MECHANISM OF HYPERCHOLESTEROLEMIA IN ARTERIOLIPIDOSIS-PRONE RATS (ALR)

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YASUO NARA, Ph.D., AND NOBUKO IRIKAMI, Ph.D.*

The cholesterol metabolism of a newly established model for atherogenesis, named arteriolipidosis-prone rats (ALR), selected from spontaneously hypertensive rats (SHR), was investigated in comparison with a substrain of SHR (B) (nonarteriolipidosis-prone rats) and the normotensive control, Wistar-Kyoto (WK) rats. Serum cholesterol of ALR was significantly lower than that of WK. Uptake of labelled serum cholesterol by the isolated liver cells at 37°C was higher in ALR than in WK. The data for SHR (B) fell in between these two. On the other hand, on feeding a cholesterol diet, serum cholesterol increased in the order WK, SHR (B) and ALR, with the highest being ALR. The absorption of cholesterol in ALR was about twice as great as in WK and SHR (B).

In search of a better model for atherosclerosis in rats, several substrains of spontaneously hypertensive rats (SHR)1 and 7 normotensive rat strains were checked for their reactive hypercholesterolemia in response to a high-fat-cholesterol diet. The selective breeding was started from one family of SHR which showed a greater hypercholesterolemia? and the repeated selection for the first several generations resulted in the gradual increase of hypercholesterolemic responses, over 500 mg/dl in males and 800 mg/dl in females on the average even after only 1 week of high-fat-cholesterol diet feeding (Fig. 1). These selectively-bred SHR not only develop hypercholesterolemia but also acute arterial fat deposits in cerebrobasal, mesenteric, renal and other arteries within a few weeks when they are fed a hypercholesterolemic diet (Fig. 2). Such acute arterial fat deposition was in such clear contrast to the generally observed resistance to lipidemic arterial lesions in rats that this selected strain was named “arteriolipidosis-prone rats” (ALR)2-4 They are regarded as good models for lipidemic arterial lesions, while SHR and stroke-prone SHR have been widely evaluated as adequate models for hypertensive arterial lesions5,6 In the present study cholesterol metabolism was analyzed to investigate the pathogenic mechanisms of hypercholesterolemia in ALR in comparison with the cholesterol metabolism in SHR, which was studied previously7,8

Key Words:
Cholesterol
Atherogenesis
Hypertension
Arteriolipidosis-prone rats (ALR)
Spontaneously hypertensive rats (SHR)

Material and Methods
Male spontaneously hypertensive rats (SHR)1 and normotensive rats from the Wistar-Kyoto

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![Graph: Serum Cholesterol (mg/100ml)]

Fig.1. Effect of a 1-week-feeding of a hypercholesterolemic diet on the serum cholesterol level in the initial 6 generations of selectively bred arteriolipidosis-prone rats (ALR).

(WK) strain (from which SHR were derived) were used at 10 weeks of age. These SHR were the substrains of arteriolipidosis-prone rats (ALR) which had been selected for reactive hypercholesterolemia, and non-arteriolipidosis-prone rats (B). Body weight and tail 'systolic' blood pressure were 230–300g and 122–136 mmHg in WK, 205–280g and 165–190 mmHg in B, and 175–230g and 175–214 mmHg in ALR. Rats were fed on a stock diet (Funabashi F₁₁) and water ad libitum, and they were kept under standardized laboratory conditions (23 ± 2°C, humidity 50 ± 5%, automatic lighting schedule from 6 a.m. to 6 p.m.). The cholesterol diet contained 5% cholesterol and 2% cholate in the stock diet.¹⁰,¹¹

Isolation of liver cells

Liver cells were isolated by the methods of Seglen.¹² Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The portal vein was perfused with modified Hanks buffer containing 5 mM EDTA for 5 min, then with the same buffer without EDTA for 5 min and finally 0.05% collagenase with 5 mM CaCl₂ for 5–10

![Fig.2: Acute arterial fat deposits stained with Sudan III, vital-microscopically (a) or microscopically (b) noted in mesenteric arteries in arteriolipidosis-prone rats (ALR) fed on a hypercholesterolemic diet for 3 weeks.]

