94 ± 0.33 (2.48-4.98)</td>
</tr>
<tr>
<td>AAF-fed</td>
<td>3.51 ± 0.26 (1.93-6.00)</td>
<td>0.51 ± 0.04 (0.29-0.88)</td>
<td>3.46 ± 0.19 (1.93-5.15)</td>
</tr>
</tbody>
</table>

* S.A., specific activity of cholesterol-3H.
* Mean ± S.E.
' Numbers in parentheses, range of values.
* Significantly different from controls (p < 0.001).
* Significantly different from controls (p < 0.01).
' Significantly different from controls (p < 0.05).

Table 3
Correlations of cholesterol synthesis, uptake, and accumulation in livers of rats fed AAF for 1 week
Correlations (r) and their degree of significance (determined by t test) were calculated as described by Mather (17).

<table>
<thead>
<tr>
<th>Correlation</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol synthesis: cpm liver cholesterol/cpm serum cholesterol</td>
<td>0.23</td>
</tr>
<tr>
<td>Cholesterol synthesis: liver cholesterol</td>
<td>-0.68*</td>
</tr>
<tr>
<td>cpm liver cholesterol/cpm serum cholesterol: liver cholesterol</td>
<td>-0.30</td>
</tr>
</tbody>
</table>

* Significant correlation (p < 0.001).

on the high-cholesterol diet until they were killed, and the measurement of cholesterol-3H uptake was carried out over the last 4 hr of measurement of sterol synthesis, rather than 12 hr later. Since the rats were killed exactly 24 hr after AY-9944 treatment, liver levels of 7-dehydrocholesterol were used as a more accurate measure of liver sterol synthesis than 7-dehydrocholesterol in the blood, as in the previous experiment.

The rats fed AAF for 2 weeks had significantly higher cholesterol synthesis on the high-cholesterol diet than did the control rats (Table 4), indicating a considerable loss of control over cholesterol synthesis. The level of cholesterol was significantly lower in the AAF-treated livers, and they also had significantly lower levels of cholesterol-3H uptake, whether measured as relative specific activity or as relative activity.

In this experiment, daily liver sterol synthesis was measured as the amount of 7-dehydrocholesterol present in the liver 24 hr after AY-9944 treatment. This was assumed to be slightly more accurate than the use of serum 7-dehydrocholesterol, although the 2 measures were very closely related (r = 0.86).

Again, there was no inverse correlation between liver cholesterol synthesis and cholesterol-3H uptake after AAF.
treatment (Table 5), confirming that the high level of cholesterol synthesis is not due to impaired uptake of dietary cholesterol. However, there was again a highly significant inverse correlation between liver sterol synthesis and total cholesterol levels in the liver. This was not due simply to replacement of cholesterol by 7-dehydrocholesterol, since there was a similar relationship with total liver sterol (cholesterol + 7-dehydrocholesterol).

As in Experiment A, there was no positive relationship between total liver cholesterol levels and uptake of cholesterol-^3H.

DISCUSSION

The results presented in this paper show that cholesterol metabolism in the livers of AAF-treated rats is similar in many ways to that of transplantable hepatomas (6): (a) livers of AAF-treated animals lack (at least partially) inhibition of cholesterol synthesis in response to cholesterol feeding; (b) the uptake of labeled cholesterol into these livers is significantly impaired; (c) cholesterol storage in the livers of AAF-treated rats during cholesterol feeding is significantly impaired.

However, although the uptake of cholesterol-^3H was impaired by carcinogen feeding, it was still many times greater than that reported for transplantable hepatomas. Furthermore, the synthesis of cholesterol in the AAF-treated rats did not exhibit the expected inverse correlation with uptake of cholesterol-^3H. This indicates that although cholesterol uptake was defective after AAF treatment it was not the critical factor causing the loss of inhibition of cholesterol synthesis in these animals.

On the other hand, the total level of liver cholesterol was strongly correlated with cholesterol synthesis, which implies that this, rather than uptake, may be the major controlling factor for sterol synthesis in AAF-treated rats. This is in agreement with reports that in normal, cholesterol-fed rats, cholesterol synthesis is closely related to the level of liver cholesterol (3, 5), although this relationship may be altered by the presence of other regulatory mechanisms, e.g., bile duct ligation (15).