TABLE I  LIPID LEVELS IN SERUM AND LIVER OF WK, SHR (B) AND ALR

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>mg/g</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WK</td>
<td>0.89 ± 0.05</td>
<td>3.32 ± 0.08</td>
</tr>
<tr>
<td>SHR (B)</td>
<td>0.76 ± 0.05\textsuperscript{b}</td>
<td>3.04 ± 0.14</td>
</tr>
<tr>
<td>ALR</td>
<td>0.62 ± 0.04\textsuperscript{c,d}</td>
<td>3.05 ± 0.10</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WK</td>
<td>0.85 ± 0.08</td>
<td>9.44 ± 0.51</td>
</tr>
<tr>
<td>SHR (B)</td>
<td>1.19 ± 0.07\textsuperscript{a}</td>
<td>10.9 ± 0.44</td>
</tr>
<tr>
<td>ALR</td>
<td>1.56 ± 0.17\textsuperscript{b}</td>
<td>9.20 ± 0.61</td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WK</td>
<td>2.20 ± 0.05</td>
<td>30.1 ± 2.97</td>
</tr>
<tr>
<td>SHR (B)</td>
<td>1.70 ± 0.05\textsuperscript{b}</td>
<td>26.5 ± 1.01</td>
</tr>
<tr>
<td>ALR</td>
<td>1.46 ± 0.10\textsuperscript{c}</td>
<td>26.0 ± 1.30</td>
</tr>
</tbody>
</table>

Data are shown mean ± SE of 5 rats
Significantly different from WK, \( a: p < 0.05, b: p < 0.01, c: p < 0.001 \)
Significantly different from SHR (B), \( d: p < 0.01 \)

TABLE II  CHOLESTEROL LEVELS IN SERUM AND LIVER OF WK, SHR (B) AND ALR FED ON 5% CHOLESTEROL DIET FOR 2 WEEKS

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>mg/g</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>mmHg</td>
<td></td>
</tr>
<tr>
<td>WK</td>
<td>129 ± 2</td>
<td>3.71 ± 0.35</td>
</tr>
<tr>
<td>SHR (B)</td>
<td>178 ± 4</td>
<td>4.45 ± 0.42</td>
</tr>
<tr>
<td>ALR</td>
<td>193 ± 5</td>
<td>6.60 ± 0.40\textsuperscript{a}</td>
</tr>
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</table>

Mean ± SE (5 rats)
Significantly different from WK, \( a: p < 0.001 \)

min. The perfusate was aerated with the mixed gases \( \text{O}_2 \) and \( \text{CO}_2 \) (95:5) at 37\(^\circ\)C. The resected liver was minced gently and slightly, and rinsed with a cold Hanks buffer in a plastic container. All procedures were done in plastics. The isolated liver cell solution was filtered through a nylon cloth. The filtrate was centrifuged at 4\(^\circ\)C for 2 min at 50G. The supernatant was withdrawn and the cells were washed again.

**Uptake of cholesterol by liver cells**

Each incubation flask contained about 10\(^6\) liver cells in 0.5–1.0 ml of modified Ca\(^++\)-Hanks buffer (Hanks buffer with 1 mM CaCl\(_2\)) and 0.5 ml of the common, pooled, labelled serum from the rats, which were killed 10–12 hours after the peroral administration of cholesterol-4\(^{14}\)C (2 \(\mu\text{Ci}/100\text{g}, \text{body weight}) dissolved in 1 ml of olive oil. The cells were incubated for 2 hours at 4\(^\circ\)C or 37\(^\circ\)C with constant shaking in 1.5 ml of total volume. After the incubation, the cells were rinsed with Hanks solution four times at 4\(^\circ\)C. Then the total lipids of cells were extracted and washed according to Folch\textsuperscript{13} The extracts were evaporated to dryness and dissolved in the scintillation solution. A Packard Type-3385 liquid scintillation counter was used for the measurement.

**Estimation of cholesterol 7α-hydroxylation**

Liver cholesterol 7α-hydroxylation was determined by the method of Mitropoulos and Blasubramaniam\textsuperscript{14} Liver was homogenized with a Teflon homogenizer in 3 times the liver volume of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 30 mM nicotinamide, 5 mM MgCl\(_2\) and 1 mM EDTA. Twenty milligrams of microsomal pellet from the homogenate were incubated in 0.25 ml of the same buffer with NADPH generating system (1 mM NADPH, 10

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TABLE III UPTAKE OF SERUM CHOLESTEROL BY ISOLATED HEPATOCYTES

<table>
<thead>
<tr>
<th></th>
<th>Uptake of cholesterol</th>
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<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>37°C</td>
</tr>
<tr>
<td></td>
<td>nmoles/h/10⁶ cells</td>
<td></td>
</tr>
<tr>
<td>WK</td>
<td>11.3 ± 0.67</td>
<td>26.0 ± 2.91</td>
</tr>
<tr>
<td>SHR (B)</td>
<td>15.4 ± 1.30a</td>
<td>33.3 ± 3.98</td>
</tr>
<tr>
<td>ALR</td>
<td>25.5 ± 1.57b, c</td>
<td>41.5 ± 4.41a</td>
</tr>
</tbody>
</table>

Data of each group are mean ± SE of 5 rats.
This experiment was repeated twice and similar results were obtained.
Significantly different from WK, a: p < 0.05, b: p < 0.001
Significantly different from SHR (B), c: p < 0.01

TABLE IV CHOLESTEROL 7α-HYDROXYLATION

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol 7α-hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/h/mg protein</td>
</tr>
<tr>
<td>WK</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td>SHR(B)</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>ALR</td>
<td>1.00 ± 0.09</td>
</tr>
</tbody>
</table>

Data of each group are mean ± SE of 5 rats.
This experiment was repeated three times and similar results were obtained.

RESULTS

Lipid levels of WK, SHR (B) and ALR

The levels of cholesterol, triglycerides and phospholipids in serum and liver of rats used for the experiments are shown in Table I. They are usual data for rats at 10 weeks of age. Serum triglycerides were high in order of ALR, SHR (B) and WK, but serum cholesterol was high in the reverse order as partly reported previously. Phospholipid levels paralleled cholesterol levels.

When rats were fed on a 5% cholesterol diet for 2 weeks, serum cholesterol was much higher in ALR than in WK and liver cholesterol was the same in both. Data are shown in Table II.

Uptake of serum cholesterol by isolated liver cells

When isolated liver cells were incubated with labelled serum cholesterol at 37°C, the incorporation of labelled cholesterol to liver cells increased in the order WK, SHR (B) and ALR, with ALR being highest. The uptake of labelled cholesterol to the cell surface at 4°C paralleled the incorporation. The results are shown in Table III.

Cholesterol 7α-hydroxylation

The cholesterol 7α-hydroxylation in liver microsomes is shown in Table IV. Though it was not significantly different in WK, SHR (B) and ALR, it was usually higher in ALR than in the others in the three experiments.

mM glucose-6-phosphate and 5 units of glucose-6-phosphate dehydrogenase) and 10 μM, 0.05 μCi of cholesterol-4,14C (purchased from the Radiochemical Centre, Amersham, England) dissolved in Tween 80 (0.05% in the incubation mixture), for 1 hour at 37°C with constant shaking. The reaction was terminated by adding 20 ml of chloroform-methanol. The lipid fraction was extracted and washed according to Folch. The products of the incubation were separated by thin-layer chromatography on Kiesel Gel H with ethyl acetate-benzene (7:3). 7α-hydroxy-cholesterol formed was measured and statistically analyzed.

Absorption of cholesterol

Rats were fasted for 24 hours and then given orally via a stomach tube 1 g glucose, 0.5 ml corn oil and 0.2 μCi cholesterol-4,14C (per 100g) dissolved in 0.025 ml of 5% Tween 80. Since serum radioactivity increased linearly after the feeding and the absorption was much greater than the clearance over the initial 6 hours, these rats were killed for sampling plasma and liver 6 hours after ingestion. The appearance of radioactive cholesterol in serum 6 hours after ingestion was used as an indicator of the extent of gastrointestinal absorption of cholesterol. Details have been described previously. Total lipids of plasma and liver were extracted as described by Folch et al. After saponification with ethanolic KOH at 60°C for 1 hour, the nonsaponifiable lipid fraction was extracted with petroleum ether. To measure cholesterol radioactivity, an aliquot of this nonsaponifiable fraction was precipitated with digitonin. Then the cholesterol digitonin complex was thoroughly washed with acetone-ether (1:1) and next with ether. It was finally dissolved in 1 ml methanol, to which was added 9 ml of scintillation fluid.