The results presented here suggest that in the early precancerous liver there may actually be no defect in the entry of cholesterol into the cell, but rather, a defect in an intracellular cholesterol binding or storage mechanism. Such a defect would naturally lead indirectly to an apparent decrease in the entry of cholesterol-^3H into the cell. This would result in the observed difference between cholesterol uptake in AAF rats and that in the controls, and would account for the lack of any direct relationship to cholesterol synthesis. This is confirmed by the lack of a positive correlation between total-liver cholesterol levels and 4-hr entry of cholesterol-^3H in the precancerous rats.

The loss of regulation of cholesterol synthesis observed in these animals may be a result of AAF toxicity, unrelated to AAF metabolism. This is confirmed by the lack of a positive correlation between total-liver cholesterol levels and 4-hr entry of cholesterol-^3H in these animals.

Table 4

<table>
<thead>
<tr>
<th>Rats</th>
<th>7-Dehydrocholesterol (mg)</th>
<th>Cholesterol (mg/g liver)</th>
<th>7-Dehydrocholesterol + cholesterol (mg/g liver)</th>
<th>cpm/g liver:cpm/ml serum</th>
<th>S.A.* liver cholesterol:S.A. serum cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per g liver</td>
<td>Per ml serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.106 ± 0.009* (0.077–0.156)*</td>
<td>0.017 ± 0.001 (0.014–0.023)*</td>
<td>3.40 ± 0.12 (3.11–3.95)</td>
<td>3.51 ± 0.11 (3.26–4.06)</td>
<td>4.96 ± 0.31 (3.55–6.18)</td>
</tr>
<tr>
<td>AAF-fed</td>
<td>0.289 ± 0.020* (0.094–0.545)</td>
<td>0.059 ± 0.004* (0.029–0.127)</td>
<td>2.71 ± 0.10* (1.80–3.89)</td>
<td>3.00 ± 0.09* (2.34–3.98)</td>
<td>1.35 ± 0.10* (0.49–1.94)</td>
</tr>
</tbody>
</table>

* S.A., specific activity of cholesterol-^3H.
* Mean ± S.E.
* Numbers in parentheses, range of values.
* Significantly different from controls (p < 0.001).
* Significantly different from controls (p < 0.01).

Table 5

<table>
<thead>
<tr>
<th>Correlation</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg 7-dehydrocholesterol/g liver:mg cholesterol/g liver</td>
<td>−0.81*</td>
</tr>
<tr>
<td>mg 7-dehydrocholesterol/g liver:mg total sterol/g liver</td>
<td>−0.73*</td>
</tr>
<tr>
<td>mg 7-dehydrocholesterol/g liver:cpm liver cholesterol/cpm serum cholesterol</td>
<td>0.57*</td>
</tr>
<tr>
<td>mg cholesterol/g liver:cpm liver cholesterol/cpm serum cholesterol</td>
<td>−0.49*</td>
</tr>
</tbody>
</table>

* Significant correlation (p < 0.001).
* Significant correlation (p < 0.01).
* Significant correlation (p < 0.05).
carcinogenicity, but this possibility is rendered unlikely by our previous findings (12, 13) that this type of loss of control is not related to carcinogen toxicity as measured by loss of weight or mortality during carcinogen treatment. Therefore, the results of Harry et al. (6), showing the lack of cholesterol storage in transplantable hepatomas and delayed cholesterol uptake into those hepatomas, may be interpreted as 2 independent effects, only 1 of which is necessary to explain the observed loss of control.

It has been shown that the lack of inhibition of cholesterol synthesis in transplantable hepatomas reflects the unresponsiveness of the rate-controlling enzyme in cholesterol synthesis, \( \beta \)-hydroxy-\( \beta \)-methylglutaryl coenzyme A reductase (4, 14, 24). Therefore, the cholesterol storage mechanism shown here to be defective may also be the mechanism responsible for the regulation of the synthesis of this enzyme.

Finally, recent experiments in this laboratory (10) showed that some highly differentiated primary hepatomas (induced by AAF) take up cholesterol-\(^{1}H\) at a rate similar to that found in precancerous liver, i.e., 10- to 30-fold faster than the rate of uptake found in transplantable hepatomas. Therefore, if further studies demonstrate that all such primary hepatomas do exhibit a complete lack of control, then it is possible that this defective control of cholesterol synthesis, found in precancerous liver and in primary and transplantable hepatomas, is due to a defect in the binding and storage of cholesterol within the cell.

Since this particular control mechanism is defective in every transplantable and primary hepatoma ever examined (23) and also during the early stages of hepatocarcinogenesis treatment (7, 11–13, 21, 22), the loss of the cholesterol storage mechanism may be an essential characteristic of the carcinogenic process.

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