Cholesterol in plasma and the liver was determined by the method of Zak et al.
TABLE V  RADIOACTIVITY IN SERUM AND LIVER 6 HOURS AFTER ORAL ADMINISTRATION OF CHOLESTEROL-4,14C

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm x 10^3/ml</td>
<td>cpm x 10^3/mg*</td>
</tr>
<tr>
<td>WK</td>
<td>1.14 ± 0.08</td>
<td>1.35 ± 0.07</td>
</tr>
<tr>
<td>SHR(B)</td>
<td>1.06 ± 0.27</td>
<td>1.42 ± 0.36</td>
</tr>
<tr>
<td>ALR</td>
<td>1.58 ± 0.18 d,d</td>
<td>2.49 ± 0.27 b,d</td>
</tr>
</tbody>
</table>

*mg cholesterol

Data of each group are mean ± SE of 5 rats.
This experiment was repeated twice and similar results were obtained.
Significantly different from WK, a: p < 0.05, b: p < 0.01, c: p < 0.001
Significantly different from SHR, d: p < 0.05, e: p < 0.01

Intestinal absorption of cholesterol

When radioactive cholesterol appeared in serum 6 hours after its oral administration, it was used as an indicator of the gastrointestinal absorption of cholesterol. The absorption of cholesterol was significantly higher in ALR than in the others. The specific activity of radioactive cholesterol (cpm/mg) in both plasma and liver of ALR was about twice as great as in WK and SHR (B). The radioactivity of cholesterol per serum (ml) and in liver tissues (g) of ALR was about one and a half times higher than that in WK and SHR (B). The radioactive cholesterol in serum and liver had not yet stabilized 6 hours after administration. The results are shown in Table V.

DISCUSSION

Although rats are generally regarded as being resistant to arterial fat deposition — the initial stage of atherogenesis — hypertensive rats, either genetic or experimental, are rather susceptible to lipidemic vascular lesions when they are fed on hypercholesterolemic diets5,10,11,16–18 Especially, ALR selectively bred for exhibiting greater reactive hypercholesterolemia quickly develop hypercholesterolemia as well as arterial fat deposits within a few weeks on a hypercholesterolemic diet2–5 However, the serum cholesterol level is significantly decreased in ALR. The present data on intestinal cholesterol absorption and cholesterol uptake into hepatocytes very well explain the greater hypercholesterolemic response and the lower endogenous serum cholesterol level in ALR.

As shown in the present cholesterol uptake study, the incorporation of cholesterol into isolated liver cells in ALR was about twice as great as that in WK. In ALR the cholesterol synthesis in liver slices was lower than in WK? It would be therefore controlled by the feed back inhibition due to much internalization of cholesterol into the liver cells. Liver cholesterol 7α-hydroxylase tended to be a little higher in ALR than in WK. It may be activated in ALR also by more accelerated internalization of cholesterol into the hepatocytes.

On feeding a high cholesterol diet, serum cholesterol increased in the order WK, SHR (B) and ALR, with the highest being ALR. When the recovery of radioactive cholesterol in serum and liver 6 hours after ingestion was used as the indicator of gastrointestinal absorption of cholesterol, the absorption in ALR was greater than in the others. However, the primary causal relation between hypertension and cholesterol absorption was not considered, since the cholesterol absorption in hypertensive SHR (B) was not high.

Our recent studies have shown that the HDL level is significantly lower in ALR than in SHR and WK rats (unpublished data). Therefore, the atherogenic index (LDH/HDL ratio) in ALR is greater. In this connection ALR seem to be a new unique model for studies on the mechanism of atherogenesis.

Acknowledgement

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